Disentangling the influence of stress (mediators) and time on fear generalization

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"What if…?" Too often, we're thinking about the past, we're thinking about the future and we're not present in the moment. What if doing a PhD was the wrong choice? Will I actually be able to finish it? And what happens afterwards?

Not only once did I found myself wallowing in thoughts like these. And here I am, sitting at my desk, writing the acknowledgements for my PhD thesis. I still cannot fully believe it. I am proud of myself and I am overly thankful. Thankful for all the people who were there for me, the whole way and beyond. Actually, I planned to spend nine months hiking the Te Araora in New Zealand but an eye infection made me to change plans. Therefore, I applied for a PhD position at Lars' and although I wasn't his first choice for this position, I felt welcome from the very first day. So, I want to thank Lars for this opportunity and for going the way with me. And honestly, it wasn't always easy for both of us. He and his demands challenged me repeatedly and I questioned myself many times. At the same time, I may have challenged Lars as well; by telling him what I was thinking and feeling. But in the end, we made it - together. A second thank you goes to the special people of my team with whom I did not only share work, but lots of laughter and some great nights out. Thank you Lisa K. and Mario! Thank you Lisa W., Lisa D. and Nadine for becoming friends and for helping me to stand up for myself. A big thank you goes to the one and only Felix, who – no matter how much work he had to do himself – always gave me a helping hand in my analyses. Anna, a special thank you goes to you, for being there when I was really down, for sharing so many laughs, frozen yoghurts and good food with me! And finally, there are Conny and Gundi, to whom I maybe have to give the biggest thank you of all. I learned so much from you, not only about work but about myself. Thank you for being just the way you are! Last but not least, I want to take the opportunity to say thank you to my two sisters Annika and Antonia. Thank you for challenging me over and over again but always loving me. Thank you for being my family, for giving me a home, no matter where I am. And finally, I must thank my mother. For making me the person I am. I wouldn't be where I am in my life if it wasn't for her and her strength. Her unbreakable strength.

"Education is an admirable thing, but it is well to remember from time to time that nothing that is worth knowing can be taught."

Oscar Wilde

In Gedenken an Mama und Papa

„Entspanne dich. Lass das Steuer los. Trudle durch die Welt. Sie ist so schön: gib dich ihr hin, und sie wird sich dir geben.“

Kurt Tucholsky

Summary

Under normal circumstances, the confrontation of a threatening situation or stimulus leads us to experience fear. To learn from those experiences, and adapt our behavior in upcoming situations when experiencing similar threats is important to protect our organism. Importantly, some people are not able to restrict their fear in future situations, but show an exaggerated fear also to actual safe conditions. This phenomenon is called fear overgeneralization and is thought to be an important contributor to anxiety and stress-related disorders. Thus, to investigate factors that may drive fear overgeneralization is of great relevance. We conducted four studies of which three directly examined possible contributing factors (time, stress and the major stress modulators cortisol and noradrenaline) to fear generalization in healthy participants using a two-day fear generalization paradigm. Taken together, we found that fear generalization increased over time without a change in the underlying neural mechanisms. However, we did not reveal any detrimental effects of stress on fear generalization, i.e. there was no fear overgeneralization due to stress. In contrast, it seems that an increase in noradrenergic arousal retains fear memory expression in more detail, thereby promoting an adequate level of fear generalization. Since the ability to predict a threat before it occurs, which is based on prior learning experiences, plays an important role in fear generalization, we conducted a fourth study. In this study, we investigated the influence of stress on attention during predictive fear learning using an aversive version of a blocking paradigm. Results suggest that stress impairs the ability to show a preferential attentional processing of stimuli, which are predictive of a forthcoming threat, when being confronted with the concurrent processing of multiple stimuli.

Altogether, this thesis adds valuable information to the role of stress and time in fear learning, especially fear generalization. Whereas over time there seems to be an increase in fear generalization, stress does not have an additional impact, when fear learning is restricted to simple cue conditioning. However, our last study showed that stress influences attentional processing when multiple stimuli have to be processed at the same time. Therefore, I suggest that future studies should examine attentional processes in fear learning paradigms that require a simultaneous processing of multiple stimuli. This would resemble real world situations more closely and might help us to understand the development of anxiety and stress-related disorders.

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List of Abbreviations

ACC	Anterior cingulate cortex
aI	Anterior Insula
ANS	Autonomic nervous system
AR	Adrenoceptor
BLA	Basolateral amygdala
C	Control
Cort	Hydrocortisone
CR	Conditioned response
CS	Conditioned stimulus
dmPFC	Dorsomedial prefrontal cortex
EEG	Electroencephalography
ERP	Event-related potential
fMRI	Functional magnetic resonance imaging
GC	Glucocorticoid
GR	Glucocorticoid receptor
GS	Generalization stimulus
HPA	Hypothalamic-pituitary-adrenal
IU	Intolerance of uncertainty
LPP	Late positive potential
mPFC	Medial prefrontal cortex
MR	Mineralocorticoid receptor
NS	Neutral stimulus
PFC	Prefrontal cortex
PIT	Pavlovian-to-instrumental transfer
Plac	Placebo
Prop	Propranolol
PTSD	Post-traumatic stress disorder
S	Stress
SCR	Skin conductance response

SPN	Stimulus-preceding negativity
TSST	Trier Social Stress Test
UR	Unconditioned response
US	Unconditioned stimulus
vmPFC	Ventromedial prefrontal cortex
Yoh	Yohimbine

1. General introduction

> *"Man braucht vor niemand Angst zu haben. Wenn man jemanden fürchtet, dann kommt es daher, daß man diesem Jemand Macht über sich eingeräumt hat."* – *Hermann Hesse, Demian*

"You should never be afraid of people... such fear can destroy us completely. You've simply got to get rid of it, if you want to turn into someone decent. You understand that, don't you?" A quotation from Demian (1919) by the German author Hermann Hesse. Although this novel is more about the struggle of finding one's own true self, the above mentioned quote caught my attention when reading this novel and nicely illustrates the importance of the emotion of fear. Not only is fear one of a few unlearned, universal, and strong emotions that can have significant influences on our behavior and our thinking (Ekman, 1992), fear-related disorders are also among the most prominent psychological disorders (Kessler et al., 2005). The investigation of the underlying cognitive and general psychological mechanisms is therefore of great importance to prevent the development of these disorders and contribute to the development of treatment options.

1.1. Basic fear memory processes

Through associative learning processes, fear can be acquired for potentially physically and emotionally dangerous stimuli or situations. This is an important and adaptive mechanism that may help us to avoid possible harm in future situations. However, some people show persistent and inflexible responses to threatening stimuli or situations, which can lead to maladaptive behavior, which is thought to be a strong contributor to the development and maintenance of anxiety and stress-related disorders (Lissek et al., 2005).

1.1.1. Acquisition, retrieval, extinction and the return of fear

To investigate and understand which factors might modulate associative fear learning processes (on a behavioral and neural level) and play a significant role for fear-related disorders, fear conditioning procedures have been used in the laboratory (for a review see Lonsdorf et al., 2017; Mineka & Zinbarg, 2006). These fear conditioning procedures are based on the well-known classical conditioning model of Pavlov (Pavlov, 1927). The

main idea of pavlovian fear conditioning is that via associative learning, a previous neutral stimulus can elicit a fear response. For example, an experience in which there is an experience of pain represents an unconditioned stimulus (US) that naturally triggers an unconditioned response (UR), in this case fear. Now, imagine a person is walking home through a park and a tall man with a beard is approaching this person. Because there is no prior experience or association with this man, he can be seen as a neutral stimulus (NS). However, when he passes the person, he suddenly pushes him or her and steals the bag. Now, the person may associate a tall man wearing a beard with a threatening situation and the former neutral stimulus turns into a conditioned stimulus (CS). A later confrontation with fear conditioned cues, in this case a tall man wearing a beard, can result in a retrieval of the fear memory. As such, the next time the person encounters a tall man with a beard on a street, a feeling of fear might emerge again, i.e. the CS leads to a conditioned response (CR). However, when there is a repeated encounter of various tall men with beards without experiencing a negative or an aversive outcome, the fear response (CR) declines over time, i.e. extinction learning to the CS takes place. Central to the process of extinction learning is inhibitory learning, which involves learning that the CS does not signal danger anymore (Bouton, 1993; Craske, Treanor, Conway, Zbozinek, & Vervliet, 2014). Importantly, it is thought that extinction learning represents new learning rather than the erasure of an established fear memory (Kim & Jung, 2006; Myers & Davis, 2007). This is best demonstrated by experiments showing a return of fear, i.e. the reoccurring of the CR after successful extinction training (Myers & Davis, 2002). Using laboratory experiments, it is possible to measure which of the two memory traces, i.e. original fear memory or extinction memory, is dominant after fear acquisition and extinction training took place. If the CR is rather low or even absent, the extinction memory is dominant. In contrast, when the CR is strong, the original fear memory is assumed to be dominant (Lonsdorf et al., 2017).

In the past decades, a lot of research has been conducted to elucidate the underlying neural mechanisms of the different fear learning processes (Figure 1), revealing an important role for the amygdala that seems to be a highly relevant structure in the acquisition, storage and expression of conditioned fear (Kim & Jung, 2006; LeDoux, 2000). Moreover, it is thought that it plays a particular role in tracking CS-US contingencies over time and thus modulating associating changes (Büchel & Dolan, 2000). In addition to the amygdala, there are other brain regions that contribute to processes important for fear

learning. The storage of contextual fear memory information, for example, has mainly been linked to the hippocampus (Maren, Phan, & Liberzon, 2013). Furthermore, the expression of the conditioned fear has been related to the anterior cingulate cortex (ACC) and the insula (Fullana et al., 2016; Milad & Quirk, 2012). In regard to extinction learning, the amygdala and the hippocampus are found to contribute to processes similar to those during fear memory acquisition, i.e. tracking contingencies and storing contextual information (Knight, Smith, Cheng, Stein, & Helmstetter, 2004). In addition, studies suggest that the ventromedial prefrontal cortex (vmPFC) inhibits amygdala activation during extinction learning, resulting in an inhibition of the old US-CS association (Kim & Jung, 2006; Milad & Quirk, 2002).

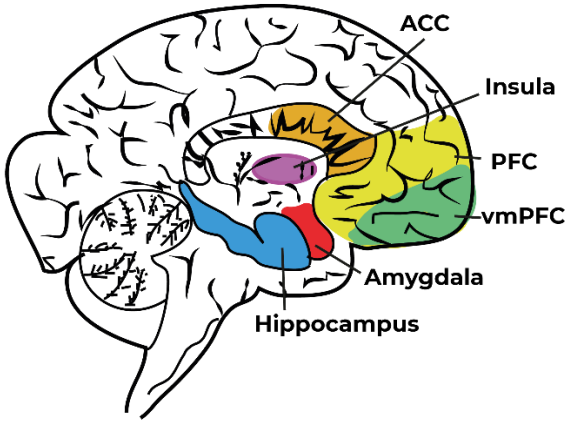

Figure 1. Schematic structure of the human brain, depicting regions relevant for fear learning.

1.1.2. Different fear conditioning procedures

Cue versus context conditioning

Regarding the above described example, this is an example of cued conditioning. The fear was associated with the cue, i.e. the tall man. However, one could also learn to associate the context, in this case the park, with fear. That would be an example of context conditioning. One speaks of cue conditioning if a specific cue is the best predictor of a forthcoming threat and of context conditioning if a specific CS and US are not paired, i.e. the context alone is the best predictor (Rescorla & Wagner, 1972). Context conditioning can arise in any context in which a US appears and it is not always easy to clearly differentiate cue from context conditioning (Lonsdorf et al., 2017). One differentiation is made in the level of predictability. If an aversive event is signaled or can be predicted by a certain cue, it is a matter of cue conditioning, while contextual conditioning develops

rather as a response to unpredictable danger (Grillon, 2002). Deficits in cued fear conditioning are thought to lead to the development of non-adaptive expectancies of aversive outcomes and an attentional bias toward generalized threat. Consequently, the organism would not be able to predict danger or safety reliably and finds itself excessively in a state of anxiety (Grillon, 2002). On a neural level, the amygdala was shown to be important for cue as well as context conditioning whereas the hippocampus has been mainly been linked to successful context conditioning (Phillips & LeDoux, 1992).

Delay versus trace conditioning

Another differentiation in fear conditioning can be made regarding the temporal relationship between the CS and the US. When the CS is present for a fixed period (delay) before the US appears and the CS and the US co-terminate, it is termed delay conditioning. Trace conditioning in contrast requires the organism to keep the CS in mind because the occurrence of the CS and the US are separated in time by a "trace" interval, i.e. an interval of time laps between the CS termination and the onset of the US (Knight, Cheng, Smith, Stein, & Helmstetter, 2004). Thus, the given example represents delay conditioning because the threatening situation (US) occurs with the tall man (CS) still being present. Studies revealed that similar brain regions are involved in both procedures, but ascribed an important role to the hippocampus in trace conditioning, as it seems to be necessary to build the memory trace for the CS (Bangasser, Waxler, Santollo, & Shors, 2006; Knight, Cheng, et al., 2004). A review of multiple patient studies furthermore suggests that fear learning can be successful on a subconscious level without cortical processing of the stimulus, which is supported by a direct thalamus-amygdala connection (Knight, Cheng, et al., 2004). This seems to be only true for delay fear conditioning, since trace conditioning requires cognitive processing of the CS-US contingency supported by the hippocampus. Furthermore, a differentiated skin conductance response (SCR), a physiological marker of arousal, was regarded as an index of contingency awareness (Hamm & Weike, 2005).

1.2. The generalization of fear

Although fear can quickly be acquired (Ohman, Erikkson, & Olofsson, 1975), threatening stimuli or situations rarely occur in the exact same manner again. Therefore, it is of great advantage to learn from past experiences and to generalize the acquired fear to similar

situations that may be threatening, simply better safe than sorry (Dunsmoor & Paz, 2015). Coming back to the example, it might be beneficial to learn from the threatening experience with the tall man in the park and to adapt the behavior, e.g. by learning self-defense or avoiding situations in which one might be alone with strangers. This is an example of adaptive fear generalization. However, imagine one does not only start to avoid situations similar to the one described but avoid being alone anywhere, or being afraid of all men with beards. This would be an example of fear overgeneralization, i.e. an exaggerated fear generalization. This would represent a maladaptive behavior, because it lets someone fear situations that are not actually threatening and affects the behavior in an overcautious manner. Importantly, the overgeneralization of fear is thought to contribute to the development and maintenance of anxiety and stress-related disorders (Dunsmoor & Paz, 2015; Lissek, 2012). To systematically investigate the strength of fear generalization, standardized paradigms and a formal analysis of fear generalization is been suggested (Dunsmoor, Prince, Murty, Kragel, & LaBar, 2011; Lissek et al., 2008). These fear generalization paradigms mostly incorporate a fear conditioning phase, in which an unpleasant US is paired repeatedly with one stimulus, thereby signaling danger (CS+), whereas another stimulus is never paired with the US, thereby signaling safety (CS-). In a subsequent test phase, similar but not identical stimuli, so-called generalization stimuli (GS), are shown. As a result, a fear generalization gradient is obtained. Normally, this gradient shows the highest responses to the original CS+ and more fear to GSs that closely resemble the CS+. With increasing perceptual distance, responses to the GS decrease, with the lowest response to the CS- (Dunsmoor, Kroes, Braren, & Phelps, 2017; Dunsmoor & Murphy, 2014; Dunsmoor, Otto, & Phelps, 2017; Lissek et al., 2008; Lissek, Bradford, et al., 2014; Lissek, Kaczkurkin, et al., 2014; Lissek et al., 2010; Morey et al., 2015; Morey et al., 2020; Shepard, 1987). Given that anxiety disorders are among the most common mental disorders (Kessler et al., 2005; Wittchen & Jacobi, 2005), it is of great relevance to identify factors that may promote the overgeneralization of fear. The next chapter gives an overview of possible modeling factors, the resulting models of fear generalization and its underlying neural mechanisms in more detail.

1.2.1. Models of fear generalization and neural mechanisms

Previous studies that investigated different possible modulating factors found that the extent of fear generalization can be influenced by the intensity of threat (Dunsmoor,

Kroes, et al., 2017), verbal instructions (Vervliet, Kindt, Vansteenwegen, & Hermans, 2010) and stimulus similarity and perception (Struyf, Zaman, Hermans, & Vervliet, 2017; Struyf, Zaman, Vervliet, & Van Diest, 2015; Zaman, Ceulemans, Hermans, & Beckers, 2019). These latter findings are particularly in line with the perceptual model of fear generalization, which assumes that fear generalization is the result of the comparison between the stored memory representation of the CS+ and a presented stimulus (Lissek, 2012; Lissek, Bradford, et al., 2014). Here, a critical role is ascribed to the hippocampus mediated process of pattern separation, i.e. representations that are similar but not identical are correctly stored in distinct, non-overlapping memory traces (Yassa & Stark, 2011). Based on the perceptual model, the extent of fear generalization depends on the perceptual similarity between the CS+ and the GS: the greater the perceptual similarity, the stronger the overlap of the hippocampal representations. As a result, this overlap increases the aversive processing in other brain areas, which are responsible for the production of the fear response (Lissek, Bradford, et al., 2014). It is assumed that, predominantly activation in the bilateral anterior insula (aI), the dorsomedial PFC (dmPFC), and the bilateral inferior parietal lobe form a positive fear generalization gradient (Figure 2).

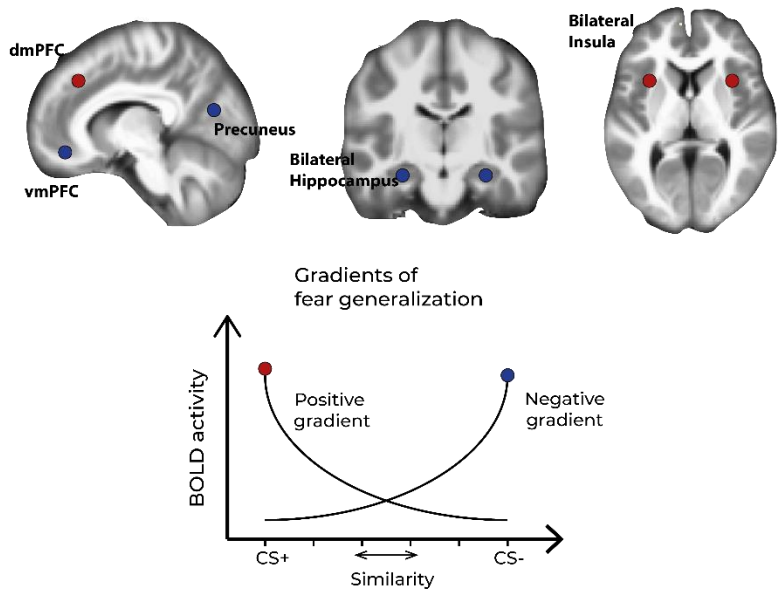

Figure 2. Fear generalization gradient. Brain areas marked with a red dot are thought to show enhanced activation towards stimuli similar to the CS+. Brain areas marked with a blue dot are assumed to show decreasing activation with increasing dissimilarity with the CS+. Thus, those areas form a positive or negative fear generalization gradient, when stimuli decrease in their similarity to the CS+. Note. Adapted from Dunsmoor, J.E., & Paz, R. (2015).

Hence, those areas reflect fear excitation. Conversely, a decrease in perceptual similarity leads to the activation of the bilateral ventral hippocampus, the vmPFC and the precuneus cortex. Those areas show inclining activation as the stimuli differentiated from the CS+, thus forming a negative fear generalization gradient (Figure 2; Dunsmoor et al., 2011; Greenberg, Carlson, Cha, Hajcak, & Mujica-Parodi, 2013a; Lissek, Bradford, et al., 2014). In particular, the model assumes that the ventromedial prefrontal activation is responsible for the inhibition of the fear response (Greenberg et al., 2013a; Lissek, Bradford, et al., 2014; Lopresto, Schipper, & Homberg, 2016). Thus, it can be assumed that, on a neural level, an appropriate degree of fear generalization requires an intricate balance of excitatory and inhibitory mechanisms of different brain regions.

Although perception seems to be important in the process of fear generalization, there is good evidence to show that an increased generalization of fear is not just the result of perceptual similarity or a mere failure in stimulus discrimination. This was shown for example by category-based fear conditioning studies, in which fear is conditioned to a class of stimuli rather than to a specific CS (Dunsmoor & Murphy, 2015). Here, studies showed that also the conception of the stimulus (i.e. a deeper/cognitive understanding of the stimulus), in addition to mere perception (Bennett, Vervoort, Boddez, Hermans, & Baeyens, 2015; Dunsmoor, Martin, & LaBar, 2012), as well as the stimulus typicality, i.e. how familiar the stimulus is (Dunsmoor & Murphy, 2014), can lead to altered fear generalization. In addition, ambiguous stimuli, which are located halfway between a CS+ and a CS-, thereby representing both threat and safety to a certain extent induced uncertainty (Onat & Büchel, 2015). As a consequence, one might speculate that uncertainty about the outcome promotes anxious behavior (Grupe & Nitschke, 2011), and contributes to the generalization of fear (Tsetsenis, Ma, Lo Iacono, Beck, & Gross, 2007). Together, these findings show that fear generalization can also be influenced by higher-order cognitive processes (Dunsmoor & Murphy, 2015), thereby challenging the perceptual model of fear generalization. Neural evidence underpinned these behavioral findings (Onat & Büchel, 2015). The authors explicitly wanted to test the prediction that certain brain areas can show fear-tuning independent from mere perception. They replicated previous findings and found similar fear-related brain regions, such as the posterior and anterior cingulate cortices, the aI, the hippocampus, and the vmPFC (Onat & Büchel, 2015). Interestingly, their results additionally demonstrated a hypersharp fear-tuning in the insula. The fear generalization gradient obtained from the insula was steeper

compared to that one from the behavioral responses, indicative of less fear generalization. In addition, the ventral inferotemporal cortex differentiated the intermediate faces from both the CS+ and the CS-, hence reflecting a rather ambiguity-based uncertainty tuning. Based on these findings, the authors support the assumption that fear generalization is not just a passively driven process by perception, but rather an active process that integrates threat identification and ambiguity-based uncertainty, ensuring an adaptive degree of fear generalization.

Further support for this theory can be seen in studies investigating the influence of intolerance of uncertainty (IU), being "an individual's dispositional incapacity to endure the aversive response triggered by the perceived absence of salient, key or sufficient information, and sustained by the associated perception of uncertainty" (Carleton, 2016, p. 31). One study that investigated the electrophysiological correlates of fear generalization could show that participants with a high IU show decreased neural attention and processing of the GS, compared to participants with a low IU (Nelson, Weinberg, Pawluk, Gawlowska, & Proudfit, 2015). Additionally, another study showed that higher IU is associated with an increased generalization from threat to safety during fear acquisition, whereas fear extinction learning was delayed (Morriss, Macdonald, & van Reekum, 2016).

Together, these results suggest that the extent of fear generalization is not solely determined by the perceptional similarity between the stimuli, but that other factors, such as uncertainty, have to be taken into account as well. Since it is beyond the scope of the current work to cover all possible contributing factors, the next section briefly discusses those possible modulating factors that were explicitly investigated in the subsequently presented studies.

1.2.2. Modulating factors: time, attention and prediction

Time

In everyday life, there is often a delay between the original situation in which a threatening stimulus was encountered and a situation in which fear generalization might occur. Time plays a crucial role in memory processes, because over time, memories naturally undergo change from a detailed to a more gist-like representation, losing precision and strength (Dandolo & Schwabe, 2018; Jasnow, Cullen, & Riccio, 2012; Winocur, Moscovitch, & Sekeres, 2007). While in the beginning, memories are specifically

stored in the hippocampus, they become more independent of the hippocampus over time and are stored in the neocortex (Nadel & Moscovitch, 1997; Winocur & Moscovitch, 2011; Winocur, Moscovitch, & Bontempi, 2010). First evidence in animals and humans suggests that this memory transformation, i.e. from a detailed to a more gist-like memory due to an increase in time, might be an important factor in the process of enhanced fear generalization (Andreatta, Genheimer, Wieser, & Pauli, 2020; Dunsmoor, Otto, et al., 2017; Pollack et al., 2018; Wiltgen et al., 2010). As such, time seems to be a crucial factor in the process of fear memory generalization, since memories naturally lose precision and strength. Furthermore, first evidence in animals underlines the importance of brain regions such as the hippocampus, the amygdala, the mPFC and the insula (Pollack et al., 2018; Wiltgen et al., 2010), all of which are important for the process of fear generalization (Dunsmoor & Paz, 2015; Lissek, Bradford, et al., 2014). However, equivalent studies that investigate the influence of time on fear generalization in humans on a neural level have yet to be carried out.

Attention and prediction

Situations are often characterized by a vast complexity, consisting of multiple concurrent visual, auditory and olfactory cues. However, we only have limited resources to perceive and process all the different information, which is why we need to select those stimuli that are most relevant for our behavior (Sternberg, Sternberg, & Mio, 2012). Subsequently, the attended stimuli will be encoded and remembered. Regarding threatening stimuli, findings suggest that attention is preferentially directed towards fear-relevant compared to fear-irrelevant stimuli (Ohman, Flykt, & Esteves, 2001) and a growing body of evidence confirms that this attentional bias also interacts with fear learning (Oehlberg & Mineka, 2011). Associative learning theories suggest that learning is preferentially driven by the predictive relationship between the stimuli. This means that attention increases when there is a discrepancy between the expected and the actual outcome and learning therefore occurs (Mackintosh, 1975; Pearce & Hall, 1980; Rescorla & Wagner, 1972). The importance of prediction for learning can be demonstrated by the well-established blocking phenomenon (Kamin, 1968). Very briefly, to investigate blocking, a stimulus (CSA) is paired with an US, becoming a CSA+. Next the CSA+ is presented together with a new stimulus (CSX) and again followed by the US, resulting in the compound stimulus CSAX+. However, learning to CSX in this stage should be strongly

reduced or blocked because the CSA+ is already a reliable predictor for the US. The CSX carries no new information, it is redundant (for a full description of the paradigm see 2.5.2 and Figure 7).

Guidance or allocation of attention is best investigated with the help of eye-tracking on a behavioral level and electroencephalography (EEG) on a neural level (Luck, Woodman, & Vogel, 2000). Using a blocking paradigm, studies revealed less attention towards the blocked compared to the non-blocked stimulus on a behavioral as well as on a neural level (Beesley & Le Pelley, 2011; Eippert, Gamer, & Büchel, 2012; Kruschke, Kappenman, & Hetrick, 2005; Le Pelley, Beesley, & Griffiths, 2014; Wills, Lavric, Croft, & Hodgson, 2007). This effect was accompanied by decreased activity in the amygdala to the blocked versus the non-blocked stimulus. Moreover, in contrast to the dorsolateral PFC, which was active at both times when a stimulus was established as predictive or non-predictive, the vmPFC was specifically active when a stimulus was established as non-predictive (Eippert et al., 2012). According to the authors, these findings are well in agreement with the vmPFC's assumed regulatory role in fear conditioning, i.e. representing inhibitory learning processes (Phelps, Delgado, Nearing, & LeDoux, 2004).

Generally, there is good evidence that indicates that attentional processes and predictability play a great role in fear learning processes, including fear generalization, mediated especially by the amygdala and the vmPFC (Greenberg et al., 2013a; Laufer, Israeli, & Paz, 2016; Lissek, Bradford, et al., 2014; Rajbhandari, Zhu, Adling, Fanselow, & Waschek, 2016). However, there is still an ongoing debate on which factors may promote attentional biases towards threatening stimuli, resulting in fear overgeneralization (Baker et al., 2019; Dennis-Tiwary, Roy, Denefrio, & Myruski, 2019). On a neural level, it might be especially useful to investigate event-related potentials (ERPs) that reflect different parts of attentional processing. To investigate fast attentional re-allocation towards relevant information, a rather early ERP such as the N2pc can be used (Eimer, 1996), whereas the P3b and the late positive potential (LPP) are more suitable to study sustained emotional processing of relevant stimuli (Mangun, 1990; Polich, 2007; Schupp, Flaisch, Stockburger, & Junghöfer, 2006). To rather investigate anticipatory attention, the analysis of the stimulus-preceding negativity (SPN) is suggested (Böcker, 2001; van Boxtel & Böcker, 2004).

The relevance of prediction as an important factor in fear generalization can be assumed by results of a recently published study in rodents (Jo, Heymann, & Zweifel,

2018). In this study, in one experiment, mice were fear conditioned to a high or a low threat intensity foot shock (US) and in a second experiment probabilistic fear conditioning took place, in which the likelihood of a moderate US to follow the CS+ decreased, while it increased for the CS- proportionally. Results revealed that both, a high intensity US and a highly predictive CS+ increased the probability for fear generalization to the CS-. The mice incorrectly assumed that both the CS+ as well as the CS- are predictive of the US. Moreover, results of in vivo recordings of dopamine (DA) neurons showed that a subset of these neurons encoded the negative valence of the threat (i.e. the threat intensity) as well as the certainty of threat prediction. With the help of optogenetics, enhancement of DA neurons activity during fear conditioning to either one of the cues (high intensity threat cues or uncertain threats) could prevent subsequent fear generalization. This resulted in the conclusion that a subset of DA neurons may encode the certainty of threat prediction and inform and update fear memory. Interestingly, Jo et al. (2018) proposed that repeated US presentation during their study may have resulted in elevated stress levels, which suppresses activity in DA neurons, impairs learning and thereby may have resulted in enhanced fear generalization. This interpretation is especially interesting, given the assumption, that stress plays a fundamental role in fear-related and anxiety disorders such as post-traumatic stress disorder (PTSD; de Quervain, Schwabe, & Roozendaal, 2017; Grillon, Duncko, Covington, Kopperman, & Kling, 2007; Pitman et al., 2012; Shin & Liberzon, 2010; Yehuda, Giller, Southwick, Lowy, & Mason, 1991). In addition, one common characteristic of these disorders is the overgeneralization of fear (Dunsmoor & Paz, 2015; Kaczkurkin et al., 2017; Lissek, 2012). Based on this potentially important link of fear generalization and stress, the following paragraphs will put an emphasis on the stress response and how it may relate to the process of fear generalization.

1.3. Stress response

Across lifespan, people are faced with numerous stressful experiences. Even if it is a very stressful, yet a traumatic experience, many people manage to deal with those situations in a good and healthy manner. However, some develop a PTSD, characterized by an overgeneralization of fear (American Psychiatric Association, 2013; Lissek et al., 2008; Yehuda, 2002). This raises the question of the underlying differences and causes that lead to PTSD in some but not in others. On a biological level, studies showed alterations in the

stress response system between people with and without PTSD, pointing to an important role of cortisol and noradrenaline (Schumacher et al., 2019; Southwick et al., 1999; Southwick et al., 1993; Yehuda et al., 1991). Before going into detail of how acute stress might interact with fear (over)generalization, the main parts of the stress response will be described in the following section to pave the way for a mechanistic understanding.

1.3.1. The autonomic nervous system and the hypothalamic-pituitary-adrenal axis

The body's stress response aims at promoting a behavior, which optimally deals with a potential threat. To this end, the physiological response constitutes mainly two interacting systems: the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA) axis (Figure 3; Ulrich-Lai & Herman, 2009).

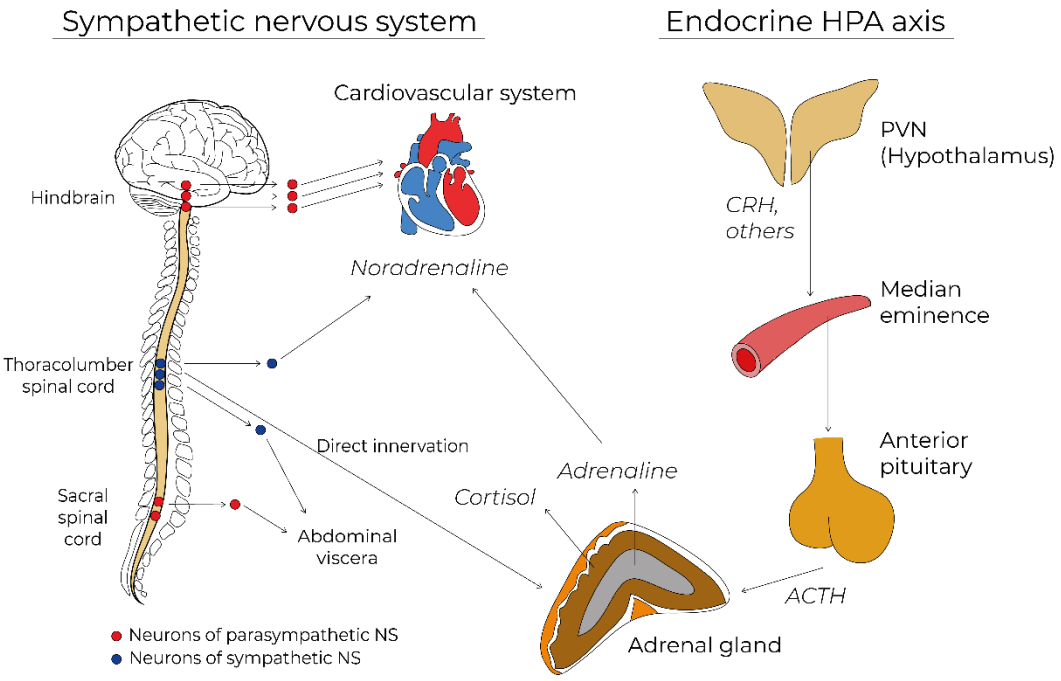

Figure 3. Autonomic nervous system (ANS) and hypothalamic-pituitary-adrenal (HPA) axes activation during stress. Acute stress activates sympathetic neurons in the spinal cord (blue dots). Ultimately, this results in an increase in levels of adrenaline (mainly from the adrenal medulla), noradrenaline (mainly from sympathetic nerves), heart rate and vasoconstriction and energy mobilization. The parasympathetic system (red dots) lead to actions, which are generally opposite to those of the sympathetic system. In regard to the HPA axis, stress exposure results in the release of hormones, such as corticotropin releasing hormone (CRH) from neurons in the paraventricular nucleus (PVN) of the hypothalamus into the median eminence. These hormones in turn act on the anterior pituitary to promote the secretion of adrenocorticotropic hormone (ACTH), which finally acts on the adrenal cortex to initiate the synthesis and release of cortisol. Note. Adapted from Ulrich-Lai and Herman, 2009.

The ANS constitutes of the sympathetic and parasympathetic activation and provides the most rapid stress response. The sympathetic activation leads to an immediate release of

catecholamines, especially adrenaline and noradrenaline (NA) from the adrenal medulla, resulting in an increased heart rate and blood pressure, preparing the organism for a 'fight-or-flight' response. Due to the counter regulating parasympathetic activation, the responses are of a short duration, enabling the organism to relax again (Joels & Baram, 2009; Ulrich-Lai & Herman, 2009). Catecholamines are not able to cross the blood-brain-barrier, but can stimulate adrenoceptors (ARs) on the vagus nerve, which afferents terminate in the nucleus tractus solitarius (Williams & Clayton, 2001). As a result, NA is released into the brain and exerts its effects particularly on the amygdala, a region that is known to play a key role in fear learning processes (Büchel & Dolan, 2000; McGaugh, Cahill, & Roozendaal, 1996; Phelps et al., 2001).

The HPA-axis activation kicks in later and is rather slow compared to the noradrenergic response. As a result of this second response, glucocorticoids (GCs) are released from the adrenal cortex, reaching their peak levels at about 20 minutes after onset of the perceived stressful experience (Dickerson & Kemeny, 2004; Kirschbaum & Hellhammer, 1994; Kirschbaum, Pirke, & Hellhammer, 1993). Free circulating GCs promote the effects of the catecholamines by mobilization of stored energy (Ulrich-Lai & Herman, 2009). In contrast to the catecholamines, they are able to cross the blood-brain-barrier, where they exert their biological action via two types of nuclear receptors: the high-affinity mineralocorticoid receptors (MRs), which are already activated at low levels of GCs and the low-affinity glucocorticoid receptors (GRs), which become activated when there is a high level of GCs, e.g. after stress (de Kloet, Joels, & Holsboer, 2005; Reul & de Kloet, 1985). Whereas GRs can be found ubiquitously in the brain, MRs are predominantly expressed in limbic regions, including the lateral septum, the central amygdala and the hippocampus, which is involved in processing emotion and memory (Joels & Baram, 2009; Ulrich-Lai & Herman, 2009). Importantly, in addition to the intracellular MRs and GRs that act in the cell nucleus as transcription factors, membrane-bound MRs were discovered, mediating fast non-genomic actions, having a lower affinity for GCs (Karst et al., 2005). The distinct affinities of the different receptor types enables the organism to adaptively react to stress and facilitates cognitive adaption under stress (Joels, Karst, DeRijk, & de Kloet, 2008). Fast activation of membrane-bound MRs boost initial stress reactions, drives appraisal of the situation and supports adaptation of the best behavioral coping strategy. This is counteracted by the slower GR-mediated activation, thereby preventing an overshooting of the initial stress response and restoring homeostasis (Joels et al., 2008).

Because of the receptors' distribution in the brain and their overlap of brain regions important for memory and learning, much research was conducted to investigate their role in modulating memory processes, underpinning the importance of GCs (Diamond, Campbell, Park, Halonen, & Zoladz, 2007; Roozendaal, 2002; Roozendaal, Okuda, de Quervain, & McGaugh, 2006; Sandi & Pinelo-Nava, 2007). It is suggested, for example that under stress, activation of membrane-bound MRs induces a rapid shift from resource-demanding 'cognitive' systems, supported mainly by the hippocampus and the PFC, to less-demanding 'habit' systems, depending particularly on the amygdala and the dorsal striatum (Vogel, Fernandez, Joels, & Schwabe, 2016). This leads to improved memory of the stressful event (Joels, Fernandez, & Roozendaal, 2011) and promotes the fast recruitment of routines, enabling the organism to respond rapidly and in a resource saving manner in a challenging situation (Schwabe & Wolf, 2013). To study the influence of stress on learning and memory in humans, different paradigms have been established (Giles, Mahoney, Brunye, Taylor, & Kanarek, 2014). Since one of them was used repeatedly in our studies, to investigate the influence of acute stress on processes such as fear generalization and attention, it will be explained in more detail in the following section.

1.3.2. Psychological stress induction in the laboratory

The Trier Social Stress Test (TSST; Kirschbaum et al., 1993), a standardized stress-induction protocol for humans, has proven to be very successful in increasing subjective stress levels, and activating both the ANS and the HPA-axis (Kirschbaum et al., 1993). The task consists of a simulated job interview and a rather difficult mental-arithmetic task. For part of the job interview, participants are asked to prepare an application speech for a job tailored to their interests within three minutes. Then, a five minute free speech follows, in which they have to indicate why they are the ideal candidate for this job, followed by the five minute mental-arithmetic task, in which participants have to count backwards from 2043 in steps of 17. Throughout both tasks, participants are standing in front of a panel of two experimenters (one man and one woman), dressed in white lab coats. They are introduced as experts in behavioral analysis to evaluate participants' performance and act in a rather reserved and non-reinforcing manner. In addition, participants are videotaped and see themselves on a screen, placed behind the panel throughout the whole procedure.

To show that this manipulation successfully induces stress, there is a non-stressful control condition, matched for the key characteristics of this task. In this case, participants have three minutes to prepare a five minute speech on a topic of their choice, which is followed by a five minute simple arithmetic task, i.e. counting forward from zero in steps of 15. They are neither evaluated by a committee, nor videotaped, but stand alone in a room during task completion.

Measurements of subjective stress ratings, blood pressure, heart rate as well as saliva samples for subsequent cortisol analysis are taken at several time points before, during and after the experimental manipulations to verify stress induction by the TSST on subjective and physiological level.

1.3.3. The influence of acute stress on cognitive processes

Memory

Stressful events are well-known to modulate learning and memory processes in general (Joels et al., 2011; Schwabe, Joels, Roozendaal, Wolf, & Oitzl, 2012) and fear learning processes, such as fear conditioning and extinction learning in particular (Jackson, Payne, Nadel, & Jacobs, 2006; Merz, Elzinga, & Schwabe, 2016; Raio, Brignoni-Perez, Goldman, & Phelps, 2014; Raio & Phelps, 2015; Simon-Kutscher, Wanke, Hiller, & Schwabe, 2019). Some studies suggest that NA and GCs act synergistically to influence learning and memory (Joels et al., 2011; Krugers, Karst, & Joels, 2012; Roozendaal et al., 2006; Schwabe, Joels, et al., 2012). As such, it is assumed that NA is an important mediator for the effects of stress to develop since studies showed that impairing effects of stress or GCs on memory retrieval can be prevented by a pharmacological reduction of noradrenergic activity (de Quervain, Aerni, & Roozendaal, 2007; Schwabe et al., 2009). However, there is also evidence for distinct roles of the individual stress mediators. The administration of GCs shortly before memory retrieval can lead to an impairment in memory retrieval (de Quervain, Nitsch, Hock, McGaugh, & Roozendaal, 2000; de Quervain, Roozendaal, & McGaugh, 1998). At the same time, acute administration of propranolol, a β-adrenergic antagonist that inhibits noradrenergic arousal but does not alter levels of GCs, also leads to impaired contextual memory retrieval (Murchison et al., 2004). Results suggest that this is particularly true for acute memory retrieval, when memory recall is still mainly dependent on hippocampal activation (Murchison et al., 2004; Schönfeld, Ackermann, & Schwabe, 2014). In addition, another study showed, that the release of NA into the

basolateral amygdala (BLA) during learning interferes with the process of systems consolidation (i.e. the memory transformation from the hippocampus to neocortical areas to promote long-term memory; Squire & Alvarez, 1995) by maintaining hippocampal involvement during memory recall, resulting in a maintenance of detailed remote memory (Atucha et al., 2017).

Taken together, study results clearly exhibit that acute stress influences processes of fear learning, promoted by either interfering with fear consolidation or by impairing fear memory recall. This might be due to an imbalance in the release of GCs and noradrenergic activation. If noradrenergic activity would be prolonged or intensified, it might be possible to counteract the impairing stress effects.

Fear Generalization

As pointed out, stress hormones act on prefrontal and medial-temporal brain sites that have also been found to be important for the process of fear generalization (Greenberg et al., 2013a; Lissek, Bradford, et al., 2014; Lopresto et al., 2016; Onat & Büchel, 2015). Further evidence in rodents and humans supports the assumption of a direct impact of stress on fear generalization (Bender, Otamendi, Calfa, & Molina, 2018; Dunsmoor, Otto, et al., 2017; Kaouane et al., 2012; Kolodziejczyk & Fendt, 2020). In mice, injections of corticosterone into the hippocampus immediately after contextual fear conditioning through a high intensity shock, resulted in fear generalization to a tone that was not a predictor of the shock. Moreover, these results were replicated with a second stressor, i.e. a restraint in a cylinder for 20 min (Kaouane et al., 2012). A more recent study adds some insight into the underlying mechanisms and suggests that GABAergic signaling in the BLA plays an important role in the generalization of contextual fear memories (Bender et al., 2018). In this study, rats were first stressed by immobilization in plastic restrainers under intense light for 60 min. Then, fear conditioning to a context with two unsigned scrambled foot shocks took place. After a minimum of 24 hours, animals were placed into a novel context and tested for fear generalization. Results showed that stressed rats compared to non-stressed rats showed enhanced fear generalization to the novel context. Follow-up experiments showed that a formation of an associative memory of context and foot shock as well as GABAergic signaling within the BLA is necessary to influence fear generalization. This conclusion was based on the results that blockade of GABA-A sites through the competitive antagonist bicuculline in the BLA before fear conditioning was

able to induce fear generalization similar to that produced by stress. At the same time, an increase of inhibition via enhancement of GABA activity prior to the stress experience, reduced the stress effect on fear generalization (Bender et al., 2018).

To best of our knowledge, there is only one study that investigated the effects of acute stress on fear generalization in humans (Dunsmoor, Otto, et al., 2017). In this study, fear conditioning took place on day 1, followed by acute stress and a subsequent test of fear generalization either immediately after fear conditioning on day 1 or 24 hours later on day 2. Results were indicative of enhanced fear generalization due to stress, only when stress was experienced one day after fear conditioning. After a 24 hour delay, stressed participants showed increased autonomic arousal and explicit shock expectancy ratings. The authors conclude that older threat memories are more prone to the effects of stress than recently formed memories, thereby increasing fear generalization. A possible explanation could be the interaction of stress effects with the decline of memory precision over time (Jasnow et al., 2012). On a neural basis, Dunsmoor et al. (2017) suggest that stress impairs hippocampal functioning, which in turn affects the process of pattern separation. At the same time stress could promote neural plasticity in the lateral amygdala. However, results partly contrast with the aforementioned animal studies, in which the drug/stress manipulation was administered before or after fear conditioning as there were no effects of stress when administered directly after fear conditioning. In addition, stress led to an overall enhanced physiological response, which makes it difficult to ascribe the effects specifically to fear generalization.

From a clinical perspective, studies in patients with PTSD also point to a role of stress in fear generalization. It was shown that an exaggerated noradrenergic activation can be associated with some symptoms of PTSD, which in turn can be reduced by β-adrenergic blockade (Southwick et al., 1999). At the same time however, a recently published study in rodents showed, that also a low-dose injection of propranolol induced PTSD-like memory impairments, i.e. fear generalization towards safe cues (Zhu et al., 2018).

Altogether, study results suggest that stress affects the process of fear generalization, whereby the exact circumstances are still to be clarified. All animal studies administered their experimental manipulation before or after fear conditioning and thereby influenced fear consolidation. In addition, most of the experimental studies so far manipulated the HPA-controlled stress response. Having said that, it should be noted that fear conditioning alone can increase the level of cortisol (Kolodziejczyk & Fendt, 2020), which is why we

suggest to investigate a fear acquisition independent influence of stress. Furthermore, the specific role of stress-induced noradrenergic activation has not yet been investigated in a sample of healthy participants. Does fear generalization change if there is only an enhancement of either GCs or noradrenergic arousal? What if there is an experience of stress but noradrenergic arousal is blocked? As can be seen from the depicted research, there are still many open questions regarding the contribution of stress or individual stress mediators on the extent of fear generalization.

Attention

To extract behaviorally relevant information, while ignoring irrelevant details, selective attention is necessary. Hereby, attention can be guided bottom-up, driven by the salience of the stimulus' property or top-down, driven by cognitive control (Buschman & Miller, 2007). Plenty of studies have shown that those attentional mechanisms rely on brain regions that are susceptible to stress and that acute stress modulates attentional processing in favor of the salience network, promoting fast detection of the threat (for a review see Hermans, Henckens, Joels, & Fernandez, 2014; Sanger, Bechtold, Schoofs, Blaszkewicz, & Wascher, 2014). However, to protect the organism, it is beneficial to predict threat in advance, which is why guidance of attention to predictive stimuli is important. On a neural level, increased attention to information with predictive value went along with an enhanced amygdala activation, whereas activation of the vmPFC was particularly enhanced for non-predictive stimuli (Eippert et al., 2012). Interestingly, both regions are thought to play a crucial role in the process of fear generalization (Asok, Kandel, & Rayman, 2019; Greenberg et al., 2013a; Greenberg, Carlson, Cha, Hajcak, & Mujica-Parodi, 2013b; Laufer et al., 2016). Although not directly tested, there is one study that suggests increased fear generalization due to an influence of stress on attentional processes (Jo et al., 2018). This increase in fear generalization was found after high threat intensity and enhanced US predictability. The authors state that due to the nature of threatening stimuli being inherently stressful, a high compared to a low threat intensity would result in a heightened level of stress. As a result, threat is not only correctly predicted based on the CS+ but also incorrectly predicted based on the CS-.

Taken together, there is good evidence to assume that there may be an interaction between stress and attention, especially in aversive predictive learning, which then may effect the degree of fear generalization, since acute stress influences brain areas that are

both relevant for attentional mechanisms and the process of fear generalization. However, to the best of our knowledge, there is no study yet that has explicitly investigated whether stress affects attention in aversive predictive learning.

1.4. Scope and aim

Although various factors have been investigated to understand what may drive fear generalization, there are still multiple factors that have not yet been investigated. How does time influence the process of fear generalization, i.e. what happens if there is a delay between fear acquisition and possible fear generalization? And is this process paralleled by a change in neural representation? Furthermore, what impact does an experience of acute stress have, when it is independent of initial fear learning? Here, it seems important to additionally disentangle the possible influence of the individual stress mediators, i.e. GCs and NA. And finally, can we see an influence of stress on attentional mechanisms during aversive predictive learning? More specifically, does stress impair the recall of previously learned information and consequently shifts the attention away from stimuli predictive for threat to stimuli that are not?

To answer these questions, four independent studies were conducted. Out of the four studies, three studies used the fear generalization paradigm as published by Onat and Büchel (2015), the crucial difference being that fear acquisition and the test of fear generalization were conducted on two separate days. This allowed us to (1) compare immediate versus delayed fear generalization and (2) analyze the influence of stress independent from initial fear acquisition. In all three studies we adopted the same procedure on experimental Day 1, i.e. a baseline and a fear acquisition phase. About 24 hours later, participants underwent a test of fear generalization. Depending on the study and research question, different manipulations preceded the test of fear generalization (Figure 4).

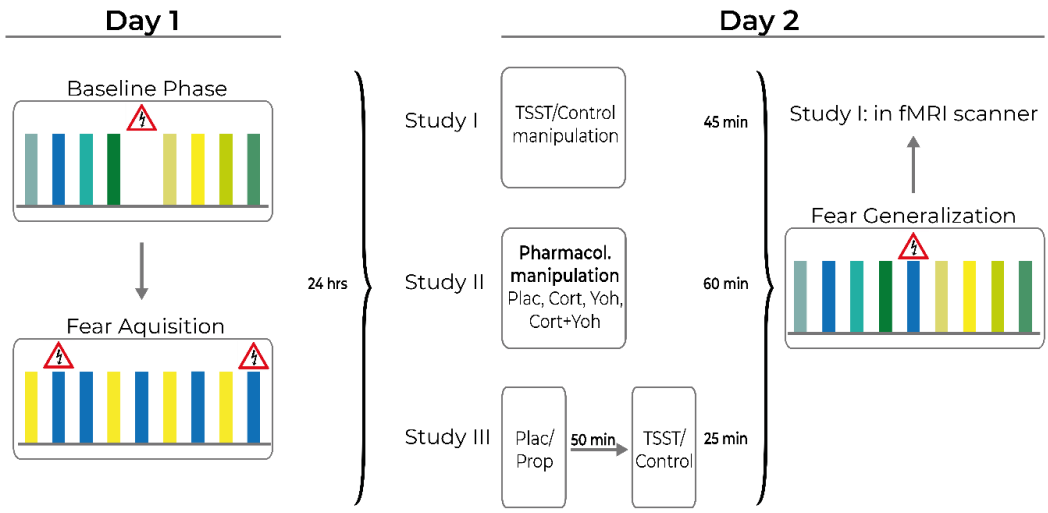

Figure 4. Study procedure of the three different fear generalization experiments. Day 1 was identical across studies. On Day 2, there was a different manipulation before the test of fear generalization, depending on the study. In Study 1, participants were assigned to a stress (TSST) or control manipulation and were later tested for fear generalization inside a fMRI scanner. In Study 2, participants either received a placebo (Plac), hydrocortisone (Cort), yohimbine (Yoh) or both drugs (Cort+Yoh). In Study 3, participants either received a placebo (Plac) or propranolol (Prop), followed by either a stress (TSST) or control manipulation. For Study 2 and 3, test of fear generalization was conducted outside the fMRI scanner.

The first study, Study I, explicitly aimed to shed more light on the neural representation of delayed fear memory generalization and compare a delayed test of fear generalization with an immediate test of fear generalization, i.e. 24 hours vs. immediately after fear acquisition, on a behavioral and neural level. To this end, the test of fear generalization on Day 2 was conducted in a functional magnetic resonance imaging (fMRI) scanner. Moreover, this study should give a first insight into a possible role of stress, which is why half of the participants underwent a stress manipulation before the test of fear generalization. Study II then aimed to disentangle possible effects of the different stress mediators. Therefore, we included four different groups receiving either a placebo, hydrocortisone, yohimbine, an alpha 2-AR antagonist to enhance noradrenergic arousal, or both drugs on Day 2. Participants of Study III were administered either a placebo or propranolol, a beta-blocker, that inhibits noradrenergic arousal, before undergoing either a stress or a control manipulation. By taking this approach, the third study aimed to answer the question whether noradrenergic arousal mediates the assumed effects of stress on fear memory generalization. The fourth and last study focused on the question, whether an acute stressor changes attentional processing and if this change has an influence on aversive predictive learning. To follow this research question, we used an

aversive blocking paradigm. After a phase of differential fear acquisition, half of the participants underwent a stress manipulation. In the following phase, the old stimuli were paired with new stimuli and blocking to one of the stimuli should occur. Finally, the last phase tested for the possible blocking effect and if blocking was affected by stress. Attentional processing was measured on a neural and behavioral level, using EEG and eye-tracking, respectively. In all of our studies, we used the TSST for stress manipulation.

Overall, this dissertation sought to investigate if and how acute stress (in particular the stress mediators cortisol and NA) and a passage of time contribute to the process of fear generalization (Study I-III) and how stress influences attentional processes during aversive predictive learning (Study IV).

2. Experimental studies

2.1. General methods: The fear generalization paradigm

Before presenting the experimental studies we conducted to test our hypotheses, the following section will describe the fear generalization paradigm (Onat & Büchel, 2015) we used in Study I-III in more detail.

The paradigm established by Onat and Büchel (2015) includes eight neutral face stimuli which differ along two dimensions on a circular perceptual similarity continuum (x-axis: identity; y-axis: gender; see Figure 5A). In this circular organization, a pair of most dissimilar faces are located on opposite sides and were later used as CS+ and CS-, respectively. The stimuli in between are quantified in their distance to the CS+ and served as GS (Figure 5B). An uncomfortable electrical stimulation to the right wrist was used as US. The paradigm comprises three phases: a baseline phase, a fear acquisition phase and a test phase (Figure).

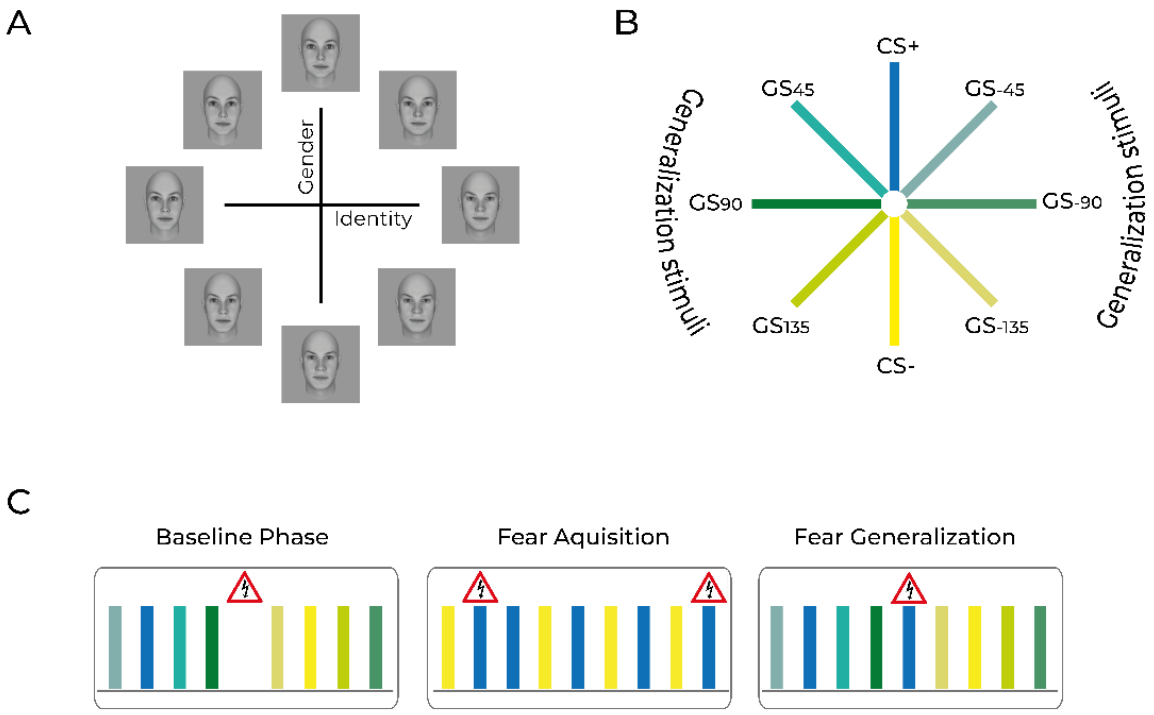

Figure 5. Fear generalization paradigm. (A) The stimuli in between the CS+ and the CS- represent the generalization stimuli (GS). (B) A pair of most dissimilar faces were on opposite sides of the circular similarity continuum. (C) The three phases of the paradigm. Note. Adapted from Onat, S., & Büchel, C. (2015).

During the baseline phase, the whole set of face stimuli were shown to the participants. This phase was conducted to assure that there are no a priori differences between the faces before fear acquisition. To maintain a comparable level of arousal due to electrical stimulation, the US was already applied during the baseline phase. Importantly however, the US was always signaled by the presentation of a shock symbol, i.e. the US was delivered in a fully predictable manner, to prevent any association of the shock with any of the faces. Next, during the fear acquisition phase, only two faces, i.e. a pair of most dissimilar faces, were presented. The US was not signaled by a shock symbol anymore, but followed the CS+ in ~23% of the trials, whereas the CS- was never followed by the US. The subsequent test phase of fear generalization was similar to the baseline phase. The complete set of faces were shown to the participants again. The shock however, was not signaled by the shock symbol but followed the CS+ in ~23% of the trials, to avoid extinction learning to the CS+. To ensure that participants were attentive, they had to react to 10 unsigned oddball trials (i.e. faces with artificially added freckles) by pressing a button. During all phases, the skin conductance response (SCR), which reflects arousal and is a common measurement in fear learning experiments (Lonsdorf et al., 2017), was

measured. In addition, after all phases each face was shown to the participants twice in a randomized order to assess the explicit US-expectancy towards every face.

To analyze fear generalization, a Gaussian function was fitted to the SCR and rating data. The function can mainly be defined through two parameters: the amplitude, which represents fear memory expression or specificity, and the width, representing the extent of fear generalization. A low degree of fear generalization, would result in a rather narrow Gaussian fear-tuning profile. In contrast, a high degree of fear generalization, would result in a rather wide and flat fear-tuning profile (Figure 6).

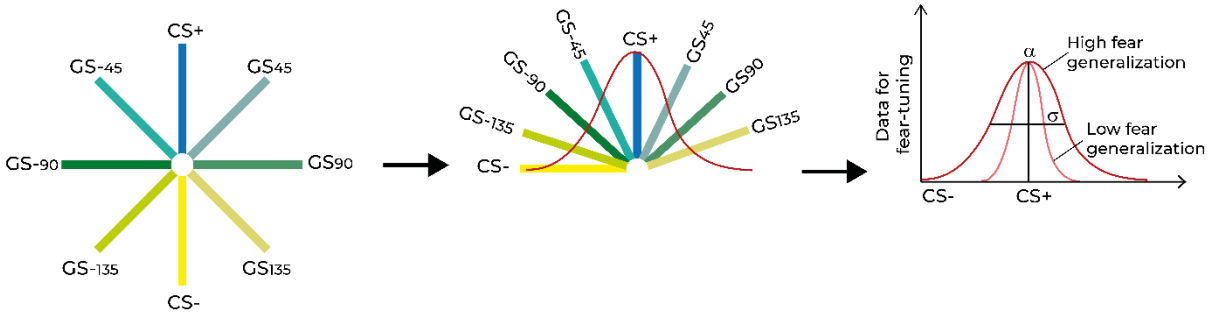

Figure 6. Gaussian fear-tuning. Gaussian fear-tuning can be described by the two main parameter: amplitude (α; strength of fear memory expression) and width (σ; fear generalization).

2.2. Study I: Neural signature of delayed fear generalization under stress

Accepted manuscript: Psychophysiology (Kausche, Zerbes, Kampermann, Büchel & Schwabe, 2021). The full manuscript can be found in Appendix A.

2.2.1. Background

It is well known that over time our memory changes from a detailed to a more gist-like representation (Dandolo & Schwabe, 2018; Jasnow et al., 2012; Winocur et al., 2007). Regarding the process of fear generalization, it is important to note, that there often is a delay between an original threatening situation and a situation in which fear generalization may occur. Moreover, threatening situations are often accompanied by acute stress; this experience of stress shortly before fear memory recall can have impairing effects (Cai, Blundell, Han, Greene, & Powell, 2006; Wolf, 2017). Up to date, it is still not known what time has on the process of fear generalization and if the neural signature of fear generalization changes due to time. We hypothesized that fear

generalization would be increased after a delayed compared to an immediate test, which should be seen on a behavioral and neural level. Moreover, we expected that acute stress would result in an even wider fear generalization.

2.2.2. Methods

Seventy-three healthy participants underwent a two-day fear generalization paradigm, testing fear generalization on Day 2 in the fMRI scanner (see 2.1 and Figure 4 and 5). Depending on the experimental group, participants either underwent a stress manipulation (in form of the TSST) or control manipulation on Day 2 prior to the test phase. On both days, we obtained US-expectancy ratings after each phase and measured SCR during all phases. On a behavioral level, we calculated fear-tuning profiles (Figure 6) based on SCR and rating data. On a neural level, we followed the procedure of Onat and Büchel (2015), investigating which brain regions can be associated with fear generalization after a delay. We then also quantified neural fear generalization by creating fear-tuning profiles for these regions. To investigate the influence of time on fear generalization, we compared our results (test of fear generalization 24 hours after fear acquisition) with the results of two previous studies (Kampermann, Wilming, Alink, Buchel, & Onat, 2019; Onat & Büchel, 2015), using the same fear generalization paradigm but testing fear generalization immediately after fear acquisition.

2.2.3. Results

Higher responses to the CS+ compared to the CS- in both measurements, i.e. SCR and rating data, confirmed successful fear acquisition for both groups on Day 1. On Day 2, successful stress manipulation was confirmed by subjective and physiological parameters, i.e. blood pressure, pulse and salivary cortisol. Importantly, behavioral data showed pronounced fear generalization for both our measurements, SCR and rating data. On a neural level, fear-tuning, i.e. higher responding towards the CS+, was found in the bilateral insula and frontal operculum, whereas safety-signaling, i.e. higher responding towards the CS-, was associated with frontal, hippocampal and temporal regions, including the vmPFC. In line with a previous study (Onat & Büchel, 2015) that investigated immediate fear generalization, the bilateral insula showed a hyper-sharp fear-tuning, i.e. a smaller width of Gaussian fear-tuning compared to the behavioral data during delayed fear generalization. To investigate the influence of acute stress on delayed fear generalization, we compared fear-tuning between our two groups. Results revealed no

additional modulating influence of acute stress on delayed fear generalization, neither on a behavioral nor on a neural level. In addition, we investigated the influence of time. Therefore, we compared results of our control participants to the results of two previous studies that investigated immediate fear generalization (Kampermann et al., 2019; Onat & Büchel, 2015). Results indicated increased fear generalization after a 24h delay. Follow-up analyses showed that this increase in fear generalization could not be explained by a change in CS+/CS- discrimination, but rather resulted from a reduced discrimination between the CS+ and the stimuli most similar to the CS+.

2.2.4. Conclusion

Based on our results, an experience of acute stress shortly before a delayed test of fear generalization does not seem to influence this process in a group of healthy participants. In addition, we suggest that the basic neural mechanisms of fear generalization remain the same, independent whether fear generalization is tested after a 24 hour delay or immediately after fear acquisition. However, with passage of time, fear generalization generally seems to increase while leaving threat-safety discrimination intact. This result was found both on a behavioral and on a neural level.

2.3. Study II: Noradrenergic stimulation increases fear memory expression

Published in: European Neuropsychopharmacology (Kausche, Zerbes, Kampermann, Müller, Wiedemann, Büchel & Schwabe, 2020). The full publication can be found in Appendix B.

2.3.1. Background

There is great consensus, that acute stress modulates fear learning and memory processes (Merz et al., 2016; Raio & Phelps, 2015; Simon-Kutscher et al., 2019). Furthermore, the two major stress mediators glucocorticoid (GC) and noradrenaline (NA) are known to exert effects on certain brain regions that are also associated with fear generalization processes (Dunsmoor et al., 2011; Lissek, Bradford, et al., 2014; Onat & Büchel, 2015). Previous studies suggest that these two mediators can act both synergistically (Joels et al., 2011; Krugers, Zhou, Joels, & Kindt, 2011; Roozendaal et al., 2006; Schwabe, Joels, et al., 2012) and distinctively (de Quervain et al., 2000; Murchison et al., 2004) to influence learning and memory. However to date, their specific and distinct effects in regard to fear generalization are still not fully understood. Therefore, we aimed to investigate the

combined effects of GCs and noradrenergic arousal, as well as their distinct roles in this process, expecting an interactive effect to influence fear generalization.

2.3.2. Methods

To test our hypothesis, that the two major stress mediators, cortisol and NA, affect fear generalization, we conducted a two-day fear generalization paradigm (see 2.1 and Figure 4 and 5). Data of 125 healthy participants, pseudo-randomly assigned to one of four groups, was analyzed. Depending on the experimental group, participants received either a placebo (Plac), 20mg of hydrocortisone (Cort), 20mg of yohimbine (Yoh), a α2-AR antagonist to increase noradrenergic arousal, or 20mg of both drugs (Cort+Yoh) prior to a test of fear generalization on Day 2. Fear-tuning profiles (compare Figure 6) were calculated for SCRs and explicit US-expectancy ratings.

2.3.3. Results

Data of SCRs and US-expectancy confirmed successful fear acquisition for all groups on Day 1. On Day 2, the measurements of blood pressure, pulse, as well as salivary cortisol validated the action of the drugs. Regarding the test of fear generalization, results of both our measurements revealed that the intake of yohimbine led to a higher fear-tuning amplitude in the SCR data and at the same time to a narrower fear-tuning curve in our rating data. A more detailed analysis revealed that yohimbine specifically increased responding to the threatening CS+, whereas it had no impact on responding to the safety signaling CS-. Thus, an increase of noradrenergic arousal through yohimbine led to enhanced fear memory expression and specificity. In addition, the perceptual discrimination ability was enhanced after yohimbine intake. However, a covariate analysis revealed that the improved discrimination ability cannot solely explain the aforementioned results. In contrast to yohimbine, we did not obtain any significant effects for hydrocortisone, but there was a trending effect for US-expectancy data which pointed to an increase in fear memory generalization after hydrocortisone administration.

2.3.4. Conclusion

In contrast to previous studies, which suggest that stress may contribute to enhanced fear generalization, our findings rather point to a role of noradrenergic arousal to sharpen fear memory. Therefore, we suggest that further studies, aiming at investigating the influence of stress on fear generalization should study both, the combined and the individual effects

of the cortisol- and NA-mediated stress response. This is in line with other studies, showing that effects of GCs and noradrenergic arousal can add to one another as well as cancel each other out (Krugers et al., 2012; Roozendaal et al., 2006). Consequently, this can have important implications for the treatment of mental disorders, in which the overgeneralization of conditioned fear is prominent.

2.4. Study III: Acute stress leaves fear generalization in healthy individuals intact

Published in: Cognitive, Affective, & Behavioral Neuroscience (Kausche, Zerbes, Kampermann, Müller, Wiedemann, Büchel & Schwabe, 2021). The full manuscript can be found in Appendix C.

2.4.1. Background

Acute stress can influence fear learning and memory and it is assumed, that a significant modulating role is assigned to noradrenergic arousal (Kausche et al., 2021b; Krugers et al., 2012; Roozendaal & Hermans, 2017; Roozendaal et al., 2006; Schwabe, Joels, et al., 2012). One previous study that investigated the influence of stress on fear generalization in humans suggest that an experience of acute stress enhances fear generalization (Dunsmoor, Otto, et al., 2017). In contrast, another fear generalization study carried out by our lab did not reveal such an influence on fear generalization (Study I) and a second previous study in which we exogenously enhanced cortisol and noradrenergic arousal rather points to a role of noradrenergic activity promoting fear memory expression (Kausche et al., 2021b). Thus, the role of stress, and the individual stress mediators in the process of fear generalization, are still far from certain. Therefore, this study aimed particularly to investigate the influence of inhibiting the noradrenergic arousal in response to stress on fear generalization.

2.4.2. Methods

To answer our research question, we invited 120 volunteers to participate in our two-day fear generalization study (see 2.1 and Figure 4 and 5). On Day 2, prior to a test of fear generalization, participants received either a placebo (Plac) or propranolol (Prop), which is a β-adrenergic antagonist that inhibits noradrenergic arousal and subsequently underwent either a stress manipulation via TSST (S) or a control (C) manipulation. Based

on this procedure, we obtained four groups: S+Plac, S+Prop, C+Plac, C+Prop. To analyze fear generalization, we calculated fear-tuning profiles (Figure 6) for SCR and rating data.

2.4.3. Results

On Day 1 participants across all groups successfully acquired fear, indicated by both SCR and explicit rating data. On Day 2, physiological data confirmed that our stress manipulation, as well as our pharmacological reduction of noradrenergic arousal, was successful. Results regarding the critical test of fear generalization revealed distinct fear generalization, which remained unaffected by both our manipulations, i.e. stress and propranolol. Using only our placebo groups and including participants of our previous fMRI study (Study I), follow-up Bayesian analysis confirmed the absence of a stress effect on fear generalization.

2.4.4. Conclusion

Taken together, our findings do not support the idea that an experience of acute stress enhances fear memory generalization in a sample of healthy individuals. In addition, a mere reduction of noradrenergic arousal did not influence fear generalization. Together with previous results of studies in patients, which show enhanced fear generalization (Kaczkurkin et al., 2017; Lissek, Kaczkurkin, et al., 2014; Lissek et al., 2010), we suggest that it might be insightful for future studies to investigate a sample of individuals who have a high vulnerability for anxiety and stress-related disorders. Investigation of such a sample may provide an answer to the question of whether there are factors that mediate the stress influence on fear generalization, resulting in fear overgeneralization.

2.5. Study IV: Blocking under stress: Sustained attention to stimuli without predictive value?

Published in: Neurobiology of Learning and Memory (Kausche & Schwabe, 2020). The full publication can be found in Appendix D.

2.5.1. Background

Associative learning theories suggest that learning occurs when there is a discrepancy between an expected and an actual outcome, whereas it does not occur when a stimulus contains no new information, also called blocking effect (Kamin, 1968; Mackintosh, 1975;

Pearce & Hall, 1980). It is assumed that attentional processes play a crucial role and explain some of the underlying mechanisms. On a behavioral level, eye-tracking studies showed that when two stimuli are presented together, a redundant stimulus receives less attention compared to a stimulus that is predictive of an outcome (Beesley & Le Pelley, 2011; Eippert et al., 2012; Kruschke et al., 2005). On a neural level, studies using EEG showed reduced attentional processing of stimuli without predictive value (Wills et al., 2007). Moreover, it is well known, that acute stress is a strong modulator of learning and memory (Schwabe, Joels, et al., 2012) and can modulate attentional processing (Hermans et al., 2014). However, whether stress may affect attentional processing in predictive fear learning is largely unknown. Focusing on the role of attentional processes, we therefore combined EEG and eye-tracking recordings with a stress manipulation during a fear conditioning paradigm, designed to probe the blocking effect.

2.5.2. Methods

In total, we analyzed data of 84 healthy young adults, tested with a between-subjects design. To test the impact of stress on the blocking effect in fear learning, participants underwent a fear conditioning paradigm consisting of three phases (see Figure 7). In the first phase fear acquisition takes place, i.e. a neutral stimulus A is paired with a shock (US), thus becoming a CSA+, and another neutral stimulus B is never paired with the US, thus becoming a CSB-. Next, depending on the group condition, participants underwent a stress (TSST) or control condition. This was followed by the blocking phase in which the blocking effect should develop. To this end, the previous introduced stimuli were additionally presented with a second, different stimulus, X and Y, forming two compound stimuli, both followed by the US, i.e. CSAX+ and CSBY+, respectively. Because previously, the CSB- did not predict the US, learning of the CSY-US association should occur. In contrast, because the CSA+ already perfectly predicted the US, this should not be the case for CSX, i.e. learning of the CSX-US association should be reduced or blocked. In the final phase, these assumptions were tested by presenting CSX and CSY individually, never followed by the US. Learning of the different stimulus-outcome associations was investigated on a behavioral (US-expectancy ratings and eye-tracking data), physiological (SCR) and neural (ERPs) level.

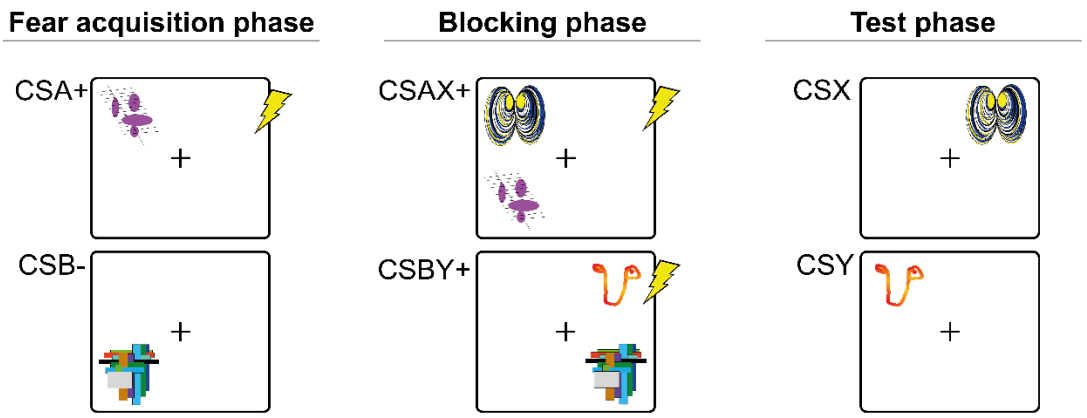

Figure 7. Aversive blocking paradigm. During the fear acquisition phase, fear learning to the CSA+ (followed by the US) takes place, whereas CSB- (not followed by the US) should be learned to be safe. In the subsequent blocking phase, compound stimuli (consisting of an old and a new stimulus) are presented and always followed by the US. Learning of the CSX-US association should be blocked, whereas learning of the CSY-US association should occur. Successful blocking is tested in the last, i.e. the test phase, in which the CSX and CSY are presented individually, not followed by the US. Note. Adapted from Eippert, F., Gamer, M., & Büchel, C. (2012).

2.5.3. Results

Results showed successful fear acquisition for the control group at all levels, as they showed higher SCRs and longer fixation durations for the CSA+ (followed by the US) compared to the CSB- (never paired with the US). Moreover, they also exhibited differential EEG responses, reflecting enhanced attention for the US predictive CS+. For the stress group, distinct brain responses also indicated successful fear acquisition, despite missing differentiation in the SCR and eye-tracking data. Successful stress induction was validated on a physiological and subjective level. During the subsequent blocking phase, in which both the new compound stimuli CSAX+ and CSBY+ were followed by a shock, eye-tracking results showed preferential attentional processing of the newly introduced CSY compared to the old CSB- when presented together. No such differentiation was observed for the CSAX+ compound. Results were the same for both groups. On a neural level however, results revealed that the stress group was impaired in its preferential early attentional processing of the predictive stimulus, as indicated by a reduced P3b. When testing for the blocking effect in the last phase, results of explicit ratings confirmed its development by lower US-expectancy ratings for the CSX compared to the CSY. This effect was irrespective of stress.

2.5.4. Conclusion

Together, our results support the general idea that the predictive value of a stimulus impacts future learning. Stimuli with a higher predictive value attract more attention resulting in further learning to these stimuli compared to those, for which learning is blocked. On a neural level, an experience of acute stress reduced this preferential processing of predictive stimuli. Not being able to restrict attention to stimuli with predictive value can lead to a missing focus on what carries important information. This can explain the negative effects of stress in anxiety disorders, in which adequate attentional processing is impaired (Mathews & MacLeod, 2005; Rudaizky, Basanovic, & MacLeod, 2014). Since our stress group did not show any impairments on a behavioral level, future studies should follow to investigate under which circumstances attentional mechanisms may also impact behavioral performance.

3. General discussion

Understanding how fear generalization evolves and how it can be influenced by internal or external factors is fundamental for scientific and therapeutic research. It could enable us to intervene in situations in which fear is no longer generalized in an adaptive manner but rather in a maladaptive one, resulting in fear overgeneralization and contributing to the development of anxiety or stress-related disorders. To fully explore all factors and consider their possible interactions is fairly impossible. However, it does not change the necessity but rather underpins the need to contribute to this goal step by step. The investigation of fear learning processes has a long history and already builds upon a large number of significant study results. Importantly, over the course of time, several factors came up repeatedly, suggesting to play a meaningful role in modulating fear learning processes, such as acute stress or time. Therefore, it seems inevitable to investigate those factors in the process of fear generalization. Clinical studies support the significance of fear generalization since an overgeneralization of fear can be seen as one key symptom in patients with PTSD (Kaczkurkin et al., 2017; Lis et al., 2020). In addition, reduced levels of GCs have been found in PTSD patients (Raglan, Schmidt, & Schulkin, 2017) and the administrations of GCs shortly after a traumatic event could be an effective preventive intervention for severe symptoms of PTSD (Astill Wright et al., 2019).

In the last three years, I dedicated most of my work to those very research question: what role does acute stress, and more specifically important stress mediators such as cortisol and noradrenaline (NA), play in the process of fear generalization? What impact might time have? And how does attention and the predictability of threat influence learning? In the following paragraph, I will first briefly summarize the results of this work and embed them into the present body of knowledge. Secondly, I will give an outlook as to what I think future research should focus on. Finally, I will draw a main conclusion and bring the experimental work into the bigger picture.

3.1. Summary and embedding of study results

To investigate the influence of stress (mediators) and time on the process of fear generalization and the interaction of attention and aversive predictive learning, we conducted four studies. Three of them used the exact same two-day fear generalization paradigm. On experimental Day 1, fear acquisition took place and on experimental Day 2, participants underwent a certain manipulation, depending on the focus of the study, before the critical test of fear generalization was performed (Figure 4). Altogether, study results revealed that when time passes, fear generalization increases and that stress does not necessarily add to this effect in a population of healthy individuals. In contrast, we found that a distinct enhancement of noradrenergic arousal strengthened fear memory expression and specificity. This is partly in contrast to previous findings in animals and humans, suggesting that stress or GCs influence the extent of fear generalization (Bender et al., 2018; Dunsmoor, Otto, et al., 2017; Kaouane et al., 2012; Kolodziejczyk & Fendt, 2020; Liu et al., 2019; Zhu et al., 2018). However, those studies differ in many aspects from our studies, which might explain the conflicting findings. To investigate this matter thoroughly, some aspects will be discussed in more detail in the following sections. Using another approach, i.e. an aversive version of the Kamin blocking paradigm (Figure 7), results of our fourth study revealed that acute stress reduced the preferential processing of predictive compared to non-predictive stimuli during fear learning. Therefore, another aim of the discussion is to compare the different approaches and to find an explanation for the question under which circumstances stress might influence the process of aversive or fear learning.

3.1.1. No influence of stress on fear generalization – different roles of stress mediators?

Very intriguingly, we did not observe an effect of acute stress on fear generalization across all our fear generalization studies (Study I-III; Kausche et al., 2021a; Kausche et al., 2021b). Based on the current literature, this partly is in contrast to what we have expected. Therefore, we need to thoroughly examine possible explanations. First of all, it seems necessary to investigate the combined and individual effects of the two main stress mediators: cortisol and NA. In particular, our results suggest that noradrenergic arousal strengthens fear memory expression and thus might work against an effect of fear overgeneralization after stress (Study II; Kausche et al., 2021b). Importantly, this result is in line with a recently published study in rodents, supporting the idea of opposite effects of noradrenergic arousal and GCs on fear memory accuracy (Roozendaal & Mirone, 2020). Whereas an increase of noradrenergic arousal resulted in a more accurate and strengthened fear memory, the administration of GCs resulted in a strengthening of generalized fear memory. The degree of generalization was however restricted, as such that rodents only showed generalization to the already known safety context but not to a completely novel one. Importantly, also in this study rats were administered yohimbine or GC after training, i.e. before the test of generalization 48 hours later. Furthermore, another study showed that a low-dose administration of propranolol immediately after fear conditioning, which inhibited noradrenergic arousal, resulted in an impairment in memory accuracy (Zhu et al., 2018). Interestingly, the authors suggest that a dose-dependent infusion of norepinephrine can induce PTSD-like memory impairments as well. This assumption is based on another study, in which they showed that rats generalized their fear responses to other cues, after a moderate increase in noradrenergic arousal. At the same time, the concurrent administration of norepinephrine and propranolol into the BLA enhanced memory for the conditioned fear (Liu et al., 2019). Clinical studies in patients with PTSD further support the role of noradrenergic activity, showing that a heightened responsivity of noradrenergic neurons (Southwick et al., 1999), as well as a reduction in norepinephrine transporter availability in the locus coeruleus (Pietrzak et al., 2013) contributes to PTSD symptoms. A slight increase of both, noradrenergic arousal and glucocorticoids, in response to a moderate stressor, however, does not influence fear generalization (Study I and III; Kausche et al., 2021a). Together, these results underline the importance of noradrenergic arousal for fear memory

specificity and point to the idea of a dose-dependent effect as to whether NA enhances or impairs fear memory specificity.

To summarize, it seems that acute stress, and in particular the stress mediators cortisol and NA, can have distinct effects in the process of fear generalization. Those can be beneficial as well as detrimental effects. Previous studies suggest that GCs might enhance fear generalization when they directly interfere with the process of fear memory consolidation (Bender et al., 2018; Kaouane et al., 2012; Kolodziejczyk & Fendt, 2020). However, when the acquisition of fear is not manipulated and the level of stress or cortisol is only enhanced shortly before a test of fear generalization, results do not point to increased fear generalization due to cortisol (Studies I-III; Kausche et al., 2021a; Kausche et al., 2021b). In addition, an increase of noradrenergic arousal seems to strengthen fear memory expression and specificity (Kausche et al., 2021b; Liu et al., 2019; Roozendaal & Mirone, 2020; Zhu et al., 2018), whereas it is still not clear if only a certain level of noradrenergic arousal leads to an increase or decrease of fear generalization.

3.1.2. The contribution of time and its interaction with stress

So far, many human studies measured fear generalization shortly after fear acquisition on the same day and were mostly conducted to compare fear generalization in healthy individuals with a population of patients (Ahrens et al., 2016; Kaczkurkin et al., 2017; Lis et al., 2020; Lissek, Kaczkurkin, et al., 2014; Lissek et al., 2010; Morey et al., 2015; Tinoco-González et al., 2015). However, the overgeneralization of fear, which is often found in anxiety or stress-related disorders (e.g. PTSD), typically relates to a threatening experience that was encountered a long time (weeks, months, or even years) ago. Therefore, the investigation of fear generalization over the course of time may contain important information for understanding the development of fear generalization.

Results of our first study suggest that there is an increase in fear generalization over time. This is in line with another very recently published study in humans, showing an increase in contextual fear generalization on an explicit level after a 24 hours delay (Andreatta et al., 2020). In contrast to the other study, we used multiple GS stimuli and were able to additionally show that this increase in fear generalization cannot be explained by an impairment in fear memory retrieval. Whereas fear memory for the original CS+ was not influenced, fear was increased for similar, generalization stimuli. This was mirrored by a wider fear-tuning for a delayed (24 hours later) compared to an

immediate test of fear generalization. Simultaneously, there was no change in neural representation and stress did not have an additional effect. These results are in contrast to another human study that investigated the influence of stress on fear generalization after a delay of 24 hours (Dunsmoor, Otto, et al., 2017). Dunsmoor and colleagues (2017) demonstrated that stress shortly before a test of fear generalization leads to an increase in the generalization of fear itself. Importantly however, stress was also followed by less precise memory of the original threatening stimulus, the CS+, indicative of a reduced memory specificity. Hence, it is possible, that it was the retrieval of memory per se that was influenced by stress and not just the process of fear generalization. Again, this partly conflicts with the results of one of our own studies (Kausche et al., 2021b) that rather showed a positive effect of noradrenergic arousal on fear memory specificity and expression.

As already discussed in the previous paragraph, the investigation of stress in combination with fear generalization may be insightful to enhance our understanding of this phenomenon. So far, all the animal studies investigating the influence of stress on delayed fear generalization rather manipulated the process of fear consolidation by either administering stress (mediators) before or directly after fear learning (Bender et al., 2018; Bueno, de Paiva, Correa, Tiba, & Fornari, 2017; Kaouane et al., 2012; Kolodziejczyk & Fendt, 2020; Krugers et al., 2020). To the best of our knowledge, there is only one study that aimed to replicate the findings of the animal studies in humans and failed to do so (Sep, Gorter, van Ast, Joels, & Geuze, 2019). In this study, fear acquisition took place immediately or 2 hours after a psychological stressor. Compared to a control group that did not experience any stress, the authors found no time-dependent effects of stress on fear generalization. Stress neither had an impact on initial fear acquisition nor on subsequent fear generalization for which was tested 24 hours later. The authors assumed that the missing effects of stress on fear generalization could be due to the long time interval between the stress experience and fear acquisition. In our studies (Study I-III), we chose a slightly different approach and left the process of fear consolidation unaffected by conducting our stress manipulation just before the test of fear generalization. We could thereby investigate possible stress effects that do not influence the initial learning of the threatening experience. As such, it was more the process of subsequent memory retrieval that was affected in our studies. Altogether, our studies repeatedly showed no influence of stress on fear generalization.

Multiple studies suggest that stress impairs memory retrieval (Buchanan, Tranel, & Adolphs, 2006; de Quervain et al., 1998; Kuhlmann, Piel, & Wolf, 2005; Roozendaal, 2002; Schönfeld et al., 2014; Schwabe & Wolf, 2014; Wolf, 2017). As such, we would have expected that an experience of stress shortly before a test of fear generalization would impair the recall of the established association and may result in an increased generalization of fear. In contrast to what was expected, our results repeatedly showed neither an impairing effect of memory retrieval due to stress nor a generalization of fear. Importantly, there are also various studies that did not show an effect of stress on memory recall. As such, some studies that explicitly investigated cued memory recall did not obtain any effect due to stress (de Quervain et al., 1998; Kuhlmann et al., 2005). This could partly explain the missing effects in our study, since we also incorporated a cued fear learning paradigm. Furthermore, studies suggest that there is a difference between cortisol responders and cortisol non-responders. Intriguingly however, whereas one study showed an impairment in memory retrieval in cortisol responders (Bentz et al., 2013), the opposite was found in another study, revealing an impairment for participants with a blunted stress response (Zoladz et al., 2014). Together, one could speculate that stress impairs a subsequent memory retrieval only under certain conditions, e.g. when cortisol response is either too low or too high. This could explain, why we did not find an effect of stress in our samples of young and healthy individuals, showing a normal and healthy stress response (Study I and III; Kausche et al., 2021a). In addition, studies carried out on patients with PTSD even showed enhanced memory retrieval after cortisol administration, indicating an altered sensitivity to cortisol (Wingenfeld & Wolf, 2015). Another possible explanation appears in a very recently published preprint (McManus, Talmi, Haroon, & Muhlert, 2020), which shows that a psychological stressor has only little influence on memory retrieval. In line with this study, it could be that a psychological stressor compared to a physiological is not potent enough to result in increased fear generalization.

To conclude, our studies (Study I-III) so far suggest that a psychological stressor experienced shortly before a test of fear generalization cannot explain the phenomenon of fear overgeneralization. Together with the literature published so far, different alternative explanations exist. The animal studies so far that showed enhanced fear generalization all affected the process of fear acquisition. Hence, it could be that it is the process of fear acquisition rather than fear retrieval that is more prone to an influence of

stress resulting in fear overgeneralization. Secondly, the exact conditions under which stress might lead to impaired fear memory retrieval are still not clear. Thirdly, it is possible that our stress manipulations were not strong enough. However, our second study, in which we administered hydrocortisone and yohimbine, also showed no effects on an enhanced fear generalization, which makes this explanation somewhat unlikely.

3.1.3. Attention

Based on our results of Study I-III, the question remains: if it is not stress, as suggested of certain studies (Bender et al., 2018; Dunsmoor, Otto, et al., 2017; Kaouane et al., 2012; Kolodziejczyk & Fendt, 2020), which factors may provoke an increase in fear generalization? According to the expectancy model of Davey (1992), the expectation of a forthcoming threat plays an important role in fear learning. It is advantageous to allocate our attention to stimuli that predict threat and thus adapt our behavior. However, when this beneficial mechanism is impaired and we (also) allocate our attention to other stimuli that do not contain any relevant information, it might lead us to associate a negative outcome with a stimulus, which would actually not be a reliable predictor of the threat. This could consequently lead to an expectation of a threatening outcome in the presence of a non-predictive stimulus.

In our fourth study, we used the Kamin blocking effect (Kamin, 1968) to investigate the influence of acute stress on predictive learning of a threat. Results showed that stress reduced the preferential neural processing of predictive compared to non-predictive stimuli. This was associated with enhanced attention allocation to non-predictive stimuli, when there is a demand of simultaneous stimulus processing. These results suggest that stress may impair efficient information processing against the background of prior experiences. Interestingly, it was recently shown that during learning, patients with PTSD compared to non-PTSD patients are slower in learning the differentiation between a threat and a safety signaling condition (Morey et al., 2020). This is in line with another study, showing that PTSD patients, compared to healthy controls, take much longer to evaluate the expectation of a threat when confronted with stimuli that are actually non-predictive of a threat but similar to the original one. Hence, they showed impairments in the processing of safety signaling cues (Lis et al., 2020). In a subsequent test of fear generalization, this resulted in an increased fear generalization towards safety signaling stimuli. In addition, this overgeneralization was particularly strong in patients who

reported widespread psychological stress and physiological responses across different domains of their lives (Lis et al., 2020).

The current findings could be traced back to an impaired recall of previously learned associations in stressed participants, resulting in a higher need for stimulus evaluation. This fits again well with the idea that stress impairs memory retrieval (Buchanan et al., 2006; de Quervain et al., 2000; de Quervain et al., 1998) but is in contrast to our fear generalization studies (Studies I-III). Importantly, between those studies and our Study IV, there was one crucial difference: participants were not confronted with the concurrent processing of multiple stimuli. In everyday life, we are mostly confronted with complex situations which require us to process much information at the same time. Therefore, it may be possible that under normal situations, stress alone will not influence fear generalization. However, when the organism is confronted with too much information to process and has too few resources to deal with the situation, this in interaction with stress can result in maladaptive fear generalization. Therefore, I suggest that future studies should investigate if the concurrent processing of multiple stimuli during fear learning and fear generalization may have an impact on our behavior. In addition, an investigation on which specific cues attention is prominently allocated to during fear learning and fear generalization could unravel underlying mechanisms of fear generalization. Consequently, this could enhance our understanding as to why some people show fear overgeneralization in contrast to others.

3.2. Outlook: Generalization of fear-related avoidance behavior

On a behavioral level, the overgeneralization of fear is often accompanied by an exaggerated avoidance behavior (American Psychiatric Association, 2013). Imagine the example from the introduction of this thesis, in which a situation was depicted of being attacked by a tall man (CS+). Now imagine that a father teaches his daughter that she can defend herself from those situations by carrying a pepper spray. This would be an instrumental behavior to avoid or in this case deal with the CS+. Ideally what follows is that when the girl later needs to walk through a park again on her own, the probability of carrying a pepper spray increases. The two learning experiences interact. The experience of the CS+ affects our instrumental responding associated with the same outcome. This is called pavlovian-to-instrumental transfer (PIT; Cartoni, Puglisi-Allegra, & Baldassarre, 2013; Geurts, Huys, den Ouden, & Cools, 2013).

It was shown very early on that aversive cues, associated with a threat, can enhance behaviors such as avoidance (Rescorla & Solomon, 1967) and in recent years, interest in this body of research has increased. To study the motivation of avoidance behavior in the laboratory, an avoidance based PIT paradigm is well suited (Geurts et al., 2013; Hebart & Glascher, 2015; Lewis, Niznikiewicz, Delamater, & Delgado, 2013). It has been suggested to distinguish between a *specific PIT,* i.e. a conditioned stimulus increases instrumental responding associated with the same outcome, and a *general PIT,* i.e. a rather non-selective enhancement of responding in the presence of a conditioned cue (Corbit & Balleine, 2005, 2011). First evidence shows that aversive stimuli increase both specific and general PIT (Nadler, Delgado, & Delamater, 2011). Moreover, it has recently been suggested that threatening cues increase active avoidance behavior to evade a negative consequence, while leaving approach behavior to an appetitive stimulus unaffected (Xia, Gurkina, & Bach, 2019). Regarding a possible influence of stress on PIT, research in this domain is still scarce and most of the studies have been conducted in the appetitive domain, with results being quite intermixed (Morgado, Silva, Sousa, & Cerqueira, 2012; Pielock, Braun, & Hauber, 2013; Pool, Brosch, Delplanque, & Sander, 2015; Quail, Morris, & Balleine, 2017; Steins-Loeber et al., 2020). Whereas one study in rats did not find an effect of stress on PIT (Pielock et al., 2013), two studies in humans revealed that stress increased the motivation to approach a reward in presence of the reward predicting cue, i.e. stress increased the *specific PIT* (Pool et al., 2015; Quail et al., 2017). However, a recently published study, that sought to investigate the impact of acute stress on conditioned substance-associated stimuli, could not replicate these findings, as acute stress did not increase the *specific PIT* effect (Steins-Loeber et al., 2020). Most importantly, there is not yet a single study that investigates the influence of stress on stimulus-motivated avoidance behavior. Previous findings suggest that an increased cortisol reactivity in response to acute stress is associated with a heightened avoidance behavior (Roelofs et al., 2009) and that stress increases behavioral inhibition and accelerates responses in the presence of high threat (Vogel & Schwabe, 2019). Interestingly, it was shown that individuals with PTSD symptoms showed a greater avoidance behavior, even in a task not associated with the experienced trauma or fear in general (Sheynin et al., 2017). Based on these results, and the fact that fear generalization is characterized by an increase in avoidance behavior, I suggest that the investigation of

the influence of stress on aversive PIT could contribute to our understanding of increased avoidance behavior associated with fear generalization.

3.3. Conclusion and future directions

The ability to generalize an acquired fear response with a threatening stimulus, to stimuli similar to the original one, is a very adaptive and important fear learning mechanism. However, patients with an anxiety or stress-related disorder often show an overgeneralization of the fear response to actual safe stimuli, making them suffer in everyday life (Dunsmoor & Paz, 2015; Dymond, Dunsmoor, Vervliet, Roche, & Hermans, 2015; Kaczkurkin et al., 2017; Lissek, 2012; Lissek, Kaczkurkin, et al., 2014; Morey et al., 2015). With increasing time, memories naturally undergo a transformation from a detailed to a more gist-like memory (Jasnow et al., 2012; Winocur et al., 2007) and we were able to show that this can also lead to an increase in fear generalization (Study I). The ability of fear generalization per se is an adaptive mechanism (for a review see Asok et al., 2019) and a mere increase in time does not explain the development of a rather maladaptive fear overgeneralization. It has been suggested that stress, mainly GCs, may increase fear generalization (Bender et al., 2018; Dunsmoor, Otto, et al., 2017; Kaouane et al., 2012; Kolodziejczyk & Fendt, 2020). However, most of the studies were conducted in animals and the only human study did not investigate the individual stress mediators, which is why we sought to fill this gap of research. In contrast to what we expected, we did not find that an experience of acute stress or the administration of stress mediators increased fear generalization in a group of healthy, young adults (Study I-III; Kausche et al., 2021a; Kausche et al., 2021b). Interestingly, we found that noradrenergic arousal was rather associated with an increased fear memory expression, hence maintaining detailed memory of the original threatening stimulus over time (Kausche et al., 2021b). In everyday life, we are always challenged to process multiple stimuli at the same time and it could be that fear generalization increases due to an imbalance of heightened attention to threatening stimuli and too little attention to stimuli, which actually signal safety. Results of our last study showed that stress indeed influenced aversive (predictive) learning, when participants were confronted with the concurrent processing of stimuli (Kausche & Schwabe, 2020). This is in line with the result that patients with PTSD suffer from a hypermnesia, i.e. an excessive consolidation of stimuli features of the traumatic event, which can result in a tendency to generalize the fear to cues sharing the same gist

(Desmedt, Marighetto, & Piazza, 2015). However it must be noted, that in this last study (Kausche & Schwabe, 2020) we did not explicitly investigate fear generalization. Therfore, the idea that the concurrent processing of multiple stimuli could impact the generalization of fear has yet to be tested.

Taken together, results of our studies illustrate a rather consistent picture, showing that a stressor, experienced shortly before a situation in which fear generalization can evolve, does not affect this process in healthy, young adults. Patients suffering from anxiety and stress-related disorders show an increased tendency for avoidance behavior. Recently, it has been suggested, that avoidance is motivated by instrumental learning rather than pavlovian learning (Cain, 2019). Since stress limits the capacity to show goal-directed behavior (Schwabe, Tegenthoff, Hoffken, & Wolf, 2012; Schwabe & Wolf, 2011), I suggest that stress may influence the possibility to show an adequate goal-directed behavior in response to a threat. It may thereby decrease the feedback of perceived self-efficacy and safety and could consequently affect the process of fear generalization, when there is a behavioral component. In our studies, participants could not do anything to avoid the threat. In addition, it is still possible that stress may affect attentional processes when confronted with threatening and safety signaling stimuli simultaneously. This may lead to a maladaptive allocation of attentional resources to threat in contrast to safety signaling stimuli which could finally result in the overgeneralization of fear. This idea may pave the way for future studies and improve our understanding of the development of anxiety and stress-related disorders.

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Appendices

Appendix A

Study I

Neural signature of delayed fear generalization under stress

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ORIGINAL ARTICLE

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Neural signature of delayed fear generalization under stress

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Abstract

Although the generalization of fear to stimuli resembling a threatening stimulus is an adaptive mechanism, fear overgeneralization is maladaptive and thought to play a key role in anxiety-related disorders. Since there is typically a delay between an initial fear experience and a situation in which fear (over)generalization may occur, we assessed delayed fear generalization and its neural signature. Moreover, as stress is known to affect fear learning, we further tested whether acute stress modulates fear generalization. Therefore, we conducted a two-day fear generalization study, with initial fear acquisition on Day 1 and a fear generalization test after a 24-hr delay in the MRI scanner. Prior to fear generalization testing, participants were exposed to a stressor or a control manipulation. Our behavioral data showed the expected generalization of fear. At a neural level, fear generalization was accompanied by increased fear-signaling for stimuli that resembled the conditioned stimulus in the bilateral insula and frontal operculum, whereas activity declined in frontal, hippocampal, and temporal regions, including the ventromedial prefrontal cortex, as stimuli became more similar to the conditioned stimulus. Importantly, stress did not modulate fear generalization, neither on a behavioral nor on a neural level. Interestingly, in an exploratory comparison to two other studies that used the same paradigm but tested generalization immediately after acquisition, we observed increased fear generalization in the delayed relative to the immediate generalization test. In sum, our results suggest that stress leaves fear generalization and its neural signature unaffected but that a temporal delay might increase the extent to which fear responses are generalized to stimuli resembling the threatening stimulus.

KEYWORDS

acute stress, fMRI, fear tuning, delay, fear generalization, insula, vmPFC

1 | INTRODUCTION

Fear triggers adaptive behaviors to avoid future threat. Because threatening stimuli rarely occur in the exact same form across situations, the generalization of fear to stimuli resembling the stimulus initially associated with danger promotes the effective avoidance of threat. Research over the past decade suggested that this process of fear generalization

is implemented by an intricate balance of excitatory and inhibitory mechanisms. In particular, whereas areas such as the insula or amygdala showed declining activity as a stimulus differentiated from the threat-related conditioned stimulus (CS+), the ventromedial prefrontal cortex (vmPFC) and hippocampus showed inclining activity as a stimulus deviated from the CS+ (Greenberg et al., 2013; Lissek et al., 2014; Lopresto et al., 2016; Onat & Büchel, 2015). Although fear generalization is generally adaptive from a survival perspective, an exaggerated generalization of fear to harmless stimuli, that is, fear overgeneralization, is maladaptive and a common characteristic of anxiety disorders or post-traumatic stress disorder (PTSD; Dunsmoor & Paz, 2015; Lissek, 2012).

Stress is known to play a key role in fear-related disorders (Pitman et al., 2012; de Quervain et al., 2017). Moreover, stress impacts fear-learning processes in general (Merz et al., 2016; Raio & Phelps, 2015) and major stress mediators, such as glucocorticoids, act on medial-temporal and prefrontal areas involved in fear generalization (Kim & Diamond, 2002; Krugers et al., 2012; Roozendaal et al., 2006; Schwabe et al., 2012). These findings suggest that stress may induce an overgeneralization of fear. In line with this idea, rodent studies showed that stress or glucocorticoids may result in increased fear generalization (Bender et al., 2018; de Quervain et al., 2017; Dunsmoor & Paz, 2015; Kaouane et al., 2012). Initial evidence from one behavioral study in humans suggests that stress increased fear generalization specifically at a 24 hr-delayed test (Dunsmoor, Otto, et al., 2017). Yet, to date, the neural underpinnings of putative stress effects on fear generalization are unknown.

Beyond potential stress-dependent changes, another factor that may modulate fear generalization is time. In fear-related disorders, there is usually a considerable delay between an initial threatening encounter and situations in which fear (over)generalization may occur. This time interval between fear acquisition and later generalization may be highly relevant because memories undergo a change from detailed to more gist-like representations over time (Dandolo & Schwabe, 2018; Jasnow et al., 2012; Winocur et al., 2007). In rodents, several studies assessed fear generalization at different delays (Asok et al., 2019) and recently enhanced cued fear memory generalization has been reported in humans as time after acquisition proceeded (Pollack et al., 2018). In contrast to the rodent literature, most human studies tested fear generalization shortly after fear acquisition (Dunsmoor, Kroes, et al., 2017; Holt et al., 2014; Lissek et al., 2008, 2014; Onat & Büchel, 2015). To the best of our knowledge, there are only two behavioral studies that explored fear generalization processes after a delay of 24 hr in humans (Andreatta et al., 2020; Dunsmoor, Otto, et al., 2017). Whereas one study focused on the influence of contextual information on fear generalization (Andreatta et al., 2020), another study suggested an increased level of fear generalization due to stress for older memories but not for recent memories, that is, a test of fear generalization after a 24-hr delay compared to an immediate test (Dunsmoor, Otto, et al., 2017). However, to what extent the neural underpinnings of immediate and delayed fear generalization differ is completely unknown.

To date, different fear generalization paradigms exist (Dymond et al., 2015), some of which focus on perceptual similarity (Lissek et al., 2008; Onat & Büchel, 2015), whereas others focus on the influence of conceptual similarity (Dunsmoor & Murphy, 2015). Although using perceptually similar stimuli, Onat and Büchel (2015) were able to show that fear generalization is not just passively driven by perceptual failure because they also found object-sensitive visual areas that rather responded to uncertainty. Here, we aimed to determine the neural signature of fear generalization 24 hr after fear acquisition and to explore its potential modulation by acute stress. In addition, we aimed to explore whether this was different from an immediate test of fear generalization, which is why we used the same fear generalization paradigm of Onat and Büchel (2015) including socially relevant stimuli. On a first experimental day, participants completed a fear conditioning procedure. Twenty-four hours later, participants underwent either a stress or a control procedure before they completed a test of fear generalization in the MRI scanner. Although our study was mainly designed to assess stress effects on (delayed) fear generalization, we also aimed to investigate time-dependent changes in fear generalization and its neural basis. To this end, we contrasted our findings with those of two previous studies that used the same experimental paradigm but without a delay between fear acquisition and generalization test (Kampermann et al., 2019; Onat & Büchel, 2015). We hypothesized that fear generalization would be increased after a 24-hr delay, relative to when tested immediately after acquisition. Furthermore, we expected that stressed participants would show an even wider fear generalization.

2 | METHOD

2.1 | Participants and experimental design

Seventy-three healthy, right-handed volunteers (34 men, 39 women) participated in this experiment. In addition to any contraindications for MRI, exclusion criteria comprised any current medication intake or physical illness, a history of any mental or neurological disorder and drug or tobacco use. Moreover, women were not tested during their menses and those taking hormonal contraceptives were excluded. All participants provided written informed consent before participation and received a monetary compensation of 60€. The study protocol was approved by the ethics committee of the Medical Association Hamburg and in accordance with the Declaration of Helsinki.

In a 2-day, between-subjects design, participants were pseudorandomly assigned to a stress group or a control group, ensuring an equal number of men and women per group. Nine participants had to be excluded from the analyses because they did not show successful (explicit) fear acquisition on Day 1 (i.e., they had a lower US-expectancy rating for the CS+ than for the CS-), which was a requirement for testing fear generalization processes 24 hr later. This left a final sample of 64 participants for behavioral data analysis (age [mean ± *SD*]: 25.5 ± 4.1 years: stress group: $n = 33$ (16 women), control group: $n = 31$ [18 women]). For fMRI analyses, 2 additional participants (both stress group) had to be excluded, due to excessive head movement (>4 mm of maximal translation (in any direction of x, y, or z) and >4.0° of maximal rotation).

The previous studies that tested fear generalization immediately after acquisition and to which we compare the present findings, included 29 participants (Onat & Büchel, 2015) and 74 participants (Kampermann et al., 2019), respectively. In these studies, participants were also young, healthy individuals and largely the same inclusion and exclusion criteria were applied.

2.2 | Fear generalization paradigm

In order to assess fear generalization processes, we used a recently introduced paradigm (Onat & Büchel, 2015). If not specified otherwise, the procedure was exactly the same as in the previous studies (Kampermann et al., 2019; Onat & Büchel, 2015). This paradigm included eight face stimuli arranged on a circular similarity continuum with two axes (x-axis: identity; y-axis: gender; Figure 1a). The two faces opposite to each other represented the most dissimilar faces and were later used as CS+ and CS-, respectively. The face stimulus chosen as CS+ was counterbalanced across participants and groups. The faces in between the CS+ and CS- represented the generalization stimuli (GS), which were quantified in their distance to the CS+ (Figure 1b). The paradigm comprised three phases: a *baseline phase*, a *fear acquisition phase*, and a *test of generalization* (Figure 1c). A moderate electric shock served as US. Face stimuli were shown for 1.5 s and, in shocked trials, the US was presented after 1.4 s and co-terminated with face offset. The mean inter-trial interval (ITI) was 3.5 s, ranging between 1.5 and 5.5 s. The ITI was slightly different (3.5 s vs. ~4 s) to the previous studies (Kampermann et al., 2019; Onat & Büchel, 2015). During the *baseline phase*, the complete set of faces was shown, to control for any a priori differences between the faces. During the *fear acquisition phase,* only two faces, that is, the most dissimilar faces, were shown. One face was followed by the US in ~30% of the trials and served as CS+, whereas the other face was never paired with the US and served as CS-. During the *test of fear generalization*, again the complete set of faces was presented. A detailed description of these phases is provided in the supplement.

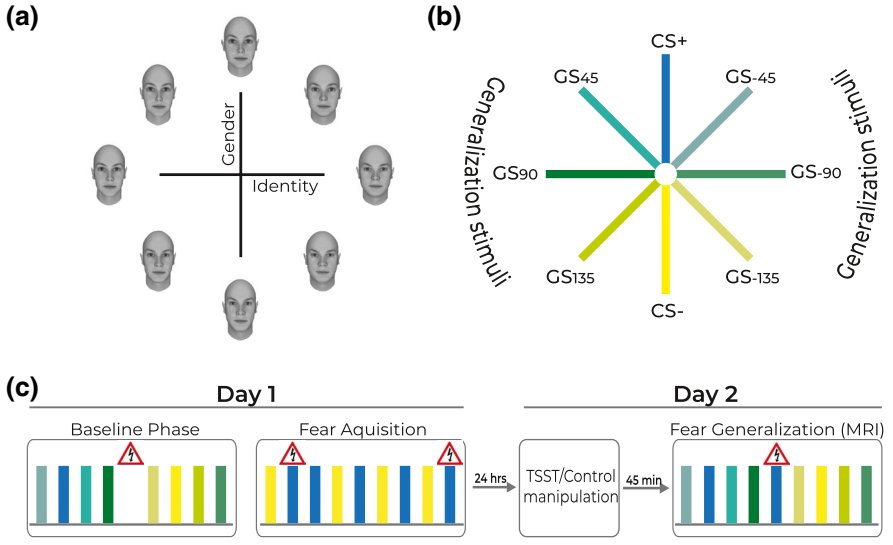

FIGURE 1 Fear generalization paradigm and stimulus organization. (a and b) There are eight different face stimuli in total, arranged on a circular similarity continuum with the axes gender and identity. The stimuli in between the CS+ and CS- represent the generalization stimuli (GS). (c) Fear generalization paradigm with three phases. On Day 1, the baseline and fear acquisition phases take place. On Day 2, the test of fear generalization follows after the stress manipulation or control condition. During the baseline phase, the complete set of stimuli (represented by colored bars) is shown to the participants and US are signaled by a shock symbol. During the fear acquisition phase, the two most dissimilar stimuli from opposite sides of the circular similarity continuum are shown to the participants, representing the CS+ and CS-. During fear acquisition, the CS+ is followed by the US in ~30% of the trials. During the test phase, again the complete set of faces is shown to the participants. To avoid extinction, there is a reinforcement rate of ~30% for the CS+ in the test phase

After each phase, each face was presented two times in randomized order and US-expectancy ratings were assessed using a visual analogue scale (VAS; anchors: "1" = certain, no shock; "10" = certain, shock) to measure explicit fear learning.

2.3 | Experimental procedure

Testing took place on two consecutive days, between 12:30 p.m. and 7:30 p.m., with fear acquisition on Day 1 and the stress manipulation and the test of fear generalization in the MRI scanner on Day 2. To induce stress, we used the Trier Social Stress Test (TSST; Kirschbaum et al., 1993), a standardized protocol for experimental stress-induction in humans that reliably increases subjective stress levels and activates both the autonomic nervous system and the hypothalamus-pituitary-adrenal axis (Kirschbaum et al., 1993). In brief, participants were asked to give a free speech and to perform a mental arithmetic task while being videotaped and evaluated by a panel of two cold, non-reinforcing experimenters. In the control condition, participants talked about a topic of their choice and performed a simple arithmetic task, while being alone in the room, without video recordings. To validate the successful stress induction, we obtained subjective ratings and physiological stress indicators, that is, blood pressure, pulse, and salivary cortisol at several time points across the experiment. For a detailed description of the task and timings of measurements, see Supporting Information.

2.3.1 | Day 1—Baseline phase and fear acquisition

Upon participants' arrival at the lab, they completed several questionnaires assessing control variables of interest (depression, Beck Depression Inventory [BDI-II; Beck et al., 1996]; anxiety, State-Trait-Anxiety-Inventory [STAI; Spielberger & Syndeman, 1994]; and chronic stress, Trier Inventory for the Assessment of Chronic Stress [TICS; Schulz & Schlotz, 1999]). After completing an unrelated, non-arousing task, the electrodes for US application and for recordings of electrodermal activity (EDA) were attached. For a detailed description of electrical stimulation and EDA analysis, see Supporting Information. Then, the individual pain threshold was determined using the QUEST procedure (Watson & Pelli, 1983), aiming at a shock intensity that was unpleasant but not painful. Next, the *baseline phase* of the fear generalization paradigm started which was immediately followed by the *fear acquisition phase*. At the end of Day 1, the pain strength rating was measured again.

2.3.2 | Day 2—Stress manipulation and test of fear generalization

About 24 hr later (range: 30 min to 3 hr), participants returned to the lab, the individual pain threshold was determined and depending on the experimental group, participants either underwent the TSST or the control manipulation. Immediately thereafter, participants were placed in the MRI scanner, completed again an unrelated, non-arousing task, before the critical *fear generalization phase* started. After the generalization test, all of the eight face stimuli were shown to the participants in a randomized circular arrangement and participants had to indicate which of the faces was followed by the shock. Outside of the scanner, participants performed a perceptual discrimination task, to check for participants' general discrimination ability (Supporting Information). At the end of Day 2, participants were debriefed and compensated for participation.

2.4 | Analysis of fear-tuning profiles

To characterize individual fear-tuning, we followed the approach of Onat and Büchel (2015) and set up a Gaussian model with two parameters (α, amplitude; σ, width), using MATLAB (Release 2016b, Natick, MA). We restricted our Gaussian model to be centered on the CS+-face. Fear-tuning profiles were calculated for z-scored skin-conductance response (zSCR) and rating data separately. For further statistical analyses, we extracted the two parameters (amplitude, width) of each profile.

2.5 | Behavioral and physiological data analysis

Statistical analyses were performed with SPSS 25.0 (IBM). Subjective and physiological data were analyzed by mixed-design ANOVAs with time and stimulus as within-subject factors and group (stress vs. control) as between-subjects factor. For simple group comparisons, independent sample *t* tests were used and for repeated measurements analyses we applied rmANOVAs. To investigate fear-tuning over time, we calculated a sharpening index (SI) by subtracting the width of the fear-tuning profile obtained for the test phase from the width of the fear-tuning profile obtained for the acquisition phase, that is, $\sigma_{Rating(Acqui)} - \sigma_{Rating(Test)}$. To analyze the perceptual discrimination ability, we calculated a discrimination score by subtracting the mean false alarm rate from the mean hit rate. Frequency of distribution was analyzed by means of Chi^2-tests and Cramer's V was used for group comparisons.

To investigate how time influenced the responding to the stimuli, we additionally calculated the mean response for the

stimuli most similar to the CS+ (|GS45|) and created a difference variable by subtracting this mean response from the CS+. In addition, we re-analyzed the behavioral results of two previous studies using the exact same paradigm in which the test phase was presented immediately after the fear acquisition phase (Kampermann et al., 2019; Onat & Büchel, 2015) and compared those results to ours.

All reported *p*-values are two-tailed, using a α-error threshold of $p = .05$. Significant main or interaction effects were pursued using the post hoc test, which were corrected for multiple comparisons. If the sphericity assumption was violated, Greenhouse-Geisser correction was applied.

2.6 | fMRI acquisition and analysis

fMRI data were acquired using a 3T MRI Scanner (Prisma, Siemens, Germany) with a 64-channel head coil. Sixty transversal slices were sequentially acquired using a T2-weighted echo-planar imaging sequence (2 s TR, 30 ms TE, 30° slice tilt, voxel size = 2 × 2 × 2 mm, 905 volumes). In addition, a high-resolution T1-weighted anatomical image was acquired (256 coronal slices, 2.5 s TR, 2.12 ms, voxel size = 0.8 × 0.8 × 0.8 mm).

Preprocessing and analysis of the fMRI data was performed using SPM12 (Wellcome Trust Centre for Neuroimaging, London). The first five functional scans were discarded, to allow for T1 equilibration. All functional volumes were motion-corrected and co-registered to anatomic images using rigid-body transformations. Both functional and structural images were normalized to the MNI standard brain. Finally, the normalized functional images were smoothed using a 4 mm FWHM Gaussian kernel.

To investigate neural fear generalization we followed the procedure described by Onat and Büchel (2015) and set up two different models. With the first model, we aimed to identify brain areas that mirrored a Gaussian shaped fear-tuning response. Therefore, we set up a linear regression model with the primary regressor representing the face onsets and two regressors of no interest (onsets of oddball trials and US trials), all of which were convolved with a canonical hemodynamic response function. In addition, we included two parametric modulators on responses evoked by our primary regressor, that is, the face stimuli, and the six realignment parameters as movement regressors. The parametric modulators were the same as in Onat and Büchel (2015) and represented (i) a Gaussian basis function and (ii) a numerical approximation of the derivative of the Gaussian function with respect to its standard deviation parameter ($dG/d\sigma$) to model a large variety of Gaussian-tuning profiles. On the individual first level, data were filtered in the temporal domain using a nonlinear high-pass filter with 128 s cut-off and we tested different combinations of the contrasts for the two parametric modulators, using a *t*-test. In line with common recommendations, we first conducted an exploratory whole-brain analysis, followed by a theory-driven analysis of a-priori defined regions of interest (ROIs; Poldrack, 2007). Those areas that exceeded a family-wise error (FWE) corrected statistical threshold of 0.05 (whole-brain) were defined as our ROIs. FWE-correction was performed without a cluster-extent threshold. Given their importance in fear generalization, we predefined the vmPFC, the insula and the amygdala as ROI and if not found on whole-brain level, we would investigate those areas with small-volume correction (SVC). In a second step, we aimed to precisely determine the activity in our ROIs to each individual face and to explicitly compare responding to CS+ versus CS-. Therefore, we set up a second linear model on the first level, that contained eight primary regressors, one for each face as well as the two regressors of no interest, again using the canonical hemodynamic response function and added the six realignment parameters as movement regressors. We extracted the eight beta-weights representing the activation levels for every individual face stimulus for each participant. Then, those beta-weights were used for the final parameterization of the fear-tuning profiles using the Gaussian-fitting procedure.

Anatomical locations were determined based on Automated Anatomical Labeling atlas (Tzourio-Mazoyer et al., 2002). At the group level, contrast images were analyzed using one-sample *t* tests and two-sample *t* tests for group comparisons. Correlations with the different stress parameters and brain regions were Bonferroni-corrected (critical *p*-value: $p/9 = .05/9 = .006$).

3 | RESULTS

3.1 | Control variables

Groups did not differ regarding their trait or state anxiety and subjective level of chronic stress (all $t \leq 1.194$; all $p \geq .237$; all $d \leq 0.301$, Table 1). However, there was a trend for a group difference in depressive mood ($t(61) = -1.958$, $p = .054$, $d = 0.494$), indicating a slightly higher degree of depressive mood in the stress group. To rule out a possible influence of depressive mood on our results, we included the BDI score as a covariate in all our analyses. Because this covariate left our results largely unaffected, we decided to report the analyses results without the covariate. In addition, we ran explorative analyses of our control variables with fear-tuning parameters for the behavioral and neural data, of which the results can be found in the Table S2. Due to the exploratory nature of this analysis, these results should be interpreted with caution.

TABLE 1 Control variables, psychophysiological and subjective measures on Day 1

Variable	Control	Stress
Control variables		
STAI-T	35.39 (1.47)	36.72 (1.57)
STAI-S	36.23 (1.31)	35.25 (1.00)
TICS	12.71 (1.31)	15.28 (1.70)
BDI-II	3.65 (0.59)	5.97 (1.02)
Experimental variables		
Salivary cortisol (nmol/L)	4.99 (0.68)	4.87 (0.53)
Systolic BP (mmHg)	122.37 (2.80)	120.74 (2.23)
Diastolic BP (mmHg)	80.82 (1.70)	81.92 (1.34)
Pulse (bpm)	81.68 (2.45)	78.88 (2.15)
Positive affect	2.91 (0.15)	2.69 (0.09)
Negative affect	1.28 (0.07)	1.21 (0.06)
Pain threshold (V)	52.75 (2.44)	53.47 (2.40)
Pain strength start	5.29 (0.36)	5.27 (0.34)
Pain strength end	5.35 (0.34)	5.09 (0.40)

Note: Data represent mean (standard error of the mean).

3.2 | Day 1: Baseline phase and fear acquisition

Before the beginning of testing on Day 1, groups did not differ in their subjective mood, salivary cortisol, blood pressure, heart rate, estimated pain threshold or pain strength rating (all $t \leq 1.292$; all $p \geq .201$; all $d \leq 0.326$, Table 1). In addition, participants experienced the US as uncomfortable from beginning until the end of testing, without differences between groups (all $F \leq 0.217$; all $p \geq .750$; all $\eta^2 \leq 0.003$).

3.2.1 | Baseline responses to face stimuli

As displayed in Figure 2a, both groups rated the faces comparably after the baseline phase (both $F \leq 0.974$; both $p \geq .328$; both $\eta^2 \leq 0.015$). There was a face stimulus main effect (F (3.4049, 211.356) = 3.197, $p = .019$, $\eta^2 = 0.049$). However, after correcting for multiple testing, no post hoc comparison approached statistical significance (all $p \geq .130$). Regarding the zSCR data, the stress group showed a slightly higher SCR than the control group during the *baseline phase* (F (1, 62) = 4.042, $p = .049$, $\eta^2 = 0.061$; Figure 2b). More importantly, however, there was no main effect of face stimulus and no group × face stimulus interaction (both $F \leq 1.725$; both $p \geq .120$; both $\eta^2 \leq 0.027$).

3.2.2 | Successful fear acquisition

Participants showed successful fear acquisition, as indicated by higher responding to the CS+ compared to the CS-, in both the subjective rating data (F (1, 62) = 507.982, $p < .001$, $\eta^2 = 0.891$; Figure 2c) and the zSCR data (F (1, 62) = 21.272, $p < .001$, $\eta^2 = 0.255$; Figure 2d). Importantly, there were no group differences in fear acquisition, neither in the rating nor in the zSCR data (all $F \leq 1.597$; all $p \geq .211$; all $\eta^2 \leq 0.025$).

3.3 | Day 2: Stress exposure and delayed test of fear generalization

Upon their arrival on Day 2, groups did not differ in subjective mood, salivary cortisol, blood pressure, heart rate or pain strength rating (all $t \leq 1.649$; all $p \geq .104$; all $d \leq 0.412$; see Table S1). With respect to the pain strength rating, participants rated the US as more painful after compared to before the *fear generalization phase* (F (1, 62) = 17.726, $p < .001$, $\eta^2 = 0.222$), independent of experimental group (both $F \leq 1.667$; both $p \geq .201$; both $\eta^2 \leq 0.026$).

3.3.1 | Successful stress-induction by the TSST

Significant changes in subjective and physiological parameters verified the successful stress induction by the TSST (Figure 3). On the subjective level, participants of the stress group felt more challenged, uncomfortable and stressed after the task than participants of the control group (all $t \geq -4.948$; all $p \leq .001$; all $d \geq 1.238$). Salivary cortisol, blood pressure, and heart rate increased from before to after the manipulation in the stress group (all $F \geq 6.251$; all $p < .001$; all $\eta^2 \geq 0.168$) but not in the control group. For the control group, there was even a significant decrease in salivary cortisol and pulse over time (both $F \geq 7.229$; both $p < .001$; both $\eta^2 \geq 0.194$). Importantly, post hoc t tests showed that groups significantly differed in their cortisol concentrations 20 min, 60 min and 110 min after the treatment, implicating significantly elevated cortisol concentrations in the stress group throughout the critical fear generalization test (all $t \geq -3.035$; all $p \leq .004$; all $d \geq 0.759$). Regarding the autonomic measurements, the stress group showed increased systolic and diastolic blood pressure compared to the control group during and 20 min after the TSST (all $t \geq -2.980$; all $p \leq .004$; all $d \geq 0.751$). The pulse was only significantly different during the stress/control manipulation ($t = -2.054$, $p = .045$, $d = 0.508$).

3.3.2 | No influence of stress on behavioral fear generalization

Twenty-four hours after fear acquisition, participants still showed intact fear memory, indicated by a higher response to the CS+ compared to the CS- in both rating and zSCR data (both $F \geq 42.465$; both $p < .001$; both $\eta^2 \geq 0.410$), without

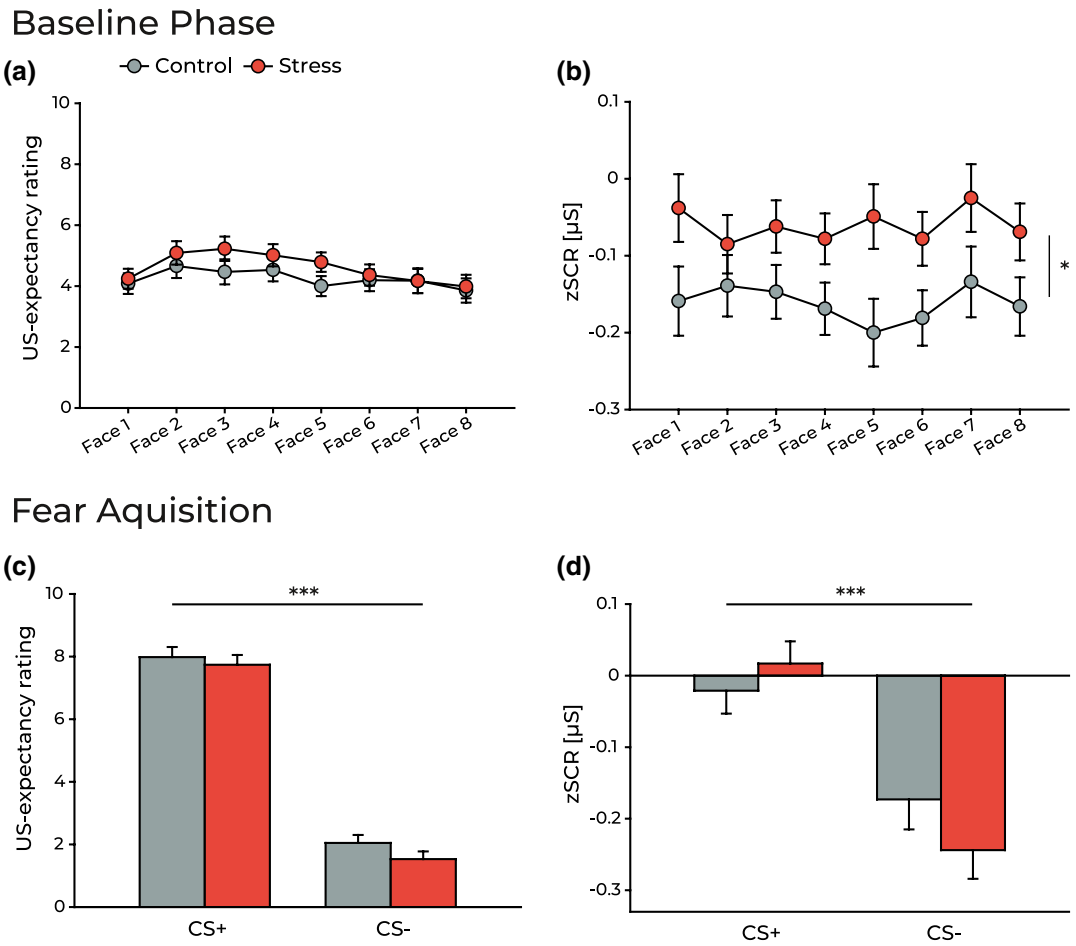

FIGURE 2 Day 1: Physiological and subjective responses to the face stimuli during the baseline and fear acquisition phases. (a) Explicit rating data as well as (b) zSCR data show no systematic a priori differences between faces and no group differences during or after the baseline phase. During and after fear acquisition, both (c) explicit US-expectancy rating data as well as (d) zSCR data show successful fear learning reflected in higher responses to the CS+ than to the CS-. Error bars represent standard errors of the mean. Asterisks denote differences between stimuli (*$p < .05$, ***$p < .001$)

any influence of group (all $F \leq 0.662$; all $p \geq .419$; all $\eta^2 \leq 0.011$). In addition, we obtained clear evidence for fear generalization, shown by fear-tuning profiles, resembling a Gaussian function, for both explicit and implicit fear data (Figure 4a,b, respectively). Statistically, an rmANOVA for the eight stimuli showed a main effect of stimulus in both measures (both $F \geq 24.794$; both $p \leq .001$; both $\eta^2 \geq 0.289$) with larger quadratic within-subject contrasts compared to linear ones (both $F \geq 45.884$; both $p \leq .001$; both $\eta^2 \geq 0.429$). Importantly, there was no main effect of group or stimulus × group interaction effect (all $F \leq 1.445$; all $p \geq .234$; all $\eta^2 \leq 0.023$), that is, stress did not modulate fear generalization. To investigate the influence of stress on delayed fear generalization in more detail, we compared the parameters of our Gaussian fear-tuning profiles obtained on Day 2 between groups. Results again showed that groups did not differ regarding their amplitude or width across measurements on Day 2 (all $t \leq 1.052$; all $p \geq .297$; all $d \leq 0.265$). Moreover, stress did not influence the change of amplitude or width of the rating data from fear acquisition to the test of fear generalization (all $F \leq 1.163$; all $p \geq .285$; all $\eta^2 \leq 0.019$).

Exploratively, we also conducted correlational analyses between the different stress mediators, that is, systolic and diastolic blood pressure, pulse and cortisol, and different fear-tuning widths. In general, there were only few significant correlations, which were not constant. These results can be found in the Supporting Information.

3.3.3 | Fear generalization requires fear reactivation

To investigate if the reminder US in the test phase had an impact on fear generalization, we calculated fear-tuning curves for reinforcement bins, representing different distances to the last US and subjected these data to a Gaussian fear-tuning analysis (Figure 5). As shown in Figure 5, a first US was necessary for a reinstatement of fear in general and consequently for fear generalization, in both groups. We hypothesized that a reminder US is necessary for the development of a Gaussian fear-tuning curve. This assumption was confirmed by rmANOVAs, showing a significant stimulus × proximity

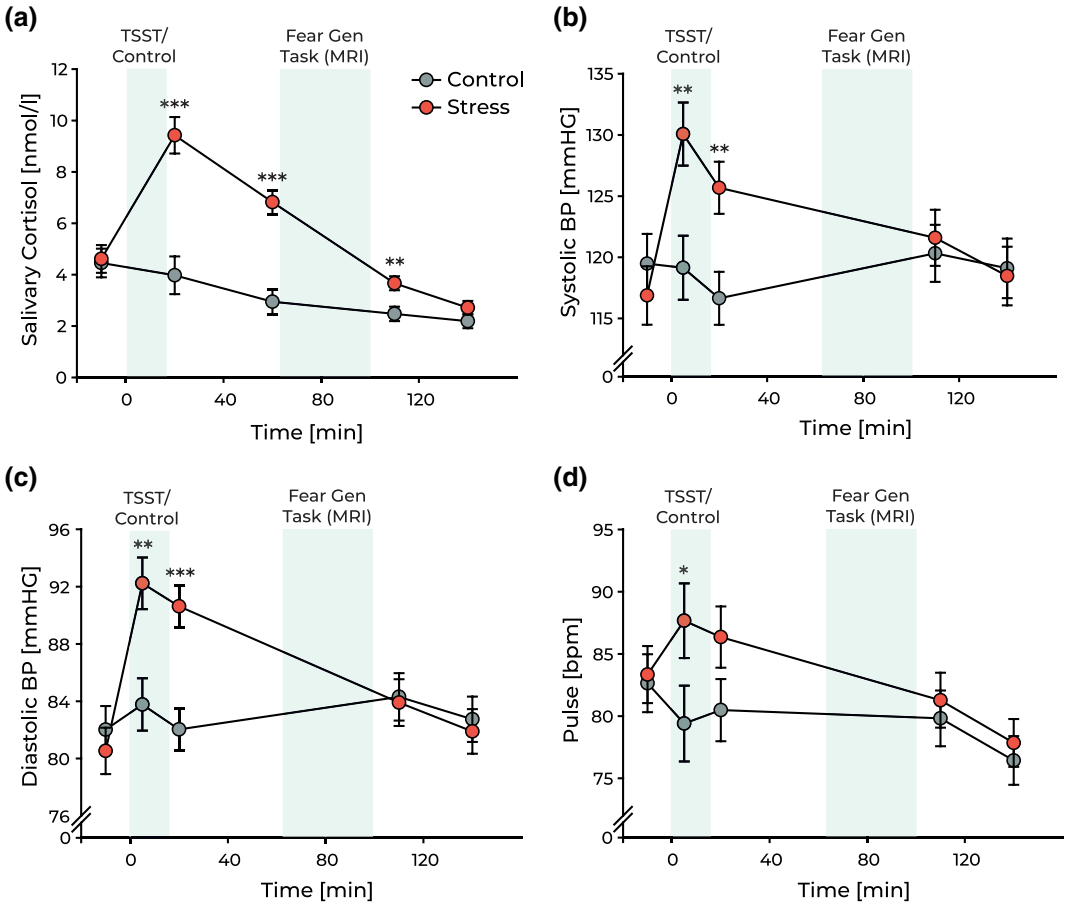

FIGURE 3 Stress manipulation check. (a) Salivary cortisol increase. (b) Systolic blood pressure increase. (c) Diastolic blood pressure increase. (d) Pulse increase. Error bars represent standard errors of the mean. Asterisks denote difference between groups. (***$p < .001$, **$p < .01$, *$p < .05$)

interaction (F (8.513, 510.783) = 1.752, $p < .001$, $\eta^2 = 0.101$). After the first US-administration during the test phase, participants showed in all proximity bins successful fear-tuning, that is, the strongest reaction toward the CS+, that decreased with increasing dissimilarity. This was confirmed by post hoc rmANOVAs for each proximity bin separately. Those showed that the reaction to the stimuli followed a quadratic trend (all $F \geq 31.579$; all $p < .001$; all $\eta^2 \geq 0.345$) compared to a linear trend (all $F \leq 5.595$; all $p \geq .021$; all $\eta^2 \leq 0.085$). In contrast, before any US was administered, the stimulus reaction rather followed a linear trend ($F = 8.388$; $p = .005$; $\eta^2 = 0.123$) instead of a quadratic one ($F = 4.073$; $p = .048$; $\eta^2 = 0.064$), suggesting that a precise fear memory was missing. Furthermore, the analyses of the Gaussian model parameters revealed a significant proximity effect for the amplitude (F (1.796, 107.779) = 12.138, $p < .001$, $\eta^2 = 0.168$) and post hoc comparisons revealed a significant lower fear reaction from before compared to after US-administration (all $p \leq .042$). These results further underpin the need for a reminder to reactivate fear-memory. Regarding the width of fear generalization, results revealed no significant effect of proximity (F (3, 180) = 0.768, $p = .513$, $\eta^2 = 0.013$). This pattern did not differ between the stress and control groups, suggesting that stress had no modulatory effects on the need of a reminder US for the development of fear-tuning (all main or interaction effects: all $F \leq 0.323$; all $p \geq .702$; all $\eta^2 \leq 0.005$).

3.3.4 | Hyper-sharp fear-tuning in brain regions beyond the insula after a 24-hr delay, irrespective of stress

To investigate the neural underpinnings of delayed fear generalization, we analyzed in a first step which brain areas showed a fear-tuning comparable to our behavioral data (i.e., following a Gaussian function). At the whole-brain level (FWE-corrected $p < .05$), several areas showed the predicted fear-tuning (Table 2), many of them overlapping with previous reports on the neural underpinnings of fear generalization (Dunsmoor et al., 2011; Greenberg et al., 2013; Lissek et al., 2014; Onat & Büchel, 2015). Most importantly, two of these regions, the bilateral insula and the right frontal operculum, showed increased activity in response to the CS+ and declining activity as the face stimuli became more dissimilar

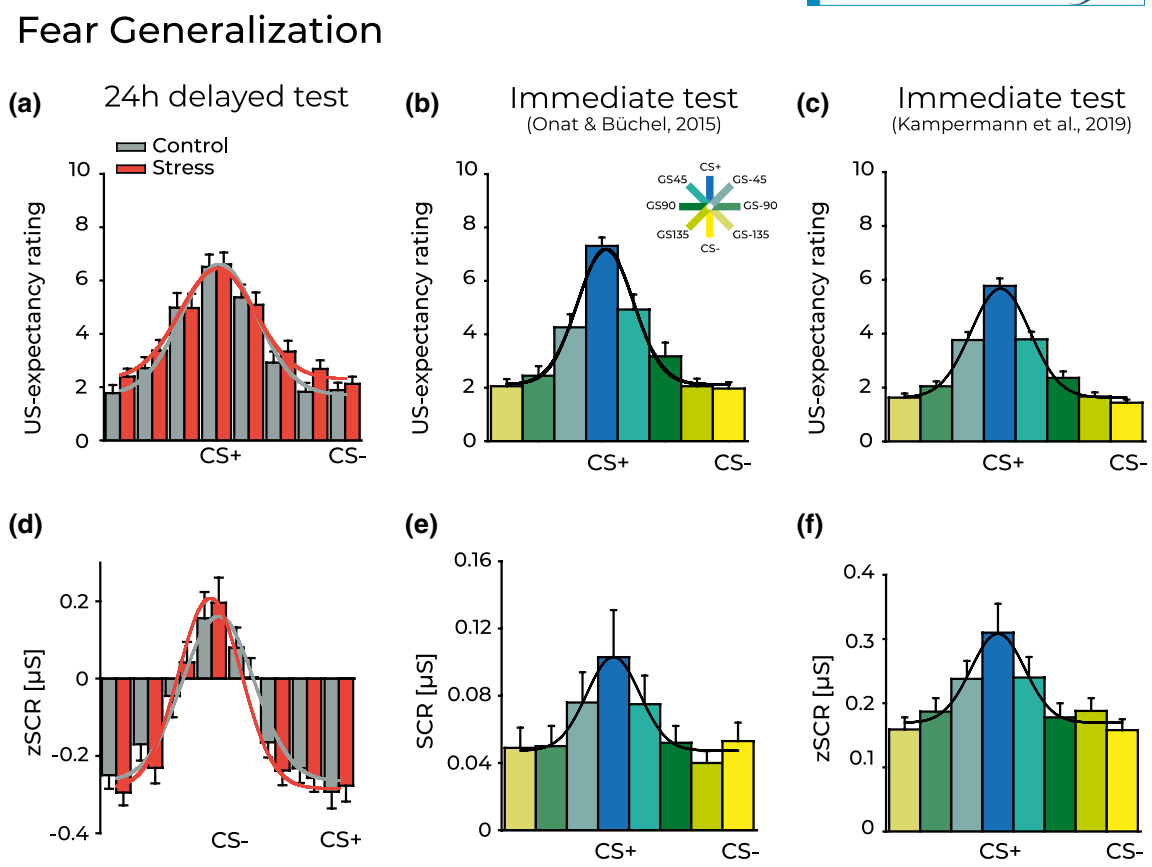

FIGURE 4 Day 2: Fear generalization phase of the different studies. Figures depict the responses to the different stimuli. Across all studies, fear-tuning is observed in (a-c) explicit fear learning, represented by US-expectancy ratings as well as (d-f) implicit fear learning, represented in electrodermal activity. For the current study (a+d) responses are depicted for the stress group and the control group separately. Error bars represent standard errors of the mean

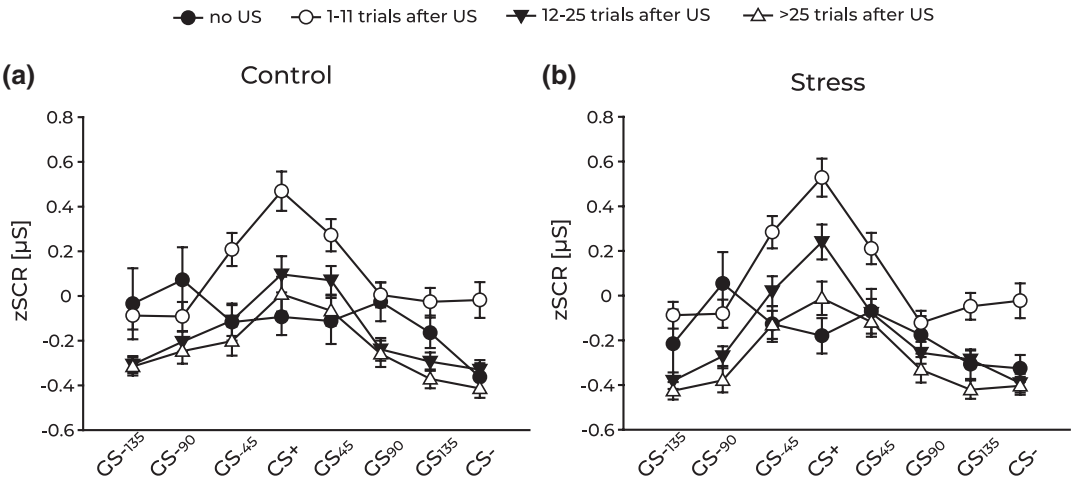

FIGURE 5 Fear-tuning dependent on US-proximity. For both the stress group and the control group, a reminder US during the test phase is necessary for the evolvement of an actual quadratic fear-tuning

to the CS+. All other regions (frontal, temporal, and hippocampal regions, angular gyrus and left precuneus) showed an inverted gauss function, that is, reduced activation to the CS+ (Figure 6a,b). Interestingly, in line with previous results (Onat & Büchel, 2015), we found a hyper-sharp tuning of the bilateral insula (i.e., smaller width compared to the behavioral data) even after a 24-hr delay. This is depicted by a significant difference of the fear-tuning width in the left insula compared to the width of rating as well as zSCR data, being narrower on the neural level (both $p \leq .042$). For the right insula, the

TABLE 2 Brain areas showing Gaussian fear-tuning

Brain region	T-value	$P_{FWE\text{-}corr}$	MNI coordinates		
			X	*Y*	*Z*
Whole-brain					
L. middle temporal gyrus	−7.56	0.000	−60	−6	−20
L. angular gyrus	−7.28	0.000	−42	−72	36
R. parahippocampal gyrus	−7.02	0.000	24	−16	−20
L. middle orbital gyrus (vmPFC)	−6.91	0.000	−6	60	2
R. angular gyrus	−6.78	0.000	48	−68	30
L. precuneus	−6.73	0.000	−6	−56	16
L. insula	6.63	0.000	−34	22	6
R. insula	6.62	0.000	34	30	6
L. middle frontal gyrus	−6.53	0.000	−26	22	50
L. parahippocampal gyrus	−6.50	0.000	0	20	−16
R. middle temporal gyrus	−5.92	0.003	62	−14	−18
R. frontal operculum	5.41	0.017	34	10	26
R. middle frontal gyrus	−5.36	0.019	26	32	46
Small-volume corrected					
R. amygdala	−3.77	0.007	20	8	−18
L. amygdala	−3.70	0.007	−18	−6	−22

differences were non-significant (both $p \geq .163$). The reversed fear-tuning pattern of the vmPFC showed a significantly narrower width compared to the zSCR data ($p = .037$), suggesting an increased neural inhibition of fear-tuning after 24 hr. Compared to the rating data, fear-tuning in the vmPFC was also narrower but this difference was not significant ($p = .190$). While all of the aforementioned results are based on a whole-brain analysis, we also performed a pre-defined ROI analysis that focused on the amygdala, an area known to play a key role in fear processing (Büchel & Dolan, 2000; Phelps et al., 2001). In line with the result of the previous study on immediate fear generalization testing (Onat & Büchel, 2015), the amygdala displayed an inversed fear-tuning curve (Figure S1).

Next, we contrasted the fear-tuning related contrast images between the stress and control groups, to investigate a possible influence of stress on the neural signature of fear generalization. On a whole-brain level with a FWE-corrected threshold, we did not observe any differences. Using SVC, we could show that participants of the stress group showed a stronger fear-tuning in the left insula ($T = 3.34$, $p_{SVC} = 0.019$ (FWE)). There was no influence of stress on any other of our ROIs (all $T \leq 2.05$, $p_{SVC} = 0.276$ (FWE)).

3.3.5 | Increased fear generalization after a 24-hr delay

Because we used the same paradigm as in two previous studies, which investigated immediate fear generalization (Kampermann et al., 2019; Onat & Büchel, 2015), we aimed to exploratively compare our results of delayed fear generalization with those of generalization tested immediately after fear acquisition. For this comparison, we only included the control group of the present study. In general, there was a wider fear generalization after a delay of 24 hr (Table 3). To investigate how fear responding changed from fear acquisition to the test of fear generalization, we first compared the sharpening index (SI) between studies (Figure 7a). Interestingly, results indicated that fear-tuning of subjective data decreased from fear acquisition to the test of fear generalization when tested on the same day but increased when fear generalization is tested 24 hr later (F (2, 130) = 5.256, $p = .006$, $\eta^2 = 0.075$). Post hoc comparisons revealed a significant difference between the current study and the non-fMRI study ($p = .002$), but only a non-significant trend in the same direction between the current study and the previous fMRI study ($p = .113$). There was no statistically significant difference between the two studies with an immediate test of fear generalization ($p = .201$).

Next, we compared the parameters of the fear-tuning profiles on Day 2 across studies. Results of the rating data for the fear-tuning width, mirrored results of the SI, showing a trend for a wider fear-tuning after a 24-hr delay (F (2, 130) = 2.791, $p = .065$, $\eta^2 = 0.041$; Figure 7b). Post hoc tests corrected for multiple comparisons revealed a significant difference between the current study and the non-fMRI study ($p = .026$) and a trend for a difference between the two fMRI studies ($p = .059$), without any statistically significant difference

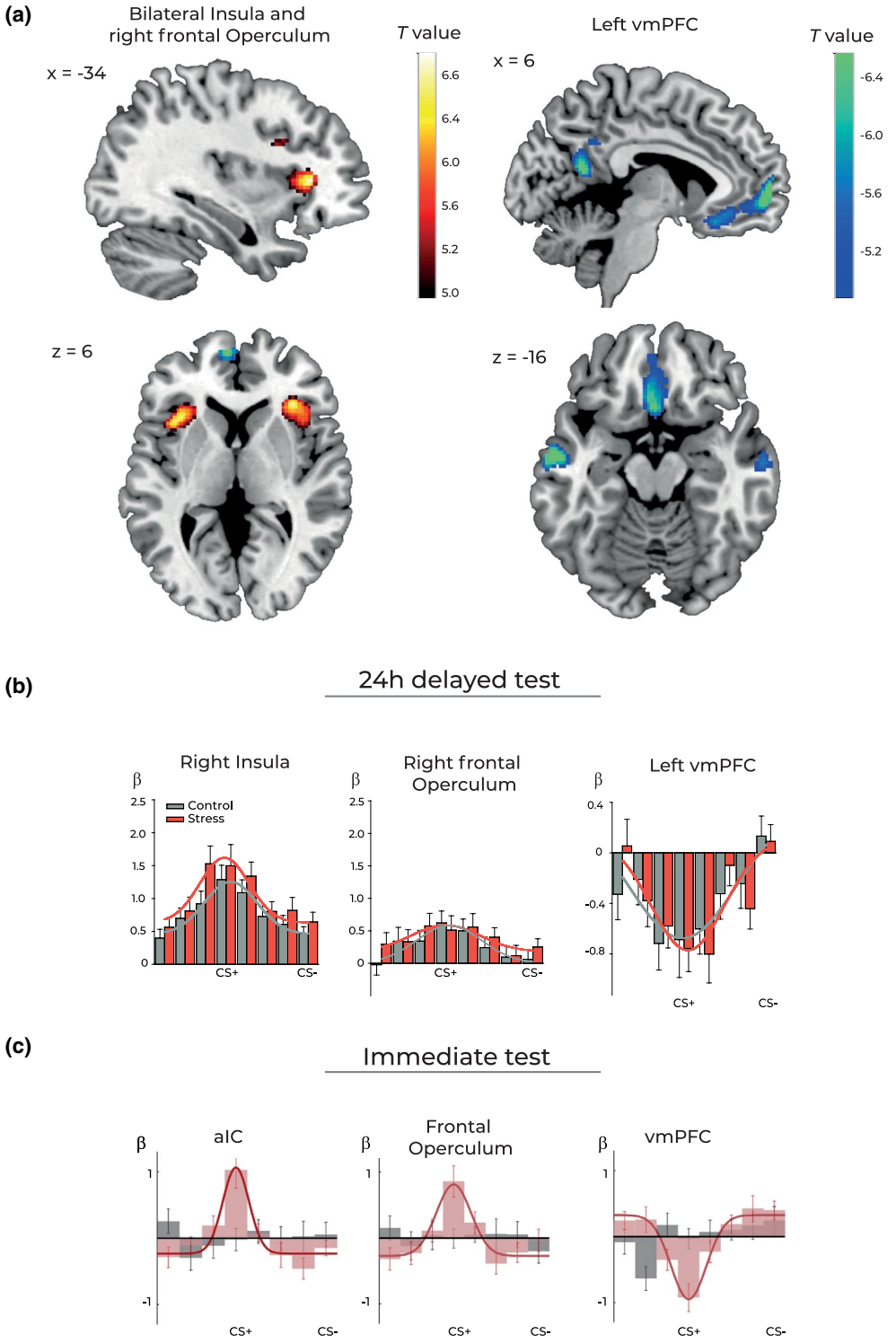

FIGURE 6 Brain areas showing Gaussian fear-tuning at the whole-brain level. (a) The bilateral insula as well as the right frontal operculum show a positive association with fear-tuning, whereas the left vmPFC is found to be negatively related to fear-tuning, thus reflecting safety tuning. Thresholded statistical maps ($p < .05$, FWE-corrected) depict fear-tuning clusters and functional maps are normalized to MNI space. (b) Fear-tuning profiles of the peak-voxel of the clusters depicted in (a) during the test of fear generalization. Bars represent the averaged neural responses across participants for each stimulus separately. The fourth bar represents the CS+, the eighth bar, the CS-. Error bars represent standard errors of the mean

between the two previous studies ($p = .966$). Regarding the amplitude, there was a significant study effect (F (2, 130) = 3.422, $p = .036$, $\eta^2 = 0.050$; Figure 7c). However, post hoc tests suggested that this effect was mainly driven by the environment of testing. Participants that were tested outside of the scanner showed a lower amplitude compared to those

TABLE 3 Comparison of fear-tuning width for behavioral and neuronal data

Variable	Current study (control group)	Onat and Büchel (2015)[1]	Kampermann et al. (2019)
$\sigma_{SCR(Test)}$	1.03 (0.48)	0.72 (0.36)	0.92 (0.61)
$\sigma_{Rating(Test)}$	0.99 (0.37)	0.78 (0.42)	0.78 (0.46)
SI	−0.13 (0.48)	0.06 (0.40)	0.18 (0.44)
$\sigma_{R.\ Insula}$	0.86 (0.55)	0.65	
$\sigma_{L.\ vmPFC}$	0.82 (0.57)	0.46	
$\sigma_{L.\ Insula}$	0.76 (0.49)		

Note: Data represent mean (standard deviation).

Abbreviations: SI = Sharpening index, that is, $\sigma_{Rating(Acqui)}-\sigma_{Rating(Test)}$.

[1]Behavioral data were re-analyzed with inference statistical analysis, why results differ to the results reported in the original paper. Data of neural fear-tuning are taken directly from Onat and Büchel (2015).

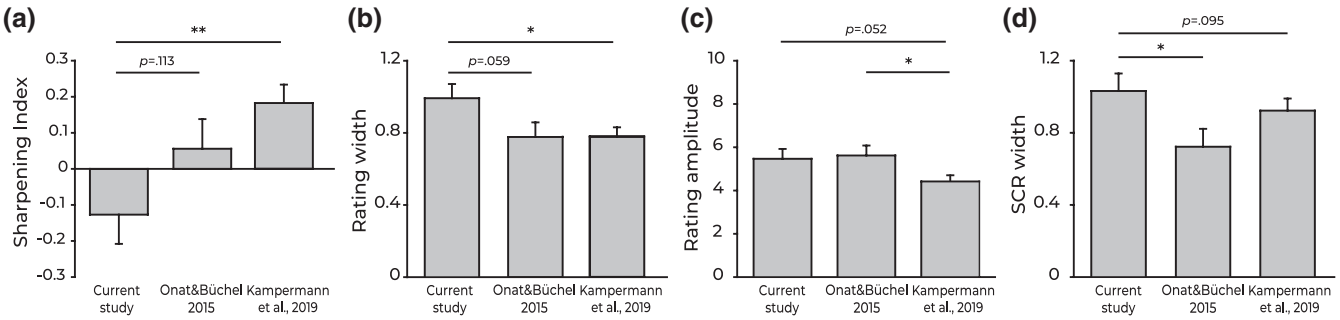

FIGURE 7 Fear-tuning results across studies. The current study tests fear generalization after a 24-hr delay, whereas the two previous studies (Onat & Büchel, 2015; Kampermann et al., 2019) tested fear generalization. (a) When fear generalization is tested after a delay compared to immediately after fear acquisition, the strength to differentiate between the CS+ compared to the stimuli most similar to it, decreases. This is revealed by a negative sharpening index (SI; $\sigma_{Rating(Acqui)}-\sigma_{Rating(Test)}$) for the current study compared to a positive SI for the studies having an immediate test of fear generalization. Furthermore, (b) the width of fear-tuning for the test of fear generalization is wider in the current study than in previous studies. (c) The amplitude of the rating data, however, shows a higher fear-tuning amplitude in both fMRI studies compared to the non-fMRI study. (d) The comparison of the fear-tuning width of the SCR data mirrors the results of the rating data, showing a broader fear generalization of the current study compared to the previous studies. Error bars represent standard errors of the mean. Asterisks denote difference between studies. (**$p < .01$, *$p < .05$)

tested in the MRI, immediately after fear acquisition ($p = .026$) and after a 24-hr delay ($p = .052$).

Because the three studies differed in the preprocessing of SCR data, which precluded a direct comparison of SCR amplitudes, we compared only the width of the fear-tuning profiles. Results again revealed a marginal effect of study (F (2, 119) = 2.612, $p = .078$, $\eta^2 = 0.042$), suggesting a trend for wider fear-tuning after a delay of 24 hr compared to an immediate test (Figure 7d). Post hoc tests revealed a significant difference between the two fMRI studies ($p = .027$) and a trend between the two previous studies ($p = .095$), but no statistically significant difference between the current study and the non-fMRI ($p \geq .355$).

In a next step, we compared the neural representation of fear generalization when tested shortly after fear acquisition versus after a 24-hr delay, by comparing the width parameter of the Gaussian fear-tuning reported after an immediate fear generalization test (Onat & Büchel, 2015) with those obtained by our delayed testing (Table 3). Descriptively, the neural fear-tuning was wider after a delay of 24 hr, mirroring the pattern of the behavioral data.

3.3.6 | Increased responding to similar stimuli accounts for broader fear generalization

To rule out that a wider fear generalization is due to a change in CS+/CS- discrimination from fear acquisition to fear generalization testing, but rather due to altered responding to stimuli most similar to the CS+, that is, the GS45 and GS-45, we compared the difference in responding to the respective stimuli across phases and studies. Because we only showed CS+ and CS- during the fear acquisition phase but obtained US-expectancy ratings for all of the eight faces, only rating data were analyzed.

For the CS+/CS- discrimination, results revealed significant main effects of phase, stimulus and study (all $F \geq 8.833$; all $p < .001$; all $\eta^2 \geq 0.119$). In addition, there was a significant phase × stimulus interaction (F (1, 131) = 8.586, $p = .004$, $\eta^2 = 0.062$), showing a decrease in responding to the CS+ ($p < .001$) but not to the CS- ($p = .085$) across phases, without any difference between studies (all $F \leq 1.961$; all $p \geq .145$; all $\eta^2 \leq 0.029$). Interestingly, the analysis of CS+/|GS45| differentiation revealed, in addition to the main effects of time, stimulus, and study (all $F \geq 7.002$; all $p \leq .001$; all $\eta^2 \geq 0.097$), a time × stimulus × study interaction (F (2, 131) = 3.079, $p = .049$, $\eta^2 = 0.045$; Figure 8). Following up on this interaction revealed that after fear acquisition, there was no statistically significant difference in CS+/|GS45| differentiation between studies (F (2, 131) = 0.978, $p = .379$, $\eta^2 = 0.015$), but a trend for such a difference after the test of fear generalization (F (2, 131) = 2.931, $p = .057$, $\eta^2 = 0.043$). After a 24-hr delay, participants did not differentiate between the CS+ and the most similar stimuli as strongly as participants did when tested immediately after fear acquisition. Statistically, this was supported by a strong trend for a difference between the two fMRI studies ($p = .051$). The other comparisons were non-significant (both $p \geq .421$).

Together, these results show that the broader fear generalization cannot be explained by a changed threat-safety discrimination but rather by a reduction in the discrimination between the threatening stimulus CS+ and the stimuli most similar to the CS+. Importantly, this reduction only occurs after a delay of 24 hr but is not found when the test of fear generalization follows immediately after the phase of fear acquisition.

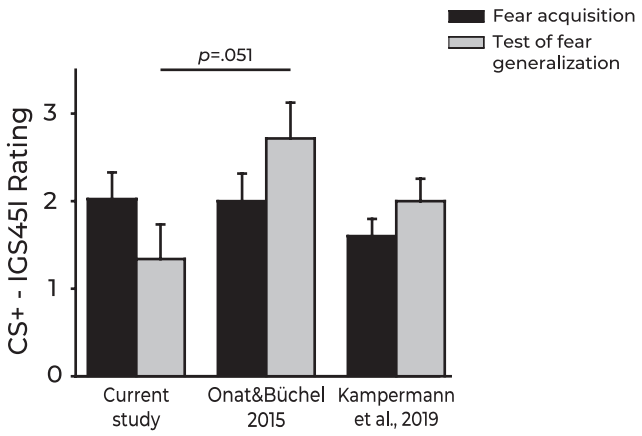

FIGURE 8 Difference in US-expectancy rating between the CS+ and the most similar stimuli, that is, |GS45|, from fear acquisition to test of fear generalization. Whereas there is no difference in CS+/|GS45| differentiation between studies during fear acquisition, the current study, which has a delayed test of fear generalization, shows a decreased differentiation strength during the test of fear generalization compared with studies having fear generalization immediately after fear acquisition

4 | DISCUSSION

Fear generalization is assumed to be a critical process in the development and maintenance of anxiety disorders (Lissek et al., 2005). While virtually all previous studies tested fear generalization shortly after fear acquisition, we investigated fear generalization and its neural underpinnings after a delay of 24 hr. Our findings showed intact fear memory and a pronounced fear generalization—both at the subjective, physiological, and neural level—after 24 hr. In addition, we determined the impact of acute stress on fear generalization. Although subjective and physiological parameters confirmed the successful stress induction, the generalization of fear was left largely unaffected by the stress manipulation. Moreover, a direct comparison of our findings to two previous studies using the same fear generalization paradigm but with the test phase presented immediately after fear acquisition (Kampermann et al., 2019; Onat & Büchel, 2015) revealed that the generalization of fear increased at the longer delay. This was reflected in a stronger responding to the stimuli most similar to the CS+.

Our neural data showed a fear-tuning profile indicative of fear generalization in the same brain regions that have been reported before during immediate fear generalization (Greenberg et al., 2013; Lissek et al., 2014; Onat & Büchel, 2015). In particular, the insula and the frontal operculum were associated with fear-signaling, showing the highest activation for the CS+, which declined for the other GSs with increasing dissimilarity to the CS+. In contrast, the vmPFC, hippocampal, middle frontal, and middle temporal regions rather reflected safety-signaling, that is, activation of these areas was associated with a strong deactivation toward the CS+, which declined with increasing similarity toward the safety-signaling CS-. It is important to note that we did not find fear-tuning in the amygdala in our whole-brain analyses. Given that the amygdala is a key structure in fear-learning processes (Büchel & Dolan, 2000), we also analyzed fear-tuning in the amygdala with an ROI-driven approach, obtaining an inversed fear-tuning curve. This is generally in line with a previous study that also did not observe significant fear-tuning in this region (Onat & Büchel, 2015). The amygdala's strong habituation effects (Breiter et al., 1996), deep location, and the fact that it is a relatively small brain structure that is difficult to image (Zald, 2003) make it particularly difficult to detect amygdala activity in a whole-brain FWE-corrected analysis. Importantly, when comparing the width of the neural fear-tuning to the previous findings observed immediately after fear acquisition (Onat & Büchel, 2015), results revealed wider neural fear-tuning curves.

How can the increased fear generalization after a delay of 24 hr be explained? One possibility might be a diminished fear memory in general. However, the CS+/CS- differentiation was comparable between the two testing days, same as

the threat/safety differentiation between an immediate and a delayed test of fear generalization, suggesting that fear memory was intact after 24 hr. In contrast to the CS+/CS- differentiation, the differentiation between the CS+ and the stimuli most similar to it changed over time. Interestingly, this differentiation also changed in the two studies that tested fear generalization immediately after fear acquisition, but in the direction of an increased differentiation, whereas we observed a diminished differentiation between the previously conditioned CS+ and the stimuli most similar to it. Support for the influence of time on a broader fear memory generalization comes from studies, suggesting that sleep plays an important role regarding a transformation process from a detailed to a more gist-like memory representation (Gais et al., 2007; Menz et al., 2013, 2016).

While the behavioral and neural fear generalization appeared to become broader after a 24-hr delay, it remained largely unaffected by stress. Acute stress shortly before the test of fear memory generalization did not alter fear memory expression or fear generalization, expressed as SCR, nor the neural underpinnings of fear generalization. This is in contrast to the only previous study that focused on the influence of acute stress prior to a test of fear generalization in humans (Dunsmoor, Otto, et al., 2017), which suggested that acute stress led to a heightened fear generalization. Previous rodent studies, however, also yielded inconsistent results. Whereas two studies found an increased fear generalization after corticosterone administration (Bender et al., 2018; Kaouane et al., 2012), another study failed to obtain any impact of corticosterone administration on the extent of fear generalization (Bueno et al., 2017). Importantly, there are some major differences between these studies and our study that may explain the different findings. First of all, the other human study used a different fear generalization paradigm (Dunsmoor, Otto, et al., 2017), using auditory stimuli instead of visual and socially relevant face stimuli. Furthermore, significantly fewer trials were administered during fear acquisition and fear generalization, with a higher reinforcement rate. Thus, fear learning was much more intense in the present study, which may have resulted in a reduced vulnerability to the stress manipulation. Moreover, while fear generalization was tested 15 to 30 min post-stress onset in the previous study, we conducted our test 60 to 95 min post-stress onset in the MRI scanner that could have resulted in a heightened arousal in general in both groups (Muehlhan et al., 2011). Our results support the idea that the environment of testing influences fear memory, specifically the amplitude of fear-tuning was heightened when participants were tested in the MRI scanner compared to outside. Together with the longer delay for testing after stress onset, this could have impeded a possible influence of stress on fear generalization to evolve. When comparing our study to the animal studies, there are crucial differences in timing and type of stress system manipulation. Most of the studies investigated the impact of corticosterone injections (Bueno et al., 2017; Kaouane et al., 2012), which is entirely different from a psychological stress manipulation which targets both the autonomic nervous system and the HPA axis and additionally increases subjective stress levels (Kirschbaum et al., 1993). Moreover, instead of manipulating stress system activity shortly before a test of fear generalization, these studies induced stress either before (Bender et al., 2018) or immediately after fear acquisition (Bueno et al., 2017; Kaouane et al., 2012). Thus, all of these studies affected the process of fear memory consolidation, which prevents a specific analysis of the impact of stress on fear memory generalization.

In line with a published review that highlights the role of conceptual knowledge for fear generalization (Dunsmoor & Murphy, 2015), Onat and Büchel (2015) suggested that fear generalization was not just passively driven by perception but was an active process, in which multiple source of information were integrated. They based their conclusion on the finding that the insula showed less generalization than behavioral responses and that the inferotemporal cortex, known to be implicated in perceptual processing, rather responded to uncertainty. However, the paradigm still included perceptually similar stimuli and we cannot fully rule out the possibility that a paradigm using higher order conditioning might have resulted in a different outcome. Furthermore, it is well known that sleep is highly relevant for the consolidation of memory (Diekelmann & Born, 2010), including fear memory (Pace-Schott et al., 2015). Thus, future studies that aim to investigate time-dependent changes in fear memory generalization should include measures of sleep quality and duration between acquisition and test sessions. Finally, it is to be noted that our explorative analysis of time-dependent changes in the magnitude and neural underpinnings of fear generalization was based on a comparison across separate studies, that is, without a random allocation of participants to experimental conditions (immediate vs. delayed test). Therefore, it cannot be fully ruled out that any differences between studies may have driven the seeming differences in fear generalization. Future studies that include explicit immediate and delayed test conditions are required to determine whether there are time-dependent changes in fear generalization.

In sum, we show that stress leaves 24 hr-delayed fear generalization and its neural signature largely unaffected. Furthermore, we provide first evidence suggesting that a delay of 24 hr results in a broader generalization of conditioned fear. This increase of fear generalization was reflected both in SCRs and the neural substrates of fear generalization. This finding may be highly relevant in the context of anxiety disorders, in which the threatening event typically dates back long in time. Based on our results, one might expect an even broader fear generalization in these long-established fear memories which may well contribute to the maintenance of the disorder. Identifying ways to interfere with old fears and strong fear generalization remains a challenge for future research.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Franziska Kausche: Data curation; Formal analysis; Investigation; Methodology; Project administration; Validation; Visualization; Writing-original draft; Writing-review & editing. **Gundula Zerbes:** Data curation; Investigation; Writing-review & editing. **Lea Kampermann:** Formal analysis; Resources; Validation; Writing-review & editing. **Christian Büchel:** Conceptualization; Resources; Software; Writing-review & editing. **Lars Schwabe:** Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Writing-original draft; Writing-review & editing.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

Supplementary Material

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Appendix B

Study II

Noradrenergic stimulation increases fear memory expression

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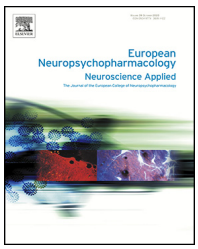

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Noradrenergic stimulation increases fear memory expression

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Pharmacological manipulation;
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Abstract
Fear responses are typically not limited to the actual threatening stimulus but generalize to other stimuli resembling the threatening stimulus. Although this fear generalization is generally adaptive, fear overgeneralization is maladaptive and assumed to contribute to anxiety disorders. Despite the clinical relevance of fear (over)generalization, how the extent of fear generalization is modulated remains not well understood. Based on the known effects of stress on learning and memory, we tested here the impact of major stress mediators, glucocorticoids and noradrenergic arousal, on fear generalization. In a laboratory-based, placebo-controlled, double-blind, between-subject design, 125 healthy participants first underwent a fear conditioning procedure. About 24 h later, participants received orally either a placebo, hydrocortisone, the α2-adrenoceptor antagonist yohimbine, leading to increased noradrenergic stimulation, or both drugs before a test of fear generalization. Skin conductance responses as well as explicit rating data revealed that yohimbine intake led to enhanced fear memory expression, i.e. an enhanced responding to the CS+ but not to stimuli resembling the CS+. Moreover, neither enhanced safety learning nor a mere enhancement of perceptual discrimination ability could explain this result. In contrast to yohimbine, hydrocortisone had no significant effect on fear memory. These findings suggest that noradrenergic arousal strengthens fear memory expression and have important implications for mental disorders in which the overgeneralization of conditioned fear is prominent.

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1. Introduction

Learning to fear potentially dangerous stimuli is highly adaptive as it helps to prevent future harm to the organism. Because threatening stimuli rarely occur in the exact same form across experiences, the generalization of fear is an important mechanism that helps us to deal with complexity (Shepard, 1987). However, an exaggerated generalization of fear to stimuli not predicting danger, i.e. fear overgeneralization, is maladaptive and may contribute to anxiety disorders or post-traumatic stress disorder (PTSD) (Dunsmoor and Paz, 2015; Lissek, 2012).

Although fear generalization is a fundamental process with important clinical implications, it is largely unclear how the extent of fear generalization is modulated. Stressful events are known to be a major modulator of learning and memory (Diamond et al., 2007; Joels et al., 2006; Quaedflieg and Schwabe, 2018; Sandi and Pinelo-Nava, 2007; Schwabe et al., 2010), including fear learning processes (Merz et al., 2016; Raio and Phelps, 2015; Simon-Kutscher et al., 2019). For instance, there is evidence that acute stress may alter fear acquisition and extinction (Jackson et al., 2006; Raio et al., 2014). Moreover, stress hormones are known to act on prefrontal and medial-temporal areas, including the amygdala, the hippocampus and the ventromedial prefrontal cortex (Joels and Baram, 2009), which are critically involved in fear generalization (Dunsmoor et al., 2011; Lissek et al., 2014; Onat and Büchel, 2015). There is initial evidence suggesting that stress may affect fear generalization processes, both in animals and in humans (Bender et al., 2018; Dunsmoor et al., 2017; Kaouane et al., 2012). However, the mechanisms involved in the impact of stress on fear generalization remain poorly understood.

The exposure to stressful events initiates a cascade of physiological changes, including the release of numerous hormones, neurotransmitters and peptides (Joels and Baram, 2009). In particular, noradrenaline and glucocorticoids are known to play key roles in the modulation of learning and memory processes (Joels and Baram, 2009; Roozendaal et al., 2006). Several studies revealed that noradrenergic arousal and glucocorticoids may act synergistically to influence learning and memory (Joels et al., 2011; Krugers et al., 2012; Roozendaal et al., 2006; Schwabe et al., 2012). In addition, there is evidence that suggests that glucocorticoids - acting in concert with noradrenergic arousal - may strengthen the noradrenergic effects (Buchanan and Lovallo, 2001; Roozendaal, 2002). On the contrary, however, there is also evidence for distinct roles of noradrenaline and glucocorticoids. For instance, glucocorticoids are known to impair memory retrieval (Cai et al., 2006; de Quervain et al., 1998, 2000) and the sensory reinstatement during a memory test (Gagnon et al., 2019) which may thus result in a less specific, more generalized fear memory. At the same time noradrenergic arousal may even facilitate certain retrieval processes (Murchison et al., 2004; Schönfeld et al., 2014), thereby preventing generalization processes. Furthermore, glucocorticoids after encoding enhance memory strength, while noradrenergic stimulation facilitates the long-term specificity of memory (Atucha et al., 2017).

This experiment aimed to investigate the impact of glucocorticoids and noradrenergic arousal on fear generalization in humans. Therefore, healthy participants underwent a differential fear-conditioning procedure on Day 1, in which one stimulus was followed by a shock (CS+), while another stimulus was never followed by a shock (CS-). Twenty-four hours later, participants received either a placebo, 20 mg hydrocortisone, 20 mg of the α2-adrenoceptor antagonist yohimbine, leading to increased noradrenergic stimulation, or both drugs before a test of fear generalization (Onat and Büchel, 2015). The distribution of fear acquisition and generalization over two days allowed us to isolate drug effects on fear memory generalization, while ruling out influences on fear acquisition and early consolidation processes.

We hypothesized that glucocorticoids and noradrenergic stimulation would exert opposite effects, resulting in enhanced fear generalization after hydrocortisone intake, represented by a wider fear-tuning function, but enhanced fear memory specificity after yohimbine intake, mirrored by an increased amplitude of the Gaussian function. Regarding the concurrent administration of hydrocortisone and yohimbine, it was hypothesized that both drugs might lead to an even further reduction in fear generalization than yohimbine alone. However, given the differential effects we expected after the administration of either drug alone, this hypothesis was more speculative.

2. Experimental procedures

2.1. Participants and experimental design

One-hundred-thirty-six healthy volunteers (68 women, age: $M = 25.41$ years, *SEM*=0.36 years) without a history of any mental or neurological disorder, current medication intake, drug or tobacco use participated in this experiment. This sample size was based on an a-priori power analysis using G*Power 3.1 [28] showing that 136 participants are sufficient to detect a medium-sized effect of $f = 0.25$ with a power of 0.95. Women were not tested during their menses and those taking hormonal contraceptives were excluded from participation. All participants provided written informed consent before taking part in the experiment and received a compensation of 60€ for study participation. The study protocol was approved by the ethics committee of the State Chamber of Physicians Hamburg and in accordance with the Declaration of Helsinki.

In a double-blind, placebo-controlled, fully crossed, between-subject design with the factors hydrocortisone (yes/no) and yohimbine (yes/no) administration, participants were pseudo-randomly assigned to one of four experimental groups: placebo (20 mg; PLAC), hydrocortisone (20 mg; CORT), yohimbine (20 mg; YOH), and hydrocortisone+yohimbine (20 mg each; CORT+YOH). To ensure full blindness, every participant received four pills that were not distinguishable. Two participants had to be excluded because of data loss during acquisition on experimental Day 1. In addition, nine participants had to be excluded from the analyses because they did not show successful (explicit) fear acquisition on Day 1 (i.e. their US-expectancy rating was not higher for the CS+ than for the CS-), leaving a final sample of 125 participants (PLAC: $n = 31$, 16 women; age: $M = 25.29$ years, SEM=0.87 years; CORT: $n = 31$, 15 women; age: $M = 24.84$ years, SEM=0.69 years; YOH: $n = 34$, 17 women; age: $M = 25.15$ years, SEM=0.71 years; CORT+YOH: $n = 29$, 15 women; age: $M = 25.62$ years, SEM=0.72 years).

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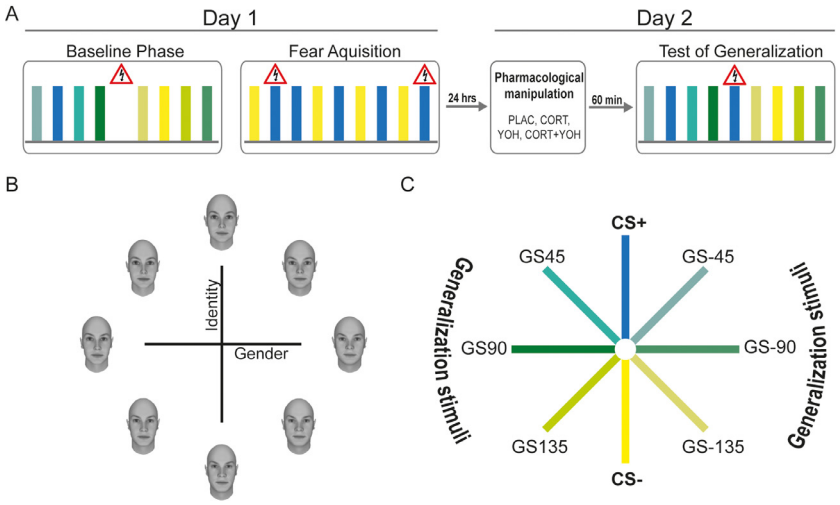

Fig. 1 Fear generalization paradigm and stimulus organization. (A) Fear generalization paradigm with three phases. The baseline and fear acquisition phases took place on Day 1, the test phase on Day 2, after the pharmacological manipulation. During the baseline phase, the complete set of stimuli (represented by colored bars) was shown to the participants and US were signaled by a shock symbol. During the fear acquisition phase, just two stimuli from opposite sides of the circular similarity continuum were shown to the participants. These stimuli represented one pair of the most dissimilar faces and were used as CS+ and CS-, respectively. During fear acquisition, the CS+ was followed by the US in ~23% of the trials. During the test phase, the complete set of faces was shown to the participants again. To avoid extinction, there was a reinforcement rate of ~23% for the CS+. (B and C) There were eight different face stimuli in total, arranged on a circular similarity continuum with the axes gender and identity. The stimuli in between the CS+ and CS- represent the generalization stimuli (GS).

2.2. General procedure and measurements

All testing took place between 1:00pm and 7:00pm on two consecutive days. On both experimental days, saliva samples were collected repeatedly using Salivette® collection devices (Sarstedt, Germany) and stored immediately after testing at −18 °C (−0.4°F). At the end of data collection, free cortisol and alpha-amylase concentrations were analyzed from saliva with a luminescence immunoassay and enzyme assay, respectively (IBL-International, Hamburg, Germany). In addition, systolic and diastolic blood pressure were obtained using a Critikon Dinamap system (Tampa, Fl, USA), with a cuff placed on the right upper arm. Potential changes in subjective mood were tracked on both testing days with a German version of the Positive and Negative Affect Schedule (PANAS; Krohne et al., 1996).

2.2.1. Day 1 - baseline phase and fear acquisition

Upon participants' arrival at the lab, baseline measurements of vital signs (i.e. systolic and diastolic blood pressure) and saliva samples were taken. Afterwards an electrode for the electrical stimulation, serving as unconditioned stimulus (US), was placed on participants' back of the right hand. Further, two electrodes for skin conductance recordings were attached to the left hand. Then, the individual pain threshold was determined using the QUEST procedure (Watson and Pelli, 1983). On a scale from 1 (no pain) to 10 (worst pain possible), participants were asked to indicate the shock intensity and we aimed to obtain a shock intensity that was unpleasant but not painful, represented by a score of 5 on the scale.

Next, the baseline phase of the fear generalization paradigm started (Fig. 1A). The paradigm contained eight face stimuli (500 × 500 pixels) arranged on a circular similarity continuum with two axes (x-axis: gender; y-axis: identity; Fig. 1B; (Onat and Büchel, 2015). The stimuli were always presented for 1.5 s. The face stimulus chosen as CS+ was counterbalanced across subjects and groups. All other faces were quantified in their distance to the CS+ on the circular similarity continuum. By having eight stimuli in a circular arrangement, this resulted in a quantification of 45° between each stimulus. The two faces opposite to each other (i.e. 180°) represented the most dissimilar faces and were used as CS+ and CS-. The faces in between represented the generalization stimuli (GS), whereby the most similar GS to the CS+ were positioned 45° next to the CS+ and the most dissimilar GS were positioned 135° away from the CS+ (Fig. 1C). During the baseline phase, the complete set of faces was shown to the participants, to control for any a priori differences between the faces. The same number of electric shocks (i.e. 10 shock trials) was administered as in the other phases to maintain a comparable arousal due to electrical stimulation throughout the task. Participants were informed that the US was always signalized by a shock symbol. This was done to ensure full predictability and prevent any association of the shock with any of the faces. Additionally, participants were asked to respond to 10 trials of oddball targets, i.e. faces with artificially added freckles. These oddball trials occurred without prior notice and served to control for attention. In total, there were 293 trials (~29 min).

During the fear acquisition phase, only two faces i.e. the most dissimilar faces, were presented. In ~23% of the trials, one face (CS+) was followed by a shock (US), resulting in 45 unreinforced CS+ trials, that later entered our analyses, whereas the other face (CS-; 44 trials) was never paired with the US. In contrast to the baseline phase, participants were informed that during this phase the US will always follow a certain face. Same as in the baseline phase, 10 oddball trials were presented to keep participants attentive. In total, there were 123 trials (~15 min). After both phases, US-expectancy ratings were assessed to measure explicit fear learning. For this purpose, we presented each face stimulus two times in randomized order and asked participants to rate for each stimulus their subjective shock expectancy using a visual analogue scale ranging from 1 (certain, no shock) to 10 (certain, shock).

At the end of Day 1 testing, pain strength rating as well as vital signs were measured again and another saliva sample was taken.

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2.2.2. Day 2 - pharmacological manipulation and test phase

At the beginning of Day 2, participants completed the State-Trait Anxiety Inventory (STAI-S; Spielberger and Syndeman, 1994) to check for differences in subjective state anxiety. Depending on the experimental condition, participants then received orally either a placebo, 20 mg of hydrocortisone, 20 mg of yohimbine or 20 mg of both drugs. Timing as well as dosage of the drug administration were chosen in accordance with previous studies (Kluen et al., 2017; Schwabe et al., 2012). Saliva samples and vital signs were collected at several time points: before drug administration, 45 min after drug intake, 60 min after drug intake, i.e. immediately before the test phase of the fear generalization paradigm, 90 min after drug intake, i.e. after the test phase, and 120 min after drug intake at the end of testing.

During a waiting period of 60 min, participants completed several questionnaires assessing control variables of interest (depression, Beck Depression Inventory (BDI-II; Beck et al., 1996); trait anxiety, STAI-T (Spielberger and Syndeman, 1994); and chronic stress, Trier Inventory for the Assessment of Chronic Stress (TICS; Schulz and Schlotz, 1999)) as well as an unrelated, non-arousing task (Zerbes et al., 2019). Then, the individual pain threshold was determined again, using the same procedure as on Day 1, before the critical fear generalization test started. In the fear generalization phase, the complete set of faces was shown to the participants again. Every face was shown ~34 times, except for the CS+ which was shown ~44 times. This was realized because only unreinforced CS+ trials later entered analysis and the US followed the presentation of the CS+ in ~23% of the trials to avoid extinction learning to the CS+. Again, 10 oddball trials were presented. Same as the baseline phase, this phase contained 293 trials (~29 min). At the end of the fear generalization phase, US-expectancy ratings were collected using the same procedure as on Day 1.

After these ratings, all of the eight face stimuli were presented to the participants as shown in Fig. 1B but in a randomized circular arrangement and participants had to indicate which of the faces was followed by the shock. Participants had to use the arrow keys to navigate around the circle and confirm their selection with the space bar. This task was self-paced.

Finally, a perceptual discrimination task was presented to assess participants' perceptual discrimination ability. In this task, participants were presented two faces one after another, each for 1.5 s and were asked to rate the faces as being the same or different. There was no time limit for the response but participants were instructed to decide quickly. Participants could use the arrow keys to select the "same" or "different" button and had to confirm their choice using the space bar. In total, the discrimination task consisted of 192 trials (each of the eight face stimuli was shown 24 times) and lasted for about 30 min.

At the end of the experiment, participants were asked to indicate which treatment they thought they received (i.e. placebo, hydrocortisone, yohimbine, both drugs or any drug) to check for successful blinding. They were then debriefed and compensated for participation.

2.3. Electrodermal stimulation and SCR analysis

The US consisted of trains of 5-ms electrical pulses at 66 Hz lasting in total 100 ms, co-terminating with the shock symbol or the face stimulus and applied via a constant voltage stimulator (STM200, BIOPAC Systems, Goleta USA) with a surface bar electrode. Electrodermal activity was recorded from the distal phalanx of the index and middle fingers of the left hand, using two 8 mm Ag/AgCl electrodes, connected to the MP-150 BIOPAC System (BIOPAC Systems, Goleta USA), assessed according to common guidelines (Boucsein et al., 2012). A deconvolution technique as implemented in Ledalab version 3.4.9 (Benedek and Kaernbach, 2010) was used to divide raw skin conductance recordings into the slowly varying tonic activity, i.e. skin conductance level, and a rather fast varying phasic activity, i.e. skin conductance responses (SCRs). As part of the procedure, skin conductance data were downsampled to a resolution of 20 Hz and optimized using four sets of initial values. The optimization procedure was used to find the best starting point for the deconvolution. To obtain the anticipatory SCRs, we derived the average phasic driver within a response window from 1 s to 4 s after stimulus onset. By setting the minimum amplitude threshold to 0.01μS, we controlled for non-responding on a trial-by-trial level. As such, trials with an amplitude smaller than 0.01μS were set to 0 and were not included when averaging the SCR. To correct for inter-individual differences, SCRs were z-transformed (zSCRs) separately for the three different phases (Ben-Shakhar, 1985). Because US- trials and CS+ trials in which a shock was presented did not enter further analyses, we excluded these trials before z-transformation. We then calculated the responses associated with the onset of individual faces at a single subject level. Finally, responses to the different stimuli were averaged and single subject fear-tuning profiles for each phase were derived (Onat and Büchel, 2015).

2.4. Analysis of fear-tuning profiles

Individual fear-tuning profiles were analyzed using MATLAB (Release 2016b, Natick, MA). To characterize the fear-tuning, a Gaussian model with two parameters (α, amplitude, i.e. the strength of fear memory specificity or expression; σ, tuning width (full width at half maximum), i.e. the strength of fear generalization) was used. We restricted our Gaussian model to be centered on the CS+-face. Fear-tuning profiles were calculated for zSCR and rating data separately. For further statistical analyses, we extracted the two parameters of each profile (Onat and Büchel, 2015).

2.5. Statistical data analysis

Statistical analyses were performed with SPSS 25.0 (IBM). To ensure that groups had not baseline differences, Day 1 data were subjected to ANOVAs with the between-subjects factor group with four levels (PLAC, CORT, YOH, CORT+YOH). As within-subject factor, we used time (start vs. end), face-number (eight levels) and stimulus (CS+ vs. CS-). Day 2 data were analyzed by means of mixed-design ANOVAs with time (five levels) and stimulus (eight levels) as a within-subject factor and hydrocortisone (yes/no) and yohimbine (yes/no) administration as between-subject factor, in order to analyze the main effect of each drug separately as well as a drug interaction effect. To explicitly compare responding to the CS+ and the most similar GS, we averaged responding of these GS and calculated the variable |GS45|. To analyze the perceptual discrimination ability, we calculated a discrimination score by subtracting the mean false alarm rate from the mean hit rate. To avoid extinction learning during fear generalization test, participants still received the US in ~23% of the trials. To investigate, if this reinforcement had an impact on fear generalization on Day 2, we calculated reinforcement bins by counting the number of trials between the US and the different GSs and CS-. We calculated the mean and grouped the time after US occurrence in three percentiles, whereby we obtained four bins: before any US had occurred, 1-11 trials after US occurrence, 12-25 trials after US occurrence, and >25 trials after US occurrence. We then performed a fear-tuning analysis for the stimuli dependent on the time of US occurrence and extracted the same parameter as for the general fear-tuning. As such, our independent variables were the between-factors group (Day 1 and baseline Day 2 analyses), hydrocortisone and yohimbine (Day 2 analyses) and the within-subject factors time, face-number and stimulus. Our

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Table 1 Physiological, endocrine, and subjective response to the pharmacological manipulation.

Variable	PLAC	CORT	YOH	CORT+YOH
Day 1				
Salivary cortisol (nmol/L)	4.00 (0.77)	4.94 (0.77)	4.55 (0.74)	5.09 (0.80)
Alpha-Amylase (U/ml)	99.48 (17.68)	108.18 (17.77)	132.39 (16.60)	106.51 (17.98)
Systolic BP (mmHG)	124.08 (2.57)	125.24 (2.57)	126.15 (2.45)	123.98 (2.65)
Diastolic BP (mmHG)	69.95 (1.82)	69.37 (1.82)	70.69 (1.74)	72.78 (1.89)
Negative affect				
Day 1	1.23 (0.05)	1.28 (0.05)	1.26 (0.05)	1.25 (0.06)
Day 2 baseline	1.20 (0.06)	1.16 (0.06)	1.20 (0.06)	1.26 (0.06)
Day 2 45 min post drug	1.14 (0.04)	1.11 (0.04)	1.15 (0.04)	1.15 (0.05)
Day 2 60 min post drug	1.14 (0.04)	1.11 (0.04)	1.14 (0.04)	1.17 (0.05)
Day 2 90 min post drug	1.17 (0.07)	1.27 (0.07)	1.24 (0.07)	1.26 (0.08)
Day 2 120 min post drug	1.06 (0.05)*	1.14 (0.05)	1.14 (0.04)	1.12 (0.05)•
Positive affect				
Day 1	2.70 (0.10)	2.71 (0.10)	2.80 (0.09)	2.72 (0.10)
Day 2 baseline	2.67 (0.11)	2.81 (0.11)	2.84 (0.11)	2.66 (0.12)
Day 2 45 min post drug	2.43 (0.12)**	2.51 (0.11)**	2.64 (0.11)*	2.41 (0.12)*
Day 2 60 min post drug	2.38 (0.12)***	2.49 (0.12)**	2.63 (0.12)	2.36 (0.13)**
Day 2 90 min post drug	2.03 (0.12)***	2.13 (0.12)***	2.38 (0.11)***	2.31 (0.13)**
Day 2 120 min post drug	2.19 (0.12)***	2.26 (0.11)***	2.31 (0.11)***	2.30 (0.12)**
Pain Threshold				
Day 1	38.53 (2.23)	46.00 (2.23)	43.73 (2.13)	42.06 (2.30)
Day 2	41.40 (1.99)	45.68 (1.99)	45.93 (1.90)	45.40 (2.06)

The table presents physiological, endocrine, and subjective responses before testing on Day 1 as well as the change over time in response to the pharmacological manipulation on Day 2. Groups did not differ in any of the measurements on Day 1 or before pill intake on Day 2. However, there were significant changes in all of the measurements in response to the pharmacological manipulation, thus confirming the action of the drugs. Data represent mean (standard error). Asterisks denote difference to Day 2 baseline: •$p < .1$, *$p < .05$, **$p < .01$, ***$p < .001$.

dependent variables were zSCR and US-expectancy rating data and the fear-tuning parameters of these data.

All reported p-values are two-tailed, using an α-error threshold of p=.05. Significant main or interaction effects were pursued using post-hoc planned comparisons, with Sidak correction if indicated. If the sphericity assumption was violated, Greenhouse-Geisser correction was applied.

3. Results

3.1. Day 1

Before the beginning of the baseline phase, the four groups differed neither in their subjective mood, physiological markers such as cortisol, alpha-amylase, systolic or diastolic blood pressure, nor in their estimated pain threshold (all $Fs \leq .749$; all $ps \geq .544$; all $\eta^2 s \leq .018$; Table 1). With respect to the pain strength rating, there was a significant time effect ($F(1113)=22.23$, $p<.001$, $\eta^2=0.164$), indicating that participants rated the US as less painful at the end of testing compared to before testing, without differences between groups (main effect of group and time × group interaction: both $Fs \leq 1.834$; both $ps \geq .145$; both $\eta^2 s \leq .046$).

3.1.1. Baseline phase

During the baseline phase, there were no main effects of group or group × face-number interaction effects, neither for the zSCR data nor for the rating data. However, for both measurements there was a face-number main effect (zSCR: $F(7847)=5.340$, $p<.001$, $\eta^2=0.042$; rating data: $F(3.097,374.724)=3.831$, $p=.009$, $\eta^2=0.031$; Fig. 2A and B). Regarding the rating data, no post-hoc comparison for individual faces reached statistical significance (all $ps>0.067$), for zSCR data face 3 elicited higher SCR compared to face 4 and face 6 (both $ps<0.002$) and face 7 elicited higher SCR compared to face 8 ($p=.017$). However, since we counterbalanced CS+ and CS- assignment across subjects and groups, the influence of this difference on our conditioning data should be negligible.

3.1.2. Successful fear acquisition

As expected, the results of the fear acquisition phase revealed a significantly higher responding to the CS+ compared to the CS-, indicated by a higher zSCR ($F(1120)=14.583$, $p<.001$, $\eta^2=0.108$) as well as a higher US-expectancy rating for the CS+ ($F(1121)=719.459$, $p<.001$, $\eta^2=0.856$). There were no differences between groups (all $Fs \leq 1.466$; all $ps \geq .227$; all $\eta^2 s \leq .035$; Fig. 2C and D).

3.2. Day 2

We obtained no group differences regarding depressive mood, chronic stress, or state anxiety (all $Fs \leq 1.134$; all $ps \geq .338$; all $\eta^2 s \leq .027$; Table 2). There was a trending group difference in trait anxiety scores ($F(3121)=2.560$, $p=.058$,

Table 2 Subjective assessments of depressive mood, chronic stress and anxiety.

Variable	PLAC	CORT	YOH	CORT+YOH
Depressive score (BDI-II)	5.07 (0.94)	5.52 (0.94)	7.12 (0.89)	5.45 (0.97)
State anxiety (STAI-S)	2.39 (0.03)	2.42 (0.03)	2.42 (0.03)	2.46 (0.03)
Trait anxiety (STAI-T)	2.02 (0.05)	2.03 (0.05)	2.19 (0.05)	2.06 (0.05)
Subjective chronic stress (TICS)	63.03 (5.72)	71.36 (5.72)	76.88 (5.47)	66.83 (5.92)

Subjective assessments of depressive mood, chronic stress and anxiety through various questionnaires reveal low levels in all of the measures and no differences between the four groups. Data represent mean (standard error).

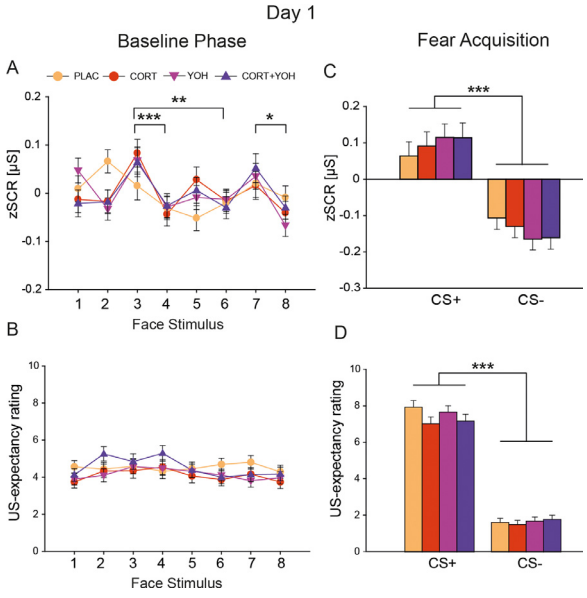

Fig. 2 Day 1: Physiological and subjective responses to the face stimuli during the baseline and fear acquisition phases. (A) zSCR data as well as (B) explicit rating data showed no systematic a priori differences between faces and no group differences during or after baseline phase. During and after fear acquisition, both (C) zSCR as well as (D) explicit US-expectancy rating data showed successful fear learning reflected in higher response to the CS+ than to the CS-. Error bars represent standard errors off the mean. Asterisks denote differences between stimuli ($^{*}p < .05$, $^{**}p < .005$, $^{***}p < .001$).

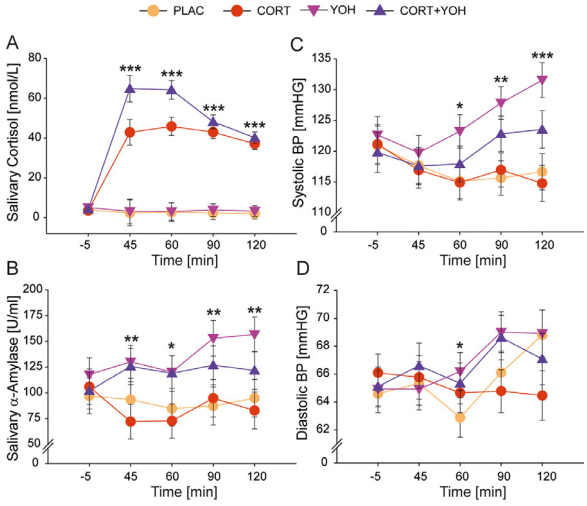

Fig. 3 Pharmacological manipulation check. (A) Salivary cortisol increase. (B) Salivary alpha-amylase increase. (C) Systolic blood pressure increase. (D) Diastolic blood pressure increase. Error bars represent standard errors or the mean. Asterisks denote difference between factors either hydrocortisone (yes/no) for salivary cortisol or yohimbine (yes/no) for alpha-amylase, systolic and diastolic blood pressure. ($^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$).

η^2=0.060). Sidak post-hoc tests indicated no significant differences (all *ps*≥.104). To ensure that trait anxiety did not modulate our results, we re-analyzed our data including the STAI-T score as a covariate, which had, however, no significant influence on our main findings. Furthermore, groups did not differ in subjective mood, cortisol, alpha-amylase, systolic or diastolic blood pressure at the beginning of Day 2, i.e. before pill intake (all *Fs*≤1.473; all *ps*≥.225; all η^2*s*≤.035; Table 1 and Fig. 3). Regarding their pain threshold, participants had in general a slightly higher pain threshold on Day 2 compared to Day 1 ($F(1121)=6.672$, $p=.011$, $\eta^2=0.052$), but rated the US as less painful ($F(1113)=12.585$, $p=.001$, $\eta^2=0.100$). Importantly however, this change from Day 1 to Day 2 was not influenced by group (all $F \leq 1.726$; all $p \leq .165$; all $\eta^2 \leq .041$). With respect to the pain strength rating only on Day 2, there was a trend for a main effect of time ($F(1114)=2.929$, $p=.090$, $\eta^2=0.025$), suggesting that groups rated the US as slightly more painful at the end of testing compared to before testing. This was not affected by our manipulation (all interactions including hydrocortisone and yohimbine: all *Fs*≤.183; all *ps*≥.670; all η^2*s*≤.001).

3.2.1. Manipulation check

Before the beginning of the fear acquisition phase, groups did not differ in subjective mood, salivary cortisol, salivary alpha-amylase and systolic or diastolic blood pressure (Table 1 and Fig. 3). Significant changes in salivary cortisol, alpha-amylase and blood pressure confirmed the action of the drugs (Fig. 3). In an ANOVA with the factors hydrocortisone, yohimbine and time point of measurement, the effectiveness of cortisol was shown by a significant time × hydrocortisone interaction effect ($F(1.542,186.602)=42.320$, $p<.001$, $\eta^2=0.259$). Post-hoc tests verified a significant increase in salivary cortisol from baseline to after hydrocortisone administration for the participants receiving hydrocortisone ($F(1.592,88.707)=36.977$, $p<.001$, $\eta^2=0.389$), whereas participants who had not received hydrocortisone

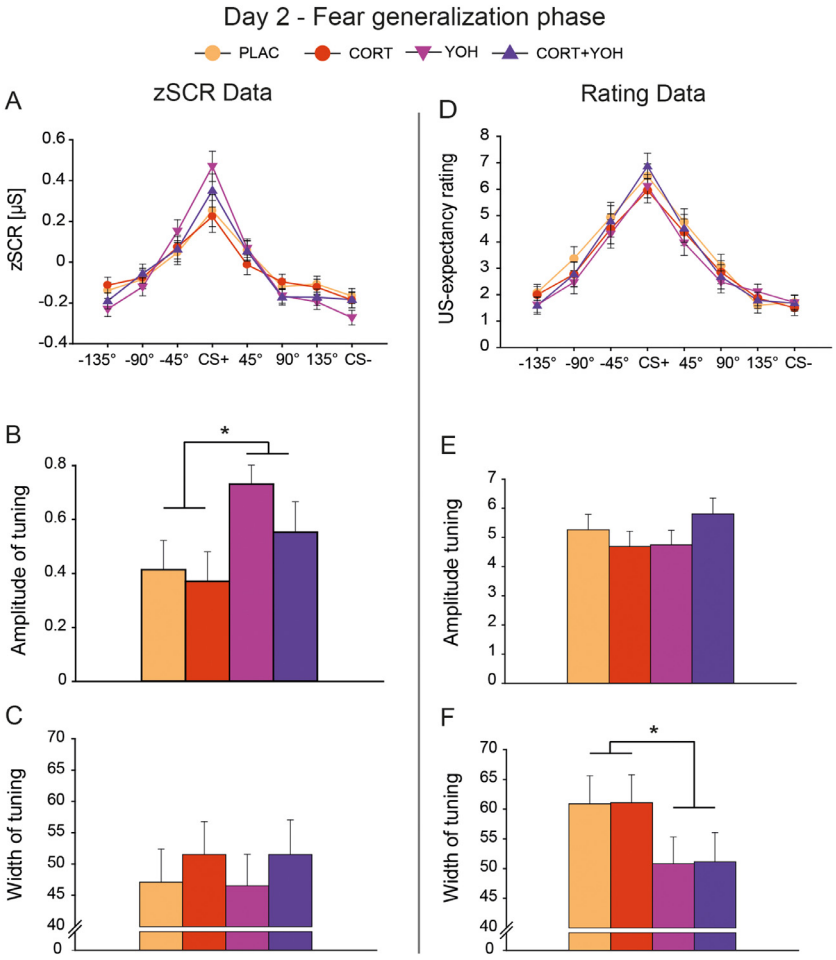

Fig. 4 Day 2: Fear generalization phase. (A) Overview of zSCR to different stimuli during the test phase. Yohimbine administration effects (B) the amplitude of Gaussian model for zSCR data as the amplitude increases. (C) There was no significant influence of yohimbine or hydrocortisone administration for the width of zSCR fear-tuning. (D) Overview of US-expectancy rating to different stimuli after the test phase. (E) There seems to be no effect of yohimbine or hydrocortisone administration on the amplitude of Gaussian model. (F) However, there is a significant influence of yohimbine administration for the width of fear-tuning, as after yohimbine administration the fear-tuning profile gets narrower. Asterisks denote difference between groups ($^*p < .05$).

even showed a decrease ($F(84.565,98.850)=6.884$, $p<.001$, $\eta^2=0.099$) for all time points after drug administration (Fig. 3).

Conversely, yohimbine administration led to significant increases in alpha-amylase as well as in systolic and diastolic blood pressure (time × yohimbine interactions: all $Fs\geq 2.887$; all $ps\leq .042$; all $\eta^2s\geq .023$). This increase was significant for participants receiving yohimbine across all variables (all $Fs\geq 3.341$; all $ps\leq .018$; all $\eta^2s\geq .052$), whereas there was a decrease for participants not receiving yohimbine (all $Fs\geq 2.046$; all $ps\leq .130$; all $\eta^2s\geq .033$; Fig. 3). For diastolic blood pressure, there was additionally a significant time × hydrocortisone interaction ($F(2.639.319.291)=3.989$, $p=.011$, $\eta^2=0.032$) and time × hydrocortisone × yohimbine interaction, which were, however, driven by the PLAC group ($F(1.764,52.917)=3.095$, $p=.060$, $\eta^2=0.094$), without a significant effect for the CORT group ($F(2.988,89.634)=1.025$, $p=.385$, $\eta^2=0.033$). In addition, participants were not aware of the administered drug. The majority (71%) guessed that they had received a placebo, without any difference between the four groups ($\chi^2(3)=3.687$, $p=.297$, Cramer's $V = 0.229$).

3.2.2. Fear generalization phase: noradrenergic arousal boosts fear memory expression

When comparing the fear-tuning parameters for the zSCR data (Fig. 4A), results showed that yohimbine increased the fear memory expression. This was indicated by a higher amplitude of the Gaussian model in both yohimbine groups, i.e. YOH and CORT+YOH compared to the groups that did not receive yohimbine, i.e. PLAC and CORT ($F(1120)=5.677$, $p=.019$, $\eta^2=0.045$; all other main or interaction effects $p\geq .374$; Fig. 4B). The strength of fear generalization, as reflected in the model width parameter, was not significantly altered by yohimbine or hydrocortisone (all main or interaction effects: all $Fs\leq .789$; all $ps\geq .376$; all $\eta^2s\leq .007$; Fig. 4C). To confirm that the yohimbine effect can be explicitly attributed to a higher responding towards the CS+ and not the similar GSs, we compared by means of an rmANOVA the

influence of our manipulation on responding to the CS+ and |GS45|. Besides a main effect of stimulus ($F(1120)=65.872$, $p<.001$, $\eta^2=0.354$) and yohimbine ($F(1120)=4.963$, $p=.028$, $\eta^2=0.040$), results showed a significant yohimbine × stimulus interaction ($F(1120)=4.287$, $p=.041$, $\eta^2=0.034$). Post-hoc t-tests confirmed that the administration of yohimbine resulted in a higher responding towards the threatening CS+ ($t(122)=-2.384$, $p=.019$, $d = 0.428$) but not the similar GSs ($t(122)=-1.427$, $p=.156$, $d = 0.256$).

Similarly, yohimbine intake led to a more specific US-expectancy rating to the CS+ (Fig. 4D). Specifically, the US-expectancy data showed a significant yohimbine effect for the width of rating data with a narrower fear-tuning curve after yohimbine intake (YOH and CORT+YOH groups) compared to groups that received no yohimbine (PLAC and CORT groups; $F(1120)=4.537$, $p=.035$, $\eta^2=0.036$; Fig. 4F). For the amplitude there were no significant effects in the subjective US-expectancy ratings (all $Fs\leq2.476$; all $ps\geq.118$; all $\eta^2s\leq.020$; Fig. 4E).

3.2.3. Noradrenergic arousal supports fear memory expression rather than safety learning

In order to test whether noradrenergic stimulation increased specifically the responding to the CS+ or safety learning, reflected in a reduced responding to the CS-, we analyzed in a next step the CS+/CS- differentiation. The results revealed a significant stimulus × yohimbine interaction ($F(1120)=4.637$, $p=.033$, $\eta^2=0.037$; without any main or interaction effects of cortisol: all $ps\geq.361$). Separate post-hoc analyses for each stimulus showed that yohimbine administration compared to no yohimbine administration specifically increased responding to the CS+ ($F(1)=5.308$, $p=.023$, $\eta^2=0.042$), without influencing the responding to the safety stimulus CS⁻ ($F(1)=2.004$, $p=.159$, $\eta^2=0.016$).

For the subjective rating data, there was a stimulus main effect ($F(1120)=297.925$, $p<.001$, $\eta^2=0.713$), but no interaction effect with yohimbine or hydrocortisone (all $Fs\leq1.867$; all $ps\geq 0.174$; all $\eta^2s\leq.015$).

3.2.4. Noradrenergic arousal boosts fear memory expression independent of the temporal distance to the threatening stimulus

To investigate how US presentation in the test phase affected fear generalization, we calculated fear-tuning curves for reinforcement bins, reflecting the distance to the last US, and applied Gaussian fear-tuning to these data (Fig. 5). The analyses of the Gaussian model parameters revealed again that the administration of yohimbine led to higher amplitudes independent of the number of elapsed trials after a US ($F(1120)=4.937$, $p=.028$, $\eta^2=0.040$), indicating once more increased fear memory expression. In contrast to yohimbine, the administration of hydrocortisone had no effects on amplitude or width (all main or interaction effects $ps\leq.129$).

3.2.5. Increased fear memory expression after noradrenergic stimulation cannot be attributed to enhanced attention or perceptual discrimination of fear-related cues

To ensure that the effect of yohimbine cannot be explained by merely enhanced attention, we analyzed responses to the oddball targets that were presented during all phases.

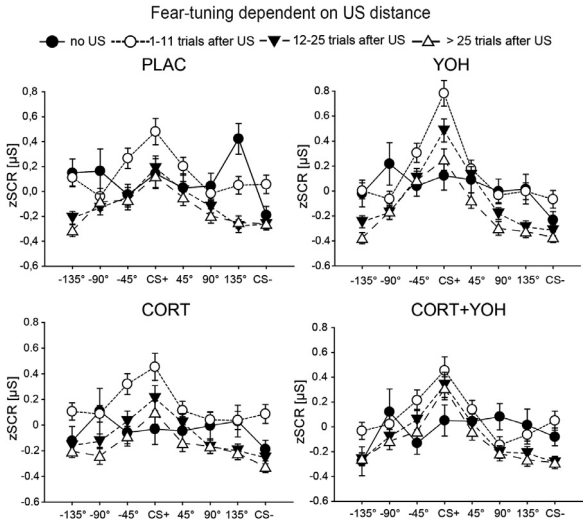

Fig. 5 Fear-tuning dependent on US distance. Descriptively, the fear-tuning curves support the statistical findings, as with the YOH Group, the strongest reaction to the CS+ can be seen, independent of time after US occurrence.

In general, participants were very attentive and reacted correctly in 98.47% of the trials, without any influence of hydrocortisone or yohimbine (all $Fs\leq1.358$; all $ps\geq.246$; all $\eta^2s\leq.011$).

Although groups did not differ in their ability to correctly identify the CS+ as assessed by the presentation of all eight faces at the end of Day 2 (both $Fs\leq1.480$; both $ps\geq.226$; both $\eta^2s\leq.012$), testing the discrimination ability between faces, results revealed an improved perceptual discrimination after yohimbine administration ($F(1121)=4.590$, $p=.032$, $\eta^2=0.037$).

To test whether the effects of noradrenergic stimulation on the expression of fear memory were due to the enhanced perceptual discrimination ability or whether the yohimbine effect goes beyond the mere discrimination ability, we re-run our analyses including the discrimination score as covariate. These analyses showed that taking the discrimination ability into account left our results largely unaffected. In particular, the yohimbine effect on the amplitude remained significant after controlling for differences in perceptual discrimination ability ($F(1119)=4.012$, $p=.049$, $\eta^2=0.034$). When directly comparing the reaction to the CS+ and CS-, there is still a trend for a stimulus × yohimbine interaction ($F(1119)=2.914$, $p=.090$, $\eta^2=0.024$). For the reinforcement bins analysis, the significant yohimbine main effect for the amplitude remains on a trend level ($F(1119)=3.316$, $p=.071$, $\eta^2=0.027$).

4. Discussion

Whereas the generalization of fear is crucial to avoid harm to the organism, fear overgeneralization is maladaptive and may contribute to anxiety disorders and PTSD (Dunsmoor and Paz, 2015; Lissek, 2012). We tested here, to the best of our knowledge, for the first time the impact of major stress mediators, i.e. noradrenergic stimulation

and glucocorticoids, on fear generalization in humans. Our results show that yohimbine intake led to a higher amplitude of fear-tuning in SCR data across a similarity continuum from CS+ to CS-, indicating that noradrenergic stimulation enhanced fear memory expression, mirrored in an increased responding to the CS+, while responding to the similar generalization stimuli and the safety signaling CS- remained unaffected by noradrenergic stimulation. In addition, effects were independent of the distance to the US, and the impact of noradrenergic stimulation on fear memory expression could not be explained by a mere increase in perceptual discrimination ability. Whereas noradrenergic arousal increased fear memory expression, there was a trend suggesting that cortisol may increase fear generalization on an explicit level.

The observed increase in fear memory expression is generally in line with previous findings suggesting that noradrenergic arousal can have enhancing effects on memory accuracy (Atucha et al., 2017; McGaugh, 2013) and is further necessary for the retrieval of recent contextual memories (Murchison et al., 2004). Previous neuroimaging studies on fear generalization showed specific responses to the CS+ and declining activity as stimuli differentiated from the CS+ in the amygdala, insula, thalamus, and striatum (Dunsmoor et al., 2011; Lissek et al., 2014; Onat and Büchel, 2015). Conversely, activity in the hippocampus and vmPFC inclined as stimuli differentiated from the CS+, suggesting an inhibition of responses to stimuli similar to the CS+ (Greenberg et al., 2013; Lissek et al., 2014; Onat and Büchel, 2015). Noradrenergic arousal has been shown to increase the activity of areas implicated in the enhanced responding to the CS+ as well as in areas involved in the inhibitory control of fear responses (Arnsten, 2009; Atucha et al., 2017; Tully and Bolshakov, 2010). However, our finding that yohimbine enhanced specifically the responding to the CS+, while it led CS- responses unaffected, might be taken as evidence that the increased fear memory expression after yohimbine intake was primarily due to enhanced activity in areas involved in CS+ responding, such as the amygdala or insula.

Traditionally, it has been argued that fear generalization is due to the perceptual similarity of the CS+ and graded versions of CS+ and CS-, assuming that the neural fear generalization is directly linked to the perceptual similarity of the stimuli (Lissek, 2012; Lissek et al., 2014). More recently, an alternative model has been proposed according to which fear generalization is an active process that may even occur when individuals are able to perceptually discriminate the CS+ (Dunsmoor and Paz, 2015; Onat and Büchel, 2015). In the present experiment, yohimbine increased participants' perceptual discrimination capacity. Critically, however, the impact of yohimbine on fear memory expression remained, at least at trend-level, when we statistically controlled for the increase in perceptual discrimination ability, thus providing further evidence that there is a fear generalization process that is at least partly independent of the perceptual discrimination capacity. Based on our results, we suggest that this active process, which maintains fear memory expression, may be shaped by noradrenergic stimulation. Although the processes of fear memory generalization and expression seem to be related (Rozeske et al., 2015), there is evidence that these are two distinct processes (Xu and Sudhof, 2013). Based on our results, we propose that the individual stress mediators have distinct effects on these two processes and disentangling glucocorticoid and noradrenaline effects on either fear generalization and fear memory expression remains a challenge for future research.

In contrast to yohimbine, hydrocortisone did not enhance fear memory expression but tended to increase fear generalization. Although this trend needs to be interpreted with caution, this finding is generally in line with the disruptive effects of cortisol on memory retrieval (Buchanan et al., 2006; Roozendaal, 2002) and more specifically, with rodent data, suggesting enhanced fear generalization after glucocorticoid administration (Kaouane et al., 2012). A cortisol-driven increase of fear memory generalization would further be in line with a previous study reporting enhanced fear memory after stress because this study tested fear generalization when stress-induced cortisol had reached peak levels while stress-induced arousal had already vanished (Dunsmoor et al., 2017).

While the present data provide initial evidence for opposite roles of noradrenaline and glucocorticoids in fear memory expression and generalization, it is important to note that there is compelling evidence for synergistic interactions of glucocorticoid and noradrenergic activity in the modulation of learning and memory (Krugers et al., 2012; Roozendaal et al., 2006). We explicitly tested for such interactions by including the CORT+YOH group. Participants in this group showed a very similar pattern as those in the YOH group, suggesting that the impact of noradrenergic impact superimposed the glucocorticoid effect. Evidence suggests that there are time-dependent effects of glucocorticoids, related to non-genomic and genomic modes of action, with rapid effects resembling those of catecholamines but opposite delayed effects (Joels et al., 2011; Schwabe and Wolf, 2014). As such, it would be interesting to test the influence of glucocorticoids at different time-intervals before the generalization test, to assess whether glucocorticoids active at longer time intervals before test would result in more pronounced effects on fear generalization or to altered interactions with noradrenergic activity.

While there was a specific increase in the response to the CS+ in our SCR data, reflected in the amplitude of tuning, our US-expectancy rating data appeared to represent fear generalization processes, reflected in the width of tuning. Previous studies suggested that SCR and expectancy ratings may reflect different types of fear memory, i.e. implicit and explicit memory systems, respectively (Manassero et al., 2019; Schultz et al., 2013). In line with our results, a recently published study found that implicit reactions, represented by the SCR, were selectively triggered by the CS+, but not by a similar stimulus. In contrast, participants were more susceptible to misidentify the same similar stimulus as the CS+ on an explicit level (Manassero et al., 2019). The authors interpret their results as a support for the two-system framework (LeDoux and Pine, 2016), that proposes a defensive survival circuit which may be mainly depending on behavioral and physiological reactions, hence fast implicit fear learning and a rather cognitive circuit, reflected in subjective experience of fear.

Finally, it should be noted that increased noradrenergic arousal after yohimbine intake might be assumed to induce

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an overall increase in SCR. However, even if there is such an effect of yohimbine on SCR per se, this should have led to an overall increase in SCR to all stimuli, which was not observed here, rather than to the stimulus-specific responses that are suggested by our analyses based on parameters of Gaussian fits.

To conclude, fear generalization is a fundamental process that allows us to deal with complexity in our environment, yet overgeneralization of fear to non-threatening stimuli may be maladaptive. We show here for the first time that noradrenergic stimulation may increase the expression of 24hrs-old fear memories and that this effect could not be explained by increases in safety learning or a mere increase in perceptual discrimination ability. These findings provide novel insights into the regulation of fear memory by major stress systems and might point to novel treatment approaches for mental disorders in which the overgeneralization of fear is a hallmark feature.

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Contributors

L.S. conceived and planned the experiment. F.M.K. and G.Z. collected the data. L.K. and C.B. contributed analysis tools. F.M.K. performed the analyses. F.M.K., L.K., C.B., J.C.M., K.W., and L.S. contributed to the interpretation of the results. F.M.K. drafted the manuscript, L.S. provided critical revisions. All authors contributed to the final version of the manuscript.

Conflict of interest

The authors declare no competing interests.

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Appendix C

Study III

Acute stress leaves fear generalization in healthy individuals intact

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Acute stress leaves fear generalization in healthy individuals intact

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Abstract

Because threatening situations often occur in a similar manner, the generalization of fear to similar situations is adaptive and can avoid harm to the organism. However, the overgeneralization of fear to harmless stimuli is maladaptive and assumed to contribute to anxiety disorders. Thus, elucidating factors that may modulate fear (over)generalization is important. Based on the known effects of acute stress on learning, which are at least partly due to noradrenergic arousal, we investigated whether stress may promote fear overgeneralization and whether we could counteract this effect by reducing noradrenergic arousal. In a placebo-controlled, double-blind, between-subjects design, 120 healthy participants underwent a fear-conditioning procedure on Day 1. Approximately 24 hours later, participants received orally either a placebo or the beta-adrenergic receptor antagonist propranolol and were exposed to a stress or control manipulation before they completed a test of fear generalization. Skin conductance responses as well as explicit rating data showed a successful acquisition of conditioned fear on Day 1 and a pronounced fear generalization 24 hours later. Although physiological data confirmed the successful stress manipulation and reduction of noradrenergic arousal, the extent of fear generalization remained unaffected by stress and propranolol. The absence of a stress effect on fear generalization was confirmed by a second study and a Bayesian analysis across both data sets. Our findings suggest that acute stress leaves fear generalization processes intact, at least in a sample of healthy, young individuals.

Keywords Stress · Fear generalization

Introduction

The experience of a threatening stimulus automatically triggers the subjective experience of fear, an adaptive emotion that helps us to avoid future harm. Because most stimuli do not occur in the exact same manner across situations, the ability to generalize fear to stimuli resembling an initial threat stimulus is highly adaptive. This fear generalization is reflected in a fear gradient, in which the fear response is highest towards the original threatening stimulus but spreads, at least in part, to similar stimuli and the lowest response is shown to the most dissimilar stimulus (Lissek et al., 2008; Lissek, Bradford, et al., 2014; Shepard, 1987). Although fear generalization may be generally adaptive, the inability to distinguish threat from safety and the overgeneralization of fear to safe stimuli are maladaptive. In particular, fear overgeneralization is thought to underlie the behavioral symptoms of fear-related disorders or posttraumatic stress disorder (PTSD; Dunsmoor & Paz, 2015; Kaczkurkin et al., 2017; Lis et al., 2020; Lissek, 2012; Lopresto, Schipper, & Homberg, 2016; Morey et al., 2020). Given that anxiety disorders are among the most common mental disorders (Kessler et al., 2005; Wittchen & Jacobi, 2005), identifying factors that may promote the overgeneralization of fear is important. Accordingly, previous studies showed that the extent of fear generalization can be influenced by the intensity of threat (Dunsmoor, Kroes, Braren, & Phelps, 2017), verbal instructions (Vervliet, Kindt, Vansteenwegen, & Hermans, 2010), the degree of anxious personality (Sep, Steenmeijer, & Kennis, 2019), and stimulus similarity and perception (Struyf, Zaman, Hermans, & Vervliet, 2017; Struyf, Zaman, Vervliet, & Van Diest, 2015; Zaman, Ceulemans, Hermans, & Beckers, 2019; Zaman, Struyf, Ceulemans, Beckers, & Vervliet, 2019). On a neural level, findings suggest that adaptive fear generalization requires an intricate balance of excitatory and inhibitory mechanisms of different brain regions.

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Specifically, studies repeatedly associated activation in the anterior insula (aI), the dorsomedial prefrontal cortex, and the bilateral inferior parietal lobe with fear excitation, whereas activation of the bilateral ventral hippocampus, the ventromedial prefrontal cortex (vmPFC) and the precuneus cortex is associated with fear inhibition (Dunsmoor, Prince, Murty, Kragel, & LaBar, 2011; Greenberg, Carlson, Cha, Hajcak, & Mujica-Parodi, 2013; Lissek, Bradford, et al., 2014; Onat & Büchel, 2015). In line with behavioral studies that showed that fear generalization can be independent of perception (Bennett, Vervoort, Boddez, Hermans, & Baeyens, 2015; Dunsmoor, Martin, & LaBar, 2012; Dunsmoor & Murphy, 2014), studies investigating the neural mechanisms showed partly sharpened fear generalization on a neural level compared to the behavioral level, indicating that processes other than perception add to fear generalization (Onat & Büchel, 2015; Stegmann, Ahrens, Pauli, Keil, & Wieser, 2020).

Stressful events—known to provoke the release of numerous hormones, neurotransmitters and peptides (Joels & Baram, 2009)—are assumed to be a driving force in fear- and stress-related disorders, such as PTSD (de Quervain, Schwabe, & Roozendaal, 2017; Grillon, Duncko, Covington, Kopperman, & Kling, 2007; Shin & Liberzon, 2010; Yehuda, Giller, Southwick, Lowy, & Mason, 1991). Two of the most prominent stress mediators are glucocorticoids and noradrenaline, both of which are known to be major modulators of learning and memory in general (Diamond, Campbell, Park, Halonen, & Zoladz, 2007; Joels, Fernandez, & Roozendaal, 2011; Quaedflieg & Schwabe, 2018; Roozendaal, Okuda, de Quervain, & McGaugh, 2006; Sandi & Pinelo-Nava, 2007; Schwabe, Joels, Roozendaal, Wolf, & Oitzl, 2012). Moreover, there also is evidence that stress affects fear learning processes (Jackson, Payne, Nadel, & Jacobs, 2006; Merz, Elzinga, & Schwabe, 2016; Simon-Kutscher, Wanke, Hiller, & Schwabe, 2019), presumably also driven by glucocorticoids and noradrenergic arousal (Krugers, Zhou, Joels, & Kindt, 2011; Merz, Hamacher-Dang, Stark, Wolf, & Hermann, 2018). Prefrontal and medial-temporal brain areas critically involved in fear generalization (Greenberg et al., 2013; Lissek, Bradford, et al., 2014; Lopresto et al., 2016; Onat & Büchel, 2015) are known to be particularly sensitive to stress and stress mediators (de Kloet, Joels, & Holsboer, 2005; Krugers, Karst, & Joels, 2012; Roozendaal et al., 2006). Furthermore, initial evidence in humans and animals suggests that stress and stress hormones may induce increased fear generalization (Bender, Otamendi, Calfa, & Molina, 2018; Dunsmoor, Otto, & Phelps, 2017; Kaouane et al., 2012; Kolodziejczyk & Fendt, 2020).

Converging lines of evidence from rodent and human studies indicate that stress effects on learning and memory rely on an interaction of noradrenaline and glucocorticoids (Krugers et al., 2012; Roozendaal & Hermans, 2017; Roozendaal, McEwen, & Chattarji, 2009; Roozendaal et al., 2006; Schwabe, Tegenthoff, Hoffken, & Wolf, 2012) and can therefore be blocked by interfering with noradrenergic (or glucocorticoid) signaling. For instance, it has been shown that the effects of stress or glucocorticoids on memory retrieval can be prevented by a pharmacological reduction of noradrenergic activity through the β-adrenoceptor antagonist propranolol (de Quervain, Aerni, & Roozendaal, 2007; Schwabe et al., 2009). Furthermore, fear learning processes per se are susceptible to the administration of propranolol, resulting for example in a reduced contextual fear conditioning or fear memory reconsolidation (de Quervain et al., 2007; Grillon, Cordova, Morgan, Charney, & Davis, 2004; Kindt, Soeter, & Vervliet, 2009). In addition, it is thought that some symptoms of PTSD rely on a heightened responsiveness of the noradrenergic system, which can be reduced by β-adrenergic blockade (Southwick et al., 1999). However, the role of noradrenergic arousal in putative stress effects on fear generalization is, to the best of our knowledge, unknown.

Therefore, the present study was designed to examine whether stress effects on fear generalization require noradrenergic arousal. To this end, participants underwent a 2-day fear generalization paradigm (Onat & Büchel, 2015), in which fear acquisition took place on experimental Day 1. Twenty-four hours later, participants received either a placebo or propranolol and underwent a stress or control manipulation before they completed the critical test of fear generalization. Furthermore, we included a task to assess participants' perceptual discrimination ability to rule out that fear generalization is merely due to insufficient perceptual discrimination. While we initially planned to test propranolol effects on stress-induced changes in fear generalization, we did not observe significant stress effects on fear generalization in the first place. We therefore added the data of a second study in which we used the exact same stress protocol and the exact same fear generalization paradigm and run a Bayesian analysis across both data sets in order to test explicitly the evidence in favor of the observed absence of a stress effect on fear generalization.

Methods and materials

Study I

Participants and experimental design

In Study I, we tested 120 healthy participants (61 women, age: M = 25.21 years, SEM = 0.35 years). This sample size was based on an a priori power analysis with the software G*Power 3.1 (Faul, Erdfelder, Lang, & Buchner, 2007) for our main hypothesis that acute stress increases fear generalization. The analysis revealed that 119 participants are sufficient to detect a medium-sized effect of f = 0.3 with a power of

0.90. Exclusion criteria were a history of any mental or neurological disorder, current medication intake, and drug or tobacco use. In addition, participants were excluded if they had any contraindications for the intake of the beta blocker propranolol. Women were not tested during their menses and those taking hormonal contraceptives were excluded from participation. All participants provided written, informed consent before taking part in the experiment and received a compensation of 60€ for participation. The study protocol was approved by the ethics committee of the State Chamber of Physicians Hamburg and in accordance with the Declaration of Helsinki.

In a double-blind, placebo-controlled, fully crossed, between-subject design with the factors condition (stress vs. control) and drug (propranolol vs. placebo), participants were pseudo-randomly assigned to one of four experimental groups: control + placebo (C+Plac), control + propranolol (C+Prop), stress + placebo (S+Plac), and stress + propranolol (S+Prop). Because successful fear acquisition is a prerequisite for testing stress or noradrenaline effects on fear generalization, we used the successful (explicit) fear acquisition on experimental Day 1 (i.e., US-expectancy rating CS+>CS-) as a predefined criterion for inclusion in the analysis. We based our criterion on the US-expectancy ratings, rather than the SCRs, because SCRs capture specifically arousal-related processes, which can only partly be used to infer fear learning (Lonsdorf et al., 2019). Based on this criterion, 11 participants had to be excluded, leaving a final sample of 109 participants (57 women; age: M = 25.29 years, SEM = 0.35 years; C+Plac: n = 28, C+Prop: n = 27, S+Plac: n = 29, S+Prop: n = 25).

Fear generalization paradigm

To assess fear generalization, we used a recently introduced paradigm (Onat & Büchel, 2015), which included eight face stimuli arranged on a circular similarity continuum along two axes (x-axis: gender; y-axis: identity; Figure 1A). The circular set-up allowed us to investigate a two-sided fear-tuning profile. The opposite points of this circle represent a pair of most dissimilar faces and served as CS+ and CS−, respectively, counterbalanced across participants and groups. In between the CS+ and CS−, stimuli represented the generalization stimuli (GS), which were quantified in their distance to the CS+ (Figure 1B). An unpleasant but not painful electric shock served as unconditioned stimulus (US). Face stimuli were shown for 1.5 sec. During shock trials, the US was presented after 1.4 sec and co-terminated with face offset. The mean intertrial interval was 3.5 sec, ranging between 1.5 and 5.5 sec. To optimally control for participants' attention to the faces, a fixation cross appeared 1 sec before stimulus onset in the middle of the screen and moved with its onset to the forehead of the face. In the middle of the trial, the cross moved to the chin of the face stimulus and disappeared with stimulus offset.

The paradigm comprised a baseline phase, a fear acquisition phase, and a test of generalization (Figure 1C). The baseline phase served as a control for any a priori differences between the faces. Therefore, during the baseline phase, the complete set of faces was shown to the participants. To maintain a comparable level of arousal due to electrical stimulation across all phases of the paradigm, the phase included 10 shock trials (i.e., the same number of US that was administered as in the fear generalization test phase). Importantly, however, the US was always signaled by a shock symbol to ensure full predictability and prevent any association of the shock with any of the faces. In total, the baseline phase contained 293 trials (each face was shown ~34 times) and lasted for approximately 29 minutes. During the fear acquisition phase, only two faces, i.e., the most dissimilar faces, were presented. In ~23% of the trials, one of the faces (CS+) was followed by the US, whereas the other face (CS−) was never paired with the US. Altogether, 123 trials were presented (duration: ~15 minutes). The reinforcement schedule was based on the study by Onat & Büchel (2015), which showed that this schedule was sufficient for participants to reliably learn the CS-US association. In the fear generalization phase, the complete set of faces was shown again to the participants. In contrast to the baseline phase, however, the US followed the presentation of the CS+ in ~23% of the trials to avoid extinction learning to the CS+. The test phase also contained 293 trials and lasted for approximately 29 minutes.

To ensure attention to the faces, participants were prompted to respond to oddball targets (faces with artificially added freckles, ~10 trials) in every phase. Moreover, after each phase, each face was presented two times in randomized order and US-expectancy ratings were assessed using a visual analog scale (VAS; anchors: "1" = certain, no shock; "10" = certain, shock) to measure explicit fear learning.

At the end of the generalization phase, all of the eight face stimuli were shown to the participants as shown in Figure 1A but in a randomized circular order. Participants had to indicate which of the faces was followed by the shock, i.e., to indicate the CS+ face.

To rule out that potential fear generalization is just based on a failure to perceptually differentiate between the faces, we also assessed participants' perceptual discrimination ability. To avoid that participants pay too much attention to differences between the faces and thereby diminishing possible effects of fear generalization, we conducted this discrimination task after the fear generalization phase. In this task, two faces were presented successively and participants were asked to

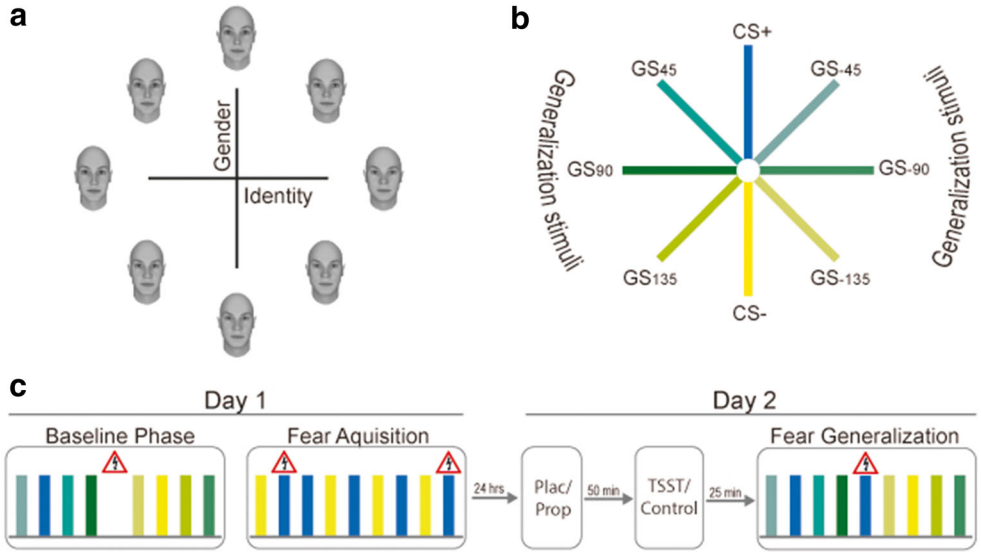

Figure 1. Fear generalization paradigm and stimulus organization. (**A** and **B**) There were eight different face stimuli in total, arranged on a circular similarity continuum with the axes gender and identity. The stimuli in between the CS+ and CS− represent the generalization stimuli (GS). (**C**) Fear generalization paradigm with three phases. On Day 1, the baseline and fear acquisition phases took place. On Day 2, the test of fear generalization followed after the pharmacological and stress/control manipulation. During the baseline phase, the complete set of stimuli (represented by colored bars) was shown to the participants and US were signaled by a shock symbol. During the fear acquisition phase, the two most dissimilar stimuli from opposite sides of the circular similarity continuum were shown to the participants, representing the CS+ and CS−. The CS+ was followed by the US in ~23% of the trials. During the test phase, again the complete set of faces was shown to the participants. To avoid extinction, the CS+ was a reinforced in ~23% of the trials.

rate the faces as being the same or different. In total, the discrimination task consisted of 192 trials.

Stress manipulation and control condition

Participants in the stress condition were exposed to the Trier Social Stress Test (TSST; Kirschbaum, Pirke, & Hellhammer, 1993), a standardized protocol for experimental stress-induction in humans that reliably increases subjective stress levels and activates both the autonomic nervous system and the hypothalamus-pituitary-adrenal axis (Kirschbaum et al., 1993). The TSST consists of a mock job interview and a mental-arithmetic task. First, participants had to prepare and deliver a 5-minute free speech, in which they applied for a job tailored to their interests, followed by a challenging 5-minute mental arithmetic task (counting backwards from 2,043 in steps of 17), while being evaluated by a rather cold and non-responsive panel of two experimenters, both dressed in white lab coats. In addition, participants were videotaped and saw themselves on a screen during task performance. Participants in the control condition gave a 5-minute talk about a topic of their choice, followed by a 5-minute simple arithmetic task (counting forward from zero in steps of 15). Neither an evaluative committee, nor a video camera, were present during the control manipulation.

To validate the successful subjective stress induction, participants rated the difficulty, unpleasantness, and stressfulness of the task on a VAS (anchors: 0 = "not at all"; 100 = "extremely"). In addition, we measured subjective and physiological stress indicators at several time points across the experiment: at the beginning of Day 2, 50 minutes after drug intake (see below), during the TSST or control manipulation (only vital signs), after the TSST/control manipulation (65 minutes after drug intake), before the test of fear generalization (75 minutes after drug intake), after the test of fear generalization (105 minutes after drug intake) and at the end of experimental Day 2 (135 minutes after drug intake). A German version of the Positive and Negative Affect Schedule (PANAS; Krohne, Egloff, Kohlmann, & Tausch, 1996) was used to track potential changes in subjective mood. Blood pressure and pulse were obtained using a Critikon Dinamap system (Tampa, FL) with a cuff placed on the right upper arm. Finally, saliva samples were collected using Salivette® collection devices (Sarstedt, Germany) and stored immediately at −18 °C (−0.4 °F) after testing. At the end of data collection, free cortisol concentrations were analyzed with a luminescence immunoassay (IBL International, Hamburg, Germany).

Pharmacological manipulation

In order to investigate the role of noradrenergic activation in potential stress effects on fear generalization, participants of the C+Prop and S+Prop groups received a small capsule which contained 40 mg of the β-adrenergic receptor antagonist propranolol 50 minutes before the stress or control

manipulation. Participants of the C+Plac and S+Plac groups received identical looking placebo capsules. Dosage and timing of the drug were chosen in accordance with earlier studies that tested the role of noradrenergic arousal in learning and memory processes (Kroes et al., 2016; Schwabe, Nader, Wolf, Beaudry, & Pruessner, 2012; Schwabe et al., 2009). To verify the action of the drug, we analyzed the change in blood pressure and pulse measurements across experimental Day 2.

Control variables

To control for individual differences in subjective chronic stress, depressive mood, and anxiety, participants completed the Trier Inventory for the Assessment of Chronic Stress (TICS; Schulz & Schlotz, 1999), the German version of the Beck Depression Inventory (BDI-II; Beck, Steer, & Brown, 1996), the German version of the State-Trait Anxiety Inventory (STAI; Spielberger & Syndeman, 1994), and the social interaction anxiety scale (SIAS; Stangier, Heidenreich, Berardi, Golbs, & Hoyer, 1999). In addition, we assessed the quantity and quality of participants' sleep over the past 4 weeks and the night between the two experimental days with a modified German version of the Pittsburgh Sleep Quality Index (PSQI; Buysse, Reynolds III, Monk, Berman, & Kupfer, 1989). To validate the successful blinding of the pharmacological manipulation, participants were asked to indicate what they thought which treatment they had received (treatment guess) at the end of the second experimental day.

General procedure

All testing took place between 1:00 pm and 7:30 pm on two consecutive days, with fear acquisition on Day 1 and experimental manipulations and the test of fear generalization on Day 2. The distribution of fear acquisition and test of generalization across 2 days allowed us to isolate stress effects on fear memory generalization, while ruling out influences on early consolidation processes.

Day 1 – Baseline phase and fear acquisition Upon participants' arrival at the lab, baseline measurements of vital signs (i.e., blood pressure and pulse), mood and saliva samples were taken. Afterwards an electrode for the electrical stimulation, serving as US, was placed on participants' back of the right hand. For skin conductance recordings, two electrodes were attached to the left hand. Then, the individual pain threshold was determined using an adaptive testing procedure (QUEST procedure; Watson & Pelli, 1983) to obtain a shock intensity for every participant individually that was unpleasant but not painful, i.e., aiming at a 5 on a scale from 1 (no pain) to 10 (worst pain possible). Next, the baseline phase of the fear generalization paradigm started, followed immediately by the fear acquisition phase (Figure 1C). At the end of Day 1 testing, the pain strength rating as well as vital signs and mood were measured again and another saliva sample was taken.

Day 2 – Experimental manipulations and test phase Same as on Day 1, at the beginning of Day 2, baseline measurements of vital signs, mood and a saliva sample were taken. Depending on the experimental condition, participants then received orally either a placebo or 40 mg of propranolol. During the latency period of 50 minutes, participants filled out the questionnaires before the TSST or control manipulation started. Then, participants completed an unrelated, nonarousing task for ~10 minutes, followed by the determination of the individual pain threshold. Next, approximately 30 minutes after stress onset, the critical test of fear generalization started, followed immediately by the perceptual discrimination task. Finally, participants were asked to indicate their treatment guess to check for successful blinding before participants were debriefed and compensated for participation.

Electrical stimulation and SCR analysis

The US consisted of trains of 5-ms electrical pulses at 66 Hz lasting in total 100 ms, applied via a constant voltage stimulator (STM200, BIOPAC Systems, Goleta USA) with a surface bar electrode attached to the back of the right hand. Electrodermal activity was recorded from the distal phalanx of the index and middle fingers of the left hand, using two 8mm Ag/AgCl electrodes, connected to a MP-150 BIOPAC System (BIOPAC Systems, Goleta USA), and assessed according to common guidelines (Boucsein et al., 2012). A deconvolution technique as implemented in Ledalab version 3.4.9 (Benedek & Kaernbach, 2010) was used to divide raw skin conductance recordings into the slowly varying tonic activity, i.e., skin conductance level, and more quickly varying phasic activity, i.e., skin conductance responses (SCRs). Skin conductance data were downsampled to a resolution of 20 Hz and optimized using four sets of initial values. To obtain the SCRs in response to the different CSs, we derived the average phasic driver within a response window from 1 s to 4 s after stimulus onset. The minimum amplitude threshold was set to 0.01 μS. Zero-responses were omitted from analyses. We calculated SCRs associated with the onset of individual faces at a single subject level, but excluded reinforced CS+ trials, to avoid confounds in SCR change due to electrical stimulation. To correct for interindividual differences, SCRs were z-transformed separately for the three different phases (Ben-Shakhar, 1985). Finally, responses to the different stimuli were averaged and single subject fear-tuning profiles for each phase were derived (Onat & Büchel, 2015; Figure 2).

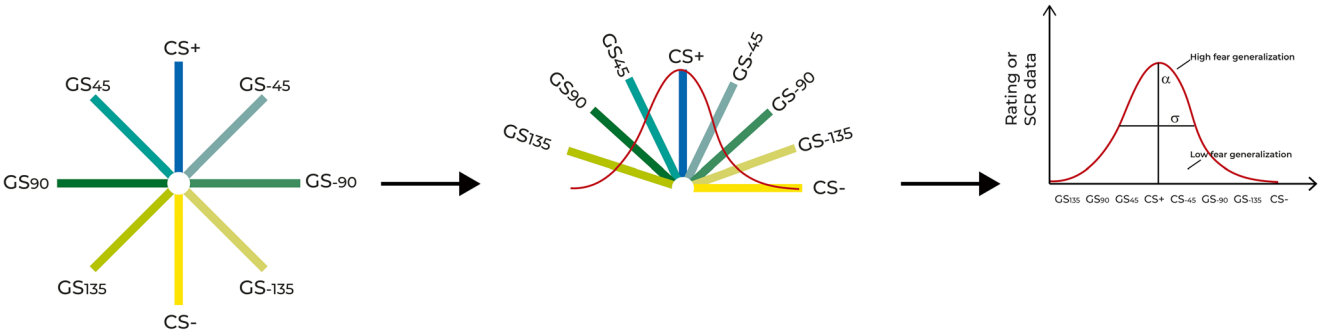

Figure 2. Gaussian-shaped fear-tuning. Bringing the circularly organized stimuli into a 2D coordinate space, it allowed us to fit a Gaussian function—defined through the parameters α (amplitude) and σ (width)—onto the individual responses to the stimuli.

Analysis of fear-tuning profiles

We expected participants to show the highest response to the CS+ and, with decreasing similarity to the CS+, decreasing responses to the other faces and thus to obtain Gaussian shaped fear-tuning profiles (Figure 2). Those individual fear-tuning profiles were analyzed using MATLAB (Release 2016b, Natick, MA). To characterize the fear-tuning, a Gaussian model with two parameters (α, amplitude, i.e., the strength of specificity; σ, tuning width (full width at half maximum), i.e., the strength of fear generalization) was used. We restricted our Gaussian model to be centered on the CS+-face. Fear-tuning profiles were calculated for zSCR and rating data separately. For further statistical analyses, we extracted the amplitude and width parameters of each profile (Onat & Büchel, 2015). For displaying our data of the fear generalization phase, we applied a Gaussian curve fitting function as implemented in SigmaPlot version 14.0 (Systat Software, Inc.).

Statistical data analysis

Statistical analyses were performed with SPSS 25.0 (IBM) and JASP 0.8.1.2 (JASP Team). Because there was no experimental manipulation on Day 1, the respective data were subjected to ANOVAs with the between-subject factor group with four levels (C+Plac, C+Prop, S+Plac, S+Prop). Data of Day 2 were subjected to ANOVAs with the two between-subjects factors condition (stress vs. control) and drug (placebo vs. propranolol). To validate the successful stress and drug manipulation, we used mixed-design ANOVAs with time as within-subject factor and the same two between-subject factors. To analyze the perceptual discrimination ability, a discrimination score was calculated by subtracting the mean false-alarm rate from the mean hit rate. To avoid extinction learning during the fear generalization test, participants still received the US in ~23% of the trials. To investigate, if this reinforcement had an impact on fear generalization on Day 2, we calculated proximity bins by counting the number of trials between the US and the different CS. We calculated the mean and grouped the proximity to US occurrence in three percentiles, whereby we obtained four bins: before any US had occurred; 1-11 trials after US occurrence; 12-26 trials after US occurrence; and >26 trials after US occurrence. We then performed a fear-tuning analysis for the stimuli dependent on the US-proximity and extracted the same parameters as for the general fear-tuning. Significant main or interaction effects were pursued using post-hoc planned comparisons, with Sidak correction if indicated. If the sphericity assumption was violated, Greenhouse-Geisser correction was applied.

To complement our inference statistical results, we additionally analyzed our main hypotheses with Bayesian statistics. This approach allows us to follow-up on possible nonsignificant results, to collect evidence for the null hypothesis, and thus to provide evidence for the presence or absence of an effect (Marsman & Wagenmakers, 2016). Because we had no specific information about our prior distribution, we chose the default Cauchy of 0.707. For paraphrasing the size of a Bayes Factor (BF), we followed the most common system, which suggest that a BF of 1-3 can be interpreted as anecdotal evidence, a BF of 3-10 as moderate evidence and a BF > 10 as strong evidence for the tested hypothesis (Jeffreys, 1961; Lee & Wagenmakers, 2013).

Study II

To determine the influence of stress on fear generalization, we include data from a second study that used the exact same behavioral paradigm as Study I in combination with a stress (TSST) or control manipulation but also included fMRI measurements, the results of which will be reported elsewhere. Seventy-three, healthy, right-handed volunteers (39 women) participated in this second study. In Study II, we also used a 2-day, between-subjects design, in which participants were pseudo-randomly assigned to the stress or control group. Applying the same predefined criterion for successful (explicit) fear acquisition on experimental Day 1 as in Study I, a final sample of 64 participants (34 women; age: $M = 25.5$ years, $SEM = 0.51$ years; Control: n = 31, Stress: n = 33) entered the data analyses. Same as in Study I, we assessed

the same baseline measurements on Day 1, followed by the baseline phase and fear acquisition phase of the fear generalization paradigm. Approximately 24 h later, participants returned to the lab, provided again baseline measurements, and because there was no pharmacological manipulation in this study, the TSST/control manipulation followed immediately afterwards. After providing another saliva sample and vital signs measurement, participants were placed into an MRI scanner and completed the test phase of fear generalization, about 60 minutes after the onset of the stress/control manipulation. Afterwards, we obtained another saliva sample and measurement of vital signs, followed by the perceptual discrimination task outside of the scanner. Finally, participants were debriefed and compensated for participation.

Electrical stimulation and recordings of SCRs were comparable to Study I. Because of MRI acquisition on Day 2, however, electrical stimulation was applied to the lower right leg on both experimental days. Furthermore, we used MRI compatible equipment on Day 2 (BIOPAC Systems, Goleta USA). SCR data analysis and analysis of fear-tuning profiles were done in the exact same manner. Because we only had one between-factor in Study II (condition: stress vs. control), statistical analyses differed slightly, but the general procedure was the same. Data of Study I (placebo groups only) and Study II were merged for the Bayesian analysis.

Results

Study I

Day 1: Successful fear acquisition

At the beginning of experimental Day 1, baseline measurements of vital signs, mood, and salivary cortisol samples revealed no differences between groups (all $F \leq 1.999$; all $p \geq 0.119$; all $\eta^2 \leq 0.056$; Table 1). The analysis of the estimated pain threshold suggested a trend for a group effect ($F(3,105) = 2.228$, $p = 0.089$, $\eta^2 = 0.060$), indicating a slightly higher pain threshold for the S+Plac group compared with the other three groups. However, this difference was not significant and more importantly, there was no group difference regarding the pain strength rating ($F(3,105) = 0.825$, $p = 0.483$, $\eta^2 = 0.023$; Table 1), suggesting that the experimental groups evaluated the electrical stimulation as equally unpleasant.

As expected, because the US in the baseline phase was always signaled by a shock symbol and not associated with a certain stimulus, there were neither main effects nor a face stimulus × group interaction effect for the zSCR data (all $F \leq 1.75.8$; all $p \geq 0.126$; all $\eta^2 \leq 0.017$; Figure 3A). Analysis of the rating data however showed a main effect of face stimulus ($F(2.97,305.94) = 3.838$, $p = 0.010$, $\eta^2 = 0.036$), without any influence of group (both $F \leq 1.186$; both $p \geq 0.319$; both $\eta^2 \leq 0.033$; Figure 3B). Post-hoc comparisons indicated that the first face stimulus was associated with a slightly lower US-expectancy ($M = 4.13$, $SD = 1.64$) than the third ($M = 4.94$, $SD = 1.89$; $p = 0.014$) and the fourth ($M = 4.86$, $SD = 1.92$; $p = 0.043$) face stimulus. However, all of these values reflect a rather high uncertainty about which stimulus is followed by a shock and there was no group main or face stimulus × group interaction effect (both $F \leq 1.1869$; all $p \geq 0.319$; all $\eta^2 \leq 0.033$).

Importantly, all groups showed successful fear acquisition, reflected in both a higher zSCR and a higher US-expectancy rating for the fear conditioned CS+ compared with the CS- (both $F \geq 30.614$; both $p < 0.001$; both $\eta^2 \geq 0.227$; Figure 2C and D, respectively), without any differences between groups (all $F \leq 2.412$; all $p \geq 0.071$; all $\eta^2 \leq 0.017$). Post-hoc *t*-tests indicated successful fear acquisition for all groups in the zSCR data (all $t \geq 2.13$; all $p \leq 0.044$; all $d \geq 0.425$) and in the rating data (all $t \geq 12.543$; all $p < 0.001$; all $d \geq 2.371$).

Day 2

Successful stress manipulation and validated drug action

Baseline measurements on Day 2 showed no differences between groups (all $F \leq 1.335$; all $p \geq 0.267$; all $\eta^2 \leq 0.037$;

Table 1. Physiological, endocrine, and subjective baseline measures on experimental Day 1

Variable	C+Plac	C+Prop	S+Plac	S+Prop
Salivary cortisol (nmol/L)	5.69 (0.64)	4.50 (0.66)	3.54 (0.65)	4.12 (0.67)
Systolic BP (mmHG)	134.25 (3.72)	140.69 (3.79)	136.76 (3.66)	136.92 (3.94)
Diastolic BP (mmHG)	76.61 (1.75)	78.48 (1.79)	78.40 (1.72)	78.22 (1.86)
Pulse (bpm)	84.77(2.88)	85.72 (2.94)	82.93 (2.83)	79.32 (3.10)
Positive affect	2.98 (0.11)	3.04 (0.11)	2.96 (0.11)	2.89 (0.12)
Negative affect	1.27 (0.08)	1.33 (0.08)	1.34 (0.08)	1.35 (0.08)
Pain threshold (V)	39.67 (2.27)	38.45 (2.31)	45.90 (2.23)	39.75 (2.40)
Pain strength	5.36 (0.35)	5.19 (0.36)	5.43 (0.35)	5.96 (0.37)

Data represent mean (standard error of the mean). Positive and negative affect represent scores of the positive and negative affect scale.

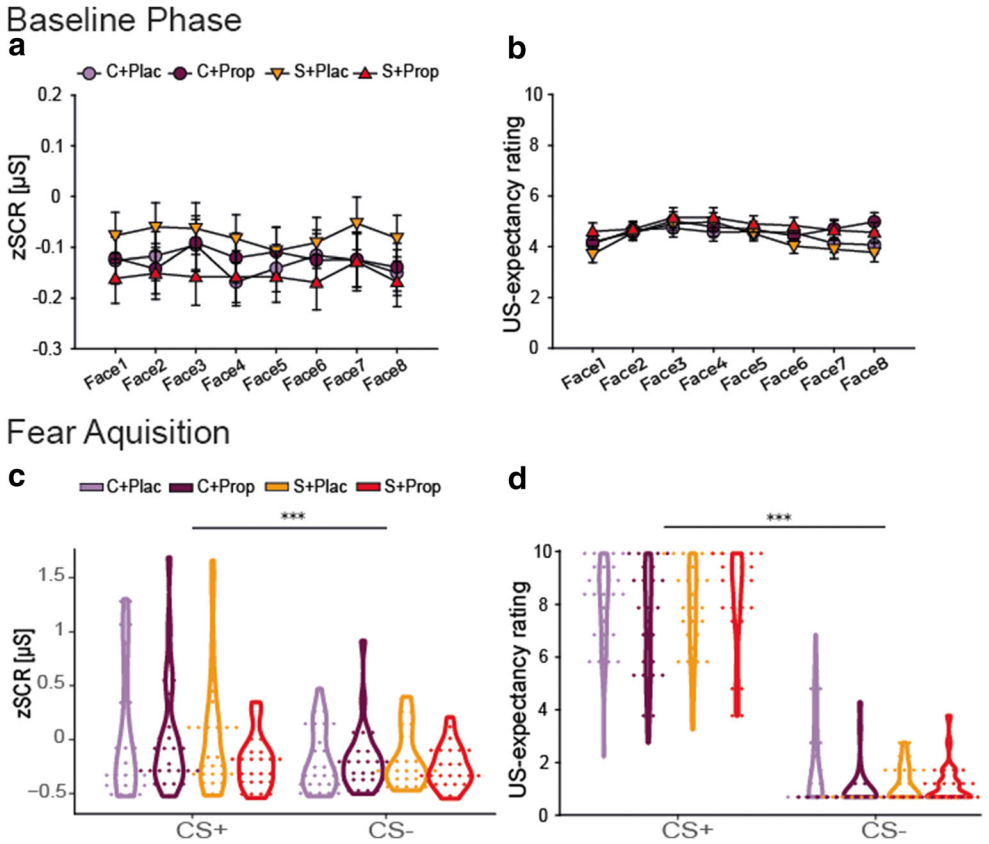

Figure 3. Day 1: Physiological and subjective responses to the face stimuli during the baseline and fear acquisition phases. (**A)** zSCR data as well as (**B**) explicit rating data showed no systematic a priori differences between faces and no group differences during or after the baseline phase. During and after fear acquisition, both (**C**) zSCR data as well as (**D**) explicit rating data showed successful fear learning reflected in higher responses to the CS+ than to the CS− in each group. Exclusion of the outliers do not affect our results. Error bars represent standard errors of the mean. Asterisks denote differences between stimuli (***$p < 0.001$).

Figure 4), except for pulse ($F(3,105) = 3.506$, $p = 0.018$, $\eta^2 = 0.091$). Post-hoc comparisons corrected for multiple testing revealed that the S+Prop group showed a significantly lower pulse than the C+Plac group ($p = 0.022$). We therefore included the baseline pulse as a covariate when analyzing treatment-related changes in pulse.

Significant changes in salivary cortisol, blood pressure, and pulse confirmed the effectiveness of our stress and drug manipulation (Figure 4). For salivary cortisol, in addition to the main effects of time and condition (both $F \geq 31.783$; both $p < 0.001$; both $\eta^2 \geq 0.236$), we obtained the expected significant time × condition interaction ($F(1.758,181.059) = 31.783$, $p < 0.001$, $\eta^2 = 0.236$) and a significant time × condition × drug interaction ($F(1.758,181.059) = 1.426$, $p = 0.019$, $\eta^2 = 0.040$). Post-hoc *t*-tests showed a significantly higher concentration of salivary cortisol for the stress group compared with the control group after the TSST/control manipulation that lasted until after the fear generalization test (all $p \leq 0.009$). Post-hoc tests for the separate time points revealed a trend for a condition × drug interaction for the time point before the test of fear generalization ($F(1,104) = 3.043$, $p = 0.084$, $\eta^2 = 0.028$), driven by a trend for higher cortisol concentrations in the S+Prop group compared with the S+Plac group ($p = 0.092$) and no difference between the two control groups ($p = 0.709$). Importantly, before the test of fear generalization started, both stress groups showed significantly higher salivary cortisol concentrations than both control groups (all $p \leq 0.029$).

The analysis of vital signs revealed for systolic and diastolic blood pressure as well as for pulse significant time × condition and time × drug interaction effects (all $F \geq 4.510$; all $p \leq 0.003$; all $\eta^2 \geq 0.042$; Figure 4), showing that the stress manipulation led to an increase in vital signs whereas propranolol decreased blood pressure and pulse. Only for pulse, there was a time × condition × drug interaction ($F(2.223,228.967) = 4.634$, $p = 0.008$, $\eta^2 = 0.043$). Post-hoc t-tests, however, confirmed that both groups that had received propranolol showed significantly lower pulse than the placebo groups (all $p \leq 0.029$).

As expected, condition—but not drug—influenced the subjective stress response. Both stress groups, irrespective of the pharmacological manipulation (all $p \geq 0.152$), rated the treatment as significantly more difficult, stressful, and unpleasant than the control groups (all $p \leq 0.001$; Table 2). In

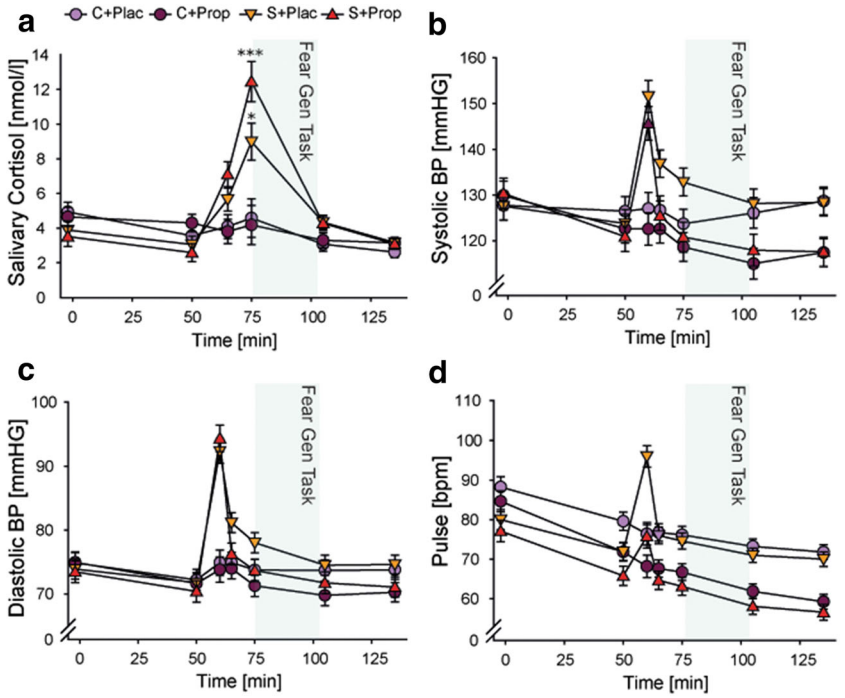

Figure 4. Pharmacological and stress manipulation check. (**A**) Salivary cortisol increased in response to the stress manipulation but was not affected by the pharmacological manipulation. (**B**) Systolic and (**C**) diastolic blood pressure, as well as (**D**) pulse increased during the stress compared with the control manipulation. However, the pharmacological manipulation resulted in reduced vital signs for the S+Prop group compared with the S+Plac group afterwards. Error bars represent standard errors or the mean. Asterisks denote difference between condition (stress vs. control). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

addition, we obtained a trend for a main effect of drug in the unpleasantness rating ($p = 0.062$). Post-hoc tests, however, revealed no significant difference between the placebo and propranolol groups ($p = 0.119$).

As on Day 1, we tested whether the stress or drug manipulation affected the pain threshold and pain strength rating. Results revealed a main effect of drug in the estimated pain threshold ($F(1,104) = 4.050$, $p = 0.047$, $\eta^2 = 0.037$) and a trend toward a main effect of condition ($F(1,104) = 3.491$, $p = 0.065$, $\eta^2 = 0.032$). After correcting for multiple comparisons, post-hoc tests revealed that the S+Plac group had a higher pain threshold than the C+Plac group ($p = 0.039$). With respect to the pain strength rating, there was a trend for a condition × drug interaction ($F(1,103) = 3.383$, $p = 0.069$, $\eta^2 = 0.032$), but post-hoc comparisons showed no significant difference between groups (all $p > 0.255$), i.e., groups experienced the electrical stimulation as comparably unpleasant.

Acute stress leaves fear generalization unaffected

Analyses of the fear-tuning parameters (amplitude and width) for the zSCR and the rating data revealed that fear memory specificity (amplitude), as well as fear generalization (width) were neither affected by condition nor by drug (all $F \leq 1.998$; all $p \geq 0.160$; all $\eta^2 \leq 0.019$; Figure 5). When specifically focusing on the differentiation ability between the fear conditioned CS+ and the safety signaling CS−, results showed a significant main effect of stimulus (both $F \geq 37.415$; both $p < 0.001$; both $\eta^2 \geq 0.265$), indicating that the acquired fear was still present. However, the absence of any main or interaction effect including the factors condition or drug (all $F \leq 2.894$; all $p \geq 0.092$; all $\eta^2 \leq 0.027$) suggested that neither acute stress nor propranolol affected the differentiation ability.

In a next step, we analyzed a possible influence of the US proximity; i.e., we aimed to test whether potential stress or drug effects might evolve only for stimuli occurring shortly after a reminder of the CS-US association (Figure 6). Analysis of zSCR data revealed a significant proximity effect ($F(1.952,202.982) = 9.361$, $p < 0.001$, $\eta^2 = 0.083$), mirroring an increased amplitude of fear-tuning when the CS occurred shortly after the US. This was confirmed by post-hoc t-tests showing a significantly higher amplitude of all fear-tuning curves after a reminder US had occurred compared to trials presented before any US had occurred (all $p \leq 0.002$). However, this effect was also neither influenced by condition nor by drug (all $F \leq 2.412$; all $p \geq 0.094$; all $\eta^2 \leq 0.023$). Analyzing the width of the fear-tuning revealed no significant main or interaction effects at all (all $F \leq 1.852$; all $p \geq 0.140$; all $\eta^2 \leq 0.018$), indicating that the width of fear-tuning remained unaffected by US-proximity for all our groups. Consequently, the lack of stress effects cannot be explained by an influence of US-proximity.

Table 2. Subjective responses on experimental Day 2

Variable	C+Plac	C+Prop	S+Plac	S+Prop
Positive affect				
Baseline	3.08 (0.13)	2.91 (0.13)	2.87 (0.12)	2.80 (0.13)
+ 50 min	2.88 (0.14)	2.63 (0.14)	2.52 (0.13)	2.61 (0.14)
+ 65 min	2.86 (0.13)	2.93 (0.13)	2.55 (0.13)*	2.60 (0.14)*
+ 75 min	2.61 (0.15)	2.54 (0.15)	2.45 (0.14)	2.49 (0.15)
+ 105 min	2.32 (0.14)	2.27 (0.14)	2.20 (0.13)	2.18 (0.14)
+ 135 min	2.31 (0.14)	2.34 (0.14)	2.28 (0.14)	2.17 (0.15)
Negative affect				
Baseline	1.21 (0.07)	1.36 (0.07)	1.30 (0.07)	1.30 (0.07)
+ 50 min	1.14 (0.05)	1.18 (0.05)	1.18 (0.05)	1.23 (0.05)
+ 65 min	1.18 (0.11)	1.20 (0.11)	2.01 (0.11)***	1.49 (0.12)***
+ 75 min	1.18 (0.09)	1.18 (0.09)	1.62 (0.09)*	1.21 (0.10)*
+ 105 min	1.27 (0.06)	1.15 (0.06)	1.36 (0.06)	1.15 (0.07)
+ 135 min	1.11 (0.04)	1.09 (0.04)	1.14 (0.04)	1.09 (0.04)
Pain threshold	43.56 (2.33)	40.65 (2.41)	49.87 (2.29)	43.22 (2.47)
Pain strength	6.19 (0.36)	5.52 (0.36)	5.89 (0.35)	6.56 (0.38)
TSST Questionnaire				
Difficulty	3.68 (0.42)	3.56 (0.43)	8.03 (0.41)***	7.48 (0.44)***
Unpleasantness	3.46 (0.43)	2.93 (0.43)	7.93 (0.42)***	6.84 (0.45)***
Stress	3.07 (0.41)	3158 (0.42)	7.69 (0.41)***	6.56 (0.44)***
Control variables				
STAI-S	35.32 (5.58)	38.48 (8.09)	38.45 (6.67)	38.60 (6.99)
STAI-T	36.07 (7.71)	36.41 (9.82)	37.69 (7.80)	38.60 (7.80)
BDI-II	5.86 (5.28)	5.47 (5.77)	8.55 (6.58)*	7.56 (3.97)*
TICS	11.54 (7.35)	12.89 (11.19)	16.00 (8.40)	14.36 (8.41)
SIAS	1.04 (0.61)	0.88 (0.66)	1.05 (0.51)	1.14 (0.56)
PSQI	7.14 (4.68)	8.07 (4.90)	8.66 (4.58)	8.44 (4.16)
Sleep quality between the days	75.25 (16.65)	70.70 (15.68)	66.86 (20.61)*	59.16 (26.22)*

Data represent mean (standard error of the mean). Positive and negative affect represent scores of the positive and negative affect scale. STAI = State-Trait Anxiety Inventory; BDI-II = Beck Depression Inventory; TICS = Trier Inventory for the Assessment of Chronic Stress; SIAS = Social Interaction Anxiety Scale; PSQI = Pittsburgh Sleep Quality Index. Asterisks denote differences between condition factor (stress vs. control) (*$p < 0.05$; ***$p < 0.001$).

Finally, we analyzed the general perceptual discrimination ability. Results of this analysis revealed a main effect of condition ($F(1,104) = 7.779$, $p = 0.006$, $\eta^2 = 0.070$), showing that both control groups (C+Plac: $M = 0.61$, $SD = 0.07$; C+Prop: $M = 0.59$, $SD = 0.07$) were better in discriminating the faces than the stress groups (S+Plac: $M = 0.57$, $SD = 0.07$; S+Prop: $M = 0.55$, $SD = 0.08$).

Control variables

The treatment guess at the end of the experiment indicated that participants were not aware of the administered drug. Most participants (74%) guessed that they had received a placebo, without any difference between the four groups ($\chi^2(3) = 4.632$, $p = 0.201$, Cramer's $V = 0.207$). In addition, we obtained no group differences in terms of state, trait, or social anxiety (all $F \leq 1.524$; all $p \geq 0.220$; all $\eta^2 \leq 0.014$; Table 2). Furthermore, groups reported a comparable quantity and quality of sleep over the 4 weeks before testing (all $F \leq 1.133$; all $p \geq 0.290$; all $\eta^2 \leq 0.011$). However, we obtained a significant main effect of condition for the quality of sleep between the two test days ($F(1,105) = 6.703$, $p = 0.011$, $\eta^2 = 0.060$), and for the level of depressive mood ($F(1,105) = 4.532$, $p = 0.036$, $\eta^2 = 0.042$), suggesting a worse night of sleep and a higher level of depressive mood in the stress group. With respect to the level of chronic stress, results also revealed a trend for a main effect of condition ($F(1,105) = 2.995$, $p = 0.086$, $\eta^2 = 0.028$), suggesting a higher level of chronic stress in participants of the stress group. To rule out that the aforementioned results are partly due to these group differences, we included

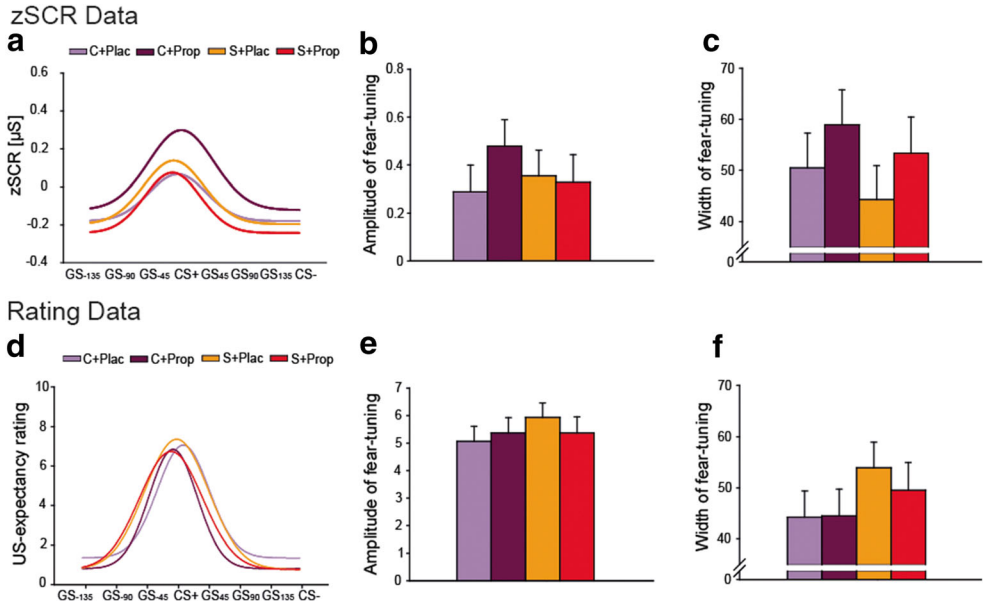

Figure 5. Day 2: Fear generalization phase. (**A**) Fear-tuning of zSCR to different stimuli during the test phase. No significant difference was seen between groups neither in (**B**) the strength of responding to the CS+ nor in (**C**) the fear generalization in the zSCR data. (**D**) Fear-tuning of US-expectancy rating to different stimuli after the test phase. Results of fear tuning of the rating data mirrored those obtained with the zSCR data. No significant difference was seen between groups neither in (**E**) the strength of responding to the CS+ nor in (**F**) the fear generalization.

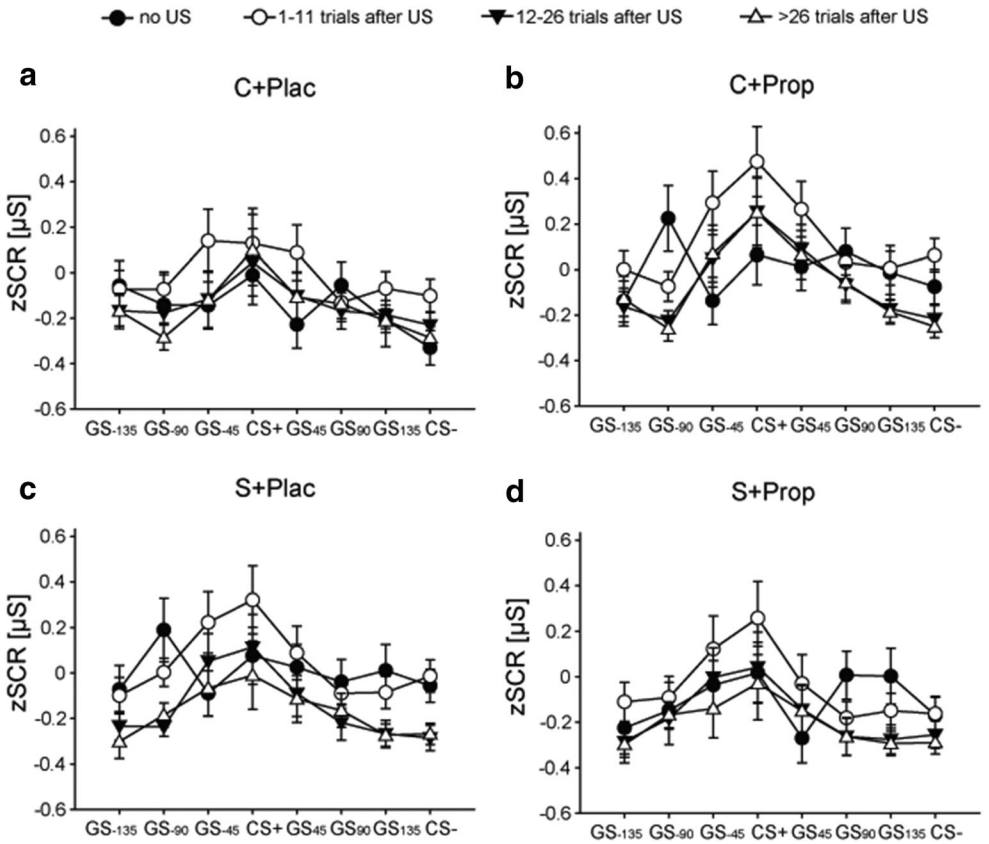

Figure 6. Fear-tuning dependent on US distance. Fear-tuning amplitude was dependent on US-proximity, i.e., all groups showed a higher fear-tuning amplitude, when they recently were reminded of the US-CS+ association. In contrast, the width of fear-tuning was unaffected by US-proximity across groups.

these variables as covariates and re-run all our analyses. Controlling for these group differences, however, left our results largely unaffected, in particular there was no evidence for any stress-induced changes in fear memory generalization (width: all $p \geq 0.143$; amplitude all $p \geq 0.178$).

Study II: summary of results

We provide here a brief summary of the results of Study II (Figure 7). The detailed results of this study, including the fMRI data which are beyond the scope of the present manuscript, will be reported elsewhere.

At baseline on Day 1, participants of the stress and control groups did not differ in any of the baseline measurements, pain threshold or intensity (all $t \leq 1.292$; all $p \geq 0.201$; all $d \leq 0.326$). During the acquisition phase, participants showed successful fear acquisition toward the CS+, indicated by a higher zSCR and subjective shock expectancy ratings for the CS+ compared with the CS− in the fear acquisition phase (both $F \geq 21.272$; both $p < 0.001$; both $\eta^2 \geq 0.255$), without any differences between groups (all $F \leq 1.597$; all $p \geq 0.211$; all $\eta^2 \leq 0.025$; Figure 6A and B).

On Day 2, groups showed comparable baseline levels of subjective mood, salivary cortisol, blood pressure, pulse, and pain strength rating (all $t \leq 1.649$; all $p \geq 0.104$; all $d \leq 0.412$). The subsequent stress induction via the TSST was successful as indicated by significant changes in subjective and physiological measurements. Participants of the stress group rated the TSST as significantly more challenging, uncomfortable, and stressful than the control group (all $t \geq -4.948$; all $p \leq 0.001$; all $d \geq 1.238$) and showed an increase in salivary cortisol, blood pressure, and heart rate from before to after the manipulation in contrast to the control group (all $F \geq 6.251$; all $p < 0.001$; all $\eta^2 \geq 0.168$; Figure 7C and D).

The fear-tuning curves obtained on Day 2 during the fear generalization phase showed no influence of the stress manipulation, neither for the amplitude nor for the width of fear-tuning for both of our measurements (all $t \leq 1.052$; all $p \geq 0.297$; all $d \geq 0.265$; Figure 7E and F). Moreover, also the successful discrimination between CS+ and CS−, indicated by a significant stimulus main effect in both zSCR and rating data (both $F \geq 42.465$; both $p < 0.001$; both $\eta^2 \geq 0.410$), did not differ between groups (all $F \leq 0.662$ all $p \geq 0.419$; all $\eta^2 \leq 0.011$). We further analyzed the influence of US-proximity and on the specific CS+/CS− discrimination. Same as in Study I, our analyses revealed a significant proximity effect for the amplitude of fear-tuning ($F(1.796,107.779) = 12.138$, $p < 0.001$, $\eta^2 = 0.168$), with post-hoc comparisons showing a significantly lower fear response from before any US occurred to all proximity bins after US administration (all $p \leq 0.042$). However, this effect was not influenced by group as there was neither a main effect of condition nor a group × proximity interaction effect (both $F \leq 0.323$; both $p \geq 0.702$; both $\eta^2 \leq 0.005$). Regarding the width of fear-tuning, Study II showed the same pattern of results as Study I, indicating

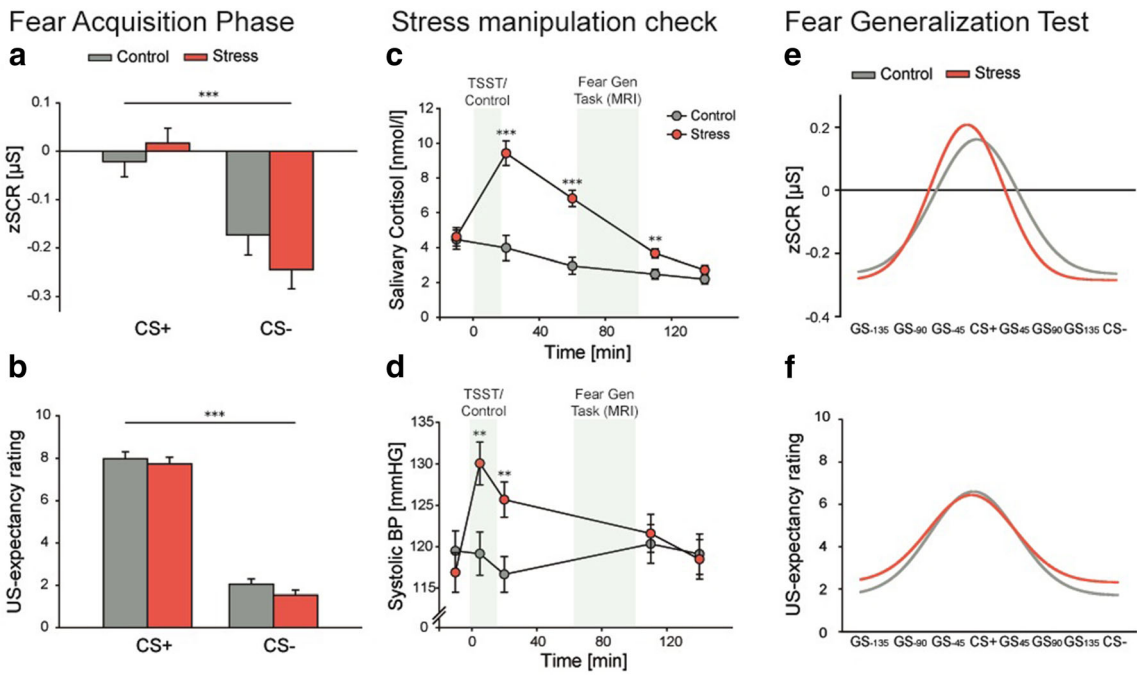

Figure 7. Results summary of Study II. Both groups showed successful fear acquisition on Day 1 in (**A**) zSCR data as well as (**B**) rating data. On Day 2, stress manipulation shortly before the test of fear generalization was successful, indicated exemplarily in an increase in (**C**) salivary cortisol and (**D**) systolic blood pressure in the stress group but not in the control group. The test of fear generalization revealed comparable fear-tuning curves for both groups across measurements, i.e., (**E**) zSCR data and (**F**) rating data.

no influence of US-proximity or group (all $F \leq 0.768$; all $p \geq 0.513$; all $\eta^2 \leq 0.013$).

Finally, in contrast to the results of Study I, analyses of the general perceptual discrimination ability revealed no significant group differences ($t(62) = 0.321$, $p = 0.750$, $\eta^2 = 0.080$).

Bayesian analysis across studies I and II provides evidence for an absence of a stress effect on fear generalization

Inference statistical results of Studies I and II converge in that they suggest that acute psychosocial stress has no influence on fear generalization in healthy participants. In order to assess the evidence in favor of the null hypothesis, we complemented these results with Bayesian analyses. Therefore, we combined the sample of Study II with the placebo groups of Study I (i.e., C+Plac and S+Plac; final sample n = 121) and analyzed the amplitude and width of our fear-tuning profiles obtained with our zSCR data and rating data with Bayesian independent samples *t*-tests. Results showed that the obtained Bayes factors for our analyses of fear-tuning amplitude and width for the zSCR and rating data provide evidence for the H_0 (Table 3). Specifically, the Bayes factors indicate that it is 4.5 (zSCR data) and 5.1 (rating data) times more likely that the amplitude of our fear-tuning profiles does not differ between the stress and the control group. In addition, it is 3.2 (zSCR) and 2.3 (rating data) more likely that also the width of the fear-tuning profiles remains unaffected by the acute stress exposure. Figure 8 depicts the sequential analysis of the data, i.e. the evidential flow for the accumulating data. This visualization suggests that the data favors rather consistently and constantly the H_0. However, it should be noted that this evidence for the H_0 ranges between moderate (width of the rating data and amplitude and width of the zSCR data) and anecdotal (amplitude of the rating data). At the same time, the error percentage of all our analyses is ≤0.013%, which suggests a high stability of the underlying numerical algorithm that was used to obtain these results.

Table 3. Results of Bayesian independent samples *t*-test

Variable	BF_{01}	Error %
zSCR data		
Amplitude of fear-tuning	4.551	0.004
Width of fear-tuning	3.225	0.001
Rating data		
Amplitude of fear-tuning	2.310	0.004
Width of fear-tuning	5.122	0.013

Discussion

Our results showed no influence of stress on fear generalization, neither in autonomic responding (SCR), nor in verbal report (US-expectancy ratings). Based on these results, we included data of a second study that differed in some aspects (e.g., MRI vs. behavioral study) but used the exact same paradigm and reanalyzed our data with Bayesian statistics to test the evidence in favor of an absence of a stress effect on fear generalization. This analysis provided evidence that stress has no impact on fear generalization in a population of young healthy individuals. Likewise, the blockade of noradrenergic arousal through propranolol left fear generalization unaffected.

In contrast to the present results, previous evidence in rodents suggested that stress may increase fear generalization (Bender et al., 2018; Kaouane et al., 2012). However, findings in rodents are also heterogeneous. Whereas one study showed fear generalization after corticosterone injection (Kaouane et al., 2012), another study did not show such an influence (Bueno, de Paiva, Correa, Tiba, & Fornari, 2017). Obviously, species differences, for instance in metabolism or brain structure, might hamper the translation of findings from rodents to humans. However, in addition to species differences, there were important methodological differences between previous rodent studies and the present study, which may account for the partly discrepant results. First, there are differences in the timing of the stress induction. Previous animal studies exposed rats to stress either before (Bender et al., 2018) or immediately after (Kaouane et al., 2012) fear conditioning, which most likely affected initial fear acquisition and/or consolidation and thus makes it impossible to disentangle these effects from potential changes in the actual generalization of fear. In the present study, we exposed participants to stress 24 h after fear conditioning. After a traumatic event, people may suffer from flashbacks, nightmares, or intrusive memories, which again result in a marked stress response and may add to an increase in fear generalization. Our delayed stress manipulation therefore enabled us to isolate these later stress effects on fear generalization from those during initial fear acquisition or consolidation. In addition, the animal studies targeted primarily the influence of stress on contextual fear generalization, and one study explicitly showed no effect of glucocorticoid injection on cued fear generalization (Kaouane et al., 2012), which is in line with the present results. Finally, it should be noted that previously reported increases in fear generalization were obtained only when threat intensities were rather high and corticosterone levels exceeded a certain threshold (Kaouane et al., 2012). This is in line with another study in humans, which showed increased fear generalization only when the US intensity was rather high compared with low (Dunsmoor, Kroes, et al., 2017).

Figure 8. Flow of evidence for H_0. With accumulating data, fear-tuning results of zSCR and rating data show rather evidence in favor of the H_0, i.e., no influence of stress on fear-tuning, in contrast to the H_1, i.e., there is an influence of stress on fear generalization. BF_{01} = Bayes Factor for the H_0. BF_{10} = Bayes Factor for the H_{01}.

In our experiment, we explicitly instructed participants to determine a pain threshold of a moderate intensity, i.e., the electrical shock should be unpleasant but not painful. Yet, the only previous study in humans that investigated the influence of stress on fear generalization used nonpainful shocks as well but did obtain a stress effect (Dunsmoor, Otto, et al., 2017). There are, however, other variables that differ between this previous study and the present studies, which may explain the different findings. While the studies differ in the modality of the CS (auditory vs. visual) and the used stressor, the most significant difference relates to the learning schedule. Compared with the earlier study, the present studies had a lower reinforcement rate (40% vs. 23%) and used considerably more trials (20 and 64 trials vs. 123 and 293 trials), both during acquisition and during the generalization test. Accordingly, fear learning may have taken longer but may have been more intense in the present study, rendering it potentially less vulnerable to a subsequent stress manipulation. This would have been in line with the finding that partial reinforcement rates, in contrast to continuous reinforcement, weaken the development of conditioning (Dunsmoor, Bandettini, & Knight, 2007). At the same time, partial reinforcement rates are assumed to prolong fear memory extinction (Lonsdorf et al., 2017).

Because we did not obtain any stress effects on fear learning, it is not surprising that in addition, there were no interaction effects of stress and propranolol. Furthermore, our results neither revealed any effects of propranolol per se. This is in contrast to previous studies that showed an influence of propranolol on fear learning processes, such as extinction learning (Burhans, Smith-Bell, & Schreurs, 2018; Chalkia,

Weermeijer, Van Oudenhove, & Beckers, 2019; but see Rodriguez-Romaguera, Sotres-Bayon, Mueller, & Quirk, 2009), fear memory reconsolidation (Kindt et al., 2009; Soeter & Kindt, 2011, 2012), or the return of fear memory (Kroes et al., 2016). However, these previous studies yielded partly inconsistent results. These inconsistencies may be due to the distinct fear learning processes under investigation, including extinction, reconsolidation, return of fear, and—in the present study—fear generalization. Moreover, it has been suggested that the administration of propranolol might primarily affect the fear-arousing aspects, reflected for instance in the startle response, but less in declarative aspects of fear memory, reflected in skin conductance responses, subjective distress, and expectancy ratings (Kindt et al., 2009; Soeter & Kindt, 2011, 2012). Furthermore, it has been shown that fear conditioning measured with the startle response is not dependent on conscious discriminative fear learning, whereas fear conditioning measure in SCR is (Sevenster, Beckers, & Kindt, 2014). In contrast to the SCR, the fear potentiated startle (FPS) does not decrease with repeated presentation of the same stimulus (Boucsein et al., 2012), and additionally, it can be evoked at other time points, independently of CS presentation, which makes it possible to compare a response to a specific CS with a baseline (Lonsdorf et al., 2017). However, studies combining SCR or FPS measurement with fMRI found a similar relationship regarding the neural underpinnings, such that the amygdala correlated with conditioned SCRs as well as conditioned FPS (MacNamara, Rabinak, Fitzgerald, Zhou, Shankman, Milad, & Phan, 2015; van Well, Visser, Scholte, & Kindt, 2012). Thus, differences in the obtained measures of fear might account for the discrepant findings between studies, and it cannot be completely ruled out that there might have been an influence of stress and/or propranolol in the present study if we had included additional measures, such as the startle response.

On a neural level, it has been shown that stress mediators act mainly on the hippocampus, amygdala, and prefrontal cortex (for a review see McEwen, Nasca, & Gray, 2016), all of which are known to play an important role in the process of fear generalization (Dunsmoor & Paz, 2015; Lissek, Bradford, et al., 2014; Onat & Büchel, 2015). One previous study in animals directly injected glucocorticoids into the hippocampus and found an increase in fear generalization only in contextual fear learning but not in cued fear learning (Kaouane et al., 2012). Results of another study that specifically investigated cued fear generalization (Pollack, Bezek, Lee, Scarlata, Weingast, & Bergstrom, 2018) are in line with our results, as they found an increase in fear generalization with passing time. In addition, their results suggest that cued fear generalization is, in part, dissociable from contextual fear generalization. Based on these results, one could assume that stress may have a higher impact on contextual fear generalization compared with cued fear generalization, which might explain the lack of a stress effect in our studies.

Finally, our results were not only consistent across ("declarative") measures, i.e., shown in our SCR data as well as in our US-expectancy rating data, but also across independent experiments. A Bayesian analysis across these independent studies supported the conclusion that acute stress does not affect fear generalization in a population of healthy, young individuals. However, multiple studies in patients suffering from anxiety or stress-related disorders, such as generalized anxiety disorder (Lissek, Kaczkurkin, et al., 2014), social anxiety disorder (Ahrens et al., 2016), or panic disorder (Lissek et al., 2010) or PTSD (Kaczkurkin et al., 2017), showed a broader fear generalization gradient compared with healthy controls, supporting the idea of fear overgeneralization as a transdiagnostic marker across multiple fear-related disorders (Dymond, Dunsmoor, Vervliet, Roche, & Hermans, 2015). At the same time, there is broad evidence that stress impacts these fear-related disorders (de Quervain et al., 2017). Therefore, while we obtained no effect of acute stress on fear generalization in healthy individuals, there may well be an important effect in vulnerable populations, such as individuals at high-risk for anxiety disorders or PTSD. If stress increases fear generalization in these populations, testing whether a blockade of noradrenergic arousal might counteract this stress-induced fear overgeneralization would be highly relevant.

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Appendix D

Study IV

Blocking under stress: Sustained attention to stimuli without predictive value?

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Blocking under stress: Sustained attention to stimuli without predictive value?

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ABSTRACT

Learning is blocked when a stimulus is followed by an outcome that is identical to what was expected and thus contains no new information. This classic 'blocking' effect exemplifies that learning is driven by the predictive value of stimuli, which in turn should guide the allocation of attentional resources. Stress is known to be a powerful modulator of learning and memory. However, whether stress may affect attentional processing during predictive learning is largely unknown. Here, we combined electroencephalography and eye-tracking with an experimental stress manipulation and a fear conditioning paradigm designed to probe the blocking effect, to determine if and how stress impacts efficient attentional processing during predictive learning. Participants' explicit ratings indicated, irrespective of stress, a blocking effect. The control group further showed preferential attentional processing of predictive vs. unpredictive stimuli, reflected in differential fixation durations and a differential N2pc. Stress abolished this differentiation and led even to sustained attention, indicated by higher late positive potentials, to stimuli with low predictive value. Moreover, stress resulted in an overall increase in the P3b during the blocking phase, suggesting increased attentional processing, presumably due to impaired access to previously learned associations. Together, our results suggest that while control participants paid particular attention to predictive stimuli and reduced attention to unpredictive stimuli, in line with the classic blocking effect, stress before learning reduced this preferential processing. Thus, the present findings highlight the role of attention allocation for predictive fear learning and suggest that stress may impair efficient information processing against the background of prior experiences.

1. Introduction

Learning to predict significant events in the environment is crucial for survival. Associative learning theory suggests that such learning is driven by the predictive relationship between two stimuli and that learning should only occur if a discrepancy between an expected and actual outcome, i.e. a prediction error, is encountered (Mackintosh, 1975; Pearce & Hall, 1980; Rescorla & Wagner, 1972). The critical relevance of the predictive relationship between stimuli for learning is demonstrated by the classic blocking phenomenon (Kamin, 1968): when a neutral stimulus A is repeatedly followed by an unconditioned stimulus (US), the fully predictive stimulus A becomes a conditioned stimulus (CSA). If a new stimulus X (CSX) is added to the CSA and the compound CSAX is also repeatedly followed by the US, conditioning to the CSX is strongly reduced (or blocked). The CSX has no predictive value as the US can be fully predicted based on the CSA alone, thus there will be no new learning to the CSX. In contrast, if another stimulus B is never followed by the US, stimulus B is a non-predictive stimulus (CSB) for this outcome. If another stimulus Y is added to the CSB and the compound stimulus CSBY is followed repeatedly by the US, learning to the CSY should occur because it is predictive of the US, i.e. it contains new information. Here, we aimed to investigate attentional processes that are critical for the blocking effect and whether the blocking phenomenon may be affected by acute stress.

The blocking effect has been repeatedly demonstrated in humans (Balaz, Gutsin, Cacheiro, & Miller, 1982; Beesley & Le Pelley, 2011; Eippert, Gamer, & Buchel, 2012; Luque, Vadillo, Gutierrez-Cobo, & Le Pelley, 2018; Tobler, O'Doherty, Dolan, & Schultz, 2006; Wills, Lavric, Croft, & Hodgson, 2007; but see Maes et al., 2016) and several studies aimed at investigating its cognitive and neural basis. Based on the existing literature, we assume that attentional processes may be involved in the blocking effect. In particular, previous eye-tracking studies suggested less allocation of attentional resources to the redundant stimulus compared to a predictive one, when presented together (Beesley & Le Pelley, 2011; Eippert et al., 2012; Kruschke, Kappenman, & Hetrick, 2005; Le Pelley, Beesley, & Griffiths, 2014; Wills et al., 2007). Further

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evidence for altered attentional processing depending on the informational value associated with a stimulus comes from two studies using electroencephalography (EEG; Sanchez-Nacher, Campos-Bueno, Sitges, & Montoya, 2011; Wills et al., 2007). For instance, stimuli that contained no predictive value and to which learning was therefore blocked were shown to be associated with reduced early event-related potentials (ERPs), suggesting reduced attentional processing (Wills et al., 2007). Moreover, a functional magnetic resonance imaging (fMRI) study revealed decreased amygdala activity to a blocked versus non-blocked CS in fear conditioning, suggesting less fear learning to the blocked stimulus. Additionally, different parts of the prefrontal cortex, i.e. dorsolateral prefrontal cortex (dlPFC) and ventromedial prefrontal cortex (vmPFC), appear to be differently involved in the acquisition of the blocking effect. Specifically, whereas the vmPFC was specifically active when conditioned stimuli were established as predictive for an outcome, the dlPFC was active when conditioned stimuli had to be established as both predictive or non-predictive (Eippert et al., 2012). Together, these studies provide first evidence that the allocation of attentional resources plays an important role in the development of the blocking effect and that the blocked stimulus may attract less attention. However, the few studies that used EEG to study the blocking effect so far used reward learning paradigms and the only study assessing the neural basis of the blocking effect in fear learning used fMRI, which is less well suited to assess fast attentional processes (Woodman, 2010). Thus, in aversive learning the attentional processing of stimuli depending on their predictive value remains not well understood.

Moreover, to date it remains unclear which factors determine the extent to which we efficiently process stimuli based on their informational value and, more specifically, to which extent learning to stimuli with low predictive value is blocked. Research over the past decades has demonstrated that acute stress is a major modulator of cognitive processing in general and learning and memory in particular (Diamond, Campbell, Park, Halonen, & Zoladz, 2007; Joels, Fernandez, & Roozendaal, 2011; Lupien, McEwen, Gunnar, & Heim, 2009; Roozendaal, 2002; Schwabe, Joels, Roozendaal, Wolf, & Oitzl, 2012; Vogel, Fernandez, Joels, & Schwabe, 2016). Furthermore, stress and stress hormones are known to affect the activity of the amygdala and prefrontal areas (de Voogd, Klumpers, Fernandez, & Hermans, 2017; Lovallo, Robinson, Glahn, & Fox, 2010; Pruessner et al., 2008; Schwabe, Tegenthoff, Hoffken, & Wolf, 2012; Wirz, Reuter, Felten, & Schwabe, 2018; for a review see Arnsten, 2009), which are critically involved in the blocking effect (Eippert et al., 2012; LeDoux, Cicchetti, Xagoraris, & Romanski, 1990), and to modulate attentional processing (Hermans, Henckens, Joels, & Fernandez, 2014; Schwabe & Wolf, 2010). However, so far, the effect of stress on aversive predictive learning and the blocking effect, in particular has not been investigated yet. Based on findings showing that acute stress interferes with prefrontal cortex functioning (Arnsten, 2009; Bogdanov & Schwabe, 2016; Qin, Hermans, van Marle, Luo, & Fernandez, 2009) and the efficient use of prior knowledge (Buchanan, Tranel, & Adolphs, 2006; de Quervain, Roozendaal, & McGaugh, 1998; Kluen, Nixon, Agorastos, Wiedemann, & Schwabe, 2017; Vogel, Kluen, Fernandez, & Schwabe, 2018a, 2018b), we hypothesized that stress would impair the efficient allocation of attention based on the predictive value of a stimulus and hence reduce the blocking effect. In particular, we expected that the eye-tracking data would reveal differential effects during the acquisition of blocking, when two stimuli were presented at the same time. Specifically, we expected reduced attention to the CSX in controls relative to stressed participants, reflecting the successful blocking effect for this stimulus. Regarding the EEG data, we expected in anticipation of a shock an increased SPN in the initial conditioning phase for the CSA, for which participants learned that this stimulus will be followed by a shock, relative to the CSB. For the blocking phase, we did not have specific hypotheses for the newly introduced compound stimuli. For the final test phase, we expected reduced early attentional processing towards the blocked stimulus CSX, mirrored by the N2pc and heightened late attentional processing, mirrored by the P3b and LPP for the control group. Furthermore, we expected that for the stress group these effects would be diminished.

Thus, the present experiment aimed to examine (i) how attentional resources are allocated during aversive predictive learning and which neural mechanisms are involved in this process and (ii) whether acute stress modulates the blocking effect. Therefore, participants completed first a classical fear acquisition phase in which one stimulus (CSA) was paired with an unpleasant shock (i.e. US), whereas another stimulus was never paired with a shock (CSB). Afterwards, participants underwent either a stress or control manipulation, followed by a blocking phase in which CSA and CSB were presented together with a new stimulus (CSAX and CSBY, respectively) and both compounds were paired with the US. Thus, a blocking effect should develop for the CSX, paired with the fully predictive CSA, but not for the CSY. Whether the CSX and CSY acquired the potency to elicit a fear response was tested in a final phase, in which CSX and CSY were presented individually. In order to track the development of a blocking effect and related attentional processing, we measured EEG and eye-tracking. We focused on several ERPs that are associated with attentional and anticipatory mechanisms and may therefore be relevant in the context of the blocking effect. Specifically, we focused on the N2pc, reflecting fast attentional reallocation towards relevant information (Eimer, 1996), the P3b and the late positive potential (LPP) that are associated with sustained emotional processing of task-relevant stimuli (Mangun & Hillyard, 1990; Polich, 2007; Schupp, Flaisch, Stockburger, & Junghöfer, 2006) and the stimulus-preceding negativity (SPN), that is considered to be an indicator of anticipatory attention (Böcker, Baas, Kenemans, & Verbaten, 2001; van Boxtel & Böcker, 2004). Because electrodermal activity (EDA) is a widely used indicator of fear learning (Lonsdorf et al., 2017), we measured EDA throughout the learning task. In particular, we expected an increased EDA to the CSA compared to the CSB, as an indicator for successful fear learning and a reduced EDA to the CSX compared to the CSY, as an indicator for successful blocking. Additionally, we expected the stress group to show a higher EDA towards the CSX compared to the control group, representing a failure in successful blocking.

2. Methods and materials

2.1. Participants and experimental design

Eighty-eight healthy men and women between 18 and 35 years of age participated in this experiment. Four participants had to be excluded due to technical failure (n = 2) or because they did not complete the learning task (n = 2), thus leaving a final sample of 84 participants (44 women; mean age = 25.79 years; SD = 4.34 years). Participants were screened for the following eligibility criteria before testing: right-handedness, Body Mass Index between 19 and 26 kg/m^2, no intake of medication, no current or lifetime mental disorders, no current or history of drug abuse. In addition, we excluded smokers and women taking hormonal contraceptives as both factors may affect the endocrine stress response (Kudielka, Hellhammer, & Wust, 2009; Rohleder & Kirschbaum, 2006). Menstrual cycle phase in women did not differ between stress and control group (stress: 10 in follicular phase, 9 in luteal phase; control: 14 in follicular phase, 6 in luteal phase; $\chi 2(1) = 2.077$; $p = .150$). The study protocol was approved by the Ethics Committee of the Faculty of Psychology and Human Movement at the University of Hamburg. All participants provided written informed consent and received a monetary compensation (35 €) for participation.

In a between-subjects design, participants were pseudo-randomly assigned to a stress or control condition, ensuring an equal number of men and women in both groups (22 women, 20 men in each group).

2.2. Stress induction and control manipulation

Participants in the stress condition were exposed to the Trier Social Stress Test (TSST; Kirschbaum, Pirke, & Hellhammer, 1993). The TSST is a standardized stress-induction protocol for humans that reliably increases subjective stress levels and activates both the autonomic nervous system and the hypothalamus-pituitary-adrenal axis (Kirschbaum et al., 1993). Briefly, the TSST mimics a job interview, consisting of a 5-minute public speech in which participants have to indicate why they are the ideal candidate for a job tailored to their interests as well as a 5-minute mental-arithmetic task (counting backwards from 2043 in steps of 17). Throughout the TSST, participants were standing in front of a panel of two experimenters, introduced as experts in behavioral analysis, who were dressed in white lab coats, acted in a rather reserved and non-reinforcing manner, and evaluated participants' performance continuously. In addition, participants were videotaped and saw themselves on a screen, placed behind the panel, while performing the two tasks.

In the control condition, participants gave a 5-minute talk about a topic of their choice and performed a simple arithmetic task (counting forward from zero in steps of 15), without being evaluated by a committee or videotaped.

To validate the successful stress induction by the TSST, subjective stress ratings, measurements of blood pressure and heart rate as well as saliva samples for subsequent cortisol analysis were taken at several time points before, during and after the experimental manipulation. Subjective ratings were assessed with three visual analogue scales (VAS; anchors: 0 = "not at all"; 100 = "extremely") on which participants rated the difficulty, unpleasantness and stressfulness of the task. Measurements of blood pressure and heart rate were taken using a Critikon Dinamap system (Tampa, FL, USA), with a cuff placed on the right upper arm. Saliva samples were obtained with Salivette® collection devices (Sarstedt, Germany) and stored immediately after testing at −18 °C (−0.4°F). At the end of data collection, free cortisol concentrations were analyzed from saliva samples with a luminescence immunoassay (IBL-International, Hamburg, Germany).

2.3. Associative learning task

In order to test the impact of stress on the blocking effect, we employed a paradigm that had been used before to study blocking effects in appetitive (Tobler et al., 2006; Waelti, Dickinson, & Schultz, 2001) as well as aversive conditioning (Eippert et al., 2012). In this paradigm, eight colored, abstract visual stimuli displayed on white background served as CSs and an unpleasant electrical shock as US. For each participant, one of the stimuli was randomly assigned to one out of four possible CS types (CSA, CSB, CSX and CSY; see below). The intensity of the US was individually set to a level that was experienced as unpleasant but not painful (see below).

On each trial, participants saw either a single CS, presented in one of the four corners of the screen (randomized), or a CS compound, consisting of two stimuli that appeared both either on the left or right of a fixation cross, for 5 s. For those CSs that were paired with the US, a train of three 2 ms electrical pulses (separated by 50 ms) was presented 4.7 s after CS onset. Between trials there was an interval (ITI) of 3–7 s, during which the black fixation cross stayed on the screen and participants were instructed to fixate on the cross. In order to keep participants attentive, we further implemented a simple attentional control task, requiring participants to indicate via a button press on ten percent of the trials whether the CS appeared on the left or right side of the fixation cross. Due to technical failure, responses in this attentional control task were not recorded for nine participants. The basic trial procedure was practiced in 12 trials before the start of the actual learning task. In this training phase, four stimuli not used in the main task were presented and no US was applied.

The actual learning task consisted of three phases (Fig. 1). Throughout all phases, CS presentation order was pseudorandomized with the constraint that no CS could occur more than twice in a row. The first phase was the fear acquisition phase in which participants were presented the CSA, which was always paired with the US (100 percent reinforcement), and the CSB, which was never paired with the US. During the acquisition phase, which lasted about 15 min, the CSA and CSB were presented 30 times each.

In the second phase, which took about 20 min, the blocking effect should develop. Therefore, the CSA that previously always co-terminated with the US was now additionally presented together with a new stimulus X to form the compound stimulus CSAX. The CSB, which was never paired with the US during the initial fear acquisition phase, was now additionally presented together with the new stimulus Y, thus forming the compound stimulus CSBY. Each compound was presented 30 times, with pseudorandomized position of the individual stimuli in the compound (top or down; see Fig. 1). Both compounds were always paired with the US (100 percent reinforcement). Since the CSA reliably predicted the US during conditioning, the CSX had no predictive value, consequently learning to the new stimulus CSX should be blocked. In contrast, learning to the CSY should occur because the CSB was never paired with the US before. To maintain the CS-US association acquired during initial conditioning, CSA and CSB were presented also 15 times each alone, with the same contingency as during conditioning (i.e. CSA always and CSB never paired with the US). To induce a rather elemental mode of processing (instead of a configural mode), the spatial distance of the CSs in a compound was maximized (Eippert et al., 2012; Glautier, 2002; Livesey & Boakes, 2004).

The blocking effect was tested in a final phase, which took about 30 min and in which the CSX and CSY were presented individually, i.e. without the CSA and CSB, respectively, 60 times each and without the US. The presentation of CSX and CSY was pseudo-randomly intermixed with the presentation of the CSA and CSB (each presented 15 times, with the same contingency as during conditioning) and the compound stimuli CSAX and CSBY (each presented 30 times, both always paired with the US). Thus, each single CS and CS compound was presented 60 times in total. In line with previous conditioning studies, a new phase always started with the presentation of a known CS-type to facilitate the transition between the different phases (Eippert et al., 2012; Hinchy, Lovibond, & Ter-Horst, 1995).

At the end of the task, participants' contingency awareness was assessed by presenting each stimulus again individually. Participants were instructed to indicate on a VAS (anchors: 0 = "Certain, no shock", 100 = "Certain, shock") whether the respective CS was paired with the US in the experiment.

2.4. Study procedure

In order to control for the diurnal rhythm of cortisol, all testing took place in the afternoon between 1 and 8 pm. Upon their arrival in the lab, participants provided written informed consent and completed questionnaires assessing depressive mood, subjective chronic stress, and anxiety (Beck Depression Inventory (BDI-II); Beck, Steer, & Brown, 1996; Trier Inventory for the Assessment of Chronic Stress (TICS); Schulz & Schlotz, 1999; State-Trait Anxiety Inventory (STAI); Spielberger & Syndeman, 1994, respectively). Afterwards, participants were prepared for the EEG, eye-tracking and SCR measurements. In addition, the electrode for shock administration was attached to the right lower leg. Next, participants provided a first saliva sample for subsequent cortisol analysis, their vital signs (blood pressure, heart rate) were measured, and they completed a German questionnaire assessing subjective mood (Mehrdimensionaler Befindlichkeitsfragebogen (MDBF); Steyer, Schwenkmezger, Notz, & Eid, 1994). After these baseline measurements, the individual pain threshold was determined. We aimed at reaching a moderate level of pain (unpleasant but not painful). Participants received an electric shock and should rate its painfulness on a numerical rating scale (anchors: 0 = "no pain",

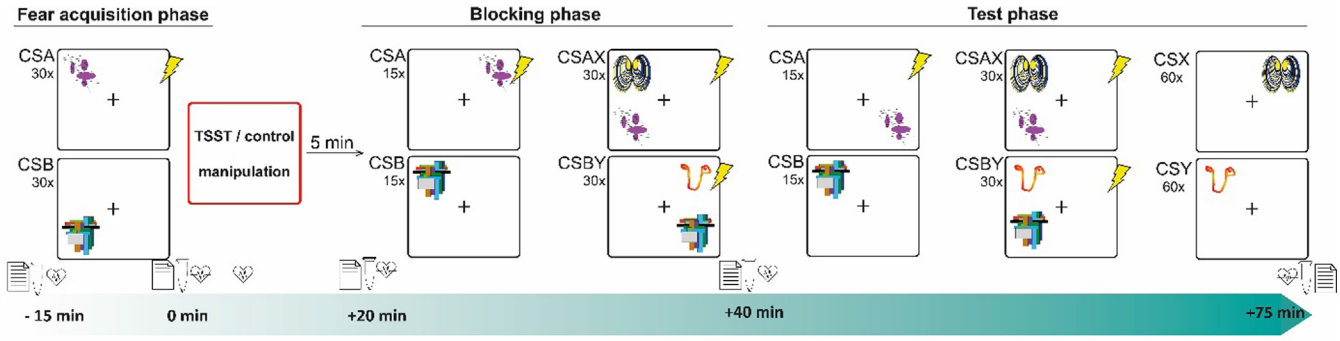

Fig. 1. Blocking paradigm and stress measurements over time. In the initial fear acquisition phase, two stimuli were presented at an equal rate. A CSA was always followed by the US, whereas a CSB was never paired with the US. During the second phase, the blocking phase, in addition to CSA and CSB, two compound stimuli CSAX and CSBY were introduced, comprised of the old stimuli and two new stimuli. Compound stimuli were continuously followed by the US. Contingencies for the CSA and CSB stayed the same as in the fear acquisition phase. In the final test phase, the CSX and CSY were presented individually, never followed by the US. In addition, CSA, CSB and the compound stimuli CSAX and CSBY were presented, with the same contingency as introduced. In addition, the time points are depicted when cortisol, ANS and subjective measures were taken.

10 = "worst pain imaginable"). After having rated a shock twice with a rating of 5, the mean of the two measures was taken as individual pain threshold. To further promote the development of a blocking effect, an additivity and submaximality manipulation followed (Beckers, De Houwer, Pineno, & Miller, 2005; Eippert et al., 2012; Mitchell & Lovibond, 2002). This was done by presenting two stimuli separately (different from those used in the associative learning task) for 5 s, both co-terminated with a shock (intensity equals the individual pain threshold). Afterwards, the two stimuli were presented as a compound for 5 s and co-terminated with a shock of an intensity that was previously determined as being twice as painful as the individual pain threshold. This procedure should inform participants that the outcome of conditioned stimuli may be additive and that receiving a shock stronger than the individual pain threshold is possible (although in the actual experiment the shock intensity always stayed the same). Next, the eye-tracker was calibrated applying a 12-point calibration and validation procedure before the acquisition phase of the associative learning task started (the calibration procedure was repeated before each phase of the task). After the acquisition phase, participants provided another saliva sample and their vital signs and mood were assessed. This was followed by either the TSST or control manipulation in a different room. Back in the testing room, participants completed the VAS-based subjective stress ratings and a MDBF, provided a third saliva sample and their vital signs were measured. About five minutes after the stress/control manipulation, the second phase (blocking phase) of the learning task started. Afterwards, another saliva sample was collected, and vital signs and mood were assessed. This was followed by the final phase of the learning task, the test phase. At the end of the learning task, a final saliva sample was taken as was a last measurement of vital signs and mood. Finally, all the electrodes were removed, participants were debriefed, compensated and thanked for their participation.

2.5. Manipulation check and behavioral data analysis

Analyses of behavioral performance, physiological and subjective stress responses were performed with SPSS 25.0 (IBM), using a α-error threshold of $p = 0.05$. Significant main or interaction effects were pursued using *post-hoc* planned comparisons, with Sidak correction if indicated. If the sphericity assumption was violated, Greenhouse-Geisser correction was applied. Physiological stress responses (i.e. cortisol response, blood pressure and heart rate) were subjected to a repeated measures analysis of variance (ANOVA), with the between-subjects factor group (control and stress) and within-subjects factor time (time points of measurement). To further test whether the observed stress effects were mainly driven by stress-induced cortisol, we subdivided our stress group into cortisol responders (baseline to peak increase > 1.5 nmol/l) and cortisol non-responders (baseline to peak increase < 1.5 nmol/l; Schwabe, Bohringer, Chatterjee, & Schachinger, 2008). Subjective stress ratings were assessed with a univariate ANOVA and mood assessments were subjected to a repeated measure ANOVA as was the US-contingency rating. For four participants (two of each experimental group), subjective stress ratings were missing, so were the measurements of mood for three participants (one of the stress group, two of the control group). To assess task compliance, we calculated the number of missed responses to the attentional control task. For nine participants, no responses were recorded due to technical failure.

2.6. Shock administration and SCR analysis

Shock administration was performed using a constant voltage stimulator (STM200, BIOPAC Systems, Goleta USA) and consisted of a train of three 2 ms pulses (separated by 50 ms) which were delivered to the participant's right lower leg via a surface bar electrode.

EDA was recorded from the distal phalanx of the index and middle fingers of the left hand, using two 8 mm Ag/AgCl electrodes, connected to the MP-160 BIOPAC System (BIOPAC Systems, Goleta USA). The EDA can be divided into the slowly varying tonic activity which is represented by the skin conductance level (SCL) and a rather rapidly varying phasic activity, mirrored by the SCRs, which we were interested in. From the raw skin conductance recordings, the SCRs were computed using a continuous decomposition analysis as implemented in Ledalab version 3.4.9 (Benedek & Kaernbach, 2010). Specifically, we were interested in the anticipatory SCR within a response window from 0.5 s to 4 s after stimulus onset. Anticipatory SCR refers to the SCR that is expected to evolve in anticipation of a consequence to a certain stimulus, independent of any other influence but the immediately preceding stimulus. Importantly, the US always occurred exactly 5 s after stimulus onset, thus leaving the anticipatory SCR unaffected by the shock itself. The minimum amplitude threshold was set to 0.01 μS. Because of a too low SCR, two participants were lost for the SCR analysis. From the other participants 64.11% of all trials over all three phases entered the analyses. Due to group differences in the baseline phase (see below), we computed ΔSCR, by subtracting the SCR to CSB from the SCR to CSA, thus mirroring the response difference to CSA vs. CSB and included this difference score as a covariate in all further analyses.

2.7. Eye-tracking recordings and analysis

Eye-tracking data were acquired with an EyeLink 1000 Plus (SR Research) device using the desktop mount installation and recorded from the right eye. We predefined four regions of interest by a rectangle

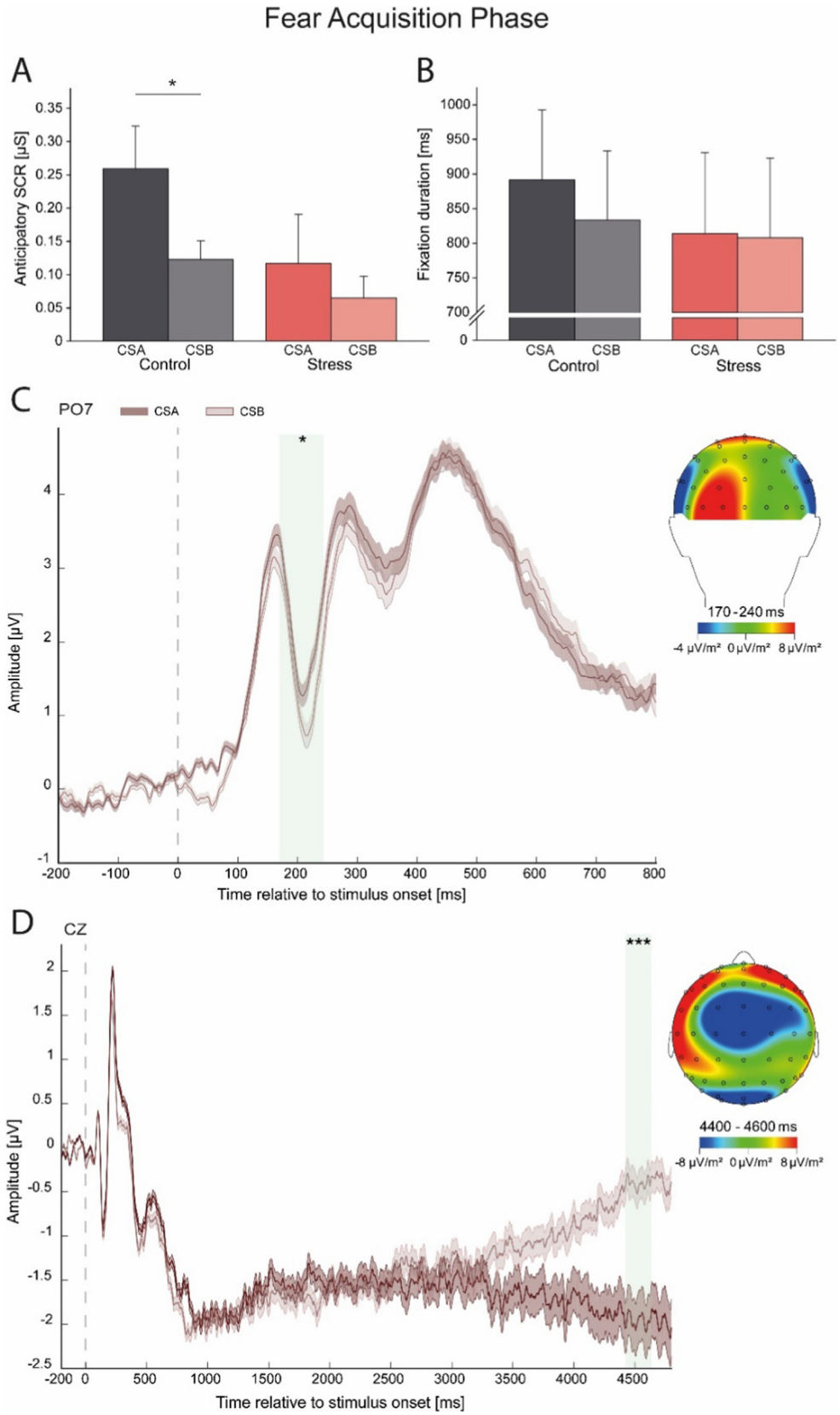

Fig. 2. Fear acquisition phase. (A) Participants' mean anticipatory SCRs were higher for the shocked CSA vs. the non-shocked CSB, in particular in the control group. (B) Participants' spend more time fixating the CSA compared to the CSB, in particular in the control group. (C) Participants' N2pc and (D) SPN was in both groups higher for the CSA compared to the CSB. Error bars and shaded error bars represent standard error. Asterisks denote difference between CSA and CSB: *$p < .05$, **$p < .01$, ***$p < .001$.

of 530 × 532 pixels in the four corners of the screen. The Data Viewer software (SR Research) was used to extract the total fixation duration participants spend on each CS, either presented individually or in a compound, in each trial and the sum of first saccades made to the different CS types when presented in a compound. To ensure the same starting point for each first saccade in every trial, the subsequent trial started only when participants had fixated on the fixation cross for at least the last second of the ITI. In addition, we excluded saccades from the analysis that occurred earlier than 150 ms or later than 1000 ms after CS onset. For the analysis of the fixation duration, we calculated a cumulative fixation duration on every CS (presented alone and presented in a compound) within a time window from 150 to 5000 ms after stimulus onset. For the analysis of first saccades to one of the compound stimuli, we computed a mean sum score for each CS type. Due to group differences in the fixation duration to CSA and CSB in the baseline phase (see below), we computed a ΔFixDur variable, subtracting the fixation duration to CSB from the fixation duration to CSA, thus mirroring the difference in fixation duration to CSA vs. CSB and included this difference score as a covariate in all further analyses. For statistical analysis, we used either paired *t* tests or repeated measures ANCOVAs. For one participant of the stress group eye-tracking data were missing, leaving eighty-three participants for eye-tracking analyses.

2.8. EEG recordings and analysis

EEG data were acquired with a BioSemi Active Two electrode system at 2048 Hz (BioSemi, Amsterdam, the Netherlands). Brain electrical activity was recorded from 64 Ag/AgCl electrodes including two mastoids according to the 10–20 electrode reference system. All sites were referenced to Cz. A bipolar horizontal and vertical electrooculography (EOG) was recorded from the epicanthus of each eye and the supra- and infraorbital positions of the right eye, respectively. Raw data was processed offline with BrainVision Analyzer 2.1 (Brain Products, Gilching, Germany). After down sampling the data to 512 Hz, a band-pass filter was applied with high and low cutoffs of 0.1 Hz and 30 Hz, respectively. Because data of the electrode sites Iz, P9 and P10 was too noisy and the electrode at O2 was damaged for the last ten participants, we excluded those electrodes from further pre-processing. Then, an ocular intercomponent analysis (ICA) was conducted and data was re-referenced to the average activity of all electrodes. Continuous EEG data were segmented into epochs with a length of 5000 ms (−200–4800 ms with respect to stimulus onset) and baseline-corrected with respect to the 200 ms pre-stimulus interval. Epochs were excluded if at any EEG electrode the following criteria were exceeded: a maximal voltage step of ± 75 μV, a maximal allowed absolute difference of 200 μV and lowest allowed activity of 0.1 μV within 1000 ms intervals. For each participant, separate ERP averages were computed for each stimulus for each phase. Based on previous stress, blocking and conditioning studies (Bublatzky & Schupp, 2012; Cuthbert, Schupp, Bradley, Birbaumer, & Lang, 2000; Nelson, Weinberg, Pawluk, Gawlowska, & Proudfit, 2015; Sanchez-Nacher et al., 2011; Sanger, Bechtold, Schoofs, Blaszkewicz, & Wascher, 2014; Weymar, Schwabe, Löw, & Hamm, 2012) and corroborated by visual inspection of the grand-averaged ERPs and topographical maps of the different waveforms, the chosen electrode sites and the time windows for component analyses were set as follows: 170–240 ms (N2pc at P5, P6, PO3, PO4, PO7, PO8, POz, Oz), 300–450 ms (P3b at P7, P8, PO7 and PO8), 400–1000 ms (LPP at C1, C2, Cz, CP1, CP2, CPz) and 4400–4600 ms (SPN at C1, C2, Cz). Mean amplitude measures were separately submitted to repeated measures ANOVA including the factors electrode site and group. Separate ANOVAs were performed for each component (N2pc, P3b, LPP and SPN). One participant from the stress group had to be excluded from EEG data analysis because of missing data. In addition, only participants who contributed at least 80% of trials after artifact rejection were included in the EEG analyses. This resulted in an exclusion of 19 participants for phase one (stress: $n = 9$, control: $n = 10$), 6 participants for phase two (stress: $n = 2$, control: $n = 4$) and 8 participants for phase three (stress: $n = 4$, control: $n = 4$).

3. Results

3.1. Fear acquisition phase

3.1.1. SCR data

Successful fear acquisition should be reflected in stronger responding to the CSA, which was paired with the US, than to the CSB, which was never paired with the US. We assessed fear acquisition at three levels: SCR, eye-tracking and ERPs. Furthermore, we obtained explicit shock expectancy ratings at the end of the task (see below).

SCR analysis revealed a significant stimulus main effect ($F(1,80) = 4.03$, $p = .048$, $\eta^2 = 0.048$), indicating that the CSA elicited a significantly higher SCR than the CSB, representing successful fear acquisition (Fig. 2, A). However, there was also a significant stimulus × group interaction ($F(1,80) = 4.54$, $p = .036$, $\eta^2 = 0.054$), suggesting that whereas the control group showed stronger SCR responding to the CSA than to the CSB ($t(39) = 2.14$, $p = .038$, $d = 1.202$), the stress group did not ($t(41) = -0.186$, $p = .853$, $d = 0.309$). In order to check for possible habituation effects which might have been stronger for the stress group and therefore resulted in a non-successful discrimination, we divided the acquisition phase into two halves. However, the pattern of results stayed the same. We found a significant stimulus main effect ($F(1,80) = 4.36$, $p = .040$, $\eta^2 = 0.052$) and a significant stimulus × group interaction ($F(1,80) = 4.51$, $p = .037$, $\eta^2 = 0.053$) replicating the results of the overall analysis.

3.1.2. Eye-tracking data

In order to make sure that participants paid a comparable amount of attention to the CSA and CSB, which is an important requirement for the development of a reliable blocking effect, we analyzed the fixation duration participants spend on each of the two stimuli. As expected, we did not find a significant stimulus main effect ($F(1,81) = 0.08$, $p = .773$, $\eta^2 = 0.001$), indicating that both stimuli got the same amount of attention. However, there was a significant stimulus × group interaction effect ($F(1,81) = 3.98$, $p = .049$, $\eta^2 = 0.047$), showing that participants in the control group spend more time fixating the CSA than fixating the CSB ($t(41) = 2.44$, $p = .019$, $d = 0.374$), whereas participants in the stress group did not ($t(40) = -0.96$, $p = .344$, $d = 0.149$; see Fig. 2B).

3.1.3. ERP results

At brain level, we found a significant main effect of stimulus type for the N2pc ($F(1,61) = 4.23$, $p = .044$, $\eta^2 = 0.065$), showing that the N2pc was more negative for the CSA compared to the CSB, which might reflect a higher degree of early attention to the threat stimulus CSA compared to the safe stimulus CSB (Bublatzky & Schupp, 2012; see Fig. 2 C). Interestingly, there was also a significant stimulus main effect for the SPN ($F(1,62) = 14.99$, $p < .001$, $\eta^2 = 0.195$), showing that the SPN was more negative for CSA compared to CSB, which might point to an anticipatory preparation for the CSA co-terminating with the US (Bocker, Baas, Kenemans, & Verbaten, 2004; see Fig. 2D). For the LPP, we did not find any significant difference between stimuli (all $p > .417$). There were no group differences for any of the ERPs (all $p > .275$), indicating that the CSA vs. CSB differentiation that was observed in the N2pc and SPN was equally strong in the stress and control groups.

Together, these data show (i) successful fear acquisition in the control group, both at the SCR, eye-tracking and brain level and (ii) successful fear acquisition in the stress group shown in differential brain responses to CSA and CSB, despite no differentiation in the SCR and eye-tracking data.

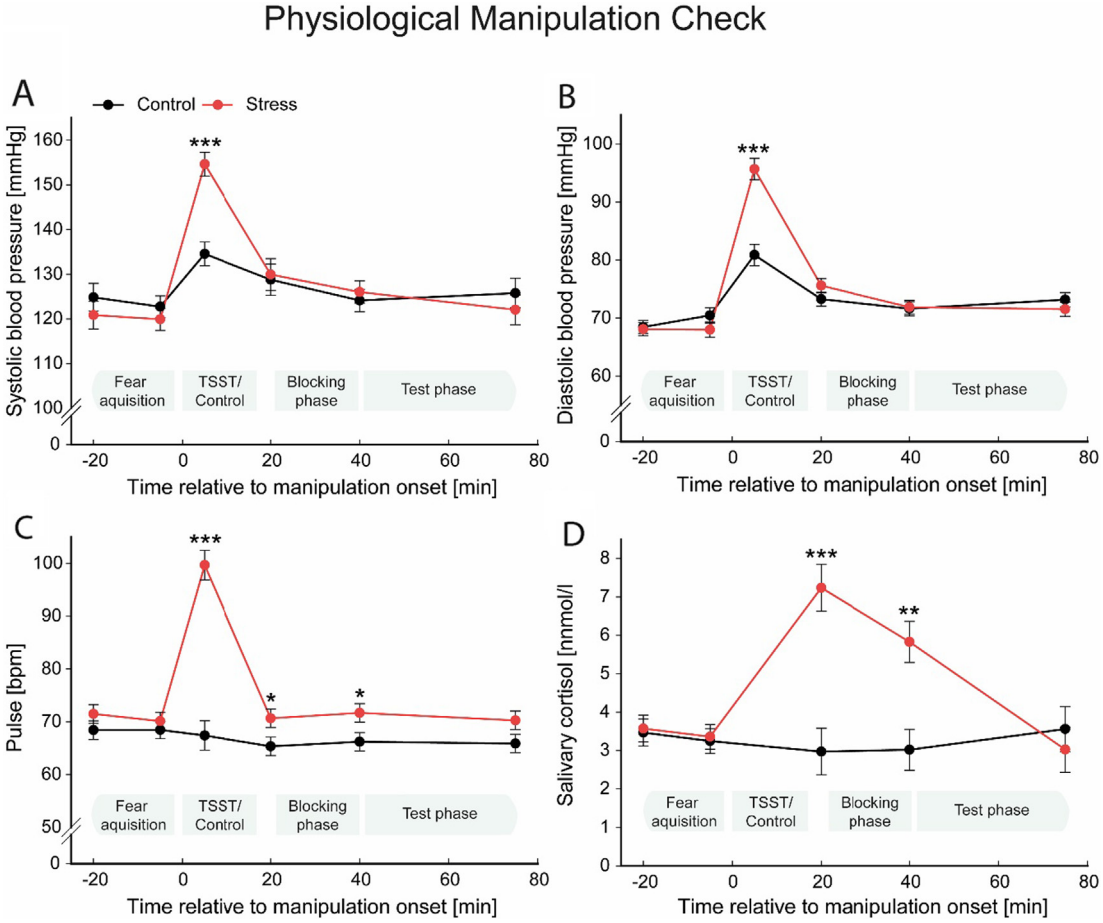

Fig 3. Autonomic and endocrine response to the psychosocial stressor. Successful stress manipulation as indicated by higher (A) mean systolic blood pressure, (B) mean diastolic blood pressure and (C) mean pulse during the TSST for participants of the stress compared to the control group. (D) In addition successful stress induction was shown by participants' mean salivary cortisol response, that was higher 20 and 40 min post TSST for the stress compared to the control group. Error bars represent mean standard error. Asterisks denote difference between control and stress group: *$p < .05$, **$p < .01$, ***$p < .001$.

3.2. Successful stress induction

Subjective, autonomic and endocrine changes confirmed the successful stress induction by the TSST (Fig. 3). Compared to the control group, participants in the stress group experienced the experimental manipulation as significantly more difficult ($t(78) = -3.753$, $p < .001$, $d = 0.839$), unpleasant ($t(78) = -3.398$, $p = .001$, $d = 0.760$) and stressful ($t(78) = -3.538$, $p = .001$, $d = 0.791$; see Table 1). At the autonomic level, there was a significant time × group interaction for systolic blood pressure ($F(3.92,321.22) = 13.539$, $p < .001$, $\eta^2 = 0.142$), diastolic blood pressure ($F(2.57,210.38) = 25.743$, $p < .001$, $\eta^2 = 0.239$) and pulse ($F(1.73,142.11) = 65.33$, $p < .001$, $\eta^2 = 0.44$). As shown in Fig. 3, systolic and diastolic blood pressure as well as pulse were significantly higher in the stress group than in the control group during the experimental manipulation (all $p < .001$), whereas groups did not differ in these measures at baseline (all $p > .219$). The pulse remained even significantly higher for the stress compared to the control group until 40 min after the treatment (20 min post stress: $t(82) = -2.14$, $p = .035$, $d = 0.467$; 40 min post stress: $t(82) = -2.25$, $p = .027$, $d = 0.490$). Finally, there was also a significant increase in salivary cortisol in response to the TSST (group × time interaction: $F(2.52,206.82) = 15.842$, $p < .001$, $\eta^2 = 0.162$). Although groups had comparable cortisol concentrations before the experimental manipulation ($t(82) = -0.210$, $p = .834$, $d = 0.046$), cortisol concentrations were significantly elevated in the stress relative to the control group 20 min after treatment onset, when the associative learning task started, as well 40 min after treatment onset (both $p < .001$, $d = 1.085$ and $d = 0.812$, respectively). By applying the predefined criterion for cortisol responders and cortisol non-responders (Schwabe et al., 2008), we obtained n = 25 cortisol responders and n = 17 cortisol non-responders. Since analyses of the two stress groups did not yield any significant difference in any of the baseline measures (all $p \geq 0.227$), we decided to conduct all further analyses with our two groups, i.e. control and stress.

Table 1
Subjective stress ratings and assessments of depressive mood, chronic stress and state anxiety.

	Control	Stress
Subjective stress assessments		
Difficulty	5.30 (1.26) ***	22.35 (4.36)
Unpleasantness	6.08 (1.49) **	22.15 (4.48)
Stressfulness	5.78 (1.32) **	22.50 (4.53)
Control variables		
Depressive score (BDI-II)	5.11 (0.91)	4.83 (0.833)
Subjective chronic stress (TICS)	13.98 (1.50)	12.45 (1.31)
State anxiety (STAI-S)	37.19 (1.09)	35.52 (0.88)

Data represent mean (standard error). Asterisks denote difference between Control and Stress group.
** $p < .01$.
*** $p < .001$.

3.3. Acquisition of the blocking effect

3.3.1. SCR data

Directly after the stress or control manipulation, participants underwent the blocking phase of the associative learning task. Because of group differences during the acquisition phase, we included the difference variable ΔSCR as a covariate. In terms of SCR, participants did not differentiate between the CSAX and the CSBY ($F(1,79) = 0.94$, $p = .335$, $\eta^2 = 0.012$), without any differences between groups (stimulus × group interaction: $F(1,79) = 0.15$, $p = .704$, $\eta^2 = 0.002$, main effect group: $F(1,79) = 0.116$, $p = .735$, $\eta^2 = 0.001$). This was expected because both compounds were paired continuously with the US.

3.3.2. Eye-tracking data

During the blocking phase, it is more informative to investigate the extent of attention participants paid to the individual parts of the compounds (e.g., CSA and CSX in compound CSAX). Therefore, we analyzed the fixation duration and the number of first saccades to the different parts. For the CSAX compound, we observed that participants fixated the previously shocked CSA and the new stimulus CSX for a comparable time ($F(1,80) = 0.49$, $p = .488$, $\eta^2 = 0.006$), without differences between groups (no interaction effect or main effect of group; both $p > .273$). For the CSBY compound, however, the new stimulus CSY attracted significantly longer fixation durations than the old stimulus CSB ($F(1,80) = 8.65$, $p = .004$, $\eta^2 = 0.098$), again without differences between groups (stimulus × group interaction: $F(1,80) = 1.95$, $p = .167$, $\eta^2 = 0.024$, main effect group: $F(1,80) = 0.18$, $p = .669$, $\eta^2 = 0.002$). When formally testing for interaction effects, we found a main effect for old (CSA, CSB) vs. new (CSX, CSY) stimuli ($F(1,80) = 7.94$, $p = .006$, $\eta^2 = 0.090$), indicating that participants were spending significant more time on the new stimuli in the compounds (i.e. CSX and CSY). Furthermore, we observed a non-significant trend for a compound × old/new × group interaction ($F(1,80) = 2.84$, $p = .096$, $\eta^2 = 0.034$). Post-hoc tests revealed that the control group fixated the CSY part significantly longer than the CSB part of the compound ($t(41) = -3.02$, $p = .004$, $d = 0.467$) but showed no difference in fixation duration to the individual parts of the CSAX compound ($t(41) = 0.21$, $p = .834$, $d = 0.032$, respectively), in line with the blocking effect. In contrast, participants of the stress group did not show such a differentiation (both $p > .151$; Fig. 4A).

When analyzing the number of first saccades, indicating fast attentional processes, we observed, for both compounds, that the new stimulus (i.e. CSX in CSAX and CSY in CSBY) attracted more first saccades than the old one ($F(1,74) = 10.90$, $p = .001$, $\eta^2 = 0.128$ and $F(1,77) = 10.31$, $p = .002$, $\eta^2 = 0.118$, respectively), irrespective of the experimental group (all $p > .401$; Fig. 4B).

3.3.3. ERP results

For the N2pc component, there was no main effect of stimulus but a strong trend towards a stimulus × group interaction ($F(1,73) = 3.54$, $p = .064$, $\eta^2 = 0.046$). A post-hoc t test revealed that the control group showed a more negative N2pc to CSAX than to CSBY ($t(37) = -2.64$, $p = .012$, $d = 0.429$). The stress group, in contrast, did not show such a differentiation ($t(38) = 0.80$, $p = .428$, $d = 0.128$; Fig. 4C). In addition, we obtained a similar pattern of results for the individual presentation of the CSA and CSB. Specifically, this analysis revealed a trend towards a stimulus × group interaction ($F(1,73) = 3.16$, $p = .080$, $\eta^2 = 0.042$) and post-hoc t test revealed that the control group showed a more negative N2pc to CSA than to CSB ($t(37) = -2.50$, $p = .017$, $d = 0.406$), thus replicating the results of the acquisition phase. The stress group in contrast, did not show such a differentiation ($t(38) = 0.63$, $p = .535$, $d = 0.100$). For the compound stimuli CSAX and CSBY, the later components, LPP and SPN, remained unaffected by stimulus type and group (all $p > .203$). We further replicated to some extent the findings of the acquisition phase as there was no difference in the LPP but a trend for a main effect of stimulus type for the SPN, indicating a more negative SPN for the CSA compared to the CSB ($F(1,75) = 3.05$, $p = .085$, $\eta^2 = 0.039$).

As displayed in Fig. 4C, groups differed also in the P3b, which evolved between 250 and 500 ms, with its maximum at parietal electrodes. A stimulus × electrode × group repeated measures ANOVA yielded a group main effect, indicating that the stress group showed, irrespective of the stimulus type, a significantly larger P3b compared to the control group ($F(1,73) = 5.73$, $p = .019$, $\eta^2 = 0.073$).

3.4. Test of blocking effect

3.4.1. SCR data

To test for a possible blocking effect, we compared the responses to CSX and CSY in the test phase, in which these stimuli were presented individually and never co-terminated with the US. Our results showed no differential SCRs to the CSX and CSY, in none of the groups (all $p > .219$). Because of possible habituation and/or extinction effects of the SCR, as already seen in previous fear conditioning studies (Bach, Flandin, Friston, & Dolan, 2009; Eippert et al., 2012), we further analyzed the initial response (defined as the response to the first presentation) to the CSX and CSY, i.e. before any extinction could have occurred. Again, we did not obtain a different response to CSX vs. CSY in none of the groups (all $p > .290$), in line with the habituation account (Fig. 5A).

3.4.2. Eye-tracking data

Next, we investigated the eye-tracking data. Although we were primarily interested in the responses to CSX and CSY not presented in a compound, we also analyzed the fixation duration and number of first saccades using the compound stimuli. Because participants were explicitly instructed to fixate the stimuli, for single stimulus presentations they had no choice which stimulus to fixate, why we did not expect to find any differences between CSX and CSY presented individually. Results indicated no different responses to the stimuli of the CSBY compound. Neither regarding the number of first saccades (all $p > .180$) nor regarding the fixation duration (all $p > .378$). Interestingly, the analysis for the CSAX compound revealed a significant stimulus × group interaction for the number of first saccades ($F(1,80) = 5.58$, $p = .021$, $\eta^2 = 0.068$) and a trending stimulus × group interaction for the fixation duration ($F(1,80) = 3.30$, $p = .073$, $\eta^2 = 0.040$). Control participants showed more first saccades and longer fixation duration to the informative stimulus CSA compared to the non-informative stimulus CSX whereas stressed participants showed the opposite pattern, suggesting successful blocking for the control group but not for the stress group (see Fig. 5C & D).

3.4.3. ERP results

Thus, most informative in terms of the actual blocking effect in the test phase were the ERP data. We were particularly interested in whether CSX and CSY attract a different amount of early or late attention. The N2pc analysis revealed a main effect of stimulus type ($F(1,74) = 4.74$, $p = .033$, $\eta^2 = 0.060$), indicating a more negative N2pc for CSX than CSY (Fig. 5C). Moreover, for the LPP we obtained a significant stimulus main effect ($F(1,74) = 4.41$, $p = .039$, $\eta^2 = 0.056$) as well as a significant stimulus × group interaction ($F(1,74) = 6.90$, $p = .010$, $\eta^2 = 0.085$). Post-hoc t tests revealed that the control group did not show a different LPP to CSX than to CSY ($t(37) = -0.39$, $p = .699$, $d = 0.063$), whereas the stress group showed a significant higher LPP to CSX than to CSY ($t(37) = 3.21$, $p = .003$, $d = 0.520$), indicating a sustained attention to the blocked stimulus CSX in contrast to the non-blocked stimulus CSY (Fig. 5D). The SPN analysis revealed no main or interaction effect (all $p > .262$).

3.4.4. Explicit fear learning and blocking

At the end of the test phase, we showed each of the four stimuli

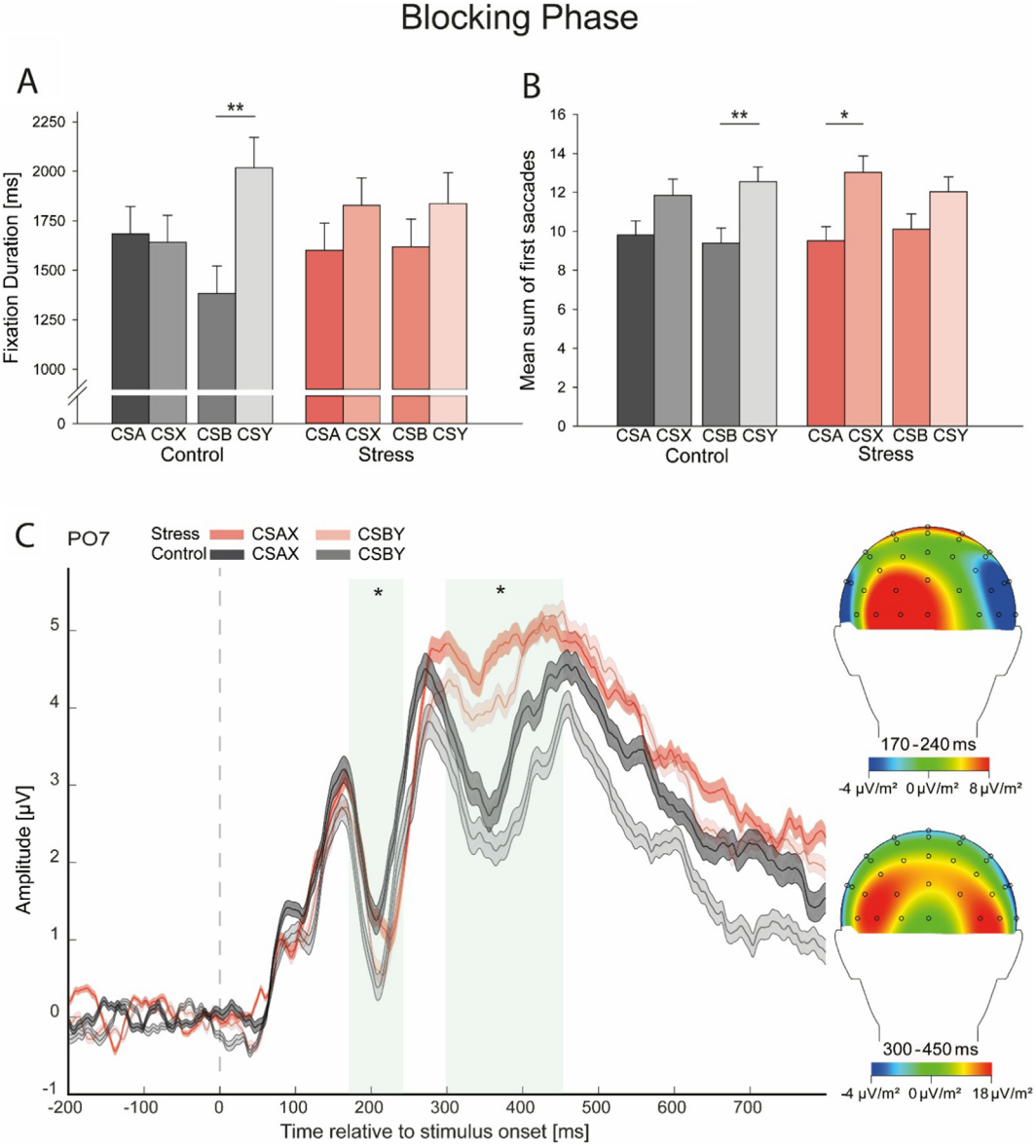

Fig. 4. Blocking phase. (A) Participants' mean fixation duration was higher for the new CSY compared to the old CSB in the CSBY compound, especially in the control group. No differentiation was found for the individual parts of the CSAX compound. (B) Participants' made more first saccades to the new stimuli (i.e. CSX and CSY) compared to the old stimuli (i.e. CSA and CSB) when presented in a compound, in particular participants of the control group regarding the CSBY compound and participants of the stress group regarding the CSAX compound. (C) Participants of the control group showed a more negative N2pc for the CSAX compared to the CSBY; participants of the stress group showed a higher P3b for the compound stimuli in general. Error bars and shaded error bars represent standard error. Asterisks in behavioral measurements denote difference between stimuli for each group; for N2pc the asterisk reflects the difference between stimuli for the control group only: for P3b, the asterisk shows the main group effect: $*p < .05$, $**p < .01$.

again individually and asked participants about their CS-US contingency awareness. When comparing CSA and CSB, there was a main effect of stimulus type ($F(1,82) = 77.66$, $p < .001$, $\eta^2 = 0.486$) and no interaction effect or main effect of group, indicating that participants were aware of the CS-US contingency, without differences between groups. Moreover, we compared the rating for CSX vs. CSY and obtained a trend towards a main effect of stimulus type ($F(1,82) = 3.72$, $p = .057$, $\eta^2 = 0.043$): Participants associated the CSY more strongly with the US then with the CSX (mean rating: 30.27, $SE = 3.11$ vs. 37.56, $SE = 3.49$, respectively), in line with a blocking effect. In addition, we found a trend towards a main effect of group ($F(1,82) = 2.80$, $p = .098$, $\eta^2 = 0.033$): The stress group tended to show a stronger US-CS association in general for CSX and CSY compared to the control group ($F(1,82) = 2.80$, $p = .098$, $\eta^2 = 0.033$; Fig. 6).

3.4.5. Analysis of a subsample showing robust fear acquisition in the SCR

Although the neural signature of fear acquisition was comparable in the two groups and the explicit ratings indicated successful fear learning in both groups, the SCR and eye-tracking data reported above suggested that the stress and control groups might have differed already in initial fear acquisition, i.e. before the actual stress manipulation took place. To control for these differences, we included the respective baseline differences as a covariate in all further analyses. Furthermore, we analyzed in an additional analysis only participants of the stress and control groups that showed a robust fear acquisition effect, i.e. stronger SCRs to CSA vs. CSB across the acquisition phase. We ran all our analyses again in this reduced sample (stress: n = 17, control: n = 20). In short, in this reduced sample both groups showed a higher SCR and more attention, expressed as longer fixation durations, to the CSA than to the CSB, indicative of successful fear acquisition, without any

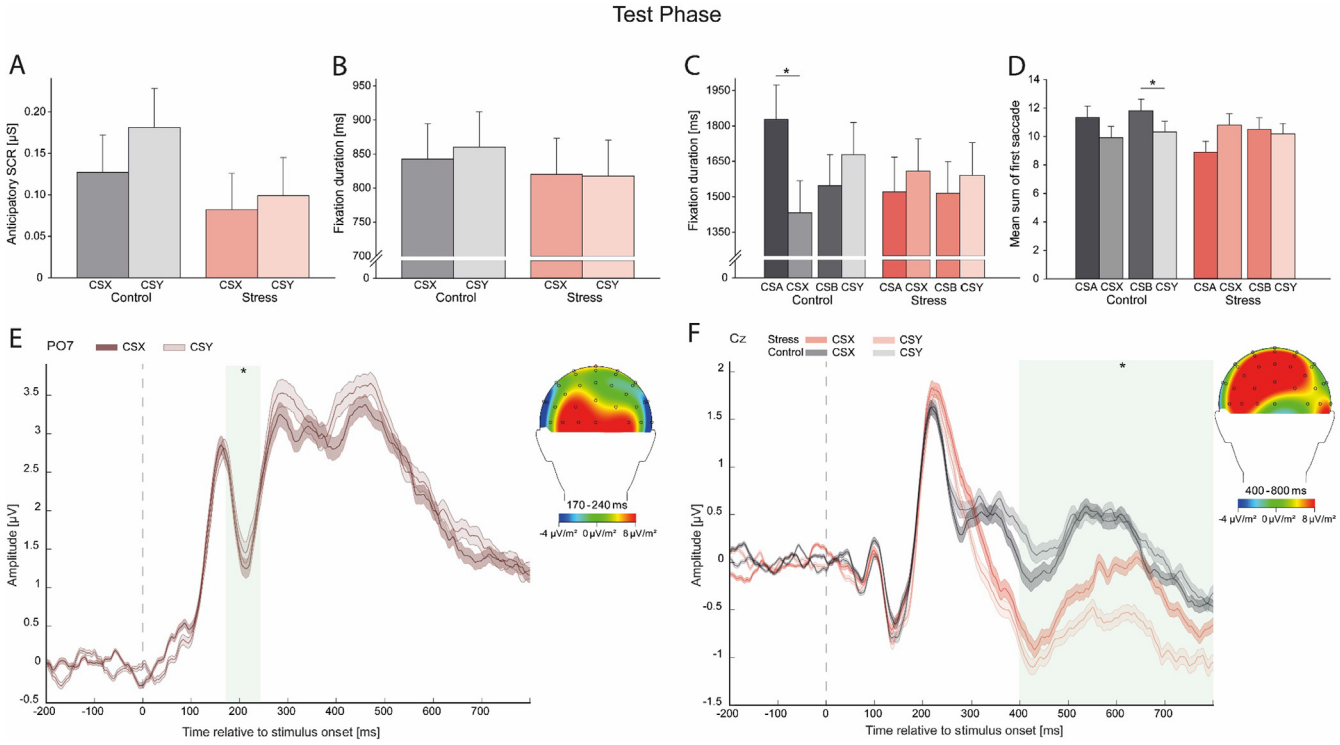

Fig. 5. Test phase. (A) Participants of both groups showed a higher mean anticipatory SCR to the initial presentation of CSX vs. CSY but did not differ significantly. (B) Participants of both groups did not show a different mean fixation duration to CSX vs. CSY. Participants of the control group fixated the predictive CSA longer (C) and showed less first saccades towards the blocked CSX of the CSAX compound compared to the stress group (D). (E) Both groups showed no significant difference regarding in their N2pc in regard to CSX vs. CSY. However, (F) the control group showed a higher LPP to the CSX and CSY compared to the stress group. Error bars and shaded error bars represent standard error. Asterisks denote difference between CSX and CSY: *$p < .05$.

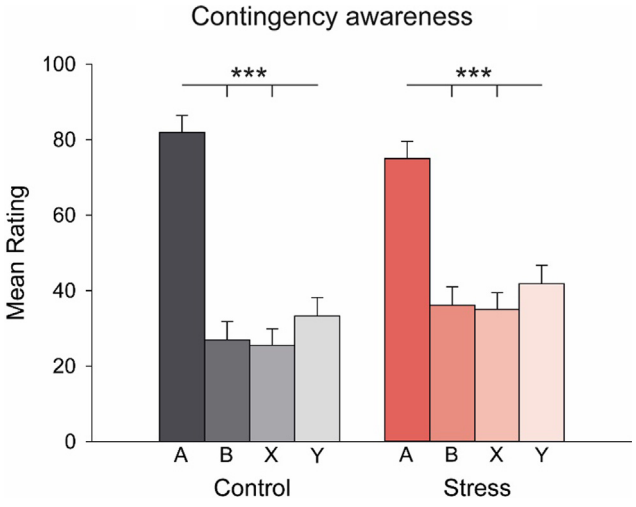

Fig. 6. Explicit rating after test phase. (A) Participants' mean US-association for each stimulus type. Asterisks and dagger denote difference between stimuli: ***$p < .001$.

differences between groups. Notably, however, in this reduced sample the N2pc response to the CSA was stronger in control than in stressed participants. During the blocking phase, the SCR, eye-tracking and ERP data for the reduced sample were largely comparable to the data obtained in the whole sample. In general, participants allocated a higher degree of attention to the newly introduced stimuli when being presented in compounds. Moreover, stressed participants tended to show more first saccades to the uninformative CSX than to the CSA, whereas control participants did not. The ERP results were identical to those found in the whole sample, i.e. a more negative N2pc to the CSAX vs. CSBY and CSA vs. CSB in the control group compared to the stress group and no effects on the later components LPP and SPN. In the test phase, we now obtained a trending difference between control and stress group, that is control participants tended to show stronger SCRs to the CSY than to the CSX, whereas the stress group did not. For the eye-tracking data, we obtained also a differentiation for the CSBY compound, i.e. that participants of both groups spend more time fixating the new stimulus CSY compared to the old CSB. In contrast, participants show longer fixation durations to the old, informative CSA compared to the CSX, independent of experimental manipulation. ERP results for the test phase were completely identical to those of the whole sample. The CSX attracted more early attention compared to the CSY in both groups as indicated by a more negative N2pc. In addition, the LPP result suggests sustained attention to the previously blocked stimulus only for the control group. In sum, this additional analysis indicates that the pattern of results observed for the whole sample remains largely unchanged when analyzing only participants showing a differential SCR to CSA and CSB. For details of these additional analyses and the referring statistics, please see the supplemental material.

3.4.6. Control variables

Depressive mood, subjective chronic stress, and anxiety levels of our sample were all rather low. Groups did not differ in these variables (all $p > .145$; see Table 1). Furthermore, we assessed general attention to the task and found that attention was overall very high (92.4% correct answers), without differences between groups ($t(73) = 0.13$, $p = .540$, $d = 0.125$).

4. Discussion

Contemporary learning theory assumes that learning depends on the predictive relationship between stimuli, rather than on the mere temporal contiguity (Mackintosh, 1975; Pearce & Hall, 1980; Rescorla &

Wagner, 1972). Although this assumption is supported by a plethora of studies (for a review see Le Pelley, Mitchell, Beesley, George, & Wills, 2016), the neural and attentional mechanisms involved in predictive learning are not fully understood, in particular in aversive learning. Moreover, while it is by now well-established that acute stress can modulate learning and memory (Diamond et al., 2007; Schwabe, Joels, et al., 2012), it remains unclear whether acute stress may modulate predictive processes in the context of aversive learning. Thus, we studied here the impact of stress on the blocking phenomenon, a classic effect demonstrating the predictive nature of learning. In order to elucidate the neural and attentional mechanisms involved in blocking and its potential modulation by stress, we combined a fear learning paradigm with EEG measurements, SCR recordings and eye-tracking. Our results showed a blocking effect at the behavioral level, reflected in lower US expectancy ratings for the blocked compared to the non-blocked stimulus. In line with our hypothesis of differential attentional allocation depending on the predictive value of stimuli, our eye-tracking data revealed higher sustained attention to the predictive compared to the blocked stimulus. Both, our eye-tracking and EEG data indicated that stress led to sustained attention to stimuli with low predictive value, suggesting that stress may impair the ability to efficiently use prior knowledge to guide learning.

Our behavioral and eye-tracking data corroborate previous findings showing (i) that (explicit) aversive learning depends on the predictive relationship between events (Eippert et al., 2012; Sanchez-Nacher et al., 2011) and (ii) that the amount of attention allocated to a stimulus depends on its predictive value (Eippert et al., 2012; Luque, López, Marco-Pallares, Càmara, & Rodríguez-Fornells, 2012; Wills et al., 2007). Previous fMRI evidence further showed stronger amygdala responses to predictive compared to non-predictive stimuli (Eippert et al., 2012). The rather sluggish fMRI signal, however, is not well suited for the investigation of fast attentional processing in predictive learning. Equipped with a significantly higher temporal resolution, our EEG data showed differential processing of predictive vs. non-predictive stimuli in the N2pc and LPP. During the blocking phase, when learning to the new (but uninformative) stimulus CSX should be blocked and learning to CSY should evolve, non-stressed controls showed a heightened N2pc to the CSAX compound compared to the CSBY compound. This is in the line with the assumption that the N2pc is thought to reflect covert attention (Eimer, 1996; Luck & Hillyard, 1994), which might be primarily guided by the previously acquired relevance of a stimulus. Moreover, it is in line with the filtering hypothesis (Luck & Hillyard, 1994) assuming that the N2pc is more negative for task relevant items when suppression of distractor items is necessary. In this case, it can be assumed that successful filtering of the CSX of the CSAX was implemented by the control group to guide attention towards the relevant CSA whereas such filtering for the CSBY would not have been advantageous. This idea is further supported by the fact that participants from the control group still showed a significant differentiation between the previously learned threatening CSA and the safety signaling stimulus CSB, i.e. the N2pc was more negative for the CSA than for the CSB, whereas the stress group did not show this differentiation. In further support of our idea, when testing for the blocking effect in a third phase, participants from the control group showed longer fixation durations and more first saccades to the predictive stimulus compared to the non-predictive stimulus when presented in a compound, suggesting successful blocking to the non-predictive stimulus. In contrast, participants from the stress group did not show this differentiation.

Somewhat surprisingly, we did not find evidence for increased attentional processing of the non-blocked stimulus in our data in the test phase of the learning task, as an earlier study did (Wills et al., 2007). This discrepancy may be partly explained by methodological differences between this earlier and the present study. In particular, we used a pavlovian fear conditioning protocol, whereas this previous study used an instrumental learning protocol, which may have required participants to be generally more attentive than it was the case in our experiment, thus increasing its sensitivity to detect neural attentional differences. Moreover, in contrast to the present study, this previous study investigated the N1, a component thought to reflect perceptual discrimination processing (Vogel & Luck, 2000). We did not focus on this early component because it is highly refractory and its reliable measurement would require significantly more trials than feasible in a fear learning task (Woodman, 2010). In addition, we were more interested in attention related components that are sensitive for top-down modulated processes, due to our aim to investigate the influence of stress.

Predictive learning requires that prior experiences are retrieved and translated to the ongoing learning situation. There is an extensive literature showing that stress can impair the retrieval of previously acquired information (de Quervain et al., 1998; Roozendaal, 2002; Schwabe, Joels, et al., 2012). Moreover, stress appears to hinder the integration of new information and stored knowledge (Kluen et al., 2017; Sanger et al., 2014; Vogel et al., 2018a, 2018b). The present data show – to the best of our knowledge – for the first time that stress may modulate aversive predictive learning, in general, and the blocking effect in particular. More specifically, our eye-tracking data showed that stress abolished the attentional discrimination between predictive and unpredictive stimuli in a compound, which was observed in non-stressed controls. Furthermore, stress led, during the blocking phase, to a higher P3b compared to the control group. This component is thought to be related to attention-driven comparisons between task-relevant stimuli and assumed to reflect the evaluation of the current stimulus with the representation of previous stored information (for a review see Polich, 2007). Additionally, the P3b is thought to be mediated by the release of noradrenaline from the locus coeruleus (Nieuwenhuis, Aston-Jones, & Cohen, 2005), which is also part of the stress mediated influence on memory processes (McGaugh, 2000). An increased P3b for the stress group may be indicative of an increased overall attention to and evaluation of the compound stimuli. This increase in overall attentional processing may be interpreted as an indication of impaired recall of previously made experiences (i.e. learning to CSA and CSB), thus requiring stressed participants to spend more resources evaluating the new compound stimuli. Our data further showed a heightened LPP for the blocked stimulus compared to the non-blocked stimulus in the stress group relative to the control group, which may further point to sustained attention to irrelevant stimuli after stress (Cuthbert et al., 2000; Hajcak & Olvet, 2008; Schupp et al., 2006). This latter finding is also in line with the idea that stress disrupts later stages of attentional processing associated with the evaluation of task-relevant information (Shackman, Maxwell, McMenamin, Greischar, & Davidson, 2011). Based on previous research on the LPP and its relevance in emotional processing, we would have expected an increased LPP towards the CSA compared to the CSB also in the fear acquisition phase. There was however no increased LPP in the acquisition phase but only later in the test phase. One potential explanation for this finding is that most of the previous LPP research compared attention towards affective and neutral pictures, whereas we used here an aversive conditioning paradigm with shock administration (Cuthbert et al., 2000; Schupp et al., 2000; Weinberg & Hajcak, 2010). Furthermore, some authors suggest that earlier components mirror fast attentional capture while later components more elaborative processes must take place (Dieterich, Endrass, & Kathmann, 2016; Lin et al., 2015). The fully deterministic reinforcement rate that we used here may have reduced the need for such elaborative processes because there was no uncertainty.

In contrast to other fear learning studies, we did not record explicit fear learning ratings on a trial-by-trial basis during the task but at the end of the experiment to avoid a possible influence on implicit measures such as SCR (Kroes et al., 2016; Phelps et al., 2001; Raio, Carmel, Carrasco, & Phelps, 2012). This, however, reduces the sensibility of the explicit rating data to some extent. Although we obtained a blocking effect in the explicit ratings measured at the end of the task, the absence of a blocking effect in SCR measurement was somewhat unexpected and

is in contrast to previous studies investigating the blocking effect (Hinchy et al., 1995; Lovibond, Siddle, & Bond, 1988). However, the study that introduced the blocking paradigm we used here, obtained also no blocking effect in the SCR (Eippert et al., 2012). The absence of a blocking effect in the SCR in this paradigm may be due to its long duration and the continuous reinforcement for the CSA and both compounds (i.e., CSAX and CSBY), both of which may have led to a strong habituation effect making a differentiation between the stimuli more difficult (Bach et al., 2009; Eippert et al., 2012). In addition, the SCR results are of limited information because only the control group showed a significant differentiation between CSA and CSB, indicating successful discrimination learning, whereas the stress group only showed a non-significant descriptive discrimination. To control for this baseline difference in further analyses, we included ΔSCR as covariate in all of the following analyses. Without changing the pattern of result, the ANCOVAs for the SCR did not reveal any significant results in the blocking or test phase. However, when analyzing only those participants who showed a robust fear acquisition effect in the SCR in the first place (see supplementary results), we did obtain evidence for a blocking effect in the SCR when comparing the initial presentation of CSX vs. CSY, suggesting that the blocking effect in the SCR may indeed depend on the overall SCR level, although such single trial comparisons can only be interpreted with caution.

Finally, it should be noted that the stress system was still activated during the test phase, as reflected in elevated cortisol concentrations, and that one might thus argue that stress affected primarily retrieval processes during the test phase. We chose this study design, in which one stage followed immediately after another, to be as close as possible to the study in which this specific blocking paradigm was introduced (Eippert et al. (2012)). Extending the interval between the blocking stage and stress/control manipulation on the one hand and the test phase on the other hand might have diluted potential blocking effects or resulted in stress effects on blocking consolidation. Moreover, the test phase included stimuli from the previous stage, and we found no effect on, for example, the CSA or CSB. These findings speak against a general retrieval deficit after stress and we consider a retrieval deficit specifically for blocking-related stimuli rather unlikely.

To conclude, we examined here the neural and attentional processes involved in predictive fear learning and tested whether acute stress may modulate the efficient processing of information against the background of prior knowledge. Our results show that attentional resources were allocated depending on the predictive value of a stimulus and, most importantly, that stress interferes with predictive learning, most likely through interfering with the efficient use of prior knowledge during learning. This stress-induced deficit was reflected in the absence of a differential N2pc for compound stimuli during the blocking phase. In addition, the stress group showed a heightened P3b for both compound stimuli, which may suggest that the impaired access to previously learned associations requires participants to spend more resources on the evaluation of stimuli, irrespective of its actual predictive value. This idea is further supported by the stress-induced increase in the LPP for the CSX compared to the CSY, which may point to sustained attention to non-predictive stimuli. The reduced processing efficiency of stimuli that were not directly relevant to the stressor may be due to a prioritized processing and consolidation of the stressful encounter itself, thus leaving less resources for processing competing events (Joels, Pu, Wiegert, Oitzl, & Krugers, 2006; Schwabe, Joels, et al., 2012). This prioritization, however, may amplify biases in predictive processing that are thought to contribute to stress-related mental disorders, such as posttraumatic stress disorder (Homan et al., 2019).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nlm.2020.107158.

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