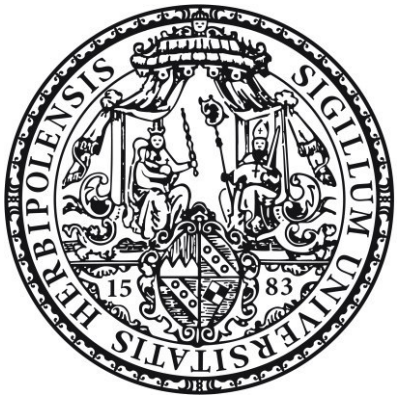

Modulating the Fear Network:

Preclinical Studies on Prefrontal Cortex Stimulation

Modulation des Furchtnetzwerkes:
Vorklinische Studien zur Stimulation des Präfrontalkortex

Doctoral thesis for a doctoral degree
at the Graduate School of Life Sciences,
Julius-Maximilians-Universität Würzburg,
Section *Neuroscience*

submitted by
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Potsdam

Würzburg, 2015

Submitted on: ……..05.10.2015…….……..

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I hereby confirm that my thesis entitled *"Modulating the Fear Network: Preclinical Studies on Prefrontal Cortex Stimulation"* is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

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Hiermit erkläre ich an Eides statt, die Dissertation *"Modulating the Fear Network: Preclinical Studies on Prefrontal Cortex Stimulation"* eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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Structure of the present thesis

The present thesis is composed of four studies which were accomplished within a collaborative research center (SFB-TRR 58) on “Fear, Anxiety, and Anxiety Disorders” funded by the German Research Foundation (DFG). With regard to the interdisciplinary concept of the SFB-TRR 58 linking basic and clinical research, the manuscripts included in this dissertation comprise preclinical studies on fear conditioning and fear extinction in healthy human participants, which are intended to contribute to an improved treatment of clinical anxiety. They were conducted between June 2010 and December 2013 at the Department of Psychiatry, Psychosomatics and Psychotherapy of the University of Würzburg, Germany.

The present thesis is going to illuminate the cross-species validity of the fear and extinction model by guidance through the following structure: In the *first part*, neural mechanisms involved in fear and extinction primarily obtained from research in rodents will be reviewed. The hereby provided hypotheses are then integrated into the existing findings on the neural mechanisms in humans, exemplified by the first manuscript which provides evidence for the prefrontal cortex (PFC) to be engaged during fear extinction. In the *second part*, the neural mechanisms involved in the failure of fear extinction will be summarized with regard to the amygdala-PFC interplay. Based on rodent research concerning manipulations of the PFC activity via electrical microstimulation, the second manuscript addresses the question whether transcranial magnetic stimulation (TMS) provides a useful intervention to enhance PFC activity related to extinction in humans. The *third part* is going to address fear generalization as another key feature of clinical anxiety. By adhering to the translational structure, the third manuscript will be introduced by reviewing rodent research regarding the generalization of extinction. In line with the enhancement of PFC activity via TMS, it deals with the generalization of extinction training from one stimulus to another. A comprehensive general discussion of all findings at *the end* of this thesis is intended to guide future directions in the understanding of neural dysfunctions across anxiety disorders thereby considering the heritability of anxiety exemplified by a fourth manuscript about the impact of a candidate gene for pathological anxiety, the *NPSR1* gene polymorphism, on cognitive emotion regulation. The thesis will be ended up with strengths and weaknesses of the studies, which are then used to conclude and provide an outlook on future clinical directions based on the presented preclinical investigations.

"Dissertation Based on Several Published Manuscripts"

Statement of individual author contributions and of legal second publication rights

Publication (complete reference): **Guhn, A.,** Dresler, T., Hahn, T., Mühlberger, A., Ströhle, A., Deckert, J. & Herrmann, M. J. (2012). Medial prefrontal cortex activity during the extinction of conditioned fear: an investigation using functional near-infrared spectroscopy. *Neuropsychobiology, 65*(4), 173-182. doi: 10.1159/000337002					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design	**AG**	TD	MJH		
Data Collection	**AG**				
Data Analysis and Interpretation	**AG**	TD/TH	AM		
Manuscript Writing	**AG**	TD	MJH	JD	AS

Publication (complete reference): **Guhn, A.,** Dresler, T., Andreatta, M., Müller, L. D., Hahn, T., Tupak, S. V., Polak, T., Deckert, J. &. Herrmann, M. J. (2014). Medial prefrontal cortex stimulation modulates the processing of conditioned fear. *Frontiers in Behavioral Neuroscience, 8*, 44. doi: 10.3389/fnbeh.2014.00044					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design	**AG**	TD	MJH/JD		
Data Collection	**AG**	TP			
Data Analysis and Interpretation	**AG**	MA	TD	TH	SVT
Manuscript Writing	**AG**	LDM	TD	JD	MJH

Publication (complete reference): **Guhn, A.,** Domschke, K., Müller, L. D., Dresler, T., Eff, F., Kopf, J., Deckert, J., Reif, A. & Herrmann, M. J. (2015). Neuropeptide S receptor gene variation and neural correlates of cognitive emotion regulation. *Social Cognitive and Affective Neuroscience*. doi: 10.1093/scan/nsv061					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design	**AG**				
Data Collection	FE	AR	**AG**		
Data Analysis and Interpretation	**AG**	KD			
Manuscript Writing	**AG**/KD	LDM	TD	JD/MJH	AR/JK

Publication (complete reference): **Guhn, A.**, Dresler, T., Müller, L. M., Ströhle, A., Deckert, J. & Herrmann, M. J. Evidence for partial generalization of fear extinction after dorsolateral prefrontal cortex stimulation: Correlation with skin conductance and prefrontal fNIRS activity. Unpublished manuscript.					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design	**AG**	MJH			
Data Collection	**AG**	LDM			
Data Analysis and Interpretation	**AG**	TD/LDM	JD	MJH	AS
Manuscript Writing	**AG**	LDM/TD	JD	MJH	AS

I confirm that I have obtained permission from both the publishers and the co-authors for legal second publication.
I also confirm my primary supervisor's acceptance.

Anne Guhn 05.10.2015 Würzburg

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Doctoral Researcher's Name Date Place Signature

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List of Abbreviations

ACC	anterior cingulated cortex
AS	anxiety sensitivity
BLA	basolateral complex of the amygdala
BOLD	blood oxygenation level-dependent
BNST	bed nucleus of the stria terminalis
CBT	cognitive behavioral therapy
Ce	central nucleus of the amygdala
CR	conditioned response
CS	conditioned stimulus
CS+	reinforced conditioned stimulus
CS+E	extinguished, reinforced conditioned stimulus
CS+U	un-extinguished, reinforced conditioned stimulus
CS-	un-reinforced conditioned stimulus
dACC	dorsal anterior cingulated cortex
DCS	D-cycloserine
dlPFC	dorsolateral prefrontal cortex
EDA	electrodermal activity
EEG	electroencephalogram
EMG	electromyography
FDA	Food and Drug Administration of the U.S.
FPS	fear-potentiated startle
fMRI	functional magnetic resonance imaging
fNIRS	functional near-infrared spectroscopy
GABA	gamma-Aminobutyric acid
GAD	generalized anxiety disorder
GS	generalization stimulus
HHb	deoxygenated hemoglobin
HPA	hypothalamo-pituitary-adrenal axis
IL	infralimbic cortex
iTBS	intermittent theta burst stimulation
ITC	intercalated amygdala neurons
LTD	long-term depression
LTP	long-term potentiation
MEG	magnetoencephalography
MEP	motor-evoked potential
MNI	Montreal Neurological Institute (brain template)
mPFC	medial prefrontal cortex
NMDA	*N*-methyl-$_D$-aspartate
NPS	neuropeptide S

NPSR1	neuropeptide S receptor 1 gene
NS	neutral stimulus
O_2Hb	oxygenated hemoglobin
PD	panic disorder
PET	positron emission tomography
PL	prelimbic cortex
PTSD	post-traumatic stress disorder
rCBF	regional cerebral blood flow
rTMS	repetitive transcranial magnetic stimulation
SAD	social anxiety disorder
SCR	skin conductance response
tDCS	transcranial direct-current stimulation
TMS	transcranial magnetic stimulation
UCR	unconditioned response
UCS	unconditioned stimulus
vlPFC	ventrolateral prefrontal cortex
vmPFC	ventromedial prefrontal cortex

Summary

Pavlovian fear conditioning describes a form of associative learning in which a previously neutral stimulus elicits a conditioned fear response after it has been temporally paired with an aversive consequence. Once acquired, the fear response can be extinguished by repeatedly presenting the former neutral stimulus in the absence of the aversive consequence. Although most patients suffering from anxiety disorders cannot recall a specific conditioned association between a formerly neutral stimulus and the feeling of anxiety, the produced behavioral symptoms, such as avoidance or safety behavior to prevent the anticipated aversive consequence are commonly exhibited in all anxiety disorders. Moreover, there is considerable similarity between the neural structures involved in fear and extinction in the rodent and in the human. Translational research thus contributes to the understanding of neural circuitries involved in the development and maintenance of anxiety disorders, and further provides hypotheses for improvements in treatment strategies aiming at inhibiting the fear response.

Since the failure to appropriately inhibit or extinguish a fear response is a key feature of pathological anxiety, the present preclinical research focuses on the interplay between the amygdala and the medial prefrontal cortex (mPFC) during fear learning with particular regard to the prefrontal recruitment during fear extinction and its recall. By firstly demonstrating an increased mPFC activity over the time course of extinction learning with functional near-infrared spectroscopy, the main study of this dissertation focused on repetitive transcranial magnetic stimulation (rTMS) as brain stimulation technique suitable to enhance extinction learning. Since hypofrontality is assumed to underlie the maintenance of pathological anxiety, rTMS application revealed an increased mPFC activity, which resulted in a decreased fear response on the behavioral level both during extinction learning as well as during the recall of extinction 24 hours later and in the absence of another stimulation. The following attempt to improve the generalization of extinction with rTMS from an extinguished stimulus to a second stimulus which was reinforced but not extinguished was at least partially evidenced. By revealing an increased prefrontal activity to the non-extinguished stimulus, the active and the placebo rTMS condition, however, did not differ on behavioral parameters. These preclinical findings were discussed in the light of genetic and environmental risk factors with special regard to the combination of a risk

variant of the neuropeptide S receptor 1 gene polymorphism (*NPSR1* rs324981) and anxiety sensitivity. While the protective homozygous AA genotype group showed no correlation with anxiety sensitivity, the *NPSR1* T genotype group exhibited an inverse correlation with anxiety sensitivity in the presence of emotionally negative stimuli. In light of other findings assuming a role of the *NPSR1* T allele in panic disorder, the revealed hypofrontality was discussed to define a risk group of patients who might particularly benefit from an augmentation of exposure therapy with rTMS.

Taken together, the presented studies support the central role of the prefrontal cortex in fear extinction and suggest the usefulness of rTMS as an augmentation strategy to exposure therapy in order to decrease therapy relapse rates. The combination of rTMS and extinction has been herein evidenced to modulate fear processes in a preclinical approach thereby establishing important implications for the design of future clinical studies.

Zusammenfassung

Die Furchtkonditionierung nach Pavlov beschreibt einen assoziativen Lernmechanismus bei dem ein ursprünglich neutraler Stimulus nach wiederholter kontingenter Darbietung mit einem aversiven Stimulus zu einer konditionierten Furchtreaktion führt, die darauffolgend allein durch den nun konditionierten Reiz ausgelöst werden kann. Obwohl die meisten Angstpatienten keine initiale Reiz-Reaktionsverbindung erinnern können, gelten die Mechanismen der Furchtkonditionierung als Erklärungsmodelle für die Entstehung und Aufrechterhaltung von Angststörungen. Evidenz erhalten sie zudem durch den Einsatz und die Wirksamkeit expositionsbasierter Methoden in der Behandlung von Angststörungen. Ihnen liegt die Extinktion einer erworbenen konditionierten Reaktion zugrunde, bei der der konditionierte Reiz wiederholt ohne seine erwartete aversive Konsequenz dargeboten wird. Dies führt in der Folge zu einer abnehmenden Furchtreaktion. Da die neuronalen Strukturen, die in den Erwerb und die Extinktion einer konditionierten Furchtreaktion involviert sind, weitgehend speziesübergreifend sind, lassen sich aus Tiermodellen wertvolle Hypothesen zur Verbesserung bestehender Behandlungsstrategien mit dem Ziel der Reduktion der erworbenen Furchtreaktion generieren.

Eine unzureichende Inhibition bzw. Extinktion der Furchtreaktion gilt als Charakteristikum von pathologischer Angst. Die im Rahmen dieser Dissertation vorgestellten Studien beschäftigen sich mit dem zugrundliegenden neurobiologischen Ungleichgewicht

zwischen der Amygdala und dem Präfrontalkortex, das als ursächlich für die Aufrechterhaltung pathologischer Angst vermutet wird. Zunächst wird hierbei eine Untersuchung vorgestellt, bei der die zunehmende Beteiligung des Präfrontalkortex‘ über den Verlauf eines Extinktionstrainings erstmals mit der funktionellen Nahinfrarot-Spektroskopie dargestellt werden konnte. Da zunehmende Evidenz auf eine unzureichende präfrontale Kortexaktivierung bei pathologischer Angst hindeutet, beschäftigt sich die Hauptstudie dieser Dissertation mit der Fragestellung, ob die Aktivität des Präfrontalkortex‘ mit Hilfe der repetitiven transkraniellen Magnetstimulation (rTMS) gesteigert werden kann. In Analogie zu tierexperimentellen Untersuchungen konnte in einer Gruppe gesunder Probanden nach einer Stimulation mit rTMS verglichen mit einer Placebobedingung eine verringerte Furchtreaktion gezeigt werden, die auch während des Abrufs des Extinktionsgedächtnis nach 24 Stunden und unabhängig von einer erneuten Stimulation noch nachweisbar war. In einem nächsten Schritt wurde, wiederum in Anlehnung an tierexperimentelle Studien, die Generalisierung eines Extinktionstrainings auf einen ebenfalls konditionierten, aber nicht extingierten Stimulus untersucht. Hierbei zeigte sich eine partielle Bestätigung der Hypothesen. So konnten zwar auf behavioraler Ebene keine Gruppenunterschiede zwischen einer aktiven und einer Placebobedingung detektiert werden, in der aktiven Gruppe ließ sich 24 Stunden nach der Stimulation jedoch eine erhöhte präfrontale Kortexaktivierung auf den nicht-extingierten Stimulus zeigen. Diese Studienergebnisse werden auf Basis einer weiteren Arbeit zu Gen-Umwelt-Einflüssen diskutiert. Hierbei konnte eine Konstellation bestehend aus der Risikovariante (T Allel) des Neuropeptid S Rezeptor Gens (*NPSR1* rs324981) und einer erhöhten Angstsensitivität im Unterschied zu einer homozygoten AA Genotyp-Gruppe mit einer verringerten präfrontalen Kortexaktivierung auf negative emotionale Stimuli assoziiert werden. Unter Einbezug des literarischen Kontexts zu *NPSR1* und dem Auftreten der Panikstörung legen diese Ergebnisse nahe, dass insbesondere solche und ähnliche Risikogruppen von einer Augmentationsstrategie mit rTMS profitieren könnten.

Zusammenfassend bestätigen die vorliegenden Studien die Rolle des Präfrontalkortex bei der Furchtextinktion und legen den Einsatz der rTMS für die Verbesserung der Expositionstherapie nahe. Aus diesen präklinischen Arbeiten werden Hinweise für die Umsetzung von klinischen Studien generiert, die über die Augmentation von Exposition mit rTMS zu einer Rückfallreduktion bei der Therapie von Angststörungen beitragen könnten.

1. Fear and Extinction

1.1 Fear Conditioning – an Historical Abstract

The beginning of the last century constituted the basis of a learning theory that further along the line grew into the foundation of behaviorism as the dominating school of psychology in the mid of the 20th century. It was Ivan P. Pavlov, a russian physiologist, who discovered the "conditioned reflex" when he investigated the physiological activity of the cerebral cortex in dogs. By measuring salivary secretion he recognized that "if the intake of food by the animal takes place simultaneously with the action of a neutral stimulus [such as a metronome] which has been hitherto in no way related to food, the neutral stimulus readily acquires the property of eliciting the same reaction in the animal as would food itself" (Pavlov, 1927, p. 26). He described the conditioning of a formerly neutral stimulus (NS, the metronome) not eliciting a specific response, to an unconditioned stimulus (UCS, food) as an associative learning process since after several pairings the formerly NS (conditioned stimulus, CS) comes to elicit the salivary secretion (conditioned response, CR) without the appearance of the UCS.

In order to demonstrate the empirical evidence on classical conditioning in humans, John B. Watson and his assistance Rosalie Rayner investigated the conditioning of a fear response in an 11 months old child, *Albert B.*, which made history as *the story of Little Albert*:

> Albert's life was normal: he was healthy from birth and one of the best developed youngsters ever brought to the hospital …. His stability was one of the principal reasons for using him as a subject in this test …. the infant was confronted suddenly and for the first time successively with a white rat, a rabbit, a dog, a monkey, with masks with and without hair, cotton wool, burning newspapers, etc. …. At no time did this infant ever show fear in any situation. (Watson & Rayner, 1920, p. 1-2)

Watson and Rayner fostered an experimentally induced emotional reaction by striking a hammer upon a suspended steel bar in the back of the child. The noise made Albert cry

quickly. When the experimenters subsequently paired the noise of the steel bar with a white rat, with which the child used to play without any signs of discomfort, it took seven joint stimulations until the rat alone produced an emotional response:

> The instant the rat was shown the baby began to cry. Almost instantly he turned sharply to the left, fell over on left side, raised himself on all fours and began to crawl away so rapidly that he was caught with difficulty before reaching the edge of the table. This was as convincing a case of a completely conditioned fear response as could have been theoretically pictured. (p. 5)

Watson and Rayner (1920) concluded that many of the phobias in psychopathology are "true conditioned emotional reactions" (p. 14). The existing information that have been published about Albert's real identity as well as the observations that were made during the experiments will be given during the course of this thesis. Since 1920, the improvements in understanding the principles of fear and its inhibition tremendously increased, particularly during behaviorism which dominated decades of research. Even quite recently, modern technologies shed light on the neurobiological mechanisms underlying the behavioral fear response, its maintenance as well as its reduction.

1.2 The Neural Mechanisms of Fear Conditioning and Extinction

In the last decades the number of publications in the field of fear and anxiety has increased exponentially (Milad & Quirk, 2012). The following paragraph thus is intended to basically review the main findings with special regard to anatomical and functional connectivity between the amygdala, the medial prefrontal cortex (mPFC) and to some extent the hippocamus, since the included manuscripts of this dissertation will consistently refer to these regions. Beyond this rather simplified description the reader is referred to comprehensive reviews in the field, such as those by Pape and Pare (2010), Maren and Quirk (2004) or Quirk and Mueller (2008).

The neurobiology of fear is primarily linked to a key structure at the anterior medial portion of each temporal lobe: the amygdalae (in the following only the singular is used). The amygdala is composed of different nuclei, each with distinctive connections with various

subcortical as well as cortical regions. Interconnections within the amygdala form three important subsystems to aquire and express pavlovian fear memory, i.e. the basolateral complex (BLA), the central nucleus (Ce) and the intercalated (ITC) cell masses (Pape & Pare, 2010). The BLA is comprised of the lateral, basolateral and basomedial nuclei and collectively receives sensory information from the thalamus, the hippocampus and the cortex depending on the sensory modality of the CS. The Ce as the second subsystem constitutes the main output region for the conditioned fear response (CR). It sends descending projections to various brain stem structures initiating the physiological responses of fear: pathways through the periaqueductal gray evoke emotional behaviors typically associated with the fight-or-flight reaction, those through the lateral hypothalamus evoke autonomic responses such as hypertension and tachycardia, and those through the bed nucleus of the stria terminalis evoke hormonal responses by activating the hypothalamo-pituitary-adrenal axis (HPA) and by an increased glucocorticoid secretion. The ITC cell masses as the third subsystem receive projections from the mPFC, notably the infralimbic (IL) cortex, and provide feed-forward inhibition to the Ce. This neuron population will be illuminated in more depth below in the context of fear extinction.

The formation and storage of the CS-UCS association during pavlovian conditioning depends particularly on conditioning-related plasticity in BLA neurons (Quirk, Repa, & LeDoux, 1995). Resorting to the story of *Little Albert*, the contingent sensory inputs from the white rat (the to-be-conditioned stimulus, CS) and the noise of the steel bar (unconditioned stimulus, UCS) converge in the BLA and lead to changes in synaptic transmission. In contrast to a pseudo-conditioning when CS and UCS are unpaired, the contingent inputs from both stimuli cause changes in thalamo-amygdala synaptic transmission during which a temporally correlated pre- and postsynaptic activity results in presynaptic release of glutamate followed by a postsynaptic depolarization, a process that is called long-term potentiation (LTP): The release of glutamate triggers numerous intracellular processes, e.g. the Ca2+ binding to *N*-methyl-$_{D}$-aspartate (NMDA) receptors, the excitation of a protein kinase which in turn activates transcription factors hence influencing RNA and protein synthesis, altogether enhancing the availability of new proteins which lastly increase the amygdala's sensitivity to the CS (Rodrigues, Schafe, & LeDoux, 2004). Thus, after several pairings the former input of the neutral stimulus (white rat) is now sufficient to drive BLA output that in turn triggers the conditioned fear response via Ce activity (e.g. crying, crawling away from the rat) even if the

UCS (noise) is not presented. The NMDA receptor-dependent LTP is suggested to cause enhanced CS-elicited spike firing in a population of glutamatergic BLA neurons, called “fear neurons” (Herry et al., 2008) which have been found by using electrophysiological recordings of amygdaloid neuronal activity (Quirk et al., 1995). Interestingly, in differential fear conditioning[1] in which a second CS is signaling safety (CS-) these neurons actually exhibit decreased spike firing evidencing discriminative plasticity (Collins & Pare, 2000).

As the amygdala manifests its reputation for being the core region of fear learning there is in turn considerable evidence for the medial prefrontal cortex (mPFC) to be crucial for its extinction. Fear extinction refers to the observation that a conditioned fear response gradually decreases if the CS is repeatedly presented without the UCS. During both processes, fear conditioning and extinction, the amygdala and the mPFC exhibit strong interactions most likely mediated by reciprocal synaptic connections (Morgan, Romanski, & LeDoux, 1993; Rosenkranz, Moore, & Grace, 2003). Lesions to the mPFC demonstrated an impaired retrieval of extinction, i.e. a spontaneous recovery from the CR, while the acquisition of extinction was unaffected (Morgan et al., 1993; Quirk, Russo, Barron, & Lebron, 2000). According to Heidbreder and Groenewegen (2003) the rat mPFC can be cytoarchitectonically divided into four distinct regions, i.e. medial precentral cortex, anterior cingulated cortex (ACC), prelimbic (PL) and infralimbic (IL) prefrontal cortex. In order to enlighten the complex mechanisms as well as involved brain circuits affecting the inhibition of the fear response, the following overview focuses on different stages of forming the extinction memory, i.e. acquisition, consolidation and retrieval (cf. Quirk & Mueller, 2008).

Investigating a combination of in vivo single-unit recordings and targeted pharmacological inactivation in behaving mice, Herry et al. (2008) identified two distinct populations of neurons in the BLA correlating with a behavioral discrimination between a CS+ and a CS+ which has undergone extinction. ‘Fear neurons’ were found to exhibit an increased spike firing to a CS+ during and after fear conditioning, while ‘extinction neurons’ exclusively respond to an extinguished CS+. A rapid switch in the balance of the activity of these neural populations has been found to be essential for triggering behavioral transitions during fear and extinction. Interestingly, although co-localized within the same nucleus, both populations are differentially connected with the hippocampus and the mPFC: Hippocampal

[1] In contrast to simple fear conditioning designs in which only one CS is presented and associated with a UCS, for differential fear conditioning studies at least two CS were used. Thereby, the term CS+ refers to the stimulus which is reinforced by a UCS whereas the term CS- refers to the non-reinforced stimulus.

input selectively targets fear neurons whereas no connections were found between the hippocampus and extinction neurons. Regarding the evolutionary perspective that it is better to fear than not to fear, hippocampal input to the BLA fear neurons may dominate the retrieval of extinction memory allowing for fear renewal after a particular CS+ has undergone extinction. On the other hand, extinction neurons are reciprocally connected with the mPFC whereas fear neurons only exhibit input from the mPFC. Since BLA inactivation did not affect extinction memory retrieval once the extinction is acquired, Herry et al. (2008) suggested BLA fear and extinction neurons to facilitate the induction of synaptic plasticity in other parts of the brain, notably the mPFC, where the consolidation and storage of the extinction memory seems to be initiated. Hereby, IL and PL have been found to exhibit opposing functions: While microstimulation of the IL together with the presentation of the CS inhibited freezing behavior indexing the CR, microstimulation of the PL increased freezing, thereby demonstrating a bidirectional control over fear expression (Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006). The PL thus is suggested to mediate the maintenance of the fear response after it has been initiated (Burgos-Robles, Vidal-Gonzalez, & Quirk, 2009). In support of the aforementioned lesion and inactivation studies, electrophysiological recordings from IL neurons found an increased response to the CS+ following extinction training (Milad & Quirk, 2002). This fear inhibition seems to be achieved via a combination of excitatory projections sent by the IL to ITC cells as well as input from the “extinction neurons” of the BLA, both subsequently cause a reduced response of the Ce to the CS+ (Likhtik, Pelletier, Paz, & Pare, 2005; Quirk, Likhtik, Pelletier, & Pare, 2003). Extinction thus results when the inhibitory mPFC influence on the Ce outcomes the excitatory influence of the BLA fear neurons to the Ce (cf. Quirk, Garcia, & Gonzalez-Lima, 2006). As outlined for the acquisition of the fear memory, the consolidation of the extinction memory likewise involves NMDA receptor-dependent plasticity in the IL, physiologically resulting in enhanced mPFC activity by non-reinforced presentations of the CS+ (Burgos-Robles, Vidal-Gonzalez, Santini, & Quirk, 2007).

Comparable to fear acquisition, fear extinction constitutes an active learning process and can be therefore characterized as a fragile state: A learnt fear response can either reappear with the passage of time (spontaneous recovery) or can return when the CS is presented in a different context (renewal) or can be re-activated following an unexpected UCS delivery (reinstatement). These phenomena support the assumption that extinction

does not result from the erasure of a fear memory but rather results from a competition between the initial CS-UCS association and the new CS-noUCS memory in order to capture behavioral control (Bouton, 2002; Pavlov, 1927). They further demonstrate a key role of the hippocampus in the retrieval of extinction memory. Hippocampus lesions have been shown to interfere with conditioning to a context whereas conditioning to a cue such as a light or a tone CS was intact (Phillips & LeDoux, 1992). The retrieval of extinction is thus expected to activate inhibitory circuits within and between the amygdala, the mPFC as well as the hippocampus. A simplified graphic account of the neural fear and extinction network is illustrated in figure 1.

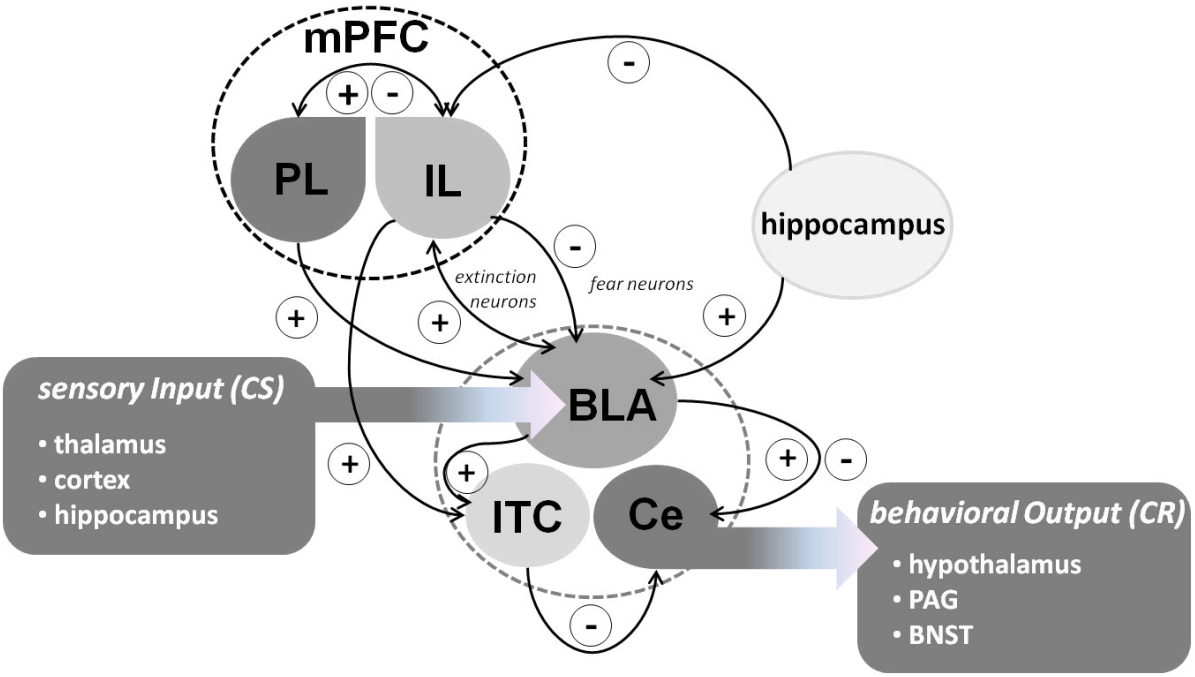

Figure 1. The Neural Fear Network. The model represents the numerous excitatory (+) and inhibitory (-) projections within the amygdala (depicted in the center) as well as between the amygdala and the medial prefrontal cortex (mPFC) and the hippocampus, respectively, which are involved in the acquisition and extinction of conditioned fear. BLA: basolateral, BNST: bed nucleus of the stria terminalis, Ce: central nucleus, CS: conditioned stimulus, CR: conditioned response, ITC: intercalated cell masses, IL: infralimbic cortex, PAG: periaqueductal gray, PL: prelimbic cortex.

1.3 Human Findings

The aforementioned findings obtained from rodent data remarkably influenced the generation of specific hypotheses about neural circuits in humans as manifested in the number of increased publications since 1990 for animal and human studies in this field (Milad & Quirk, 2012). Although modern technologies drag behind the variety of methods

available to elucidate neural structures in animals, translational similarities allow for testing specific assumptions about neural circuits involved in humans.

Consistent with the comparative animal data, neuroimaging findings in humans revealed a core fear network for fear acquisition and extinction centered on the amygdala (Sehlmeyer et al., 2009). Likewise the brain lesion experiments in rats conducted by Phillips and LeDoux (1992), bilateral amygdala damage in humans has been found to block the ability to acquire a CR independent of the declarative memory of the conditioning experience associated with an intact hippocampus (Bechara et al., 1995). By the end of the last century, the first studies using functional magnetic resonance imaging (fMRI) have been initiated investigating pavlovian fear conditioning in healthy human participants in a way that resembled the experimental setup exhibited in animal research. LaBar et al. (1998) used a simple discrimination procedure with two visually presented squares serving as a CS+ which was co-terminated with a brief electric shock (UCS) applied to the wrist of the participants, and a control CS- never paired with the UCS. The authors found an increased differential amygdala blood oxygenation level-dependent (BOLD) response (CS+ minus CS-) during the early acquisition phase. Büchel et al. (1998) circumvented the possible confound of the unconditioned response initiated by the UCS by separating the CS+ (neutral faces) into 50% reinforced trials, i.e. time-locked CS+ and UCS (an aversively loud tone) presentations, and 50% non-reinforced trials during which only the CS+ was presented. Those intermittent reinforcement procedures have been shown to slow learning rates (Gottlieb, 2005) and delay extinction (Haselgrove, Aydin, & Pearce, 2004). Again, a differentially increased response to the CS+ was found in the amygdala (Büchel et al., 1998). Furthermore, activation in motor-related brain regions were found and interpreted as behavioral fear response, i.e. the attempt to escape the aversive situation, paralleling the descending projections from the amygdala to the brain stem. Interestingly, both studies found differential CS+-related amygdala activity to decrease over time evidencing other brain regions to inherit memory consolidation and retrieval (Büchel et al., 1998; LaBar et al., 1998).

With regard to fear extinction a similar response emerged, i.e. again the amygdala showed an increased response to CS+ during the early phase paralleled by activation of the mPFC, more precisely the anterior cingulated cortex (ACC; LaBar et al., 1998). The retrieval of extinction memory on a subsequent day was found to correlate with ventromedial prefrontal cortex (vmPFC) activity (Phelps, Delgado, Nearing, & LeDoux, 2004) as well as

vmPFC cortical thickness (Milad et al., 2005) which again can be integrated with the knowledge derived from rodent data since the vmPFC is anatomically thought to be the putative homologue of the rodent IL (cf. Milad et al., 2007). Consistent with the opposing roles of PL and IL in the maintenance and inhibition of the amygdala's fear response in rodents (Vidal-Gonzalez et al., 2006), higher resting amygdala metabolism in healthy humans predicted activation of the dorsal ACC (dACC) during extinction training and deactivation of the vmPFC during extinction recall (Linnman et al., 2012). It was further shown that extinction recall context-dependently co-activated the hippocampus (Kalisch et al., 2006; Milad et al., 2007).

In order to demonstrate mPFC engagement during fear extinction by using an optical imaging technique that has so far not been investigated in a common composition of a pavlovian fear conditioning and extinction design in humans, the first manuscript of this thesis is now being introduced. Before presenting this study, two methods of the experiment are going to be highlighted depicting dependent variables of the fear CR which have been consistently used in almost all the here presented studies: Skin conductance response and functional near-infrared spectroscopy.

1.3.1 Excursus: Skin Conductance Response (SCR)

Deriving from electrodermal activity (EDA) measurable changes in skin conductance at the surface provide a common psychophysiological indicator of emotional behavior (Critchley, 2002). During fear conditioning the learnt CS-UCS association elicits a sympathetic nervous system activity in response to the CS+ causing increased secretion of eccrine sweat glands. A marginal current that is steadily passed between two electrodes applied to the hand or feet of a participant is used to record the electrical resistance typically emerging with a latency window of 1 to 3 seconds after stimulus presentation. The phasic increase of this electrical resistance in response to a cue is called skin conductance response (SCR) as opposed to the skin conductance level evidencing a more tonic activity (Dawson, Schell, & Filion, 2000). The positive correlation between neural activation in the amygdala with SCR during fear conditioning (Furmark, Fischer, Wik, Larsson, & Fredrikson, 1997; LaBar et al., 1998; Phelps et al., 2004) as well as the failure of conditioned SCR in amygdala-damaged patients (Bechara et al., 1995) evidenced SCR as an appropriate index of fear conditioning. Furthermore, the generation and control of EDA is besides limbic structures subserved by a

variety of brain regions assigning to prefrontal and parietal cortices (Tranel & Damasio, 1994). Thus, SCR during extinction learning and extinction retrieval has been shown to correlate with prefrontal cortex activity as well (e.g. Phelps et al., 2004). In accordance with the aforementioned neuroimaging findings, extinction success as indexed as the change in CR from early to late extinction trials positively correlated with vmPFC activity during extinction retrieval (Phelps et al., 2004). Since the studies included in this dissertation focus on the prefrontal cortex leaving amygdala activity untracked, SCR as dependent CR measurement thus promises to represent an appropriate variable to index the fear response.

1.3.2 Excursus: Functional Near-Infrared Spectroscopy (fNIRS)

Functional NIRS is an optical neuroimaging method quantifying changes in hemoglobin content and oxygenation that are related to neurovascular coupling. Obrig and Villringer (2003) as well as Scholkmann et al. (2014) provide comprehensive overviews; regarding these references at this point only the basic principles and a selection of studies is presented.

Neurovascular coupling refers to the increased regional cerebral blood flow (rCBF) and the increased regional cerebral blood volume that is required for transporting glucose and oxygen during neural activity. These processes are accompanied by an increase in the concentration of oxygenated hemoglobin (O_2Hb) and a decrease in the concentration of deoxygenated hemoglobin (HHb, Wolf et al., 2002). Since both chromophores exhibit characteristic optical properties (i.e. specific absorption spectra), concentration changes within the rCBF allow indirect conclusions about the neural activity in this region. It is thus comparable to the BOLD response measured with fMRI. FNIRS apparatuses, such as the ETG-4000 continuous-wave Optical Topography System (Hitachi Medical Corporation, Japan) which was used in all four studies, operate with near-infrared light in a range between 700 and 900 nm since most biological tissues are transparent in this so called optical window. By emitting near-infrared light onto the scalp, the number of photons absorbed or scattered by brain tissue namely varies with the changes in the chromophores' concentration. Passing the tissue following an elliptical pathway, the amount of back-scattered near-infrared light detected at the surface thereby offers information about cortical oxygenation. These changes in light intensity are quantified by using the modified Beer-Lambert law which

calculates the relative concentration as a function of the total path length: By taking into account a scattering dependent light intensity loss parameter, it assumes light scattering to remain stable over time, so that detected changes in light intensity must be attributed to relative changes in the chromophores' concentration. Since continuous-wave systems do not allow the quantification of the total amount of O_2Hb and HHb, the relative change of both chromophores respectively is obtained in relation to experimental manipulations, for instance the presentation of a CS+ and a CS- as in the subsequently presented study.

Compared to other neuroimaging modalities fNIRS has several advantages. As the electrochemical mechanisms during neural activity lead to changes in the magnetic and electrical fields which can also be (summed across numerous synchronously activated neurons) measured by using electroencephalography (EEG) or magnetoencephalography (MEG), fNIRS additionally provides information about the anatomical location throughout the cortex. The spatial resolution of fNIRS systems is approximately 1 cm^2 (Bunce, Izzetoglu, Izzetoglu, Onaral, & Pourrezaei, 2006). The interoptode distance between the light emitter and the detector further determines the depth of penetration (Villringer & Chance, 1997) with the 3 cm distance of the ETG-4000 System expecting to reach a penetration depth of up to 1.5 cm (Quaresima, Bisconti, & Ferrari, 2012; Strangman, Boas, & Sutton, 2002). In contrast to fMRI and positron emission tomography (PET) which allow a much more precise anatomical location of approximately 1 mm^2 even for subcortical areas (Bunce et al., 2006), the higher temporal resolution of fNIRS with a sampling rate of 10 Hz vs. 0.5 Hz for most of the fMRI studies, combines spatial and temporal advantages of EEG/MEG and fMRI/PET imaging modalities (Strangman et al., 2002). In order to integrate findings obtained by the different techniques, a virtual spatial registration method has been developed in which optode positions (referring to light emitters and detectors) of a specific holder, e.g. a 3 x 11 array as used in the presented studies, were registered onto the Montreal Neurological Institute (MNI) space typically referred to in fMRI studies (Tsuzuki et al., 2007). FNIRS is less susceptible to movement artifacts allowing participants to talk or move during the experiment (e.g. Dieler, Tupak, & Fallgatter, 2012; Egetemeir, Stenneken, Koehler, Fallgatter, & Herrmann, 2011) and it is applicable for patients who may avoid the fMRI environment for instance due to claustrophobic anxiety (e.g. Dresler et al., 2011). Furthermore, fNIRS can be applied cost-effectively, safe and with only minimal preparation beforehand while still exhibiting a sufficient reliability (Plichta et al., 2006). However, fNIRS is limited by its spatial

resolution being only moderately in contrast to fMRI which restricts its application to parts of the cortex located directly under the skull, while subcortical regions such as the amygdala or the hippocampus are not assessable. Nonetheless, the spatial resolution is sufficient to differentiate between distinct prefrontal regions such as medial, ventrolateral (vlPFC) and dorsolateral PFC (dlPFC) thereby raising the question whether fNIRS is suitable to detect the proposed prefrontal engagement during fear extinction that has been proposed by a vast majority of animal and human findings outlined above.

1.4 Study 1: Investigating Fear Extinction by using fNIRS

The first study included in this dissertation aimed at investigating prefrontal cortex activity during the extinction of conditioned fear by means of fNIRS for the first time. With regard to the prefrontal involvement found during extinction learning in the existing fMRI studies (e.g. Linnman et al., 2012; Milad et al., 2007), a likewise increase in mPFC activity was hypothesized to be detectable from early to late extinction trials. To provide a short preview to the main research focus of this dissertation, the study further aimed at defining a target region for the application of repetitive transcranial magnetic stimulation (rTMS) to augment extinction training. Since both techniques, fNIRS and rTMS, are likewise restricted to cortical structures, it was assumed that the obtained results could be used to define a target region for the intended excitatory rTMS application provided that fNIRS is suitable to detect prefrontal involvement during extinction learning.

The next chapter is going to focus on prefrontal stimulation with rTMS, but initially the first study will be presented in its individual manuscript form. The license for using the material for the purpose of the present dissertation is given.

Original Paper

Neuropsychobiology

Neuropsychobiology 2012;65:173–182
DOI: 10.1159/000337002

Received: October 24, 2011
Accepted after revision: January 30, 2012
Published online: April 26, 2012

Medial Prefrontal Cortex Activity during the Extinction of Conditioned Fear: An Investigation Using Functional Near-Infrared Spectroscopy

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Key Words
Extinction learning · Fear conditioning · Medial prefrontal cortex · NIRS

Abstract
The majority of fear conditioning studies in humans have focused on fear acquisition rather than fear extinction. For this reason only a few functional imaging studies on fear extinction are available. A large number of animal studies indicate the medial prefrontal cortex (mPFC) as neuronal substrate of extinction. We therefore determined mPFC contribution during extinction learning after a discriminative fear conditioning in 34 healthy human subjects by using functional near-infrared spectroscopy. During the extinction training, a previously conditioned neutral face (conditioned stimulus, CS+) no longer predicted an aversive scream (unconditioned stimulus, UCS). Considering differential valence and arousal ratings as well as skin conductance responses during the acquisition phase, we found a CS+ related increase in oxygenated haemoglobin concentration changes within the mPFC over the time course of extinction. Late CS+ trials further revealed higher activation than CS– trials in a cluster of probe set channels covering the mPFC. These results are in line with previous findings on extinction and further emphasize the mPFC as significant for associative learning processes. During extinction, the diminished fear association between a former CS+ and a UCS is inversely correlated with mPFC activity – a process presumably dysfunctional in anxiety disorders.

Introduction

Fear is an aversive emotional state which at moderate levels proves biologically useful by enabling effective detection of threat and automatic activation of defensive behaviour [1]. Anxiety disorders such as post-traumatic stress disorder, panic disorder and phobias are characterized by increased fear levels that might contribute to a generally impaired ability of fear extinction [2–4]. In order to model the development and maintenance of anxiety disorders, learning theories – notably conditioned fear reactions and their extinction – have been widely applied and particularly validated with regard to the effectiveness of exposure-based treatment in psychotherapy [2].

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In a classical pavlovian fear conditioning paradigm, an initially neutral stimulus, such as a tone, is paired with an aversive event (unconditioned stimulus, UCS), e.g. an electric shock, and comes to elicit the so-called conditioned response (CR, e.g. freezing) itself after several pairings. In the absence of the UCS, the CR gradually disappears in response to the conditioned stimulus (CS, tone), i.e. its amplitude and frequency decrease [2]. This decrease of the CR is referred to as extinction and is thought to be the experimental foundation of exposure therapy applied to anxiety patients. During extinction learning, the acquisition of inhibitory memories is assumed to compete with excitatory memories formed during fear conditioning and thereby suppress the CR [3, 4]. These interactions are neuroanatomically mirrored in subcortical as well as cortical structures which have been closely investigated in a variety of lesion studies [e.g. 5, 6] as well as single-cell recording [e.g. 7, 8] and stimulation studies [e.g. 9, 10] in animals. Above all, the amygdala has been shown to be notably involved in the expression and acquisition of conditioned fear [11], also in humans [12]. In intracellular in vivo recordings in rats, Rosenkranz et al. [7] demonstrated enhanced activity of the lateral amygdala while presenting conditioned affective stimuli whereas bilateral amygdala lesions prevented the acquisition of CR [13, 14]. Lesions to the medial prefrontal cortex (mPFC) on the other hand have been shown to generate an increased resistance to extinction as well as a high rate of spontaneous recovery while the acquisition of fear CR remained unaffected [6, 15, 16]. During extinction learning a rapid switch in the activity of two distinct populations of basal amygdala neurons seems to be essential for activating behavioural alterations [8]. According to Herry et al. [8], these 'extinction neurons' are bi-directionally connected with mPFC neurons that might mediate the consolidation of extinction memory. The 'fear neurons' in turn seem to be depressed if the predictive ability of the CS for danger is weakened through successful extinction training [5]. Paralleling these findings, mice that underwent extinction training in an investigation using fluorodeoxyglucose displayed elevated prefrontal cortex activity. Moreover, mice with higher prefrontal activity showed less CR and in turn mice not receiving extinction training demonstrated significantly more stable amygdala activity [17].

Although the neural mechanisms of extinction learning in humans are less well characterized than for animals [12], the general pattern of brain activation during fear and extinction learning seems to be essentially the same [18]. A variety of pavlovian conditioning studies in humans actually found amygdala involvement during fear conditioning [19–27]. Accordingly, mPFC activity could have been associated with extinction learning [20, 23, 24, 26, 28] and further linked with decreasing amygdala activity [23, 24, 29–31]. However, although extinction is thought to be crucial for understanding and improving psychotherapy, in 2009, Sehlmeyer et al. [12] merely found seven studies directly focussing on neurobiological correlates of extinction learning. Frequently but even inconsistently found was prefrontal engagement. More recently these findings were enriched by studies concentrating on trait anxiety in fear extinction. These studies revealed comparable results to the aforementioned animal studies [7, 10] relating to mPFC-amygdala coupling. Anxious participants displayed enhanced amygdala activity during extinction learning that correlated negatively with mPFC involvement, indicating delayed inhibitory learning or rather generally reduced extinction [26, 28]. Irrespective of extinction learning, in anxious subjects, Indovina et al. [32] demonstrated an insufficient recruitment of the prefrontal cortex to downregulate fear in a safety context, and Bishop et al. [33] showed reduced mPFC activity when anticipating threat. Taken together, these findings clarify extinction as a form of new learning which is hence prone to behavioural instability and emphasise the importance of a better understanding of extinction mechanisms. To date, the limited number of available studies on mPFC activity and extinction learning in humans impedes gathering insides into the mode of action in psychotherapy.

The present investigation therefore focused on contribution of the mPFC during extinction learning in a classical discriminative fear conditioning paradigm by using functional near-infrared spectroscopy (fNIRS). NIRS is an optical imaging method to non-invasively and in vivo investigate tissue such as the brain, muscle and others. It enables measuring concentration changes in oxygenated (O_2Hb) and deoxygenated (HHb) haemoglobin which are accompanied by increases in cerebral blood volume [34]. To our knowledge, fNIRS has never been used to investigate contribution of the mPFC to extinction learning. However, fNIRS has been successfully applied to measure changes in O_2Hb concentration within the mPFC during emotional tasks [e.g. 35]. The frontal positioning of the NIRS probe set enables the investigation of medial Brodmann areas 9 and 10 bilaterally extending to the dorsolateral prefrontal cortices and is hence covering the mPFC. Participants in the present study performed a fear conditioning paradigm in which one of two neutral faces (CS) was paired with an aversive scream (UCS). Immedi-

ately after establishing the fear conditioning they underwent an extinction training in which the originally neutral stimulus was repeatedly presented without the UCS. By dividing the so-called within-session extinction phase into an early and late component, we hypothesised increasing mPFC activity to the CS+ as described by others [25, 26, 36].

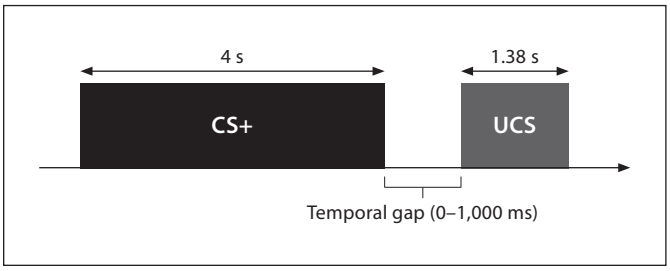

Fig. 1. Temporal arrangement of CS+ and UCS.

Methods

Participants

Thirty-five healthy volunteers (17 females, 18 males; mean age 24.7 years, standard deviation (SD) 3.32, range 20–32 years) participated in the study. All subjects were screened for current mental health using the German version of the Mini International Neuropsychiatric Interview (MINI [37]) and for right-handedness according to the Edinburgh Handedness Inventory [38] before the experiment. We further assured that all females used oral monophasic contraceptives and that they were not in their pill-off phase when participating in the experiment in order to exclude changes in hormonal levels which have been demonstrated to influence conditioned fear acquisition as well as extinction recall [39]. Psychology students were excluded to exclusively investigate paradigm-naive volunteers. For this reason, 1 male participant (age 31 years) was not considered for further data analyses because of familiarity with the procedure. Subjects were reimbursed with 7 Euros for participation in an experimental setting lasting 60 min.

They were recruited through online advertisement and gave written informed consent in accordance with the Declaration of Helsinki in their most recent version from 2008. All procedures were approved by the ethical review board of the medical faculty of the University of Würzburg (Protocol ID 151/10) and were performed in the facilities of the department of Psychiatry, Psychosomatics and Psychotherapy of the University of Würzburg.

Experimental Paradigm

The differential fear conditioning paradigm investigated in the present study consisted of three experimental phases (habituation, acquisition and extinction). Two colour photographs of neutral male faces selected from the NimStim set of facial expressions [40] served as conditioned stimuli (CS) and a scream of 95 dB adapted from the International Affective Digital Sounds [41] was used as UCS. During the habituation, each stimulus was presented 8 times without the UCS. The following acquisition phase comprised 30 trials in total, i.e. 15 CS– and 15 CS+ trials in which 12 CS+ were paired with the UCS and the remaining 3 CS+ trials rested unpaired (reinforcement rate 80%) in order to decelerate the acquisition of conditioned fear and to extend its extinction. The CS– was never paired with the UCS. The extinction phase consisted of 18 CS– and 18 CS+ presentations without the UCS. Faces were presented for 4,000 ms and counterbalanced as CS+ and CS– to each subject, so that both faces were equally often selected as CS+ and CS–. The scream lasted 1,380 ms and appeared in a jittered time interval of 0–1,000 ms after the CS+ offset (fig. 1). Inter-trial intervals ranged from 10 to 16 s and consisted of a white fixation cross on a black screen. CS presentations were randomized within all three experimental phases.

In order to adjust the NIRS probe set to cover concentration changes in cerebral haemoglobin while passing through the extinction, we implemented a short break after the acquisition phase lasting 2–4 min. We did not change any contextual parameter such as the lighting conditions or the subjects' sitting position to ensure within-session extinction. All subjects were informed that during the experiment one of two male faces is paired with an aversive loud scream, whereas the second face is never presented together with a scream. Apart from their knowledge of the break to adjust the NIRS probe set, they were not informed about the experimental phases and in particular the UCS absence during the extinction phase. In order to eliminate intense novelty responses to the UCS and thereby risking dropouts during the paradigm, subjects were presented once with the scream before the experiment and were asked to determine the level of aversiveness on a scale ranging from 0 for 'not unpleasant' to 10 for 'extremely unpleasant'. While passively regarding the faces during the examination, subjects had to field a total of seven valence and arousal ratings of the CS to maintain attention and ensure successful conditioning over the whole experiment, i.e. approximately 30 min: after the habituation phase and 3 times during acquisition and extinction, respectively. Therefore, we used the Self-Assessment Manikin [42] and a 9-point Likert Scale ranging from 1 for 'unpleasant' to 9 for 'pleasant' and 1 for 'not arousing' to 9 for 'highly arousing'. We additionally assessed contingency awareness on CS+ and UCS and implemented three expectancy ratings during the extinction phase. Expectancy ratings were formulated as 'How likely is a reappearance of the scream in your opinion?' and were assessed by a 9-point Likert Scale ranging from 0 to 100% conviction of UCS recurrence.

These expectancy ratings are expected to represent a kind of prediction error, i.e. the higher the prediction error was, the higher the expectation of recurring UCS towards the following items is. The paradigm was presented using Presentation® Version 12.2 software (Neurobehavioral Systems, Inc., Albany, Calif., USA). All responses were given by keyboard presses with the right hand.

SCR and NIRS Measurements

We assessed skin conductance responses (SCR) to CS+ and CS– during the whole experiment in order to ensure effective fear conditioning as well as extinction. SCR is regarded as an index for emotional responses associated with automatic arousal [43] and therefore an indicator of successful conditioning. It was assessed with two Ag/AgCl electrodes attached to the thenar eminence of the subjects' left palm. SCR were recorded using a GSR sensor

(Brain Products GmbH, Munich, Germany) constantly delivering 0.5 V across both electrodes and a 72-channel QuickAmp amplifier (Brain Products GmbH) with a sampling rate of 1,000 Hz and a notch filter of 50 Hz. Data were acquired and saved via Vision Recorder Version 2.0 software (Brain Products GmbH) and towards data collection processed by the appropriate Vision Analyzer software (Brain Products GmbH). Herewith, time series were filtered at 1 Hz and segmented into experimental phases as well as single CS+ and CS– trials. Each segment was further baseline corrected 1,000 ms prior to the onset of the stimuli and characterised by the peak response of the SCR signal between 1 and 4 s after stimulus onset. Artefact rejection was conducted manually for all 82 trials per subject.

In order to investigate regional cerebral blood flow in the mPFC during extinction learning, we examined changes in O_2Hb by using fNIRS. fNIRS measurements are based on differential absorption of near-infrared light due to O_2Hb and HHb concentration changes that arise through neurovascular coupling mirroring the metabolic demands of the nervous system. Illuminating the brain surface through the intact scalp and skull, near-infrared light reflected from deep tissue layers is received by a photodetector that is fixed some centimetres apart from the light emitter. fNIRS measurements are comfortable for the subjects because of fewer motion restrictions and no noise disturbance; it has a high temporal resolution (<1 s) and can be easily combined with other neuroimaging techniques or physiological measurements [44]. Further, more detailed information about the fundamentals of fNIRS is provided elsewhere [e.g. 45, 46]. We opted to restrict the fNIRS measurement to the extinction phase for two reasons: first, our experiment focused particularly on extinction learning and not fear acquisition, and second, we know from experience that subjects in fNIRS settings exceeding a time period of 20 min without a break might feel more and more uncomfortable. fNIRS signals were measured with the continuous-wave system ETG-4000 (Hitachi Medical Co., Tokyo, Japan) using a 3 × 11 channel array of optodes consisting of 16 photodetectors and 17 light emitters resulting in 52 channels in total. The ETG-4000 operates with two different wavelengths (695 ± 20 and 830 ± 20 nm) and its frequency is modulated for wavelengths and channels to prevent crosstalk. In order to reliably position the probe set, the lowest-row centre optode is typically placed on the Fpz position at the frontal region of the head extending symmetrically towards positions T3 and T4 according to the International 10–20 system for EEG electrode placement [47]. The interoptode distance of 30 mm enables measurements approximately 15–25 mm beneath the scalp [48]. Signals were acquired with a sampling rate of 10 Hz and transformed into values for changes in the concentration of O_2Hb.

Statistical Methods

All statistical analyses were performed using PASW Statistics 18 (SPSS Inc., Chicago, Ill., USA) and Matlab software (Version 7; MathWorks Inc., Natick, Mass., USA). Whenever we had directed hypotheses, one-tailed tests at a significance level of $p < 0.05$ were performed (otherwise two-tailed). Valence and arousal ratings were analysed separately using repeated-measures analyses of variance (ANOVA) with two within-subject factors: stimulus (CS+, CS–) and phase (habituation, acquisition, extinction). For the repeated ratings derived from the acquisition and extinction phases, values were averaged. In case of significant stimulus × phase interactions post hoc t tests were performed. Non-sphericity was considered applying Greenhouse-Geisser correction.

Before performing statistical analyses for the SCR data, we log-transformed all peak amplitudes (SCR + 1) to normalize the distribution and further scored responses <0 μS as zero in order to adequately characterize non-responses to the CS. Afterwards we separated the existing 18 CS+ and 18 CS– extinction trials into early (n = 9) and late (n = 9) responses to compare SCR and fNIRS parameters. SCR data were analysed with repeated-measures ANOVA with stimulus (CS+, CS–) and phase (habituation, acquisition, early extinction, late extinction) as within-subject factors. A significant interaction of both factors was further assessed with one-tailed post hoc t tests at a significance level of $p < 0.05$ due to our directed hypotheses in fear and extinction learning. In relation to our assumptions concerning the fNIRS signal changes, we expected that successful extinction will be indicated by a decrease in SCR to the CS+ during the time course of extinction training.

Analogous to the procedure for SCR analyses, fNIRS signals were divided into an early and late phase, each consisting of nine trials. Because of our interest in signal changes occurring in response to the CS+ offset and accordingly the anticipated UCS onset, all trials were time-locked to the jitter mean, i.e. 4,500 ms after CS+ onset, and screened for artefacts. O_2Hb changes were preprocessed by applying a low-pass filter of 0.5 Hz and a cosine filter correcting for low-frequency signal drifts. In a next step, functional data were modelled by four regressors (CS+ early, CS– early, CS+ late, CS– late) (online supplementary fig. 1; see www.karger.com/doi/10.1159/000337002). Events per condition were further modelled as δ functions and convolved with a gaussian hemodynamic response function at a peak time of 6.5 s. Time series were analysed by applying a general linear model approach [49] using Matlab Version 7 software (MathWorks Inc.). The resulting β estimates per condition and subject served as parameter set for subsequent testing. According to our hypothesis of an increasing O_2Hb concentration towards early and late extinction trials for CS+ compared to CS–, we determined differential β values for CS+ and CS– each and contrasted these differences by using paired t tests: $[(CS+_{late} - CS+_{early}) - (CS-_{late} - CS-_{early})]$. Correction for multiple comparisons across probe set channels were performed by using a cluster permutation approach. Specifically, we compared the cluster size of significantly active channels (at $p < 0.1$ for each channel) to the distribution of cluster sizes expected under the null hypothesis (adapted for the 2-d fNIRS case from Wager et al. [50]). To obtain the null distribution, we performed 10,000 permutation tests across all channels given a single channel p value < 0.1. Activation was thus considered significant if the probability of obtaining this cluster size under the null hypothesis was $p < 0.05$. According to a probabilistic map (http://www.jichi.ac.jp/brainlab/virtual_regE.html#AnatomLabel) we provide MNI coordinates (x, y, z) of significant fNIRS channels to allow for integration of our results across imaging methods.

SCR and fNIRS data were tested for significant correlations using Pearson's correlation coefficient and one-tailed tests due to our expectations of negative correlations between SCR and O_2Hb within fNIRS channels or even channel clusters during the extinction phases. For exploratory purposes, we examined the influence of expectancy and SCR as well as fNIRS data to consider the influence of prediction error (two-tailed tests).

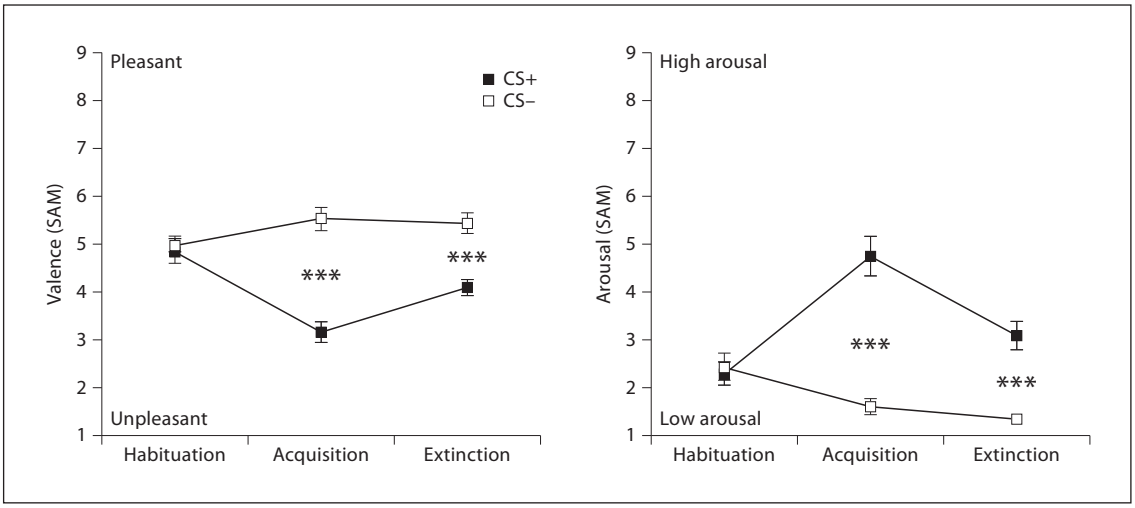

Fig. 2. Valence and arousal ratings. Assessment by using the Self-Assessment Manikin (SAM [42]). Significantly different values for CS+ and CS– per experimental phase (mean + SEM) are depicted as *** to indicate a significance level of $p < 0.001$.

Results

As expected, subjects rated the UCS as quite unpleasant (mean 7.8, SD 1.3, range 4–10) indicating that the scream of 95 dB was aversive enough to induce fear conditioning. All 34 participants reported awareness of the CS-UCS contingency at the end of the acquisition phase and displayed a significant linear decrement of the UCS expectancy ratings during the extinction phase (linear trend test: $F(1, 33) = 24.53$, $p < 0.001$) from 78% after the first third, over 62% after the second third to 52% at the end of the experiment. Concerning the valence and arousal ratings, the repeated-measures ANOVA yielded main effects for stimulus (valence: $F(1, 33) = 29.37$, $p < 0.001$; arousal: $F(1, 33) = 43.25$, $p < 0.001$) and phase (valence: $F(1.5, 52.7) = 15.33$, $p < 0.001$; arousal: $F(1.5, 50.8) = 13.98$, $p < 0.001$) as well as significant stimulus × phase interactions (valence: $F(1.3, 42.3) = 23.49$, $p < 0.001$; arousal: $F(1.3, 41.4) = 34.02$, $p < 0.001$). As expected, post hoc t tests revealed that CS+ and CS– were equally evaluated after the habituation phase, both as neutral and sparsely arousing, but were differentially rated during conditioning. Herein, CS+ ratings were significantly lower in valence ($t_{33} = 6.51$, $p < 0.001$) and higher in arousal than for CS– ($t_{33} = 7.52$, $p < 0.001$). Comparing acquisition and extinction phase, the decrement for CS+ arousal and the increase for CS+ valence became significant (arousal: $t_{33} = 7.02$, $p < 0.001$; valence: $t_{33} = 6.5$, $p < 0.001$) although the differential ratings persisted during extinction learning (valence: $t_{33} = 5.55$, $p < 0.001$; arousal: $t_{33} = 6.13$, $p < 0.001$; fig. 2).

For SCR analyses we had to exclude 2 female subjects who did not display any fluctuations in their responses to either CS or UCS across the whole experiment. Analyses of the remaining sample of 32 subjects revealed similar results as for subjective ratings indicating a successful conditioning during acquisition and additionally suggesting extinction learning within the last experimental phases (fig. 3). The 2 × 4 repeated-measures ANOVA showed significant main effects for stimulus ($F(1, 31) = 15.82$, $p < 0.001$) as well as phase ($F(3, 93) = 6.74$, $p < 0.001$) and again a significant interaction ($F(3, 93) = 4.51$, $p = 0.005$). Post hoc t tests demonstrated significantly higher SCR amplitudes to CS+ than CS– during acquisition ($t_{31} = 4.87$, $p < 0.001$), which significantly diminished during the extinction phases (paired t test for SCR to CS+ during acquisition compared to early extinction: $t_{31} = 4.15$, $p < 0.001$, and late extinction: $t_{31} = 3.77$, $p < 0.001$). Differences between CS+ and CS– remained significant during the time course of extinction (early extinction: $t_{31} = 1.93$, $p = 0.032$; late extinction: $t_{31} = 2.5$, $p = 0.009$). However, SCR amplitudes during the extinction phase returned to habituation level (early extinction: $t_{31} = 0.55$, $p = 0.293$; late extinction: $t_{31} = 0.69$, $p = 0.248$) and further did not change significantly through early and late extinction trials ($t_{31} = 0.33$, $p = 0.746$).

For the fNIRS data we did not have to exclude channels from analyses due to little motion artefacts in the

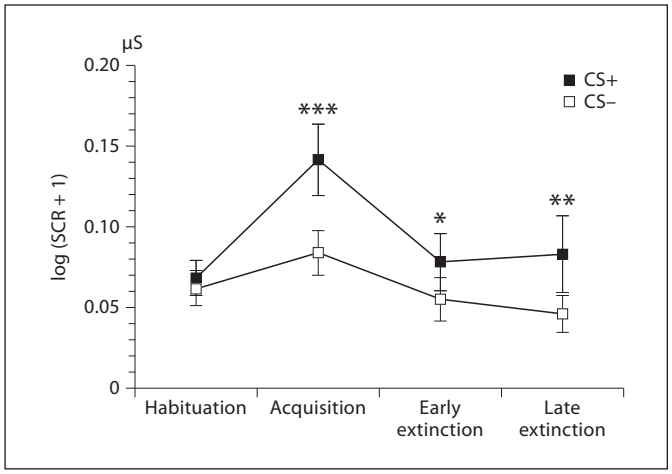

Fig. 3. ANOVA results for SCR to CS+ and CS–. As expected, SCR to CS+ trials increased during conditioning and decreased during extinction indicating successful conditioning and extinction. Responses to the CS– remained unchanged through the experiment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

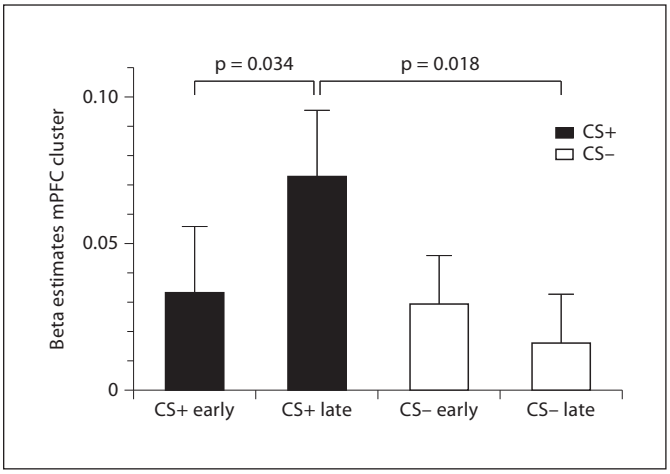

Fig. 4. Beta estimates of the regressors CS+ early, CS+ late, CS– early and CS– late. Depicted are cluster means and standard errors of the cluster means.

frontal region as expected. We found four significant probe set channels for which our analysed *t*-contrast [$(CS+_{late} - CS+_{early}) - (CS-_{late} - CS-_{early})$] revealed significant results, that is the difference between early and late trials was larger for CS+ than CS– (channel 35: $t_{33} = 2.26$, $p = 0.016$ [x = 27, y = 68, z = 9]; channel 27: $t_{33} = 2.22$, $p = 0.017$ [x = –13, y = 68, z =20]; channel 26: $t_{33} = 1.92$, $p = 0.032$ [x = 15, y = 68, z = 21]; channel 47: $t_{33} = 1.79$, $p = 0.042$ [x = 15, y = 71, z = –3]). Eleven other channels revealed significant results by trend and are mentioned for completeness (channel 20: $t_{33} = 1.64$, $p = 0.055$; channel 16: $t_{33} = 1.62$, $p = 0.057$; channel 24: $t_{33} = 1.62$, $p = 0.058$; channel 36: $t_{33} = 1.61$, $p = 0.059$; channel 19: $t_{33} = 1.58$, $p = 0.062$; channel 21: $t_{33} = 1.57$, $p = 0.066$; channel 5: $t_{33} = 1.57$, $p = 0.063$; channel 37: $t_{33} = 1.53$, $p = 0.068$; channel 29: $t_{33} = 1.5$, $p = 0.072$; channel 45: $t_{33} = 1.42$, $p = 0.083$; channel 51: $t_{33} = 1.4$, $p = 0.086$). Ten of the aforementioned probe set channels resulted in one single significant cluster ($p < 0.03$; channels 35, 27, 26, 47, 16, 24, 36, 5, 37, 45); no other cluster reached the significance threshold. Figure 4 pictures mean β values for this cluster by separating into CS+ and CS– as well as early and late trials according to our analysed *t*-contrast (see above). The cluster is being composed of a significant increase of CS+ trials during the early and late extinction phase ($t_{33} = 1.89$, $p = 0.034$) and significant exceeding O_2Hb values between CS+ and CS– trials during the late extinction phase ($t_{33} = 2.2$, $p = 0.018$). We neither found differences between CS– trials across the two phases ($t_{33} = 0.9$, $p = 0.187$) nor between CS+ and CS– early trials ($t_{33} = 0.23$, $p = 0.41$; fig. 4, 5).

As depicted in figure 5b, the hemodynamic responses approximately started according to the expected UCS onset (around 5,000 ms) and revealed no differences during CS presentation. Hemodynamic responses starting at the expected UCS onset during extinction learning seem to reflect a prediction error, i.e. the expected UCS did not occur.

Correlations

Correlation analyses concerning SCR and O_2Hb in the sample of 32 subjects revealed a negative correlation between mean cluster O_2Hb values for late CS+ trials and the difference score between early and late SCR to CS+ ($r = –0.327$; $p = 0.034$). Subjects who displayed decreasing SCR to CS+ from early to late trials as it is expected during successful extinction, showed higher β values within late extinction phase indicating higher activity within the mPFC. In order to adequately compare SCR and fNIRS data during the same time interval, we additionally performed SCR peak detection during the extinction phase in a later interval between 5.5 and 8.5 s after CS presentation. This segment corresponded to the analysed fNIRS segment, which was assumed to mark the hemodynamic response function onset predicting the UCS. Even if we changed the analysed SCR segment in this way, the negative correlation between the difference score of late and early SCR responses and the cluster activity during the

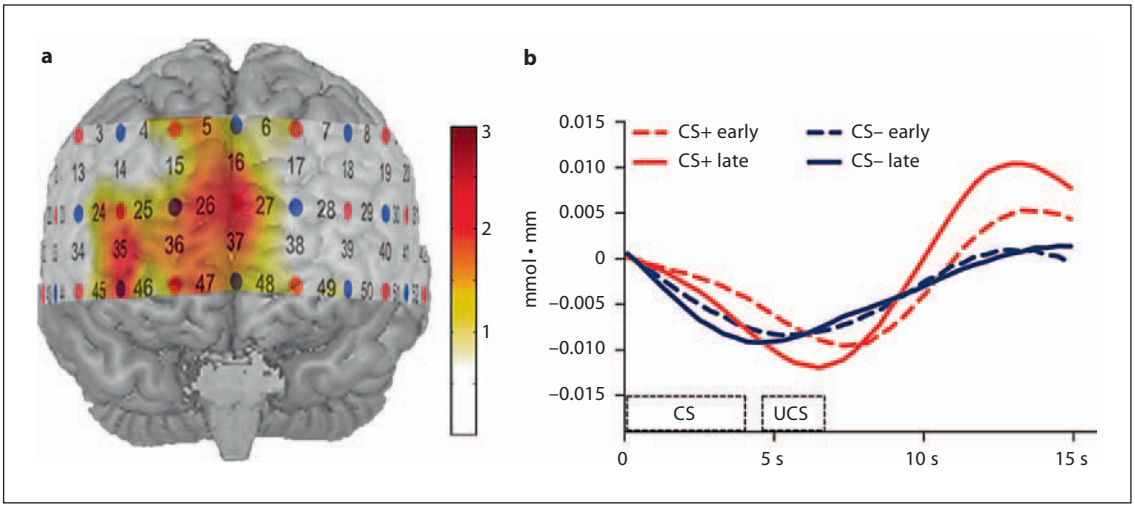

Fig. 5. a T-map superimposed on a standard brain. The comparison 'CS+ late vs. CS+ early' revealed significant O_2Hb concentration changes in the mPFC cluster at a peak time of 6.5 s after CS+ offset. **b** Time series of all regressors.

late extinction phase remained significant (r = –0.324; p = 0.035). The exploratory analyses concerning the recurrence of the UCS revealed that only the second out of three expectancy ratings correlated significantly with SCR to CS+ trials within the early (r = 0.37, p = 0.037) and late extinction phase (r = 0.4, p = 0.022), suggesting that subjects who subjectively tend to resist to extinction learning show appropriately higher SCR values during the extinction phases and herewith demonstrate less well extinction learning than participants who did report more certainty towards the disappearance of the UCS.

Discussion

In the present study, 34 healthy subjects underwent a fear conditioning paradigm with two neutral faces as CS and a loud, aversive scream as UCS to examine the time course of extinction learning by analysing concentration changes in O_2Hb across early and late extinction trials. SCR and valence as well as arousal ratings were assessed to ensure successful conditioning.

We found significantly different valence and arousal ratings for CS+ and CS– trials as well as SCR data after the habituation phase. CS+ presentations evoked lower valence ratings and appropriately higher arousal ratings for CS+ than CS– as well as higher SCR amplitudes. During the extinction phase, fNIRS data displayed a significant increase in response to CS+ trials from early to late extinction within one cluster of 10 probe set channels covering the mPFC. The cluster activity elicited by CS+ trials further exceeded CS– trials during late extinction while β values for CS– showed no significant difference across both extinction phases. To our knowledge, this is the first study investigating fear extinction by using fNIRS. This optical imaging method is restricted to the cortical surface and therefore cannot directly be compared to methods with higher spatial resolution, e.g. fMRI. Nonetheless, our findings are in accordance with previous imaging results confirming an mPFC contribution during within-session extinction [e.g. 25, 26, 36].

In order to critically review our results, we have to mention some inconsistencies of the data and will follow to discuss these aspects in light of the current literature. First one might argue that valence and arousal ratings as well as SCR data did not reflect successful extinction learning. We did find strong conditioning effects for all variables, but irrespective of the UCS absence during the extinction phase, participants continued to rate the former CS+ as significantly more unpleasant and more arousing than the CS–. However, there are other studies that found this kind of resistance to extinction in verbal reports [e.g. 25–28], and besides, UCS expectancy ratings showed a constantly decreasing expectancy across the extinction phase, reflecting that our participants did unlearn the CS-UCS association. Differential verbal ratings for CS+ and CS– might reflect lasting aversiveness of the UCS that prolonged throughout extinction trials, but on

the physiological level we do see an altered fear processing. SCR levels during early and late extinction decreased significantly from acquisition and further reached the habituation level as it is defined for successful extinction learning. Above all, at the rate of extinction and acquisition the number of trials is comparable to former studies [23, 27] or even contains a higher number of extinction trials [25–28].

Another constraint of our study is related to the mPFC activity we associated with extinction learning. A recently published review by Etkin et al. [51] argued that mPFC activity during extinction learning might reflect remnants of fear conditioning because studies on fear appraisal and sympathetic arousal also found mPFC engagement while generating fear responses. If mPFC activity would indeed reflect an explicit threat evaluation, one would have expected a decrease in activity from early to late trials contrarily to the increasing mPFC activity we found in the present study. Beta values for CS+ and CS– also started on an equal level during early extinction, we thus argue that mPFC activity in our study reflects extinction learning rather than a fear response. This is in line with the already explained successful induction of extinction, immanent in our UCS expectancy ratings and SCR data. Moreover, correlations between SCR and O_2Hb values emphasise the expected top-down control executed by the mPFC as subjects who exhibited a greater SCR decrement from early to late extinction phase also revealed higher β values and thereby more activity in the mPFC during the late CS+ condition. A study investigating fear conditioning as a form of prediction error learning does also confirm our assumption. Spoormaker et al. [52] examined CS+ trials in which no UCS was administered and found increased activity in ventromedial, dorsolateral and orbitofrontal regions as neuronal correlates of this so-called negative prediction error. The absence of negative consequences therefore seems to be associated with prefrontal engagement that would also fit explanations of fear extinction [52]. This might also explain the timing of our hemodynamic response function. The temporal gap between CS+ and UCS presentations enabled us to examine the onset of the expected neuronal response towards the anticipated UCS during the extinction phase rather than the CS onset. The mentioned negative prediction error is existent if the UCS did not occur against one's expectation. This mPFC-coupled learning process could only start in the absence of the UCS and not in the beginning of the CS presentation. Linnman et al. [53] investigated neuronal responses on shock delivery in a fear conditioning paradigm and found increased engagement of the dorsal anterior cingulate cortex, a region corresponding to the mPFC, during the non-delivery of an expected UCS. This finding fits our results as well, although it restricts comparability to available studies on fear extinction that did not provide such temporal information about CS and UCS. Future studies taking these differences into account would certainly contribute to a better understanding of temporal interactions such as the functional mPFC-amygdala coupling.

One major limitation of our study is the restricted application of fNIRS during the extinction phase. We discussed our result of significant mPFC activity as successful extinction learning, but in fact we cannot strictly obviate mPFC contribution during the acquisition phase. Numerous animal studies highlighted prefrontal contribution during extinction learning, i.e. when the CR is already acquired. Moreover, a systematic review about neuroimaging literature on human fear conditioning by Sehlmeyer et al. [12] did not find support for mPFC involvement during fear conditioning. Thus, it appears reasonable to restrict our fNIRS measurement exclusively to the extinction phase.

Secondly, we have to admit that we did not investigate mPFC activity during extinction retention, i.e. 24 h after the initial fear conditioning. The present study was not intended to compare mPFC engagement on the acquisition and recall of extinction. We well know that the ventromedial prefrontal cortex frequently found in animal studies is involved in recall of extinction rather than the initial acquisition [16], on the other hand there are the aforementioned studies in humans that found mPFC activity already during within-session extinction. It might be possible that long-term storage of extinction memory is supported in other for example more dorsal situated brain regions as Gottfried and Dolan [24] already speculated. Future studies therefore have to consider the consolidation of extinction memory by implementing a second extinction training after a delay period.

A third limitation relates to context changes which might have occurred through attaching the fNIRS probe set. In this connection we have to consider context dependency of extinction learning suggesting a return of fear by presenting the formerly CS+ again in the initial context, i.e. without the fNIRS probe set. We would again like to stress the fact that we kept all other parameters constant during the short break for attaching the probe set to minimize context effects. However, future studies using fNIRS for investigations of prefrontal activation during extinction learning could overcome this limitation by implementing shorter fear conditioning paradigms to assess all experimental phases.

Conclusions

The present study revealed increasing mPFC activation to CS+ trials during extinction that was different from that for a CS– which displayed no change across early and late extinction learning. Based on these findings, we propose mPFC activity during extinction learning to reflect better regulation of CR expression. The increase of prefrontal contribution from early to late extinction trials seems to be associated with changes in the associative significance of CS+ and UCS. Increasing associative strength might thereby rely on amygdala activity, decreasing associative strength appears to be inversely correlated with mPFC activity. Patients suffering from anxiety disorders or even high trait-anxious subjects have been characterized by increased CR to threat cues and reduced extinction [54]. Thus, they show deficient associative learning and accordingly deficient recruitment of amygdala and mPFC [26, 28].

Future studies have to examine cortical-subcortical interactions in more detail to ascertain strategies to affect mPFC activity in the treatment of anxiety disorders. Combined methods such as fNIRS and fMRI would further provide complementary results [44] by offering both high temporal as well as high spatial resolution. Here, a more precise definition of mPFC subregions involved in extinction learning might open up prospects to strengthen prefrontal areas, and transcranial magnetic stimulation could for instance be such a tool [55]. Transcranial magnetic stimulation is as restricted to the cortical surface as fNIRS. The use of fNIRS for mapping the prefrontal cortex is therefore not contradictory by searching innovative treatment options for facilitating extinction learning or even exposure therapy.

Acknowledgments

The authors would like to thank Juliana Rost for her support in participant recruiting and data acquisition. We also thank Michael M. Plichta for his ideas in data analyses and modelling, Evelyn Glotzbach who helped us with analysing the SCR data as well as Wilma Harnisch for calibrating our sound system.

This publication was funded by the German Research Foundation (DFG, SFB TRR 58, C04 project). The funding source had no role in study design, data collection and analyses, decision to publish, or preparation of the manuscript.

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Supplementary Material

Original Paper (Copyright © 2012 by S. Karger AG, Basel)

Medial Prefrontal Cortex Activity during the Extinction of Conditioned Fear: An Investigation Using Functional Near-Infrared Spectroscopy

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Neuropsychobiology 2012;65:173-182 (DOI: 10.1159/000337002)

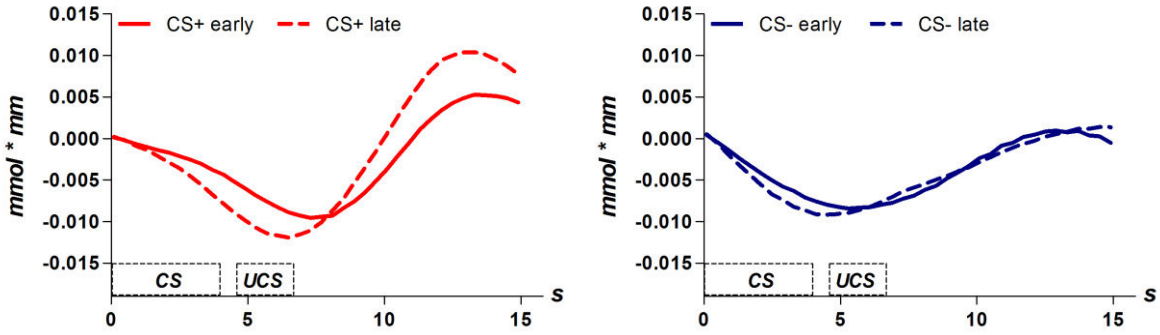

Figure 6. Time series of the four regressors, separated into CS+ and CS- trials.

2. The Neural Mechanisms Involved in the Failure of Fear Extinction

Returning to the story of *Little Albert*, Watson and Rayner (1920) noticed that "Unfortunately Albert was taken from the hospital the day the above tests were made. Hence the opportunity of building up an experimental technique by means of which we could remove the conditioned emotional responses was denied us." (p. 12). However, they formulated hypotheses on how to remove the CR, i.e. (1) confronting *Albert* with the feared objects until habituation would come in resembling a "fatigue of reflex" (p. 12), (2) insisting *Albert* to imitate "constructive activities around the object" (p. 12) resembling Banduras concept of observational learning becoming famous 50 years later (Bandura, 1971) or (3) establishing a "recondition" (p. 12) by pairing the CS with candy or a "simultaneous stimulation of erogenous zones" (p. 12) - a comment that was probably formulated with a wink to Sigmund Freud's essays on the theory of sexuality that got much attention at the same time and were clearly striking the behavioristic concept of conditioned emotional responses.

Nowadays it is widely accepted that extinction does not simply comprise the "fatigue of [a] reflex" but rather the acquisition of a second meaning associated with the CS, i.e. a CS-noUCS association, which is competing with the original CS-UCS memory trace (Bouton, 2002). Deficient extinction learning has been argued to underlie the persistence of fear in pathological anxiety[1] (e.g. Graham & Milad, 2011). The following section will thus review the recent understanding of pathological anxiety by summarizing the evidence of animal and human research. Deficient extinction retrieval is thought to explain the persistence of anxiety disorders and might further account for the fact that approximately a quarter of the patients do not respond properly to pharmaceutical or psychotherapeutic treatments although the applied extinction procedures represent the gold standard in the treatment of

[1] Throughout this dissertation, the expressions 'pathological anxiety' and 'pathological fear' are used synonymously. Several lines of research, however, indicate the discrimination between a more long-lasting state of sustained fear referred to as 'anxiety' and a phasic component in response to a specific threat referred to as 'fear'. Both responses have been evidenced to involve different limbic structures, i.e. phasic fear was associated with amygdala activity while sustained fear (anxiety) was associated with the BNST (see Davis, Walker, Miles, & Grillon, 2010 for a review). This conceptual distinction between fear and anxiety is beyond the scope of this dissertation. Nevertheless, it can be stated that the reviewed literature on pathological anxiety herein focuses on fear-related disorders such as post-traumatic stress disorder, phobias as well as panic disorder rather than anxiety-related disorders such as generalized anxiety disorder.

anxiety disorders (Foa, 2000; Scholten et al., 2013). In combination with the increased understanding of the neurobiological circuits being engaged in extinction learning, noninvasive brain stimulation techniques will further be introduced as a promising tool for priming and augmenting psychotherapy (Bajbouj & Padberg, 2014).

2.1 Pathological Anxiety

There is considerable evidence that the mechanisms of fear and extinction learning are preserved across species (cf. Milad, Rauch, Pitman, & Quirk, 2006). The aforementioned fear conditioning animal studies thus guided the search for functional dysregulations within limbic and frontal regions that could account for the inappropriate and persistent fear responses found in patients suffering from anxiety disorders. In this regard, fear conditioning mostly resembles post-traumatic stress disorder (PTSD, Pitman, 1997) insofar that the experience of intense fear (formerly unconditioned response, UCR) during the trauma is associated with cues or stimuli (CS, formerly neutral) in the traumatic environment which later elicit a CR. The PTSD symptoms namely (1) the persistent re-experience of the trauma (intrusion), (2) the avoidance of trauma-reminders, (3) negative alterations in mood and cognition, and (4) an increased arousal and reactivity (American Psychiatric Association, 2013) are assumed to reflect an inability to extinguish an acquired conditioned fear response (c.f. Milad et al., 2006). Concerning group differences between traumatized persons with and without PTSD symptoms, during fear acquisition an increased conditionability was reported to manifest in a higher heart rate and SCR to CS+ versus CS- trials (Orr et al., 2000), greater fear-potentiated startle responses (FPS, Norrholm et al., 2011) and an increased attention bias toward threat stimuli during fear acquisition as well as during extinction (Fani et al., 2012). This heightened “bottom-up” impact of the amygdala seems to be further evidenced by the elevated fear responding to safety cues (CS-) in anxiety disorders (Lissek et al., 2005), for instance in PTSD patients compared to trauma controls (e.g. Glover et al., 2011; Norrholm et al., 2011) and in panic disorder (PD) patients compared to healthy samples (e.g. Lissek et al., 2009; Lueken et al., 2013). However, a recently published up-dated meta-analysis of classical fear conditioning studies in anxiety disorders did not support differences between patients and healthy controls in fear acquisition (Duits et al., 2015). The existing findings more consistently refer to the failure to extinguish fear, i.e. PTSD patients but similarly PD patients (e.g. Michael, Blechert, Vriends, Margraf, & Wilhelm, 2007)

continue to show a higher differential fear response during extinction (Fani et al., 2012; Norrholm et al., 2011; Orr et al., 2000; Wessa & Flor, 2007). Interestingly, results of a twin study suggest the deficits in extinction recall to represent an acquired PTSD sign rather than a vulnerability factor increasing the risk of PTSD after trauma experience (monozygotic twins exposed vs. non-exposed to vietnam combat with vs. without PTSD, Milad et al., 2008).

Referring to the involved brain circuits the psychopathology is thus assumed to be either related to hyper-responsiveness of the amygdala ("bottom-up") to threat cues or a deficient inhibitory influence of the mPFC ("top-down") or both. In a two-day discriminative fear conditioning and extinction paradigm Milad et al. (2008; 2009) exhibited no SCR differences between PTSD patients and trauma-exposed non-PTSD controls during acquisition and extinction learning, though the extinction recall on day two was impaired. According to previously mentioned studies in healthy volunteers (cf. Milad et al., 2005; Milad et al., 2007; Phelps, Delgado, Nearing, & LeDoux, 2004) PTSD patients were found to show decreased activation in vmPFC and hippocampus as well as increased dACC activity during the early phase of an extinction recall test (Milad et al., 2009). However, due to the variability of studies and materials the results are not as consistent. Bremner et al. (2005) for instance showed increased amygdala activation in PTSD patients compared to healthy controls even during fear acquisition as well as decreased orbitofrontal and mPFC activation during extinction learning as against the recall of extinction (cf. Milad et al., 2008; 2009), the latter was inversely correlated with anxiety symptoms.

Besides the fear conditioning paradigm, a meta-analysis on symptom provocation studies in anxiety disorders further underline the hyper-responsiveness hypothesis of the amygdala as well as the insula[2] in patients suffering from PTSD, social and specific phobia compared to healthy controls (Etkin & Wager, 2007). In patients with PD and agoraphobia we similarly found hyper-responsiveness of these two regions when anticipating agoraphobia specific pictures (Wittmann, Schlagenhauf, John, Guhn, et al., 2011), even though the increased activity of the amygdala failed statistical significance in a larger sample and in comparison to healthy controls (Wittmann, Schlagenhauf, Guhn, et al., 2014). Based on the animal fear conditioning results reviewed above Gorman et al. (Gorman, Kent, Sullivan, & Coplan, 2000) established a neurocircuitry model for PD centered on the

[2] The insulae belong to the cerebral cortex and have been found to exert diverse rolls in emotions. Notably the anterior portion of the insula has been implicated in the anticipation and perception of pain of shock during a fear conditioning study focusing on the neural basis of the UCR (Linnman, Rougemont-Bucking, Beucke, Zeffiro, & Milad, 2011).

functional coupling between the amygdala and the mPFC. In a comprehensive literature search, we thus investigated the published neuroimaging studies on PD since 2000 in the light of the core regions of this model (Dresler, Guhn, et al., 2013): The most consistent findings arise from case studies of spontaneous panic attacks in which hyper-responsiveness of the amygdala has been found to occur in concert with a decreased frontal activity (e.g. Dresler et al., 2011). According to the fear conditioning model these findings are of particular interest since panic attacks once initiated the PD (CR), are assumed to be triggered through either internal or external stimuli (CS). On the basis of the literature consensus we concluded Gorman's neuroanatomical hypothesis to still represent an appropriate neurocircuitry model with special regard to the central involvement of the amygdala, even though the hypofrontality hypothesis was rather scarcely supported probably due to the large methodological variability (Dresler, Guhn, et al., 2013).

Further evidence to prove the mPFC-amygdala coupling arises from treatment studies since different treatment strategies have been supposed to act on different brain levels, i.e. pharmacotherapy affecting the amygdala and the brain stem whereas cognitive-behavioral therapy (CBT) acting on the regulatory mPFC regions (Gorman et al., 2000). Of particular interest for the purpose of this dissertation thereby is exposure therapy, which is usually one integrated component of the treatment with CBT. Exposure therapy incorporates extinction procedures since patients are exposed to trauma-associated cues or situations (CS) in the absence of negative reinforcement comparable to fear extinction procedures used in the laboratory. After several exposure sessions, successfully treated patients are supposed to have consolidated a CS-noUCS memory that can be retrieved subsequently by re-confrontation with the CS thereby gradually reducing the patient's fear of a specific cue over time. Accordingly, PTSD patients revealed reduced reactivity of the autonomous nervous system to traumatic cues (CS) after a trauma-focused CBT compared to waitlist conditions as assessed by heart rate reactivity, systolic blood pressure and electromyogram (Zantvoord, Diehle, & Lindauer, 2013). However, the findings on functional changes after treatment with regard to the hypothesized regions, namely the amygdala and the mPFC, are not as consistent. The studies by Peres et al. (2007) and by Goldin et al. (2013) argue for the mPFC-amygdala coupling since PTSD and social anxiety disorder (SAD) patients revealed an increased mPFC activity and decreased amygdala activity with symptom improvements correlating with higher PFC and less amygdala activity after trauma-focused

CBT (PTSD, Peres et al., 2007) and CBT focusing on cognitive reappraisal of negative self-beliefs (SAD, Goldin et al., 2013). For specific phobia and PD, on the other hand, the findings more strongly point towards the hypothesis of a normalized activity of a hyper-responsive amygdala from pre- to post-treatment, without evidencing the supposed causal increase of the mPFC top-down activity (Goossens, Sunaert, Peeters, Griez, & Schruers, 2007; Kircher et al., 2013). However, the small number of neuroimaging studies did not allow drawing conclusions on whether pathological fear originates from hyper-responsiveness of the amygdala or hypo-responsiveness of the mPFC (Milad et al., 2006; Zantvoord et al., 2013). Either way, striking findings in the understanding of the mPFC in animals inspired the idea of facilitating the inhibitory influence of the mPFC for controlling an overactive amygdala.

2.2 From Rodents to Humans: Prefrontal Cortex Stimulation

Evidence for the enhancement of mPFC neurons in order to exert an increased top-down control over the amygdala during fear originates from translational research in rodents. In 2002, Milad and Quirk published a set of experiments which consistently evidenced the mPFC to signal extinction memory. They demonstrated IL neurons to fire to a tone CS exclusively when rats were recalling extinction. A time-locked electrical stimulation of these IL neurons in rats not having undergone extinction training likewise elicits extinction effects, i.e. rats treated in this way showed less freezing behavior (CR). The authors thus assumed microstimulation to resemble extinction-induced IL tone responses that simulate extinction memory, an assumption which was further supported by the finding that stimulated rats even exhibited a facilitated extinction recall 24 hours after the stimulation (Milad & Quirk, 2002). Since 2002, the effect of high-frequency microstimulation on fear extinction in rodents has been replicated several times, thereby (1) specifying the time window for stimulation to start at CS onset extending to less than 1 s of the CS presentation time (Milad, Vidal-Gonzalez, & Quirk, 2004), (2) defining the contingency between CS and IL stimulation to be important since an unpaired stimulation did not differ from no stimulation (Baek, Chae, & Jeong, 2012; Kim, Jo, Kim, Kim, & Choi, 2010) and (3) estimating the necessity of stimulating IL neurons versus microstimulation of PL neurons which evoked the opposite effect (Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006). The mechanism of IL microstimulation could have been further identified to cause an inhibition of Ce output neurons (Quirk, Likhtik, Pelletier, & Pare, 2003), via the activation of γ-aminobutyric acid

(GABA) receptors at ITC cells that receive direct mPFC inputs and project to the Ce (Likhtik, Pelletier, Paz, & Pare, 2005).

In order to bring the results on electrical microstimulation together with the findings on increased amygdala and decreased mPFC activity found for PTSD as well as other anxiety disorders outlined above - though not consistently – transcranial magnetic stimulation (TMS) has been suggested to represent a potential technique for the likewise treatment of pathological fear in humans (Milad & Quirk, 2002; Milad et al., 2006). In a double-blind placebo-controlled study, PTSD patients undergoing 10 daily treatment sessions with high-frequent repetitive TMS over the right dlPFC (10 Hz, 80 % motor threshold) relative to a 1 Hz and a placebo stimulation exhibited therapeutic effects regarding improvements in PTSD symptom severity, anxiety as well as depression scores that remained stable for at least two weeks after the last treatment session (Cohen et al., 2004). Referring to a recently published meta-analysis, TMS has proven its effectiveness in the treatment of PTSD, however, there is no clear consensus with regard to the setting for optimally applying TMS, the region of stimulation or the protocols that should be used since low and high-frequency stimulation has been shown to exert beneficial but controversial results (Karsen, Watts, & Holtzheimer, 2014). With regard to the rodent experiments in which IL stimulation was only effective when paired with an extinction training of the CS (Baek et al., 2012; Kim et al., 2010), the combination of TMS with trauma-related stimuli (CS) might be more advantageous (Baek et al., 2012). In fact, repetitive TMS (rTMS) applied to the dlPFC in combination with imaginal exposure versus placebo stimulation leaded to a decrement in hyperarousal symptoms and hormonal serum changes in nine refractory PTSD patients (Osuch et al., 2009). In order to study rTMS as potential adjunct to augment the extinction memory formed during exposure therapy, the following study investigated the combination of rTMS and fear extinction on extinction recall in healthy probands for the first time. Before presenting this study, the fundamentals of TMS will be explained in more depths and FPS will be introduced as another dependent variable to measure the conditioned fear response.

2.2.1 Excursus: Transcranial Magnetic Stimulation (TMS)

TMS is a non-invasive method delivering an electric field through the intact scalp by operating with a brief high-current magnetic pulse that is produced in a coil of wire (Hallett, 2000). By passing a rapidly changing current, the intense localized magnetic field outside the

scalp produces an electric current in the brain region under the magnetic coil following Faraday's principles of electromagnetic induction. The induced electrical current produces a depolarization of cellular membranes thereby forcing target neurons to fire action potentials. Depending on the utilized stimulators and coils cortical neurons at a depth of 1.5 to 2 cm beneath the scalp can be reached in this way (Epstein, Schwartzberg, Davey, & Sudderth, 1990). A deeper insight into the physical principles of TMS is provided by Siebner and Ziemann (2007).

TMS can be delivered in single pulses or in trains of TMS pulses referred to as repetitive TMS (rTMS) modulating brain activity beyond the time of stimulation (Hallett, 2000). Electrophysiological studies revealed rTMS induced changes in cortical excitability typically expressed as an increased or decreased size of motor-evoked potentials (MEP) depending on the stimulation protocol (Pascual-Leone et al., 1998). Although TMS cannot reach subcortical brain structures directly, neuroimaging studies point to neuronal responses of TMS that are not only restricted to the stimulation site but also exhibit wide-spread activations even of contralateral homologues cortical areas (e.g. Ilmoniemi et al., 1997), owing to the initial action potential of cortical neurons that can spread transsynaptically through entire neural circuits (Pascual-Leone et al., 1998). However, this not only complicates finding the right stimulation site, a variety of stimulation parameters has to be considered such as the intensity and frequency of stimulation, the number of pulses per train as well as pulses in total, the number of trains per session and the inter train intervals and furthermore the question whether the effects were evaluated in terms of a valid control condition (Fitzgerald, Brown, & Daskalakis, 2002). Thus, the existing results are quiet heterogeneous and at present prohibiting to draw conclusions concerning the treatment of anxiety disorders (Lefaucheur et al., 2014; Zwanzger, Fallgatter, Zavorotnyy, & Padberg, 2009). However, in the field of depression TMS has already been approved by the US FDA for treatment-refractory patients in 2008 and the German Institute of Medical Documentation included rTMS in the guidelines for good clinical practice in 2014. The enhancement of fear extinction memory in the laboratory and within healthy participants was claimed as a first step in order to subsequently consider rTMS as potential adjunct to psychotherapy also in anxiety patients (Marin, Camprodon, Dougherty, & Milad, 2014). The following study was intended to follow this idea, thereby trying to guide future directions concerning the

standardization of the above mentioned parameters for clinical trials comprising exposure plus rTMS interventions for the treatment of pathological anxiety.

It is rather unresolved by which mechanism rTMS influences brain activity, however there is converging evidence that the long-lasting effects of rTMS originate from altered synaptic strengths (synaptic plasticity) through processes like LTP and long-term depression (LTD, Hallett, 2000), i.e. high rTMS frequencies above 5 Hz have been shown to induce LTP whereas low frequencies (≤ 1 Hz) induce LTD (cf. Hoogendam, Ramakers, & Di Lazzaro, 2010). Keeping in mind that memory formation is likewise built through LTP, the combination of rTMS and extinction learning (or in a therapeutic way: exposure session) might result in a summation of synaptic plasticity that might compensate for a deficient extinction memory formation in pathological anxiety.

Concerning the usage of rTMS in clinical and experimental settings an international workshop on its risk and safety reached consensus on ethical and safety guidelines advising to be attentive to contraindications for people with (1) metal anywhere in the head excluding the mouth, (2) cardiac pacemakers and implanted medication pumps, (3) intracardiac lines such as a serious heart disease or an increased intracranial pressure cause of an increased risk in the event of a seizure, (4) pregnancy, (5) usage of tricyclic anti-depressants, neuroleptic agents and other drugs that lower the seizure threshold and (6) a family history of epilepsy. (7) Children are further not recommended to get treated with TMS (Wassermann, 1998). In accordance with these guidelines a review on adverse effects in rTMS studies with stimulation of non-motor cortical areas concluded rTMS to be “very safe” (p. 468) with headaches representing the most common adverse effect following frontal stimulation although headache has also been induced by placebo-stimulation making it thus difficult to establish a direct relationship between rTMS and headache (Machii, Cohen, Ramos-Estebanez, & Pascual-Leone, 2006).

2.2.2 Excursus: Fear-Potentiated Startle Response (FPS)

The FPS response is a short-latency defensive reflex consisting of an eyelid-closure and a contraction of facial and skeleton muscles that can be elicited in all mammals (Landis & Hunt, 1939 as cited in Davis, Walker, & Lee, 1997). FPS responses have been shown to be augmented in the presence of aversive stimuli including fear-conditioned stimuli (Hamm & Vaitl, 1996) or unpleasant pictures while they are attenuated in the context of positive

stimuli (Lang, Bradley, & Cuthbert, 1990) thereby highlighting its potential as an index of fear for the usage in translational research. Typically measured with surface electromyographic (EMG) electrodes in human startle blink research, even weak contractions of the orbicularis oculi muscle can be recorded that occur with a latency of about 10 ms.

Interestingly, in comparison to non-PTSD Vietnam combats PTSD patients were found to show elevated FPS responses anticipating the administration of an electric shock even when the shock electrodes were not attached. This finding was assumed to reflect an anxiogenic response to an unpredictable stressful environment (Grillon, Morgan, Davis, & Southwick, 1998) representing a specific psychophysiological correlate of PTSD and also PD as against generalized anxiety disorder (GAD, Grillon et al., 2008). Since FPS responses has been further shown to provide a more sensitive measure of heightened fear responses than did SCR (at least in PTSD patients, Glover et al., 2011), the following manuscript aimed at investigating both measures as indexes for fear responses. Methodological issues concerning response elicitation, recording, quantification and reporting was oriented on the guidelines for human startle eyeblink studies (Blumenthal et al., 2005).

2.3 Study 2: TMS Effects on Conditioned Fear

The aim of the present study was to investigate the impact of high-frequency rTMS (10 Hz) on the processing of conditioned fear. With regard to the inconsistent results on high- and low-frequency stimulation in the treatment of anxiety disorders, the administration of high-frequency rTMS was favored for the following reasons: first, it was comparable to the 10 Hz frequency Milad and Quirk used in 2002; second, it is in line with the assumption of induced excitability of cortical neurons since the stimulation was intended to increase the inhibitory mPFC influence; and third, it follows the recommendation of high-frequency stimulation that has been claimed for PTSD treatment on the basis of the currently existing attempts (cf. Lefaucheur et al., 2014; Paes et al., 2011). With regard to the uncertainty of the proper stimulation site, the present study was oriented on the results of the previous study in which a prefrontal cluster depicting increased mPFC activation during extinction learning was detected by the usage of fNIRS (study 1).

Compared to a sham stimulated control group, active stimulation was expected to diminish CR expression during extinction learning and extinction recall due to increased mPFC activation. In order to verify an rTMS influence on several levels, different dependent

variables indexing conditioned fear were implemented, i.e. skin conductance response (SCR) and fear-potentiated startle response (FPS) as psychophysiological measures, fNIRS as an index of neural activity as well as self-reports representing learning on a conscious level. The results have been first published in *Frontiers in Behavioral Neuroscience* in the here presented form.

frontiers in
BEHAVIORAL NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 18 February 2014
doi: 10.3389/fnbeh.2014.00044

Medial prefrontal cortex stimulation modulates the processing of conditioned fear

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The extinction of conditioned fear depends on an efficient interplay between the amygdala and the medial prefrontal cortex (mPFC). In rats, high-frequency electrical mPFC stimulation has been shown to improve extinction by means of a reduction of amygdala activity. However, so far it is unclear whether stimulation of homologues regions in humans might have similar beneficial effects. Healthy volunteers received one session of either active or sham repetitive transcranial magnetic stimulation (rTMS) covering the mPFC while undergoing a 2-day fear conditioning and extinction paradigm. Repetitive TMS was applied offline after fear acquisition in which one of two faces (CS+ but not CS−) was associated with an aversive scream (UCS). Immediate extinction learning (day 1) and extinction recall (day 2) were conducted without UCS delivery. Conditioned responses (CR) were assessed in a multimodal approach using fear-potentiated startle (FPS), skin conductance responses (SCR), functional near-infrared spectroscopy (fNIRS), and self-report scales. Consistent with the hypothesis of a modulated processing of conditioned fear after high-frequency rTMS, the active group showed a reduced CS+/CS− discrimination during extinction learning as evident in FPS as well as in SCR and arousal ratings. FPS responses to CS+ further showed a linear decrement throughout both extinction sessions. This study describes the first experimental approach of influencing conditioned fear by using rTMS and can thus be a basis for future studies investigating a complementation of mPFC stimulation to cognitive behavioral therapy (CBT).

Keywords: fear conditioning, memory consolidation and extinction, learning, transcranial magnetic stimulation (TMS), medial prefrontal cortex (mPFC)

INTRODUCTION

The extinction of conditioned fear describes the decrement of conditioned responses (CR) after repeatedly presenting a formerly conditioned stimulus (CS) that no longer predicts an unconditioned stimulus (UCS). Extinction learning, memory consolidation and recall of extinction memory have been found to represent different stages of the extinction process, which is also supported by a distinct cortico-limbic functionality (Quirk and Mueller, 2008). At the beginning of the extinction learning, the amygdala shows a profound activation increase to the CS which decreases throughout extinction learning while ventro medial prefrontal cortex (vmPFC) activation meanwhile increases. This reversed amygdala-vmPFC correlation has been shown to reduce the expression of the conditioned fear response. Heightened vmPFC activation thereby inhibits the amygdala's expression of fear during successful extinction recall, i.e., when the already consolidated extinction memory is retrieved (Etkin et al., 2011; Linnman et al., 2012). VmPFC contribution thus appears to be a precondition for sufficient consolidation and later recall extinction memory in animals (Quirk and Mueller, 2008) as well as in humans (Phelps et al., 2004; Kalisch et al., 2006).

Due to homologous prefrontal structures in the rodent and human brain (Milad and Quirk, 2012), results obtained from fear-conditioned animals can be transferred to fear modulation in humans. This is of interest since deficient fear modulation is seen in patients suffering from anxiety disorders (e.g., see Bremner et al., 2005; Milad et al., 2009). A meta-analysis verified that patients with anxiety disorders generally show stronger CR during extinction relative to healthy controls (Lissek et al., 2005). This appears to be caused by a failure of consolidating and recalling extinction memory that most likely originates from a mPFC dysfunction (Rauch et al., 2006; Etkin, 2012).

Since exposure therapy as an effective treatment for anxiety disorders (Foa, 2006) represents the implementation of extinction, it is of clinical relevance to improve extinction learning and extinction memory consolidation. In this regard, manipulations of memory consolidation processes have been established in cross-species translational research. Pharmacologically, D-cycloserine (DCS), a partial N-methyl-D-aspartic acid (NMDA) agonist, has been shown to facilitate fear extinction in rats (Walker et al., 2002; Ledgerwood et al., 2005), which initiated the usage of DCS to augment exposure therapy in patients with

anxiety disorders (e.g., Ressler et al., 2004). Acute DCS administration during symptom provocation has been shown to increase prefrontal cortex activity in phobic patients (Aupperle et al., 2009) confirming the reported mPFC dysfunction in anxiety disorders. However, the additional beneficial effects of DCS are rather small when provided in combination with an effective treatment such as cognitive behavioral therapy (CBT; Siegmund et al., 2011) Thus, DCS is suggested to be exclusively indicated for treating severely impaired patients (Siegmund et al., 2011; Klumpers et al., 2012). Moreover, experimental conditioning studies in healthy volunteers failed to show benefits of DCS on extinction learning or extinction recall (Guastella et al., 2007; Klumpers et al., 2012) thereby contradicting the above mentioned animal results (e.g., Walker et al., 2002). A different strategy to improve fear extinction is to electrically stimulate prefrontal regions involved in extinction memory consolidation. In this regard, Milad and Quirk (2002) demonstrated a facilitated extinction in rats that underwent high-frequency stimulation of the infralimbic cortex (IL)—the homolog of the vmPFC in the rat brain. Compared to non-stimulated controls, these rats showed immediate CR attenuation during extinction learning, which persisted to an extinction recall test conducted 24 h later (see also Kim et al., 2010). This inhibitory effect of IL stimulation was ascribed to a reduced responsiveness of output neurons in the central amygdala (Quirk et al., 2003). Thus, electrical stimulation of mPFC structures in rats facilitated extinction learning and extinction recall. So far, it is unclear whether stimulation of homologous regions in humans could have likewise beneficial effects. In this regard, transcranial magnetic stimulation (TMS) represents a suitable method for the translation from animal to human studies (Etkin, 2012).

TMS is a non-invasive technique for stimulating the human cerebral cortex using a brief high-current pulse applied via an electromagnetic coil placed above the scalp (Hallett, 2000). Depending on the stimulation parameters the produced magnetic field can either inhibit (<1 Hz) or excite (>5 Hz) a focal cortical area, most likely by inducing changes in synaptic plasticity linked to learning and memory (Hoogendam et al., 2010). TMS in its repetitive form (rTMS) is able to produce effects beyond the time of stimulation and exceeding the targeted area (Ilmoniemi et al., 1997; Guse et al., 2010). Baeken et al. (2010) investigated one session of 10 Hz rTMS applied to the right dorsolateral prefrontal cortex (dlPFC) in healthy volunteers while passively viewing emotional faces. They found a significant attenuation of right amygdala activation when evaluating negatively valenced stimuli. The use of rTMS as a method to facilitate extinction has been already proposed a decade ago (Milad and Quirk, 2002), but was not accomplished so far.

The aim of the present study was to investigate whether high-frequency rTMS (10 Hz) can modulate the processing of conditioned fear. Based on the results of a previous study in which mPFC contribution during extinction learning was measured with functional near-infrared spectroscopy (fNIRS), a prefrontal cluster depicting increased mPFC activation during extinction learning was targeted (Guhn et al., 2012). Compared to a sham stimulated control group, active stimulation was expected to diminish CR expression during extinction learning and extinction recall due to an increased mPFC activation. In order to verify a rTMS influence on several levels, we implemented different dependent variables indexing conditioned fear, i.e., fear-potentiated startle (FPS) and skin conductance responses (SCR) as psychophysiological measures, fNIRS as an index of neural activity as well as self-reports representing learning on a conscious level. The results of this study could be the basis for investigating the adjunct impact of rTMS to CBT in patients with anxiety disorders.

MATERIALS AND METHODS

SUBJECTS

Eighty-eight healthy, TMS-naïve volunteers (43 men) were recruited from a large sample collected at a Collaborative Research Center (SFB-TRR 58) of the Universities in Münster, Würzburg and Hamburg, Germany, as well as internet announcements. They were screened for current mental health and right-handedness by using the Mini International Neuropsychiatric Interview (M.I.N.I., Sheehan et al., 1998) and the Edinburgh Inventory (Oldfield, 1971). All female volunteers were additionally screened for a regular menstrual cycle and the non-usage of any hormonal contraceptives for at least 3 months prior to measurement. In order to account for facilitating effects of estrogen on extinction learning (Glover et al., 2012) and extinction recall (Milad et al., 2010; Zeidan et al., 2011), women only participated in the experiment during their early follicular phase (defined as the first 5 days of a 28-day cycle) when estradiol and progesterone levels are low. Contraindications regarding the TMS safety guidelines (Wassermann, 1998) such as epilepsy, use of pacemakers or pregnancy were assured. Participants gave written informed consent in accordance with the Declaration of Helsinki in its latest version from 2008. All procedures were approved by the ethics committee of the University of Würzburg.

Three female subjects dropped out due to the experience of discomfort while receiving TMS application and were thus not considered for further data analysis. Demographic data of the remaining $N = 85$ participants are presented in **Table 1**. None of the reported variables reached statistical significance for group comparisons between active and sham TMS (student's t-test, $p > 0.05$). Group differences can therefore be interpreted in terms of TMS effects.

DESIGN

The paradigm consisted of four phases divided into familiarization, fear acquisition and extinction learning on day 1 and a test for extinction recall on day 2 (see **Figure 1**). Two male neutral faces served as conditioned stimuli (CS; Tottenham et al., 2009) and an aversive scream of 95 dB served as unconditioned stimulus (UCS; IADS, Bradley and Lang, 1999). Volunteers were first familiarized with both CS by presenting each face eight times without the UCS. During the following fear acquisition phase consisting of 32 CS presentations one neutral face (CS+) was randomly followed by the UCS in 50% of trials whereas the other face (CS−) never preceded the UCS. Both extinction phases (day 1 and 2) consisted of 40 trials in total (20 CS+, 20 CS−) without UCS presentations. CS stimuli were presented for 6000 ms duration separated by jittered inter trial intervals (ITI) of 5000–8000 ms displaying a fixation cross. The UCS lasted

1380 ms and followed CS+ offset after a jittered temporal gap of 0–1000 ms (Guhn et al., 2012). The assignment of CS+ and CS− was counterbalanced across subjects and stimuli were presented in a pseudo-randomized order such that maximally three similar faces followed each other. Presentation® version 12.2 software (Neurobehavioral Systems, Inc., Albany, CA, USA) was used for presenting the paradigm.

REPETITIVE TRANSCRANIAL MAGNETIC STIMULATION (rTMS)

Following the fear acquisition phase, subjects received one offline session of either active or placebo (sham) rTMS prior to performing extinction learning on day 1. Stimulation was applied via a round coil (MMC-140 Parabolic) of a Medtronic MagPro X100 stimulator (Medtronic MagPro, Düsseldorf, Germany) to a cluster within the medial prefrontal cortex. The coil was positioned in the middle of the cluster which was identified by marking channel 26 of the NIRS probe set corresponding to the MNI coordinates $x = 14.5$, $y = 68.3$, $z = 21.3$ (according to http://www.jichi.ac.jp/brainlab/virtual_registration/Result3x11_E.html). This channel represents the center of the mPFC activation cluster for which we found an increase in oxygenated hemoglobin concentration over the time course of extinction learning in a prior study (Guhn et al., 2012). Inter-subject variance was considered by assigning Fpz according to the 10-20 EEG system (Jasper, 1958). Emanating from Fpz, channel 26 was marked resulting in slightly varying positions for TMS coil positioning based on the participants individual head sizes. The upper edge of the coil was tilted 2 cm away from the scalp in order not to stimulate the premotor cortex; the handle of the coil was pointed upwards. The rTMS protocol was adapted from Baeken et al. [2010; stimulation intensity of 110% of the individual resting motor threshold (RMT), 10 Hz stimulation frequency, 40 trains of 4 s duration (1560 pulses), inter train intervals of 26 s], who found an amygdala attenuation in response to negative stimuli after one rTMS session. For the present study, this protocol was selected corroborating the intention that it should impact the fear circuit in the same way, i.e., the proposed increased prefrontal top–down modulation of subcortical systems, in particular the amygdala. Sham rTMS was applied using a placebo coil (MC-P-B70 Placebo) which appeared similar in placement and acoustic properties to the active coil but had a magnetic shield embedded limiting the amount of the magnetic field. In order to control for the proposed facilitatory effects of active rTMS, fNIRS was used to monitor blood oxygenation as an index of functional brain activity in the mPFC directly following the stimulation, i.e., during extinction learning, and during extinction recall on day 2 (see below). The TMS protocol and the subsequent attachment of the NIRS probeset resulted in a time lag of approximately 25 min between the fear acquisition and extinction learning phase.

Table 1 | Sample description.

		Active group	Sham group
Sex	Males	21	22
	Females	19	23
Age	*M* ± *SD*	23.9 ± 3.0	24.6 ± 4.5
Education (years)	*M* ± *SD*	12.7 ± 0.6	12.9 ± 0.4
UCS intensity[a]	(0–10)	6.5 ± 1.6	6.3 ± 2.1
STAI[b]	Trait	36.8 ± 6.8	33.9 ± 7.1
	State	36.7 ± 6.9	36.1 ± 9.1
PANAS[c] I	Positive affect	2.95 ± 0.5	3.08± 0.5
	Negative affect	1.23 ± 0.3	1.20 ± 0.2
PANAS[c] II	Positive affect	2.70 ± 0.6	2.68 ± 0.6
	Negative affect	1.17 ± 0.3	1.22 ± 0.3
N		40	45

[a] *UCS intensity determined the subjective level of aversiveness of the scream used as unconditioned stimulus (UCS) on a scale ranging from 0 for "not unpleasant" to 10 for "extremely unpleasant."*

[b] *State-Trait Anxiety Inventory (Laux et al., 1981).*

[c] *Positive and Negative Affect Scale (Krohne et al., 1996), I indicate the first investigation before the experiment, II the second investigation after completing study day 1.*

PROCEDURE

On the day of stimulation (day 1), subjects were first familiarized with the experimental design and asked to answer questionnaires concerning mood (Positive and Negative Affect Scale, PANAS; Krohne et al., 1996) and anxiety (State-Trait Anxiety Inventory,

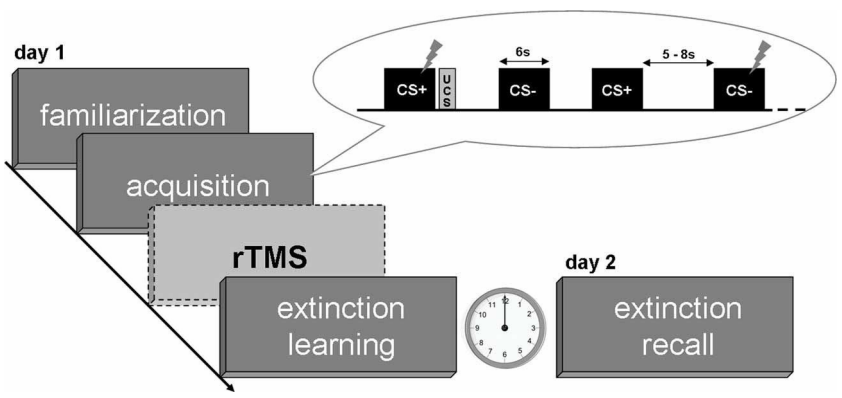

FIGURE 1 | Experimental design. Flashes indicate startle stimuli during CS presentations as primary measure of the conditioned response.

STAI; Laux et al., 1981). Subsequently, they were introduced to the TMS machine by identifying the individual RMT defined as the lowest stimulation intensity capable of inducing a visible finger movement at least 5 times out of 10 single pulses over the right hand area of the primary motor cortex. TMS application and all measurements were conducted in a sound-attenuated, electrically shielded and air-conditioned cabin. Subjects were prepared for the experiment by attaching headphones and electrodes for startle potentiation and skin conductance recordings (see below). They were instructed about the separation of the experiment into three parts: (1) in the first half of the experiment they are confronted with two neutral faces on the computer screen as well as two auditory sounds (familiarization and fear acquisition), (2) subsequently the rTMS application to their forehead while sitting still on a chair, and (3) immediately after the stimulation the second half of the experiment again consisting of faces and auditory stimuli (extinction learning). Subjects were not instructed about the CS+/UCS contingency or the UCS absence during the extinction phase. At the end of day 1 the PANAS was assessed a second time to evaluate a potential rTMS impact on mood (Tupak et al., 2013).

On day 2, subjects had to answer a self-construed questionnaire concerning rTMS side effects based on Wassermann (1998) ("Did you experience any adverse side effects after the rTMS yesterday? If yes, please mark which kind of discomfort you experienced and how long it lasted."). They were prepared for physiological recordings and underwent the test for extinction recall while the instruction resembled that of day 1. TMS was not applied a second time. Afterwards subjects were unblinded to the rTMS condition and were paid for participation.

Conditioned fear responses (CR) were assessed by FPS, SCR, fNIRS, and subjective valence and arousal ratings for CS+ and CS−.

FEAR-POTENTIATED STARTLE (FPS)

The eyeblink component of the startle reflex was measured by recording electromyographic (EMG) activation of the right orbicularis oculi muscle. Two 5 mm Ag/AgCl disc surface electrodes were positioned approximately 1 cm below the pupil and 1 cm below the lateral canthus of the right eye (impedance $<5\,k\Omega$). A third electrode was placed at the right mastoid and served as isolated ground. The acoustic startle stimuli consisted of a 50 ms burst of white noise with 40 ms plateau and 5 ms rise and fall time at intensities of 100 dB (sound pressure level, SPL) delivered binaurally via in-ear headphones. No background sound was presented. Startle probes were delivered in half of the trials (4000 ms after CS onset) and ITI (randomly between 3000 and 5000 ms). EMG activity was recorded via a 72-channel amplifier (QuickAmp, Brain Products GmbH, Munich, Germany) and sampled at 1000 Hz. Data was acquired, saved and analyzed with Vision Recorder/Analyzer Version 2.0 (Brain Products GmbH, Munich, Germany). The EMG-signal was filtered with a 28 Hz high-pass and a 500 Hz low-pass filter (time constant 0.0057 s, 24 dB per octave). A notch filter was applied to control for components caused by (electro-)magnetic interference. After rectification signals were smoothed using a 50 ms moving average filter. Each segment was baseline-corrected 50 ms prior to the startle probe onset. Startle amplitudes were further defined as peak magnitudes (in microvolt) from the corrected EMG signal between 21 and 200 ms following probe onset. Artifact rejection was performed manually for every single peak. Startle non-responders on either one or both days were identified by mean magnitudes of less than 5 μV per day and excluded accordingly ($n = 14$). Another male subject had to be excluded due to a nystagmus, which made startle blink recording impossible. In order to allow for inter-individual differences, absolute blink magnitudes were normalized using *z*-standardization (Blumenthal et al., 2005). ITI startle probes were further utilized as control condition for CS+ and CS− by converting startle magnitudes during each CS presentation (X) into *Z* scores using the ITI mean and standard deviation per phase ($Z_{CS} = (X_{CS} - M_{ITI})/SD_{ITI}$); (e.g., Bonnet et al., 1995; Blumenthal et al., 2005).

SKIN CONDUCTANCE RESPONSE (SCR)

SCR was assessed by using two Ag/AgCl electrodes attached to the thenar eminence of the subjects' left palm. Measurements were acquired via a 72-channel amplifier and a Galvanic Skin Response (GSR) sensor which constantly delivered a 0.5 V current (Brain Products GmbH, Munich, Germany). The sampling rate was set to 1000 Hz. SCR recording and analyses were performed with Vision Recorder/Analyzer Version 2.0 (Brain Products GmbH, Munich, Germany). Offline, raw data were first high-pass filtered with 1 Hz and a notch filter of 50 Hz and afterwards segmented into CS+ and CS− trials that were baseline-corrected 1000 ms prior to CS onset. SCR were characterized by peak responses in a time window of 1 to 5 s after CS onset. Artifact rejection was performed manually for every single trial. Similarly to the FPS analyses SCR data were z-transformed across both days without the first four respective CS trials in order to account for inter-individual differences. Six non-responders had to be excluded and were thus not considered for further analysis.

FUNCTIONAL NEAR-INFRARED SPECTROSCOPY (fNIRS)

Functional NIRS is based on near-infrared light of different wave lengths that is emitted to the cortical surface by means of sensors attached to the participant's forehead and thereby measures local changes of blood oxygenation. A detailed description can be found elsewhere (Obrig and Villringer, 2003). Oxygenation concentration was measured with the continuous wave system ETG-4000 (Hitachi Medical Co., Tokyo, Japan) using a 3×11 array which covered the prefrontal cortex. The interoptode distance was set to 3 cm. Signals were acquired with a sampling rate of 10 Hz. The method was included in order to discuss FPS, SCR, and rating results in the light of rTMS induced mPFC activation within the targeted fNIRS channels. We hypothesized that if rTMS modulates the processing of conditioned fear, it will correlate with higher mPFC activation in the cluster for which we found a signal increase from early to late extinction learning in a previous study (Guhn et al., 2012). Accordingly, we time-locked the onset of the signal to the jitter mean, i.e., 6500 ms after CS onset, and manually screened for artifacts due to head movement or technical problems. Signals were further processed by applying a cosine filter of 0.5 Hz correcting for low-frequency signal drifts. The four regressors (CS+ early, CS+ late, CS− early, CS− late) were modeled as delta functions and convolved with a gaussian hemodynamic response function at 6.5 s peak time.

Time series for blood oxygenation (O_2Hb) during both extinction sessions were then assessed by applying a general linear model approach. Beta estimates for stimulus (CS+, CS−) by phase mean (extinction learning early, extinction learning late, extinction recall early, extinction recall late) between groups (active, sham) were investigated by using repeated measures analyses of variance (ANOVA).

SUBJECTIVE RATINGS

Subjective CS+ and CS− ratings were assessed through self-assessment manikins (SAM; Bradley and Lang, 1994) for valence and arousal at different time points during the experiment: after familiarization and twice during/after fear acquisition, as well as during/after both extinction sessions. Subjects were asked to indicate whether a face was perceived as pleasant or unpleasant and whether it induced arousal or not on a 9-point Likert Scale.

STATISTICAL ANALYSIS

Demographic data such as age and years of education were compared between groups with student *t*-tests. Psychometric data (UCS-intensity, PANAS, and STAI scores) were analyzed by using the Mann–Whitney-*U*-test, rTMS side effects by using Fisher's Exact Probability Test.

For FPS and SCR analyses, subjects were first characterized by CS+ and CS− responses during the acquisition phase. We analyzed paired (CS-UCS) as well as unpaired (CS-noUCS) CS+ trials since UCS followed the CS with a short temporal gap, i.e., the analyzed segment did not include the actual UCS delivery. Subjects who did not show higher responses for CS+ than CS− were not considered for further TMS group comparisons due to non-successful fear conditioning (e.g., Phelps et al., 2004). Likewise 22 subjects (13 women) had to be excluded. Potential group differences on a descriptive or psychometric level (age, UCS-intensity, STAI-T, STAI-S; PANAS) were accounted for and did not reveal any significant results. CS trials were averaged for each stimulus (CS+, CS−) per phase (acquisition, extinction learning, extinction recall) and statistically evaluated using repeated measures ANOVA with stimulus and phase mean as within-subject factors and group (active, sham) as between-subject factor. A p-value < 0.05 was considered significant; Greenhouse Geisser correction was applied in case of non-sphericity. *Post-hoc t*-tests were used when (1) stimulus × phase × group interactions proved to be significant or (2) stimulus × phase interactions proved to be significant without significant group effects; in the second case *post-hoc t*-tests were conducted within groups. Additionally, we analyzed gender effects for FPS and SCR data and tested for significant interactions between gender and TMS group. A short theoretical background and discussion of these results is provided in the supplement.

RESULTS

FEAR POTENTIATED STARTLE (FPS)

The final sample consisted of $n = 21$ (13 women) subjects receiving active and $n = 24$ subjects (12 women) receiving sham stimulation. ANOVA revealed significant main effects for stimulus [$F_{(1, 43)} = 15.35, p < 0.001$], the interaction of stimulus × phase [$F_{(1.5, 66.5)} = 5.7, p = 0.009$] and a trend-wise significant stimulus × phase × group interaction [$F_{(1.5, 66.5)} = 2.92, p = 0.074$].

As expected, *t*-tests revealed significant differences between CS+ and CS− trials during acquisition within both groups ($p < 0.001$), but revealed sustained CS+/CS− discrimination for sham only, i.e., higher FPS responses for CS+ than for CS− for both extinction learning [$t_{(23)} = 2.3, p = 0.031$] and extinction recall [$t_{(23)} = 2.44, p = 0.023$; **Figure 2**]. CR for both groups in time course are provided in **Figure 3**. In order to statistically analyze these group differences during the experimental phases we continued to separate each extinction session into an early and a late phase consisting of 10 trials each for which we used the CS+/CS− differences. A one-way ANOVA examining the effects of phase (acquisition, early extinction learning day 1, late extinction learning day 1, early extinction recall day 2, late extinction recall day 2) on FPS magnitudes revealed a trend-wise significant main effect of phase for the active group [$F_{(2.3, 46.7)} = 2.98, p = 0.054$]. This is composed of a negative linear trend [$F_{(1, 20)} = 4.19$, $p = 0.054$]: FPS responses decreased proportionately through all phases while the sham group neither showed a significant main effect of phase ($p > 0.79$) nor significant trends. **Figure 4** shows the time course of the difference scores (CS+ minus CS−) throughout the five phases.

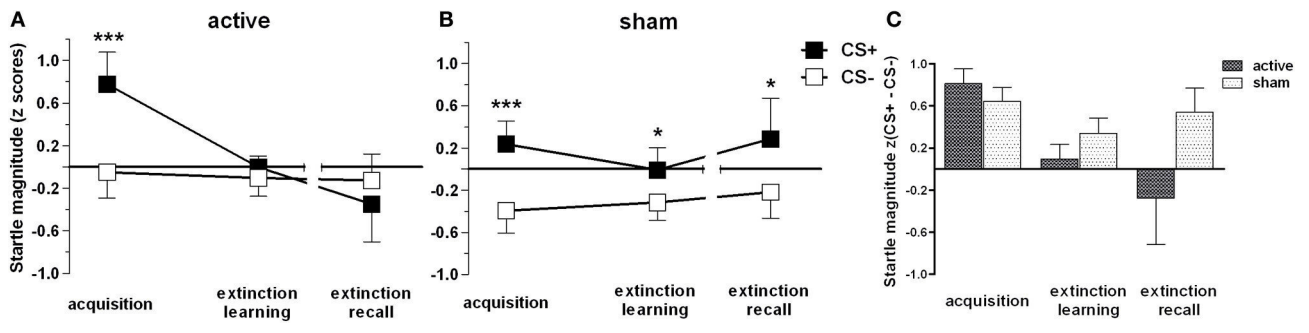

FIGURE 2 | Fear-potentiated startle magnitudes for CS+ and CS− trials for active (A) and placebo (B) group and the difference score (C) accordingly. In all experimental phases mean responses and standard errors of the mean (SEM) are depicted. Asterisks indicate significant differences (*$p < 0.05$, ***$p < 0.001$). **(C)** illustrates CS+ and CS− trials as difference scores to indicate that groups did not differ in their conditioned response during the acquisition phase [independent *t*-contrast: $t_{(43)} = 1.47$, $p > 0.05$].

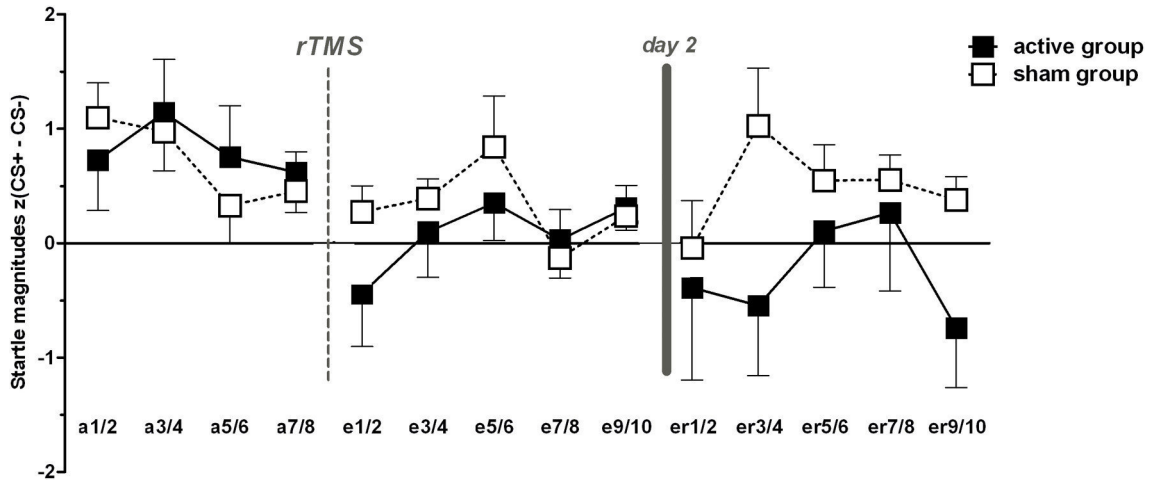

FIGURE 3 | Conditioned responses (*CS+ minus CS−*) indexed by fear potentiated startle magnitudes for acquisition (a), extinction (e), and extinction recall (er). For reasons of clarity two trials were averaged for each phase, respectively. Note that in the middle of each phase online valence and arousal ratings were conducted. Error bars indicate the standard error of the mean (SEM).

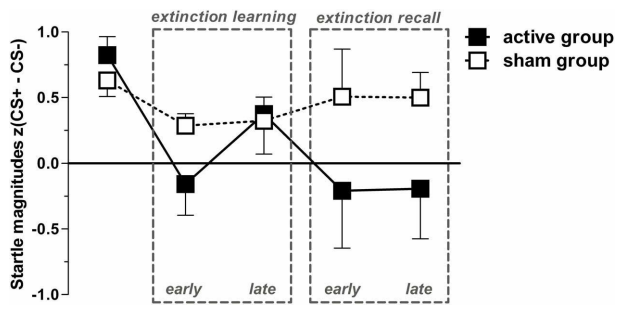

FIGURE 4 | Conditioned responses (*CS+ minus CS−*) for both groups indexed by Fear-potentiated startle magnitudes (mean + SEM). The active group exhibit a significant linear trend through both extinction phases indicated by a proportionately CR decrement from fear acquisition (first data point) throughout both extinction phases. For the active group, all dependent *t*-tests ($p_{\text{one-tailed}} < 0.05$) for acquisition with each extinction phase showed significant results, except for acquisition vs. late extinction day 1 which revealed only a trend-wise significant *p*-value ($p < 0.1$). For the sham group, only the early extinction day 1 compared to the acquisition phase revealed a significant difference thereby indicating extinction learning; all other scores resemble the acquisition phase ($p > 0.05$).

SKIN CONDUCTANCE RESPONSE (SCR)

The final sample for SCR analyses consisted of 47 subjects, $n =$ 26 active (15 women) vs. $n = 21$ sham group (9 women). The three-way ANOVA revealed significant stimulus [$F_{(1,\ 45)} = 26.28$, $p < 0.001$], phase [$F_{(1.6,\ 74)} = 7.62$, $p = 0.001$] and stimulus × phase interaction effects [$F_{(2,\ 90)} = 14.84$, $p < 0.001$]. Group did not influence main or interaction effects ($p > 0.1$). Both groups showed successful discrimination during acquisition ($p < 0.001$). Notably, the sham group still showed the CS+/CS− discrimination sustained during extinction learning [$t_{(20)} = 2.11$, $p =$ 0.047] while the active group displayed no significant CS+/CS− differences ($p > 0.9$; **Figure 5**).

SUBJECTIVE RATINGS

In order to keep the sample constant we examined self-reports only for subjects who were analyzed either for FPS or SCR data ($n = 62$, see **Table 2**). This sample did not differ from the non-conditioners ($n = 23$) in any of the assessed descriptive or psychometric measures.

All subjects indicated successful fear acquisition as evident from significant main effects for stimulus [valence: $F_{(1,\ 60)} = 8.16$, $p = 0.006$; arousal: $F_{(1,\ 60)} = 27$, $p < 0.001$], phase [valence: $F_{(2.6,\ 157.7)} = 18.31$, $p < 0.001$; arousal: $F_{(3,\ 180)} = 42.22$ $p < 0.001$] and significant stimulus × phase interactions [valence: $F_{(2.3,\ 137.7)} = 11.8$, $p < 0.001$; arousal: $F_{(1.9,\ 114)} = 15.67$, $p < 0.001$]. CS+ and CS− were equally evaluated during familiarization [valence: $t_{(61)} = 0.27$, $p = 0.790$; arousal: $t_{(61)} = -0.18$, $p = 0.857$] but self-reports diverged significantly during fear acquisition, in that CS+ was rated as more unpleasant [$t_{(61)} = 4.79$, $p < 0.001$] and evoked higher arousal [$t_{(61)} = -5.57$, $p < 0.001$] than CS−. This significant discrimination persisted over both extinction learning [valence: $t_{(61)} = 2.05$, $p = 0.044$; arousal: $t_{(61)} = -4.76$, $p < 0.001$] and extinction recall [valence: $t_{(61)} = 2.33$, $p = 0.023$, arousal: $t_{(61)} = -4.19$, $p < 0.001$] although CS+ valence increased [$t_{(61)} = -6.88$, $p < 0.001$] and CS+ arousal decreased in the course from acquisition to extinction [$t_{(61)} = 7.67$, $p < 0.001$] again resulting in familiarization-like levels ($p > 0.1$).

In order to account for rTMS induced group differences we conducted a three-way ANOVA examining effects of stimulus by phase with only two levels (extinction learning, extinction recall) between groups. We found a significant stimulus × phase × group interaction for arousal [$F_{(1,\ 60)} = 4.33$, $p = 0.042$]. The active group ($n = 32$) discriminated significantly less between CS+ and CS− while the sham group ($n = 30$) persisted to evaluate CS+ as more arousing than CS− [$t_{(53.8)} = -2.01$, $p = 0.043$] resembling the FPS and SCR results (**Figure 6**). Valence ratings revealed no group differences. The three-fold interaction did not

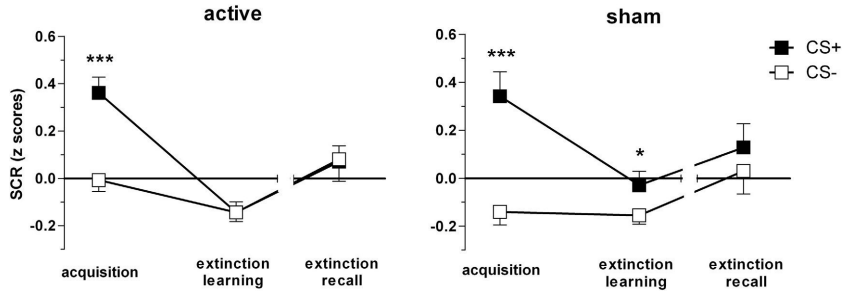

FIGURE 5 | Skin conductance responses (SCR) for CS+ and CS− trials during acquisition, extinction learning on day 1, and extinction recall on day 2, per group, respectively. Depicted are means and standard errors of the mean. Asterisks indicate significant differences (*$p < 0.05$, ***$p < 0.001$).

Table 2 | Subsample of successful conditioned volunteers for data analysis of the subjective ratings.

		Active group	Sham group
Sex	Males	15	16
	Females	17	14
Age	$M \pm SD$	23.81 ± 3.2	24.43 ± 3.5
UCS intensity	(0–10)	6.23 ± 1.6	6.47 ± 2
STAI	Trait	36.84 ± 6.9	34.5 ± 7.68
	State	37 ± 7.35	37.3 ± 10.4
N		32	30

Successful conditioning was defined by a higher CR on CS+ vs. CS− trials during the fear acquisition phase. None of the reported variables reached statistical significance for group comparisons.

reach statistical significance ($p > 0.2$) for the whole sample ($N = 85$), including conditioners *and* non-conditioners.

FUNCTIONAL NEAR-INFRARED SPECTROSCOPY (fNIRS)

We neither found significant group differences during extinction learning nor during extinction recall in the sample of $n = 62$ which was used for the subjective ratings. Exploratorily we analyzed the subsample of volunteers fulfilling the requirements for the analysis of both FPS *and* SCR ($n = 12$ active and $n = 13$ sham, two data sets were not included into the analysis due to an insufficient signal quality) since those participants were believed to have the strongest conditioning response regarding the consistency across measurements. However, we are well aware that the results have to be regarded cautiously. For the cluster reported in our pilot study (10 medial prefrontal channels expanding to the right hemisphere: 5, 16, 24, 26, 27, 35, 36, 37, 45, 47) the active and sham group differed in the amount of O_2Hb in response to CS+ during the early extinction learning phase for which the active group displayed a higher signal than the sham group [student *t*-test: $t_{(23)} = 2.65$, $p_{\text{one-tailed}} = 0.008$]. While there was no signal change from the early to the late phase in the active group, the sham group showed a trend-wise significant signal increase [$t_{(23)} = -1.61$, $p_{\text{one-tailed}} = 0.067$] resembling the signal increase reported in the previous study. During the extinction recall on day 2 there were no within or between-group differences (see **Figure 7**).

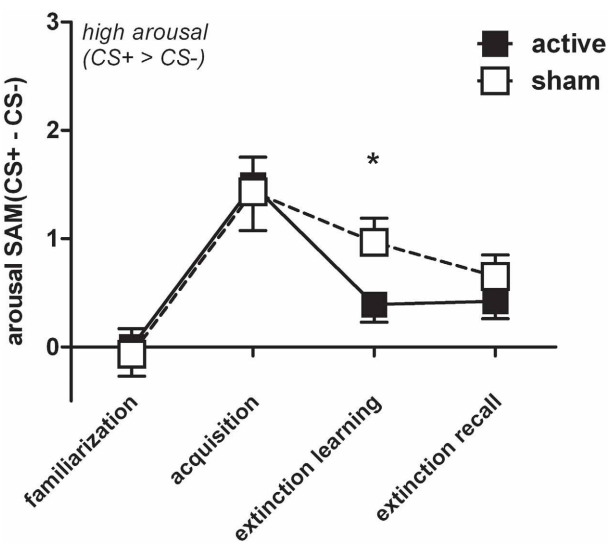

FIGURE 6 | For reasons of visualization arousal difference scores (*CS+ minus CS−*) were depicted (M + SEM) during familiarization, acquisition, extinction learning, and extinction recall for the active ($n = 32$) and the sham group ($n = 30$). Groups significantly differed during extinction learning (*$p < 0.05$), i.e., sham showed higher arousal for CS+ than CS− trials.

SIDE EFFECTS

Side effects were assessed using a questionnaire which contained previously published rTMS side effects such as headache, neck pain, dizziness, drowsiness, nausea, speech, or sleep problems, problems to concentrate, paraesthesia, seizures, muscle contraction, faint, local discomfort at the stimulated site and ear noise (Wassermann, 1998). Subjects were asked to evaluate these side effects in their intensity and duration before unblinding them regarding the TMS group. For completeness, the $n = 3$ females who dropped out due to rTMS discomfort were included in the analysis ($N = 88$). Overall, rTMS was well tolerated. Twenty-two subjects (25%) reported side effects, therefrom 10 subjects of the sham group. Type of side effects per group are depicted in **Table 3**, no other side effects were quoted. Headaches as the most prominent side effect lasted less than 1 h in 11 subjects; 5 subjects complained about headaches for less than 6 h and 2 for less than

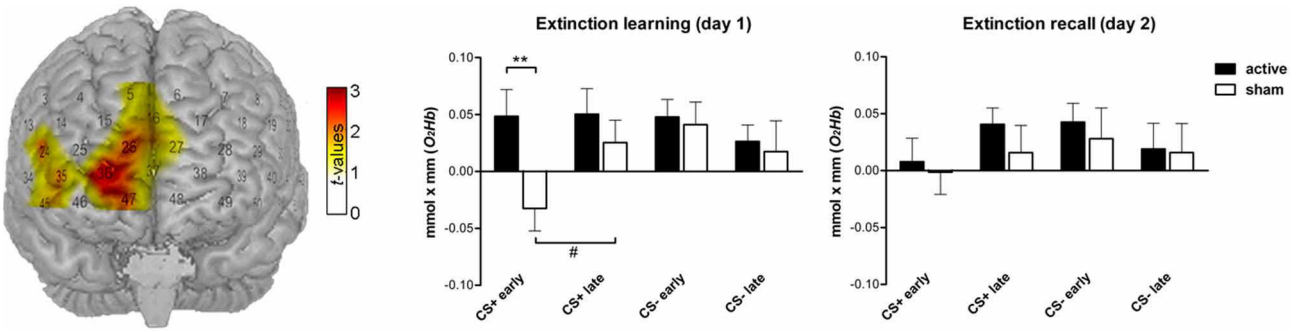

FIGURE 7 | Functional NIRS results (O_2Hb) during both extinction phases ($n = 25$). Left: T-map superimposed on a standard brain. During the early extinction learning the active group showed a higher signal for CS+ than the sham group in a cluster of 10 probeset channels covering the medial prefrontal cortex. The bar charts in the middle and on the right depict the corresponding beta estimates for CS+ and CS− trials ($^{**}p_{one-tailed} < 0.01$). The sham group showed a trend-wise significant ($^{\#}p_{one-tailed} < 0.1$) signal increase from early to late extinction learning in response to CS+ trials while the active group persisted to show a high concentration level.

Table 3 | Frequencies of quoted rTMS side effects.

	Active group ($n = 43$)	Sham group ($n = 45$)
Headaches	9	9
Neck pain	0	5
Drowsiness	1	2
Problems to concentrate	0	2
Local discomfort (forehead)	2	3

12 h. There was neither a significant group difference concerning the overall frequency of side effects nor the type of side effects ($p > 0.49$), except for neck pain which was trend-wise quoted more frequently by the sham group ($p = 0.056$). Altogether, the results demonstrate that subjects were actually TMS-naïve.

Possible mood changes caused by rTMS were evaluated using PANAS × group repeated measures ANOVA. Positive affect showed a significant main effect [$F_{(1, 86)} = 45.94$, $p < 0.001$] indicating that subjects rated their affect prior to the experiment as more positive than afterwards. Negative affect did not change. The group interaction did not reach statistical significance, i.e., rTMS did neither induce negative nor positive mood changes ($p > 0.1$).

DISCUSSION

In the present study, one session of high-frequency rTMS was applied to the mPFC in healthy, TMS-naïve subjects who underwent a 2-day discriminative fear conditioning and extinction paradigm. In order to increase a top-down regulation of the mPFC thereby modulating the processing of conditioned fear, facilitatory rTMS was administered offline before an extinction learning phase. Consistent with our hypothesis, the active group displayed diminished CS+/CS− discrimination during extinction learning (day 1) as evident from FPS data and to a smaller extend from SCR as well as from subjective arousal ratings. Moreover, rTMS had a persisting effect on extinction recall (day 2) as seen with FPS while the sham group revealed higher conditioned fear responses to CS+ than to CS− trials and reported higher arousal for CS+ during extinction learning. This study describes the first experimental approach of influencing conditioned fear by using rTMS.

Resembling the animal data of prefrontal electrical stimulation (Milad and Quirk, 2002; Kim et al., 2010), we found significant group differences for active vs. sham stimulation during extinction learning (FPS, SCR, and arousal ratings) and extinction recall (FPS). While IL stimulation studies in rats revealed the most prominent results during extinction recall, such a *sustained* effect of rTMS in the present study was limited to the FPS data. Hereby the CS+ responses linearly declined from high FPS magnitudes during acquisition to low magnitudes during late extinction recall without the prominent fear return typically emerging when subjects are confronted with the former CS+ a day after the extinction learning (Bouton, 2002). Quirk et al. (2003) provided a probable explanation for likewise results by showing that mPFC stimulation in animals inhibited central amygdala output neurons and thereby reduced the conditioned fear. In this regard, the here applied active mPFC stimulation should have increased the activity of amygdaloid intercalated cells, resembling a top–down mechanism (Quirk and Beer, 2006; Milad et al., 2007). With regard to the startle response which is mediated by a neural pathway that directly originates from the amygdala (Davis et al., 1997), the improved extinction recall as indexed by the FPS data could thus represent attenuated amygdala activation. This interpretation of our findings is consistent with results of amygdala attenuation following dlPFC stimulation while processing negative pictures using the same rTMS protocol (Baeken et al., 2010). Moreover, the results that we obtained for fNIRS point toward higher O_2HB values for the active compared to the sham group which confirms the interpretation of increased mPFC activity through rTMS.

Based on these experimental results in healthy volunteers, rTMS might be a promising complementing therapeutic tool in anxiety patients when combined with exposure therapy, which is based on the principles of extinction learning and extinction

memory recall. Pathological anxiety and even anxiety-related personality traits in healthy subjects have been associated with hyper-reactivity of the conditioned amygdala response and deficient prefrontal recruitment. An impaired inhibition of the amygdala through the mPFC is hereby suggested to cause enhanced vulnerability to pathological anxiety and risk for relapse (Sehlmeyer et al., 2011). According to the present results, rTMS in combination with exposure therapy might effectively inhibit the amygdala response via an increased prefrontal cortex activity. As mentioned before, the pharmacological intervention with DCS was able to increase PFC activity in phobic patients while it was surprisingly unable to show facilitation effects on experimental fear extinction in healthy subjects (Guastella et al., 2007; Klumpers et al., 2012). Therefore, it is most likely that the present rTMS effect on extinction memory would be even more marked in patients with anxiety disorders showing overall heightened fear reactions and diminished fear extinction.

The exact underlying neurophyisological mechanisms of rTMS remain unclear. Hallett (2000) and Hoogendam et al. (2010) propose that rTMS influences the consolidation of learning by modifying excitatory synaptic efficacy or neuronal synchrony. By comparing high- and low-frequency stimulation in mice using an offline approach, successful extinction learning was associated with long-term potentiation (LTP) while long-term depression (LTD) resulted in the return of conditioned fear (Herry and Garcia, 2002). High-frequency rTMS over 10 consecutive days in rats was further associated with a lasting increase of prelimbic levels of the brain-derived neurotrophic factor (BDNF), a neuroplasticity marker involved in LTP (Gersner et al., 2011). Thus, in the present study rTMS might have either promoted prefrontal LTP during extinction learning as well or interfered with LTP during the consolidation of the fear memory. In order to enlighten which learning phase was actually modulated, future studies should consider a 3-day design in order to be able to separate fear acquisition and extinction learning into consecutive days. Thereby, the memory stage which is influenced by rTMS, i.e., fear or extinction memory could be disentangled.

The present study has a number of limitations which need to be considered when interpreting the results. First of all, the comparability with findings of animal studies regarding the mPFC-amygdala interplay is limited by the fact that the present design used an offline rather than an online TMS approach in which the stimulation is applied time-locked to CS presentations (Milad and Quirk, 2002). However, an offline TMS approach enabled us to assure that participants indeed exposed themselves to the magnetic field. Prefrontal TMS affects face muscles which commonly irritates TMS-naïve participants at the beginning. Therefore, in an online approach participants might avoid the stimulation in case of discomfort by moving the head slightly away from the coil. Instead of an online stimulation a TMS protocol inducing long lasting effects up to 30 min was selected (George et al., 1996). The TMS coil positioning in the present study was further not identical to the electrical IL stimulation in the rat studies. Due to the coil size, the limited stimulation depth to the cortex and the high stimulation intensity the vmPFC as homologues region to the IL was not selected as rTMS target region. According to a pilot study a more dorsal part of the mPFC was referred to as target region since this region was associated with an increased activity to CS+ trials in an extinction learning session (Guhn et al., 2012). In order to proof the targeted mPFC region, inhibiting the mPFC via low-frequency rTMS should result in prohibited or at least decelerated fear extinction (Herry and Garcia, 2002) which future studies should confirm.

With regard to the data analysis it has to be further mentioned that the number of volunteers who showed higher CR to CS+ than to CS− after the fear acquisition phase was limited regarding the whole sample. This was the result of a methodological challenge we had to face: In contrast to anxiety patients, healthy volunteers exhibit a fast and efficient extinction learning and extinction recall (e.g., see Milad et al., 2008, 2009). In order to resemble deficient extinction learning, the extinction process had to be decelerated. This was achieved by reducing the CS+/UCS pairings during the fear acquisition phase. The UCS in average only followed every second presentation of the CS+ (50% reinforcement rate) and thereby became a less predictable signal for UCS occurrence leading to a prolonged resistance to extinguish the CS+. Investigating interventions on extinction learning in healthy participants raise the question of how to establish optimal circumstances in which an intervention such as rTMS can show advantages. While we constituted decelerated extinction in healthy participants, we had to face the problem of non-conditioners not adapting to the danger signaling properties of the CS+ and/or the safety signaling properties of the CS−. Based on findings by Van Well et al. (2012) we therefore decided to exclude these participants accepting a higher number of non-considered data sets. Comparing neural substrates between conditioners and non-conditioners in an instructed fear paradigm (reinforcement rate 75%), Van Well et al. found significant group differences in stimulus differentiation between CS+ and CS− as well as differential stimulus peak activations within the amygdala and other regions. This shows that conditioners exhibited higher peak activations for CS+ compared to CS−. Furthermore, amygdala activation significantly correlated with FPS and thereby supports FPS as reliable and specific index of fear. Assuming that rTMS interacts with memory consolidation via the mPFC-amygdala top-down regulation, our hypothesis could not have been tested in participants who did not established fear, which was defined as a positive CS+/CS− discrimination during fear acquisition.

In conclusion, our results indicate that rTMS provides a non-invasive and well-tolerated therapeutic tool as evidenced by the low frequency of side effects which can modulate the processing of conditioned fear in healthy human subjects. Therefore it can serve as a basis for future studies investigating the precise learning stage, i.e., fear vs. extinction memory and its respective causal mechanisms. Additionally future studies can use these results to investigate the effect of rTMS on fear extinction in patients with anxiety disorders as well as its proposed beneficial effect in combination with psychotherapy.

ACKNOWLEDGMENTS

The present study was funded and supported by the German Research Foundation (SFB-TRR 58, Project C04 and RTG 1253/1) and the University of Wuerzburg in the funding programme

Open Access Publishing. Thomas Dresler was partly supported by the LEAD graduate school [GSC1028], a project of the Excellence Initiative of the German federal and state governments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnbeh.2014.00044/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 August 2013; accepted: 29 January 2014; published online: 18 February 2014.

Citation: Guhn A, Dresler T, Andreatta M, Müller LD, Hahn T, Tupak SV, Polak T, Deckert J and Herrmann MJ (2014) Medial prefrontal cortex stimulation modulates the processing of conditioned fear. Front. Behav. Neurosci. ***8****:44. doi: 10.3389/fnbeh.2014.00044*

This article was submitted to the journal Frontiers in Behavioral Neuroscience.

Supplemental Information

Medial prefrontal cortex stimulation modulates the processing of conditioned fear

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Frontiers in Behavioral Neuroscience, 8, 44. doi: 10.3389/fnbeh.2014.00044

Gender effects

Epidemiological studies agree on the fact, that women are more susceptible to develop mood and anxiety disorders (Kessler et al., 2005). The incidence of all anxiety disorders such as posttraumatic stress disorder, panic disorder, agoraphobia, social anxiety, and phobias is two to three times higher for women compared to men (Seeman, 1997). Interestingly, gender differences arise starting with the reproductive years of women suggesting that sex hormones play an important role in the development and maintenance of anxiety disorders (Seeman, 1997).

To date, the number of publications controlling for or manipulating the hormonal status in females is quiet low (Farrell et al., 2013). Existing studies however do point towards an important role of the estrogen level which varies throughout the menstrual cycle i.e. being low at the beginning and high at the end of the follicular phase (mid cycle). Rat studies comparing females in these two phases of the estrous cycle in a classical pavolovian fear conditioning study revealed differences particularly during extinction recall (Milad et al., 2009;Zeidan et al., 2011): Females in the late follicular phase showed a comparable extinction recall than males while females in the early follicular phase exhibited an impaired performance. Translational studies in humans revealed comparable findings (Milad et al., 2010) and further point to specific gender effects in brain regions correlating with emotional learning (Zeidan et al., 2011). Due to the fact that the amygdala and the ventromedial prefrontal cortex (vmPFC) contain a relatively high number of estrogen receptors (Goldstein et al., 2001), the neural reactivity at these sites, which are involved in the stress response (Goldstein et al., 2010) and more precisely in fear extinction (Merz et al., 2010;Zeidan et al., 2011), is modulated by estrogen. Thus, the investigation of sex hormones enables the possibility to adjust therapies for women depending on their current state within the menstrual cycle. Since the present investigation focuses on the effects of repetitive transcranial magnetic stimulation (rTMS) on extinction learning and extinction recall in premenopausal naturally cycling women, gender effects will be considered for the data analysis. All women were recruited in the early follicular phase which was defined as the first five days of a regular menstrual cycle. Additionally, they did not take oral contraceptives for at least three month prior to the measurement. Based on the existing literature, low estrogen levels are hypothesized to cause deficits in extinction recall. Therefore, a possible compensating rTMS effect is tested.

Statistically, gender was integrated as a second between-subject factor in a stimulus (CS+, CS-) x phase (acquisition, extinction learning, extinction recall) by group (active, sham) repeated measurements ANOVA. The resulting sample distributions with regard to the dependent variables FPS and SCR are depicted in Table 1.

Table S1. Sample distributions for FPS (left) and SCR data (right) with regards to the TMS group (active, placebo) and gender (male, female).

FPS	♂	♀	
active group	8	13	21
placebo group	12	12	24
	20	25	

SCR	♂	♀	
active group	11	15	26
placebo group	12	9	21
	23	24	

FPS: Fear-potentiated startle response; SCR: Skin conductance response

For the *FPS data*, the ANOVA revealed a significant interaction between gender and stimulus [$F(1,41) = 13.24$, $p \leq .001$], which resulted from a significantly higher conditioned response in women compared to men during the fear acquisition phase [$t(40.2) = 2.12$, $p = .04$]; no gender differences were observed for both extinction phases. Moreover, group and gender did not interact significantly. On a descriptive level, both genders show lower conditioned responses in the active group than in the placebo group (Figure S1).

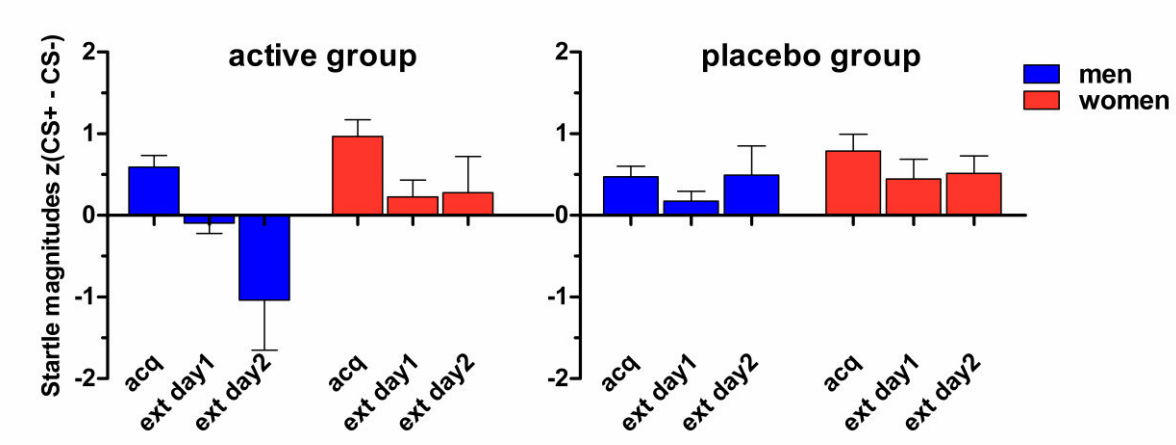

Fig. S1. Conditioned responses (CS+ minus CS-) indexed for fear potentiated startle magnitudes in men and women divided for TMS group (active, placebo). Depicted are phase means for acquisition (acq), extinction learning (ext day1) and extinction recall (ext day2); error bars indicate the standard error of the mean (SEM).

For the *SCR data*, adding the gender to the analysis resulted in a significant interaction for stimulus x phase x group x gender [$F(2,86) = 4.42$, $p < .05$]. In order to elucidate this four-fold interaction we divided the sample into two subsamples for men and women and calculated a stimulus x phase by group ANOVA respectively (for results see Table S2). Both subsamples showed the expected stimulus x phase interaction representing successful fear conditioning and fear extinction, however only in women this interaction was trend-wise impacted by TMS stimulation. Thus, while women seem to benefit from a prefrontal stimulation, stimulation group did not reveal a significant interaction for men since the descriptive illustration points towards a successful recall of extinction memory even without stimulation (Figure S2). However, post-hoc tests did not confirm the facilitated extinction learning and extinction recall statistically in women statistically [$t(22) < 1.3$, $p > .1$], probably due to the low statistical power.

Table S2. SCR results for a stimuli x phase x group ANOVA, analyzed for men and women respectively.

gender	factor	F	df	*p*
men	stimulus	14.17	1, 21	.001
	phase	7.1	2, 42	.002
	stimulus x phase	10.69	2, 42	<.001
	stimulus x phase x group	2.0	2, 42	.149
women	stimulus	11.65	1, 22	.002
	phase	5.24	2, 44	.009
	stimulus x phase	3.93	2, 44	.027
	stimulus x phase x group	2.43	2, 44	.099

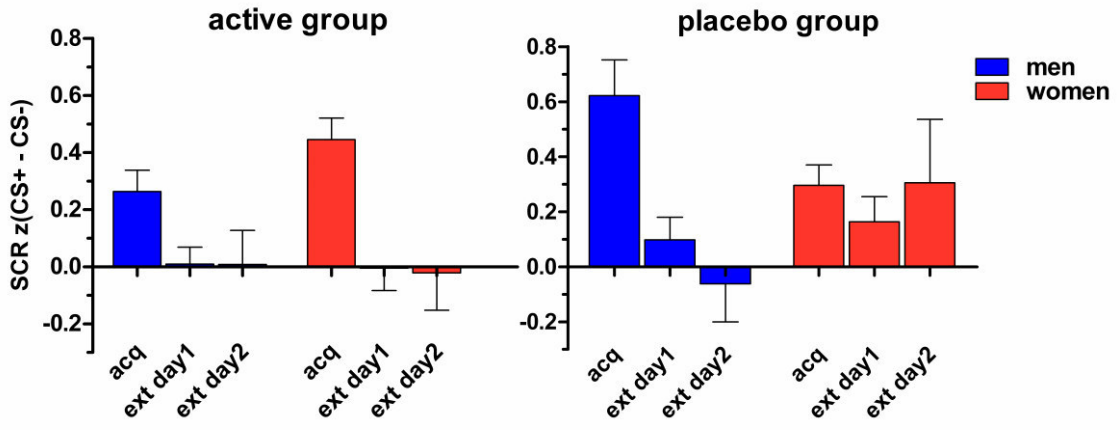

Fig. S2: Conditioned responses (CS+ minus CS-) indexed for skin conductance responses (SCR) in men and women divided for TMS group (active, placebo). Depicted are phase means for acquisition (acq), extinction learning (ext day1) and extinction recall (ext day2); error bars indicate the SEM.

To summarize, the analyses of gender effects revealed opposing results for FPS and SCR data. While there was no significant interaction between gender and TMS session for FPS data, the consideration of gender seemed to improve the TMS impact on the SCR data by showing an additional profit for women. Thereby, the SCR results are in line with the facilitating role of estrogen on extinction recall (Zeidan et al., 2011): In the placebo group, women show a numerically larger conditioned response during extinction recall, which is consistent with an impaired recall of the safety memory. Therefore, TMS might constitute a treatment option which could compensate for the reduced prefrontal top-down control in early cycle women when estradiol is low. However, given that we did not find a significant group x gender interaction in the FPS data, which was the main outcome variable of the present study, the SCR results have to be regarded with caution and necessarily has to get replicated in an independent and numerously larger sample.

Nonetheless, when considering rTMS as a possible treatment option the simultaneous consideration of gender and hormonal status in women might be promising. Exposure therapy as the clinical application of fear extinction for instance could be scheduled when the estrogen level is high. However, if this is not possible, exposure therapy could be combined with rTMS in order to compensate for low estrogen, for instance in women taking oral contraceptives which reduces endogenous cycling estradiol levels.

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3. The "Problem" of Fear Generalization

Even Watson and Rayner (1920) discovered that conditioned fear responses can be transferred to objects which exhibit similarities to the original conditioned stimulus. *Little Albert* who showed initially a conditioned fear response to a white rat was systematically confronted with other furry stimuli, e.g. a rabbit, a dog, a fur coat and a Santa Claus mask, all producing similar responses of withdrawal as did the rat.

The phenomenon of fear generalization has gained much attention in the pathology of anxiety disorders, particularly PTSD and PD, since a variety of CS with anxiogenic valence occur coincident with the former UCSs (PTSD: trauma, PD: panic attack) thus acquiring the strength of eliciting a CR. The generalization of anxiety and moreover a lack of generalization of extinction is assumed to further contribute to the persistence of anxiety disorders (Bouton, Mineka, & Barlow, 2001). The following paragraph is intended to outline overgeneralization as a pathogenic marker of anxiety as well as the importance of the generalization of extinction in order to manifest a stabile CS-noUCS memory. By evidencing brain stimulation techniques to facilitate the transfer from one extinguished CS to another in animals, the adaptation of such a design for humans is presented within the third manuscript included in this dissertation.

3.1 Overgeneralization in Pathological Anxiety

Generalization refers to the observation that stimuli resembling the CS can acquire the property of triggering a CR, often appearing over the time course of anxiety disorders. Learned fear of a particular situation in which the initial panic attack or the trauma (UCS) occurred might transfer to other situations exhibiting similarities. Moreover, autonomic fear responses during the UCS, e.g. an increased heart rate, might similarly generalize to everyday life experiences, such as climbing stairs, which might then elicit resembling sensations hence inducing a panic attack or the re-experience of the trauma.

In order to test the observed clinical characteristics of enhanced fear generalization in anxiety patients, Lissek et al. (2008) established a laboratory fear conditioning paradigm employing a different number of rings with gradually increasing sizes thus being different from the differential (CS+, CS-) paradigms outlined above. For half of the participants the

largest ring represented the CS+ which was paired with an electric shock (UCS) and the smallest ring served as CS- never paired with an aversive consequence. For the other half of the participants CS+ and CS- were reversed. The intermediately sized rings constituted generalization stimuli (GS) not paired with electric shocks that showed constantly decreasing similarity from one extreme to the other (CS+ to CS-). By assessing FPS responses healthy controls revealed the highest CR on CS+ and gradually decreasing CR as the presented stimuli become less similar to the CS+, with CS- producing the lowest CR as expected (Lissek, Biggs, et al., 2008). Patients suffering from PD, in contrast, displayed a different response pattern revealing abnormalities in inhibitory fear mechanisms: They showed generalization to rings (GS) with up to three out of five units of dissimilarity to the CS+ while healthy controls responded with equally high levels of FPS only on GS constituting one unit of dissimilarity (Lissek et al., 2010). Overgeneralization has been further proven to dissociate within different anxiety disorders since patients suffering from GAD showed abnormal levels of fear conditioning and generalization (Lissek, Kaczkurkin, et al., 2014) whereas patients with social anxiety disorder (SAD) did not (Lissek, 2011, as cited in Lissek, 2012), corresponding to the clinical observation that SAD patients merely fear social situations instead of the specific threat of an electric shock that was applied during the test (cf. Lissek, Levenson, et al., 2008 for enhanced CR on socially relevant UCS in SAD). The inability to inhibit fear responses to safety cues further emerged in differential fear conditioning studies (CS+ vs. CS-) with numerous reports on elevated CR to CS- stimuli in PD (e.g. Lissek et al., 2009; Lueken et al., 2013) and PTSD (Jovanovic et al., 2009), e.g. as opposed to depression (Jovanovic et al., 2010). Interestingly, PTSD patients with poor inhibition of CS- during the end of fear acquisition even demonstrated delayed extinction of fear in response to CS+ during an extinction phase (Norrholm et al., 2011) hence proposing (1) a relationship between overgeneralization and the persistence of pathological fear and (2) similar neural mechanisms to be involved in safety learning and the extinction of danger cues.

Neurobiologically, there is indeed evidence for conditioned generalization to engage the same brain circuits which emerged indispensable for fear learning and fear extinction, i.e. simplified the amygdala, the vmPFC and the hippocampus. Particularly the hippocampus demonstrated its importance in fear generalization by revealing increased activity with increasing similarity between the GS and the CS+ (Lissek, Bradford, et al., 2014). It encompasses a mediating role by comparing current information to representations of

previous stimuli that are stored elsewhere (Otto & Eichenbaum, 1992). GS resembling the CS+ revealed both an increased connectivity from the hippocampus with the amygdala and the insula corresponding to fear excitation, and decreased functional coupling between the hippocampus and the vmPFC in turn corresponding to less fear inhibition (Lissek, Bradford, et al., 2014). In conjunction with these results, PD patients not responding to CBT exhibited elevated activation of the hippocampus, the amygdala and the pregenual ACC during CS- trials compared to treatment responders as well as a decreased functional mPFC-amygdala coupling that did not change with treatment (Lueken et al., 2013), thus probably representing a sub-group of patients who might benefit from "novel extinction facilitators" (Norrholm et al., 2011, p. 561), such as rTMS, arising from translational research.

3.2 Rodent Findings on the Generalization of Extinction

With regard to the NMDA receptor dependency of the amygdala and the vmPFC during fear acquisition and extinction (Burgos-Robles, Vidal-Gonzalez, Santini, & Quirk, 2007), D-cycloserine (DCS), a partial NMDA agonist, has been found to facilitate fear extinction in rats in comparison to a saline control infusion: The administration of DCS either before (DCS infusion into the BLA, Walker, Ressler, Lu, & Davis, 2002) or after extinction training (subcutaneous DCS injection, Ledgerwood, Richardson, & Cranney, 2003) dose-dependently decreased FPS responses to CS not interfering with subsequent learning or renewal of fear (subcutaneous DCS injection; Ledgerwood, Richardson, & Cranney, 2005; Woods & Bouton, 2006) thus implicating DCS to affect consolidation processes of the extinction memory. Interestingly, a post-extinction DCS administration further affected the generalization from the extinction of one CS to another that did not undergo extinction (Ledgerwood et al., 2005). Carry-over effects of DCS on humans revealed rather inconsistent results with no benefits on extinction of classically conditioned fear in healthy controls (Klumpers et al., 2012) but treatment effects in patients suffering from anxiety disorders (e.g. Aupperle et al., 2009; Ressler et al., 2004), at least for patients exhibiting a high symptom severity (Siegmund et al., 2011).

The idea of facilitating the generalization of extinction by cognitive enhancers arising from the rodent literature seems significantly important for an improved treatment of anxiety patients. Thus, although exposure therapy are referred to as the gold standard, a lasting improvement on anxiety symptoms with regard to reduced relapse highly depends on

(1) numerous exposure sessions with regard to the duration, frequency and intensity of fear (Cain, Blouin, & Barad, 2003) as well as (2) a high variability of extinction in terms of different stimuli and contexts (Bouton, 2002; Lang & Craske, 2000). Searching for extinction facilitators which might advance the transfer from one exposure session to another might thus improve treatment, particularly in those patients who did not respond as quickly (cf. Norrholm et al., 2011) or exhibit relapse of symptoms after a while.

The basis of using rTMS for treatment studies is to understand the precise effects of rTMS on different mechanisms of extinction. While study 2 has contributed to the missing laboratory evidence for the ability of rTMS to facilitate extinction recall, study 3 now aims at shedding light on the effects of rTMS on extinction generalization. Since rTMS is assumed to influence synaptic plasticity via NMDA-dependent mechanisms likewise DCS approved in rats, rTMS might provide an equally suited tool to increase mPFC top-down control from an extinguished CS to a non-extinguished CS. Before presenting the study, the next paragraph will outline an experimental paradigm suitable to test this hypothesis.

3.3 How to Investigate the Generalization of Extinction in Humans?

In contrast to the generalization of fear by using GS that resemble the original CS+ but are not UCS-reinforced, investigating the generalization of extinction requires primarily the conditioning of similar stimuli to the same UCS in order to acquire at least two fear memories. By extinguishing only one out of these two CS+, both stimuli differ with regard to the acquisition of fear and extinction memory, i.e. while the CS+ which underwent an extinction training subsequently activates a fear (CS-UCS) as well as an extinction memory (CS-noUCS), the non-extinguished CS+ only exhibits a fear memory. In the following the term CS+E is referred to the reinforced stimuli that underwent extinction training and CS+U will be used for the reinforced stimuli that did not undergo extinction. In a recall test 24 hours later in which both stimuli are presented non-reinforced, CS+U is suggested to evoke the highest CR since only fear learning is recalled while the CS+E activates fear as well as extinction memory thereby exhibiting an intermediate CR. The implementation of a third stimulus (CS-) which has never been paired with the UCS is assumed to evoke the lowest CR since there is neither a fear nor an extinction memory recalled. By using a similar design for the investigation of neural correlates of fear extinction recall Milad et al. (Linnman et al., 2012; Milad et al., 2007) found increased vmPFC activation to CS+E relative to CS+U accompanied

by hippocampus activation which was related to a manipulation of context since fear conditioning was performed in a different context (A) than extinction training as well as extinction recall (B), referred to as ABB-design. VmPFC and hippocampus activity further positively correlated with a psychophysiological extinction recall index as assessed by SCR.

In order to test whether the extinction memory of the CS+E can be generalized to the CS+U by means of rTMS, in the following study rTMS was administered offline subsequently to the extinction training hence following the post-extinction DCS administration in rats (Ledgerwood et al., 2003, 2005) as well as the reputation for DCS usage subsequently to exposure sessions in humans (Mohr & Schneider, 2015).

3.4 Study 3: TMS Effects on the Generalization of Extinction

Figure 2 displays the experimental setting for the investigation of rTMS effects on the generalization of extinction adapted from Milad et al. (2007, without a manipulation of context). According to the decreased CR in response to CS+ following high-frequent mPFC versus sham stimulation (study 2) as well as the findings of DCS-enhanced extinction generalization in the rat (Ledgerwood et al., 2005) a high-frequent rTMS protocol was hypothesized to increase the inhibitory top-down control over the amygdala's fear expression in contrast to a rTMS control condition (vertex stimulation). Thus, a decreased CR to CS+U accompanied by higher prefrontal activation during a generalization test conducted 24 hours after the stimulation was revealing. The following manuscript has not been submitted for publication by the date of submission of this dissertation.

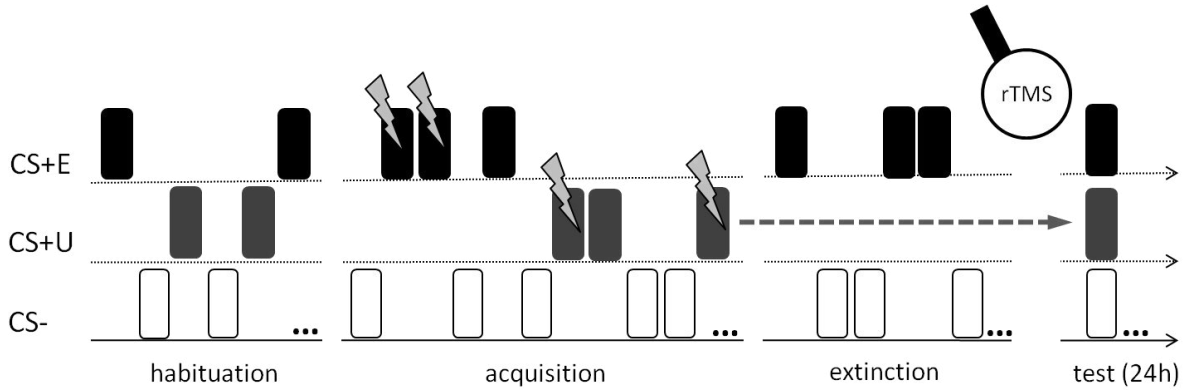

Figure 2. Experimental Setup of Study 3 for the Investigation of Generalized Extinction. Whereas CS+E and CS+U represented conditioned stimuli which were paired with an aversive consequence (UCS) during the fear acquisition phase, CS- remained unreinforced throughout all four experimental phases. Both CS+ differed with regard to the extinction phase in which only the CS+E was presented. Subsequently, rTMS was applied to increase extinction memory consolidation. A generalization test with all three CS was conducted 24 hours later. CS+E, extinguished stimulus; CS+U, un-extinguished stimulus.

Evidence for partial generalization of fear extinction after dorsolateral prefrontal cortex stimulation: Correlation with skin conductance and prefrontal fNIRS activity

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Number of words: 4367

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Abstract

The last decades of research demonstrated cross-species similarities in the interplay between the amygdala and the prefrontal cortex (PFC) for acquiring and extinguishing fear memories, thereby increasing our understanding of pathological anxiety and its treatment with exposure therapy. In order to obtain a robust extinction memory, brain stimulation techniques are speculated to enhance PFC functioning while down regulating the amygdala's fear response. The present study investigated the generalization of extinction learning from one extinguished conditioned stimulus (CS+E) to a non-extinguished second conditioned stimulus (CS+U) after prefrontal stimulation in a between-group design: Intermittent theta burst stimulation (iTBS) was applied to the left dorsolateral PFC (dlPFC) versus the vertex constituting the placebo condition. By contrasting the responses to both CSs in a generalization test 24 hours later, the CS+U revealed significantly higher arousal reports than CS+E and CS-. This was mirrored by higher skin conductance responses to CS+U than to the CS+E as well as the CS- and a trend-wise higher functional NIRS signal in the dlPFC most likely demonstrating remnants of the fear memory. Stimulation groups differed with regard to dlPFC activity, i.e. the active group exhibited an increased prefrontal engagement in response to the CS+U which was interpreted as a partial generalization of the extinction training. However, since there were no behavioural group differences, results were discussed in the light of a combined iTBS- extinction training as well as the stimulation time point which might constitute the important factor for preventing relapse of fear.

Introduction

Establishing a robust fear extinction memory is one of the most relevant topics in fear extinction learning and memory research. A conditioned stimulus (CS) that has been ascertained as a reliable predictor of an aversive event (unconditioned stimulus, UCS) through contingent pairings loses its predictive value when it is repeatedly presented in the absence of the aversive consequence. This effect of extinction learning is largely dependent on the formation of a new memory, rather than the simple deletion of the original fear memory trace (e.g. Bouton, 2002; Pavlov, 1927). Although the clinical application of extinction learning is quite effective in the treatment of anxiety disorders (Hofmann & Smits, 2008), relapse after successful exposure therapy is not uncommon. It occurs since the first-learned information (CS-UCS) acquires a second "meaning" through extinction (CS-noUCS) which is competing with the first learning trace causing behavioral instability (Bouton, 2002). In order to prevent relapse, experimental manipulations have been identified which are intended to improve behavioral fear extinction. One line of research implicates the stimulation of brain regions most likely involved in the consolidation of fear extinction memory. Based on translational research in animals and humans the amygdala and the ventromedial prefrontal cortex (vmPFC) have been shown to be critically involved in the acquisition and recall of extinction memory across species. The functional correlation between the amygdala and the vmPFC during extinction recall is in line with the idea that the vmPFC mediates extinction by suppressing the amygdala output via activation of inhibitory intercalated cells (Linnman, Zeidan, Pitman, & Milad, 2012; Quirk, Likhtik, Pelletier, & Pare, 2003). Sufficient vmPFC activation has been found to exhibit a precedent condition to consolidate and later express extinction memory in the human (Kalisch et al., 2006; Phelps, Delgado, Nearing, & LeDoux, 2004) and animal literature (Quirk & Mueller, 2008). Remarkably, high-frequent electrical micro-stimulation of the vmPFC in rats revealed a significantly lower conditioned response (CR) to a fear conditioned stimulus, even in an extinction recall test conducted 24 hours later and without a second stimulation (e.g. Kim, Jo, Kim, Kim, & Choi, 2010; Milad & Quirk, 2002; Quirk et al., 2003). Recently, we showed that one session of high-frequent repetitive transcranial magnetic stimulation (rTMS) of

the medial prefrontal cortex was associated with decreased CR in healthy human subjects in a similar vein (Guhn et al., 2014). Participants underwent a two-day discriminative fear conditioning protocol in which one neutral face (CS+) was paired with an aversive scream (UCS) while another was intended to function as a safety signal (CS-). Prior to perform the extinction training on day 1, a placebo-controlled offline rTMS protocol was administered to the medial prefrontal cortex of the participants. During the subsequent extinction training, the active group showed lower fear-potentiated startle (FPS) and skin conductance responses (SCR) to the former CS+ than the placebo group. The FPS effect outlasted to an extinction recall test on day 2 of the experiment. Thus, as rTMS has been shown to successfully enhance fear extinction its effects might further extend beyond the stimulus used during the treatment, i.e. fear extinction interventions should generalize to stimuli that are not part of the primary extinction training. Generalization is a highly desired therapeutic outcome aiming at maintaining effects beyond different feared situations or objects. On an experimental level, study designs have been implemented in which two CSs are fear-conditioned with the same US while only one CS gets subsequently extinguished (CS+E). By contrasting the responses to both CSs in an extinction recall test, behavioral responses to the extinguished CS (CS+E) represent extinction memory while behavioral responses to the second conditioned stimulus which is not extinguished (CS+U) represent fear memory recall. Ledgerwood and colleagues (2005) used this procedure to assess the effect of a psychopharmacological intervention with D-cycloserine, a partial N-methyl-D-aspartate (NMDA) receptor agonist acting at NMDA receptors. They found less fear responding to the non-extinguished CS in the DCS-treated rats compared to a control group treated with saline thereby reasoning DCS to enhance the generalization of fear extinction. Accordingly the aim of the present study was to investigate whether a prefrontal stimulation applied subsequently to the extinction of one out of two CS+ would similarly generalize to the extinction of the non-extinguished CS+U in a generalization test 24 hours later. Recall of the extinguished CS+E is expected to manifest in a smaller CR as behaviorally evidenced in decreased skin conductance reponses (SCR) as well as neurofunctionally in increased prefrontal activation. Intermittent theta burst stimulation, developed by Huang and colleagues (2005), was shown to produce increased cortical excitability outlasting the

comparatively short application time by up to 60 minutes (Wischnewski & Schutter, 2015). Similarly to the DCS effects, the aftereffects of TBS also depend on NMDA receptor activity (cf. Hoogendam, Ramakers, & Di Lazzaro, 2010). With regard to the successful interference with fear memory consolidation after stimulation of the left dorsolateral prefrontal cortex (dlPFC, Asthana et al., 2013) this region was targeted for the stimulation since the location of the vmPFC prohibits a direct stimulation with a non-invasive magnetic field. Moreover, the dlPFC has been linked to cognitive regulation of conditioned fear responses and has been shown to be anatomically and functionally linked with the vmPFC (Delgado, Nearing, Ledoux, & Phelps, 2008). In line with Ledgerwood et al. the active iTBS-group was hypothesised to show less fear in response to a non-extinguished CS than a placebo group owing to an increased dlPFC activity as measured with self reports, skin conductance responses (SCR) and functional near-infrared spectroscopy (fNIRS) .

Methods

Participants

Fifty-two healthy volunteers (29 women, 23 men) were recruited through local internet announcements. They were screened for the absence of mental disorders by using the Mini International Neuropsychiatric Interview (M.I.N.I., Sheehan et al., 1998), right-handedness, and contraindications concerning MRI measurements and iTBS according to safety guidelines (Wassermann, 1998; e.g. ferromagnetic material, cardiac pacemakers, pregnancy). Four participants had to be excluded due to left-handedness (n = 1), technical reasons during iTBS application (n = 2) and one measurement was stopped since the participant fell asleep. After participants were given a complete description of the study and its procedures, written informed consent was obtained in accordance with the Declaration of Helsinki in its latest version from 2008. An anatomical MRI scan was obtained before participating in the intended experiment in order to acquire a structural sequence necessary for the neuronavigation-based iTBS protocol. Afterwards, the participants were

invited to two consecutive study days to perform the experiment. All procedures were approved by the ethics committee of the University of Würzburg, Germany.

Paradigm

The experimental protocol was administered over two separate days. It consisted of a modified version of a paradigm used by Milad and colleagues that has demonstrated the vmPFC to be activated in response to an extinguished CS (CS+E) while a non-extinguished CS (CS+U) revealed a vmPFC deactivation (Linnman, Zeidan, Furtak, et al., 2012; Milad et al., 2007). On the first day, participants were made familiar with pictures of three male faces with neutral expressions (source: Tottenham et al., 2009) during a habituation phase in which the faces were presented four times each. In the fear acquisition phase two faces were paired with an aversive scream (UCS) at a partial reinforcement rate of 75 %. One of these was extinguished during the following fear extinction phase (CS+E) while the other was not (CS+U). A third CS was never paired with an aversive UCS (CS-). The fear acquisition phase consisted of 32 trials in total, 8 CS+E, 8 CS+U and 16 CS- presentations. All CS were presented for 6 s duration, the UCS co-terminated with CS+ trials and lasted 1380 ms. The scream was adapted from the International Affective Digital Sounds (Bradley & Lang, 1999) and was delivered at 97 dB binaurally through in-ear headphones. After a short break of approximately one minute in which participants answered valence and arousal ratings as well as a question concerning the awareness of the CS+UCS contingency, the extinction learning phase started consisting of 16 trials in total, 8 CS+E and 8 CS-. Day 2, representing the intended generalization test, consisted of an extinction recall phase, in which all three CS were presented resulting in 8 CS+E, 8 CS+U and 16 CS- trials. No UCS was delivered during or before the onset of the recall phase.

According to the protocol of Milad et al. (2007), the CS+ presentations across all experimental phases were sequential. All trials of one CS+ (the to-be-CS+U or the to-be-CS+E) were presented first, followed by all trials of the second CS+ due to the authors' experience that this sequential arrangement induced the most effective conditioning. The CS- was presented intermixed with both CS+. The mean inter-trial interval (ITI) was 11 s (range: 10 to 12). Presentation® software version 14.1

was used to present the paradigm and record self-reports. Participants were instructed to notice whether the CS and the UCS were related in any way. They were not informed about the non-occurrence of the UCS during the extinction and the generalization test. An instructed fear conditioning paradigm was preferred making successful fear acquisition more likely. Participants who did not correctly identify the CS- as safety cue were excluded from data analysis (n = 4). Sample characteristics of the remaining participants are shown in table 1. All experimental phases were conducted while SCR recordings were being acquired; on day 2 prefrontal cortex oxygenation was obtained additionally by means of functional near-infrared spectroscopy (fNIRS).

Table 1. Sample characteristic.

	dlPFC group (verum)	**vertex group (placebo)**
n (f/m)	22 (13/9)	22 (12/10)
age in years (SD)	26.7 (4.4)	25.5 (3.7)
education in years (SD)	12.95 (0.2)	13
mean hours day1-day2 (SD)	24.4 (1.3)	24.3 (1.4)
mean rating UCS (SD)	7.3 (1.2)	7.4 (1.2)
STAI trait anxiety	31.7 (7.1)	32.7 (7.5)
STAI state anxiety	33.4 (4.9)	35.5 (5.8)

Intermittent theta burst stimulation (iTBS).

At the end of day 1, an iTBS was administered intended to parallel the consolidation of the extinction memory. Participants were randomized into two groups: left dlPFC stimulation and vertex stimulation representing the control condition. TBS was applied with a circular coil (MCF-75, 65 mm diameter) by a Medtronic MagPro X100 stimulator (Medtronic MagPro, Düsseldorf, Germany). The iTBS consisted of 200 high-frequency triple-bursts (50 Hz), delivered in trains of 2s TBS (5-Hz theta rhythm) that was followed by a 8 s rest period repeated every 10 s for a total of 600 pulses (190 s). According to a review by Hoogendam et al. (2010) the effects of TBS are expected to last for a time period of 6 to 60 minutes after the end of the stimulation. In order to localize the stimulation site, a T1-weighted MRI was acquired for all subjects prior to the experiment. The stimulation site,

landmarked as the anterior part of the left middle frontal gyrus, was determined by using a neuronavigation system [LOCALITE GmbH, St. Augustin, Germany]. Participants were instructed to leave their eyes open and remain silent during the stimulation.

Behavioral, Psychophysiological and Functional Measures

Self-reports. CS valence and arousal ratings were assessed as behavioral indices of the CR after each experimental phase. At the end of the extinction training only CS+E and CS- had to be rated. Likert scales ranging from 1 “unpleasant” and “no arousal” to 9 “pleasant” and “high arousal” were used. In order to account for conscious awareness of the CS+UCS pairings at the end of the acquisition phase and thereby collecting the intended study sample, participants were asked to select the face which was not followed by the scream out of the presented three faces.

Skin conductance response (SCR). Recording and analysis was performed by use of the same equipment as described previously (Guhn et al., 2014; Guhn et al., 2012). In brief, peak responses in a time window between 1000 and 5000 ms following the baseline corrected (-1000 ms till CS onset) CS presentations were defined as CR. Visual artifact inspection identified n = 5 (n = 3 dlPFC, n = 2 vertex) non-responders, who were not considered for further data analyses. One further data set (dlPFC group) was lost due to a technical problem during the recording. SCR were z-standardized. The first trial of each phase was discarded in order to account for the fact that knowledge about the meaning of the CS could not have been achieved.

Functional near-infrared spectroscopy (fNIRS). Functional NIRS is an optical imaging technique operating with light from the near-infrared spectrum which is penetrating brain tissue and thereby delivering information about local blood oxygenation changes of the cortex (for more details please refer to Obrig & Villringer, 2003). The continuous wave system ETG-4000 with a 3 x 11 array of optodes, an interoptode distance of 3 cm and a sampling rate of 10 Hz (Hitachi Medical Co., Tokyo, Japan) was used to capture prefrontal cortex oxygenation during the generalization test on study day 2. With regard to the iTBS application on the day before, blood oxygenation was measured as an index of the hypothesized group differences in the fNIRS signal of optodes covering the left dlPFC.

Based on previous fNIRS results all signals were first visually inspected for technical or motion artifacts. Thereby five data sets had to be excluded due to technical errors with the recording and artifacts in channels covering the dlPFC. Afterwards the signal onset was time-locked to the end of the CS presentation, i.e. 6000 ms after the CS onset, and preprocessed with a cosine filter of 0.5 Hz to account for low-frequency signal drifts (cf. Guhn et al., 2012). A correlation-based signal improvement algorithm developed by Cui et al. (2010) was applied to the fNIRS data resulting in one integrated signal for both chromophores per channel. By differentiating between early (first four CS+E and CS+U respectively and first 8 CS- trails) and late trials (last four CS+E and CS+U respectively and last 8 CS- trials) six regressors were modeled as delta functions and convolved with a Gaussian hemodynamic response function at 6.5 s peak time. The mean of four channels covering the left dlPFC was calculated and defined as the region of interest since the iTBS was applied above this region (#8, #18, #19, #29; cf. Tupak et al., 2013). The corresponding channels in the right hemisphere (#3, # 13, #14, #24) were defined as the control region in which no group differences were expected. By applying a general linear model approach beta estimates of all regressors were analyzed by calculating a repeated-measurements ANOVA with stimulus (CS+E, CS+U, CS-) and phase (early, late) between groups (active, sham).

Statistical Analysis. Data analyses were performed with SPSS (Version 22, IBM Corporation, Armonk, NY) and Matlab software (Version 7; MathWorks Inc., Natick, Mass., USA). Sample characteristics were inspected for group differences by Student *t*-tests. None of the variables listed in table 1 reached the significance level of $p < .05$. For self-reports and SCR data, repeated-measurements ANOVAs were conducted in order to test for fear and extinction learning, at first. Stimulus (CS+E, CS+U, CS-) and phase (habituation, acquisition, and generalization test) were defined as within-subject factors; group (active iTBS, placebo) served as between-subject factor. Since only the intended interaction effect with group was expected for the generalization test on day 2, a repeated-measures ANOVA was then performed separately, consisting of stimulus (CS+E, CS+U, CS-) as single within-subject factor and group as between-subject factor.

Results

1. Self-reports.

Analyses revealed significant main effects for stimulus [valence: $F(2,84) = 5.68$, $p < .01$, arousal: $F(2,84) = 13.22$, $p < .001$] and phase [valence: $F(2,84) = 18.55$, $p < .001$; arousal: $F(1.6,67.7) = 22.72$; $p < .001$] and a significant stimulus x phase interaction of both valence [$F(4,168) = 13.81$, $p < .001$] and arousal reports [$F(2.8,117.4) = 17$ $p < .001$], but no effects involving the stimulation group ($F \leq 1.35$, $p \geq .255$). During the fear acquisition phase both CS+ revealed significantly lower valence [$T(43) \geq 5.55$, $p < .001$] and higher arousal scores than the CS- [$T(43) \geq 6.63$, $p < .001$]. While valence scores for both CS+ significantly increased from acquisition to extinction and generalization, the CS+U revealed significantly higher arousal reports during the generalization test [$T(43) = 2.21$, $p < .05$, see figure 1]. The ANOVA for the generalization test did not reveal significant interactions with group ($p > .48$).

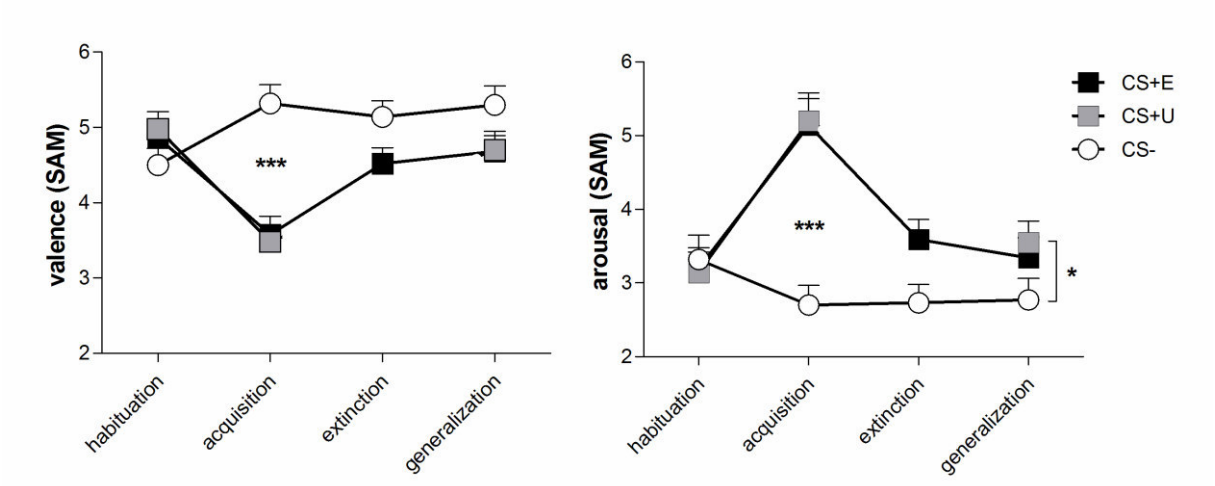

Fig. 1. Self-reports. Valence and arousal assessed on Likert-scales ranging from 1 for "unpleasant" and "no arousal" to 9 for "very pleasant" and "high arousal". The connecting line between acquisition and generalization phase (day 2) for the CS+U is missing since only the CS+E was extinguished on day 1.

2. Skin conductance response.

The ANOVA revealed a significant main effect of stimulus [$F(2,72) = 3.17$, $p < .05$] and a marginally significant main effect of phase [$F(1.2,41.5) = 2.92$, $p = .06$], while neither the stimulus x phase interaction ($p = .27$) nor interactions with group reached statistical significance (see figure 2). The

ANOVA for the generalization test revealed a trend wise significant main effect [$F(2,72) = 2.65$, $p = .077$], which was composed of marginally significant differences between the CS+U and the CS- [$T(37) = 1.93$, $p = .061$] and between the CS+U and the CS+E [$T(37) = 1.8$, $p = .074$], while CS+E and CS- did not differ ($p = .8$). Group did not reveal a significant interaction with stimulus.

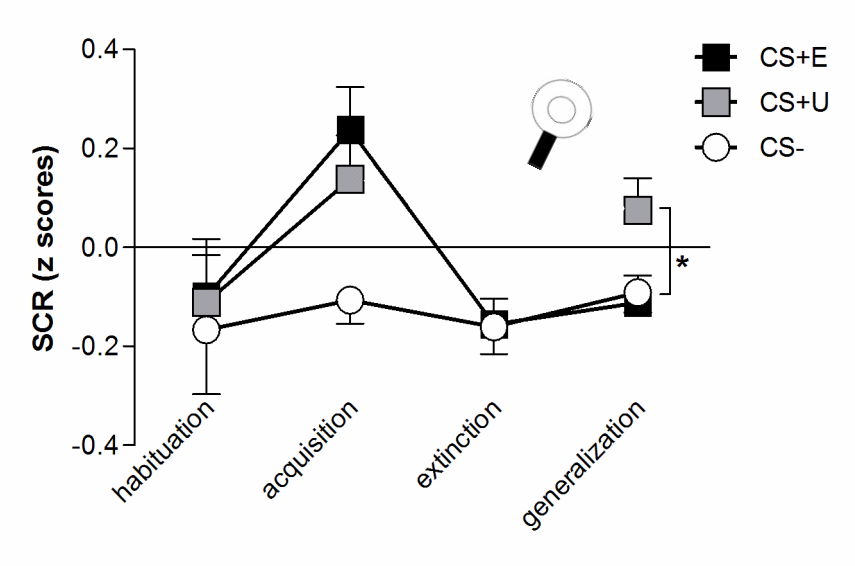

Fig. 2. Skin conductance responses (SCR). Z-standardized mean SCR (+SEM) for stimuli which were paired with the UCS during the acquisition phase (CS+E, CS+U) and the unpaired CS-. Again, the connecting line between acquisition and generalization phase (day 2) for the CS+U is missing since only the CS+E was extinguished on day 1.

3. Functional near-infrared spectroscopy (fNIRS) data

ANOVA for the left dlPFC revealed a trend wise significant interaction between stimulus and group [$F(2,72) = 2.61$, $p = .096$]. Since phase did not show significant main or interaction effects early and late trials were averaged and compared between groups. Post-hoc t-tests revealed marginally significant differences between all three CS only within the active group. Here, the fNIRS signal in response to the CS+U exhibited the largest dlPFC involvement as compared to the CS+E [$T(17) = -1.89$, $p = .076$] as well as the CS- [$T(17) = 1.84$, $p = .084$]. There were no significant differences within the vertex group. The only between-group comparison reaching a trend-wise significance level was a higher fNIRS signal for the CS+U in the dlPFC group [$T(36) = 1.76$, $p = .086$, see figure 3], while

responses to CS+E and CS- did not differ. ANOVA for the right dlPFC as control region did not reveal any significant main or interaction effects.

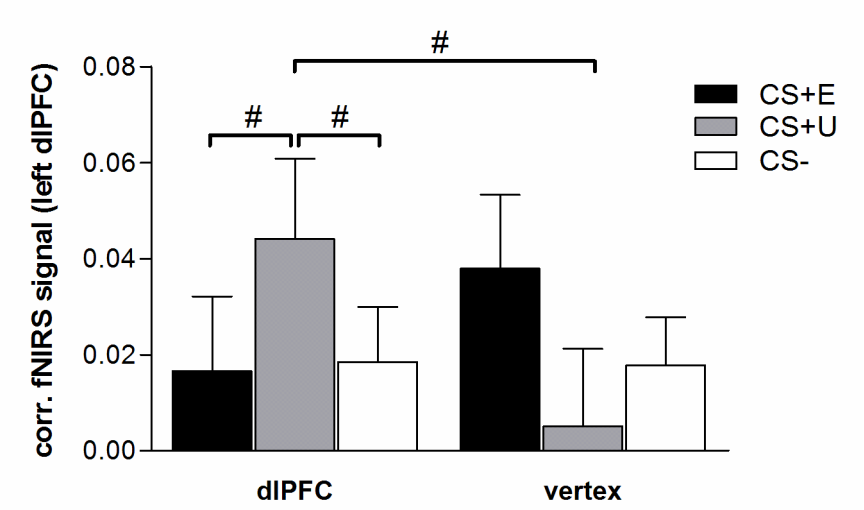

Fig. 3. Corrected fNIRS signal of the left dlPFC ROI during the generalization test for stimulation groups (dlPFC vs. vertex control stimulation). Displayed are means and SEM. The asterisks indicate trend-wise differences ($p < .1$).

Discussion

The present study was conducted in order to assess the generalization of extinction training to a non-extinguished conditioned stimulus after prefrontal intermittent theta burst stimulation. Healthy human subjects were fear-conditioned to two neutral face stimuli of which only one was extinguished during an immediate extinction training session. 24 hours later both CS+ were presented to investigate the generalization of the extinction training as indexed by self-reports and skin conductance responses. Functional NIRS was assessed in order to detect group differences concerning hemoglobin concentration changes in the stimulation site, the left dlPFC.

As intended, the non-extinguished CS+U exhibited higher arousal self-reports and a higher skin conductance response than the extinguished CS+E thereby demonstrating both the maintenance of the conditioned fear response for CS+U as well as the recall of extinction memory for CS+E. The significantly higher SCR to the CS+U was comparable to the results observed by Milad et al. (2007) from whom the present paradigm was modified. Regarding differences concerning the iTBS

intervention, groups did not differ on the behavioral level. However, compared to the vertex stimulated group (placebo) the actively stimulated group showed a trend-wise higher fNIRS signal in the left dlPFC to the CS+U while the responses to the CS+E and the CS- were similar. Following our hypothesis of reduced fear responses to the non-extinguished CS+U in the dlPFC group, the present results however disconfirm the assumption of a generalized extinction towards the psychophysiological fear response as indexed by self-reports and SCR but may confirm a partial generalization effect on the neural level as indexed by fNIRS. Since the psychophysiological and neurobiological results are inconsistent, the following discussion will be comprised of a pro-con debate on two explanations, i.e. iTBS did facilitate vs. iTBS did not facilitate the generalization of extinction.

In favor of the first alternative, the actively stimulated group showed the expected higher signal to the CS+U in the left dlPFC compared to the vertex stimulation group. This is assumed to represent an increased top-down modulation of the fear response due to the dlPFC stimulation following the extinction training with the CS+E on the previous day. Regarding the DCS effects in the rat experiments, iTBS can be speculated to enhance the consolidation of the extinction memory probably by devaluation of the UCS (cf. Baker, McNally, & Richardson, 2012; Ledgerwood et al., 2005). It has been argued that the presentation of the CS+ without the UCS during extinction nonetheless activates a representation of the UCS thereby decreasing the UCS' affective value owing to habituation. UCS devaluation and extinction training have been demonstrated to rely on overlapping mechanisms, both evidenced to be NMDA-dependent (Storsve, McNally, & Richardson, 2010). Since CS+E and CS+U were fear conditioned to the same UCS, the extinction training with the CS+E might have similarly resulted in UCS devaluation. With reference to the supposed iTBS influences on memory consolidation via NMDA-dependent mechanisms (cf. Hoogendam et al., 2010), the subsequently applied iTBS can be similarly suggested to increase UCS devaluation so that the dlPFC activity in response to the CS+U during the generalization test 24 hours later represent a heightened top-down modulation of the fear response. However, in line with this argumentation one would have expected to find a similar dlPFC activity for both CS+ during the generalization test but in

fact, the CS+U revealed a significantly higher signal than the CS+E. Furthermore, the iTBS groups did not differ with regard to their fear responses to the CS+E on the behavioral level considering the interpretation of a generalized extinction effect to the non-extinguished CS+U via UCS devaluation as merely speculative. Future investigations using fMRI which allows for a better spatial resolution of the PFC than fNIRS are needed to prove the suggested explanation. It is further advised to examine anxiety patients with this paradigm since owing to the supposed hypofrontality in these patients (e.g. Milad et al., 2009) the enhancement of the iTBS effect on prefrontal activity might be more pronounced. In this case, behavioral group differences should become visible.

The alternative, i.e. the iTBS intervention did not facilitate the generalization of extinction, namely contradicts earlier investigations in our lab in which (1) prefrontal stimulation significantly influenced conditioned fear responses tested in immediate extinction as well as extinction recall (Guhn et al., 2014) and (2) catholdal stimulation with tDCS inhibited fear consolidation in response to a CS+ that was similarly not extinguished prior to the stimulation (Asthana et al., 2013). However, an important modification in the present study was related to the timing of the stimulation. While in our first study the time point between the fear acquisition and the fear extinction was favored for the stimulation, in the present investigation the stimulation was intended to parallel the consolidation of extinction memory, i.e. it was conducted after the extinction phase. However, the failure of group differences even with regard to the extinguished CS+ (CS+E) points towards the conclusion that the stimulation should be carried out in relation to the *following* extinction learning, not afterwards. This interpretation contradicts the finding that DCS showed facilitating effects both before extinction training and afterwards in rodents (Ledgerwood et al., 2005), but supports a finding on rTMS intervention in a clinical trial in which offline rTMS coupled with a *subsequent* imaginal trauma exposure in patients suffering from a refractory post-traumatic stress disorder (PTSD) revealed decreased hyperarousal symptoms compared to a placebo stimulation (Osuch et al., 2009). An artificially boosted prefrontal activity (e.g. via rTMS) preceding learning is thought to facilitate the formation of a new memory in the target region by interacting with the activity subsequently induced during the task. This might also explain why iTBS in patients suffering from panic disorder did

not result in higher prefrontal activation after receiving 15 iTBS sessions above the left dlPFC (Deppermann et al., 2014) since iTBS was applied independent of any prefrontal demand. With regard to the usefulness of DCS as augmentation strategy to exposure therapy administered prior to treatment (e.g. Ressler et al., 2004), iTBS might be equally useful as add-on strategy.

In conclusion, the present study comprises partial evidence for a generalization effect of iTBS on extinction training, but needs to be replicated with regard to the optimal timing (i.e. preceding the extinction training) to apply stimulation. In order to discriminate whether iTBS is then affecting fear memory or extinction memory consolidation, both learning phases should be furthermore performed in different learning contexts (cf. Milad et al., 2007). Besides the optimal timing to apply stimulation, the appropriate methods that have been used to impact memory consolidation are on debate (cf. Marin, Camprodon, Dougherty, & Milad, 2014; Pirulli, Fertonani, & Miniussi, 2013), i.e. in the last years positive results on the facilitation of fear extinction has been published by using different techniques for instance deep brain stimulation (DBS, Rodriguez-Romaguera, Do-Monte, Tanimura, Quirk, & Haber, 2015) or vagus nerve stimulation (e.g. Pena, Engineer, & McIntyre, 2013). The inhibition of subcortical regions that show hyperfunction in response to fearful stimuli like the amygdala (e.g. Goossens, Sunaert, Peeters, Griez, & Schruers, 2007) would represent another possible target region for the augmentation contrasting the here intended increase of activity in prefrontal regions associated with the acquisition of extinction memory and its consolidation. More studies are warranted to understand the mechanisms behind the augmentation with brain stimulation techniques in order to gain insight for which patients these techniques might provide a more sufficient treatment outcome keeping in mind that some patients remain symptomatic after having completed the existing treatment strategies.

Acknowledgement

The present study was funded by the German Research Foundation (DFG: SFB-TRR-C06) and the Research Training Group RTG 1253/2. Thomas Dresler was partly supported by the LEAD graduate school [GSC1028], a project of the Excellence Initiative of the German federal and state governments.

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4. General discussion

4.1 How Did the Story of *Little Albert* Continue?

Watson and Rayner (1920) speculated that *Albert's* fear "responses in the home environment are likely to persist indefinitely, unless an accidental method for removing them is hit upon" (p. 12), but since *Albert* was taken from the hospital unexpectedly and before the experimenters could perform the proposed strategies of "re-conditioning" (cf. chapter 2), it is some kind of a mystery what ever happened to *Little Albert*. Did he continuously suffer from a persistent and generalized anxiety disorder to all furry objects resulting in an isolated life?

The real identity of *Little Albert* has long intrigued researchers and even these days the debate does not seem to be concluded. In 2009, Beck et al. came up with the name *Douglas Merritte*, who – regarding their argumentation – shared many characteristics with the descriptions of *Albert*; amongst other things his time of birth, the fact that he used to live in the John Hopkins Hospital for almost a year where his mother was employed as a wet nurse as well as some physical resemblances, many of them allegedly corresponding to the descriptions Watson made of "*Albert B.*" (cf. Watson & Rayner, 1920). With regard to the medical records of *Douglas Merritte* which Beck et al. assembled, pediatric neurologists were subsequently asked to analyze the video material which has been made of the experiments on *Little Albert* – probably more or less unaware which famous child they are investigating. They raised an remarkable conclusion: *Douglas* aka *Albert* probably suffered from several severe medical conditions such as a congenital obstructive hydrocephalus as indicated by observable behavioral and neurological deficits (Fridlund, Beck, Goldie, & Irons, 2012). This in turn threw up doubts on the health and robustness by which Watson claimed to have selected his subject, thereby aggravating his disreputability in terms of the unethical experiments on *Albert*. The mismatch also lead Russell Powell (2011) to re-analyze the argumentation of Beck et al. (2009) concluding that the evidence for *Little Albert's* supposed identity as *Douglas Merritte* was not sufficiently proofed. He and his coworkers hence posted the name *William Albert Barger*, who was born at the same time as *Douglas Merritte* but, in contrast, was described as remarkably healthy and well-developed just like Watson had claimed it for *Albert* (Powell, Digdon, Harris, & Smithson, 2014). In contrast to *Douglas*

Merrittee who died at the age of six due to his neurological disorder, *William Albert Barger* used to live a long life until his death in 2008, unfortunately before researchers could have identified him as *Little Albert*. However, in 2014, his niece Dorothy Parthree, gave an interview to a newspaper, *The Chronicle of Higher Education*, telling that her uncle did not like dogs in fact but beyond that used to have a good life without pathological fears. She was not able to remember her uncle talking about fear experiments that he was forced to do when he was a baby, probably evidencing that not even *Albert* remembered them on his own (Bartlett, 2014).

Provided that *William Albert Barger* was actually *Little Albert*, this opens up a discussion on protective factors that might have prevented *Albert* from developing a persistent anxiety disorder owing to his stability and fearlessness. By introducing the so-called "intermediate phenotype concept" in the fourth manuscript, vulnerability factors associated with a risk gene for anxiety will be exemplified. A summary of the results of all included studies then constitutes the main part of the general discussion by focusing on the respective strengths and weaknesses. Based on these findings the last part is hence directed on research questions and hypotheses arising from the presented studies.

4.2 Excursus: The Concept of Intermediate Phenotypes of Anxiety

Genetic factors have been attributed to explain one third of the variance emerging from inter-individual differences obtained from fear conditioning and extinction studies in humans (Hettema, Annas, Neale, Kendler, & Fredrikson, 2003). In order to locate a specific risk gene or a risk gene constellation which is associated with an anxiety disorder, the consideration of so-called "intermediate phenotypes" has been proven to be advantageous. Intermediate phenotypes are defined as neuropsychological traits or neurobiological markers, such as fear learning or increased amygdala reactivity, which link genetic risk variants to psychiatric diseases. They are considered to be closer to the underlying genetic risk factor than the overall categorical disease phenotype of psychiatric disorders (Flint & Munafo, 2007). By using imaging techniques to reveal intermediate phenotypes, the so called "imaging-genetics approach" has pointed to genetically driven alterations in several neurotransmission systems in pathological anxiety, which have been shown to mediate emotional processing in the brain fear circuit (Domschke & Dannlowski, 2010). An outline of the numerous findings on risk genes contributing to the pathogenesis of anxiety is beyond

the scope of this dissertation. The interested reader is thus referred to comprehensive reviews in the field: Regarding genetic association studies on fear conditioning and extinction in particular (Lonsdorf & Kalisch, 2011), concerning clinical genetic studies on anxiety disorders in general (Smoller, 2015) as well as clinical genetic studies on anxiety disorders with special regard to the imaging-genetics approach (Domschke & Dannlowski, 2010).

In order to exemplify the usefulness of intermediate phenotypes for the understanding of pathological anxiety, the following study examined the impact of a functional polymorphism in the neuropeptide S receptor 1 gene (*NPSR1* rs324981 A/T) associated with anxiety on cognitive emotion regulation with regard to anxiety sensitivity (AS) representing a neuropsychological intermediate phenotype. AS refers to the fear comprising the consequences of anxiety, for instance bodily sensations which are misattributed as being harmful hence inducing anxiety (Reiss, Peterson, Gursky, & McNally, 1986). These anxiety-related personality traits are regarded to represent biological predispositions (Rauch, Shin, & Phelps, 2006) closely related to pathological fear and anxiety (e.g. Schmidt, Zvolensky, & Maner, 2006). It has been demonstrated that *state anxiety*, referring to a transitory affective state (cf. Spielberger, Gorsuch, & Lushene, 1970 for state and trait anxiety), was associated with elevated fear responses during extinction (Vriends et al., 2011) and further interfered with the top-down control over threat-related distracters, i.e. high-anxious participants showed less prefrontal recruitment (Bishop, Duncan, Brett, & Lawrence, 2004). Similarly, Sehlmeyer et al. (2011) found high *trait anxiety*, which represents a relatively enduring personality trait, to be associated with prolonged and exaggerated fear responses during the late phase of fear extinction as evidenced by an increased amygdala and a decreased dACC activation. The authors hence suggest high-anxious subjects to be at a higher risk for fear relapse since they seem to be unable to maintain the inhibitory mPFC activity during the extinction process. To some extent contradictory, Barrett and Armony (2009) reported prefrontal recruitment of the subgenual ACC during fear extinction to be positively associated with trait anxiety assuming a compensatory mechanism for a hyper-responsive amygdala in high-anxious subjects. However, all studies assert resemblance to the altered mPFC activity reported for PTSD patients (Etkin & Wager, 2007). The additional consideration of anxiety-related personality traits as an intermediate phenotype is thus

assumed to elucidate why some individuals develop pathological anxiety for instance after trauma while others do not (e.g. Milad et al., 2008).

The fourth manuscript closely focuses on this line of argumentation and is intended to contribute to the knowledge on risk constellations that increase the vulnerability for anxiety disorders. By using an imaging-genetics approach the associations between a genetic risk polymorphism (*NPSR1*) and AS on cognitive emotion regulation in healthy participants was examined. NPS has been demonstrated to be related to anxiety in animals (e.g. Xu et al., 2004) and humans, both in healthy participants (e.g. Dannlowski et al., 2011; Glotzbach-Schoon et al., 2013) as well as in PD patients (Domschke et al., 2011; Donner et al., 2010; Okamura et al., 2011). The risk variant of the *NSPR1* gene polymorphism, the T-allele, is thereby suggested to impair emotion regulation strategies which are necessary to cope with negative emotions, probably mediated through AS since T-allele carriers have been further identified to exhibit an increased AS (Klauke et al., 2014).

Interestingly, the mechanisms of cognitive emotion regulation have been shown to overlap with the neural structures involved in fear extinction (cf. Etkin, Egner, & Kalisch, 2011; Schiller & Delgado, 2010), e.g. the instruction to regulate the participant's feeling in the anticipation of a UCS resulted in a decreased amygdala activity and an increased prefrontal activity (Delgado, Nearing, Ledoux, & Phelps, 2008). Besides the amygdala and the mPFC, the lateral PFC, notably the dlPFC, has been shown to be additionally engaged during cognitive emotion regulation (Ochsner & Gross, 2008). However, since there is no direct connection between the dlPFC and the amygdala, the inhibitory influence of the dlPFC on the amygdala function that underlies a decreased CR is engaged via connections from the dlPFC to the vmPFC (Delgado et al., 2008), thus relying on similar mechanisms as fear learning and extinction.

4.2.1 Study 4: Multi-level Effects of Genotype and Anxiety on PFC Activity

By means of fNIRS, dlPFC activity was examined during a working memory task composed of emotionally negative or positive as well as neutral pictures in healthy subjects who were further examined concerning AS. With regard to the reviewed hypofrontality in pathological anxiety (see chapter 2.1), *NPSR1* was expected to influence cognitive emotion regulation presumably mediated through AS.

OXFORD

Social Cognitive and Affective Neuroscience, 2015, 1–8

doi: 10.1093/scan/nsv061
Original article

Neuropeptide S receptor gene variation and neural correlates of cognitive emotion regulation

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Abstract

The neuropeptide S (NPS) and its receptor NPSR have captured attention in the pathogenesis of anxiety disorders. Here, a functional polymorphism in the *NPSR1* gene has been linked to deviant cortico–limbic interactions in response to negative stimuli. While healthy T allele carriers exhibited increased amygdala and prefrontal cortex activity, panic disorder patients carrying the T risk allele displayed hypofrontality possibly reflecting insufficient prefrontal inhibition of limbic reactivity. In order to study multi-level effects of genotype and anxiety, prefrontal cortex activity during an emotional n-back task was measured in 66 volunteers genotyped for the *NPSR1* rs324981 A/T variant (AA homozygotes *vs*. T allele carriers) by means of functional near-infrared spectroscopy. For a high working memory load (3-back), T allele carriers showed a signal increase to negative pictures in the dorsolateral and medial prefrontal cortex while AA homozygotes displayed a signal decrease. Since groups did not differ on skin conductance level and behavioral parameters, this effect in the risk group in line with results from fMRI studies is speculated to represent an adaptive mechanism to compensate for presumably increased subcortical activity driven by an overactive NPS system. However, anxiety sensitivity correlated negatively with prefrontal activity in T allele carriers possibly suggesting a decompensation of the adaptive compensatory upregulation.

Key words: neuropeptide S; *NPSR1*; emotional working memory; anxiety; fNIRS

Introduction

In the last few years, the neuropeptide S (NPS) system has captured much attention as a promising novel pathomechanism of anxiety disorders (Tsuzuki *et al*., 2007). NPS administration in mice has been shown to produce anxiolytic-like effects in a battery of behavioral tests: NPS significantly increased the exploration of less protected or brighter areas in the open field (Xu *et al*., 2004; Jüngling *et al*., 2008), prolonged the time mice spent in the light zone of a light–dark box as well as within the open arms of the elevated plus maze (Xu *et al*., 2004; Jüngling *et al*., 2008), and dose-dependently reduced the number of marbles that were buried in the marble burying task (Xu *et al*., 2004; Vitale *et al*., 2008). In addition, NPS demonstrated arousal-promoting effects as indicated by an increase in locomotor activity and wakefulness (Xu *et al*., 2004). Pharmacologically, NPS binds to a G-protein-coupled receptor (NPSR) that stimulates intracellular calcium concentrations and cyclic adenosine monophosphate accumulation (Reinscheid *et al*., 2005). These NPS receptors are widely distributed in the central nervous system with highest expressions in the cortex, thalamus, hypothalamus and the amygdala (Xu *et al*., 2004; Reinscheid and Xu, 2005). The effects on synaptic transmission to and within the

Received: 15 July 2014; **Revised:** 12 March 2015. **Accepted:** 8 May 2015

amygdala are of particular relevance since an increased glutamatergic synaptic transmission to intercalated GABAergic neurons in the amygdala has been identified to accompany the effects of NPS administration on mice behavior (Jüngling *et al.*, 2008).

While NPS is associated with anxiolytic-like effects in the rodent model, investigation of the NPS system in humans revealed divergent but nonetheless anxiety-related results: The human gene coding for the NPS receptor (*NPSR1*) on chromosome 7p14 contains an A/T single nucleotide polymorphism (SNP, rs324981) leading to an amino acid exchange ($Asn^{107}Ile$), with the T allele (^{107}Ile) conferring a 10-fold increased *NPSR1* expression and NPS efficacy at the receptor (Reinscheid *et al.*, 2005). The more active T allele was consistently found to be overrepresented in patients with panic disorder (Okamura *et al.*, 2007; Donner *et al.*, 2010; Domschke *et al.*, 2011). The T allele was also associated with increased autonomic arousal as evident in a heightened heart rate and more intense symptom reports during a behavioral avoidance test (Domschke *et al.*, 2011). Paralleling these findings, healthy T allele carriers showed significantly higher fear ratings in a Pavlovian conditioning experiment than AA homozygotes (Raczka *et al.*, 2010). The *NPSR1* T allele was further found to be associated with significantly elevated anxiety sensitivity (AS)—reflecting the tendency to cognitively (mis-)interpret anxiety-related bodily sensations (Reiss *et al.*, 1986) and constituting an intermediate phenotype and risk factor of pathological anxiety (Schmidt *et al.*, 1997, 1999, 2006)—in healthy probands in interaction with early life stress (Klauke *et al.*, 2012) as well as in patients with panic disorder (Domschke *et al.*, 2011).

Anxious individuals have been shown to be highly susceptible to emotionally loaded material (Bar-Haim *et al.*, 2007), resulting in a loss of concentration and impairments in executive functioning for the actual task, which has been linked to a reduced recruitment of top-down control mechanisms in the brain fear circuit (Bishop *et al.*, 2004). In imaging genetics approaches, *NPSR1* gene variation has been reported to drive a deviant cortico–limbic interaction potentially reflecting dysfunctional emotional processing. Healthy T risk allele carriers showed significantly increased amygdala activation along with increased dorsolateral prefrontal cortex (dlPFC), orbitofrontal cortex (OFC) and dorsal anterior cingulate (ACC) activity when passively watching fearful face stimuli. This increased prefrontal activation was suggested to represent a compensatory increased top-down regulation of amygdala activity evoked by negative emotional stimuli (Dannlowski *et al.*, 2011). Conversely, in a sample of panic disorder patients investigated with a similar task of passive emotion perception, the *NPSR1* T allele group showed decreased prefrontal cortex activity which was discussed as insufficient prefrontal inhibition of limbic activity in clinically manifest pathological anxiety (Domschke *et al.*, 2011).

To explicitly study multi-level effects of genotype and anxiety levels on cognitive emotion regulation, this study investigated healthy volunteers for their response to an emotional n-back task depending on the functional *NPSR1* A/T SNP (rs324981) by means of functional near-infrared spectroscopy (fNIRS), skin conductance level (SCL) and behavioral data. Based on the findings reviewed above, negative pictures were hypothesized to induce an increased prefrontal recruitment detectable with fNIRS in carriers of the more active T risk allele. On the other hand, increased AS, as an intermediate phenotype of pathological anxiety, was hypothesized to lead to a decompensation of this adaptive prefrontal upregulation in *NPSR1* T risk allele carriers as expressed by lower prefrontal recruitment during the processing of negative emotional stimuli.

Materials and methods

Participants

Sixty-six healthy Caucasian volunteers (female = 33, male = 33; mean age = 25.36 ± 4.8; years of education = 12.91 ± 0.5) participated in this study. They were recruited through online advertisements and screened for current mental health using the Mini International Neuropsychiatric Interview (Sheehan *et al.*, 1998) and for right handedness using the Edinburgh Handedness Inventory (Oldfield, 1971). In order to assess *NPSR1* genotype group differences on state and trait measurements of anxiety, the state version of the State-Trait-Anxiety Inventory (STAI; Laux *et al.*, 1981) and the Anxiety Sensitivity Index (ASI; Reiss *et al.*, 1986) were administered (STAI state anxiety = 32.23 ± 6; ASI = 13.42 ± 8.5). All participants signed written informed consent before taking part in the experiment and were reimbursed with 15 Euro. The study was approved by the Ethics committee of the University of Würzburg, Germany, and was conducted in accordance with the declaration of Helsinki in its latest version from 2008.

Genotyping

Genotyping of the functional *NPSR1* rs324981 A/T (Asn107Ile) polymorphism was performed according to published protocols (e.g. Bishop, 2009; Domschke *et al.*, 2011, 2012). In brief, DNA isolated from venous blood samples was amplified by PCR using the primers F: 5′-GAA GGA AAA AAA TTA AAA ATG AAC CTC CCC AGG ATT TCAT and R: 5′-TTC TAC CCA GGA GAA AGC GGG CAG TTT GAT GCA, resulting in an amplicon size of 353 bp. Standard PCR was carried out in a 20-ml volume containing 45–60 ng of genomic DNA, 10 pmol of each primer, 200 mM dNTPs, 0.4U Taq DNA Polymerase (Eppendorf, Hamburg, Germany), 50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris–HCl (pH 8.4). After a 5-min denaturation, 35 cycles were carried out consisting of 30 s at 94°C, 30 s at 66°C and 60 s at 72°C, followed by a final extension time of 10 min at 72°C. Amplicons were digested with TasI (Fermentas, St Leon-Rot, Germany) (1 U), separated for 2 h on a 15% polyacrylamide gel and visualized by silver-staining. Due to genotyping failure in two probands, a sample of $N = 64$ remained for further analyses. Hardy–Weinberg criteria, as calculated by the online program DeFinetti (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl; Wienker TF and Strom TM), were fulfilled for genotype distribution (AA = 28, AT = 30, TT = 6, $P = 0.78$). For further analyses, *NPSR1* genotypes were grouped according to functionality and on the basis of previous studies assuming a dominant role for the T risk allele (AA *vs* AT/TT; Raczka *et al.*, 2010; Domschke *et al.*, 2011). The groups are further referred to as AA homozygotes on the one hand and T allele carriers for participants with at least one T allele (AT/TT genotype carriers) on the other hand.

Emotional n-back task

The task consisted of 90 colored photographs derived from the Emotional Picture Set (EmoPicS; Wessa *et al.*, 2010). Based upon the normative data provided for the EmoPicS database, they were selected according to their valence and arousal in order to group (a) 30 pleasant and (b) 30 unpleasant pictures with moderately high arousal and (c) 30 neutral pictures inducing only little to no arousal. Pleasant pictures depicted athletic activities, children

and couples in love (excluding erotic scenes). Unpleasant pictures depicted war scenarios showing injured or crying people, and neutral pictures mostly depicted people reading or walking without any emotional expression. All pictures illustrated people excluding merely sheer artificial or naturalistic content. Pictures were presented in nine blocks counterbalanced by three different working memory load manipulations namely 1-back, 2-back and 3-back. Each emotional category was thus presented once in all three n-back levels. The sequential arrangement of blocks was pseudorandomized in three different versions to prevent learning effects and habituation to the emotional picture content. Versions were counterbalanced across participants. Each block had a duration of 60 s and consisted of 30 pictures of which six were target trials. Pictures were presented for 500 ms followed by an inter-trial interval of 1500 ms depicting a black screen. The n-back level of each block was announced by an instruction slide and was started individually by the participant. Blocks were separated by a resting period of 30 s in which participants were instructed to relax.

To become familiar with the task, participants practiced each n-back level beforehand with pictures, which were not selected for the actual task. They were instructed to respond as fast and accurate as possible by button presses, irrespective of the emotional picture content. After the experiment, all participants evaluated the pictures regarding valence and arousal on two Likert scales ranging from 1 for 'very unpleasant' to 9 for 'very pleasant' and 1 for 'no arousal' to 9 for 'high arousal'.

Skin conductance level

SCL was measured by using two Ag/AgCl electrodes which were attached to the hypothenar eminence of the left hand. Recording was performed via the Vision recorder software (Brain Products GmbH, Munich, Germany), which operates with a sampling rate of 1000 Hz. Data were offline low-pass filtered at 12 Hz to correct for signal drifts. Each block was further baseline corrected 500 ms before block onset. Due to the response latency of the SCL, signal blocks were analysed 4 s after trigger onset. Mean activity of the resulting 56 s segments was calculated.

Functional near-infrared spectroscopy

Prefrontal cortex activation was measured by means of fNIRS, a non-invasive optical imaging technique which is explained in detail elsewhere (Obrig and Villringer, 2003). In brief, light from the near-infrared spectrum penetrating biological tissue is inducted to the skull by light emitters and gets partly absorbed in depth up to 2.5 cm of the cortex (Hoshi *et al.*, 2005) by oxygenated (O_2Hb) and deoxygenated (HHb) hemoglobin. The amount of reflected light at the surface can be detected, providing thus cortical concentration changes of O_2Hb and HHb. With regard to neurovascular coupling, neural activation is associated with increasing O_2Hb and decreasing HHb theoretically correlating perfectly negative (Cui *et al.*, 2010).

Hemoglobin concentration changes were measured with the continuous wave system ETG 4000 (Hitachi Medical Co., Tokyo, Japan) operating with two different wavelengths (650 ± 20 and 830 ± 20 nm). In order to cover the whole prefrontal cortex a 52-channel array consisting of 17 light emitters and 16 photo detectors was used. The middle detector in the lowest row was positioned on Fpz according to the 10-20 EEG system (Jasper, 1958), the lateral optodes extended approximately to T3 and T4. The interoptode distance was set to 3 cm. Data were recorded with a sampling rate of 10 Hz.

Data analysis

Preprocessing and statistical analysis were performed by using Matlab (2009a, The MathWorks Inc., MA, USA), Vision Analyzer 2.0 (Brain Products GmbH) and SPSS version 21 (IBM SPSS Statistics, Munich, Germany). On the behavioral level, accuracy was calculated as the ratio of hits and correct rejections to total number of trials (cf. Grimm *et al.*, 2012). Moreover, mean reaction times on hits were investigated. These parameters and SCL were analysed by repeated measurements (ANOVA) with working memory load (1-, 2-, 3-back) and emotion (positive, neutral, negative pictures) as within-subject factors and group (*NPSR1* genotype AA, T) as between-subject factor. Significant interaction effects were further elucidated by post-hoc Student's *t* tests at a significance level of $P < 0.05$ (two-tailed). In addition, *t*-contrasts were referred as Pearson's correlation coefficients (r_{con}) in order to provide an effect size with $r_{con} > 0.5$ characterizing large effects (e.g. Rosnow and Rosenthal, 2005). Non-sphericity was considered by applying the Greenhouse Geisser correction.

Based on the assumption that O_2Hb and HHb should be negatively correlated, a correlation-based signal improvement algorithm developed by Cui *et al.* (2010) was applied to the fNIRS data resulting in one integrated signal of both chromophores per channel (for previous studies using this algorithm please refer to e.g. Müller *et al.*, 2014; Tupak *et al.*, 2014). These signal changes were processed by applying a low-pass filter of 0.5 Hz and a cosine filter correcting for low-frequency signal drifts. The resulting nine segments had duration of 50 s starting 10 s after block onset. They were baseline corrected by using the time window of 5–4.5 s before block onset which represents the inter block resting period before participants were instructed with the following n-back condition. In order to analyse working memory load by emotion effects five regions of interest were defined (ROIs, see Figure 1): right dlPFC (channels 4, 14, 15, 25), left dlPFC (7, 17, 18, 28), right ventrolateral prefrontal cortex (vlPFC: 35, 45, 46), left vlPFC (39, 49, 50) and the medial prefrontal cortex (mPFC: 16, 26, 27, 37). The ROIs were chosen according to probabilistic registration methods (Tsuzuki *et al.*, 2007) and practical considerations. The dlPFC channels cover the middle frontal gyrus, the vlPFC channels the inferior frontal gyrus. The mPFC channels comprise the closest channels in the vicinity of the interhemispheric fissure above the medial PFC. According to the behavioral data analyses, ROIs were statistically evaluated by repeated measurements ANOVA with working memory load (1-, 2-, 3-back), emotion (positive, neutral, negative) and hemisphere (right, left) as within-subject factors and group (*NPSR1* genotype AA, T) as between-subject factor. fNIRS results were further correlated with measurements of anxiety depending on *NPSR1* genotype by calculating Pearson's (ASI) and Spearman's (STAI) correlation coefficients. To keep the amount of correlations as low as possible, only positive (mean positive – mean neutral) and negative picture blocks (mean negative – mean neutral) were analysed. Correlations were further evaluated concerning significant ($P_{one\text{-}tailed} < 0.05$) group differences by using the Fisher *r*-to-z transformation.

Results

Picture ratings

As expected, ANOVA revealed a main effect of valence [$F(1.8,113.1) = 763.56$, $P < 0.001$], with significant differences between all picture categories (positive > neutral > negative)

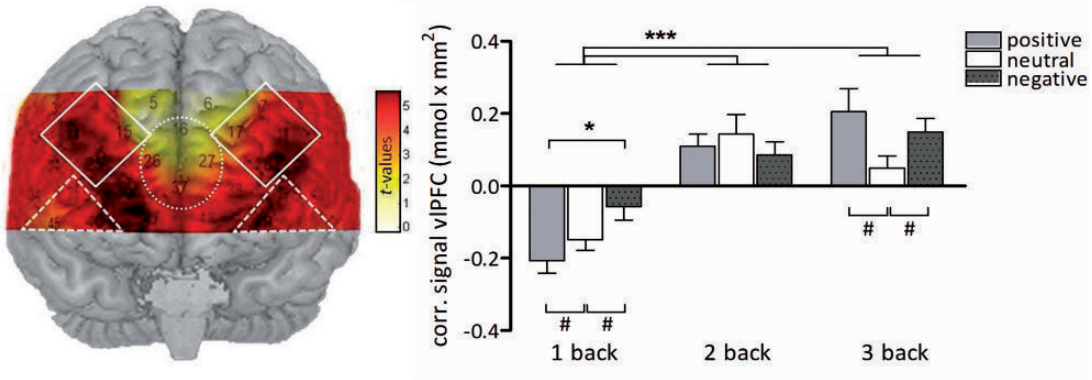

Fig. 1. Left: T-map superimposed on a standard brain showing the signal increase with increasing task demands (3-back *vs* 1-back, FDR corrected). Geometrical figures depict the ROI: dlPFC (rhombs), vlPFC (triangles), mPFC (oval). Right: Task-evoked corrected signal changes for the vlPFC in the whole group of $N = 66$. Asterisks indicate significant differences concerning a *P*-value which is either uncorrected ($^{\#}P < 0.05$) or corrected for multiple comparisons ($^{*}P_{corr} < 0.006$). The significant main effect of condition is indicated by $^{***}P < 0.001$.

[$t(64) \geq 18.8$, $P < 0.001$, $r_{con} \geq 0.92$]. Arousal also revealed a significant main effect [$F(2,128) = 206.65$, $P < 0.001$], with positive and negative pictures showing an equally high arousal [$t(64) = 1.78$, $P > 0.05$], while both significantly differed from the neutral picture category [$t(64) \geq 16.89$, $P < 0.001$, $r_{con} \geq 0.82$]. *NPSR1* genotype group did not reveal significant main or interaction effects.

Behavioral results

ANOVA revealed a main effect of condition [$F(1.4,87.2) = 132.52$, $P < 0.001$] and a significant condition × emotion interaction [$F(3,185.9) = 2.79$, $P < 0.05$]. To further elucidate this interaction, accuracy scores depending on emotional category were compared on every n-back level resulting in significantly higher accuracy scores for positive *vs* neutral pictures in the 3-back condition [$t(63) = 2.28$, $P < 0.05$, $r_{con} = 0.28$]. *NPSR1* genotype group did not reveal significant main or interaction effects.

Reaction times revealed a main effect of condition [$F(1.7,104.4) = 238.5$, $P < 0.001$], i.e. the more difficult the task the longer the reaction times [3-back > 2-back: $t(63) = 18.7$, $P < 0.001$; 2-back > 1-back: $t(63) = 13$, $P < 0.001$; $r_{con} > 0.85$]. Neither emotion nor genotype group showed significant main or interaction effects.

SCL results

Six participants were excluded as non-responders; data of two other participants were lost due to a technical problem during recording. ANOVA revealed significant main effects of condition [$F(1.8,95.1) = 6.88$, $P < 0.05$] and emotion [$F(2,108) = 6.11$, $P < 0.05$] and a marginally significant condition × emotion interaction [$F(1.7,91.3) = 3.17$, $P < 0.1$]. Medium and high working memory load were associated with an increased SCL [dependent *t*-test 1-back *vs* 2-back: $t(55) = -3$, $r_{con} = 0.38$; 1-back *vs* 3-back: $t(55) = -3.27$, $r_{con} = 0.4$; $P < 0.01$], and positive picture blocks evoked higher responses than neutral and negative blocks [positive *vs* neutral: $t(55) = 3.29$, $r_{con} = 0.41$; positive *vs* negative: $t(55) = 2.99$, $r_{con} = 0.37$; $P < 0.01$]. Positive pictures evoked a higher SCL than neutral pictures in the 1-back condition and also a higher SCL than negative pictures in the 2-back condition [$t(55) \leq 4.17$, $r_{con} \leq 0.49$, $P \leq 0.005$, Bonferroni corrected]. *NPSR1* genotype group-dependent analysis did not result in significant interactions.

fNIRS results

Whole group results (N = 66)

For the dlPFC, working memory load revealed a significant main effect [$F(1.8,112.2) = 12$, $P < 0.001$] which manifested in a linear signal increase from 1- via 2- to 3-back [linear trend test for condition: $F(1,63) = 18.17$, $P < 0.001$], and was more pronounced in the right than the left hemisphere [main effect of hemisphere: $F(1,63) = 10.96$, $P < 0.001$]. For the mPFC, condition exerted a main effect as well [$F(2,130) = 6.83$, $P < 0.01$], again showing a linear signal increase with difficulty [$F(1,65) = 10.95$, $P < 0.01$]. The working memory-related main effect was also present in both vlPFCs [$F(2,130) = 31.43$, $P < 0.001$], again with marginally higher values for the right hemisphere [main effect for hemisphere: $F(1,65) = 5.72$, $P < 0.05$]. In addition, a significant interaction between condition and emotion was discerned [$F(3,194.7) = 4.66$, $P < 0.01$] showing negative pictures to evoke a higher fNIRS signal than positive pictures at the 1-back level [paired sample *t*-test: $t(65) = 3.44$, $P_{corr} < 0.006$, $r_{con} = 0.39$, see Figure 1].

Results stratified for NPSR1 genotype

Due to genotyping failure in two participants, a sample of $N = 64$ remained for further analyses which however did not affect the results of the whole sample. Genotype groups (AA = 28 *vs* T = 36) did not differ in terms of sex, age, level of education and measures of anxiety (Table 1).

The interaction between working memory load and emotion revealed significant *NPSR1* genotype group differences for the dlPFC [$F(4,248) = 3.32$, $P < 0.05$] and the mPFC ROI [$F(4,248) = 5.2$, $P < 0.001$]. In order to disentangle the condition × emotion × hemisphere by *NPSR1* genotype group interaction for the dlPFC, post-hoc *t*-tests were performed and revealed significant group differences for the condition × emotion interaction in the left hemisphere. Here, the interaction with genotype group mainly consisted of a different activation pattern for positive and negative pictures in the 3-back condition. AA homozygotes displayed a higher fNIRS signal for positive pictures than T allele carriers [independent *t*-test: $t(62) = 2.22$, $P < 0.05$, $r_{con} = 0.27$], while T allele carriers showed a higher signal to negative pictures than AA homozygotes [$t(62) = 2.45$, $P < 0.05$, $r_{con} = 0.3$]. A comparable reciprocal activation pattern was evident in the mPFC: again, AA homozygotes showed a marginal signal increase for positive pictures along with increasing working memory load [3-back working memory load: $t(62) = 1.89$, $P < 0.1$, $r_{con} = 0.23$], whereas T allele carriers showed a signal increase

Table 1. Sample characteristics

	All	*NPSR1* AA	*NPSR1* T	P[a]
Sex (m/f)	33/31	15/13	18/18	0.806
Age	25.36 ± 4.8	25.5 ± 5.7	25.25 ± 4.2	0.839
Education (years)	12.91 ± 0.5	13	12.83 ± 0.7	0.211
ASI	13.37 ± 8.6	11.39 ± 7.8	14.92 ± 9	0.105
STAI state	32 ± 5.9	32.11 ± 5.9	31.92 ± 6	0.900

ASI, Anxiety Sensitivity Index; STAI state, state version of the State Trait Anxiety Index. Calculated are means and SEM.

[a]P-values indicate between-group differences as calculated by independent Student's *t* tests or Chi-square test

for negative pictures with increasing difficulty [3-back working memory load: $t(62) = 2$, $P \leq 0.05$, $r_{con} = 0.25$; Figure 2]. There was no group difference in response to neutral pictures, neither in the left dlPFC nor the mPFC ROI [$t(62) \leq 1.2$, $P < 0.05$].

The two ROIs for which significant interactions with *NPSR1* genotype were found were further analysed for correlations with ASI and STAI anxiety scores in both groups. Here, in T-risk allele carriers, but not in AA homozygotes, AS was significantly related to the fNIRS signal in the mPFC for negative ($r = -0.45$, $P < 0.01$) as well as for positive ($r = -0.35$, $P < 0.05$) pictures, in that *NPSR1* T allele carriers with high ASI scores showed significantly less mPFC activation. Accordingly, the ASI score was by trend inversely correlated with left dlPFC activation in response to negative pictures in T allele carriers only ($r = -0.31$, $P = 0.062$). All correlation coefficients significantly differed between groups ($z \geq 1.68$, $P < 0.05$) indicating a specific relationship between prefrontal activation and AS for the T allele group (Figure 3). In addition, in T allele carriers, but not in AA homozygotes, STAI state anxiety revealed an inverse correlation with mPFC ($r = -0.41$, $P < 0.05$) and left dlPFC activation ($r = -0.41$, $P < 0.05$) in response to negative pictures. Due to the fact that ASI scores and STAI state anxiety were significantly correlated, we conducted a partial correlation between STAI state anxiety and ROI activity while controlling for the ASI effect. This analysis confirmed the aforementioned results (mPFC: $r_{part} = -0.47$, dlPFC: $r_{part} = -0.52$, $P < 0.005$). However, correlation coefficients did not significantly differ between groups ($z \leq 1.01$, $P > 0.1$) emphasizing a specific relationship between AS and not STAI state anxiety and prefrontal activity in the T allele group.

Discussion

This study investigated the effects of *NPSR1* gene variation on emotional working memory by means of an emotional n-back task consisting of three working memory load conditions (1-, 2- and 3-back) and three picture categories (positive, neutral and negative pictures). While genotype groups (AA homozygotes *vs* T allele carriers) did not differ with regard to behavioral parameters such as accuracy and reaction times or skin conductance, the analyses of hemoglobin concentration changes in the prefrontal cortex measured with fNIRS revealed genotype-specific results in the mPFC and the left dlPFC: In the high working memory load condition (3-back), T allele carriers showed a signal increase in response to negative pictures and a signal decrease in response to positive pictures, while AA homozygotes displayed a reciprocal pattern. When additionally considering AS, a high ASI was associated with significantly decreased mPFC and left dlPFC activation in T allele carriers.

From previous studies it is known that the ability to control and modulate emotional responses depends on a cortical top-down modulation of the limbic system. For instance, Hariri *et al.* (2000) reported an inverse relationship between the prefrontal cortex and the amygdala in conscious semantic processing of emotional stimuli with the PFC exerting a modulating effect on emotional experience. In this study, *NPSR1* T-risk allele carriers showed significantly increased prefrontal activation (mPFC, dlPFC) in response to negative pictures in the highest working memory load condition, demanding utmost cognitive control. Given converging evidence for the more active *NPSR1* T allele to constitute a risk factor for panic disorder, to be associated with increased autonomic arousal and heightened fear conditioning (Okamura *et al.*, 2007; Donner *et al.*, 2010; Raczka *et al.*, 2010; Domschke *et al.*, 2011) and to drive higher amygdala activation along with increased dlPFC, OFC and dorsal ACC activity in response to fearful faces in healthy probands (Dannlowski *et al.*, 2011), the presently observed higher prefrontal engagement in response to negative emotional stimuli in T allele carriers may be interpreted as an adaptive compensatory engagement counterbalancing a presumably increased subcortical activity as conferred by an overactive NPS system. According to Dannlowski *et al.* (2011), the increased prefrontal activity might either be associated with an increased subjective experience of negative emotions or reflect an increased emotion regulation to cope with the requirements of the working memory task. Since the interaction between emotion and cognition can be considered as a competition for attentional resources (Vytal *et al.*, 2012), negative stimuli might have captured more attention than positive ones, probably on the basis of a threat-related attentional bias in anxious individuals (Bishop *et al.*, 2004; Bar-Haim *et al.*, 2007). However, in this study this was not evidenced on the behavioral level since accuracy reached a score of at least 93% even in the 3-back condition suggesting a ceiling effect. Neurobiologically, high arousal has been referred to an increased activity in both the amygdala and the dlPFC during an emotional working memory task (Perlstein *et al.*, 2002). Orientation toward threat associated stimuli in *NPSR1* T allele carriers in this study is also supported by the T allele carriers' tendency to over-interpret the harmfulness of aversive events and increased harm avoidance (Raczka *et al.*, 2010; Domschke *et al.*, 2011). In contrast, *NPSR1* AA homozygotes, i.e. non-risk allele carriers, displayed decreased prefrontal activation in response to negative and increased activation in response to positive pictures in the 3-back working memory load condition, which is in line with healthy participants exhibiting the same pattern in emotional word n-back tasks (Grimm *et al.*, 2012; Kopf *et al.*, 2013) as well as in an emotional picture detection task (Perlstein *et al.*, 2002). In accordance with Kopf *et al.* (2013), who found decreased prefrontal cortex activity on negatively valenced and increased activity on positively valenced word stimuli for the 2-back and 3-back conditions, the observed activation patterns in response to positive and negative pictures strongly point to a valence effect not confounded by arousal, as in this study positive and negative pictures were selected to achieve a comparable level of moderate arousal. Neutral pictures, which did not require the regulation of emotions but rather require mere working memory demands, did not result in significant group differences neither in the dlFPC nor in the mPFC. This supports the notion that differences observed in tasks with valenced pictures, which did show group differences for prefrontal activity, indeed are due to emotion regulation and not mere working memory processes. While the existing literature primarily focuse on top-down modulation of negative or

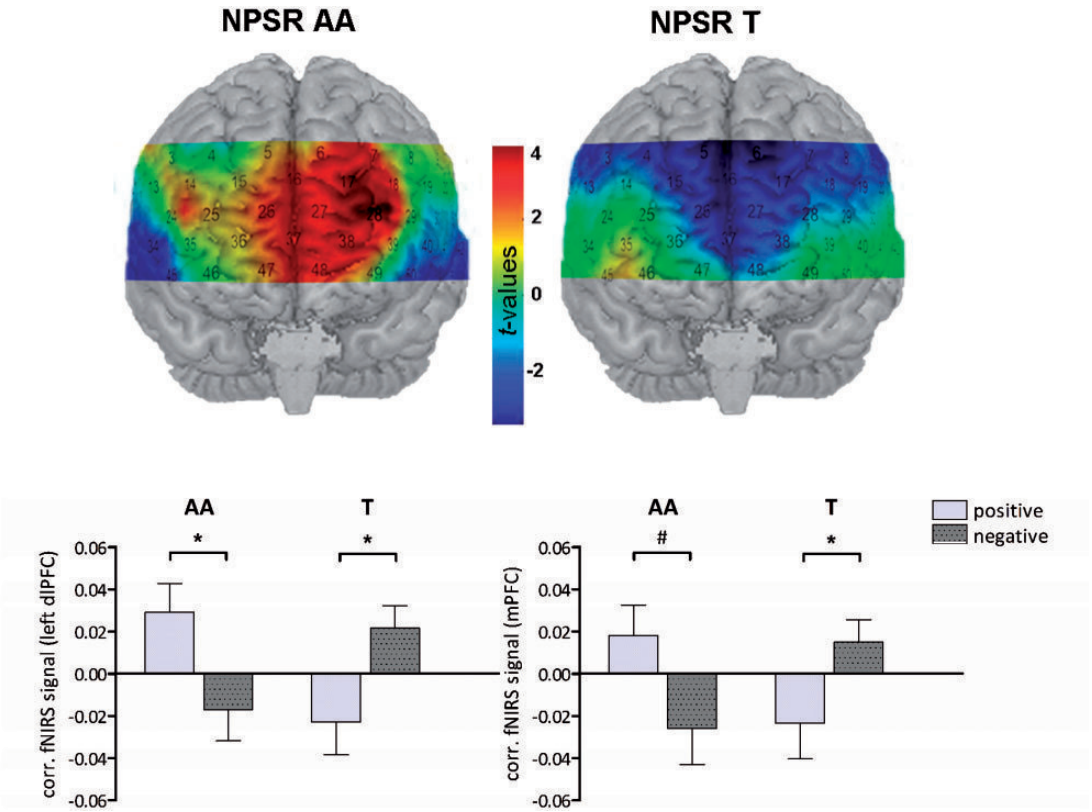

Fig. 2. Above: within-group fNIRS signals for prefrontal activation during the 3-back working memory load for positive *vs* negative pictures stratified for *NPSR1* genotype. While AA homozygotes (left) showed an increased fNIRS signal in regions covering the mPFC and left dlPFC, T allele carriers (right) showed deactivations in these areas. Depicted are *t*-values for all 52 channels ($P_{uncorr.} \leq 0.05$). Below: Corrected fNIRS signal changes in the left dlPFC (left) and mPFC (right) for positive and negative pictures (3-back condition) showing a significant interaction with *NPSR1* genotype (AA *vs* T). While in AA homozygotes the fNIRS signal was increased for positive pictures, T allele carriers responded to negative pictures with a signal increase. Depicted are means and SEM. Asterisks indicate significant differences ($^{*}P < 0.05$), the rhomb mark indicates a trendwise significant result ($^{\#}P < 0.1$).

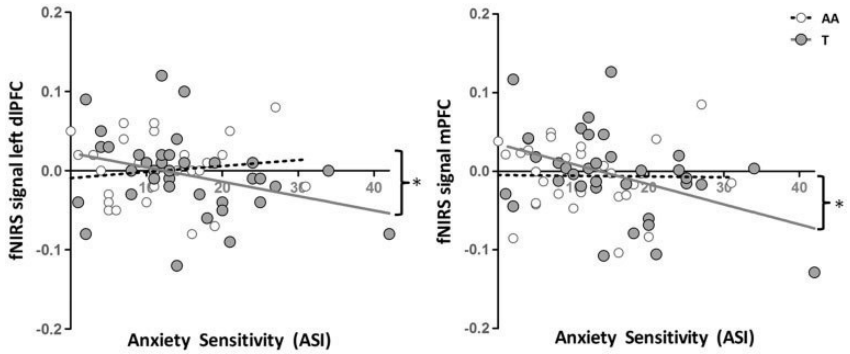

Fig. 3. Scatterplots showing *NPSR1* genotype (AA *vs* T) dependent significant correlations of left dlPFC activation (left) and mPFC activation (right) with anxiety sensitivity measured by the ASI in response to negative pictures. Asterisks indicate significant group differences for correlation coefficients ($z \geq 1.68$, $P < 0.05$).

aversive stimuli, the PFC involvement in response to positive emotions is rather under examined. Perlstein *et al.* (2002) proposed dlPFC activity in response to positive pictures to reflect activity of an appetitive system that is able to enhance cognitive functioning through an increased prefrontal dopamine turnover. In line with this notion, the tendency to over interpret the harmfulness of aversive events in T risk allele carriers (Raczka *et al.*, 2010) might also hinder positive pictures from being processed. Together with our finding of decreased prefrontal activity to positive pictures, future studies investigating *NPSR1* might also focus on deviant perception of positive emotions.

Interestingly, when additionally considering measures of anxiety (AS, state anxiety), AS and state anxiety were negatively correlated with mPFC and left dlPFC activation in response to negative pictures in *NPSR1* T allele carriers. Notably, both genotype groups in this study did not differ on anxiety measures *per se* (i.e. ASI and STAI, see Table 1) most probably reflecting the fact that healthy volunteers who were free of a diagnosis with anxiety disorders were investigated. However, in T risk allele carriers, the correlation between ASI and prefrontal activation was evident, while in AA homozygotes the correlation coefficient was close to zero (see Figure 3). Increased subclinical

anxiety thus was interpreted to lead to a decompensation of the previously adaptive compensatory upregulation of mPFC/dlPFC activity in healthy *NPSR1* T risk allele carriers. This is of interest since AS has been shown to be highly predictive of anxiety disorders especially panic disorder (Schmidt *et al*. 1997, 1999, 2006). The proposed maladaptive mPFC/dlPFC hypoactivity potentially reflecting an insufficient cortical top-down modulation during emotional processing (cf. Bishop *et al*., 2004; Bishop, 2009) against the background of a combined genetic and clinical-risk factor constellation is in line with previous reports of the *NPSR1* T allele being associated with increased AS in healthy probands with increased early adversity (Klauke *et al*., 2012) and panic disorder patients (Domschke *et al*., 2011). Furthermore, this interpretation is supported by the observation of decreased dlPFC activation in response to negative emotional stimuli in patients with clinically manifest panic disorder carrying the *NPSR1* T risk allele (Domschke *et al*., 2011). It thus can be speculated that the *NPSR1* T allele does not constitute a risk factor for pathological anxiety *per se*, since in healthy probands the assumed higher amygdala activity is suggested to be compensated by an upregulation of the PFC. This is not surprising considering the high prevalence of the *NPSR1* T allele. However, when *NPSR1* T allele carriers additionally exhibit high AS, this cognitive vulnerability to anxiety is suggested to impair the top-down modulation of the PFC entailing an increased risk of panic disorder. Thus, subclinical anxiety might impair the upregulation of the PFC to compensate for a subcortical fear response in T risk allele carriers potentially constituting a vulnerability factor for the development of panic disorder.

The following limitations have to be taken into account: The above interpretations of the results of our fNIRS study in an emotional n-back task are only justified in conjunction with functional MRI studies in complimentary emotional tasks. Imaging studies need to prove the speculated compensatory engagement of the PFC in the T group by demonstrating an increased functional coupling with the amygdala in the context of downregulating negative stimuli. Beyond, trial-by-trial valence reports should be included in such follow-up studies to further define the emotion regulation processes. Future investigations in larger, independent samples are warranted to replicate the suggested combined risk factor constellation. In particular also, this study was underpowered to investigate the described interaction between *NPSR1* and gender as an additional between-subject factor (cf. Domschke *et al*., 2011). The present sample consisted of university students and graduates. This might have been the reason why genotype groups did not show differences on the performance level although performance deficits can be expected when prefrontal compensation has reached its limit (cf. Siegmund *et al*., 2011).

In conclusion, this multi-level investigation of prefrontal cortex activity during emotional working memory and the interaction with premorbid anxiety supports a strong role of NPS and its receptor in the genetic and neural underpinnings of anxiety and anxiety disorders. In conjunction with comparable findings they may stimulate future studies exploring the potential of therapeutic agents targeting the NPS system in anxiety disorders (cf. Ionescu *et al*., 2012; Lukas and Neumann, 2012).

Acknowledgements

We thank M. Wessa and colleagues for providing the EmoPics.

Funding

This study was funded and supported by the German Research Foundation (SFB-TRR 58, Project B06 to A.R., Project C02 to K.D. and J.D., Project C06 to M.J.H., Project Z02 to J.D. and A.R.). J.D., M.J.H. and A.R. received support by the DFG and Länder funds RTG 1256/2 'gk emotions' and the Comprehensive Heart Failure Center Würzburg funded by the BMBF (Project 01EO1004). T.D. was partly supported by the LEAD graduate school [GSC1028], a project of the Excellence Initiative of the German federal and state governments.

Conflict of interest. None declared.

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4.2.2 Integration of Study 4 into the Findings on Pathological Anxiety

The presented study suggests those subjects who exhibit a specific risk factor constellation, i.e. the *NPSR1* T genotype and high AS, to be at an increased risk to develop pathological anxiety although the investigated participants were free of any anxiety diagnosis by the time of the study. The consideration of AS as an intermediate phenotype related to the cognitive vulnerability to anxiety is further in line with Raczka et al. (2010) who suggested catastrophizing overinterpretations of fear reactions to represent the link between the *NPSR1* T-allele and the development of PD. Without considering AS, the T risk allele was associated with compensatorily increased dlPFC activity similar to previous results (Dannlowski et al., 2011). The existent risk factor combination of AS and the *NPSR1* T genotype, though, resulted in a diminished prefrontal activation that is suggested to cause a reduced top-down control over the amygdala. The neurofunctional mechanism underlying this increased vulnerability to anxiety is thus assumed to be based on the same mPFC-amygdala coupling that has been evidenced to rely on fear learning and fear extinction as well as its alterations in anxiety disorders.

Eventually, the consideration of intermediate phenotypes is assumed to guide treatment strategies for a better therapy outcome (Domschke & Dannlowski, 2010). The presented study for example may suggest patients at increased risk for PD due to the AS/*NPSR1* risk constellation to benefit from an enhanced treatment, for instance more cognitive interventions (cf. Raczka et al., 2010) or more exposure sessions (cf. Vriends et al., 2011) or – in order to return to the main topic of this dissertation - an augmentation of exposure sessions with rTMS.

4.3 What Did We Learn from Fear Extinction and its Enhancement via rTMS?

The following part is directed to summarize and combine the presented studies concerning the involvement of the mPFC during extinction learning (study 1), the impact of prefrontal rTMS on extinction learning and extinction recall (study 2) as well as the impact of prefrontal rTMS on the generalization of extinction from one CS to another (study 3). The main findings of these studies can be outlined as follows:

Study 1: Over the time course of an extinction training, O_2Hb concentration in response to CS+ vs. CS- trials significantly increased in a cluster of probe set channels covering the mPFC. Prefrontal activity was negatively correlated with SCR, i.e. successful extinction as evidenced as a decrease in CR from early to late CS+ extinction trials was paralleled by an increase in mPFC activity.

Study 2: One session of high-frequent rTMS above the mPFC cluster identified in study 1 revealed diminished CR in an actively stimulated group vs. placebo during subsequent extinction training as well as during the recall of extinction memory 24 hours later. Confirming the interpretation of an enhanced top-down control over the amygdala, the active group showed a higher O_2Hb concentration in the mPFC than the placebo group during extinction learning.

Study 3: The effect of rTMS on the generalization of an extinction training from one CS+ (CS+E) to another CS+ which was not extinguished (CS+U) was partially supported by an increased fNIRS signal to the CS+U in the left dlPFC, though on the behavioral level the actively stimulated group did not differ from a placebo condition.

The inclusion of study 4 into this dissertation concerning the above presented intermediate phenotype concept was intended to open up future perspectives about the application of rTMS by considering specific risk factor constellations known to increase the vulnerability for anxiety disorders or impede with extinction learning, respectively:

Study 4: The T risk variant of the *NPSR1* gene polymorphism was associated with increased prefrontal activity to emotionally negative stimuli during an emotional working memory task and is thereby suggested to represent a compensatorily increased top-down control over a hyper-active amygdala due to an over-active NPS system. However, the consideration of AS leads to a decompensation of the top-down control in terms of a decreased prefrontal activity which may be speculated to increase the vulnerability for anxiety disorders.

4.3.1 Strengths of the Studies

The most important strength of the studies included in this dissertation is their origination from translational research on fear conditioning and fear extinction in animals. It is this knowledge about fear and extinction learning mechanisms outlined in chapter 1 which

enables the formation of hypotheses for the understanding of pathological anxiety in the first place (Milad & Quirk, 2012). All the herein presented studies thereby contain preclinical human research conducted with healthy volunteers, nonetheless, the obtained results can be transferred to anxiety disorders thus contributing to the understanding of neural mechanisms involved in pathological fear learning and anxiety. They were further oriented towards the search for treatment strategies to augment exposure therapy for the treatment of anxiety disorders, thereby fulfilling two main requests: (1) the combination of rTMS *and* trauma-related (CS) stimuli as originated from the animal literature (Baek et al., 2012), and (2) the establishment of laboratory evidence for the enhancement of extinction memory before considering rTMS as an adjunct to psychotherapy in clinical models (Marin et al., 2014). In this regard, the studies 1 to 3 were built upon each other as follows:

First, *study 1* aimed at investigating mPFC involvement during fear extinction by the usage of fNIRS. While fNIRS was neither evidenced before nor recommended owing to its limitations for examining the fear network (see chapter 4.3.2), it was nonetheless appropriate for the purpose of defining a target region for the intended prefrontal enhancement by rTMS (study 2) since both, fNIRS as well as rTMS are comparably restricted to the cortex (cf. Epstein et al., 1990; Strangman et al., 2002). Consequential, the interpretation of the fNIRS results was certainly impeded by missing previous fNIRS studies on extinction learning; however, the applied fear conditioning and extinction paradigm was designed relying on earlier investigations using fMRI with regards to the employed stimuli, the stimulus duration as well as the number of stimulus presentations and thereby enables the integration of the results into the literary context. Further confirmation for the mPFC cluster which was found to be involved during extinction learning arose from study 2 in which the increase of O_2Hb concentration from early to late extinction trials was replicated for the sham group who did not undergo an active rTMS.

By having defined an appropriate rTMS target region*, study 2* then was conducted to investigate the facilitation of extinction through rTMS. With regard to the size of the mPFC cluster of 10 probe set channels found in study 1, a rTMS round coil was used, which produces a larger magnet field than the more frequently used figure-of-eight coils which target a focal and localized magnetic field of approximately one square centimeter (Hallett, 2000). Moreover, the stimulus parameters of the fear conditioning and extinction paradigm were kept constant, with the exception of implementing a 25 minutes break for applying the

rTMS as well as a second extinction phase in order to test for extinction recall, which resembled the first extinction training in terms of the stimuli used as well as the number of stimulus presentations. Study 2 further convinced by the usage of a number of dependent variables indexing the CR. Recording SCR as well as FPS and moreover self-reports of valence and arousal aimed at indirectly assessing amygdala activation in response to the fear-conditioned stimuli since on the one hand, the induced fear responses (CR) are known to be generated through projections from the amygdala to the brainstem (LeDoux, 2003) and on the other hand, fNIRS did not allow for a direct investigation due to its restriction to cortical structures (Strangman et al., 2002). Strikingly, all dependent variables did point in the same direction namely towards a facilitation of extinction, notably extinction learning. This substantiated the conclusion that extinction learning was modulated by an increased prefrontal activation through rTMS, notwithstanding the slightly differing sample sizes mostly due to the unconditionability of a minority of participants. In terms of the homogeneity of the study sample, only premenopausal naturally cycling women were recruited for study 2. Even though it complicated the recruitment of probands, this ensured the exclusion of one confounding factor, since estrogen has been shown to influence fear extinction (e.g. Glover et al., 2012). Thus, the female participants did not take oral contraceptives for at least three month prior to participation in the study and were investigated only during the early follicular phase defined as the first five days of a regular menstrual cycle. The low estrogen levels during this phase have been associated with extinction deficits in female rats (Milad, Igoe, Lebron-Milad, & Novales, 2009), healthy women (Milad et al., 2010) as well as in women with PTSD (Glover et al., 2012). They have been further evidenced to resemble the stress response system activated in the male brain during fear extinction recall (Milad, Goldstein, et al., 2006) and during the processing of emotionally stimulus material (Goldstein, Jerram, Abbs, Whitfield-Gabrieli, & Makris, 2010). Since study 2 aimed at rTMS effects on fear modulation, the controlling for hormonal status opens up the analysis of a gender by rTMS interaction which revealed partial evidence for women to additionally benefit from rTMS (for more details please refer to the supplementary material of study 2).

Subsequently, *study 3* aimed at the generalization of extinction through rTMS as derived from the DCS administration in rats. Since the generalization from an extinguished CS+ to a non-extinguished CS+ was only partially evidenced by a higher dlPFC involvement

for the non-extinguished stimulus, this study however attracts attention with regards to the time point of application when rTMS might be an advantageous add-on to exposure therapy. While comparing the rTMS application before undergoing an extinction training (study 2) with the rTMS application thereafter (study 3), the evidence arising from both studies point towards an application *prior* to the extinction training probably due to a summation of synaptic plasticity induced through rTMS and the subsequent learning (see chapter 4.4). However, particularly study 3 has to face several limitations and its results need to be thus regarded cautiously. The following part on the weaknesses of the studies will now attend to these and other general limitations.

4.3.2 Weaknesses of the Studies

For particular limitations of each study please refer to the subsequent discussion sections of each manuscript. This part is rather intended to discuss limitations that apply to the majority of the included studies of this dissertation.

The most impeding limitation of all studies (except study 4) that emerged during the scientific peer-review process when submitting the manuscripts to international journals was the usage of fNIRS for the study of fear. As outlined above, the usage of fNIRS did have advantages with respect to the aims of studies 2 and 3, however, its disadvantages have to be mentioned likewise. The penetration depth of the near-infrared light when travelling through the skull and the brain tissue beneath is restricted to about 1.5 cm (Quaresima et al., 2012; Strangman et al., 2002). Thus, hypotheses are limited to cortical regions thereby disregarding deeper situated brain structures. However, as outlined in chapter 1, the mechanisms identified to be engaged during fear learning and fear extinction besides the mPFC basically comprise the amygdala as well as the hippocampus. The investigated research questions regarding the top-down control over the amygdala's fear response thus remain hypothetically. In order to overcome this limitation all studies aimed at indirectly investigating amygdala activity by assessing psychophysiological responses known to be generated by neural pathways originating from the amygdala (FPS, Davis et al., 1997) or correlating with amygdala activation (SCR, e.g. Furmark et al., 1997). However, the proposed regulation of the amygdala through an increased top-down control of the mPFC during extinction (study 1) and through rTMS (studies 2 and 3) has to be replicated and quantified by means of imaging techniques which exhibit a higher spatial resolution than fNIRS and thus

enable the investigation of limbic structures. The usage of fMRI for instance would allow for testing the proposed mPFC-amygdala interplay by conducting connectivity analyses.

Accompanying the restrictions of fNIRS, the second limitation refers to the assumption that rTMS facilitated extinction (studies 2 and 3). While searching for adjuncts to exposure-based therapy in individuals suffering from anxiety disorders two different memory processes constitute targets, i.e. either the decrement of the *fear memory* or the enhancement of the *extinction memory* (Marin, Lonak, & Milad, 2015). The interpretation of the results of study 2 is based on the modulation of *extinction* learning; however, the experimental setups of this study as well as that from *study 3* did not allow disentangling both memory processes from each other. In order to clarify which precise memory process has been influenced through rTMS, the application of rTMS should be investigated in a placebo-controlled design without performing the extinction training. If actively stimulated participants then respond with a lower CR when re-confronted with the CS+ as compared to a placebo group, an inhibition of the fear memory consolidation would be most likely. However, a rat study on rTMS effects on fear extinction which was published while study 2 was in process found 10 Hz rTMS to reveal reduced freezing as well but only in conjunction with an extinction training (Baek et al., 2012). RTMS performed independently of an extinction training (but unfortunately in a different experimental chamber than that of the fear conditioning) did not result in a lower CR when compared to rats that were just handled (control condition) instead of receiving rTMS. The activation of the fear or trauma memory thus seems to be a precondition for modulating the fear responses by means of rTMS. Since this study did neither investigate the sole rTMS effect in the fear environment which would have activated the fear memory in favor of the explanation of an inhibiting effect of the fear memory over the enhancement of the extinction memory, it remains an open question which learning memory was modulated. However, concerning the clinical implementation both explanations result in the same favored outcome, i.e. reduced fear responses. They would merely differ with regard to the need for additionally perform an exposure session after having reactivated the fear memory. So far, there is evidence for rTMS application in conjunction with trauma reminders (e.g. Baek et al., 2012; Osuch et al., 2009) against the conventional rTMS treatment without attempting the combination with exposure therapy in clinical trials (e.g. Deppermann et al., 2014). Future studies are thus needed to encounter the optimal temporal pairing of brain stimulation with cognitive activation.

Study 3 investigated rTMS applied subsequently to extinction training and failed to find behavioral evidences for an enhanced extinction recall even to the extinguished CS+E. However, the comparability of the studies 2 and 3 is unfortunately hampered by the manipulation of three parameters within study 3, notably the stimulation site (mPFC cluster in study 2 vs. left dlPFC in study 3), the rTMS protocol (10 Hz rTMS in study 2 vs. iTBS in study 3), as well as the time point of rTMS application (prior to extinction training in study 2 vs. subsequent to extinction training in study 3). Although the stimulation site was chosen with regard to the inhibition of fear consolidation after cathodal transcranial direct-current stimulation (tDCS) above the left dlPFC (Asthana et al., 2013), the evidence for an enhanced effect of iTBS on cognitive functions of prefrontal regions is sparse (Grossheinrich et al., 2009). It would have been more elaborated to retain the mPFC stimulation site as well as the 10 Hz protocol and solely alter the time point of stimulation relying on animal literature which comprised the basis for this study idea (cf. Ledgerwood et al., 2003; Ledgerwood et al., 2005). While considering for these limitations, the following part is intended to derive conclusions from all above reported study results for the clinical context.

4.4 Conclusions: From the Preclinical to the Clinical Model

Extinction learning and extinction recall rely on an increased mPFC recruitment (studies 1 and 2) which has been evidenced to down-regulate amygdala reactivity in response to feared stimuli causing decrements in fear expression (CR). Brain stimulation via rTMS demonstrated to influence prefrontal activity thereby modulating fear (studies 2 and 3). These preclinical results give rise to the development of improvements on treatment strategies for anxiety disorders since patients suffering from pathological anxiety are characterized by deficits in the presented fear mechanisms. Most patients do benefit from exposure therapy which relies on the principles of fear extinction; however, genetic as well as environmental factors have been found to influence extinction hence suggesting patients with an unfavorable (intermediate) phenotype (trait variables: e.g. the T genotype of the *NPSR1* gene with high AS, study 4; state variables: e.g. low estrogen, supplement of study 2) to profit less from exposure therapy. Additional brain stimulation with rTMS is thus suggested to compensate for deficits in acquiring and consolidating extinction memory as well as for deficits in the generalization of extinction (study 3) and can therefore possibly enhance the therapeutic success.

Although the precise rTMS working mechanism remains to be determined, there is evidence suggesting alterations in synaptic strengths via LTP as an explanation for the modulation of learning. During extinction an increased spike firing of mPFC neurons has been shown to cause reduced amygdala reactivity (Likhtik et al., 2005; Quirk et al., 2003). The consolidation of the extinction memory has been further demonstrated to rely on NMDA receptor-dependent plasticity (Burgos-Robles et al., 2007). RTMS, which is also influencing the NMDA receptor-dependent plasticity even up to 60 minutes after the stimulation, is thus able to enhance learning processes such as extinction learning (study 2) as well as the consolidation of extinction (study 3). The stimulation is suggested to produce a depolarization of mPFC neurons (study 2) and dlPFC neurons (study 3) which elicits the firing of action potentials while the same neurons are simultaneously engaged in learning/consolidating. The outcome of this summation of neuronal activity may now result in heightened synaptic plasticity which in turn increases the top-down control of the mPFC over the amygdala. This assumption corresponds to the increased prefrontal activity that was found during extinction learning and 24 hours after the stimulation (studies 2 and 3). With regard to the fear network model illustrated in chapter 1, figure 3 displays the here proposed mechanism of rTMS to improve fear extinction learning as well as its consolidation.

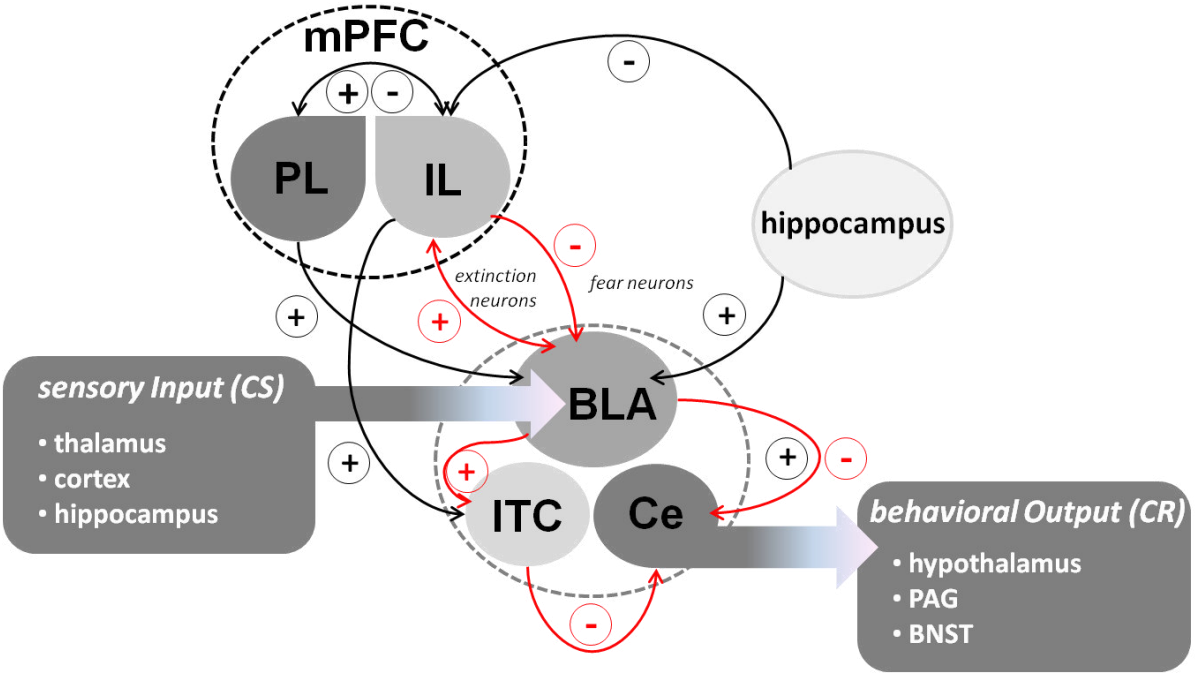

Figure 3. The Neural Circuits of Fear Extinction. The increased top-down control of the amygdala (depicted in the center) is highlighted in red. It is suggested that the mPFC inhibits fear neurons and activates extinction neurons of the BLA which both in turn inhibit Ce output, either directly or via activation of inhibitory ITC cells hence resulting in a reduced CR. BLA: basolateral, BNST: bed nucleus of the stria terminalis, Ce: central nucleus, CS: conditioned stimulus, CR: conditioned response, ITC: intercalated cell masses, IL: infralimbic cortex, PAG: periaqueductal gray, PL: prelimbic cortex.

In order to combine this preclinical model of facilitated extinction through rTMS with the clinical context, the next step should involve phobic patients undergoing an exposure session with previous rTMS application targeting the mPFC. Regarding the outlined extinction deficits in anxiety disorders both on the behavioral level as well as the distorted cortico-limbic interaction on the neural level (see chapter 2.1), anxiety patients are suggested to benefit even more from an augmentation of extinction with rTMS. As one example, simple phobias, such as the fear of heights (acrophobia), are considered as true conditioned emotional reactions and as such require the habituation of fear which is accomplished through extinction for their recovery. Phobias thereby demonstrate a suitable candidate for transferring the obtained preclinical results onto a clinical level. Regarding the fact that rTMS and DCS are thought to rely on similar mechanisms, the proposed clinical application for a combination of rTMS with exposure can be designed with regard to a randomized, double-blind and placebo-controlled study with DCS by Ressler et al. (2004). In this study subjects suffering from acrophobia were treated with two exposure sessions using virtual reality while investigating the impact of DCS administered prior to exposure. Interestingly, this combination significantly reduced acrophobia symptoms on all outcome parameters, although limited by missing symptom improvements in the control group which would have been expected due to the mere exposure. However, a recently published meta-analysis did not replicate DCS effects for the treatment of anxiety disorders (Ori et al., 2015), which underlines the necessity for alternative augmentation strategies such as rTMS. Therefore, the investigation of patients with acrophobia during a combined rTMS and exposure session is highly recommended, either in virtual reality or in real-life. The herein presented studies contribute to a number of requirements needed to implement such a clinical study design: *first*, they demonstrated the mPFC to constitute a suitable target region for the rTMS application; *second*, they determined the time point of the stimulation which should be scheduled prior to the exposure session and in relation to the activation of the fear memory (e.g. being confronted with heights in the case of acrophobia); *third*, they favored the usage of a rTMS round coil to cover a large region of the prefrontal cortex until a more precise target has been identified (Milad, Rauch, et al., 2006); and *fourth*, they suggested a suitable placebo condition such as the usage of a placebo rTMS coil similar to the active coil in placement and acoustic properties or a vertex stimulation in order to compare the augmentation of exposure therapy with rTMS with the standard exposure

therapy. The ethical board of the University of Würzburg already approved this study design and so the results of this ongoing study are awaited with great anticipation.

In the same way as the above presented studies contribute to some elementary questions regarding the modulation of extinction learning processes they also build the fundament for future clinical trials which will be able to further clarify remaining questions. These involve for example the mechanistic explanations for the enhancement of fear extinction via rTMS as well as whether an inhibition of fear or a facilitation of extinction underlies the obtained findings. However, most importantly these studies contribute to the improvement of psychotherapy for anxiety patients especially for those who bear an increased risk of reduced therapeutic responding.

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List of Publications

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