Modulators of Prefrontal Fear Network Function:

An Integrative View

Modulatoren präfrontaler Furchtnetzwerkfunktion:

Ein integrativer Ansatz

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Submitted by

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Start where you are.

Use what you have.

Do what you can.

Arthur Ashe (1943-1993)

Structure of the present thesis

The present thesis is based on several manuscripts describing four different studies. All studies were conducted within a collaborative research center (SFB TRR 58) with the focus on translational research on "Fear, Anxiety, and Anxiety Disorders" funded by the German Research Foundation (DFG). All of the presented data were collected between November 2008 and April 2012 at the Department of Psychiatry, Psychosomatics and Psychotherapy at the University of Würzburg, Germany. Measurements focused exclusively on healthy control subject and each study was approved by the local ethics committee and in accordance with the declaration of Helsinki.

Basically, the thesis is structured into four parts. **First**, a general *Theoretical Introduction* is provided that highlights the neurobiological models on which all studies were based and which gives an overview into earlier important research on fear network function and prefrontal regulation during the processing of fear-relevant stimuli. **Second**, more detailed information about the principal research methods is presented in the following section entitled *Introduction into the Methods of the Present Research*. The **third** part includes all four studies, each with a separate and study-specific abstract, introduction, methods section, discussion, and conclusion. Between studies, a short *Transition* is provided in which the impact and results of the former study on the research questions and design of the following study is discussed. **Fourth**, a comprehensive *General Discussion* of all findings was meant to link the results of all four studies under different aspects to finally come to a *Conclusion* regarding the regulatory function of the prefrontal cortex within the fear network.

Such an extensive research would not have been possible without the help of many other skilled and trained researchers whose individual contributions to each single study are listed on the following page.

"Dissertation Based on Several Published Manuscripts"

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Sara V. Tupak April 3, 2013 Würzburg

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Summary

Regulating our immediate feelings, needs, and urges is a task that we are faced with every day in our lives. The effective regulation of our emotions enables us to adapt to society, to deal with our environment, and to achieve long-term goals. Deficient emotion regulation, in contrast, is a common characteristic of many psychiatric and neurological conditions. Particularly anxiety disorders and subclinical states of increased anxiety are characterized by a range of behavioral, autonomic, and neural alterations impeding the efficient down-regulation of acute fear. Established fear network models propose a downstream prefrontal-amygdala circuit for the control of fear reactions but recent research has shown that there are a range of factors acting on this network. The specific prefrontal cortical networks involved in effective regulation and potential mediators and modulators are still a subject of ongoing research in both the animal and human model.

The present research focused on the particular role of different prefrontal cortical regions during the processing of fear-relevant stimuli in healthy subjects. It is based on four studies, three of them investigating a different potential modulator of prefrontal top-down function and one directly challenging prefrontal regulatory processes. Summarizing the results of all four studies, it was shown that prefrontal functioning is linked to individual differences in state anxiety, autonomic flexibility, and genetic predisposition. The T risk allele of the neuropeptide S receptor gene, a recently suggested candidate gene for pathologically elevated anxiety, for instance, was associated with decreased prefrontal cortex activation to particularly fear-relevant stimuli. Furthermore, the way of processing has been found to crucially determine if regulatory processes are engaged at all and it was shown that anxious individuals display generally reduced prefrontal activation but may engage in regulatory processes earlier than non-anxious subjects. However, active manipulation of prefrontal functioning in healthy subjects did not lead to the typical behavioral and neural patterns observed in anxiety disorder patients suggesting that other subcortical or prefrontal structures can compensate for an activation loss in one specific region.

Taken together, the current studies support prevailing theories of the central role of the prefrontal cortex for regulatory processes in response to fear-eliciting stimuli but point out that there are a range of both individual differences and peculiarities in experimental design that impact on or may even mask potential effects in neuroimaging research on fear regulation.

Zusammenfassung

Tagtäglich sind wir gefordert, die Kontrolle über unsere unmittelbaren Gefühle und Bedürfnisse zu bewahren und diese zu regulieren. Die effektive Kontrolle unserer Emotionen ermöglicht es uns, uns unserer Umgebung und Gesellschaft anzupassen und langfristige Ziele zu erreichen. Defizitäre Emotionsregulation, im Gegensatz, charakterisiert eine Reihe von psychiatrischen und neurologischen Erkrankungen. Vor allem Angststörungen und subklinisch erhöhte Ängstlichkeit zeichnen sich durch eine Reihe von behavioralen, vegetativen und neuronalen Abweichungen aus, welche sich störend auf die effiziente Furchtregulation auswirken. Gängige Modelle des Furchtnetzwerks gehen davon aus, dass Furchtreaktionen durch eine top-down Verschaltung von Präfrontalkortex und Amygdala reguliert werden. Neure Studien jedoch haben gezeigt, dass dieses Netzwerk durch eine Reihe von Faktoren beeinflusst wird. Die spezifischen präfrontalen kortikalen Netzwerke, die an einer effektiven Regulation beteiligt sind und deren potentielle Mediatoren und Modulatoren sind jedoch noch immer Gegenstand heutiger Forschung, sowohl im Tier-, als auch im Menschenmodell.

Der Fokus der vorliegenden Arbeit richtete sich speziell auf die Rolle verschiedener Regionen des Präfrontalkortex während der Verarbeitung furchtrelevanter Reize bei gesunden Probanden. Die Arbeit basiert auf vier Studien, von denen drei jeweils einen potentiellen Modulator präfrontaler top-down Funktion näher untersuchten, während jene regulatorischen Prozesse in einer weiteren Studie gezielt manipuliert wurden. Zusammenfassend konnte gezeigt werden, dass die Präfrontalfunktion mit individuellen Unterschieden in Ängstlichkeit, vegetativer Flexibilität und genetischer Prädisposition assoziiert ist. So wurde beispielsweise das T Risikoallel des Neuropeptid S Rezeptor

Gens, ein erst kürzlich entdecktes Kandidatengen für pathologisch erhöhte Ängstlichkeit, speziell während der Darbietung furchtrelevanter Reize mit geringerer Präfrontalkortex Aktivierung in Verbindung gebracht. Des Weiteren konnte gezeigt werden, dass die Art der Verarbeitung im Wesentlichen bestimmt, ob überhaupt regulatorische Vorgänge in Gang gesetzt werden und dass insbesondere ängstliche Probanden eine allgemein verminderte präfrontal Aktivierung zeigen. Die Ergebnisse deuten jedoch auch darauf hin, dass diese regulatorischen Prozesse bei Ängstlichen möglicherweise früher aktiviert werden als bei weniger Ängstlichen. Das aktive Eingreifen in die Präfrontalfunktion bei Gesunden führte jedoch nicht zu den typischen neuronalen und Verhaltensmustern, wie sie bei Patienten mit Angststörungen beobachtet werden, was wiederum die Annahme nahe legt, dass andere subkortikale oder präfrontale Strukturen für eine Aktivitätsverringerung in einer bestimmten Region kompensieren können.

Zusammenfassend kann gesagt werden, dass die vorliegenden Ergebnisse aktuelle Theorien einer zentralen Rolle des Präfrontalkortex in Bezug auf regulatorische Prozesse während der Konfrontation mit furchtrelevanten Reizen untermauern, jedoch auch zeigen, dass es eine Reihe an individuellen Charakteristika und Feinheiten im jeweiligen experimentellen Design gibt, die potentielle Effekte in Bildgebungsstudien zur Furchtregulation beeinflussen oder sogar maskieren können.

Theoretical Introduction

Fear is considered to be one of the basic human emotions that can be recognized worldwide independently of the cultural background (Ekman, 1988, 1992). Fear mobilizes our bodies in life-threatening situations, which is referred to as 'the fight-flight response', but sometimes also leads to complete immobilization, termed 'freezing'. Under certain circumstances, both reactions must have been proven beneficial for survival by our ancestors (Marks and Tobeña, 1990). The evolutionary perspective demonstrates that anxiety and fear reactions are not just negatively connoted. However, if elevated anxiety becomes disabling to the individual causing psychological or physical distress or an inability to participate in everyday life, it is likely that the criteria of one of the pathological states summarized under the general heading of 'anxiety disorders' (i.e., panic disorder, agoraphobia, posttraumatic stress disorder, generalized anxiety disorder, social anxiety disorder, specific phobia, and obsessive-compulsive disorder) are fulfilled. Anxiety disorders are not rare: According to estimated prevalence rates, every third individual is affected by at least one disorder once in a lifetime (life time prevalence: 29% [women: 33%; men: 22%]; 12-months prevalence: 23% [women] and 13% [men]; Kessler et al., 2005; McLean et al., 2011). The lifetime prevalence of anxiety disorders even exceeds that of mood disorders (20.8% according to Kessler et al., 2005). Generally, women are more often affected than men except for social anxiety disorder (SAD; McLean et al., 2011) and 41% of patients receive no current form of treatment (Kroenke et al., 2007).

Based on these estimations, it seems reasonable to track the etiological factors of increased states of anxiety. In recent years, much effort has been put into finding the genetic contributors for elevated anxiety because twin studies indicated heritabilities of up to 0.32 and 0.48 for generalized anxiety disorder (GAD) and panic disorder (PD), respectively (Hettema et al., 2001). But also for non-pathological states of increased anxiety, such as anxiety sensitivity and SAD related cognitions, estimated heritability was found to be relatively high (Stein et al., 1999, 2002).

The emerging field of imaging genetics research incrementally suggests a range of candidate genes that may pose individuals at an increased risk of developing certain anxiety disorders through

their modulatory effects within the central nervous system (CNS), particularly on the limbic system (Domschke and Dannlowski, 2010; Domschke and Deckert, 2009; Domschke and Reif, 2012). These novel approaches are promising to gain a better understanding of brain function in clinically anxious but also healthy populations.

Apart from genetics, functional imaging research on both healthy individuals and anxiety disorder patients has given an idea about the core neural networks involved during fear, anxiety, and their efficient or dysfunctional regulation (e.g., Bishop et al., 2004; Bishop, 2007, 2009; Eldar et al., 2010; Kalisch et al., 2006; Miller et al., 2005; Robinson et al., 2012), but has also shown that there are still a bunch of open questions to answer. Especially the role of the prefrontal cortex (PFC) and its regionally dependent, distinctive functions during the processing of emotional stimuli, and fear-relevant stimuli in particular, received increasing attention in recent years (Dresler et al., 2013; Etkin, 2010; Etkin et al., 2011; Ochsner and Gross, 2005). Generally, a top-down function of the PFC has been suggested (Berkowitz et al., 2007) but there exist also studies indicating that some regions such as the dorsomedial parts of the PFC (DMPFC) are associated with the generation rather than inhibition of fear responses (see Etkin et al., 2011 for a review). Moreover, not only the individual genetic profile has a modulating effect on fear network activation, also individual differences in physiological flexibility (Appelhans and Luecken, 2006; Lane et al., 2009; Thayer and Lane, 2009), state and trait anxiety (e.g., Bishop et al., 2004; Bishop, 2009) have been associated with changes in prefrontal processing of emotional stimuli. The aim of the present research was to evaluate some of those important individual differences that determine and shape particularly the regulatory function of PFC activation in more detail. Beyond that, the assumed regulatory function of the PFC and common experimental tasks used in functional imaging were challenged.

The present work consists of four studies, all focusing on the interplay between the PFC and one or more important variables that are decisive or reflective of its function. A down-regulatory PFC activation on the subcortical fear network that involves primarily the amygdala and brainstem was the central hypothesis for the entire line of research that is presented here. First, a review on the

general theoretical background of the entire work is given in the following sections, starting with the basic neurobiological models of fear and anxiety and a broad overview over two particular lines of research on fear processing which are highly relevant for the current work. Second, a brief summary over the most outstanding candidate genes for anxiety disorders is provided and third, in the remainder of the introduction, the general research questions and hypotheses for each individual study are presented. For more specific and detailed theoretical information about each study the reader is referred to the according manuscript. Until now, two of the presented studies have been published in international peer-reviewed journals (*study 1*: Tupak et al., 2013a; *study 3*: Tupak et al., 2013b); the other two studies are presented in manuscript form.

Prefrontal Top-Down Regulation of Limbic Structures

A literature search for the neural correlates of fear and anxiety results almost inevitably in these two brain areas: the PFC and amygdala (e.g., Davis et al., 2009; Etkin et al., 2011; Hariri et al., 2003; Kim et al., 2011a; Miller et al., 2005; Phelps et al., 2004; Robinson et al., 2012; Somerville et al., 2012). There exist a range of neurobiological theories dealing with the interplay between these two structures during emotional processing, and fear processing in particular, most of them proposing a top-down regulation through the PFC (Berkowitz et al., 2007; Bishop, 2007; Davidson, 2002; Öhman, 2005). Although neurobiological and neuroimaging research have shown that there are many other regions mediating and complementing this complex interplay, such as the insula (Dresler et al., 2013; Paulus and Stein, 2006), hippocampus (Bannerman et al., 2004), or the bed nucleus of the stria terminalis (BNST; Straube et al., 2007; Walker et al., 2003), most functional neuroimaging studies focused on the processing of fear-relevant stimuli in these two areas.

Anatomically, pathways have been found between the amygdala and several, primarily prefrontally located, brain areas such as the orbitofrontal cortex (OFC), posterior MPFC, anterior lateral PFC, cingulate cortex, and insula with the densest bidirectional projections between the amygdala and OFC and posterior MPFC (Ghashghaei et al., 2007; Stein et al., 2007a). As expected, the

functional coupling between the PFC and amygdala also influences the efficacy of emotional regulation. Effective reappraisal, for example, is related to the connectivity between the amygdala, OFC, and DMPFC (Banks et al., 2007). Interestingly, resting state analyses showed that the inverse relationship between PFC and amygdala anatomically differs dependent on trait anxiety. While high anxious subjects displayed an inverse relationship between ventral MPFC (VMPFC) and amygdala, low anxious subjects are characterized by a comparable relationship between DMPFC and amygdala. Vice versa, the respective regions were either uncorrelated or even positively associated (Kim et al., 2011a). Dissociating roles have been ascribed to ventral and dorsal regions of the anterior cingulate (ACC) and MPFC with the dorsal regions primarily being associated with the expression and generation of fear while ventral parts were rather found to have down-regulatory function on limbic and physiological fear reactions (Etkin et al., 2011).

The involvement of prefrontal and limbic regions during the processing of fear-relevant stimuli has been investigated in a range of functional imaging studies using passive viewing (e.g., Guyer et al., 2008; Thomas et al., 2001), anticipation of threat (e.g., Drabant et al., 2011; Holtz et al., 2012; Straube et al., 2007), and emotional regulation tasks (e.g., Banks et al., 2007; Goldin et al., 2008; Phan et al., 2005). While simple perceptual tasks like passive viewing of fearful faces or threatening pictures predominantly led to increases in amygdalar activation (Lange et al., 2003; Thomas et al., 2001; Whalen et al., 2001), more complex tasks involving a cognitive component recruited prefrontal areas (Lange et al., 2003; Ochsner et al., 2009) and were often associated with simultaneous amygdalar attenuation (Hariri et al., 2000; Hariri et al., 2003; Phan et al., 2005). However, several studies found no amygdala activations to fear-relevant stimuli at all (e.g., Schäfer et al., 2005). Interestingly, the offset of a threat cue signaling the application of an electric shock has been linked to an inverse relationship between VMPFC and limbic activation although the threat cue itself elicited no increase in amygdala activation (Klumpers et al., 2010).

But not only simple processing of threatening information has been associated with activation changes in these areas, also fear learning and memory, referring to fear acquisition and

extinction have been shown to involve the amygdala and PFC regions (e.g., Delgado et al., 2008; Maren and Quirk, 2004; Phelps et al., 2004). Beyond that, also intended cognitive forms of emotional regulation such as reappraisal were associated with prefrontal top-down regulation of the limbic system whereas the suppression of negative affect led to increases in amygdala activation (e.g., Goldin et al., 2008; Ochsner and Gross, 2005). Cognitive reappraisal of aversive pictures, for instance, has been found to activate particularly DMPFC, lateral PFC, and dorsal ACC (dACC) in a functional magnetic resonance imaging (fMRI) study by Phan et al. (2005) with the latter being linked to simultaneous decreases in limbic system activation.

According to Bishop (2007; 2008), The interplay between amygdala and the PFC not only influences fear learning but also attentional and interpretative processing of fear-relevant information. A closer look on the literature focusing on attentional prioritization in anxious and non-anxious populations and studies investigating the regulatory function of elaborate cognitive processing of emotionally negative stimuli is provided in the following section. Before that, a short review about the antagonistic PFC-amygdala relationship in pathological states of anxiety is given with a specific focus on PD. Compared to other anxiety disorders, imaging studies of PD are of particular interest when searching for the neural correlates of acute anxiety and its regulation because panic symptomatology can be provoked even in healthy control subjects (Benkelfat et al., 1995; Ehlers et al., 1986; Vasa et al., 2009) and there are certain traits, such as increased anxiety sensitivity, that are considered to be predictive and –although in a weaker form – quite similar to some of the symptoms in PD (Donnell and McNally, 1990; McNally, 2002; Schmidt et al., 2006). It is likely that the neural processes which are dysfunctional in PD are functionally the same that are involved during acute fear and the control of anxiety in non-pathological samples.

Neuroscientific Models of Anxiety and Anxiety Disorders

Neuroscientific models of anxiety disorders can provide valuable information about how fear-relevant information is processed in the brain and how dysfunctions in those areas can lead to

symptoms of increased fear and anxiety. As for PD, one of the most cited models is the neuroanatomical hypothesis from Gorman, Liebowitz et al. (1989) and its revised version from 2000 (Gorman et al.). In this model, the central nucleus of the amygdala (ceA) plays a critical role for the overly sensitive reactions of the autonomic nervous system. The ceA is thought of as the central point for incoming sensory information via the anterior thalamus and has multiple efferents towards the brainstem (parabrachial nucleus, locus coeruleus, periaqueductal grey) and hypothalamus (lateral and paraventricular nuclei). A dysfunctional regulation of the ceA via its afferents (thalamus, PFC, insular, and primary somatosensory cortex) is suggested to cause the typical misinterpretation of bodily symptoms during arousal in PD. The effects of selective serotonin reuptake inhibitors (SSRI) can be nicely explained by the model of Gorman et al. (2000): By increasing the level of 5-HT in the synaptic cleft, noradrenergic activity in the locus coeruleus is decreased which leads to an attenuation of cardiovascular symptoms. Likewise, persistent SSRI treatment dampens activation of the hypothalamic-pituitary-adrenal (HPA) axis.

Recently, this model has been reviewed and amended to include findings from recent structural and functional imaging research (Dresler et al., 2013). The authors point out that there is no clear-cut evidence for an overly sensitive amygdala in PD patients. Rather, the focus of interest on other brain regions (i.e., PFC and insula) has increased during the past ten years, however, yielding controversial findings. Given that the PFC is a comparably large part of the fear network, it is reasonable that ambiguous results have been found for its various parts. While some studies reported weaker prefrontal control, others observed regional hyperactivation (Dresler et al., 2013). The ambiguous findings in patient studies point towards the need for a better understanding of regional differences in PFC function. Basic research on fear processing can help to gain further insight into the specific role of prefrontal functioning and may elucidate its potential modulators.

Some attempts have been made to categorize the anxiety disorders into those displaying high vs. low prefrontal functioning based on their most prominent symptoms. It has been suggested that those primarily characterized by excessive worrying and rumination (e.g., GAD and

obsessive compulsive disorder [OCD]) show rather high PFC activation while those that are characterized by sudden onsets of acute fear (i.e., PD and posttraumatic stress disorder [PTSD]) display rather hypoactive PFC responses (Berkowitz et al., 2007). Similarly, others suggested a dissociation within limbic areas differentiating between disorders characterized by either elevated phasic fear reactions (e.g., phobias) vs. the ones that are associated with sustained levels of enhanced fear (e.g., PTSD and PD; Grillon et al., 2008; Grillon et al., 2009). Phasic fear reactions cause a rapid activation of the amygdala which fades quickly after removal or disappearance of the threatening stimulus. Sustained fear reactions also elicit an initial amygdalar activation but in addition they are characterized by a slow but longer-lasting BNST activation (Alvarez et al., 2011; Davis et al., 2009).

Also subclinical states of elevated anxiety were linked to altered activation of the fear network (Bishop, 2009). Especially studies on healthy subjects with increased trait anxiety or anxiety sensitivity (AS) may serve as an intermediate step for the investigation of the fear network. AS was positively related to panic symptomatology during pharmacological challenges in both control subjects and PD patients and has been found to decrease following cognitive behavioral therapy (McNally, 2002). During a facial matching task, subjects with high AS and trait anxiety displayed increased amygdalar and insular activation (Stein et al., 2007c). A positive correlation between AS and insular activation was also found in both healthy control subjects and subjects with specific phobia in an fMRI study by Killgore et al. (2011). Particularly the insula has been linked to interoceptive processes which might account for the increased attentional focus on bodily reactions in subjects with elevated AS (Paulus and Stein, 2006). Apart from the insula, also ACC and OFC activation were positively correlated with trait anxiety whereas regulatory regions, i.e., MPFC and dorsolateral PFC (DLPFC), showed a negative correlation (Schäfer et al., 2009). These findings suggest that research on healthy individuals can provide meaningful information 1) about the fear network in general and 2) about the neural correlates that are significantly altered in pathological states of anxiety.

The Impact of Fear-Relevant Stimuli on PFC Activation

One attempt to investigate the neural structures of the human fear network in functional imaging research is to present stimuli with a task-irrelevant but emotionally salient meaning and to ask subjects to evaluate certain stimulus characteristics. In this way, it is assured that stimuli are actively processed as compared to for example passive viewing paradigms and the focus of attention can be shifted from neutral aspects to affective attributes depending on the particular research question. In this section, a brief review is presented on two particular lines of research that attempt to disentangle the role of the PFC within the fear network: research on attentional control and implicit emotion regulation.

The Attentional Bias

Highly salient information is preferentially processed, even at early pre-attentional stages (Eldar et al., 2010). Further, it has been hypothesized that this attentional prioritization happens at the amygdalar level (Compton, 2003) although controversial findings exist (Bishop, 2007). Even if the valence of a presented stimulus is task-irrelevant, attention automatically shifts if the meaning is of emotional relevance to the subject. Such shifts have been termed 'attentional bias' and manifest themselves through variations in response latencies to emotional when compared to neutral stimuli (Bar-Haim et al., 2007). The direction of this deviation depends on the task and sample characteristics. In the dot-probe task, for instance, attention to threat commonly facilitates processing in trials during which subjects have to respond to a dot-probe replacing a previously presented threatening cue compared to a neutral cue (Lipp and Derakshan, 2005; Mogg et al., 1997). As such, the task gives information about what stimuli are preferentially attended (Bar-Haim et al., 2007). Other tasks, like the emotional Stroop task (Williams et al., 1996), are based on the assumption that attention is bound by emotional stimulus valence which in turn leads to a delay in processing. These tasks also provide information about difficulties in disengagement (Cisler and Koster, 2010). As for fear-relevant stimuli, the attentional bias has been shown to be greater among

anxious individuals and anxiety disorder patients (Amir et al., 2002; Bishop, 2008). Most studies on healthy subjects showed that the behavioral effect seems to be limited to patients or subclinical samples and cannot be found among non-anxious subjects (see Bar-Haim et al., 2007 for a review; Thomas et al., 2007). However, there are single studies reporting an attentional bias also among non-stratified healthy subjects (Dresler et al., 2009b; Lipp and Derakshan, 2005).

If the attentional bias is larger in anxious individuals, the neural structure that controls it might be functionally relevant for the respective psychopathology. Apart from facilitated attentional processing of threat and a difficulty in disengagement from those stimuli, Cisler and Koster (2010) added a third component, attentional avoidance, and suggested that while threat detection occurs automatically, attentional avoidance, just like delayed disengagement, represents at least to some extend a strategic process and must therefore depend on PFC function. In addition, they argue that attentional avoidance is primarily driven by emotional regulation and that difficulties in disengagement rely on attentional control. Browning et al. (2010) examined the effects of attentional training in a group of control subjects and found that training participants to avoid a threatening linguistic stimulus led to delayed processing of fearful facial expressions in a subsequent task. This delay in reaction times was accompanied by an increase in right lateral PFC activation. These findings support the theory of Cisler and Koster (2010) and demonstrate that threat avoidance, which constitutes a common symptom among anxiety disorders (American Psychiatric Association, 2000), is accompanied lateral PFC activation.

Imaging studies on the attentional bias towards negative (mostly fear-relevant or threatening) stimuli have indeed shown that in anxious populations attentional control is less efficient and accompanied by lower prefrontal top-down regulation (for a review see Bishop, 2008). However, anxiety is not linked to a general dysfunctional top-down inhibition: Recent studies reported that, in anxious individuals, attention to threat may also be accompanied by a positive relationship between DMPFC and amygdala (Robinson et al., 2012) supporting suggestions of a functional dissociation between dorsal and ventral MPFC and ACC with the former having a fear-

generating and the latter having a fear-regulatory function (Etkin et al., 2011). With respect to tasks of emotional conflict, the DMPFC has been specifically linked to the evaluation and detection of emotional conflict whereas regulatory roles were ascribed to ventral ACC and MPFC (Etkin et al., 2011). The regulatory role of the lateral PFC has been emphasized by the neurocognitive model of selective attention to threat (Bishop, 2007). According to this model, attentional conflict detection occurs at the level of the rostral ACC but efficient performance, requiring a disengagement from task-irrelevant emotional information, depends primarily on the activation of lateral PFC (Bishop, 2007; Bishop, 2008).

As for the present work, the first three studies were based on an emotional conflict task to investigate potential modulators and generators of the attentional bias towards fear-relevant linguistic stimuli in healthy subjects. Therefore, literature on this specific behavioral paradigm, the emotional Stroop task, is reviewed and presented in some more detail with reference to the according studies in the methods section of the present work. *Study 4* diverged from the previous ones regarding the behavioral paradigm. In this study, a more specific focus was set 1) on the circumstances that might elicit an attentional bias in healthy subjects and 2) on differences in prefrontal regulation between tasks of simple perceptual processing of threatening stimuli (such as during the emotional Stroop task) and those of more elaborate cognitive processing. Particularly this latter type of processing has been categorized as a strategy of implicit emotional regulation (Gyurak et al., 2011).

Implicit Emotion Regulation

Implicit emotion regulation is defined as an automatic stimulus driven process that occurs primarily without conscious insight or even completely unintended. It differs qualitatively from explicit emotion regulation which is characterized by more or less awareness and deliberate activation (Gyurak et al., 2011; Koole and Rothermund, 2011). Whereas research on explicit emotional regulation of negative affect (e.g., reappraisal, attentional deployment, suppression, or

active down-regulation) seems relatively straightforward with well-defined experimental tasks and designs (e.g., Goldin et al., 2008; Gross, 2007; McRae et al., 2009; Ochsner et al., 2002), implicit emotion regulation has been addressed by a multitude of different methods and hypothetical considerations with less clear-cut definitions and theoretical considerations ranging from automatic processes such as habituation and extinction learning to more cognitively driven top-down processes (see Gyurak et al., 2011 for an overview; Koole and Rothermund, 2011).

Apart from the main focus on attentional biases and their top-down modulation *(studies 1-3)*, *study 4* of the current work compared different types of emotional processing and particularly challenged the hypothesis of prefrontal top-down control during simple perceptual processing of fear-relevant stimuli. To do so, the match-label task was adapted and modified from an earlier fMRI study (Hariri et al., 2003) which was very similar to affect labeling tasks used by others to investigate the neural correlates of cognitive evaluation of affect (Creswell et al., 2007; Hariri et al., 2000; Lieberman et al., 2007). While affect labeling (i.e., ascribing the adequate emotional label to a facial expression) has been suggested to elicit implicit emotional regulation (Gyurak et al., 2011) in terms of a down-regulation of limbic system activation (Creswell et al., 2007; Hariri et al., 2000; Lieberman et al., 2007), the labeling of non-emotional stimulus characteristics (e.g., gender) has rarely been proposed as an automatic emotional control strategy. Compared to gender labeling, affect labeling produced stronger increases in prefrontal activation that were also linked to amygdalar attenuation (Lieberman et al., 2007). However, when compared to simple perceptual processing (i.e. deciding which of two simultaneously presented pictures matches an identical target), even non-emotional labeling led to an activation increase within ventrolateral PFC (VLPFC) that was linked to a simultaneous amygdala decrease (Hariri et al., 2003). These earlier findings gave rise to the hypothesis that even the cognitive evaluation of non-emotional stimulus characteristics of fear-relevant stimuli can induce prefrontal top-down activation attenuating emotional reactions to the stimulus. However, this earlier research never tested whether their results are specific to emotionally negative stimuli. This lack of evidence was addressed by *study 4* through the inclusion of a neutral

control condition (see *Supplement A* for an illustration of the experimental conditions and *Supplement B* for detailed information about stimulus material). Findings from this research have also important implications for the interpretation of the first three *studies (1-3)* because it seems yet unclear whether tasks addressing the attentional bias towards threat, in particular the emotional Stroop task, also initiate emotional regulation.

Genetic Modulation of Fear Network Function

In recent years, the number of studies focusing on candidate genes for anxiety disorders massively increased (Domschke and Reif, 2012). It is assumed that possessing one or two copies of a so-called risk allele goes along with an increased risk for developing a psychopathological condition. According to vulnerability-stress models (Ingram and Luxton, 2005), these risk allele carriers might react more sensitively to environmental stressors than homozygous non-risk allele carriers. For states of elevated anxiety, such gene x environment interactions have already been shown for the 5-hydroxytryptamine transporter-linked polymorphic region (5-HTTLPR; Stein et al., 2007b), the neuropeptide S receptor gene (*NPSR1*; Klauke et al., in press), and the brain-derived neurotrophic factor gene (BDNF Val66Met polymorphism; Gatt et al., 2009), whereas no significant interaction was found for the 5-HT $_{1A}$ receptor gene (5-HTR1A; Chipman et al., 2010). Apart from those, functional single-nucleotide polymorphisms (SNPs) on the catechol-O-methyltransferase gene (COMT val158met polymorphism; Domschke et al., 2007; Domschke et al., 2004), the neuropeptide Y (NPY; Sah and Geracioti, 2012), and NPY Y5 receptor gene (Domschke et al., 2008a) have been suggested to play a potential role in the etiology of several anxiety disorders, particularly in PD (Domschke and Dannlowski, 2010; Domschke and Deckert, 2009; Domschke and Reif, 2012; Jacob et al., 2010). In addition, various studies demonstrated differential effects on the processing of fear-relevant stimuli and fear learning as a function of genetic variation in these genes (Dannlowski et al., 2011; Domschke et al., 2010; Domschke et al., 2008b; Lonsdorf et al., 2009).

Study 3 of the present thesis focused specifically on the *NPSR1* rs324981 genotype as a potential modulator of prefrontal activation during emotionally conflicting stimuli. The state of the art of science regarding *NPSR1* and its contribution to fear and anxiety research is described in detail in the introduction of *study 3*.

Research Questions and Hypotheses

The overall aim of the present research was to further elucidate different variables modulating PFC activation or being modulated by the PFC during the processing of particularly fear-related or threatening stimuli. Based on previous literature, it was postulated in all of the four studies that processing of fear-relevant stimuli activates down-regulating PFC areas in healthy control subjects and that this activation is attenuated by genetic, autonomic, and personality factors linked to increased anxiety. Further, it was hypothesized that active inhibition of the PFC by means of repetitive transcranial magnetic stimulation (rTMS) in healthy subjects in turn leads to neural and behavioral patterns similar to those observed in anxious individuals. From a bottom-up perspective, it was tested whether autonomic flexibility in terms of heart rate variability (HRV) can in turn provide valuable information about prefrontal functioning during emotional and cognitive control. Finally, the effects of processing type (perceptual vs. cognitive) on behavioral, autonomic, and neural correlates were investigated.

The present work was thus based on an integrative model of fear processing taking physiological, genetic, and current state variables into account (*figure 1*). At the core of this model, the basic components of the fear network - the PFC, amygdala, and brainstem - are supposed to determine the final outcome (behavior and autonomic fear response). This illustration is of course a simplistic version of a fear network that in fact encompasses several other CNS structures as discussed before. Of empirical relevance for the present research, however, are these three structures: The PFC, because its activation constitutes the central variable of interest in all four studies, the amygdala and the brainstem because they are considered to directly govern autonomic

and behavioral outcome as assessed by means of skin conductance (*studies 3 and 4*), HRV (*study 2*), response errors and latencies (*all studies*).

First of all, the simplicity of this model was challenged in *study 1* of the present work by actively interfering with PFC function through the application of inhibitory TMS. By means of the virtual lesion technique, it was aimed to lower PFC activity and to subsequently investigate the effects on neural and behavioral processing of fear-relevant stimuli. According to the model (*figure 2a*), lowered PFC activation causes a stronger downstream signal via the amygdala and brainstem leading to an increased fear reaction to fear-relevant stimuli.

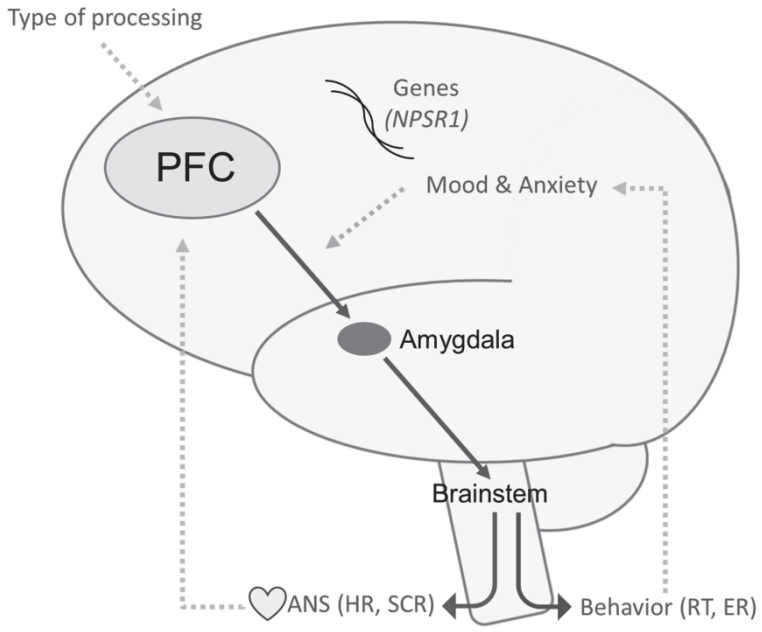

Figure 1: Schematized illustration of the investigated variables and their assumed effects on the basic components of the fear network
Inhibitory top-down prefrontal cortex (PFC) activation is assumed to cause an attenuation of the amygdalar down-stream signal via the brainstem. As a consequence, a disinhibition of the amygdala is hypothesized to cause changes in behavior, leading for example to a stronger attentional bias (higher error rates [ER] and reaction times [RT]), or to an increased activation of the autonomic nervous system (ANS) eliciting a physiological fear response (e.g., accelerated heart rate [HR] and skin conductance responses [SCR]). These output variables act in turn, via feedback loops, on the fear network. Several variables are suggested that act directly or indirectly on the interplay between PFC and amygdala (e.g., genes, state variables, and the way in which stimuli are processed).

In the next step, *study 2* focused on the output of the autonomic nervous system (ANS), particularly HRV. More detailed models exist of the interplay between the PFC and heart suggesting a default fear reaction to ambiguous or fear-relevant stimuli that is under constant control of the PFC via the subcortical path and vagus nerve (Appelhans and Luecken, 2006; Thayer and Lane, 2009). These models are based on a similar down-stream network of brain regions as common theories about the fear network. It has been hypothesized that parasympathetic activation as reflected by HRV may serve as a trait index for general PFC activation during both emotional and cognitive top-down regulation (Thayer et al., 2009; Thayer and Lane, 2009). If there was evidence for a relationship between autonomic flexibility (HRV), anxiousness, and PFC functioning this would further strengthen

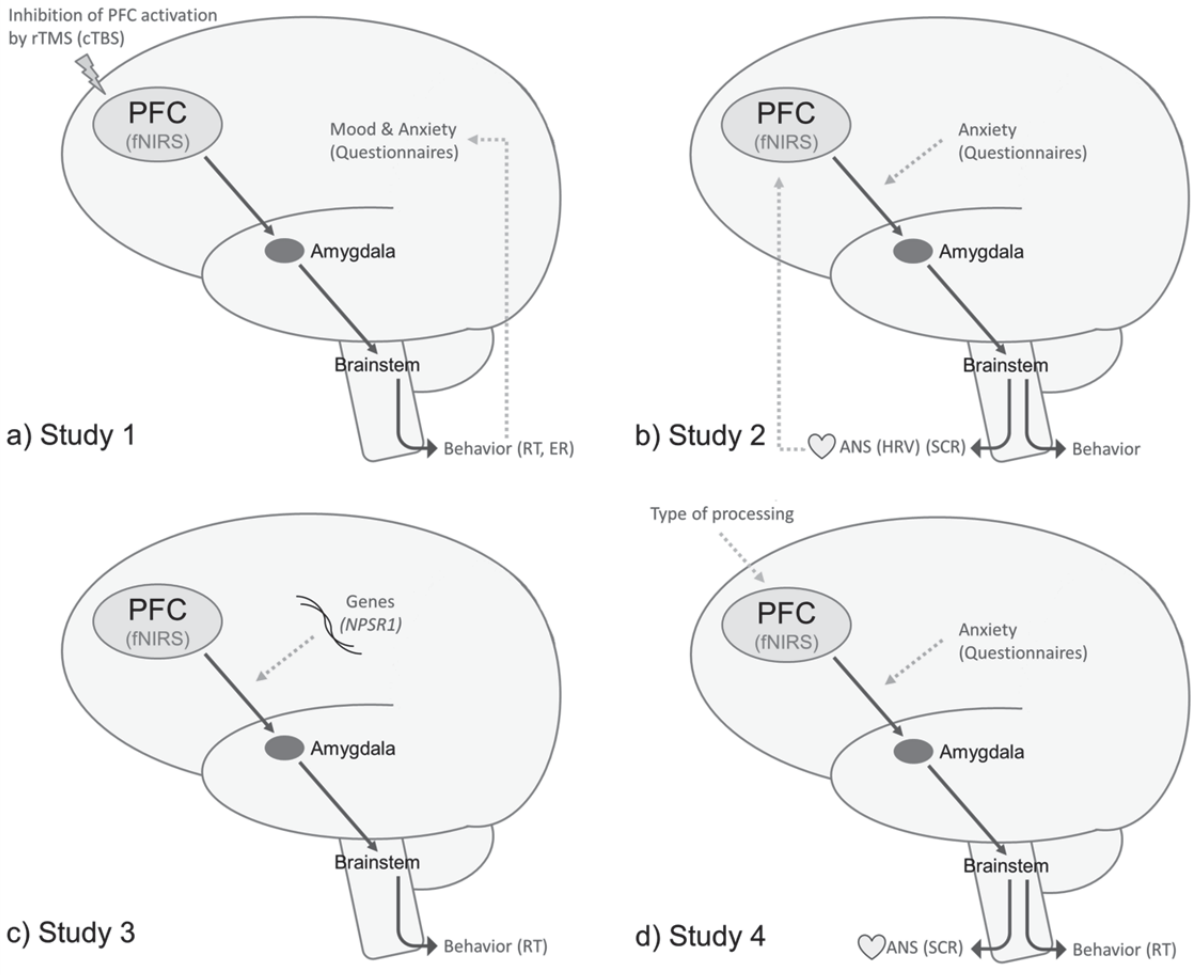

Figure 2: Investigated variables and assumed pathways of all studies

the idea of neocortical modulation of the subcortically driven fear response (*figure 2b*).

The function of all structures on brain level is logically driven by neurochemical processes modulating neurotransmission and these processes in turn are hardly determined by the genetic makeup of the individual. Therefore, as a third variable of interest, a recently suggested candidate gene for PD (Domschke et al., 2011), the *NPSR1* rs324981 gene has been tested regarding its potentially modulating effects on PFC activation, arousal and behavior in response to particularly fear-relevant stimuli in *study 3* (*figure 2c*). The genetic basis determines the effectiveness of each neural module within the fear network and there is evidence that *NPSR1* affects two of the most important ones, the PFC and amygdala (Dannlowski et al., 2011; Domschke et al., 2011; Raczka et al., 2010). Until now, it was, however, not tested whether its PFC modulating effects are specific to the processing of particularly emotionally interfering stimuli and not interfering stimuli in general. *Study 3* aimed at disentangling this latter question.

Finally, the fourth variable - impacting on the neural and in turn also autonomic and behavioral components - that is included in the model is the way in which fear-relevant stimuli are processed by the individual (*figure 2d*). Functional imaging research on the processing of emotional stimuli made use of a great variety of behavioral paradigms whose results are often compared with each other yielding controversial findings in terms of prefrontal up- or down-regulation. Particularly the comparison of tasks that are primarily based on perceptual processing compared to those requiring cognitive processing has led to differential findings (Hariri et al., 2000; Hariri et al., 2003). It is obvious that the way in which subjects are instructed to process fear-relevant stimuli has a great impact on PFC activation and in turn also on behavior and psychophysiological measures. *Study 4* compared two different kinds of processing: simple perceptual vs. more elaborative cognitive processing of threatening stimuli. Further, it was tested in how far state anxiety influences both processes.

Table 1: Summary of methods and hypotheses

	Study 1 PFC Inhibition	**Study 2 HRV as an index**	**Study 3 Genetics**	**Study 4 Processing**
Manipulation	PFC inhibition through rTMS	High vs. low HRV	*NPSR1* genotype	Cognitive vs. perceptual processing
Task	Emotional Stroop	Combined Stroop[1]	Combined Stroop[1]	Match-Label task
Methods	fNIRS, rTMS	fNIRS, HRV, SCR	fNIRS	fNIRS, SCL
ROI	DLPFC	DLPFC	DLPFC & MPFC	VLPFC
Hypotheses:				
PFC	↓	High: ↑ Low: ↓	Risk genotype: ↓	Perceptual: ↑ Cognitive: ↑↑
ANS measures	(↑)	High: ↓ Low: ↑	Risk genotype: (↑)	Perceptual: ↑ Cognitive: ↓
Behavior	↑	High: ↓ Low: ↑	Risk genotype: ↓	Perceptual: ↑ Cognitive: ↓

↑: Increase (↑): Increase is assumed but not measured
↓: Decrease (↓): Decrease is assumed but not measured
[1] Hypotheses for the combined Stroop refer particularly to the attentional bias within the emotional part. In *study 2*, the hypotheses are identical for the classical part. In *study 3*, it is assumed that there are no group differences in the classical part of the task.
ANS: Automatic nervous system; **DLPFC:** Dorsolateral prefrontal cortex; **fNIRS:** Functional near-infrared spectroscopy; **HRV:** Heart rate variability; **MPFC:** Medial prefrontal cortex; **NPSR1:** Neuropeptide S receptor 1; **PFC:** Prefrontal cortex; **ROI:** Region of interest; **rTMS:** repetitive transcranial magnetic stimulation; **SCL:** Skin conductance level; **SCR:** Skin conductance response; **VLPFC:** Ventrolateral prefrontal cortex

A summary of all methods, investigated variables, and hypotheses of the present studies is presented in *table 1*. For more detailed information about the theoretical background, derivation of hypotheses, methods and experimental manipulations, the reader is referred to the according manuscript. However, there exists a great deal of overlap between the studies regarding their principal methods. Therefore, a short introduction into the theoretical background of the emotional Stroop task and functional near-infrared spectroscopy (fNIRS) is provided in the following sections.

Introduction into the Methods of the Present Research

The exact methodological approach of each study is explained in detail in the methods section of the according article. This section provides a more detailed insight into the theoretical backgrounds of the Stroop paradigm and fNIRS since both constitute key elements of the present studies. Moreover, the Stroop task used in *studies 2 and 3* fundamentally differed from the version used in *study 1*. A brief review on the task and the methodology of fNIRS, their advantages and limitations, and the current methodological variations between studies in case of the Stroop task is given in the following sections. A description of the match-label paradigm used in *study 4* can be found in the respective article.

The Stroop Task

A Stroop task was used in *studies 1-3*. More specifically, in *study 1* an emotional Stroop task was presented, whereas a combined version of the original classical Stroop task and its emotional counterpart was designed for *studies 2 and 3*. The following sections provide a brief overview over the theoretical and practical background of both versions and the methodological variations between the present studies.

Classical Version

The Stroop task was firstly described as early as in 1929 (Jaensch, 1929). In 1935, John Ridley Stroop published the first description of the original task in English which was republished in 1992 by the same journal (Stroop, 1992). Since then the article has been cited more than 8000 times and entering “Stroop task” as a search term yields more than 20,000 findings (according to scholar.google.com, last access on March 25, 2013). However, first evidence for the idea that reading is a highly optimized process which appears to occur faster than object and color naming had been provided already in the late nineteenth century by James McKeen Cattell and Wilhelm Wundt (according to MacLeod, 1991).

Several modifications of the original task led to the version that is currently known as the classical Stroop task, which has been widely used in experimental psychology and neuroscience research (MacLeod, 1991; Vanderhasselt et al., 2009). Today, the conventional Stroop design comprises two conditions with varying degrees of stimulus interference. Typically, color words are presented in different font colors and the subject is asked to name the font color of the presented word aloud or to indicate it by pressing a corresponding button ignoring the meaning of the presented word itself. In the congruent condition, color words are presented in their corresponding font color (e.g., the word "red" shown in red font color). During the incongruent condition, color words are displayed in a font color other than that of the presented word (e.g., the word "red" shown in blue font color). Whereas congruent trials, in which word and font color are matched, are characterized by relatively fast processing, incongruent trials have been shown to slow down response latencies (Redding and Gerjets, 1977; Stroop, 1992). This slowdown has been come to know as the classical Stroop or Stroop interference effect, an effect that has presented itself as highly reliable and robust across studies (MacLeod, 1991; MacLeod, 1992; Siegrist, 1997).

The classical Stroop task, however, was not of primary interest for the current research and was mainly included as a control task for the generally interfering effects of stimuli eliciting a response conflict between task-relevant and task-irrelevant stimulus characteristics. The main focus was set on emotionally interfering stimuli as presented in the emotional Stroop task.

Emotional Version

The idea of an emotional Stroop task version came up in the mid-eighties (Gotlib and McCann, 1984; McKenna, 1986; Watts et al., 1986; Williams and Nulty, 1986). In these first experiments it was observed that words which had personal and emotional relevance to the subject led to longer reaction times than those which were unrelated. As such, the emotional Stroop task represents an emotional conflict task measuring the attentional bias (i.e., prolongation of response latencies) towards emotional, mostly fear-relevant, stimuli.

A great deal of research using the emotional Stroop focused on anxiety disorder patients. In one of the first studies, it was found that spider phobics performed worse when phobia-related words were presented but not when they had to react to general threat words. Even more, psychotherapeutic interventions reduced the degree of interference caused by phobia-related stimuli (Watts et al., 1986). An extensive review had been published ten years later by Williams et al. (1996) indicating the great impact the emotional Stoop task has had in clinical research. The authors reported emotional Stroop interference particularly for anxious populations ranging from samples with increased trait anxiety to clinical populations with PD, PTSD, GAD, OCD, SAD, and specific phobia.

Controversial findings, however, have been reported for healthy control samples. Particularly for the word-color version derived from the original Stroop task, most studies found no particular attentional bias (e.g., Mohanty et al., 2007; Phaf and Kan, 2007). Others argued that the task has some peculiarities, which, if controlled for, can unmask the effect also in healthy subjects. There exists evidence, for instance, indicating that emotional Stroop interference exerts its effect primarily on the subsequent trial (McKenna and Sharma, 2004; Waters et al., 2003). Others reported that arousal accounts for most of the differences in reaction times (Dresler et al., 2009b). The suitability of the emotional Stroop task as a measure of behavioral emotional conflict has been critically discussed (Algom et al., 2004; Buhle et al., 2010), an issue that will be raised again in the discussion in more detail. Apart from inconsistencies regarding behavioral measures, neuroimaging studies reported profound interference effects on brain level in both anxious and non-anxious subjects (e.g., Compton et al., 2003; Dresler et al., 2012a). The theoretical rationale of the current work was principally based on these neural effects described below.

Neural Correlates of the Stroop Task

To ease the direct comparison between the classical and emotional Stroop tasks, Compton et al. (2003) performed an fMRI study using both versions and additionally implemented two stages of

varying interference in both tasks. Their results showed that particularly the DLPFC seem to be critically involved during interfering trials in both tasks. Activation in this area was found to be increased for the contrasts between incongruent vs. neutral and emotional vs. neutral color-word stimuli. Moreover, the DLPFC response was even higher when response-eligible trials were compared to non-eligible trials and when high arousing negative words were compared to low arousing negative words.[1] These DLPFC effects could not be ascribed to a general effect of emotional valence since no such results were obtained for positive words (Compton et al., 2003). In another study comparing both tasks, a dissociation between dorsal and rostral ACC (rACC) was observed with increased rACC activation for the emotional Stroop contrast (negative > neutral) and increased dACC activation for the classical Stroop contrast (incongruent > neutral; Mohanty et al., 2007). In this study, activations in both ACC regions also accounted for a large amount of variation within DLPFC activation. Both studies (Compton et al., 2003; Mohanty et al., 2007) investigated healthy control subjects indicating that neural responses profoundly differed between conditions of the emotional Stroop even in those samples.

Based on the findings of the former study, *studies 1-3* of the present work focused primarily on the DLPFC as a region of interest (ROI); first, because the DLPFC seems to be critical for both Stroop versions and varying degrees of emotional and non-emotional interference, allowing to test the specificity of the experimental manipulations in *studies 2 and 3* to emotional compared to cognitive control. Second, because of the excellent accessibility of this region compared to for example the ACC when using fNIRS. Furthermore, both tasks have been successfully applied in fNIRS research reporting similar results for the classical Stroop task in lateral (Schroeter et al., 2002; Schroeter et al., 2004) and inferior PFC (Ehlis et al., 2005). The emotional Stroop version has been used in a single case study evaluating the therapeutic potential of rTMS in PD (Dresler et al., 2009a)

[1] Response-eligibility in this study (Compton et al., 2003) referred to whether the font color of some of the presented color word stimuli shown in the blocks of incongruent stimuli was also part of the response set which consisted of the following colors: Red, yellow, green, and blue. For example, in response-eligible trials the word “red” was displayed in blue font color. In non-eligible trials, none of the presented color word stimuli was part of the response set (e.g., the word “violet” displayed in blue font color). Both conditions (eligible vs. non-eligible), however, included only incongruent color-word pairs, thus varying the degree of cognitive interference.

on which several of the hypotheses of *study 1* were based (for more information the reader is referred to the introduction of this study).

Methodological Differences between Studies

In *study 1*, subjects performed an emotional Stroop task while a combined version of both the emotional and classical Stroop task was used in *studies 2 and 3* (*figure 3*). The differences in experimental setup between studies are listed in *table 2*, however, for a detailed description the reader is referred to the methods section of the according article. A list of the emotional Stroop stimuli used in *studies 1-3* can be found in *Supplement C*.

The stimulus material of the emotional Stroop paradigm as used in *study 1* has been tested before in several PD patient studies by Dresler and colleagues (Dresler et al., 2012a; Dresler et al., 2009a; Dresler et al., 2012b). A detailed description of the selection procedure has been reported elsewhere (Dresler, 2011). To briefly summarize, all fear-relevant stimuli were chosen out of a pool

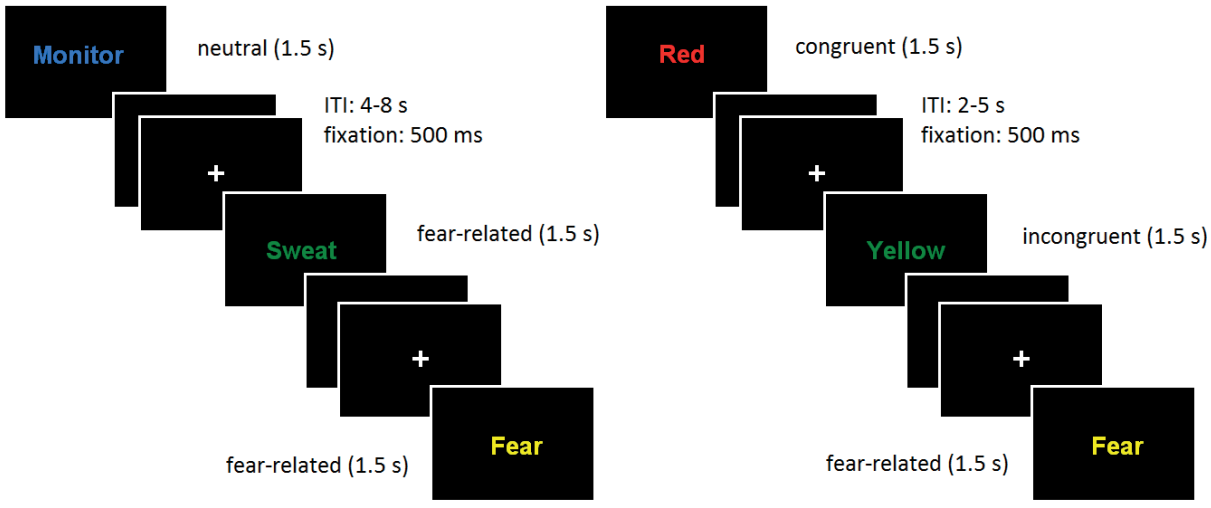

Figure 3: Example trials of the emotional Stroop *(study 1)* and combined Stroop task *(studies 2 and 3)*
The left figure shows a series of trials of the emotional Stroop task as presented in *study 1;* the right figure depicts a series of trials of the combined Stroop task as presented in *studies 2 and 3*. Regarding the presentation mode, stimulus and fixation times were identical for both paradigms while the average inter-trial interval (ITI) was shorter for the combined Stroop task. *Study 1* (left) used neutral and fear-related words while in *studies 2 and 3* (right) neutral, fear-related, congruent and incongruent color words were presented.

Table 2: Variations in experimental design between *study 1* and *studies 2 and 3*

	Study 1	Studies 2 and 3
Number of conditions	2	4
Experimental design	Event-related	Event-related
Number of different font colors	4	3
Number of response buttons	4	3
Fingers used for responding	Both index and middle fingers	Non-dominant right index, middle and ring finger
Number of words per condition	Neutral: 15 Fear-relevant: 15	Neutral: 15 Fear-relevant: 15 Congruent: 16 Incongruent: 16
Number of times each stimulus is presented in total	4	3
Number of trials per condition	60	Congruent: 48 Incongruent: 48 Neutral: 45 Fear-relevant: 45
Total number of trials	120	186
Stimulus presentation time	1.5 s	1.5 s
Length of fixation (preceding each trial)	0.5 s	0.5 s
Inter-trial interval	4-8 s (jittered)	2-5 s (jittered)
Minimum trial length	6 s	4 s
Maximum trial length	10 s	7 s
Total length of the experiment	Average[1]: 16 min	Average[1] 17 min

[1] The total length of the experiment was not exactly the same for all subjects due to the jittered inter-trial interval in both versions and could vary approximately 2-3 min from average for each participant.

of items that had been judged by experts in the field, who were familiar with the diagnostic criteria of PD, on a 10- point Likert scale according to their relevance for PD patients (a score of 9 indicated very high relevance, a score of 0 indicated no relevance at all). Out of the words that yielded scores above 6, 15 were chosen and matched with neutral words yielding scores beneath 2 on the same scale according to the number of letters, syllables, and frequency within written and spoken language (see Baayen et al., 1995 for frequency estimates; Dresler, 2011).

The color word stimuli used in the classical part of the Stroop task in *studies 2 and 3* were ‘Gelb’, ‘Rot’, and ‘Grün’ (i.e., the German words for ‘Yellow’, ‘Red’, and ‘Green’). A comparison of methodological variations between *study 1* and *studies 2 and 3* can be found in *table 2*. In the latter two, the inter-trial interval (ITI) was shorter (jittered from 2 to 5 s) than in *study 1* (jittered from 4 to 8 s). Because the number of trials had increased due to the inclusion of the classical Stroop variant, the ITI was shortened to keep the total measurement time beneath 20 min. A shorter ITI in event-

related fNIRS studies decreases the amplitude of O_2Hb but still leads to reliable experimental results (Schroeter et al., 2004).

Functional Near-Infrared Spectroscopy

In a first series of experiments in animals and a human subject, Jöbsis (1977) successfully tested the potential of light from the near-infrared spectrum for non-invasive imaging of cerebral hemodynamic activation. This has been considered to mark the starting point of fNIRS research for the study of brain-function relationships and technologies and apparatuses have steadily improved since then (Obrig and Villringer, 2003).

Measuring hemodynamic activation at a certain position on the human scalp requires at least two fNIRS probes, a light emitter and a photo-detector. From the light emitter light in the near-infrared (NIR) range is sent through the underlying scalp and tissue into the cortex. The measurement depth of fNIRS depends on the inter-optode distance with increasing depth for increasing distances (Quaresima et al., 2012; Villringer and Chance, 1997). With an inter-optode distance of 3 cm as for the ETG-4000 continuous-wave Optical Topography System (Hitachi Medical Corporation, Japan) which was used in all of the four present studies, the light is assumed to reach a depth of approximately 1.5 cm (Quaresima et al., 2012; Strangman et al., 2002a). The pathway along which the NIR light travels through the scalp and brain equals the shape of a banana and as such a large portion of the NIR light leaves the skull in a circle around its entrance position, i.e. the light emitting diode. Although an uncertain portion of the light gets lost due to scattering, the amount of NIR light that leaves the skull at the position of the detecting probe offers valuable information about cortical oxygenation. More precisely, NIR light is differentially absorbed by oxygenated (O_2Hb) and deoxygenated hemoglobin (HHb). Consequently, the detected signal reflects changes in both chromophores over time. Because the pathlength factor, which refers to the path along which the NIR light travels from the emitter to the detecting probe, is unknown for continuous wave systems, it is the relative change in O_2Hb and HHb from one experimental manipulation to the other that

provides parameters for statistical inference. This explains the suitability of fNIRS for cognitive and affective neuroscience research but also shows that continuous wave systems cannot be used for a measure of absolute chromophore concentration. For this purpose there exist other apparatuses based on time and frequency-domain approaches described elsewhere (Obrig and Villringer, 2003; Wolf et al., 2007).

FNIRS has several **advantages** over other functional imaging methods. *First*, it is relatively robust against movement artifacts which allows for measurements without head fixation in a sitting position. Measurements are even possible when the subject is moving or speaking (Dieler et al., 2012; Tupak et al., 2012) and can be conducted in a natural setting without much noise. *Second*, the temporal resolution is relatively high (10 Hz for the present studies). *Third*, the preparation time is fairly short (about 5 min) as compared to for example electroencephalography (EEG) or positron-emission tomography (PET). These advantageous in turn lead to a higher willingness to participate in fNIRS experiments in first place and greater compliance and little drop-outs later, particularly in populations who may experience the entire measurement procedure as more distressing such as children and infants (e.g., Baird et al., 2002), psychiatric or neurological patients (Dieler et al., 2012). *Fourth*, fNIRS has no side effects. Subjects fulfilling exclusion criteria of other functional imaging measurements, e.g. for ethical or safety reasons, might well participate in fNIRS studies (e.g., pregancy; Roos et al., 2011). *Fifth*, the record of two complementary chromophores (O_2Hb and HHb) improves the signal-to-noise ratio. A valid hemodynamic response to an external stimulus causes an increase of O_2Hb and a simultaneous decrease of HHb. Thus, these two parameters ideally reach a highly negative correlation and correlation coefficients between both offer a useful way to reduce the amount of artifacts within the signal (Cui et al., 2010). The application of such a correction method is described in more detail in the methods section of *study 4*.

As every functional imaging method, fNIRS has also two important **limitations**. *First*, its spatial resolution is moderate (i.e., 3 cm for the current studies) compared to fMRI (mm range) and restricted to those parts of the cortex that lay directly under the skull. Subcortical structures or

medially located cortical regions (e.g., ACC) are not assessable by fNIRS. *Second*, the measurement principle assumes a constant skin blood flow of the scalp and forehead (in case of prefrontal recordings). A recent study, however, has shown that the brain-derived fNIRS signal can be distorted by task-related changes in skin blood flow (Takahashi et al., 2011). The authors found that during a verbal fluency task particularly measures over the forehead are affected. The results of this study show that fNIRS studies require a careful experimental design to control for muscular artifacts in the forehead.

Taken together, fNIRS offers adequate spatial resolution to differentiate between distinct parts of the prefrontal cortex (PFC) like the dorsolateral, medial, and ventrolateral PFC. Therefore, the method is well suited to investigate the research questions at hand, given that the results are interpreted with caution taking the above mentioned limitations into account. The fNIRS setup was identical in all of the present studies using a 52-channel system that covered large parts of the prefrontal lobe including anterior, dorsal, lateral, and ventral PFC (for a graphical illustration see figure 1 in the manuscript of either *study 1 or 3*).

Psychophysiological Measures as an Index for Fear Network Activation

When functional imaging techniques are not assessable, measures of ANS activation can provide valuable information about emotion-associated limbic brain activity. Psychophysiological techniques most often named in this context include for instance the startle probe (Gajewska et al., 2013; Grillon and Davis, 1997), skin conductance (Linnman et al., 2012), heart and respiratory rate (Evans, 2010; Gianaros and Sheu, 2009; Lane et al., 2009). Generally, fear responses are assumed to be mediated through a functionally connected subcortical fear network including the amygdala, hypothalamus, and brainstem (Lang et al., 2000) as presented in *figure 1*.

In the present studies, heart rate variability (HRV) and skin conductance responses (SCR) or levels (SCL) were recorded to gain insight into ANS activation during the processing of fear-relevant stimuli on the one hand *(study 4)* and ANS-CNS interaction on the other hand *(study 2)*. Most

importantly, however, was the use of physiological measures to indirectly gain information about amygdala activation and the subcortically driven fear response because the amygdala cannot be tracked by fNIRS. For this purpose, skin conductance was recorded during *studies 2 and 4*. An increase in perspiration in response to stressful events leads to improved conductance when a small electric current is applied to the skin, known as SCR. The SCR has been shown to be strongly associated with activation changes in the amygdala (Furmark et al., 1997; Lang et al., 2000).

A direct assessment of the predictive potential of psychophysiological measures for brain activation and function was the aim of *study 2*. Here, HRV was recorded to test if autonomic flexibility (i.e., increased HRV) can serve as an index for prefrontal function during the processing of cognitively and emotionally interfering stimuli in the combined Stroop task. For this study, hypotheses were based on the neurobiological model provided by Thayer and colleagues (2009; Thayer and Lane, 2009) suggesting that the heart rate is constantly inhibited by the PFC via the amygdala and its projections to various brainstem target areas such as the parabrachial and dorsal vagal motor nuclei, the nucleus of the solitary tract, nucleus ambiguous, and the caudal and rostral ventrolateral medulla (Thayer and Lane, 2009).

In the following, all four studies are presented in their individual manuscript form *(studies 2 and 4)* or in published format *(studies 1 and 3)*. For detailed information on the theoretical background, hypotheses, methods, results, and discussion of results, the reader is referred to the appropriate section of the individual article. The introductory remarks until this point were meant to serve as a comprehensive view on the existing literature and overall methods relevant for the work as a whole. Similarly, an integrative discussion of all results is provided in the remainder following *studies 1-4*. Theoretical considerations that were crucial for the progress and changes in experimental strategy from one study to the next are shortly described in between in the sections entitled *Transition*.

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◆ Human Brain Mapping 34:150–157 (2013) ◆

Inhibitory Transcranial Magnetic Theta Burst Stimulation Attenuates Prefrontal Cortex Oxygenation

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Abstract: Recent studies highlighted the great potential of newly established theta burst stimulation (TBS) protocols for non-invasive human brain stimulation studies using transcranial magnetic stimulation (TMS). While intermittent TBS over the primary motor cortex was found to potentiate motor evoked potentials, continuous TBS led to profound attenuations. Although numerous studies investigated the impact of TBS on motor cortex function, yet, only few imaging studies focused on its effects in other brain areas. Particularly for the prefrontal cortex, it is unclear whether TBS has similar effects compared to application over motor areas. In the current study continuous TBS was applied to either the left or right dorsolateral prefrontal cortex in a sample of healthy subjects. Changes in prefrontal oxygenation were measured during an emotional Stroop task by means of functional multi-channel near-infrared spectroscopy (fNIRS) before and after stimulation. Results showed bilaterally decreased prefrontal oxygenation following inhibitory stimulation of the left prefrontal cortex but no behavioral effect. No such alterations were observed following right-hemispheric or sham stimulation. The results of the current study are in line with earlier findings and additionally demonstrate that also prefrontal oxygenation can be impaired by continuous TBS. *Hum Brain Mapp 34:150–157, 2013.* © 2011 Wiley Periodicals, Inc.

Key words: DLPFC; emotional Stroop task; NIRS; prefrontal cortex; TBS; TMS

INTRODUCTION

About 25 years ago, Barker et al. [1985] showed that motor-evoked potentials (MEPs) of the hand and leg can be elicited through magnetic stimulation of the primary motor cortex, marking the beginning of human brain stimulation research. During transcranial magnetic stimulation (TMS), a brief electrical current is passed through a coil

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thereby generating a magnetic field perpendicular to it. Placed on the head, this field penetrates the underlying skull and tissue and impacts neuronal activity within the cortex [Hallett, 2000]. Directly observable reactions can be elicited by single pulse TMS, e.g., MEPs when applied to primary motor cortex [Barker et al., 1985], visual phosphenes over primary visual cortex [Meyer et al., 1991], and disturbances in speech production over Broca's [Pascual-Leone et al., 1991] and Wernicke's area [Knecht et al., 2002]. Longer lasting alterations of focal neuronal activity are accomplished through repeated application of magnetic pulses (known as repetitive (r)TMS). Low-frequency (≤1 Hz) rTMS is commonly assumed to induce temporary neuronal inhibition whereas high-frequency (≥10 Hz) stimulation has been shown to have excitatory effects [Hallett, 2000]. An exception is the recently developed theta burst stimulation (TBS) protocol by Huang et al. [2005]. Emanated from years of electrophysiological animal research [e.g., Hess and Donoghue, 1996; Hyman et al., 2003], they transferred the findings of induced long-term depression or potentiation following electrical stimulation within the theta-frequency range of rodents' hippocampal and primary motor neurons to human research. Two protocols were evolved, an intermittent (iTBS) and a continuous TBS (cTBS) paradigm. Although both protocols partly consist of high-frequency stimulation (50 Hz), cTBS has been found to have attenuating effects on MEPs when applied to primary motor cortex, while MEP amplitudes were potentiated following iTBS. A third protocol, intermediate TBS (imTBS), produced no specific effects. A general advantage of those TBS protocols is the duration of their effects (∼60 min for cTBS and ∼20 min for iTBS) that greatly exceed the relatively short time of application [< 1 min for cTBS and <4 min for iTBS; Huang et al., 2005].

To date, only few studies investigated the effects of TBS by means of neuroimaging methods such as electroencephalography [EEG; Grossheinrich et al., 2009], positron emission tomography [PET; Ko et al., 2008], functional magnetic resonance imaging [fMRI; Hubl et al., 2008], and functional near-infrared spectroscopy [fNIRS; Mochizuki et al., 2007]. Most research, however, focused on primary motor cortex since effects in those areas are directly assessable by? electromyography [EMG; for a review see Cárdenas-Morales et al., 2010].

The primary purpose of the current study was to investigate whether cTBS impedes prefrontal activity and if this cortical inhibition can be tracked by multi-channel fNIRS. FNIRS measures regional cortical changes in oxygenated (O_2Hb) and deoxygenated hemoglobin (HHb), both of which are assumed to reflect ongoing brain activity [for detailed information see Jöbsis, 1977; Obrig and Villringer, 2003; Strangman et al., 2002a]. Using fNIRS for the detection of hemodynamic TBS effects has two main advantages. First, preparation time is relatively short (<10 min) which assures that TBS effects are not outlasted by the measurement procedure. Second, it has no side effects thereby enabling repeated-measurement designs. In addition, it is relatively insensitive to movement artifacts [Schecklmann et al., 2010] and measurements can be conducted in a quiet and natural environment without head fixation.

As indicated by Mochizuki et al. [2007], the inhibitory effects of cTBS exert their effects primarily in terms of reduced O_2Hb levels. The authors observed decreased oxygenation in the contralateral site of stimulation and neighboring areas (i.e. cTBS over primary sensory cortex led to reduced O_2Hb signals in contralateral primary sensory and motor cortices). Similar results in terms of an ipsilateral decrease in the blood oxygen level dependent (BOLD) response were obtained in an fMRI study following cTBS over the right frontal eye field [Hubl et al., 2008]. To date, those are the only studies investigating hemodynamic effects of TBS.

Our secondary aim was to assess whether prefrontal cortex (PFC) inhibition in turn leads to specific interference during emotional word processing. Hemodynamic changes were recorded during the performance of an emotional Stroop task [Williams et al., 1996]. In this task, subjects indicated the font color of anxiety and neutral words while reaction times were recorded. A specific attentional bias with increased latencies for emotionally negative (e.g., threatening, disorder-related) compared to neutral words has repeatedly been shown for various kinds of anxiety disorders [e.g., Lundh et al., 1999; Williams et al., 1996] and subjects with elevated trait anxiety [for a review see Bar-Haim et al., 2007]. Among healthy subjects, such bias was found less reliably [e.g., Dresler et al., 2009b; Thomas et al., 2007]. On the neuronal level, processing of negative words activated the dorsolateral PFC [DLPFC; Compton et al., 2003]. Moreover, the PFC seems to represent a critical structure exerting top-down control during this task [Dresler et al., 2009a]. D'Alfonso et al. [2000] applied low-frequency rTMS to either the left or right PFC prior to a pictorial emotional Stroop task. Inhibition of the right but not left PFC resulted in an attentional bias towards angry faces. This result is also supported by recent fMRI studies that evaluated the potential top-down control of prefrontal structures on the limbic system to regulate negative emotions [Ochsner et al., 2004] and anxiety [for a review see Berkowitz et al., 2007; Kalisch et al., 2006]. Especially during cognitive processing of threatening stimuli, PFC and amygdala activity seem to be inversely related [Hariri et al., 2000, 2003].

To summarize, in the current study we applied active or sham cTBS to either the left or right DLPFC in a sample of healthy subjects and measured both behavioral and hemodynamic reactions before and directly after stimulation. We expected to observe a significant decrease in O_2Hb levels following active TBS. Further, we hypothesized that PFC inhibition will cause impaired processing of anxiety words during an emotional Stroop task. More precisely, we assumed that active cTBS will lead to a potentiated attentional bias towards anxiety words in terms of increased reaction times and error rates.

MATERIALS AND METHODS

Subjects

In total, 51 right-handed volunteers participated in the current study (34 females, mean age: 23.14 ± 2.59 years). Except for one participant, who reported a dysthymic episode in the past, none of the subjects had a history of psychiatric or neurological illness according to a self-report screening questionnaire based on the Structured Clinical Interview for DSM-IV-TR Axis I Disorders [First et al., 2002]. Exclusion criteria were current pregnancy or rTMS contraindications (i.e., epilepsy, heart disease, magnetizable implants within the head).

Because rTMS over frontal areas may impact mood [Dearing et al., 1997], state anxiety, state anger, and mood were monitored before and after the experiment by means of the state subscales of the State-Trait Anxiety Inventory [STAI; Laux et al., 1981], State-Trait Anger Expression Inventory [STAXI; Schwenkmezger et al., 1992], and Positive and Negative Affect Schedule [PANAS; Krohne et al., 1996]. To assess anxiety sensitivity, subjects filled in the German version of the Anxiety Sensitivity Index [ASI; Alpers and Pauli, 2001].

All participants gave written informed consent and were TMS-naive. The present study was approved by the ethics committee of the University of Wuerzburg and is in accordance with the declaration of Helsinki from 2008. The sample was randomly divided into three subgroups: cTBS over the left DLPFC ($N = 16$), right DLPFC ($N = 16$), or as a control group, sham cTBS ($N = 19$) counterbalanced for stimulation side.

Emotional Stroop Task

We presented 15 neutral and 15 anxiety words in a computerized emotional Stroop task using Presentation software (Neurobehavioral Systems, Albany, CA). Anxiety words were related to bodily symptoms and cognitions of acute fear (e.g., fear, danger, or dizziness). Words of the two conditions were comparable regarding the number of letters, number of syllables, and frequency in German written and spoken language (CELEX database). Each word was presented once in four different font colors (i.e., blue, green, yellow, and red), resulting in 120 trials in total. Trials were arranged in a random event-related design lasting between 6 to 10 s and started with a fixation cross for 500 ms. Stimulus duration was 1.5 s and the interstimulus interval randomly jittered between 4 and 8 s. Participants had to indicate the font color by pressing a corresponding button using their index and middle fingers of both hands. Color-button assignment was counter-balanced across subjects and measurements. In advance, subjects practiced the appropriate color-button assignment during 20 practice trials by responding to meaningless letter strings ('XXXXXXXX').

All subjects were measured twice, once at baseline before the application of cTBS and once afterwards. Following cTBS, half of the subjects directly performed the second run of the emotional Stroop task while the other half firstly performed another cognitive task for 9 min. The results of this task are not the focus of the present study and will be reported elsewhere. The time interval between the end of stimulation and the second run never exceeded 45 min.

FNIRS

Blood oxygenation parameters were recorded with a 52-channel continuous wave system (ETG-4000 Optical Topography System; Hitachi Medical Corporation, Tokyo, Japan). A flexible 3 × 11 channel probe set was strapped over the subjects' forehead so that the middle probe of the bottom row was positioned over Fpz according to the international 10–20 system for electrode placement [Jasper, 1958; Fig. 1]. Near-infrared light of two different wavelengths (695 ± 20 and 830 ± 20 nm) was emitted through the skull and underlying cortex by 17 semiconductor lasers. Relative changes in the reflected light were detected by 16 neighboring photo-detectors at a temporal resolution of 10 Hz. Inter-probe distance was 3 cm. The measured signal was transformed into relative changes of O_2Hb by a modified Beer-Lambert Law. Further details about the fundamentals of the fNIRS signal are described elsewhere [Obrig and Villringer, 2003].

CTBS

Following the emotional Stroop task, TMS was applied with a figure-of-eight coil (MC-B70, 80 mm diameter) by a Medtronic MagPro X100 stimulator (Medtronic MagPro, Duesseldorf, Germany). CTBS consisted of 200 high-frequency triple-bursts (50 Hz), delivered every 200 ms (5-Hz theta rhythm), summing up to 600 pulses in total [see Huang et al., 2005]. Stimulation sites were electrode positions F3 (left DLPFC) or F4 (right DLPFC) according to the international 10–20 system for electrode positioning [Jasper, 1958]. Active left- and right-hemispheric stimulation was applied at 80% resting motor threshold as measured over the respective left and right primary motor cortex. Sham cTBS was applied at 60% resting motor threshold by tilting the coil by 45°.

Data Analysis

Data analysis was performed using MATLAB (MathWorks, Natick, MA) and PASW Statistics (SPSS, Chicago, IL). The level of significance was set to $P = 0.05$ and statistical trends were reported for $P \leq 0.10$. Data were analyzed using the general linear model. For post hoc tests either one- or paired-sample t tests were used. If necessary, a Bonferroni correction was applied. For O_2Hb activation maps the false discovery rate (FDR) correction was used.

TABLE I. Sample characteristics

	Prior to cTBS			Following cTBS		
	Sham cTBS	Left cTBS	Right cTBS	Sham cTBS	Left cTBS	Right cTBS
Anxiety sensitivity (ASI)	14.9 (10.0)	16.1 (6.9)	13.9 (8.5)	—	—	—
State anxiety (STAI)	41.5 (4.3)	40.2 (6.6)	40.3 (3.4)	39.8 (4.9)	38.8 (6.1)	37.2 (8.8)
State anger (STAXI)	10.6 (1.6)	10.7 (1.4)	10.8 (1.6)	10.3 (0.7)	10.1 (0.3)	10.6 (1.3)
Positive affect (PANAS)	29.2 (4.4)	29.2 (4.6)	29.1 (3.9)	26.3 (5.5)	29.4 (8.0)	23.9 (6.2)
Negative affect (PANAS)	11.6 (1.7)	11.1 (1.3)	12.5 (2.2)	11.3 (2.2)	10.8 (1.4)	11.4 (1.8)

Mean scores and standard deviations for all groups prior to and following cTBS.

Trait Anxiety and Mood

Possible TMS induced changes in state anxiety, state anger, and positive and negative affect were analyzed by performing repeated measures analyses of variance (ANOVA).

Behavioral Data

Mean reaction times were calculated for all correct trials in each condition separately. Responses beneath or above two standard deviations from the mean were excluded from further analyses. In addition, error rates were taken as a second indicator for a potential emotional Stroop effect. Baseline data were analyzed by paired-sample t tests. CTBS effects were in turn investigated by performing repeated measures ANOVA. One subject was excluded from behavioral analyses because of a 33% error rate. All other subjects ($N = 50$) correctly identified at least 85% of all trials.

FNIRS

Data analysis focused on changes in O_2Hb because it has been shown that O_2Hb reacts more sensitively than HHb to changes in cerebral blood flow and TBS [Hoshi et al., 2001; Mochizuki et al., 2007; Strangman et al., 2002b]. Ten data sets were excluded after visual inspection and quality ratings by three of the authors due to insufficient data quality. Three additional subjects could not be included due to data loss resulting in final sample sizes of 13 (sham cTBS), 11 (left active cTBS), and 14 (right active cTBS) subjects.

A cosine and a moving average filter (time window: 5 s) were applied to remove slow drifts and the high frequency portion of the data. Hypothesis testing was performed on estimated beta weights calculated by using an ordinary least squares regression model. A Gaussian hemodynamic response function was fitted to the data with a peak time of 7.5 s [for details see Plichta et al., 2006, 2007]. To test whether frontal areas were sufficiently activated during the emotional Stroop task, O_2Hb activation maps were constructed by calculating one-sample t tests against zero for every channel and condition separately and paired-sample t tests for the contrast between conditions. For further analyses, a priori determined bilateral DLPFC channels were averaged and taken as dependent variable (right DLPFC channels: 3, 13, 14, 24; left DLPFC channels: 8, 18, 19, 29; Fig. 1).

Exploratory analysis indicated a trend toward unexpected hemodynamic baseline differences between groups which could not be explained by any other collected data (demographics or psychometric data). Consequently, we added the grouping variable for baseline fNIRS analyses by means of repeated measures ANOVA. To compare hemodynamic activity between baseline and post-TBS measurements, repeated measures ANOVAs were performed for each experimental group separately to obviate possible confoundation due to baseline differences.

RESULTS

Trait Anxiety and Mood

Means and standard deviations for all questionnaires are displayed in Table I. A "Time" (pre vs. post cTBS) × "Group" (sham vs. left active vs. right active cTBS) ANOVA revealed a significant "Group" × "Time" interaction for the positive affect subscale of the PANAS ($F_{(2,48)} = 3.60$, $P < 0.05$). Post-hoc t tests showed a significant decrease of positive affect following right active ($P < 0.05$) and sham cTBS ($P < 0.05$) but not following left active stimulation (Fig. 2). This decrease was also reflected by a significant main effect of "Time" ($F_{(1,48)} = 10.42$, $P < 0.05$). Contrary, the same analysis of negative affect and state anxiety resulted in significant main effects of "Time" ($F_{(1,48)} = 5.13$, $P < 0.05$ for negative affect; $F_{(1,48)} = 7.69$, $P < 0.01$ for state anxiety), indicating decreases following TBS over all groups. No further interaction or group effects were observed. Analysis of state anger revealed no significant effects.

Behavioral Data

At baseline, paired-sample t tests showed an emotional Stroop effect for error rates ($t_{(49)} = 8.64$, $P < 0.001$) but not reaction times. Subjects made significantly more errors during the presentation of anxiety compared to neutral words.

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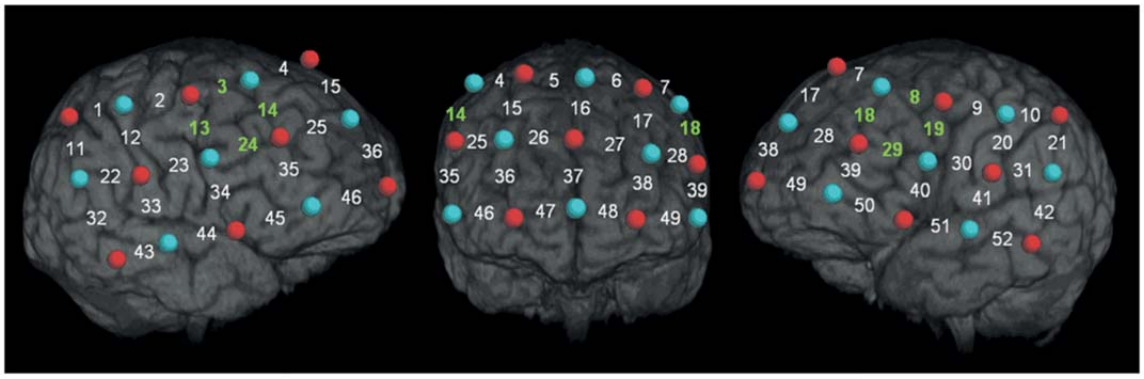

Figure 1.

Position of probes and channels mapped onto an MR scan of one single subject. Red dots represent light emitters and blue dots represent light detectors. Between every emitter and detector O_2Hb levels were measured by the respective channel. Green channel numbers correspond to ROI channels covering the DLPFC.

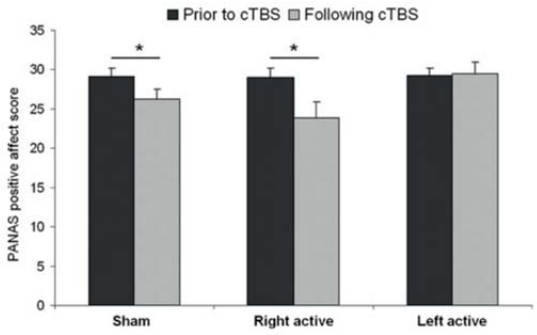

Figure 2.

Changes in positive affect (PANAS) over the course of the experiment. Error bars depict the standard error of the mean.

For the analysis of baseline compared to post measures, a significant effect of "Condition" ($F_{(1,47)} = 129.60$, $P < 0.001$) was again observed for error rates following cTBS in a three-factorial "Time" × "Condition" × "Group" repeated measures ANOVA. Subjects made more errors indexing anxiety compared to neutral words. In contrast, a trend for "Condition" was also found for reaction time measures ($F_{(1,47)} = 3.39$, $P < 0.10$) indicating that subjects tended to respond faster to anxiety words. Significant main effects for reaction times were also found for the factors "Group" ($F_{(2,47)} = 3.37$, $P < 0.05$) and "Time" ($F_{(1,47)} = 5.23$, $P < 0.05$). Subjects responded generally faster during the baseline measurement and subjects in the control group were significantly faster than those in the left-hemispheric cTBS group ($P < 0.05$). However, no interaction was observed.

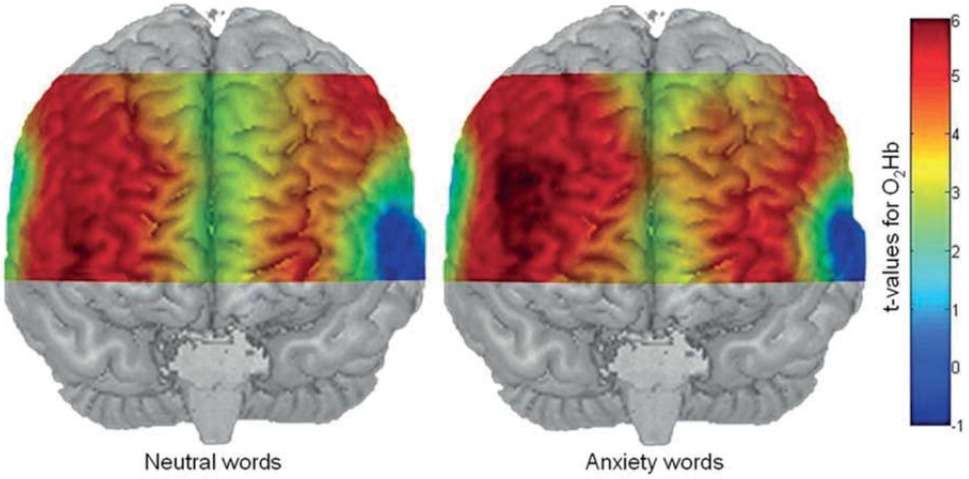

Figure 3.

Activation maps show increased levels of O_2Hb during baseline measures. Maps were superimposed on a standardized brain template.

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FNIRS

One-sample *t* tests against zero over all subjects showed widespread increases in prefrontal oxygenation for both conditions at baseline. During the presentation of neutral and anxiety words significant O_2Hb increases were found in 41 and 42 channels, respectively. Particularly, bilateral dorsolateral, bilateral anterior orbitofrontal, and right ventrolateral areas were recruited during the task (Fig. 3). All ROI channels were significantly activated ($P < 0.001$). No significant oxygenation patterns were observed for the contrast between both conditions.

A "Condition" × "Group" ANOVA for repeated measures revealed no significant interaction or main effect of condition prior to stimulation. However, a significant group difference was found ($F_{(2,35)} = 3.78$, $P < 0.05$). In the sham stimulation group subjects showed higher levels of O_2Hb compared to the active stimulation groups (sham > right: $P = 0.05$; sham > left: $P = 0.10$). Therefore, for post measures, a "Time" × "Condition" ANOVA for repeated measures was calculated for each group separately. No significant main or interaction effects were found for the sham or right active cTBS groups. However, for the left active cTBS group a significant main effect of "Time" was observed ($F_{(1,10)} = 7.30$, $P < 0.05$), indicating markedly reduced O_2Hb levels following cTBS (Fig. 4).

DISCUSSION

The results of the present study illustrate that cTBS applied to the left DLPFC had a measurable inhibitory effect on bilateral hemodynamic activity. Moreover, positive mood was significantly affected in this group. However, contrary to our hypotheses, no behavioral effects were found following PFC inhibition.

The current results are in line with the only previous fNIRS study investigating TBS effects on sensory, motor, and premotor cortices [Mochizuki et al., 2007]. Similar to our findings, the authors found attenuating cTBS effects on O_2Hb in areas contralateral to the site of stimulation (no measures were recorded for ipsilateral stimulation sites). Our results are also comparable to earlier findings of a suppressed BOLD signal response following stimulation of the frontal eye field in an fMRI study by Hubl et al. [2008]. Although comparability between the fMRI BOLD and fNIRS O_2Hb response is limited, since the BOLD signal seems to be more related to HHb signal changes [for a review see Steinbrink et al., 2006], cTBS has apparently significant influence on the hemodynamic response.

Though left active cTBS had an attenuating effect on hemodynamic activity, we observed no inhibition following right active stimulation. A similar asymmetric inhibitory impact of cTBS on the DLPFC was also reported in a PET study by Ko et al. [2008]. Modulations of striatal dopaminergic activity and behavioral performance during a set-shifting task were only found following cTBS over the left but not right DLPFC. This asymmetric effect might have been due to left-hemispheric dominance since all subjects in this study and the current experiment were right-handed. Another explanation is that both studies used completely different methodological approaches and results might have been caused by different underlying neural and metabolic processes. Finally, without knowledge about subjects' individual anatomy we cannot ascertain whether the appropriate region was sufficiently targeted during stimulation. Regarding the small sample size, it is possible that cTBS affected regions other than the right DLPFC. Contradicting this hypothesis is the observation that F3 and F4 correspond to the medial frontal gyrus and cover parts of the left and right DLPFC, respectively [Herwig et al., 2003]. Even if cTBS might have been applied to a neighboring brain region, effects on this area were measured by fNIRS since the calculated ROI also covered F3 and F4. A neuronavigation system might, however, be of profound benefit to further studies addressing these questions.

The finding of a bilateral reduction of O_2Hb is not surprising taking the inter-hemispheric connections into account and also earlier observations of a delayed but similar effect of TMS on the contralateral site of stimulation [Ilmoniemi et al., 1997].

Apart from the neurovascular effect, results show a significant positive impact of left DLPFC inhibition on mood. Positive affect markedly decreased during the course of the experiment in the right active and sham cTBS groups. This was not unexpected and probably due to fatigue and the long-lasting experimental procedure. Contrary, no such decrease was present in the left-hemispheric TBS group which showed stable levels on this scale. Considering the therapeutic potential of rTMS for mood disorders, this observation is interesting but on the other hand questionable since mood enhancing effects were primarily found for either excitatory high-frequency rTMS over the

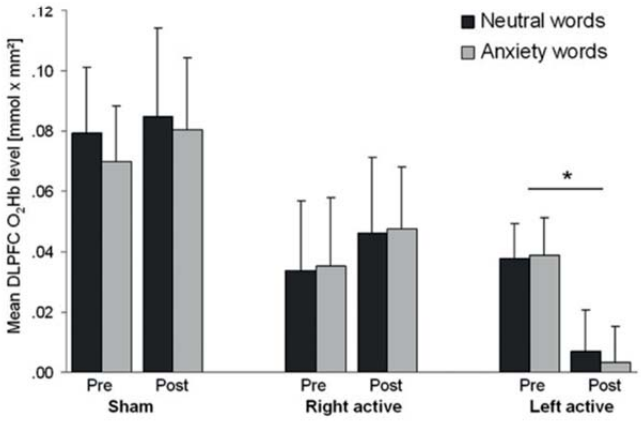

Figure 4.
Changes in DLPFC O_2Hb levels following active and sham cTBS. Error bars depict the standard error of the mean O_2Hb signal.

left PFC or inhibitory low-frequency rTMS over the right PFC [Gershon et al., 2003; Loo and Mitchell, 2005]. On the other hand, even high-frequency rTMS might have beneficial interfering effects on neuronal activity.

Contrasting our hypothesis, we could not support findings emphasizing an important functional role of the PFC for top-down control during the processing of fear-relevant stimuli although subjects made more errors in response to anxiety words. Anxiety compared to neutral words led to no additional recruitment of the DLPFC and inhibitory TBS caused no behavioral effects. One possible explanation for this finding might be that subjects successfully compensated for the interfering cTBS effects. It is likely that other cortical or subcortical areas were recruited to compensate for the lack of prefrontal control. Furthermore, the precise networks involved during the task could not be tracked by fNIRS due to limited spatial resolution. As a result, changes in the amygdala or anterior cingulate cortex (ACC) were not monitored. Both structures have been assumed to play a role during the emotional Stroop task although even PET and fMRI findings are inconsistent regarding their involvement [Compton et al., 2003; George et al., 1993; Isenberg et al., 1999; Whalen et al., 1998].

Furthermore, stimulus material displayed mainly symptoms of acute fear and anxiety thereby being particularly relevant for anxiety sensitive subjects. But regarding psychometric data, the present sample was little anxious at all. The sample's ASI scores were four points below the norm scores according to Peterson and Reiss [1992]. One can assume that the words were striking enough to draw attention away from the task but did not substantially activate the fear-network, at least in healthy subjects. In addition, evidence exists that both anxiety sensitivity and trait anxiety can affect the performance of emotional Stroop tasks [Richards et al., 1992; Stewart et al., 1998].

CONCLUSION

To conclude, we showed that hemodynamic activity was significantly reduced following cTBS over the left but not right DLPFC. This attenuation was present at both the actual site of stimulation and the contralateral brain region. However, no behavioral effects were observed following PFC inhibition. Future studies might preferentially focus on subjects with elevated or pathologically enhanced anxiety levels in order to challenge the regulatory top-down function of the PFC.

To the best of our knowledge, the current study showed for the first time that also prefrontal cTBS led to significant decreases in the fNIRS signal.

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Transition 1: From the Top to the Bottom…

In the previous *study 1*, the experimental manipulation consisted of directly interfering with PFC activity and to investigate the consequences of such manipulation on both neural and behavioral level. The methodological strategy thus followed a top-down approach by interfering at the very top of the fear circuit to test the functional role of the PFC during the processing of fear-relevant stimuli. The results of *study 1* showed that although PFC activation was significantly lowered following left sided cTBS, this had no impact on behavioral outcome but a positive effect on affect. Regarding the model in *figure 1* in the introduction, state measures of affect seem to not only act on the PFC-amygdala circuit but are also modulated by changes in PFC activation suggesting a bidirectional relationship between mood and PFC function.

In the next study, a reversed approach was employed. In s*tudy 2* it was tested whether ANS output can be used to infer valuable information about PFC functioning. Evidence exists, showing that autonomic flexibility as measured by the individual HRV might serve as an index for both effective emotional and cognitive regulation. Most of this research, however, relied on behavioral measures and only few functional imaging studies directly tested the hypothesis of a functional relationship between PFC and HRV (Åhs et al., 2009; Lane et al., 2009; Matthews et al., 2004; Thayer et al., 2012). Compared to *study 1*, the independent variable (HRV) was not directly manipulated in this study. Though individual HRV may change over time (e.g., through exercise or disease), this parameter resembles rather a trait marker and does not allow for a temporary experimental manipulation. Therefore, the sample was divided by a median split into subjects with low and high HRV to test whether the degree of ANS flexibility offers information about PFC functioning. Evidence for a functional relationship would support the idea that ANS activation underlies top-down regulation by the PFC and that HRV can in turn serve as an index for PFC functioning when imaging techniques are unavailable or contraindicated.

Dysfunctional neural and behavioral inhibition in subjects with low heart rate variability: The role of state anxiety

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Abstract

Substantial evidence indicates that the prefrontal cortex has inhibitory top-down influence on autonomic processes. An activation decrease in prefrontal areas causes a simultaneous attenuation in vagal tone and thus parasympathetic inhibition which leads to a subsequent acceleration of heart rate and decreased heart rate variability (HRV). Both low prefrontal activation and low HRV have been associated with deficits in emotional and cognitive regulation. In the present study, 54 low and 54 high HRV subjects performed a combined emotional and cognitive Stroop task while hemodynamic activity was measured by means of 52-channel functional near-infrared spectroscopy. Results showed that high HRV was associated with increased activity in the dorsolateral prefrontal cortex (DLPFC), particularly during incongruent trials. Low HRV, on the other hand, was linked to higher error rates indicating less efficient response inhibition capacities. State anxiety was higher in low HRV subjects and correlated negatively with DLPFC activation. This inverse relationship was most prominent during trials with threatening content.

The present study indicated dysfunctional cognitive but not emotional regulation in subjects with low HRV. However, state anxiety correlated negatively with DLPFC activation particularly in the presence of threatening stimuli, thereby potentially affecting HRV in an indirect manner.

1. Introduction

Over the past two decades, heart rate variability (HRV) has been increasingly discussed as a robust index for both physical and mental health (Appelhans and Luecken, 2006; Rajendra et al., 2006). There is clear evidence that low HRV is not only associated with but also increases the risk for cardiovascular diseases, diabetes, and overall morbidity (La Rovere et al., 2003; Rajendra et al., 2006). From an evolutionary perspective, HRV represents a quantifiable index in how successful an organism's autonomic nervous system reacts to even subtle changes in the inner and outer environment. This automatic process is the result of a fine-tuned interplay between the central nervous system, afferent and efferent nerves, and muscles controlling the heart. Both sympathetic and parasympathetic pathways descending from the medulla up- and down-regulate the heart rate through motor and vagal input (Brownley et al., 2000). According to the neurovisceral integration model of Thayer and Lane (2009), the heart is under constant indirect control of the prefrontal cortex (PFC). Inhibitory gamma-aminobutyric acidergic (GABAergic) projections emerging within the PFC are assumed to down-regulate the amygdala thereby impeding activation of sympathetic excitatory pathways originating from the rostral ventrolateral medulla. A disinhibition of the central nucleus of the amygdala causes an increase in sympathetic activity in this pathway and a simultaneous attenuation of parasympathetic vagal inhibition originating from the nucleus ambiguous and dorsal vagal motor nucleus, leading to an acceleration of heart rate. The authors stated that higher PFC activation causes higher variation in heartbeat intervals and - as a consequence - that HRV must be closely linked to cognitive and emotional regulation (Thayer et al., 2009; Thayer and Lane, 2009).

The association between high HRV and better performance on cognitive tasks is supported by several studies using Stroop (Hansen et al., 2003), working memory, continuous performance or monitoring tasks (Hansen et al., 2003; Luft et al., 2009). Hansen et al. (2004) experimentally attenuated HRV by aerobic detraining and observed a decline in performance on a range of executive tasks. Similarly, performance and HRV improved after aerobic training in a group of elderly subjects (Albinet et al., 2010).

Since both cognitive and emotional regulation are linked to increased activity in the anterior cingulate (ACC), medial prefrontal (MPFC), orbitofrontal (OFC), and dorsolateral prefrontal cortex (DLPFC), efficient emotional regulation is associated with high HRV according to the model of Thayer and Lane (2009). This is supported by a study of Pauls and Stemmler (2003) who found that a defensive coping style had an attenuating effect on HRV during experimentally induced fear. Both repressors (i.e. subjects who reported low anxiety but scored high on a social desirability scale) and high anxious subjects (i.e. high anxiety but low scores on the social desirability scale) also showed smaller respiratory sinus arrhythmia (RSA) amplitudes when compared to truly low anxious subjects (who scored low on the social desirability scale; Fuller, 1992). RSA describes the HR changes that occur during one breathing cycle and is considered to be one major part of HRV. In the presence of alcohol cues, abstinent alcoholics showed HRV increases compared to control subjects but less overall HRV. Moreover, there was an inverse relationship between HRV and self-reported compulsive drinking behavior (Ingjaldsson et al., 2003). High alcohol consumption in healthy subjects was also related to low HRV (Thayer et al., 2006). A recent study showed that the high frequency portion (HF-HRV) in subjects scoring low on a neuroticism scale increased when they actively down-regulated their emotions in response to negative stimuli compared to just passively viewing them (Di Simplicio et al., 2012). Particularly HF-HRV, in contrast to low frequency HRV (LF-HRV), is associated with vagally mediated parasympathetic activation (Rajendra et al., 2006).

Pathological states of anxiety are also highly associated with low HRV levels. Accumulated evidence exists linking low HRV to anxiety disorders, in particular panic disorder (Klein et al., 1995; McCraty et al., 2001; Yeragani et al., 1993), phobic anxiety or specific phobia (Bornas et al., 2005; Kawachi et al., 1995), and generalized anxiety disorder (Thayer et al., 1996). Apart from pathological anxiety, both trait and state anxiety were found to be inversely related to HRV (Miu et al., 2009; Shinba et al., 2008). Hypofrontality, which has been discussed as a neurobiological marker for acute states of anxiety and panic (Berkowitz et al., 2007; Dresler et al., 2009a; Dresler et al., 2011), might

account for the overall lower HRV found in those patients. However, most HRV studies reported no measures of state or trait anxiety.

So far, only few imaging studies have investigated the prefrontal impact on the inverse relationship between HRV and the effectiveness of cognitive or emotional regulation as it has been hypothesized previously (Thayer et al., 2009; Thayer and Lane, 2009). Matthews et al. (2004) found that HF-HRV positively correlated with left ventral ACC activity during a counting Stroop task. Activation increases in the MPFC, insula, caudate nucleus, and periaqueductal grey were also found to be related to higher HF-HRV in an emotion induction experiment (Lane et al., 2009). However, activation changes were observed regardless of whether the induced emotion was positive or negative. Åhs et al. (2009) found positive correlations between HF-HRV and ACC, MPFC, DLPFC, and caudate nucleus activation in social phobics during a social stress test. A recent meta-analysis of imaging studies in the field came to the conclusion that HRV is primarily linked to activation changes within the MPFC and amygdala (Thayer et al., 2012).

The aim of the current study was to evaluate the inhibitory role of the PFC during cognitive and emotional regulation in healthy low compared to healthy high HRV subjects by means of functional near-infrared spectroscopy (fNIRS). We therefore used a combined emotional and classical (cognitive) Stroop task (Stroop, 1935; Williams et al., 1996) using interfering and non-interfering color, neutral, and emotional words related to cognitions and physical reactions of acute anxiety. Both types of interference are known to elicit prefrontal regulatory control necessary for successful task performance (Compton et al., 2003; Ehlis et al., 2005). By using a similar task, Johnsen et al. (2003) found an increased attentional bias towards interfering stimuli in dental phobics with low HRV. We hypothesized reduced prefrontal activation in low HRV subjects during trials that require enhanced regulation in the presence of distracting stimulus information (incongruent color and emotional word content). Furthermore, we investigated the relationship between HRV, DLPFC activity, and state anxiety and discussed whether anxiety levels might serve as a higher-ranking factor for regulatory PFC activity and consequently also HRV.

2. Materials and Methods

2.1 Subjects

The present study was approved by the ethics committee of the University of Wuerzburg and all procedures were in accordance with the declaration of Helsinki from 2008. Informed consent was given by each of the 119 subjects who were mostly recruited from a larger pool of subjects who were screened for physical and mental health beforehand by a trained clinical psychologist. Five subjects were excluded from further analyses due to critical scores on either the Panic and Agoraphobia Scale (PAS score > 9; Bandelow, 1997) or Beck Depression Inventory (BDI-II score > 20; Beck et al., 1996) on the day of measurement, indicating mild forms of current panic disorder and moderate to severe depression symptoms. Sixteen participants with BDI-II scores between 9-13 (indexed as minimal depression) and four with scores between 14-19 (mild depression) were not excluded since they indicated no depressed mood over the past four weeks in a brief psychiatric screening questionnaire. Another subject reported current psychopharmacological treatment of generalized anxiety disorder and was therefore excluded. For five additional subjects no HRV data could be recorded because of technical failure. To analyze effects of anxiety, subjects filled in the State-Trait Anxiety Inventory (STAI; Spielberger et al., 1970). Altogether, data of 108 right-handed subjects were analyzed (see *table 1* for sample characteristics).

Table 1: Sample characteristics

	Low HRV (N=54)	High HRV (N=54)	p
sex (m/f)[a]	19/35	20/34	.84
education[a, b]	6/43/4/1	3/49/2/0	.18
SDNN (ms)	43.27 ± 7.55	73.53 ± 14.01	<.001
Age (years)	25.07 ± 5.22	24.19 ± 2.47	.91
BDI II	4.63 ± 4.03	3.91 ± 3.91	.34
ASI	13.63 ± 5.22	14.67 ± 7.21	.36
trait anxiety (STAI)	36.68 ± 7.66	34.71 ± 8.10	.15
state anxiety (STAI)	36.44 ± 7.69	34.22 ± 6.00	.08

[a] Mean values ± standard deviation (range); p values are given for non-parametric Mann-Whitney-U tests or chi² tests for variables sex and education.

[b] Education according to the German school/university system: university/(Fach-)Abitur/Mittlere Reife/not applicable (university = university graduate, (Fach-)Abitur ~ high school (high level), Mittlere Reife ~ high school (moderate level)).

ASI: Anxiety Sensitivity Index; BDI: Beck Depression Inventory; HRV: Heart rate variability; SDNN: standard deviation of the normal-to-normal heartbeat intervals; STAI: State-Trait Anxiety Inventory

2.2 Combined Stroop task

Subjects performed a combined emotional and classical Stroop task while prefrontal hemodynamics, HRV, skin conductance responses (SCR), and behavioral data (error rates and reaction times) were recorded. Anxiety, neutral, incongruent and congruent color words were presented by Presentation software (Neurobehavioral Systems, Albany, CA) on a black screen either in red, green or yellow font color. Subjects had to indicate the font color by pressing a corresponding button with their right index, middle or ring finger. Anxiety words were related to bodily sensations and cognitions of acute fear (e.g. dizziness, heart attack, panic) and were matched to neutral words with regard to frequency within German language, number of letters and syllables. Each trial started with a 500 ms fixation cross followed by 1.5 sec stimulus presentation and a randomly jittered inter-stimulus interval of 2 to 5 sec. Each of the neutral and anxiety words was shown once in each font color (45 trials per condition). For congruent and incongruent trials 16 stimuli were presented for each font color (48 trials per condition). In total, the task comprised 186 trials and the measurement duration varied between 16 to 18 min. All trials were presented randomly in an event-related design. Prior to the experiment, subjects completed 20 practice trials with meaningless letter strings to learn the appropriate color-button assignment.

2.3 HRV

Pulse intervals were recorded on a beat-to-beat sampling rate using the volume-clamp method (Peñáz, 1973; Finometer® Midi, Finapres Medical Systems, Netherlands). All data were analyzed by means of Kubios HRV (version 2.0, Biosignal Analysis and Medical Imaging Group, University of Eastern Finland). Time domain based HRV was defined as the standard deviation of the normal-to-normal heartbeat intervals (SDNN) for the first 15 min of the experiment starting with the first stimulus. This was done because of the randomly jittered inter-stimulus interval which caused differences in total measurement time of up to 2 min across subjects. The sample was divided into

high and low HRV groups according to a median-split of the SDNN *(table 1).* Apart from SDNN, groups differed trend-wise in state anxiety.

2.4 FNIRS and SCR

We used a 52-channel ETG-4000 Optical Topography System (Hitachi, Medical Corporation, Tokyo, Japan) to measure changes in oxygenated (O_2Hb) and deoxygenated hemoglobin (Hbb) concentration by means of near-infrared light within the prefrontal cortex. During fNIRS, near-infrared light in the range of 695 ± 29 nm and 830 ± 20 nm is sent through cortical tissue and blood vessels. The reflected amount of light is continuously (10 Hz sampling frequency) captured by photo-detectors placed on the head and transformed online by a modified Beer-Lambert Law (for details see Plichta et al., 2006). Brain activity, commonly associated with increased cerebral blood flow, is linked to increases in O_2Hb and simultaneous decreases in HHb (Obrig and Villringer, 2003). A 3x11 probe set with 17 light emitting laser diodes and 16 detectors was placed over the forehead thereby covering most of the PFC, large parts of motor and premotor cortex, minor parts of the temporal and sensory cortex, and supramarginal gyrus. Detailed information about probe set placement can be found elsewhere (Tupak et al., 2013).

SCRs were recorded at the middle phalanxes of the non-dominant left ring and little finger by means of two Ag/AgCl electrodes and amplified at a sampling rate of 2000 Hz (QuickAmp 72, Brain Products, Munich, Germany). At the same time, event-related potentials were recorded at four midline scalp positions that were not covered by the fNIRS probe set. However, these data were not included in our hypotheses and will be reported elsewhere.

2.5 Statistical analysis

Reaction times were averaged per condition excluding trials beneath or above two standard deviations from the mean. Error rates and reaction times were further analyzed using repeated measures analyses of variance (ANOVA).

FNIRS data were first corrected using a moving average filter with a time window of 5 sec. Estimated beta weights were calculated by an ordinary least squares regression model with a peak time of 6.5 sec after stimulus onset (Plichta et al., 2007). For exploratory contrasts between groups for individual conditions, channel-by-channel one-way ANOVAs were performed and Dubey/Armitage-Parmar (D/AP; Sankoh et al., 1997) corrections were applied to control for multiple testing. For further analyses, bilateral DLPFC channels 3, 8, 13, 14, 18, 19, 24, and 29 were pooled to form one region of interest (ROI) for each parameter (O2Hb and Hbb; see Tupak et al., 2013).

SCR data were filtered offline with a 1 Hz low-pass filter and transformed from mV into µS. Time segments from –1 to 7 sec were averaged only for correct trials and baseline corrected for a time interval of 1 sec before stimulus onset. Peaks were detected in a time window of 1.5 to 7 sec. To control for inter-individual variability, we applied a log transformation before data were entered into a general linear model.

All data were analyzed using Matlab (v. R2008a, The Math Works, Natick, MA), Vision Analyzer (Brain Products, Munich, Germany), and SPSS (v. 19, IBM SPSS Statistics, Munich, Germany). The alpha level of significance was set to .05 and to .10 for trends. Error rates, reaction times, estimated ROI beta weights, and SCR data were analyzed with separate Stroop (classical vs. emotional) x interference (interfering vs. non-interfering) x group (low vs. high HRV) repeated measures analyses of variance (ANOVA). Since most variables were not normally distributed, we used non-parametric Wilcoxon and Mann-Whitney U post hoc tests. In case of significant Stroop x interference interactions, planned contrasts were calculated to solely compare conditions within each Stroop task (incongruent vs. congruent and anxiety vs. neutral words).

Further, Spearman correlations were calculated between HRV, DLPFC activation, and state anxiety. To elucidate the relationship between state anxiety and frontal oxygenation in more detail, state anxiety was again correlated with DLPFC activity for each condition separately. Bonferroni corrections were applied to control for multiple comparisons.

3. Results

3.1 Behavioral data

All subjects identified more than 80% of all trials correctly and were included into further analyses. Analyses of error rates revealed significant main effects of Stroop ($F_{(1,106)}$=30.49, p<.001), interference ($F_{(1,106)}$=41.51, p<.001), and HRV group ($F_{(1,106)}$=4.90, p=.03). Significant interactions were found for the factors Stroop x interference ($F_{(1,106)}$=29.77, p<.001) and trends for the Stroop x HRV ($F_{(1,106)}$=3.18, p=.08) and Stroop x interference x HRV interaction ($F_{(1,106)}$=3.54, p=.06). Subjects made significantly more errors during incongruent compared to congruent trials (z=-6.15, p<.001) but not during anxiety compared to neutral trials (z=-.60, p=.55). Post hoc analyses of the three-way interaction revealed no differences in processing individual conditions between subjects with high and low HRV. Both groups showed a cognitive interference bias (low HRV: z=-5.06, p<.001; high HRV: z=-3.61, p<.001) but no emotional interference. However, low HRV subjects made generally more errors (7.57 ± 6.22) than high HRV subjects (5.22 ± 5.01; U=1012.00, p=.006). For exploratory

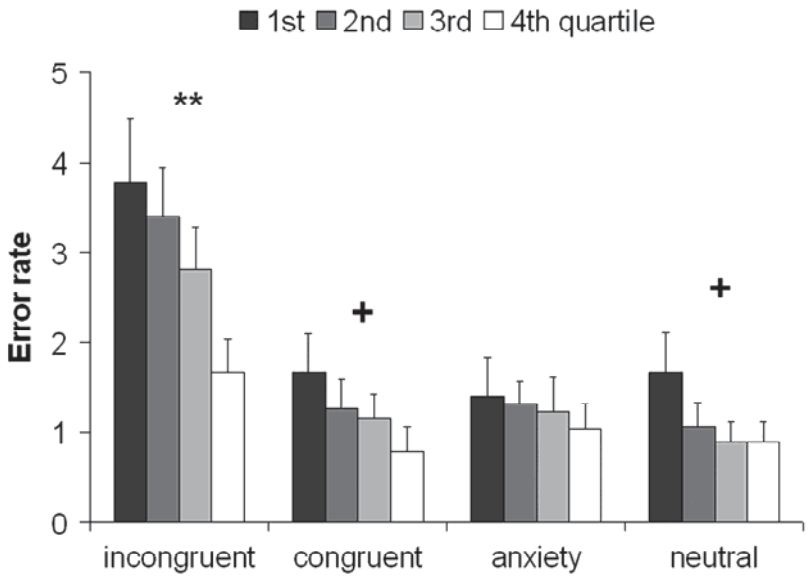

Figure 1: Error rates
The figure depicts linear trend tests over all conditions (** p<.01, + p<.10). Subjects were divided into quartiles based on their individual heart rate variability (HRV). From highest (fourth quartile) to lowest HRV (first quartile), error rates increased in a linear fashion for all trial types except for anxiety trials for which no linear trend was observed.

reasons, we further divided the complete sample into quartiles of HRV and performed a linear trend test over the error rates of each condition. Significant linear trends were found for incongruent (p=.005), congruent (p=.07), and neutral words (p=.06) but not anxiety words (p=.42; *figure 1*).

Analyses of reaction times indicated significant main effects of Stroop ($F_{(1,106)}$=12.62, p=.001), interference ($F_{(1,106)}$=131.39, p<.001), and an interaction between both factors ($F_{(1,106)}$=126.17, p<.001). Again an interference bias was present for the classical (incongruent > congruent, z=-8.83, p<.001) but not for the emotional Stroop task (z=-.75, p=.45).

3.2 fNIRS

3.2.1 O_2Hb

ROI analyses by means of a Stroop x interference x HRV group ANOVA showed significant effects of Stroop ($F_{(1,106)}$=6.15, p=.02), interference ($F_{(1,106)}$=6.96, p=.01), HRV group ($F_{(1,106)}$=4.92, p=.03), and Stroop x interference ($F_{(1,106)}$=5.31, p=.02). Processing of incongruent compared to congruent words led to increased activity (Z=-4.06, p<.001), while anxiety and neutral words equally

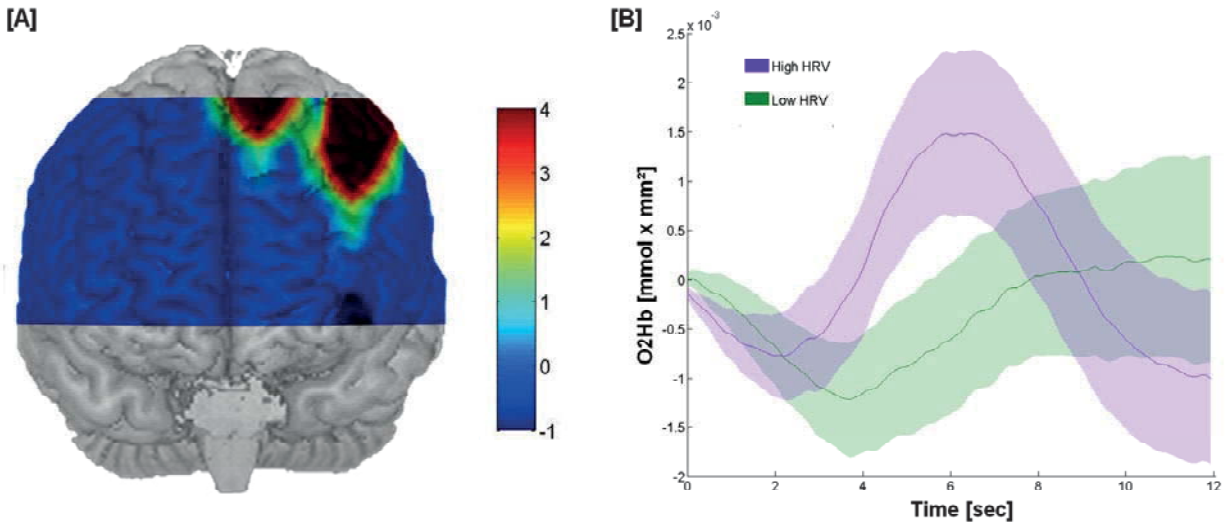

Figure 2: Prefrontal activation in high versus low HRV subjects
[A] Oxygenated hemoglobin (O_2Hb) contrast between heart rate variability (HRV) groups for incongruent color word trials (F-statistic). High HRV subjects showed increased left dorsolateral prefrontal activity compared to low HRV subjects. [B] Example of the average O_2Hb response curve for incongruent trials in one channel over the left superior frontal gyrus (channel 6). Compared to high HRV, low HRV subjects displayed a flattened hemodynamic response with a later onset.

activated the DLPFC (Z=-.30, p=.76). Generally, high compared to low HRV subjects showed higher DLPFC activation (U=1129.00, p=.04). However, when each condition was contrasted separately over all channels, it became apparent that this group difference was only present during incongruent color words and was restricted to the left DLPFC (channels 6, 8, and 18; *figure 2*). For congruent trials, increased activation was observed in one channel (11) over the right sensorimotor cortex and no significant differences were seen for neutral or anxiety words.

3.2.2 HHb

The same ANOVA applied to HHb beta values resulted in significant effects for Stroop ($F_{(1,106)}$=3.24, p=.08), interference ($F_{(1,106)}$=24.32, p<.001), and Stroop x interference ($F_{(1,106)}$=5.93, p=.02). Larger HHb decreases were observed for incongruent compared to congruent words (Z=-4.73, p<.001) and also for anxiety compared to neutral words (Z=-2.23, p=.03). However, no significant difference was seen between high and low HRV subjects ($F_{(1,106)}$=1.79, p=.18). For contrasts between groups over all conditions and channels, larger decreases were found in high HRV subjects in the right PFC (channel 25) for congruent and neutral words, in the left DLPFC (channel 29) for incongruent words, and in the left ventral PFC (channel 49) for congruent and incongruent words.

3.3 SCR

A Stroop x interference x HRV group repeated measures ANOVA revealed no HRV effects but a main effect of interference ($F_{(1,106)}$=6.19, p=.01) and a Stroop x interference interaction ($F_{(1,106)}$=17.63, p<.001). Incongruent compared to congruent words elicited higher SCRs (Z=-4.49, p<.001) whereas no difference was found for anxiety compared to neutral words (Z=-.40, p=.69).

3.4 State anxiety

As depicted in *table 1*, state anxiety differed between HRV groups by trend. Spearman correlations, however, revealed no explicit linear relationship between HRV and state anxiety (r_s=-.10, p=.45) or HRV and DLPFC activation (O_2Hb: r_s=.11, p=.38; HHb: r_s=-.08, p=1.0). However, a

significant negative correlation was found between state anxiety and DLPFC O_2Hb (r_s=-.22, p=.03) but not HHb (r_s=-.13, p=.56) across all conditions. Moreover, state anxiety correlated negatively with DLPFC O_2Hb measures during all conditions except for incongruent trials (incongruent: r_s=-.10, p=.66; congruent: r_s =-.20, p=.08; anxiety: r_s=-.27, p=.008, neutral: r_s=-.20, p=.08). Correlations bear on one-sided tests because all variables were associated in the direction that was hypothesized beforehand.

4. Discussion

The present findings support earlier studies which showed a direct link between diminished PFC activity and lower HRV (e.g. Ahern et al., 2001; Lane et al., 2009). We measured generally higher DLPFC O_2Hb increases in high HRV subjects and group contrasts revealed that this higher activation was most prominent during incongruent trials. This suggests more efficient neural inhibition in the presence of highly interfering stimuli in high HRV subjects. Also for HHb measures, larger PFC activation was observed in high HRV subjects but only in single channels. These effects were also located within prefrontal regions but rather unspecific regarding conditions and disappeared within ROI analyses. Behavioral measures also supported the assumption of deficient regulatory PFC activation in low HRV subjects. The lower the HRV, the higher error rates were found during all but anxiety trials. We found, however, no correlational relationship between HRV and DLPFC activity. Taking state anxiety levels into account, it became apparent that increased state anxiety resulted in lower PFC activation during all conditions except for incongruent word stimuli. Interestingly, this correlation was strongest during trials presenting anxiety words. State anxiety was also higher in the low HRV group.

In general, the present results are in line with the model of Thayer and Lane (2009) stating that prefrontal brain regions have indirect inhibitory influence on efferent nerves regulating the heartbeat. Our results also favor a link between HRV levels and cognitive neural and behavioral inhibitory processes as proposed by the model. In contrast, we could not find a specific emotion regulation deficit in low HRV subjects. One possible explanation for that might be that the selected

anxiety words were too weak with respect to emotional intensity and arousal to cause emotional interference in healthy subjects as reflected by comparable error rates, reaction times, and SCR for both anxiety and neutral words. Moreover, validity and reliability of the emotional Stroop task and their potential confounders have been critically discussed by others (Algom et al., 2004; Dresler et al., 2009b; McKenna and Sharma, 2004). On the contrary, fNIRS data showed an emotional interference effect between neutral and anxiety words. In the presence of emotional interference, DLPFC HHb measures decreased whereas no effect was seen for O_2Hb, a finding that has been reported for O_2Hb previously (Tupak et al., 2013). Given that the a priori assumption of a valid emotional Stroop task was potentially not fulfilled, no definite conclusion regarding the relationship between emotional regulation and HRV can be drawn from our results. For this reason, we restricted interpretation of this part of the data to overall performance and DLPFC activity disregarding the emotional valence of the stimuli. In this way, support for better cognitive regulation in high HRV subjects in terms of lower error rates could also be found for neutral but not anxiety words. It is striking that both groups performed equally on just this emotion condition. Contradicting behavioral results were also found in an earlier combined Stroop task study by Johnsen et al. (2003) who found particularly increased response latencies in high relative to low HRV subjects for incongruent and threatening words.

In accordance with behavioral measures, we observed a classical Stroop effect in terms of increased SCR and DLPFC activation during incongruent compared to congruent color words. Again, group differences were present for overall activation levels but not specific to a certain condition within DLPFC ROI analysis. Exploratory whole-probe set analyses of O_2Hb levels, however, revealed that this group effect was only present in the left DLPFC during the incongruent color condition though smaller ROI effects in the remaining conditions might have been erased by correcting for multiple comparisons. We assume that this effect might be stronger rather than unique for trials with high attentional interference. No such specification was seen for HHb parameters.

Taken together, low HRV seems to be linked to dysfunctional regulatory processes, which is also reflected on the neural level. Several studies showed that impulsive behavior is associated with

less activation in prefrontal brain areas (Horn et al., 2003; Kopf et al., 2012). Both impulsive behavior and deficient response inhibition are core symptoms of attention-deficit/hyperactivity disorder (ADHD; American Psychiatric Association, 2000), a disorder that is characterized by diminished prefrontal brain activation during cognitive tasks (Ehlis et al., 2008; Schecklmann et al., 2008).

Although several studies showed an association between low HRV and elevated anxiety levels (e.g. Fuller, 1992; Miu et al., 2009), so far no study has investigated the relationship between all three variables (HRV, anxiety, and brain activation). Consistent with earlier findings, low HRV subjects in our sample also displayed higher state anxiety. In contrast, we could not replicate previous findings of a negative correlation between HRV and state anxiety. In fact, a significant negative correlation was only found between state anxiety and DLPFC activation with the strongest effect during the presentation of anxiety words. This poses the question whether anxiety may serve as a higher-order factor interacting with prefrontal cortex activation which in turn up- or down-regulates vagal inhibition of the heart. The neurobiological circuit controlling the heartbeat strongly resembles the fear circuit accounting for the neural correlates of acute anxiety as particularly present in panic disorder (Dresler et al., 2013; Gorman et al., 2000). The neuroanatomical hypothesis of panic disorder, for example, describes a hierarchical system consisting of three main entities: MPFC and ACC, amygdala, and brainstem. Apart from HR, this model also explains how other vegetative symptoms such as respiratory rate and perspiration are up-regulated through increased amygdala activity which is directly projecting to various brain stem nuclei. At the top of this fear circuit, ACC and MPFC serve to exert inhibitory control on these subcortical structures. Once disinhibited, the amygdala elicits a multitude of the vegetative symptoms that accompany acute fear (Gorman et al., 2000). A recent revision of the original model further includes the insula, hippocampal and parahippocampal areas as important parts of this network (Dresler et al., 2013). The neurovisceral integration model (Thayer and Lane, 2009) might be thought of as one part of this model that focuses exclusively on HRV. As shown in our study, state anxiety influences PFC activation and thus probably also the degree of amygdalar disinhibition which in turn triggers the autonomic nervous

system. As such, both environmental factors and personality traits should indirectly impact HRV and as a consequence physical well-being. This assumption is supported by previous work linking certain personality traits with low HRV (Di Simplicio et al., 2012; Fuller, 1992) or showing that changing daily routines can alter HRV (Hansen et al., 2004).

Dividing subjects into low and high HRV groups according to measures that have been recorded during performance of the task poses an important limitation of the present study. High and low HRV might have been the consequence of processes that were elicited by the Stroop task itself rather than a reliable trait marker. Making more errors might have increased arousal and HR thereby decreasing HRV. Likewise, fatigue might have been a confounder to the data. Subjects with chronic fatigue show lower HRV (Stewart, 2000) and fatigue is associated with increased LF- and decreased HF-HRV (Zhang and Yu, 2010). On the other hand, fatigue also causes worse performance during cognitive tasks and might have accounted for the higher error rates found in the low HRV group. Nevertheless, the effects found in these studies referred to spectral measures of HRV, no significant relationship was seen between fatigue and the SDNN in a study by Tran et al. (2009). Worse cognitive performance was also linked to low baseline HRV instead of measures collected during task performance (Hansen et al., 2003). These previous results support the conclusion that cognitive regulation is more effective among high HRV individuals and cannot solely be explained by the factors described above.

5. Conclusion

The present results provide further evidence for a link between PFC activation and HRV as posed by the neurovisceral integration model (Thayer et al., 2009; Thayer and Lane, 2009). Subjects with low HRV displayed less prefrontal activation and reacted more impulsively to cognitively interfering stimuli. However, we suggest including state anxiety as a higher-order factor into the model since our findings showed that state anxiety is inversely related to prefrontal activity and

increased in low HRV individuals. Future studies might further explore the influence of personality states and traits on PFC function and as a consequence also on HRV.

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Transition 2: From the Changeable to the Unchangeable…

The findings of *study 2* showed that ANS activation can offer valuable information about overall prefrontal activation and to a certain degree also about cognitive top-down regulation but is not directly correlated with the PFC. Moreover, it was again found that the PFC is rather associated with affect, because DLPFC activation correlated negatively with anxiety. The results are thus similar to those of *study 1* because in both studies a critical relationship was found between overall PFC activation and affective state measures but not between PFC activation and behavioral output to fear-relevant stimuli. Both studies thus support the notion of a critical role of the DLPFC for affective regulation, although with ambiguous results. While in *study 1* decreased DLPFC function had a beneficial effect on mood in terms of no decrease in positive affect, it was linked to higher state anxiety in *study 2*. Those seemingly contradictory findings and the missing link to behavior are discussed in detail in the general discussion section later on.

With regard to the following study, *study 3*, it is important to consider the present and earlier research on HRV which has found that 1) HRV crucially linked to cognitive and behavioral regulation (Hansen et al., 2004) and 2) that HRV can be increased through physical exercise (Schuit et al., 1999). Furthermore, improving physical fitness has beneficial effects on executive functioning (Albinet et al., 2010) and thus perhaps also on emotional regulation. This means that top-down regulation mediated by the PFC can be improved by increasing ANS flexibility. Consequently, effective PFC function depends to a certain degree on the individual lifestyle. Genetic modulators of PFC function, in contrast, are out of personal control. Although yet no single gene has been suggested to necessarily cause pathological anxiety, a range of candidate genes have been identified that seem to crucially impact on the fear network (Domschke and Dannlowski, 2010). These genes have been hypothesized to accumulate with environmental stressors, according to the diathesis-stress model (Ingram and Luxton, 2005), and pose certain individuals at an increased risk for developing an anxiety disorder. One of these genes, the *NPSR1* rs324981 gene has recently been shown to be related to PD (Domschke et al., 2011) and to significantly alter fear network activity in both PD patients and

healthy controls (Dannlowski et al., 2011; Domschke et al., 2011; Raczka et al., 2010). However, only few studies (i.e., Domschke et al., 2011; Raczka et al., 2010) examined PFC functioning during the processing of fear-relevant stimuli in humans and until now it is not clear whether alterations in PFC function in *NPSR1* risk allele carriers are specific to fear-relevant stimuli. To address this question, *study 3* investigated cognitive and emotional top-down regulation in *NPSR1* risk and non-risk allele carriers by means of the combined Stroop task.

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Neuropeptide S receptor gene: Fear-specific modulations of prefrontal activation

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ABSTRACT

Since central administration of neuropeptide S (NPS) has been shown to exert anxiolytic effects on rodent behavior in a number of studies, genetic variants of its cognate G-protein coupled receptor (NPSR1) became the focus of several recent human studies on anxiety and anxiety disorders. The T allele of rs324981, which goes along with enhanced receptor function, was associated with panic disorder, increased anxiety sensitivity in healthy subjects, attenuated prefrontal brain activation and elevated amygdala responses to fear-relevant stimuli. To investigate whether prefrontal attenuations in rs324981 T allele carriers are specific to fear-relevant stimulus content and cannot be attributed to a generally higher interference of emotional stimuli, 92 subjects performed a combined cognitive and emotional Stroop task while oxygenation changes in the prefrontal cortex were recorded using functional near-infrared spectroscopy. Results showed a specific *NPSR1* gene activation modulation in response to fear-relevant word stimuli. Only A-homozygotes displayed an emotional Stroop effect in terms of increased activation to fear-relevant stimuli in medial and dorsolateral prefrontal cortex. Specifically, activation in the fear-relevant condition was higher in A-homozygotes as compared to T allele carriers while no group differences were found during neutral, congruent or highly interfering incongruent color word presentation. The current results are in line with earlier imaging genetic studies and suggest a potential protective function of the *NPSR1* rs324981 A/A genotype against pathologically enhanced anxiety that might be explained by stronger reflective prefrontal regulation over the subcortical fear response.

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Introduction

A growing body of both animal and human research points to a potential etiological role of neuropeptide S (NPS) and its cognate G-protein coupled receptor (NPSR1) for the development of anxiety disorders and exaggerated fear responses in general (Domschke et al., 2011; Pape et al., 2010). Most prominent regions for NPS precursor and NPSR mRNA expression, as first described by Xu et al. (2004), are the brainstem and hypothalamus, respectively, while NPSR mRNA is also highly expressed within the hippocampus, cortex, and amygdala. By binding to the NPSR, NPS elicits increases in intracellular calcium and cyclic adenosine monophosphate (Reinscheid et al., 2005). Central NPS administration had anxiolytic effects on rodent behavior in a number of tests including the open field, light–dark box, elevated plus/zero maze, four-plate test, and marble-burying test (Jüngling et al., 2008; Leonard et al., 2008; Xu et al., 2004). Ionescu et al. (2012) recently developed a method to apply NPS intranasally with similar effects in the light–dark box test, particularly in mouse strains with high innate anxiety behavior. Conversely, application of an NPS receptor antagonist (SHA 68) increased anxious behavior in the open field test (Jüngling et al., 2008). NPS also impacted behavior in a fear conditioning experiment: When administered directly into the lateral and basolateral amygdala, NPS led to accelerated fear extinction learning in mice (Jüngling et al., 2008).

Due to increasing evidence for an important anxiolytic role of NPS within the central nervous system, genetic variants of the NPS receptor

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attracted attention particularly for human studies on anxiety and anxiety disorders. A single nucleotide polymorphism (rs324981 A/T) in the human *NPSR1* gene on chromosome 7p14 results in an Asn–Ile exchange at position 107. The T allele (coding for Ile^{107}) leads to increased agonist efficiency, while binding affinity remains unaffected (Reinscheid et al., 2005). Previous studies indicated an underrepresentation of the A/A genotype among patients with panic disorder regardless of the ethnical background but with ambiguous gender effects (Domschke et al., 2011; Donner et al., 2010; Okamura et al., 2007). Consistently, anxiety sensitivity, a trait that has been linked to an increased risk for panic disorder and panic attacks (Schmidt et al., 1997; Schmidt et al., 2006), was found to be elevated among T allele carriers with panic disorder (Domschke et al., 2011) and healthy homozygous T allele carriers in interaction with environmental factors such as childhood abuse and recent negative life events (Klauke et al., in press). Further, the T allele was associated with higher physiological arousal during a behavioral avoidance test (Domschke et al., 2011), a tendency to over-interpret the harmfulness of aversive stimuli (Raczka et al., 2010), and interacted with caffeine administration in an emotion-modulated startle paradigm (Domschke et al., 2012).

Taken together, contradictory findings were obtained comparing human and animal studies, even though NPS has been consistently associated with anxiety-related characteristics in both species. While in humans, increased agonist efficiency on the NPS receptor was linked to increased anxiety, NPS administration in rodents had anxiolytic effects. Currently, there is no definite explanation for this discrepancy but it has been suggested that arousal might be an influential mediator (Domschke et al., 2011). NPS administration in mice also led to increases in arousal (Xu et al., 2004) and the misinterpretation of arousal-related bodily symptoms as being potentially harmful is in turn a core characteristic of panic disorder and augmented anxiety sensitivity (Clark et al., 1997; McNally, 2002).

Until today, the impact of *NPSR1* genotype on human brain function has been investigated by three functional magnetic resonance imaging (fMRI) studies (Dannlowski et al., 2011; Domschke et al., 2011; Raczka et al., 2010). Domschke et al. (2011) reported lower activation in the anterior cingulate (ACC), dorsolateral prefrontal (DLPFC), and orbitofrontal cortex (OFC) during the presentation of fearful faces in panic disorder patients with at least one T allele. Using a similar paradigm, Dannlowski et al. (2011) found higher basolateral amygdala activation to fearful and angry facial stimuli in healthy T allele carriers. Considering the T allele as a risk factor for exaggerated fear responses and anxiety disorders, these findings are in line with common theories about the neural substrates of fear, anxiety, and their regulation, which posit a down-regulatory function of prefrontal over subcortical limbic structures (Berkowitz et al., 2007; Dresler et al., in press). In a fear conditioning experiment, Raczka et al. (2010) observed higher dorsal ACC and dorsomedial PFC responses to CS+ stimuli during the acquisition phase in T allele carriers. A critical regulatory function has been ascribed particularly to the medial PFC (MPFC) during fear extinction (Guhn et al., 2012; Milad and Quirk, 2002), whereas dorsal ACC activation seems to index the magnitude of the fear response (Milad et al., 2007).

In summary, both human and animal studies provide profound evidence for a mediating role of NPS and NPSR on physiological, neural, and behavioral correlates of fear and anxiety. The present study focused on how particularly PFC function is modulated by *NPSR1* genotype in the presence of fear-relevant stimuli in a group of healthy subjects. Therefore, we measured DLPFC and MPFC responses to fear-relevant relative to neutral word stimuli by means of functional near-infrared spectroscopy (fNIRS) in an emotional Stroop paradigm. The emotional Stroop task and its variations are supposed to assess emotional regulation through attentional processes (Gyurak et al., 2011; Koole, 2009; Todd et al., 2012). Reading is a highly automated process in adults that can slow down task-related performance (e.g., indicating the font color of a presented word) when comprising task-irrelevant information. In addition, negative compared to neutral valence of a word stimulus is thought to capture attention thereby increasing response latencies and error rates, particularly in anxiety disorder patients when exposed to disorder-related word stimuli (Williams et al., 1996) whereas results remain ambiguous for healthy individuals (Dresler et al., 2009b; Tupak et al., in press). The disparity between behavioral or neural reactions to emotional vs. neutral stimuli is supposed to indicate the degree of attentional distraction due to emotional interference.

To rule out simple interference effects and to differentiate between emotional and cognitive regulation, we extended the task by adding the classical Stroop variant that comprises congruent and incongruent color word stimuli. Both tasks (emotional and cognitive) have been shown to elicit prefrontal activation during high interference trials (Compton et al., 2003; Ehlis et al., 2005). Particularly the DLPFC is activated by incongruent color–word pairs and has therefore been interpreted as a top-down regulator for resolving task conflict (MacDonald et al., 2000) regardless of whether the interference derived from response or semantic conflict alone (van Veen and Carter, 2005). The MPFC seems to be involved during cognitive conflict tasks as well (Ochsner et al., 2008) although it has been found to be more characteristic for emotional than cognitive Stroop trials (Compton et al., 2003). According to the neural network model by Cohen et al. (1990), the interplay between lateral PFC and MPFC, particularly ACC, is crucial for resolving task conflict. Monitoring roles were ascribed to ACC and parts of adjacent MPFC, while goal-directed behavior is represented in lateral PFC (Buhle et al., 2010).

If NPSR plays a specific role in fear processing, modulations of PFC activity should be observed only during the emotional (fear-relevant vs. neutral) but not during the cognitive component (incongruent vs. congruent) of the task. Based on previous findings (e.g., Domschke et al., 2011; see above), we hypothesized diminished activation in these areas among T allele carriers during the presentation of fear-relevant stimuli only.

Materials and methods

Subjects

The study was approved by the ethics committee of the University of Würzburg, Germany, and is in accordance with the declaration of Helsinki in its latest revision. All 100 participants were recruited from a larger pool of subjects ascertained within the framework of a Collaborative Research Center (SFB TRR 58) located at the Universities of Würzburg, Münster, and Hamburg, Germany. These subjects were previously screened for mental axis I disorders, drug abuse, pregnancy, handedness, and severe medical conditions by a trained clinical psychologist (described previously by Klauke et al., 2011; Klauke et al., in press). Twenty ml EDTA blood samples were taken for genotyping. Inclusion criteria comprised right-handedness, German as mother tongue, Caucasian descent, and written informed consent. Exclusion criteria comprised mental axis I disorders, current psychopharmacological treatment, and scores above 13 and 8 on the Beck's Depression Inventory (BDI-II; Beck et al., 1996) and the Panic and Agoraphobia Scale (PAS; Bandelow, 1997), respectively, at the day of fNIRS measurement. Based on these criteria, eight subjects were excluded leaving a total sample size of 92 participants (Table 1).

All subjects were part of a larger group of 121 subjects participating in a study investigating the prefrontal neural correlates of heart rate variability (HRV). The results of this study will be reported elsewhere. However, we hereby make clear that the data analyses of the current study rely on a subset of the data acquired in this initial study using the same behavioral paradigm and methods described here. Because *NPSR1* data were not available for all subjects of this initial study, data analyses of the current study therefore rely on this smaller subsample. We consider the methodological approach of analyzing the gross of data twice in this case as a minor limitation because both analyses

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Table 1
Sample characteristics.

	All	A/A	A/T	T/T	*p*
Age	24.38 ± 3.46	24.53 ± 2.94	24.52 ± 4.19	23.65 ± 2.39	.610
Sex[a]	31/61	8/22	13/29	10/10	.203
Education[b]	6/85/1	3/27/0	1/40/1	2/18/0	.563
BDI-II	3.71 ± 3.58	2.97 ± 3.63	3.62 ± 3.73	5.00 ± 2.90	.141
PAS	.46 ± .94	.63 ± 1.19	.38 ± .82	.35 ± .75	.458
ASI	13.45 ± 7.40	12.17 ± 6.50	14.10 ± 8.42	14.00 ± 6.37	.518

Means ± standard deviations and *p* values of one-way analyses of variance testing for significant between-group differences are given for variables age and scores on the Beck's Depression Inventory (BDI-II), Panic and Agoraphobia Scale (PAS), and Anxiety Sensitivity Inventory (ASI). For variables sex and education, group sizes and *p* values of chi-squared tests are presented ([a] male/female; [b] graduates/undergraduates/not specified).

focused on independent research questions with a priori stated hypotheses based on previous findings. There is no known relationship between *NPSR1* and HRV. Also, the re-use of data for secondary analyses is a common procedure for the conduction of meta analyses and is the underlying principle of data archives which provide open access for researchers not involved in the primary study. However, to prevent potential redundancy, the present study focused on the genetic modulation of brain activation and performance only and does not go into further detail with regard to the general task dependent activations which are described in the original study. However, for completeness, all significant results will be reported.

Genotyping

Genotyping of *NPSR1* rs324981 was performed according to previously published protocols (Domschke et al., 2011). The sample was divided into three groups: A/A ($N = 30$ [32.6%]), A/T ($N = 42$ [45.7%]), and T/T ($N = 20$ [21.7%]). The distribution of genotypes did not deviate from Hardy–Weinberg equilibrium ($\chi^2 = .53$, $p = .47$) nor was there any significant difference between groups regarding demographic and questionnaire data (Table 1).

fNIRS

The principles of optical topography and fNIRS data analysis have been described in detail by others (e.g., Obrig and Villringer, 2003; Plichta et al., 2007; Plichta et al., 2006). Frontal oxygenated (O_2Hb) and deoxygenated hemoglobin (HHb) changes were measured with an ETG-4000 Optical Topography System (Hitachi, Medical Corporation, Tokyo, Japan) and a 52-channel probe set consisting of 17 laser diodes and 16 photo-detectors. The probe set covered large parts of the forehead including medial and dorsolateral PFC (Fig. 1). Measures were recorded with a sampling frequency of 10 Hz and subsequently transformed by a modified Beer-Lambert law. A cosine and moving average filter with a time window of 5 s were applied. Estimated beta weights for O_2Hb and HHb were determined by means of an ordinary least squares regression model assuming a peak of the hemodynamic response at 6.5 s following stimulus onset.

Task

The task consisted of a combination of the classical cognitive Stroop task (Stroop, 1935) and its modified emotional version (Williams et al., 1996). During the cognitive part, 48 congruent and 48 incongruent color words were presented on a black computer screen. During the emotional part, 15 neutral and 15 fear-relevant words were shown three times each, totaling 45 trials of each condition. Words were presented in red, green or yellow font and participants were instructed to indicate the font color by pressing a corresponding button. During congruent trials the depicted word matched the font color, while during incongruent trials there was a discrepancy between word and color (e.g., the word "green" shown in red font color). This latter condition is known to cause cognitive interference as indicated by increased response latencies because the task requires suppressing the highly automated process of reading (Stroop, 1935). Emotional interference, on the other hand, is elicited by emotional words. It is assumed that emotional compared to low arousing neutral words bind attention which in turn leads to increased error rates and response latencies (e.g., Dresler et al., 2009b; Tupak et al., in press). Fear-relevant words referred to cognitive and bodily symptoms of acute fear as particularly experienced by panic disorder patients during a panic attack (e.g., death, heart attack, dizziness). Neutral and fear-relevant words were matched according to the number of syllables, letters, and frequency in German language.

All 186 trials were presented event-related and in random order. Each stimulus was presented for 1500 ms and preceded by a fixation cross of 500 ms. A random jitter between 2000 and 5000 ms was used for inter-trial intervals.

Statistical analysis

Data analyses were performed using Matlab (v. R2008a, The Math Works, Natick, MA) and SPSS (v. 19, IBM SPSS Statistics, Munich, Germany).

Regions of interest (ROI) included bilateral dorsolateral and medial prefrontal cortices. Beta estimators of four fNIRS channels over each hemisphere (right: 3, 13, 14, 24; left: 8, 18, 19, 29) were pooled for each condition to gain one parameter for DLPFC activation. Similarly,

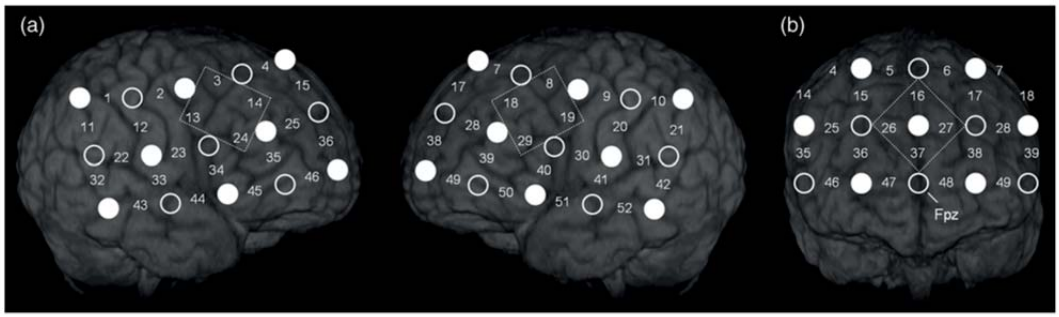

Fig. 1. FNIRS probe set and regions of interest. Schematic side and front views on the fNIRS probe set and analyzed regions of interest (ROI). Light emitters and detectors are depicted by filled and open white circles, respectively. Measurement channels are presented by their respective channel number. Eight dorsolateral prefrontal cortex channels, four covering each hemisphere (right: 3, 13, 14, 24; left: 8, 18, 19, 29), were pooled to form the first ROI (a). The second ROI (b) consisted of four medial prefrontal cortex channels (16, 26, 27, 37). Position Fpz refers to the international 10–20 system of electrode placement for electroencephalography (Jasper, 1958).

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the average of four frontopolar channels (16, 26, 27, 37) was taken for MPFC activation analyses (Fig. 1).

fNIRS O_2Hb and HHb, error rates (ER), and reaction time (RT) data were entered into two separate interference (interfering, non-interfering) × group (A/A, A/T, T/T) repeated measures analyses of variance (ANOVA) for each part of the combined Stroop task (emotional and cognitive). Reaction times deviating more than two standard deviations from the mean, above 1500 ms or beneath 250 ms were excluded from further analysis. Post hoc analyses were performed using Wilcoxon tests for non-normally distributed measures (i.e., error rates), independent and paired-sample *t*-tests. If necessary, a Bonferroni correction was applied. Results will be reported for one-sided post hoc tests and at significance levels of $p = .05$ and $p = .10$ for trends. Based on previous evidence, we expected higher prefrontal activation (i.e., increased O_2Hb and decreased HHb) in A-homozygotes compared to T allele carriers and, with respect to task performance, higher ER and RT in T compared to homozygous A allele carriers (see e.g., Dannlowski et al., 2011; Domschke et al., 2011). Regarding the type of task, we hypothesized higher activation, RT, and ER for interfering compared to non-interfering trials (fear-relevant > neutral and incongruent > congruent). To further investigate the relationship between anxiety sensitivity and fear-specific brain activation, contrast values between activation to fear-relevant vs. neutral words were correlated with ASI scores.

Since initial analysis of the present data was of exploratory nature focusing on three other genetic variants (catechol-O-methyltransferase [*COMT*], serotonin receptor 1A [*5-HT1A* -1019C > G], and serotonin transporter [*5-HTTLPR*] polymorphisms; the respective results will be reported elsewhere), main group and group interaction effects were further investigated only if they were still significant following Bonferroni correction ($p \leq .0125$, or $p \leq .025$ for trends) to handle these multiple comparisons within the same dataset. However, all reported effects reached this stringent level of significance anyway.

Because initial recruiting aimed at stratifying subjects for the *COMT* val158met polymorphism and scores on the Anxiety Sensitivity Index (Peterson and Reiss, 1992), equal group distribution within the *NPSR1* sample was checked by means of a chi-squared test and one-way ANOVA, respectively. Both proved to be not significant ($\chi^2 = 4.00$, $p = .41$; $F_{(2,89)} = .66$, $p = .52$). Although not statistically significant, groups seemed to differ in terms of depressive symptoms (BDI; Table 1). We therefore reran all ROI analyses including BDI scores as a covariate to rule out potential confounding effects. All group × interference effects as reported below remained unchanged in terms of significance and post hoc analyses, indicating no confounding effect of depressive symptoms on *NPSR1* effects. Interference effects changed from significant to non-significant for DLPFC O_2Hb during the cognitive part of the Stroop and vice versa for MPFC HHb during the emotional part.

Results

Task performance

All *NPSR1* groups performed equally on both tasks as indicated by the absence of any main or interaction effects of group on ER and RT. Main interference effects were found for the cognitive part of the combined Stroop task only (ER: $F_{(1,89)} = 37.41$, $p < .001$; RT: $F_{(1,89)} = 130.05$, $p < .001$). Subjects made more errors and reacted more slowly during incongruent than congruent trials (ER: $Z = -5.82$ $p < .001$; RT: $t_{(91)} = 11.93$, $p < .001$).

DLPFC

Emotional Stroop task

A group (A/A vs. A/T vs. T/T) × interference (fear-related vs. neutral) repeated measures ANOVA resulted in a significant interaction between both factors ($F_{(2,89)} = 10.36$, $p < .001$) and no main effects for O_2Hb measures. Increased DLPFC activation was found for fear-related compared to neutral words in the A/A group ($t_{(29)} = 3.84$, $p < .001$). No significant difference was found among homozygous T allele carriers whereas heterozygous subjects, in contrast, displayed a trend towards a reversed O_2Hb activation pattern (fear-related < neutral: $t_{(41)} = .15$, $p = .07$; Fig. 2). However, because we expected greater activation to fear-related words, one-sided post hoc testing may seem inappropriate in this case.

Contrasts between groups showed significantly higher O_2Hb levels to fear-related words in the A/A group when compared to the A/T group ($t_{(70)} = 2.62$, $p = .02$) and T/T group by trend ($t_{(48)} = 2.12$, $p = .06$). No differential DLPFC response to fear-related words was found between homozygous and heterozygous T allele carriers. All groups reacted equally to neutral words, revealing no statistically significant effect (Fig. 2).

A significant HHb effect was found for the factor interference ($F_{(1,89)} = 11.75$, $p = .001$) with larger HHb decreases for fear-related compared to neutral words ($t_{(91)} = 2.99$, $p = .002$).

Cognitive Stroop

A group (A/A vs. A/T vs. T/T) × interference (incongruent vs. congruent) repeated measures ANOVA resulted in a significant main effect of interference ($F_{(1,89)} = 7.69$, $p = .007$) for O_2Hb measures. Incongruent compared to congruent words elicited increased DLPFC activation ($t_{(91)} = 3.24$, $p = .001$) with no specific deviant response pattern in any group (Fig. 2). A similar effect of interference was also found for HHb measures ($F_{(1,89)} = 19.92$, $p < .001$) indicating larger decreases for incongruent compared to congruent words ($t_{(91)} = 5.08$, $p < .001$). *NPSR1* had no impact on DLPFC functioning during the cognitive Stroop task.

MPFC

Emotional Stroop task

A significant interaction between group and interference was also seen for MPFC O_2Hb measures ($F_{(2,89)} = 5.61$, $p = .005$). Separated by group, increased activation for fear-related compared to neutral words was seen in homozygous A allele carriers only ($t_{(29)} = 3.64$, $p < .001$) whereas T allele carriers displayed similar activation patterns. Group comparisons revealed higher MPFC O_2Hb levels in the A/A compared to the A/T group ($t_{(70)} = 2.30$, $p = .04$; Fig. 3).

No significant results were found for HHb measures in MPFC channels.

Cognitive Stroop

Neither O_2Hb nor HHb measures showed any significant main or interaction effects for the factors group and interference (Fig. 3).

Fear-specific activation and anxiety

Overall, there was no significant correlation between ASI scores and fear-relevant DLPFC or MPFC activation. Split by *NPSR1* genotype, only the correlation between ASI scores and DLPFC HHb for the contrast between fear-relevant vs. neutral words in the heterozygous group reached the threshold for a statistical trend ($r = .-30$, $p = .06$). Visual inspection of the corresponding scatterplot, however, suggested that this correlation was mainly caused by an outlier and after removing the according subject, the correlation disappeared ($r = .-13$, $p = .42$).

Discussion

The current study investigated whether carriage of one or two T risk alleles of the *NPSR1* gene specifically impacts on the processing of fear-relevant linguistic stimuli and not simply high interference stimuli in general. The results were in favor of our hypotheses and in

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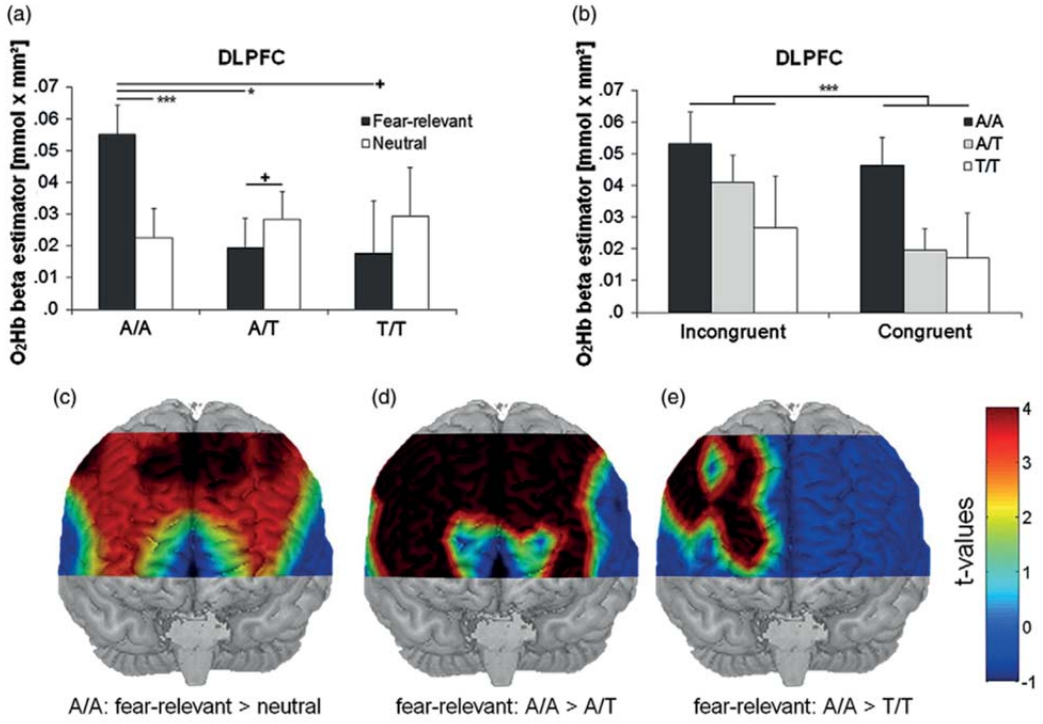

Fig. 2. Effects of *NPSR1* genotype on dorsolateral prefrontal cortex activation. *NPSR1* genotype effects on oxygenated hemoglobin (O_2Hb) levels within dorsolateral prefrontal cortex (DLPFC) during the emotional (a) and cognitive (b) Stroop task. O_2Hb whole probe set contrasts showed large prefrontal activation for fear-relevant vs. neutral words in A/A carriers (c). Compared to A/T carriers, A/A carriers displayed higher signal changes during the presentation of fear-relevant words in superior medial, dorsolateral, and ventral prefrontal areas (d). Compared to T/T carriers, A/A carriers showed higher activation in right DLPFC and medial prefrontal cortex (e). + significant by trend, $^{*}p<.05$, $^{***}p<.001$.

accordance with previous findings. Subjects with the A/A genotype displayed an emotional Stroop effect in terms of higher activation in both DLPFC and MPFC, while carriers of one or two T alleles showed similar or even reversed activation to fear-relevant and neutral stimuli. Moreover, group contrasts between A/A genotype and heterozygous and partly homozygous T allele carriers revealed that prefrontal activation was specifically increased in the A/A group during the fear-relevant condition, while there were no genotype effects on high cognitive interference or low interference trials in general. Thus, prefrontal modulation by *NPSR1* genotype seems to be specific to the processing of fear-relevant stimuli. No particular activation differences were observed between heterozygous and homozygous T allele carriers. For most of the discussion, we therefore refer to both of these groups as a whole, including subjects carrying either one or two T alleles ('T allele carriers'), and split them up only if they differentially reacted to the experimental manipulation.

The functional mechanism behind the elevated fear-specific prefrontal activation in A-homozygotes remains to be discussed. Considering T allele carriers to be at increased risk for panic disorder (Domschke et al., 2011), it seems that increased prefrontal activation in response to a threatening context in A/A genotype carriers is beneficial and might thus confer resilience against anxiety-related states or panic disorder in particular. Accordingly, decreased prefrontal brain activity was associated with less efficient emotion regulation (Goldin et al., 2008; Wager et al., 2008) and panic disorder (Dresler et al., 2009a, 2011). Hariri et al. (2000, 2003) showed that, in the presence of threatening stimuli, higher prefrontal activation was inversely related to the corresponding amygdala response. Based on our findings and the previous literature we therefore assume that T allele carriers possess a less efficient reflective prefrontal regulation system with decreased inhibitory control over the subcortical fear response as initiated by the amygdala with its direct projections to brainstem areas

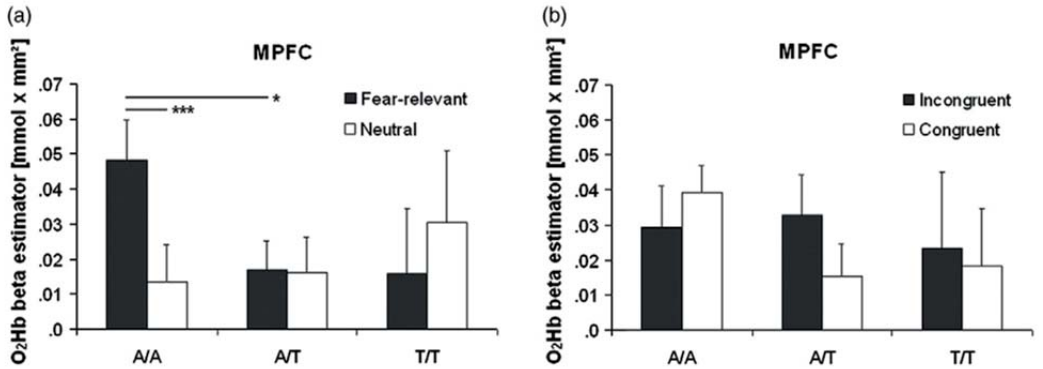

Fig. 3. Effects of *NPSR1* genotype on medial prefrontal cortex activation (MPFC). *NPSR1* genotype effects on oxygenated hemoglobin (O_2Hb) levels within medial prefrontal cortex (MPFC) during the emotional (a) and cognitive (b) Stroop task. $^{*}p<.05$, $^{***}p<.001$.

(e.g., Liddell et al., 2005). These individuals might react with increased arousal and amygdala activation to threatening stimuli as previously shown (Dannlowski et al., 2011) and might simultaneously fail to inhibit this response. Such a dysfunctional neural regulation system in T allele carriers might expose them to an increased risk for panic disorder and to experience bodily sensations as more harmful (Domschke et al., 2011; Raczka et al., 2010). Also, their individual threshold for clinically relevant anxiety might be lower compared to A-homozygotes. This hypothesis is supported by the finding that childhood mistreatment in combination with the T/T genotype affected anxiety sensitivity to a greater extent than one of these factors alone (Klauke et al., in press). As an alternative explanation, it should be considered whether the A/A genotype might in turn be characterized by too little anxious behavior in general. Future studies might for example investigate sensation seeking or risk behavior in these individuals. Further, by using fNIRS in the present study, it was not possible to track amygdalar reactions or other structures of the limbic system beneath the outer cortex. Consequently, network activity was not assessable which leaves our assumption hypothetical. Also, groups did not differ in terms of anxiety sensitivity which was a precondition for analyzing *NPSR1* effects on the one hand but difficult to explain in the light of emotion regulation efficiency on the other. A third limitation refers to the fact that DLPFC activation to fear-relevant stimuli differed only by trend between the homozygous groups. Most reasonably, this lack of significance may have been caused by larger variation in the T/T group due to the smaller sample size. Still, interpretations must be considered with caution in this case.

In contrast to its impact on neural activation patterns, *NPSR1* genotype had no influence on behavioral performance during the emotional Stroop. One possible explanation might be that the fear-relevant stimuli used in the current study were not significant enough to cause attentional binding in healthy subjects. Inclusion criteria were relatively strict; hence the sample consisted mainly of healthy young subjects without any psychopathological features. The emotional interference might thus have been too subtle to fully tap regulatory potential necessary for proper task performance but still great enough to uncover genetic influences on neural activity. Furthermore, the existence of an emotional Stroop effect in healthy populations has been questioned before (Algom et al., 2004; Dresler et al., 2009b; Egloff and Hock, 2001). Moreover, even more generally speaking, neural measures of brain activity have been discussed to be more sensitive to subtle modulations of information processing than relatively 'crude' behavioral parameters, thus reflecting the impact of respective manipulations — even in the absence of behavioral effects — in a meaningful way (Wilkinson and Halligan, 2004). Nevertheless, the lack of a behavioral effect poses a difficulty regarding our interpretation that less reactive PFC activation in T allele carriers might reflect less efficient emotion regulation as we cannot rule out that increased activation in the A/A group reflects a greater demand of regulation rather than greater efficacy. Our data provided no information about the effectiveness of emotional regulation and therefore our conclusions are based partly on earlier work describing increased fear responses and anxiety in T allele carriers (Dannlowski et al., 2011; Domschke et al., 2011; Klauke et al., in press). Given these findings, however, it seems unlikely that an elevated fear response in T allele carriers indicates a smaller demand for top-down regulation.

The results of previous fMRI and the current fNIRS study showed that *NPSR1* modulated brain activation is specific to threatening stimuli (facial, linguistic or unspecific fear-conditioned) and suggest that it cannot be attributed to increased stimulus arousal or interference alone (Dannlowski et al., 2011; Domschke et al., 2011; Raczka et al., 2010). *NPSR1* genotype had no effect on PFC activation during the cognitive Stroop task and the processing of highly interfering incongruent color word stimuli. However, both parts of the Stroop task referred to differential aspects of information conflict and cannot be compared directly (Buhle et al., 2010). First, cognitive interference in the task depicts a very different process from that of emotional interference. During cognitive interference trials, it is the automaticity of reading which is assumed to strongly interfere with performance of the task. During emotional interference trials, however, performance is thought to be hampered by a loss of attention, which is eventually diverted away from the task due to the fear-relevant stimuli. Thus for the cognitive part, it is an active process (i.e., reading) that interferes whereas during the emotional part, the valence of the stimulus interferes by catching attention which would be needed for proper task performance. Second, methodological issues arise when comparing color words with emotional words. Words of both conditions are neither matched for letters, syllables nor frequency in written/spoken language. In addition, each color word is presented much more frequently during the task than each neutral and fear-relevant word. Since novelty of stimuli is known to alter frontal neural activation (Matsumoto et al., 2007; Yamasaki et al., 2002), effects that arise from a direct comparison cannot solely be attributed to cognitive vs. emotional interference alone. We therefore analyzed both types of interference separately in the current study and could not directly compare whether one was superior to the other in any group.

Of particular interest are also the divergent findings between fNIRS parameters within the DLPFC. *NPSR1* effects were exclusively found for O_2Hb while HHb measures indicated an emotional Stroop effect in terms of stronger activation to fear-related stimuli over all groups. Considering that both chromophores should ideally show a negative correlation, it is surprising that the A/T group displayed an opposite response pattern with larger O_2Hb levels for neutral words but stronger HHb decreases for fear-related words. However, HHb has been shown to be less sensitive to changes in cerebral blood flow than O_2Hb (Hoshi et al., 2001; Strangman et al., 2002). Nevertheless, future studies are needed to further disentangle these chromophore specific effects.

Previous imaging genetic studies reported *NPSR1* gene modulations in the amygdala, ACC, OFC, dorsolateral and dorsomedial PFC response during the processing of fear-relevant stimuli (Dannlowski et al., 2011; Domschke et al., 2011; Raczka et al., 2010). The present study added the most anterior parts of the MPFC to the list of critical structures of the fear circuit that are influenced by the *NPSR1* gene during emotion regulation in addition to fear acquisition in a classical conditioning paradigm (Raczka et al., 2010). However, while MPFC activation was found to be increased among T allele carriers in the latter study, the present results indicate the contrary. These findings seem reasonable given that Raczka et al. focused on fear conditioning, in particular fear acquisition, while the present study investigated emotion regulation. Prefrontal activation during fear acquisition has been reported before (Sehlmeyer et al., 2009) and is considered to be of evaluative nature (Etkin et al., 2011), potentially indexing the extent of the fear response, while prefrontal activation during fear extinction and fear regulation is considered to inhibit subcortical parts of the fear network (Hartley and Phelps, 2009; Sotres-Bayon and Quirk, 2010). Rodent fear conditioning studies suggested that different parts of the MPFC play either an inhibitory or excitatory role in the presence of fear-relevant stimuli. Particularly more dorsal subdivisions might react functionally similar to the amygdala through direct excitatory projections between both structures while the more ventrally located infralimbic areas were implicated in fear regulation (Quirk and Beer, 2006; Sotres-Bayon and Quirk, 2010). Generally, MPFC activation was rather related to successful fear extinction (Milad and Quirk, 2002).

In future studies, the investigation of different types of emotion regulation and fear processing strategies, such as reappraisal vs. suppression or simple perceptual vs. elaborate cognitive processing, may provide more detailed insight into how specifically *NPSR1* affects function in this area and whether its modulation can be functionally distinguished from other prefrontal regions like the DLPFC and ACC.

Conclusion

To summarize, the current results showed that both medial and dorsolateral PFC activation are modulated by the *NPSR1* gene in the presence of fear-relevant stimuli that require emotional regulation for efficient behavioral performance. This modulation was fear-specific and could not be attributed to stimulus interference or cognitive regulation in general. The results are in line with earlier work suggesting that the *NPSR1* T allele might be accompanied by a higher vulnerability for the development of anxiety disorders or accentuated anxiety-related traits and that the A/A genotype, on the other hand, might have a protective function. Our work shows that *NPSR1* gene variation affects the functionality of a reflective prefrontal regulation system which in turn might impact on the down-regulation of subcortical fear responses.

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Study 3: Neuropeptide S receptor gene: Fear-specific modulations of prefrontal activation
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Transition 3: From Perceptual Processing to Implicit Emotional Regulation

Study 3 has shown that there are factors that inherently affect PFC activation to particularly fear-relevant stimuli. This implicates that the subjective experience and regulation of fear and anxiety is to a certain degree already determined at birth. The results suggest that some individuals may be better in regulating their emotional responses simply because their neural substrates offer a greater potential compared to those carrying one or two risk alleles of certain candidate genes.

Nevertheless, although fear processing may be crucially influenced by such intrinsic factors such as genes, years of research on emotion regulation have shown that the cognitive attributes that are ascribed to fear-eliciting stimuli and the way particular situations are interpreted are important for how threatening they are perceived (Gross, 2007; Hartley and Phelps, 2009; Ochsner and Gross, 2008). Particularly anxiety disorder patients tend to interpret certain situations as extremely threatening which are perceived as being rather harmless by non-anxious individuals (Clark et al., 1997; Margraf and Ehlers, 1989; McNally, 1999). It is this interpretational bias that is also addressed by cognitive behavioral therapy (CBT) as a treatment of pathological anxiety (Tobon et al., 2011).

Cognitive processing of fear-relevant stimuli thus seems to be crucially affected by elevated anxiety. Many functional imaging studies investigating the fear network, however, use experimental tasks that are primarily based on bottom-up processing. In contrast to emotion regulation tasks, typical paradigms of anxiety research often require passive viewing of fear-relevant stimuli. In others, participants are asked to process stimuli based on perceptual characteristics and often attention is diverged from the actual meaning of the stimulus such as in the dot-probe or emotional Stroop task. To understand the neural dysfunctions underlying anxiety disorders, basic research on healthy individuals may therefore strengthen its focus on interpretational processes using tasks of explicit or implicit emotional regulation (Gyurak et al., 2011; Koole and Rothermund, 2011). In the final *study 4* of this research, PFC regulation and its effects on arousal and behavior were investigated using an implicit emotion regulation task that directly compared perceptual processing of threat with interpretational processing in a group of healthy control subjects. The results of this work highlight

important distinctions between both processes for PFC function that should be considered particularly in attentional bias studies. The findings of *study 4* are critically related to the previous results in the overall discussion.

Implicit emotion regulation in the presence of threat: Neural and autonomic correlates

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Abstract

Efficient emotion regulation is essential for social interaction and functioning in human society and often happens without direct intention and conscious awareness. Cognitive labeling of stimuli based on certain characteristics has been assumed to represent an effective strategy of implicit emotional regulation whereas processing based on simple perceptual characteristics (e.g., matching) has not. Evidence exists that the ventrolateral prefrontal cortex (VLPFC) might be of functional relevance during labeling by down-regulating limbic activity in the presence of threatening stimuli. However, it remained unclear whether this VLPFC activation was particularly specific to threat because previous studies focused exclusively on threatening stimuli. In the current study, 35 healthy participants labeled or matched both threatening and neutral pictures while undergoing 52-channel functional near-infrared spectroscopy. Results showed increased VLPFC activation during labeling of threatening but not neutral pictures. No increase in prefrontal activation was detected during matching. Moreover, skin conductance increased equally for both valence conditions during initial phases of labeling whereas during matching stronger increases were found for threatening stimuli. Although a general inverse relationship between VLPFC function and skin conductance was not confirmed, both were negatively correlated during matching of threatening pictures in subjects with high state anxiety. It was concluded that the VLPFC plays an essential role during implicit emotion regulation. Further, even simple perceptual processing seems to engage regulatory top-down activation in anxious individuals.

1. Introduction

Emotion regulation refers to the ability to handle distressing or inappropriate feelings by using appropriate emotion regulation strategies. The most frequently mentioned strategies in this context include reappraisal and suppression or distraction (Gross, 2002; Kalisch et al., 2006) while reappraisal appeared to be the most effective one (Gross and John, 2003; John and Gross, 2004). However, emotion regulation does not necessarily require conscious awareness and can occur without insight. Gyurak et al. (2011) differentiated between these two kinds of emotion regulation, as being either explicit or implicit. While reappraisal and suppression represent strategies of explicit emotion regulation, other strategies are applied implicitly and occur outside of awareness without conscious intention. As an example, the authors refer to affect labeling as a cognitive strategy of implicit emotion regulation.

Labeling has been initially investigated in two functional magnetic resonance imaging (fMRI) studies to differentiate between the neural correlates of simple perceptual compared to more elaborate cognitive processing (Hariri et al., 2000; Hariri et al., 2003). In these studies, the authors presented threatening visual stimuli (i.e. angry/fearful faces or threatening pictures) to healthy subjects. Subjects either matched the presented target picture to one of two simultaneously presented pictures of which one was identical to the target or they labeled the according picture with one of two possible descriptions referring to the meaning or content of the stimulus. In one study (Hariri et al., 2000) affective labels were used while in the other (Hariri et al., 2003) labels referred to neutral characteristics of the presented picture. However, results were comparable between both studies: Matching threatening stimuli was associated with increased amygdalar and thalamic activation, whereas labeling elicited activations in VLPFC, anterior cingulate cortex (ACC), and Broca's area. Moreover, activity in amygdala and prefrontal activation was negatively correlated, suggesting that in the presence of threatening stimuli, emotional regulation of the subcortical limbic fear response is governed by the PFC (Hariri et al., 2000; Hariri et al., 2003). This finding is in line with earlier functional neuroimaging studies that identified the PFC and amygdala as core brain structures

involved during emotional regulation (Kim et al., 2011b). As discussed by Lieberman et al. (2007), affect labeling partly resembles reappraisal, although reappraisal was rather associated with activation increases in right anterolateral PFC (Kalisch et al., 2005), whereas, similar to affect labeling, self-distraction was linked to activation increases in left lateral PFC (Kalisch et al., 2006). The most important distinction between both processes, however, is that reappraisal refers to explicit emotion regulation, whereas affect labeling represents an implicit emotion regulation process (Gyurak et al., 2011; Koole and Rothermund, 2011).

While a lot of evidence points towards a regulatory role of the PFC during cognitive emotional regulation, no scientific consensus has been reached with regard to the obligatory unconditional response of the amygdala to emotionally salient stimuli, particularly threatening or fear-related stimuli (Bishop, 2008). Many studies reported a functional connectivity between both structures during emotion regulation (for recent reviews see Gyurak et al., 2011; Kim et al., 2011b). Recent studies showed that a response of the amygdala is more likely to occur following transient emotional provocation but is not sustained over longer periods of emotional stimulation (Alvarez et al., 2011; Somerville et al., 2012). Moreover, activation in ventromedial prefrontal cortices (VMPFC) was negatively associated with this transient amygdala response (Somerville et al., 2012) and is assumed to have a regulatory function (Etkin et al., 2011). Connectivity between VMPFC, dorsomedial PFC (DMPFC), and amygdala is also influenced by state anxiety with positive VMPFC-amygdala correlations in low anxious and negative correlations in high anxious individuals at rest. In contrast, low anxious subjects displayed an inverse relationship between DMPFC and amygdala. Functional connectivity in these areas was also found to correlate with trait anxiety with less pronounced effects (Kim et al., 2011a).

Until today, only few functional imaging studies directly compared simple perceptual bottom-up with more elaborate top-down processing of threatening or fear-relevant stimuli (e.g., Hariri et al., 2000; Hariri et al., 2003; Lieberman et al., 2007). It is possible that the effects found in those studies might primarily be due to the higher cognitive load and linguistic demands of labeling

compared to matching. The idea that prefrontal activation during affect labeling results from cognitive and linguistic top-down processes has been addressed before in an fMRI study by Lieberman et al. (2007). To solve this problem, the authors varied the labels subjects ascribed to facial stimuli. In the experimental condition affective labels were used, in the control condition gender labels. Thus in the first condition, attention was directed at the stimulus meaning and in the second it was directed at affect-independent stimulus properties alone. Their results revealed that affect labeling elicited higher right VLPFC activation than gender labeling and can thus not be due to higher cognitive load per se.

The specificity of VLPFC activation with respect to stimulus valence, however, has never been investigated in detail. Earlier studies used exclusively stimuli of negative valence (i.e., fear, anger, threat) but interpreted their findings as being either specific to the particular valence at hand (Hariri et al., 2000; Hariri et al., 2003) or independent of the affective valence at all (Lieberman et al., 2007). The present study aimed at identifying the role of the VLPFC during implicit emotion regulation of particularly threatening stimuli more precisely by using functional near-infrared spectroscopy (fNIRS). To this end, we adapted the original affect labeling paradigm by Hariri et al. (2003) and added additional conditions using neutral pictures to simultaneously investigate the effects of valence (threatening vs. neutral) and to control for the higher cognitive load of the labeling as compared to the matching condition. We aimed at investigating whether VLPFC activation during labeling was due to cognitive picture evaluation alone or specific to implicit regulation of salient emotional stimuli, in this case threatening pictures. Likewise, we assessed whether top-down processing of threatening stimuli leads to lower autonomic responses in terms of skin conductance. We referred to the skin conductance level (SCL) as an indirect measure of amygdalar reactivity because only cortical activation changes can be targeted by using fNIRS. We hypothesized that perceptual processing of threatening compared to neutral pictures elicits an amygdalar reaction which in turn causes SCL increases. In contrast, elaborate cognitive processing of threat during labeling was hypothesized to increase regulatory VLPFC activity, thereby down-regulating the amygdalar response leading to

smaller valence effects (threat > neutral) in terms of skin conductance. Based on earlier findings, we assumed an inverse relationship between VLPFC activation and SCL particularly during the presentation of threatening stimuli. This negative correlation was hypothesized to be more pronounced during top-down compared to bottom-up processing and to be stronger in subjects with higher levels of state anxiety.

2. Materials and Methods

2.1 Subjects

In total, 37 subjects participated in the current study and filled in the state subscale of the State-Trait Anxiety Inventory (STAI; Spielberger et al., 1970). All except for one were right-handed. Two subjects had to be excluded because one of them reported a history of psychopathology (bulimia nervosa and major depression) and the other repeatedly fell asleep during the measurement. Data of the remaining 35 subjects (mean age: 26.46 years; SD: 6.96; 24 female) were entered into further statistical analyses.

The study was approved by the ethics committee of the University of Würzburg and in accordance with the declaration of Helsinki in its latest revision. All subjects gave written informed consent.

2.2 Task

The task was adopted from Hariri et al. (2003) but slightly modified. We selected 36 neutral and 36 threatening pictures of the International Affective Pictures System (IAPS; Lang et al., 1997). Stimuli differed significantly in terms of valence ($\bar{x}_{neutral}$=5.64 ± .90, $\bar{x}_{threat}$=3.31 ± .71; $t_{(70)}$=12.35, p<.001) and arousal ($\bar{x}_{neutral}$=3.45 ± .91, $\bar{x}_{threat}$=6.22 ± .52; $t_{(70)}$=15.94, p<.001). The task consisted of two main experimental conditions: matching versus labeling pictures. During the *matching condition*, a target stimulus was presented in the upper half of a computer screen on a black background and two pictures of the same valence condition, of which one was identical to the target, were shown

next to each other below the target. Subjects had to indicate by button press, which picture matched (i.e. was identical to) the target. During the *labeling condition*, a target stimulus was presented in the same way as during the matching condition but instead of two pictures, two labels were given below ('natural' vs. 'artificial'). Subjects were instructed beforehand to judge whether the target picture displayed rather a natural or an artificial scene. Natural scenes were defined as 'something occurring in nature without human influence' and included e.g. plants, mushrooms, landscapes or animals. Artificial scenes depicted for example tools, traffic or war scenarios and always referred to objects or situations that were 'created or caused by human beings'. Labels were presented in different colors (green for 'natural' vs. orange for 'artificial') and associated with a corresponding button (left and right, respectively) for the entire session to direct attention at picture evaluation and to minimize distraction due to reading. Similar to the original study, 20 pictures of geometrical shapes were used as a *control condition*. In contrast to an earlier version of the task (Hariri et al., 2003), shapes were presented in different colors to adjust task difficulty to the match condition because IAPS pictures were presented in color which is a perceptual characteristic that facilitates processing particularly during matching. Regarding the type of *task* (control, matching, and labeling) and stimulus *valence* (neutral and threat) the paradigm consisted of five conditions in total: control, matching neutral, matching threat, labeling neutral, and labeling threat pictures. Pictures were shown in blocks of six stimuli, each presented for 2 s without any inter-stimulus interval. Each picture was presented once as a target in each condition. In the matching condition, each picture was additionally shown as a distractor once. In total, six blocks of each condition were shown resulting in 30 blocks and a total task length of 13.2 min. Blocks and order of pictures within one block were presented in pseudo-randomized order. A second version of the task was established by reversing the block sequence. Both versions were counterbalanced over all subjects. Prior to each block an instruction was given for 2 s ('identical pictures', 'identical shapes', 'appropriate category'). A fixation cross was shown during the 12 s inter-block intervals. Subjects indicated their decision by pressing a button with the right index or middle finger.

2.3 FNIRS

We measured changes in prefrontal oxygenated (O_2Hb) and deoxygenated hemoglobin (HHb) by means of a multi-channel optical topography system (ETG 4000, Hitachi, Medical Corporation, Tokyo, Japan) applying two different wavelengths of near-infrared light (695 ± 29 nm and 830 ± 20 nm). Data was recorded at a temporal resolution of 10 Hz. The probe set consisted of 3 x 11 probes (17 light emitters and 16 light detectors), resulting in 52 measurement channels in total, covering the entire forehead. The inferior row of probes was positioned along the F1-Fpz-F2 line with the middle inferior probe placed over Fpz according to the international 10-20 system for electrode placement (Jasper, 1958). The signal was transformed online by a modified Beer-Lambert law and a moving average filter with a time window of 5 s was applied. Because neurovascular coupling is accompanied by local increases in O_2Hb and simultaneous decreases in HHb, measures of both chromophores should ideally approach a correlation of -1.0. Correlations that tend to be positive or equal to 0 may indicate noise caused by motion. A correlation based signal improvement (CBSI; Cui et al., 2010) algorithm was used to filter out spikes and to improve signal quality based on the assumed negative correlation between O_2Hb and HHb. The corrected signal no longer differentiates between O_2Hb and HHb but reflects an integrated measure of both chromophores. We will refer to this parameter as the corrected fNIRS signal in the following sections. Further, we applied a cosine filter to remove slow drifts.

Because the averaged hemodynamic response over all participants started relatively late (4 s following block onset) and was independent of the experimental condition, time segments were selected starting 4 s after block onset and lasting for 8 s (i.e. until the end of the block). Segments were baseline corrected using the first 0.5 s of each segment. The individual average over all segments of each condition was taken as the final parameter for statistical analyses.

2.4 Skin conductance levels

SCL was recorded using two Ag/AgCl electrodes, one each at the middle phalanxes of the index- and middle finger of the left hand. Recordings were amplified using a QuickAmp Amplifier (Brain Products, Munich, Germany) with a sampling rate of 1000 Hz. Data were filtered offline using a 1 Hz high cut-off filter and transformed from mV into µS. A baseline correction was applied for the time window of -3 to -2 s before the first trial of each block which refers to the 1 s time interval before the instruction for the subsequent block was presented. Each block underwent visual inspection for artifacts in the form of sudden spikes, responses starting before the instruction was given, and non-responses. Two subjects with less than three acceptable blocks per condition due to noise or motor artifacts were excluded from further analyses, leaving 33 subjects in total for SCL analysis. For correlation analysis, artifact-free blocks were averaged and the area under the curve (AUC) was defined for a segment of 12 s starting 2 s after stimulus onset. Further, the early part of the segment (first 6 s) and the late part (last 6 s of the block) were taken as separate measures to investigate SCL changes over the length of the block.

2.5 Statistical analyses

Second-level fNIRS analysis included exploratory whole probe set contrasts between conditions and more specific regions of interest (ROI) analyses based on previous findings using similar tasks (Hariri et al., 2000; Hariri et al., 2003). For whole probe set analyses, contrasts were calculated in a channel-wise manner: [1] between the matching and control condition (neutral/threat vs. control), [2] between valences in each task condition (threat vs. neutral), and [3] between tasks in each valence condition (labeling > matching). To control for multiple comparisons a Dubey/Armitage-Parmar (D/AP) correction was applied. ROI analyses focused on two single channels covering the left (channel 49) and right VLPFC (channel 46). Due to fixed inter-probe distances of 3 cm, both channels referred to the scalp area lying 3 to 6 cm away from Fpz in lateral direction. The underlying brain region was assumed to correspond to the VLPFC located within the inferior frontal gyrus.

Behavioral analyses focused on average reaction times and not error rates since performance in the labeling condition depended on subjective judgments and could therefore not be quantified. Nevertheless, incorrect trials and trials with reaction times beneath or above 2 standard deviations from the mean were excluded from averaging.

Task (matching vs. labeling) x Valence (neutral vs. threat) repeated measures analyses of variance (ANOVA) were calculated for reaction times, left and right VLPFC activation. For the analyses of SCL, Time (early vs. late segment) was inserted as an additional factor into the model. We used a log transformation to reduce inter-individual variability within the SCL data. Significant effects were reported for $p<.05$ (trends: $p<.10$). Post hoc analyses of normally distributed data were performed using paired sample t-tests, otherwise Wilcoxon signed-rank tests were applied. For reaction time data, we additionally calculated difference scores for each task (e.g., $diff_{label}$ = labeling threat – labeling neutral) and valence (e.g., $diff_{threat}$ = labeling threat – matching threat) separately. By using paired sample t-tests, we then compared whether effects of task were greater for neutral compared to threatening pictures ($diff_{threat}$ vs, $diff_{neutral}$) or whether effects of valence were greater during labeling compared to matching ($diff_{label}$ vs, $diff_{match}$).

To investigate whether VLPFC activation had an attenuating effect on subcortically driven arousal levels, we correlated the average VLPFC activation of channels 46 and 49 with SCL. The same correlational analysis was performed after subjects had been divided into low and high anxious subjects by a median-split of their individual scores on the state subscale of the STAI to test if state anxiety influences inhibitory prefrontal regulation on SCL.

3. Results

3.1 Behavioral data

On average, subjects identified more than 97% of all trials correctly with lowest error rates during matching and control trials (matching neutral: 99.7%, matching threat: 99.6%; control: 99.4%) and highest error rates during labeling of threatening stimuli (labeling neutral: 97.5%; labeling threat: 92.4%).

Task (match vs. label) *x Valence* (neutral vs. threat) repeated measures ANOVA on reaction time data revealed significant effects of *Task* ($F_{(1,34)}$=142.68, p<.001), *Valence* ($F_{(1,34)}$=65.09, p<.001), and *Task x Valence* ($F_{(1,34)}$=15.23, p<.001). Labeling compared to matching produced longer latencies, as well as threat compared to neutral pictures *(figure 1)*. This Valence effect (threat > neutral) was larger in the label ($t_{(34)}$=7.05, p<.001) compared to the match condition ($t_{(34)}$=6.02, p<.001; $diff_{label}$ > $diff_{match}$: $t_{(34)}$=3.90, p<.001; *figure 2A*). Likewise the effect of Task (label > match) was greater for threat ($t_{(34)}$=12.76, p<.001) compared to neutral blocks ($t_{(34)}$=9.97, p<.001; $diff_{threat}$ > $diff_{neutral}$: $t_{(34)}$=3.90, p<.001; *figure 2B*). Each condition differed significantly in terms of longer latencies from the control condition (p<.001).

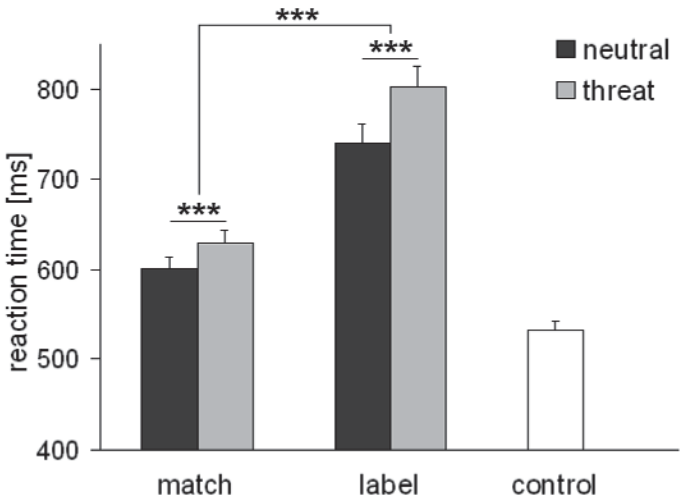

Figure 1: Reaction times
Valence effects were found for both matching and labeling. All conditions differed significantly from the control condition (p<.001).

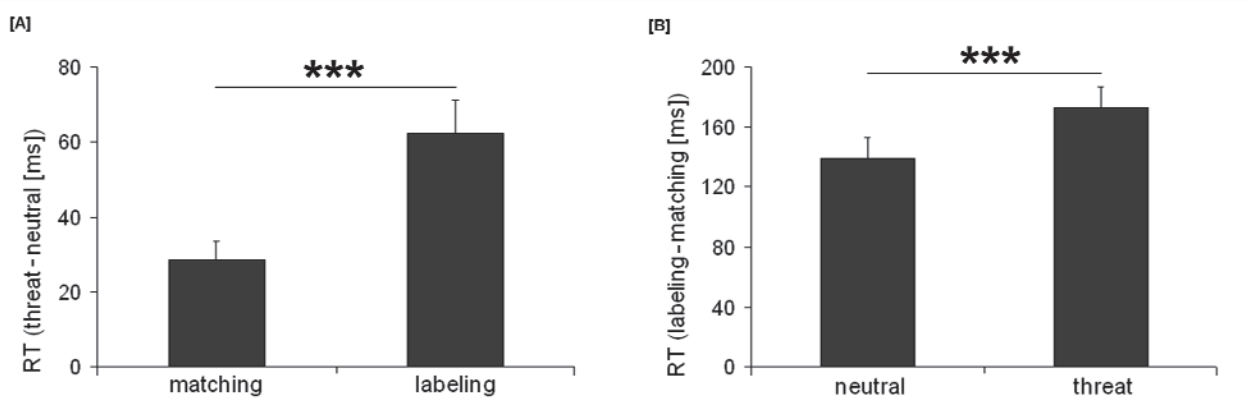

Figure 2: Comparison of valence and task effects
Valence effects in terms of slower response latencies to threatening compared to neutral words were found to be greater during labeling compared to matching [A]. The contrast between labeling and matching in terms of faster response latencies during the latter one was larger during the processing of threatening compared to neutral pictures [B].

3.2 FNIRS

Results for whole-probe set contrasts revealed a significantly increased fNIRS signal in dorsolateral and lateral ventral PFC areas for the contrasts labeling threat vs. control (channels 24, 29, 34, 39, and 50) and labeling threat vs. matching threat (channels 8, 13, 25, 35, 36, 38, 39, 46, and 49; *figure 3A*). No other contrast depicted significant differences between conditions.

Significant *Task x Valence* interactions were observed for both right ($F_{(1,34)}$=6.10, p=.019; *figure 3B*) and left VLPFC ($F_{(1,34)}$=12.49, p=.001; *figure 3C*). Non-parametric post hoc tests showed significant effects of *Valence* (threat > neutral) in terms of higher activation during labeling (left VLPFC: Z=2.92, p=.004; right VLPFC: Z=1.75, p=.080) but not matching. Labeling threatening pictures also elicited higher VLPFC activation compared to matching them (left VLPFC: Z=3.00, p=.003; right VLPFC: Z=3.59, p<.001). No activation differences were observed for neutral pictures between both types of tasks. Generally, higher VLPFC activation was found for labeling compared to matching (left: $F_{(1,34)}$=4.10, p=.051; right: $F_{(1,34)}$=13.69, p=.001), although only the label threat condition elicited significant fNIRS signal increases compared to baseline.

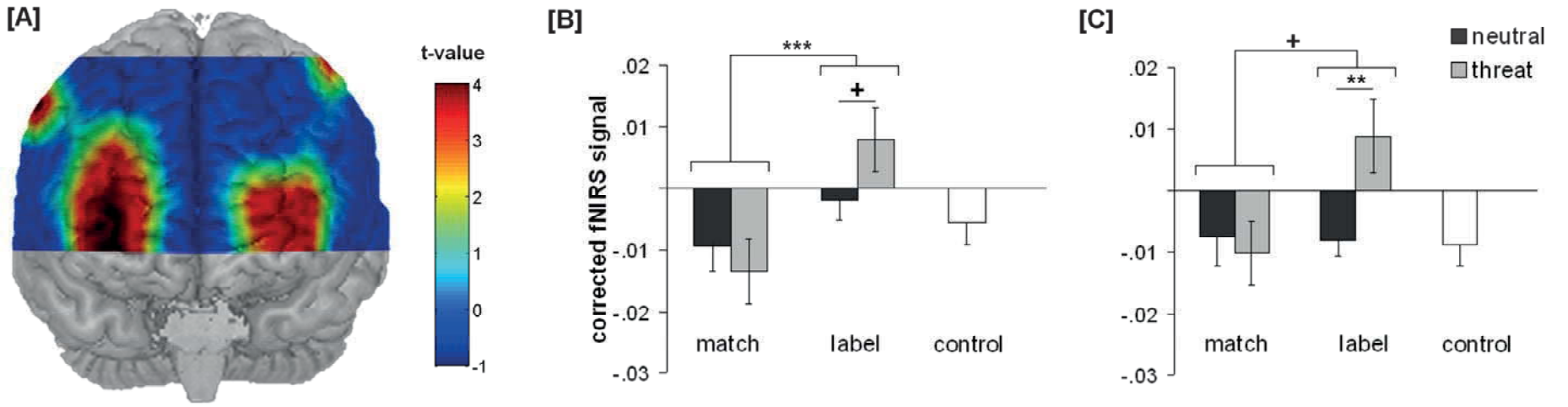

Figure 3: Prefrontal activation map and ROI analyses

A whole probe set contrast for labeling vs. matching of threatening pictures revealed significantly higher activation in nine channels over bilateral ventral and dorsolateral prefrontal cortices, including ROI channels 46 and 49 [A]. ROI analyses showed increased activation in [B] right (channel 46) and [C] left VLPFC (channel 49) only for labeling threatening pictures.

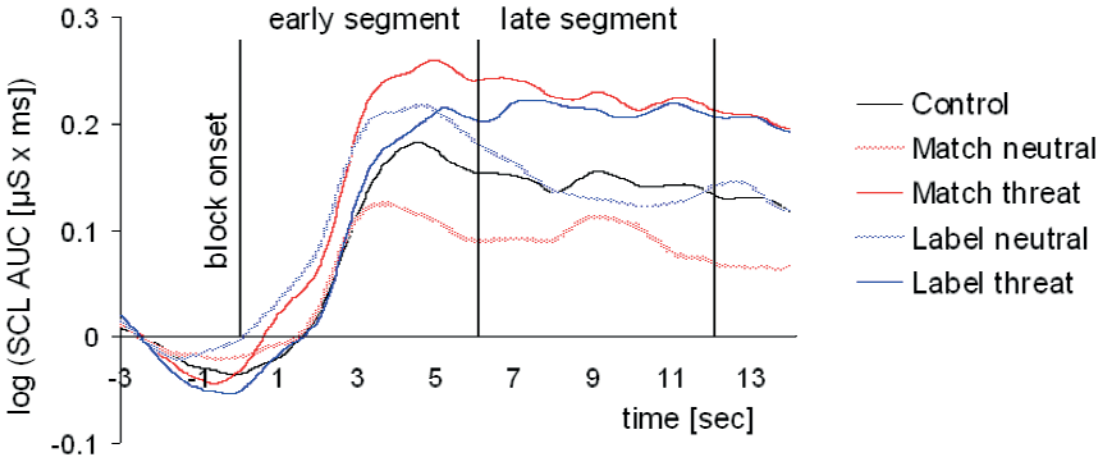

Figure 4: Time courses of skin conductance

The figure displays the time course of the averaged skin conductance over the entire block of each condition. In the early segment, a strong valence effect is seen for the matching (red) but not labeling (blue) condition. During the later segment both tasks were characterized by increased SCL to threatening pictures.

3.3 Skin conductance level

Time courses for all conditions are displayed in *figure 4*. A *Time x Task x Valence* repeated measures ANOVA resulted in significant effects for *Valence* ($F_{(1,33)}$=5.20, p=.029), *Task x Valence* ($F_{(1,33)}$=3.06, p=.090), and *Time x Task x Valence* ($F_{(1,33)}$=9.08, p=.005). To unravel this three-way interaction, a separate *Task x Valence* ANOVA was calculated for early and late segments. A significant interaction was present only during early segments ($F_{(1,33)}$=10.75, p=.002) revealing a significant effect of valence (threat > neutral) in the match ($t_{(33)}$=3.49, p=.001) but not in the label condition. Moreover, labeling neutral pictures produced a greater SCL compared to simple matching during the early segment ($t_{(33)}$=2.53, p=.016), while an equally increased SCL was found for threatening pictures. During late segments threatening pictures elicited higher SCL in both task conditions ($F_{(1,33)}$=4.20, p=.048; *figure 5*).

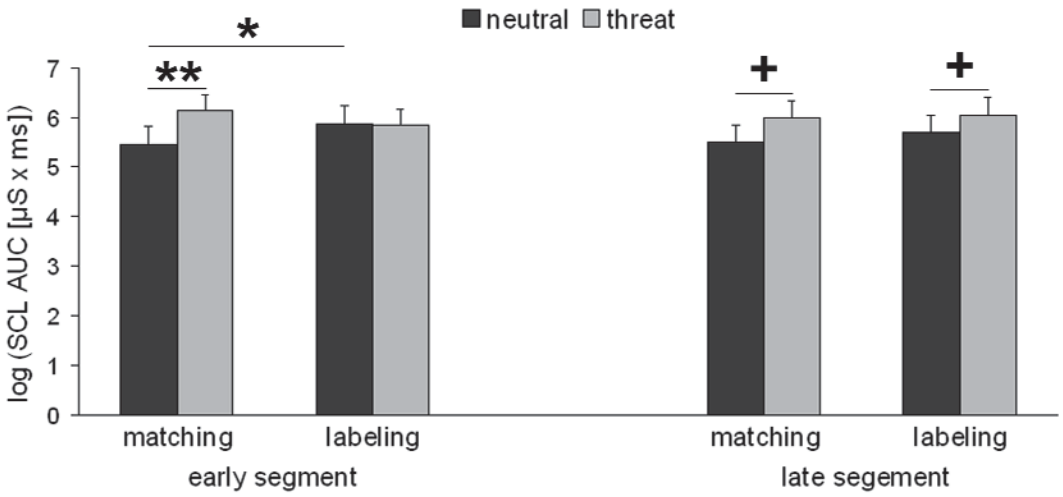

Figure 5: SCL valence effects during early and late segments
During early phases of the block, a significant valence effect (threat > neutral) was present during matching but not labeling. During later phases, valence effects were indicated for both task conditions.

Table 1: Correlation analyses between SCL and VLPFC

	Low state anxiety *(N=17)*	High state anxiety *(N=16)*
Matching neutral	.403	-.161
Matching threat	.194	**-.566**[a]
Labeling neutral	.171	.277
Labeling threat	.316	-.252

The table presents Pearson's correlation coefficients for the correlation between bilateral VLPFC activation and SCL. [a] p<.05

3.4 fNIRS-SCL Correlation

Regarding the entire sample, no significant relationship was found between VLPFC activation and SCL in any condition. Dividing the sample into high and low anxious subjects (high: N=16, $\bar{x}$ =40.00 ± 4.87; low: N=17, $\bar{x}$=30.71 ± 2.85) revealed an inverse relationship between VLPFC and SCL during matching of threatening pictures in the high anxious group only (r=-.566, p=.022, *uncorrected*). No significant correlations were present in low anxious subjects. Strikingly, correlations in this group and for each condition tended to be positive, though not significant. In high anxious subjects, however, VLPFC and SCL tended to be rather negatively correlated, except for the labeling of neutral pictures *(table 1).* The inverse VLPFC-SCL correlation in high anxious subjects was also independent of time (early segment: r=-.560, p=.024; late segment: r=-.551, p=.027, *uncorrected*). Groups did not differ with respect to age and gender ($t_{(31)}$=1.12, p=.27; Chi^2=.41, p=.52).

4. Discussion

The current results highlight the differential effects of cognitive top-down (label) compared to perceptual processing (match) of threat on neural and autonomic activity. We showed that VLPFC activation during more elaborate processing is specific to threatening stimuli and cannot simply be attributed to higher cognitive load or linguistic aspects of the task. Also, skin conductance responses differed between both types of processing. During the early phases of picture processing, only perceptual processing led to a significant valence effect in terms of increased autonomic reactions to threatening pictures. In contrast to our hypotheses, however, no general inverse relationship was

seen between VLPFC activation and SCL. Rather, perceptual processing of threat was characterized by a negative correlation between VLPFC and SCL but only in subjects displaying high state anxiety.

The present results support the hypothesis that the VLPFC is crucially involved during implicit emotional regulation. Moreover, it was shown that increased VLPFC activation during labeling was specific to the negative valence of the stimulus. In addition, this effect was accompanied by a greater attentional bias towards threatening pictures than in the matching condition. The neural valence effect is particularly interesting regarding the findings of Lieberman et al. (2007). In their version of the task, labels were changed thereby shifting the focus of attention from emotional to non-emotional aspects of the stimuli (i.e., gender). Stimuli, however, were always of negative valence. In our version of the task, labeling constantly focused on non-emotional aspects of the stimuli (i.e., natural vs. artificial) but stimulus valence varied. Strikingly, both variations seemed to have similar effects on the VLPFC with increased activation for threatening compared to neutral stimuli and also for the contrast between affect and gender labeling (Lieberman et al., 2007). Because gender labeling is similar to our threat labeling condition (negative stimuli and neutral labels) it is likely that cognitive evaluation of negative stimuli alone with a focus on non-emotional stimulus properties leads to increases in VLPFC activation. Shifting the focus on the emotional content of the stimulus (Lieberman et al., 2007), however, additionally increases this regulatory PFC activation, whereas elimination of any emotional information (neutral stimuli and neutral labels) does not elicit any reaction in this area at all. It seems that with an increasing focus on negative valence, emotional regulation is amplified. To test this hypothesis, future studies are needed which modify the task in a way that both stimulus valence and the focus of cognitive processing vary between conditions.

Initial arousal was also significantly modulated by the type of processing as indicated by SCL in the early phases of each block. As assumed, simple perceptual processing of threat led to increased SCL while no valence effect was present during cognitive evaluation. During later phases valence effects emerged also in the label condition, mainly due to a decrease of SCL in response to neutral pictures. This finding contradicts our hypothesis that with increasing prefrontal activation the

autonomic fear response gets attenuated. One possible explanation might be that subjects experienced labeling particularly at the beginning of each block as more difficult, so that the condition itself induces SCL increases independent of picture valence. Also, it has been shown previously that high cognitive load attenuates amygdalar reactions to stimuli regardless of their valence (Straube et al., 2011). Matching, in contrast, is performed comparably effortlessly from the beginning over the entire block as indicated by shorter reaction times, lower error rates, and no prefrontal activation compared to baseline.

Correlation analyses revealed an inverse relationship between VLPFC and SCL during perceptual processing of threat in high anxious subjects whereas no such relationship was seen in low anxious subjects. Although some studies indicated a link between amygdala activation and skin conductance changes (Williams et al., 2001), others could not find such a relationship (Critchley et al., 2000). First, if SCL is not driven directly by the amygdala, this might explain the lack of a significant correlation effect in our study, whereas VLPFC and amygdala activity were found to be clearly negatively correlated by others (Hariri et al., 2003). Second, other intermediary brain areas that are involved in this circuit response like the MPFC and ACC (Bishop et al., 2004; Etkin et al., 2011) might have altered the initial VLPFC downstream signal to such a degree that there is no statistical relationship to the output signal (SCL). Nonetheless, VLPFC activation and SCL were inversely correlated in high anxious subjects during matching of threatening pictures. In line with earlier findings (Kim et al., 2011a), VLPFC-SCL correlations appeared to be rather negative in high anxious subjects but tended to be positive in low anxious subjects. It is conceivable that high state anxiety lowers the threshold for stimuli to elicit a neural fear response (Bishop, 2008) and that this in turn activates the PFC as a regulatory instance. This would mean that even perceptual processing of threat leads to some form of implicit emotional regulation in high anxious individuals.

The present results showed that labeling must not necessarily involve emotional aspects of the stimulus but can also be performed on neutral properties to elicit VLPFC engagement as long as the stimulus is of negative valence. Thus, the VLPFC seems to be of crucial relevance during implicit

emotional regulation of negative affect. Nonetheless, pictures used in the current study differed from each other not only in terms of valence but also arousal. It cannot be excluded that increased VLPFC activation and SCL were caused by arousal instead of or in addition to negative valence. Future studies might overcome this limitation by including highly arousing positive pictures to control for arousal and general emotionality of the stimuli.

A second point that has to be discussed is the possibility that activation changes might have been caused by muscle contractions and skin blood flow in the forehead. A recent study showed that fNIRS measurement channels covering the lower parts of the forehead are prone to artifacts caused by changes in skin blood flow (Takahashi et al., 2011) and results obtained from these measurement sites have to be interpreted carefully. Increased task difficulty might have led to increased tension and frowning as well as increased sympathetic activation, both accompanied by an increase in regional blood flow and hence O_2Hb alterations which cannot be attributed to neural activation changes (Kirilina et al., 2012; Takahashi et al., 2011). Although subjects were explicitly instructed to keep their facial muscles as relaxed as possible, we cannot fully exclude the possibility that effects arose from unintended muscle contractions. However, we regard this as being rather unlikely because our findings line up with earlier studies using fMRI, which is not affected by this methodological limitation (e.g., Creswell et al., 2007; Hariri et al., 2003).

5. Conclusion

It was shown that the VLPFC plays an essential role during cognitive evaluation of threatening but not neutral stimuli. We interpret this activation to be of regulatory nature, inhibiting subcortical structures like the amygdala. However, also during simple perceptual processing, the VLPFC affects physiological fear responses in anxious subjects. We assume that anxious individuals engage in implicit emotional regulation even when attention is not directed at the meaning of a threatening stimulus.

6. Acknowledgement

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General Discussion

Taken together, it was shown that PFC activation to fear-relevant stimuli depends on a range of different aspects including individual differences in genetic risk factors (i.e., *NPSR1*), state anxiety, and physiological flexibility, but also extrinsic factors such as the type of processing. However, direct manipulation of task associated PFC regions did not impair attentional control in the presence of fear-relevant stimuli. The main findings of each single study can be summarized as follows:

Study 1: Inhibition of DLPFC activation is not sufficient to increase or generate an attentional bias towards fear-relevant stimuli in healthy individuals.

Study 2: Autonomic flexibility can index overall DLPFC functioning and cognitive control. No evidence was found for a relationship between HRV and prefrontal emotional regulation. Further, DLPFC activation decreases with increasing state anxiety.

Study 3: Emotional processing within MPFC and DLPFC was crucially influenced by *NPSR1* genotype, suggesting that the T risk allele causes less efficient top-down regulation to fear-relevant stimuli.

Study 4: Generally, cognitive but not perceptual processing of threatening stimuli involves regulatory VLPFC activation. Only subjects with increased state anxiety showed prefrontal regulation during perceptual processing.

The present findings have multiple implications for the relatively broad topic of prefrontal functioning during emotional processing of fear-relevant stimuli and need to be discussed in more detail according to separate aspects. *First*, different prefrontal regions were investigated across the studies: the DLPFC, MPFC, and VLPFC. The present findings are reviewed according to their individual regional contributions and responsibilities within the prefrontal fear-network in the following section. *Second*, it is discussed in how far the findings from the emotional Stroop and match-label

task are related to each other and can provide information about implicit emotional regulation. *Third*, important limitations of the present work are considered with a particular focus on the suitability of the emotional Stroop task for research on regulatory PFC activation. *Last but not least*, a summarizing integrative view on prefrontal processing of fear-relevant information in healthy subjects is given based on the preset findings. In this context, potential implications and important caveats for further research are discussed.

Regional Contributions

The DLPFC

Based on previous work, in all of the three Stroop *studies*, the focus of interest was set on the DLPFC as the crucial structure for resolving emotional and cognitive conflict (Compton et al., 2003; Compton, 2003; d'Alfonso et al., 2000; Etkin et al., 2011). Its involvement during the performance of the Stroop has been endorsed by each study in terms of significant widespread activations in that area. However, specifically increased DLPFC activation to interfering stimuli was primarily found for the incongruent color condition but less reliably for emotionally interfering words. Though of larger HHb decreases to fear-relevant compared to neutral words were found in two studies *(2 and 3)*, no statistically meaningful differentiation between emotional Stroop conditions was found for O_2Hb in none of the studies. Moreover, the results of *studies 2 and 3* are not independent from each other because both partly rely on the same data set as described in *study 3*. This means that there was no straightforward evidence for the priori assumption of regulatory DLPFC activation during this task and also only minor evidence for the existence of an attentional bias on the behavioral level (in s*tudy 1* but not *2* and *3*)[2]. The important role of the DLPFC for cognitive control and executive function has, however, nicely been replicated. The ambiguous results on neural level make the interpretation of results from *studies 1 and 2* difficult. Although it was shown in *study 1* that DLPFC activation was bilaterally and significantly reduced – at least following

[2] The limitations of the emotional Stroop task in studies on healthy subjects are discussed later in the text in the limitations section

left-hemispheric inhibitory cTBS – this had no impact on behavioral performance. On the other hand, not even the working memory aspect of the task (i.e., recall of color-button assignment) was significantly affected by the rTMS manipulation, which would have resulted in an overall increase of error rates or response latencies. The lack of a general behavioral effect following cTBS seems surprising taking the bunch of literature into account reporting a strong association between DLPFC function and working memory performance (e.g., Barch et al., 1997; Fregni et al., 2005). Similarly, the results of *study 2* are affected by the undifferentiated O_2Hb effects of the emotional Stroop. The results clearly indicated a relationship between autonomic flexibility, as indexed by HRV, and DLPFC activation in terms of decreased prefrontal activation in subjects with low HRV but beyond that, results were again found to be stronger related to the classical part of the Stroop and the overall error rate, showing that less autonomic flexibility was associated with weaker cognitive control particularly in the left DLPFC. Nevertheless, it could be shown in this study that the DLPFC has some crucial - although probably indirect - impact on ANS activation. These findings support the model of top-down regulation of the subcortical regulation of the heartbeat (Cacioppo et al., 2007; Thayer and Lane, 2009) but do not point towards a predictive function of HRV for emotional regulation.

Summarizing the results of *studies 1 and 2,* no clear-cut evidence was found for a functional role of the DLPFC during emotional conflict. However, both studies provided evidence for a link between DLPFC function and affective state. In *study 1*, bilateral DLPFC inhibition following left-hemispheric cTBS had a beneficial effect on positive affect, and in *study 2*, activation in this area was inversely correlated to state anxiety. These results seem contradictory because in the first case lower DLPFC activation was associated with less negative emotion while in the second it was linked to increased negative emotion. Yet, the results of increasing activation with decreasing state anxiety are in line with previous research (Bishop et al., 2004) while those of *study 1* are rather contradictory (Gershon et al., 2003; Loo and Mitchell, 2005). However, the network effects of rTMS are still unknown and it remains unclear whether effects on mood were caused by changes in DLPFC activation or co-(de)activations in other functionally connected brain areas. Also, a decrease in

positive affect as assessed by the Positive and Negative Affect Schedule (PANAS; Watson et al., 1988) in *study 1* represents a qualitatively very different emotional state than the more focused state anxiety subscale of the State-Trait Anxiety Inventory (STAI; Spielberger et al., 1970). In the end, correlations (DLPFC and state anxiety) provide also more precise information about the functional relationship between two variables than a post hoc t-test (DLPFC and positive affect). The finding of decreased DLPFC activation as a function of increasing state anxiety before the measurement shows how important it is to control for individual differences in functional neuroimaging research.

But not only psychometric and physiological differences were related to DLPFC functioning. Dividing subjects based on their individual genetic variation of the *NPSR1* rs324981 gene resulted in an exceptional neural pattern in homozygous A allele carriers that differed from all other subjects. In this group, increased DLPFC activation was found to fear-related compared to neutral words, a pattern that was originally expected to be seen among all healthy subjects. Regarding earlier studies on *NPSR1*, the increased activation to fear-relevant stimuli was interpreted as a protective mechanism in contrast to the undifferentiated activation patterns in T risk allele carriers that were considered to reflect a subclinical form of weakened emotional regulation.

The results of *studies 1-3* make clear that the DLPFC is crucially involved in emotional processing, also in the emotional Stroop task, but activation differences in healthy subjects might be biased or even completely masked by a range of individual differences like genotype and anxiousness. Although the DLPFC was not a region of interest in *study 4*, it is of notice that activation in this area was increased during the cognitive compared to perceptual evaluation of threatening pictures suggesting a role during implicit emotional regulation and emphasizing the importance of processing type for prefrontal fear network activation.

The MPFC

The most anterior part of the MPFC was a ROI in *study 3* because of a previous study showing *NPSR1* effects on fear learning in this area (Raczka et al., 2010). As for the DLPFC, activation

in the MPFC was specifically increased to fear-relevant words in homozygous A allele carriers while no genotype effects were observed for the cognitive part of the Stroop task. Because the MPFC has been shown to play an important role during the regulation of emotional conflict (Etkin et al., 2011), activation differences were assumed to reflect individual differences between genotypes with less efficient regulatory function in T risk allele carriers. Regarding the antagonistic roles that have been ascribed to ventral and dorsal parts, the current results rather relate to the former region since the ROI in *study 3* encompassed the entire anterior pole located directly above the OFC, thus covering the most rostral parts of the VMPFC. Only one out of four ROI channels (i.e., channel 16; *figure 1* in the manuscript of *study 3*) covered an area that can be assumed to be part of the DMPFC in most subjects. The findings, however, support the assumed down-regulatory role of the VMPFC and not that of the fear-generating role of the DMPFC (Etkin et al., 2011; Ochsner et al., 2009) because previous findings showed that particularly the AA genotype is associated with lower anxiety levels (Domschke et al., 2011; Klauke et al., in press). Apart from that, the dorsal PFC has also been found to have inhibitory effects on negative affect and limbic system activation in some studies (Phan et al., 2005; Phillips et al., 2003).

To conclude, it is important to keep in mind that the entire MPFC region could not be addressed by using fNIRS due to limited measurement depth and findings remain restricted to the most anterior cortical surface. MPFC findings cannot be related to *studies 1, 2, and 4* because only *study 3* investigated emotional processing in this area.

The VLPFC

Study 4 highlighted the importance of the VLPFC for unintended forms of emotional regulation. Like the MPFC, the VLPFC has been targeted as a ROI only in one of the four studies. In a nutshell, it was shown that the VLPFC plays a particular role during the elaborate cognitive evaluation of threatening stimuli. Activation increases were specific to this form of processing and to the negative valence of the experimental stimuli. No VLPFC activation increases were found during

simple perceptual processing of threat at all. Simultaneous with increasing VLPFC activation, an attenuation of valence effects was found on initial arousal levels, supporting the assumption of inhibitory top-down influence on the amygdala and brainstem *(figure 1)*. Furthermore, there was a negative correlation between VLPFC activation and SCL in subjects with increased state anxiety during perceptual processing of threat. This was interpreted as an augmented need for prefrontal control in the face of task-irrelevant threatening stimulus characteristics in those subjects. Such increases in VLPFC activation may reflect the top-down regulation that is needed to counteract the attentional bias towards fear-relevant stimulus information typically seen in anxious individuals (Bishop, 2008). The findings of *study 4* implicate that the VLPFC crucially guides implicit emotional regulation. The extent of implicit emotional regulation during the Match-Label and emotional Stroop task and the similarities and differences in fear processing between both behavioral paradigms are discussed in the following section.

When does implicit emotional regulation start?

The results of *study 4* emphasize the importance of the type of processing on the extent of the attentional bias in behavioral, physiological, as well as neural measures. On behavioral level, the attentional bias towards fear-relevant (threat) stimuli was present during both perceptual and more elaborate cognitive processing but more pronounced during the latter. Similarly, initial arousal to blocks of fear-relevant pictures was higher compared to neutral pictures only during perceptual processing, although later during the block, accelerated physical reactions were observed for both types of processing. In contrast, the weaker but nevertheless distinctive attentional bias during labeling was accompanied by an increase in VLPFC activation. As already discussed, in healthy subjects, cognitive evaluation of fear-relevant stimuli thus seems to activate the VLPFC while simple perceptual processing does not. In summary, and in contrast to the emotional Stroop studies, the results of this study are coherent in all parameters that have been measured and the stimuli were sufficiently threatening to induce physiological arousal and an attentional bias towards them.

Comparing the emotional Stroop task with the match-label paradigm of *study 4*, it seems questionable whether the Stroop task is suited to measure emotional top-down regulation in healthy subjects. First of all, compared to the threatening pictures of *study 4*, the fear-relevant words elicited neither an attentional bias in terms of increased response latencies for fear-relevant trials *(studies 1-3)* nor any differential physiological reaction (*study 2*). Despite the differential findings of both tasks, there exists, however, some qualitative overlap regarding the psychological processes underlying the matching trials of the match-label task and the fear-relevant Stroop trials. In the emotional Stroop task, subjects evaluate stimuli based on their color, a perceptual characteristic, which is comparable to the match condition in *study 4*. It seems reasonable that the latter task, like the emotional Stroop task, engages primarily bottom-up processes without actively encouraging the subject to process the stimulus' meaning. However, recognition of valence occurs automatically also during perceptual processing as indicated by the attentional bias found in *study 4.* Similarly, other modified versions of the emotional Stroop task using faces and words clearly showed that reading, and as such also retrieval of word meaning, happens automatically also during the emotional Stroop task as indicated by prolonged response latencies when emotionally incongruent information was presented (Egner et al., 2008; Krug and Carter, 2010). Consequently, the meaning of words of the present Stroop studies must have been realized to some extent, even if the process was primarily unintended. Furthermore, if the present stimulus material *(Supplement C)* would have been of sufficient emotional significance, it would have elicited an increase in amygdalar activation as shown in an earlier study by Isenberg et al. (1999). Activation changes in the amygdala, however, could not be measured by using fNIRS and indirect measures of the fear reaction (i.e., SCR) indicated no differential activation patterns to fear-relevant words.

Bottom-up processing of threatening or fear-relevant stimuli activates predominantly brain areas that are associated with fear generation (Ochsner et al., 2009). However, as has been postulated by LeDoux (1996, 2003), it is widely accepted that even the fast subcortical fear reaction is followed by a delayed activation of the PFC and thus top-down emotional regulation. As such,

every stimulus provoking a fear response can lead to some form of automatically activated prefrontal control. If this regulatory activation is elicited unconsciously or, at least, unintendedly such as during the present conditions, it may represent implicit emotional regulation as well as the labeling condition although probably less insistently. Although the current studies investigated exclusively control subjects, it was shown that those with high state anxiety were characterized by an increasing hypoactivation with increasing levels of anxiety (*study 2*) and, in addition, by a negative correlation between VLPFC and SCL during matching of threatening pictures (*study 4*). It is striking that this regulatory PFC activation during perceptual processing was only found in in anxious subjects[3] and not present during the labeling condition of *study 4*. However, a recent emotional Stroop study by Dresler et al. (2012a) showed an increase in activation to fear-relevant words in similar prefrontal regions (i.e., inferior and middle prefrontal gyrus) in patients with PD. It seems as if particularly bottom-up processing of fear-relevant stimuli leads to top-down control in anxious but not non-anxious subjects. Therefore, it is reasonable to assume that even perceptual processing as assessed by tasks of attentional control (e.g., emotional Stroop task) and the matching condition of the match-label task engages some form of implicit emotion regulation.

Interestingly, and in line with the previous findings, genetic risk factors for pathological anxiety also act on bottom-up processing of fear-relevant stimuli. Separating the minor group of non-risk allele carriers (A/A genotype of the *NPSR1* gene) from a larger sample of healthy control subjects revealed that non-risk allele carriers displayed an increased DLPFC response to fear-relevant words. Both findings, the prefrontal hypoactivation in anxious subjects (*study 2*) and T risk allele carriers, integrate nicely into current opinions of decreasing prefrontal control as a function of increasing anxiety (Bishop, 2007; Bishop, 2008). According to the neurocognitive model of anxiety-related selective attentional biases by Bishop (2007), state anxiety acts primarily on threat detection mechanisms depending on amygdalar functioning whereas trait anxiety influences rather attentional

[3] Here, anxiousness is used as an arbitrary term that refers to scores on the STAI subscale assessing state anxiety. These scores have only a relative meaning referring to the distribution of scores within the samples of *studies 2 and 4*. In *study 4*, for example, the terms 'anxious' and 'non-anxious subjects' refer to those subjects with scores above and below the median, respectively.

control mechanisms based on PFC activation. For *NPSR1*, it is still unclear which part of the fear circuit is directly and which part is indirectly affected by alterations in NPS since both the amygdala and PFC have been shown to be affected in earlier studies (Dannlowski et al., 2011; Raczka et al., 2010). However, integrating genetic mechanisms into the neurocognitive model of Bishop (2007), the genetic profile is rather considered to represent a trait and not state factor and might therefore act primarily on PFC function. In contrast to the non-differential findings of the other two Stroop studies, particularly the protective A/A genotype was found to be characterized by a regulatory activation pattern during the fear-relevant condition.

As for state anxiety, the neurocognitive model (Bishop, 2007) postulates that primarily amygdalar functioning is affected with increasing anxiety. It is supposed that particularly anxious subjects are characterized by a more sensitive threat detection mechanism to task-irrelevant stimulus characteristics. To explain the inverse relationship between VLPFC and physiological arousal found in *study 4*, it can be assumed that the fear-relevant stimuli during the match condition led to a greater fear response in terms of amygdalar increases in anxious compared to non-anxious subjects. This in turn required a top-down response of the PFC to ease attentional control which was needed for efficient task performance. Non-anxious subjects, in contrast, might have reacted with a less intense fear response. Consequently, they did not show PFC activation that would have been needed to down-regulate physiological arousal. To summarize, elevated anxiety may not only be characterized by a stronger attentional bias towards task-irrelevant fear-relevant stimulus information but also by subsequent automatic regulatory activation of the fear network during certain types of task. However, a direct correlation between overall PFC functioning and state anxiety in *study 2* revealed that PFC activation decreased with increasing levels of state anxiety emphasizing that anxiousness is generally rather accompanied by deficient prefrontal regulation.

All together, the present results provide significant evidence that also simple perceptual processing of fear-relevant stimuli can activate implicit emotional regulation when controlling for anxiety-related variables such as risk genes or anxiousness.

General Limitations

The particular limitations of each individual study can be found in the respective discussion sections of the published articles. This section provides a discussion of limitations that apply to the majority or to all of the present studies: 1) sample selection, 2) disadvantages of fNIRS for the study of the fear network, and 3) the emotional Stroop task.

Sample Selection

A common problem of human research refers to the standardization of the measurement situation and the unavoidable variance that exists among participants. Personal background, personality, traits, current mood, and many other variables undeniably influence the behavior of each person and can even vary from one point in time to the other. The best way to prevent these variables from confounding the experimental results is a standardized setting, careful screening of subjects, and a large sample size. As for the latter two criteria, there were some study-specific limitations that need to be considered.

Careful screening was particularly part of *studies 1-3* and participants were excluded by using previously set criteria if necessary. In *study 1*, participants were required to fill in a screening questionnaire assessing psychopathological tendencies and the sample of both *studies 2 and 3* underwent a structural clinical interview by a trained clinical psychologist in advance. The subjects of *study 4*, however, were selected less strictly due to the initial pilot character of that study. Approximately one-third of participants was a member or an associate of the Psychophysiology and Functional Imaging Lab at the Department of Psychiatry, Psychosomatics and Psychotherapy in Würzburg and included without any clinical screening. The remaining two-thirds filled in a screening questionnaire based on the Structured Clinical Interview for DSM-IV Axis I Disorders (First et al., 1996), due to which one subject was excluded. The missing data for the other third poses certainly a limitation on the present data.

For all studies, the measurement procedure was held constant in terms of order and instructions. The measurements of *study 1, 2 and 3* were performed by two different experimenters each but at random order with respect to experimental group membership so that a potential effect of experimenter should have been cancelled out.

Regarding sample sizes of the present studies only *study 1* has to be considered with some caution. The cTBS groups were relatively small and there were baseline differences between groups in PFC activation which might have confounded the results. This issue is also discussed in the according article. Sample and group sizes in *studies 2, 3, and 4* were sufficiently large if not above-average when compared to other functional imaging studies.

Finally, a comparison of results between *studies 2 and 3* is limited because analyses of the former study were performed on a subsample of the larger sample of *study 3*. Thus, results of both studies relied to a large extent on the same behavioral and fNIRS data and are therefore not independent from each other.

Disadvantages of fNIRS for the Study of the Fear Network

FNIRS was used in all of the present studies to gain insight into cortical neural activation during the processing of emotional stimuli. Besides its multiple advantages, fNIRS is also afflicted by some limitations. The depth to which the NIR light travels is restricted to about 1.5 cm for the apparatus used in the current studies (Quaresima et al., 2012; Strangman et al., 2002a). Therefore, hypotheses could only be made for brain regions lying on the surface of the frontal lobe. A great deal of the limbic and paralimbic structures which are primarily involved in emotional processing and regulation, however, were out of reach in medial and posterior direction within the frontal or deep in the temporal lobes. These include for example the ACC, amygdala, and hippocampus. Therefore, the present results provide information about prefrontal cortical functioning only and interpretations including other areas of the limbic system remain hypothetical. It was also not possible to investigate network activity between these areas and the PFC. In *studies 2 and 4*, we aimed at indirectly assessing amygdalar activation by recording skin conductance because bodily arousal in response to

fear-relevant stimuli is assumed to be generated by a subcortical fear network consisting of projections from the amygdala to the brainstem (LeDoux, 2003; Liddell et al., 2005). Nevertheless, this gives only an idea about limbic functioning but no quantitative measure. Since PFC mediated top-down inhibition of the amygdala and its modulation during emotional processing was one of the fundamental ideas for conducting the present studies, restricted measurement depth is probably the most impeding limitation.

A second limitation of fNIRS refers to the origin of the hemodynamic signal. A recent attempt to investigate the confounding effects of skin blood flow revealed that the fNIRS signal from the skin strongly correlates with the overall fNIRS signal aimed at measuring cortical hemodynamic activity (Takahashi et al., 2011). Moreover, during a verbal fluency task, the hemodynamic activity in the skin rises with increasing task difficulty making it difficult to interpret the fNIRS signal as reflecting neurovascular coupling. The results, however, varied with probe position: Signals from channels over the forehead showed highest correlations whereas those located over temporal or dorsolateral regions correlated less strongly (Takahashi et al., 2011). The authors concluded that activation changes measured over the forehead mirror changes in skin blood flow instead of brain-related hemodynamic activity. However, a greater amount of residuals was found for other frontal and temporal measurement sites and interpreted to reflect neurovascular coupling. Although the results of Takahashi et al. (2011) raise serious questions for the interpretation of the fNIRS signal, most ROI channels in the present studies were located outside of the critical forehead region and are thus considered to reflect true brain activation. Only in *study 2*, the MPFC ROI covered the area in question almost completely. Nevertheless, in this study we found interaction effects that showed differential activation patterns among various genotypes and did not reflect task difficulty. It is unlikely that skin blood flow in this study was related to *NPSR1* genotype, thereby confounding the present results.

The Emotional Stroop Task

The emotional Stroop task has been used in psychological research now for over 15 years (Williams et al., 1996) and is one of the most investigated but also hardly criticized behavioral paradigms (Algom et al., 2004; Buhle et al., 2010). In the context of the present work, three problematic issues should be considered: 1) if the emotional Stroop task can be classified as an emotion regulation task, 2) if emotional interference can be induced in control subjects, and 3) the non-equivalence with the classical Stroop task.

First, in the present work the emotional Stroop task was used because previous literature indicated that the task engages top-down control to deal with the attentional bias towards emotional stimuli (Bishop et al., 2004; Bishop, 2008). Further, as it has been postulated by Todd et al. (2012), affect-biased attention itself already reflects a habitual filtering process and can therefore be categorized as an automatic form of emotional regulation. However, as already discussed above, stimulus processing in the emotional Stroop can be assumed to rather reflect bottom-up compared to top-down processing. Similarly, the use of the emotional Stroop task as an emotion regulation task, particularly in its present form, has been questioned before (Buhle et al., 2010).

Second, the lack of behavioral *(studies 2 and 3)* and autonomic interference effects *(study 2)* in healthy subjects could be interpreted as a failure of the emotional Stroop task to cause an attentional bias toward fear-relevant stimuli. But does a lack of a quantifiable response imply that no regulatory process was activated? A behavioral non-response does not automatically mean that there was no interference; it may also be the result of effective attentional control based on a well-functioning fear network. Likewise, differential PFC activations as partly found for HHb measures in *studies 2 and 3* may indicate that top-down regulation took place. The lack of a behavioral effect may simply mean that top-down regulation was sufficient to enable efficient task performance.

Apart from that, interfering effects of fear-relevant words were observed for error rates in *study 1* indicating that word meaning caused at least some disturbance in performance. The slightly ambiguous behavioral results are in line with previous research (Dresler et al., 2012a; Phaf and Kan,

2007) and might be due to the differences in experimental design. It has been shown that the emotional Stroop effect is difficult to find in healthy subjects using event-related designs because the interfering effects of emotional words exert their slow-down effects predominantly on the following trial independent of condition (McKenna and Sharma, 2004; Waters et al., 2003). Therefore, block designs are better suited to investigate emotional Stroop interference because these carry-over effects are likely to cancel each other out in event-related designs. Since all trials in the present studies were presented in an event-related and randomized order, this is the most probable explanation for the lacking effect. Moreover, it can explain why a partial inference effect was found in *study 1* compared to the others. The task design in this study comprised only two conditions (fear-relevant vs. neutral), thereby increasing the probability that two or more trials of the same condition were presented in a row. In *studies 2 and 3*, however, this likelihood was decreased due to the additional two conditions of the classical Stroop task.

Third, the emotional Stroop effect is not the same as the classical Stroop effect. While the latter is caused by a dimensional conflict between word color and word meaning, the former represents rather an attentive bias towards salient emotional word content (Algom et al., 2004). This was shown in a smart series of small experiments by Algom et al. (2004) comparing both types of tasks. One of the main outcomes of this study was that a reversion of the task demands (word reading instead of color naming) also elicited an emotional interference effect. In the classical Stroop, in contrast, interference effects vanished with reverse instructions. More important arguments for the non-equivalence of both versions have recently been summarized by Buhle et al. (2010). For those reasons, the interfering trials of both tasks have never been directly compared in *studies 2 and 3.* The classical Stroop rather served as a separate measure of cognitive regulation in *study 2* and as a control task for the specificity of effects on fear processing in study 3.

Towards an Integrative View of PFC Function

According to the present findings, PFC functioning is altered by a number of individual intrinsic and extrinsic differences during the processing of fear-relevant stimuli. The results of the current studies showed general and functional up- and down-regulatory influence of physiological, genetic, psychological state, and task variables (*figure 4*). More specifically, state anxiety and low HRV were associated with attenuating effects on overall DLPFC activation while the type of processing and *NPSR1* genotype modulated particularly activation to fear-relevant stimuli in the DLPFC, MPFC, and VLPFC, respectively.

Going back to the hypothesized model of fear network regulation and potential modulators, it was shown that variables of qualitatively very distinct origins act on the PFC, which is assumed to

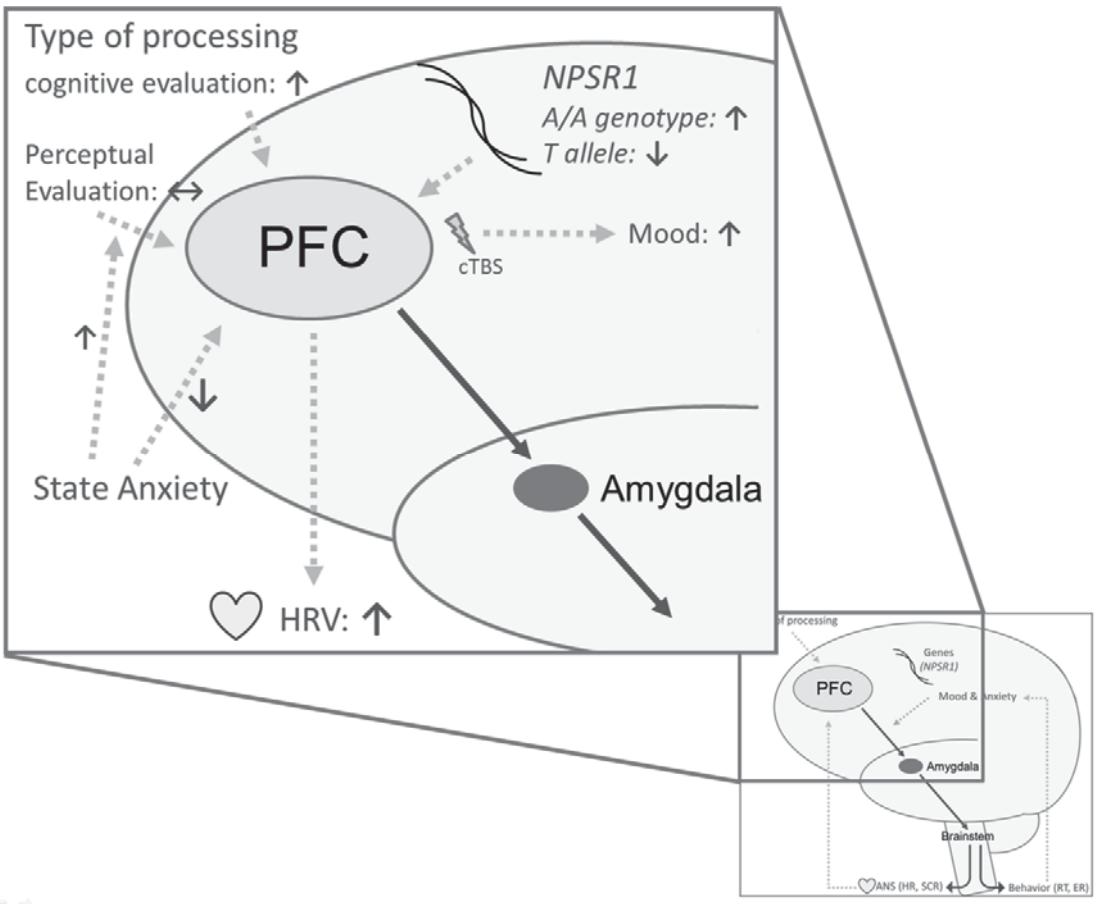


Figure 4: Modulators of prefrontal fear network function
The figure displays up- (↑) and down- (↓) regulatory influences of the investigated variables of all studies. In *study 1*, PFC activation was attenuated by means of cTBS causing changes in mood; *study 2* investigated the potential of HRV as an index for PFC functioning; *study 3* focused on *NPSR1* genotype effects; and *study 4* tested the regulatory function of different processing types.

represent the highest order structure in this hierarchical top-down system of automatic, or unintentional, emotional control. In an ideal research, all of these variables would be controlled for to gain a better understanding of the regulatory functions of the PFC during emotional processing. In reality, however, human neuroimaging studies have to deal with a lot of heterogeneity with respect to the former variables. Particularly the findings from *study 3* have shown that the degree of prefrontal control can vary depending on the individual genetic profile with risk allele carriers of the *NPSR1* gene displaying equally enhanced PFC activation to fear-relevant and neutral stimuli. Considering that according to Hardy-Weinberg equilibrium approximately 75% of the population carries the *NPSR1* risk allele, it seems hardly possible to find emotion-specific PFC changes in non-stratified populations using the current experimental design. So when we talk about such genetic risk factors for certain anxiety disorders, we actually refer to the majority of the population. Bearing in mind that there are more than just one candidate gene, the question arises as to whether it may not be more plausible to regard risk allele carriers as the ordinary and non-risk allele carriers as the ones possessing one or more protective genotypes.

While genetic profiles may constitute one factor that can mask variations in PFC activation, the type of processing that is required by the experimental design is another essential point that needs to be considered. As explained in the previous section, the emotional Stroop task in its present form seems not well suited to investigate healthy control samples. The amount of attentional binding in response to fear-relevant stimuli may be too subtle to require top-down control facilitating performance. This assumption is also supported by the lack of an effect of PFC inhibition on behavior in *study 1*. In patients, however, the emotional Stroop task has led to differential behavioral and neural effects (e.g., Becker et al., 2001; Bremner et al., 2004; Dresler et al., 2012a; Dresler et al., 2012b; Williams et al., 1996).

But also state anxiety, as shown by *studies 2 and 4*, has been associated with decreased overall prefrontal functioning but also with regulatory activation during bottom-up processing of fear-relevant stimuli. This latter finding was thought to reflect a more sensitive threat detection

mechanism in anxious individuals that in turn elicits reflective top-down control of the PFC to ease attentional deployment. Particularly anxious individuals might have difficulties to automatically disengage from task-irrelevant but emotionally salient stimulus information. In addition, an overly sensitive threat detection mechanism in those subjects might have led to attentional avoidance which is most likely reflected by increased PFC activation (Cisler and Koster, 2010). This would explain why a negative VLPFC-SCL correlation was only found in this group and not in less anxious subjects. Because attentional avoidance is considered to represent a strategic process of emotional regulation (Cisler and Koster, 2010), also bottom-up processing of fear-relevant stimuli seems to engage implicit emotional regulation at least in anxious individuals.

Compared to the results of perceptual processing alone, fear-specific valence effects were found on multiple levels (behavioral, physiological, and neural) during labeling in the entire and non-stratifies sample of healthy control subjects (*study 4*). In summary, the current findings demonstrate that the engagement of implicit emotional regulation depends not only on task instructions but varies with the sample characteristics. Although anxiety is generally associated with hypofrontality, anxious subjects might react with top-down control in situations during which non-anxious subjects do not. However, this differential neural response may be rather due to an increased need for attentional control than to a generally more efficient prefrontal top-down control.

Taken together, PFC functioning during the processing of fear-relevant stimuli was found to be reflected by higher autonomic flexibility (i.e., increased HRV), and specifically increased by the A/A genotype of the *NPSR1* gene, and elaborate cognitive evaluation of the threatening stimulus. In contrast, low HRV, the *NPSR1* T allele, and elevated state anxiety were associated with lower PFC activation. Overall, simple perceptual processing of fear-relevant stimuli (matching and Stroop task) yielded no differential neural activation patterns except for those subjects displaying higher levels of state anxiety. In this group, an increasing PFC activation was related to a decreasing subcortically mediated physiological fear response.

Outlook and Future Directions

All four studies have very specific implications for further research as discussed in the respective sections of each manuscript. Although the results of *studies 2-4* provide more specific information about potential factors acting on the fear-network than *study 1*, it is the methodological design of this study that offers probably the most interesting perspective for future research. *Study 1* showed the great potential of cTBS for research on PFC functioning in general and anxiety research in particular. By means of cTBS the functional role of different cortical areas can be directly tested. Regarding the focus of the present work, this is particularly helpful in testing which regions are functionally crucial during fear regulation compared to those that are less important or are just co-activated. The non-specificity of effects regarding fear processing in this study, were most likely due to the limitations of the emotional Stroop task for research on healthy control subjects but do not question the potential of rTMS for future research. Quite the contrary: the present findings showed that cTBS can considerably attenuate PFC functioning and therefore support the high relevance of rTMS for the study of brain-function relationships (Hallett, 2000). Based on the present study, future research may focus on experimental tasks that are known to produce robust effects also in healthy individuals, such as the match-label task of *study 4*. Inhibition of the VLPFC by means of cTBS may for example provide more information about causal relationships between this brain region and its functional role during implicit emotional regulation. Such basic research might also give direction for future treatment strategies of anxiety disorders, particularly regarding the potential of rTMS as an add-on treatment, an issue that is currently discussed in psychiatric neuroscience (Zwanzger et al., 2009).

The present research highlighted that prefrontal fear network function is impacted by a range of individual differences between subjects, particularly in basic research on non-clinical samples. In an ideal research, all those variables (e.g., genetic profile, anxiousness, and autonomic flexibility) would be controlled for. Apart from animal research, however, this is a very challenging task in neuroimaging research. In human studies, strict standardization is feasible for the

experimental setting but limited with respect to the sample. As shown in the present work, heterogeneity and individual differences can mask experimental effects and it is reasonable that the larger portion of those confounders is not yet identified. An ongoing search for other variables that are shaping, modulating, and mediating PFC function seems thus essential for a better understanding of emotional control, and, as a consequence, the deviating neural patterns observed in anxiety disorders.

Conclusion

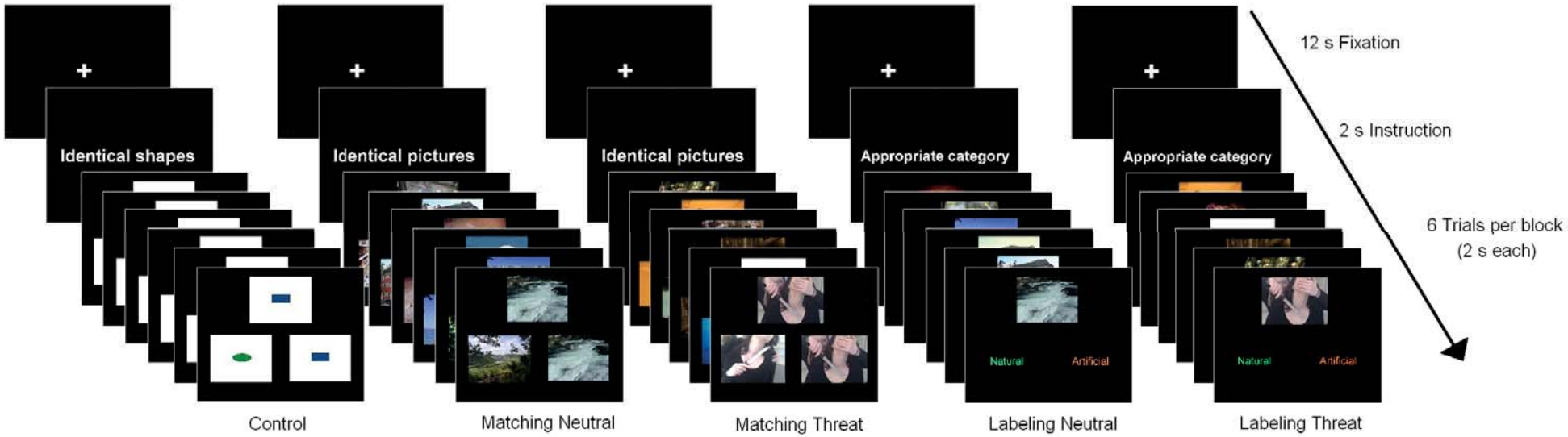
The present studies illustrated the complex role of the PFC within the fear network and presented some of the multiple specific factors that modulate its function during fear processing. It was shown that PFC activation to fear-relevant stimuli is critically influenced by individual genetic and task variables, suggesting that PFC functioning is partly intrinsically and partly extrinsically determined. More specifically, it was shown that also implicit emotional regulation of threat depends on PFC activation whereas simple perceptual processing yielded ambiguous results across the present studies. The findings highlight that the PFC is inevitably involved in fear processing but depends on multiple modulating factors that are difficult to control for as a whole in human experimental research. Furthermore, interfering with PFC activation did not particularly hamper fear processing, indicating that in healthy individuals the fear network may be very flexible and that emotional control does not exclusively depend upon one single region. Similarly, autonomic flexibility can provide valuable information about overall prefrontal activation but was not systematically related to emotional control.

Also, it was demonstrated that even bottom-up processing of fear-relevant stimuli engages implicit emotional regulation that may be too subtle to produce a distinct signal pattern in non-stratified healthy control samples but becomes evident when controlling for subclinical individual differences related to anxiety. More precisely, it was shown that subjects displaying higher state anxiety might be characterized by implicit emotional regulation even when attention was not directed at the meaning of a stimulus. This increase in top-down control during task in which no regulatory activation was seen in non-anxious subjects was interpreted to reflect attentional avoidance, a behavioral pattern that is typically observed in anxiety disorder patients. Apart from differences in individual anxiousness, there may be also certain protective factors against pathological anxiety such as the *NPSR1* genotype which has been linked to more efficient reflective prefrontal control.

All together, the present results support earlier models of decreasing prefrontal activation with increasing levels of anxiety and related states and traits but highlight the experimental precautions that must be considered when aiming to uncover the distinct prefrontal neural patterns underlying them. The present studies investigated only a few of those confounding variables and there may be many more state, trait, and environmental factors that need to be disentangled to gain insight into the complete makeup of prefrontal fear network function.

Supplement A: Experimental Task of *Study 4*

The figure below shows example blocks of all conditions of the match-label task used in *study 4*. The task was adapted and modified from the original match-label paradigm developed by Hariri et al. (2003). During control and matching trials, subjects were asked to choose from one of two simultaneously presented pictures at the bottom of the screen the one that was identical to the target picture displayed above. During labeling trials, they had to choose between two simultaneously presented labels (i.e., "natural" and "artificial") the one that correctly describes the target picture above.



For copyright reasons, apart from the control stimuli, all pictures depicted in the figure are reenacted scenes from the original stimulus material. A list of the original picture stimuli from the International Affective Picture System (IAPS; Lang et al., 1997) can be found in *Supplement B*.

Supplement B: Pictorial Stimuli of *Study 4*

Stimuli used from the International Affective Picture System (IAPS; Lang et al., 1997) for the match-label task of *study 4*. The table displays the reference numbers of all stimuli and a description of the picture.

Practice trials	**Neutral Condition**		**Threat Condition**	
	Natural	Artificial	Natural	Artificial
2870 (boy)	1333 (parrots)	2393 (factory)	1050 (snake)	2683 (war)
5260 (landscape)	1390 (bees)	2745 (supermarket)	1052 (snake)	6020 (electric chair)
5700 (mountain)	1450 (bird)	5395 (ship)	1114 (snake)	6212 (gun)
7002 (towel)	1560 (bird)	5471 (satellites)	1120 (snake)	6260 (gun)
7041 (baskets)	1670 (cow)	7000 (rolling pin)	1201 (spider)	6550 (knife)
7234 (ironing board)	1740 (owl)	7010 (basket)	1205 (spider)	6560 (gun)
7620 (plane)	1910 (fish)	7036 (harbor)	1300 (dog)	6570 (gun)
	5020 (plant)	7037 (trains)	1301 (dog)	6940 (armor)
	5250 (landscape)	7080 (fork)	1321 (bear)	8485 (fire)
	5300 (stars)	7090 (book)	1525 (dog)	9050 (plane crash)
	5530 (mushrooms)	7130 (truck)	1930 (shark)	9230 (fire)
	5534 (mushrooms)	7175 (lamp)	1931 (shark)	9404 (armor)
	5594 (landscape)	7224 (cabinet)	1932 (shark)	9495 (war)
	5750 (tree)	7500 (building)	5920 (lava)	9600 (accident)
	5781 (landscape)	7510 (building)	5940 (lava)	9622 (plane crash)
	5800 (tree)	7550 (computer)	5971 (tornado)	9911 (accident)
	5870 (clouds)	7560 (highway)	5972 (tornado)	9920 (accident)

Conditions significantly differed regarding arousal and valence. The table displays means ± standard deviations and statistical output from independent t-tests.

	Neutral	Threat	t	df	p
Arousal	3.45 ± .91	6.22 ± .52	15.935	55.614	<.001
Valence	5.67 ± .90	3.31 ± .71	12.354	66.228	<.001

Supplement C: Word Stimuli of the Emotional Stroop Task

The table below lists the original stimuli - and their corresponding translations - used in the emotional Stroop task of *study 1* and in the emotional part of the combined Stroop in *studies 2 and 3*. Stimuli presented in the neutral and fear-relevant conditions were matched with regard to their number of letters, syllables, and frequency within German language. More information about the selection procedure is reported elsewhere (Dresler, 2011).

Neutral Stimuli		**Fear-related Stimuli**	
German original	English translation	German original	English translation
Papier	Paper	Anfall	Attack
Hafer	Oat	Sorge	Worry
Fenster	Window	Kollaps	Collapse
Gesetz	Law	Gefahr	Danger
Dampfer	Steamboat	Notfall	Emergency
Parkplatz	Parking Ground	Schwindel	Dizziness
Laterne	Lantern	Atemnot	Breathlessness
Formel	Formula	Opfer	Victim
Schema	Scheme	Panik	Panic
Unterschrift	Signature	Herzinfarkt	Heart Attack
Kreis	Circle	Furcht	Fear
Knopf	Button	Angst	Anxiety
Bleistift	Pencil	Schweiß	Sweat
Monitor	Monitor	Tod	Death
Kaugummi	Chewing Gum	Katastrophe	Catastrophe

Word stimuli between the two conditions equalled with respect to the average number of syllables and letters. The table below displays means, standard deviations and test statistics from independent t-tests.

	Neutral	**Fear-relevant**	**t**	**df**	**p**
Syllables	2.13 ± .64	2.00 ± .85	.487	28	.630
Letters	7.00 ± 1.89	6.67 ± 2.23	.537	28	.662

Abbreviations

0-9

5-HTR1A: Serotonin receptor 1A
5-HTTLPR: Serotonin transporter linked polymorphic region

A

ACC: Anterior cingulate cortex
ADHD: Attention-deficit/hyperactivity disorder
ANOVA: Analysis of variance
ANS: Autono mic nervous system
AS: Anxiety sensitivity
ASI: Anxiety Sensitivity Index
AUC: Area under the curve

B

BDI: Beck's Depression Inventory
BDNF: Brain derived neurotropic factor
BNST: Bed nucleus of the stria terminalis
BOLD: Blood oxygen level dependent

C

CBSI: Correlation based signal improvement
CBT: Cognitive behavioral therapy
ceA: central amygdala
CNS: Central nervous system
COMT: Catechol-O-methyltransferase
cTBS: Continuous theta burst stimulation

D

D/AP: Dubey/Armitage-Parmar
DLPFC: Dorsolateral prefrontal cortex
DMPFC/dmPFC: Dorsomedial prefrontal cortex
DSM-IV: Diagnostic and Statistical Manual of Mental Disorders (4^{th} edition)
DSM-IV-TR: Diagnostic and Statistical Manual of Mental Disorders (fourth edition, text revision)

E

EEG: Electroencephalography
EMG: Electromyography
ER: Error rate

F

FDR: False discovery rate
fMRI: functional magnetic resonance imaging
fNIRS: functional near-infrared spectroscopy

G

GABA: Gamma-aminobutyric acid
GAD: Generalized anxiety disorder

H

HHb: Deoxygenated hemoglobin
HF-HRV: High frequency heart rate variability
HPA: Hypothalamic-pituitary-adrenal
HR: Heart rate
HRV: Heart rate variability

I

IAPS: International Affective Picture System
imTBS: Intermediate theta burst stimulation
iTBS: Intermittent theta burst stimulation
ITI: Inter-trial interval

L

LF-HRV: Low-frequency heart rate variability

M

MEP: Motor evoked potential
MPFC/mPFC: Medial prefrontal cortex
mRNA: Messenger ribonucleic acid

N

NIR: Near-infrared
NIRS: Near-infrared spectroscopy
NPS: Neuropeptide S
NPSR1: Neuropeptide S receptor 1
NPS: Neuropeptide Y

O

O_2Hb: Oxygenated hemoglobin
OCD: Obsessive-compulsive disorder
OFC: Orbitofrontal cortex

P

PANAS: Positive and Negative Affect Schedule
PAS: Panic and Agoraphobia Scale
PD: Panic disorder
PET: Positron emission tomography
PFC: Prefrontal cortex
PSNS: Parasympathetic nervous system
PTSD: Posttraumatic stress disorder

R

ROI: Region of interest
RSA: Respiratory sinus arrhythmia
RT: Reaction time

rTMS: Repetitive transcranial magnetic stimulation

S

SAD: Social anxiety disorder
SCL: Skin conductance level
SCR: Skin conductance response
SDNN: Standard deviation of the average normal-to-normal heartbeat interval
SNP: Single nucleotid polymorphism
SSRI: Selective serotonin reuptake inhibitor
STAI: State-Trait Anxiety Inventory
STAXI: State-Trait Anger Expression Inventory

T

TBS: Theta burst stimulation
TMS: Transcranial magnetic stimulation

V

VLPFC/vlPFC: Ventrolateral prefrontal cortex
VMPFC/vmPFC: Ventromedial prefrontal cortex

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