FEAR NOT – NEUROBIOLOGICAL MECHANISMS OF FEAR AND ANXIETY

Floris Klumpers

Fear Not: Neurobiological Mechanisms of Fear and Anxiety
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Cover design & layout by Matthijs Ariëns
The red glow in the brain represents the upwelling emotion in response to threat, represented by the shocks which is also a reference to the shock paradigm used throughout this thesis. The blue glow represents the prefrontal ability to control the emotion.

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Neurobiologische mechanismen betrokken bij angst
(met een samenvatting in het Nederlands)

Proefschrift

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CHAPTER 1

GENERAL INTRODUCTION

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GENERAL INTRODUCTION

Let me start this introduction by conveying an event that happened to me recently while commuting to my new office in Nijmegen. Unexpectedly, the train traffic was halted on authority of the police. While waiting at the station to hear what was going on, I overheard two security officers receiving orders to start preparations for immediate evacuation of the station. The security officers rushed off, clearly shocked by this distressing message. One other passenger that also overheard the message approaches me, to confirm what he just heard. Primed by a previous incident that had occurred that day, a worst-case scenario crosses our minds. Earlier today, there was a terrible incident in the city of Liege, Belgium. Using grenades and a firearm, a man killed at least five people and injured more than 100 people in what appears to be a desperate, random act. Immediately I awake from the drowsy, slightly depressed state that had gotten over me after I foresaw that I would spend an indefinite part of my evening at the not very cheerful train station of Arnhem.

Together with my fellow passenger, I now start scanning the environment to see what could be the cause of the alarm. I notice an unusually high number of people in uniform on the platforms, that also look around them conspicuously. While I start thinking about the quickest exits, I feel my muscles tighten. My breathing gets shallow and my mouth feels dry. My heart rate rises. Suddenly, a man comes running towards me on the platform, and I freeze, trying to figure out his intention and the best way to react. Looking at me attentively, he passes and now I see that he's carrying a portable phone in his hand. "Security guy", I suppose. Now I notice another man standing with a portable phone on the other side of the platform, and approach him to ask what the situation is. He informs me and the other worried passenger that there was a suspicious man walking on the train tracks, but that they just arrested him. There is no evacuation. This information produces a nervous smile on our faces and we chat about how wide media coverage of terrible events in recent years have made us more responsive to these kind of situations. Sinking back into my usual state, I start to think about whether I should get dinner while I wait for the next train.

Emotions may be viewed as response tendencies that result from the appraisal of important events (Frijda 1986; Gross 1998). The situation described above illustrates the emotional reaction to threat commonly known as fear. Described as one of the most basic emotions, fear is an emotional state that is common to all of us, just like anger and pleasure (Ekman 1992). Emotional response tendencies are manifested on the experiential, the behavioural and the physiological level. Thus, my reaction to the apparent threat at the train station involved a subjective feeling of nervousness and heightened arousal. Also, it involved a behavioural tendency to avoid this situation, an increase in effort expended to detect possible dangers,

and more intense reactions to potentially threatening abrupt events (the man running towards me). Finally, a set of somatic symptoms characteristic to fear were evoked, including increases in muscle tension, changes in heart rate and blood pressure, and an upregulation of the availability of energy resources. Fear may be defined as the conglomerate of all these processes that are set in action when facing threat.

This thesis centres on the neurobiological regulation of emotional reactions to threat. Besides fear, "anxiety" is often used to describe these same processes. The term "anxiety" is typically reserved for situations in which there is more uncertainty about whether a threat is present. Anxiety therefore typically refers to a more long lasting state, which is not confined to one particular situation (Barlow 2000; Davis et al 2009). Stress is another concept closely related to fear. The stress response can be defined as an organism's general response to perceiving a challenge (Lazarus 1999) or relevant environmental or physical change (Herman and Cullinan 1997). A biologically prepared state of readiness is induced with increased arousal and sharpened attention towards salient events. Also, there is preparation for action – either flight or fight. When dealing with reactions to threat, emotions and stress often co-occur and are essentially inseparable.

Fear, anxiety and stress reactions generally facilitate adaptive coping with threats. However, disorders of anxiety are also the most common form of psychiatric disease. The anxiety disorders include generalized anxiety disorder, panic disorder, specific phobia, agoraphobia, social phobia, obsessive compulsive disorder and post-traumatic stress disorder (Diagnostic and statistical manual of mental disorders, 4th edition). Taken together as a group, the lifetime prevalence for anxiety disorders may be as high as 28% in western populations (Kessler et al 2005).

Basic mechanisms of emotion can be found back in all mammals, they are thought to rely on evolutionary older brain systems that lie deep in the brain (Maclean, 1973). The neurobiological mechanisms that underlie fear and anxiety have been extensively investigated in animal research, particularly in rodents (Davis 2006; LeDoux 2003). In the last decades, the development of research techniques for the measurement of the neurobiological background of fear, anxiety and other emotional processes have provided increasing opportunities to also assess human responding. The current thesis indeed comprises a wide variety of studies regarding the fundamental biological determinants of the *human* defensive system. Neuroimaging, genetic, pharmacological, brain stimulation and behavioural techniques were used in healthy subjects and specific patient populations to shed more light on the neuroanatomical and pharmacological systems that underlie fear and anxiety.

Besides investigating the pathways involved in the generation of reactions to threat, this thesis also describes work on the downregulation of fear. Remember that in the story described above, I quite rapidly switched from my alert state in which all other tasks were halted, to a more relaxed state in which other important matters (dinner) started receiving more attention. After a threat has passed, the emotional response should be terminated to reduce unnecessary energy consumption and negative side effects of prolonged stress. The most prominent theory on how control is exerted over negative emotions and stress is that cortical structures inhibit subcortical areas in the brain that are involved in the expression of emotion (Davidson et al 2000; Ochsner and Gross 2005a; Quirk and Beer 2006; Sotres-Bayon et al 2006). The subcortical area that receives most attention in these theories is the amygdala. On the other hand, the main centre of cognitive control in the brain is considered the prefrontal cortex.

In the next section, a broad background will be offered to the research data that are the core of this thesis. The goal is to formulate a more nuanced model that identifies the most important sources and targets of threat-related emotion regulation in the brain. This model will be based on the available evidence coming from a wide range of animal and human studies. First an overview of key neuroanatomical circuits that underlie the instantiation of fear and anxiety will be given. Next, evidence for the role of prefrontal cortical areas as source of the regulation of emotion is reviewed. The final part of this introduction will be centred on the question how brain areas discussed in the first 2 sections may interact in several forms of emotion regulation in healthy humans. This will require an overview of the anatomical knowledge of how these areas are interconnected. In the end, a framework will be proposed that gives an overview of the most important neurobiological pathways for the regulation of defensive responses.

THE NEUROBIOLOGY OF NEGATIVE EMOTIONS IN RESPONSE TO THREAT

The amygdala

The amygdala is part of a brain system that has been relatively stable throughout mammalian evolution (Maclean 1973; McDonald 1998). Consequently, animal studies with rodents and primates have been very informative in elucidating amygdala function. The first indications that the amygdala was implicated in emotion came from early lesion studies conducted with monkeys. Bilateral removal of the temporal lobe in these animals was found to result in profound changes in emotional behaviour (Brown and Schafer 1888; Kluver and Bucy 1939).

Later, it was found that more selective lesions of the amygdala were sufficient to cause similar effects on emotional behaviour, including a loss of emotional reactivity, increases in tameness and exploratory behaviour and a disruption of social behaviour (Aggleton and Young 2000; Weiskrantz 1956). More recent research has indicated that distinct cell groups within the amygdala make selective contributions to emotion.

Amygdala anatomy

The amygdala consists of several cell groups or nuclei that each have distinctive characteristics in terms of function and cellular anatomy. Studying amygdala anatomy in rats, cats and primates, Macdonald (1998) distinguished three common groups of nuclei that can be found in all three species. First, the basolateral nuclear group consists of the more laterally located nuclei and comprises the basal and lateral nuclei. Secondly, the centromedial nuclei are located on the dorsomedial side of the brain and consist of the central and medial nuclei and also include a more rostrally located nucleus: the bed nucleus of the stria terminalis (BNST or extended amygdala). Although there has been a debate about whether the BNST should really be considered part of the amygdala, it is similar to the centromedial nuclei in terms of function and cell type (McDonald 2003). Finally, the superficial cortex-like nuclear group consists of the nuclei that are located on the outside of the amygdala, next to the cortex. With respect to cell type, the majority of cells in the basolateral and cortex-like groups are glutamatergic pyramidal projection neurons, while the centromedial group consists mainly of spiny-like GABAergic neurons. As McDonald (1998) already noted, according to this framework there are some cell groups within the amygdala that are not placed in any group. Particularly noteworthy is the intercalated cell mass, a distributed group of GABAergic interneurons between the basolateral and central amygdala that inhibit the central amygdala (Grace 2006).

Within the amygdala, neural activity generally is transferred from lateral to medial areas. In fact, the majority of the connections between the basolateral and centromedial group are unidirectional, projecting from the basolateral nuclei to the centromedial nuclei (Pitkanen 2000). Connections from the mammalian amygdala with other brain areas can be grouped into three systems (Price 2003). First, particularly the lateral amygdala has bidirectional connections with the main sensory cortices and thalamus. Through these connections the amygdala receives multimodal sensory information. Second, the centromedial amygdala sends output to the hypothalamus and brain stem areas to modulate visceral function. Finally, a set of connections with forebrain areas are thought to be involved in the regulation of behaviour. These involve outputs to the ventral striatum, and thalamus and reciprocal connections with the hippocampus and prefrontal cortical areas, especially medial and orbital parts (Pitkanen

2000; Price 2003). In the following sections, it will become clear that all three systems play an important role in emotions in response to threat and their regulation.

Amygdala function in negative emotion: fear and aggression studies in animals

Most of the evidence for the functional specialisation of amygdaloid nuclei in emotion has centred on the contribution of the centromedial and basolateral amygdala in fear. Fear conditioning studies in rodents have made a substantial contribution in elucidating the roles of these different nuclei in emotion by studying the acquisition and expression of fear. In general, results indicate that the centromedial groups seem to be mainly involved with the actual triggering of physiological fear responses by its connections with subcortical and brain stem areas (Davis and Whalen 2001; Kalin et al 2004). The basolateral nuclei in turn, are involved in detecting emotional material that requires a reaction and are important for signalling emotion to areas involved in cognition like the PFC and hippocampus (Davis and Whalen 2001; LeDoux 2003; Price 1999). The initiation of emotion through the amygdala may go according to the following steps. First, sensory information about noteworthy events reaches the lateral amygdala. Then, when a threat is detected, the basolateral nuclei will activate the centromedial nuclei. Finally, the centromedial nuclei will subsequently trigger the appropriate physiological emotional responses by recruiting the hypothalamus and several brain stem areas (Davis and Whalen 2001; Rosen and Schulkin 1998).

While fear is one possible reaction to threat, defensive aggression is another. As for fear, the amygdala is thought to play a central role in this type of aggression by modulating activity in downstream areas in the hypothalamus and brain stem (Blair 2004; Gregg and Siegel 2001; Nelson and Trainor 2007; Panksepp 2003). A similar mechanism may operate in aggression as in fear in which the basolateral nuclei trigger activity in the centromedial nuclei which then send their output to the target areas in the hypothalamus and brain stem.

Specifying amygdala function: integrating human and primate lesion studies

One of the drawbacks of the older lesion studies described in the first paragraph of this section is that regions directly around the target site are also damaged in the procedure. Advances in neurotoxic lesion techniques have made it possible to lesion the amygdala without damaging the surrounding areas and the white matter bundles travelling through the amygdala (Amaral 2003). More recent studies with primates that have used these techniques have found more selective deficits in emotional behaviour than the early lesion studies did (Amaral 2003;

Machado and Bachevalier 2006; Meunier et al 1999). For example, lesions of the bilateral amygdala disrupt normal fear conditioning but do not block the expression of fear-potentiated startle (Antoniadis et al 2007; Antoniadis et al 2009), a reliable defensive reaction to threat (see Box 1). Studies on the effect of amygdala lesions in humans mostly involve patients who required surgical removal of the amygdala to treat intractable epilepsy or patients with a rare genetic defect that causes calcification of the temporal lobe and specifically the amygdala (lipioid proteinosis or Urbach-Wiethe disease). In interpreting findings from these studies, it has to be taken into account that in both groups normal amygdala functioning may be already disrupted at an early stage of development leading to compensatory plasticity and functional recovery. This may be why patients with selective damage to the amygdala in general do not seem greatly impaired emotionally (Aggleton and Young 2000). However, these patients may have difficulty in recognizing potential threats. For example, facial expressions that express negative emotions may be recognized less well with amygdala damage (Adolphs et al 1994; see Rosen and Donley 2006 for review). Moreover, there is converging evidence that these patients do not learn to show fear responses when repeatedly confronted with a certain stimulus that predicts threat. Several studies have now shown that these patients show reduced acquisition of fear responses in classical fear conditioning paradigms (Bechara et al 1995; LaBar et al 1995; Weike et al 2005; see Box 2). Finally, there have been reports that these patients express less fear. For example, (case) studies have found indications for a lack of fear-potentiated startle (Angrilli et al 1996; Buchanan et al 2004; Funayama et al 2001), electrodermal activity (Asahina et al 2003) and subjective fear expression (Dellacherie et al 2011; Feinstein et al 2011).

Insights from neuroimaging

Neuroimaging studies in healthy subjects and psychiatric patients provide further evidence for the role of the amygdala in emotional processing. The amygdala has been widely found to respond to aversive stimuli, pictures of (emotional) faces and emotional states (Davis and Whalen 2001; Zald 2003). Meta-analyses of emotional processing studies in PET and fMRI confirmed the role of the amygdala in negative emotion and particularly pointed toward an important role of the amygdala in the detection of emotionally significant external stimuli (Costafreda et al 2008; Phan et al 2002). Further evidence comes from findings that increased amygdala activation corresponds to more intense emotional experience (Rosen and Donley 2006). Consistent with a role for the amygdala in initiating the bodily responses that occur during emotions, amygdala activity has been shown to be restricted to situations in which perceived threat also triggers increases in autonomic activity (Cheng et al 2006). Interestingly, amygdala activation may be linked to initiation of an emotional response but not the continuing

BOX 1. Fear-potentiated startle and other methods for indexing fear and anxiety

Fear potentiated startle (FPS) refers to the reliable increase in startle reflex intensity due to an organism's defensive state. To induce startle reflexes investigators typically present brief, loud noises during their experiment. Startle magnitude in humans is then assessed by measurement of the eye-blink component of the startle reflex, which consists of a rapid contraction of the orbicularis oculi muscle. This contraction is measured through electromyographic recording from electrodes that are placed over the orbicularis oculi, just below the eye. In animals, startle responses are usually assessed by measuring the displacement of their housing as a result of the whole-body startle. Threat induces a defensive state which is reflected in an increase in protective startle magnitude across species (Davis et al 1993; Grillon and Baas 2003; Winslow et al 2002). The neuroanatomical pathways for startle potentiation have been investigated extensively in animals and involve interactions between the amygdala and brainstem (Davis 2006; Davis and Whalen 2001). Because positive stimuli can lead to an attenuation of the startle reflex (Lang et al 1990; Vrana et al 1988), the magnitude of startle reflexes are not simply related to increases in arousal or attention (Böcker et al 2004). Moreover, modulation of startle reflexes reflects basic defensive processing and can be observed independently of subjects' conscious awareness of threat (Hamm and Vaitl 1996; Hamm and Weike 2005; Weike et al 2005). These factors set FPS apart from autonomic measures of fear such as modulation of skin conductance which typically mirrors subjective awareness and should be considered a more general measure of arousal because it is valence unspecific (Hamm and Weike 2005; Lang et al 1990). FPS was used to quantify defensive responding in chapters 2-8 of this thesis. Subjective ratings of state anxiety are also included in each chapter, but it has to be kept in mind that these ratings are liable to response bias (e.g. subjects may give socially desirable ratings and rate according to what they think is expected). Moreover, ratings that are acquired in retrospect could be less reliable due to memory inaccuracies. On the other hand, when subjects are required to rate their anxiety "online", that is on a trial-by-trial basis, this causes subjects to constantly label their emotional state during the task. This constant rating affects the emotional response (Coppens et al 2009; Lieberman et al 2007). Thus, we chose to use retrospective ratings, while keeping in mind their limitations.

subjective experience (Garrett and Maddock 2006; Marschner et al 2008; Phelps et al 2001). This is in line with results showing that amygdala activity rapidly habituates during repetitive stimulation while the emotional experience remains (Rosen and Donley 2006; Zald 2003). Another apparent dissociation between amygdala activation and emotional experience can be observed in instructed threat paradigms (see Box 2). When cues are presented that were previously identified by verbal instruction to signal threat of electric shock, the resulting state of defence is typically not accompanied by strong amygdala activation (Chua et al 1999;

BOX 2. Methods of fear induction: fear conditioning versus instructed fear

One of the most common approaches to study the biological mechanisms of fear and anxiety has been the use of fear conditioning (FC) paradigms. In FC, subjects are presented with a stimulus (the conditioned stimulus or CS) that is coupled to an aversive event that naturally elicits a defensive response (the unconditioned stimulus or UCS). Usually, the coupling of these two stimuli is achieved by always presenting the unconditioned stimulus during presentation of the conditioned stimulus. After a number of pairings, animal or human subjects learn that the CS is predictive of the US and this results in a conditioned defensive response to the CS. Subjects or animals learn that the CS is in fact a cue for danger that requires the appropriate preparations to cope with that danger. Besides the advantage that this procedure can be applied in a similar manner across species, another potential strength of FC is that it presumably models how anxiety symptoms can be acquired through experience (Mineka and Oehlberg 2008). FC is therefore an important model to study the acquisition of fear memories that may lead to psychological pathology (Lissek et al 2005). The fear conditioning approach is adopted in chapters 5 and 7 of this thesis.

While FC continues to be a successful paradigm to study fear learning, it is also a relatively complex approach to study fear. Factors such as the exact instruction given beforehand, the number of trials given to learn the association and the rate at which the conditioned stimuli are reinforced all affect the learning outcome. Moreover, there are profound individual differences in how fast subjects learn the contingencies between the threatening and neutral stimuli (Baas et al 2008). The instructed fear paradigm is a practical alternative if not these learning processes but the basic expression of fear or anxiety in response to threat stimuli is the primary aim of the study.

In this paradigm, subjects are explicitly instructed before the start of the task about the contingencies between the cues and the unconditioned stimulus. This removes variance between subjects in the learning phase by immediately making all subjects aware of which cues signal threat and safety. Moreover, UCS presentation is not required for fear expression. Because in this case there is no strong learning component to be expected during the experiment, the procedure can in principle be repeated within subjects on multiple occasions. This may be an important practical advantage for certain studies, for example pharmacological studies in which effects of a drug can be compared to placebo by administering each on a separate occasion in the same subjects. Nevertheless, one can imagine that habituation or other learning processes could have an impact on measurements when repeating very similar measurements. The question whether repeating the instructed paradigm results in stable measurements of fear is tested in chapter 6 and the instructed fear paradigm is used in the studies described in chapters 2-4 and 8 of this thesis.

Mechias et al 2010; Phelps et al 2001). Taken together, neuroimaging studies in healthy subjects indicate that the amygdala plays an important role in learning to predict threat and the initial start of the emotional response in response to external stimuli. Consequently, increased amygdala activation may be expected to be involved in psychiatric disorders that involve excessive emotional reactions. Indeed, patients with anxiety disorders show exaggerated amygdala responses. Most consistent findings are in phobia and post-traumatic stress disorder (Etkin and Wager 2007; Rauch et al 2003; Shin and Liberzon 2009), disorders that are characterized by excessive fear reactions to the perception of specific emotionally laden stimuli, in contrast to more sustained anxiety states as observed in generalized anxiety and obsessive compulsive disorder.

Characterisation of the role of the amygdala in emotion

So how is the function of the amygdala best characterized? Amaral (2003) concluded from his primate studies that the amygdala is involved in evaluating the environment for potential danger. This conclusion is supported by human work of Öhman and colleagues (2005). Behavioural and imaging studies by this group show that the amygdala is involved in the automatic detection of threat, relatively independent of consciousness. The proposed function of a threat detector is consistent with the fact that the amygdala responds to a wide range of aversive stimuli. Reactions to these stimuli can range from anxiety and disgust to a general state of disliking (Zald 2003). Davis and Whalen (2001) have also proposed a more general role for the amygdala in emotion. Reviewing evidence from a wide range of animal and human studies, they stress the role of the amygdala in producing and maintaining vigilance in response to stimuli and events that are perceived as important to the organism. These more broad definitions seem to fit the available evidence on the role of the amygdala in emotion best (Costafreda et al 2008). Importantly however, as the amygdala seems to be mainly involved in appraisal of external emotional stimuli and the following initiation of the emotional response, expression of negative emotional responses is not completely dependent on only the amygdala. Below other structures are discussed that are key areas for the expression of threat-related emotional responses.

Cortical correlates of anxiety: the anterior insula and anterior cingulate

While the amygdala is a crucial hub in the detection of external threats, the perception of the aversive internal states that may follow is another important part of negative emotions and stress. There is now abundant evidence that the anterior insula plays an important role in interoception and affective awareness (for review see Craig 2009; Kurth et al 2010; Sterzer

and Kleinschmidt 2010). Given the consistency of anterior insular activation across conditions in a range of paradigms, the insula is assumed to play a role in the perception of demanding or salient situations and in the regulation of the arousal (Craig 2009; Kurth et al 2010; Sterzer and Kleinschmidt 2010). In line with this, neuroimaging studies consistently find activation in the anterior insula during the presentation of fear eliciting cues (Drabant et al 2011; Mechias et al 2010) and arousing emotional pictures (Phan et al 2002). Importantly, the anterior insula is also consistently more reactive in anxiety disordered and anxiety prone subjects (Etkin and Wager 2007; Simmons et al 2006; Simmons et al 2010; Stein et al 2007; Straube et al 2007). This suggests that individual differences in anxiety are also associated with different levels of anterior insula activation. Of note, especially the anterior part of the insula has strong connections with the amygdala (Hoistad and Barbas 2008). The insula is thought to supply cortical somatosensory information to the amygdala but connections are bidirectional and thus the insula is also in the position to receive information about potential threat from the amygdala. Given this bidirectionality, the anterior insula may be a core hub in the brain mechanisms of fear and anxiety. In line with this, the preparedness for possible adversity that is central to anxiety (Barlow 2000) has been suggested to be driven by hyperarousal mediated by the insula (Paulus and Stein 2006).

The anterior insula seems to be part of a broader salience processing network (Seeley et al 2007) that also involves the thalamus and dorsal anterior cingulate cortex (dACC). Co-activation of the anterior insula and dACC across a range of neuroimaging studies (Craig 2009; Seeley et al 2007) suggests that these areas are closely connected in function. Activation of the dACC and anterior insula has been linked to increases in arousal, as indexed by autonomic measures such as skin conductance and heart rate (reviewed in Critchley 2005). Just like the insula, the dACC is consistently found to be activated during the presentation of fear cues (Etkin et al 2011). Moreover, the dACC also shares significant connections with the amygdala across species (McDonald 1998). Because there is also a wealth of animal lesion and stimulation data that suggest involvement of animal analogue of the dACC in fear states (e.g. see Corcoran and Quirk 2007; Vidal-Gonzalez et al 2006), the dACC is hypothesized to play a crucial role in fear expression (Etkin et al 2011; Milad et al 2007a).

Lower level brain mechanisms of negative emotions and stress: the hypothalamus and brain stem nuclei

The amygdala is dependent on several relay stations in lower parts of the brain to cause its effect on behaviour. As we have seen, the output of the amygdala through centromedial nuclei to the hypothalamus and brain stem neurons are thought to play an important role in the

initiation of emotional responses in the body. The hypothalamus is a set of nuclei located just ventral to the thalamus. It is at the head of the hypothalamo-pituitary-adrenocortical (HPA) axis, an important system in the neurocircuitry of stress responses (Herman and Cullinan 1997). Activation of the paraventricular nucleus (PVN) of the hypothalamus leads to secretion of corticotrophin releasing hormone (CRH), in turn leading to release of adrenocorticotropic hormone (ACTH) from the pituitary, which in turn leads to release of glucocorticoids from the adrenal cortex. This then leads to a variety of bodily processes that are adaptive to the organism in times of danger, e.g. an upregulation of glucose production to increase energy available for fight or flight. Next to its role in activating the HPA-axis, the amygdala is also part of a feed forward system that increases ongoing stress responses by its glucocorticoids receptors and connections to the HPA axis (Herman et al 2005). Conversely, activation of glucocorticoids receptors in the hypothalamus, pituitary, hippocampus and prefrontal cortex leads to downregulation of the stress response through a negative feedback loop.

While the hypothalamus is to some extent controlled by the amygdala, both the hypothalamus and amygdala are thought to control activation of the affective centres in the lowest parts of the brain. One of these lower level systems is the set of neuromodulatory projections that arise from monoamine brain stem and midbrain nuclei. Serotonergic, noradrenergic and dopaminergic neurons located in the midbrain exert a modulatory influence over behaviour by adjusting neurotransmission levels upstream (LeDoux 2002; Tucker et al 2000) (see Box 3). Furthermore, both hypothalamus and amygdala project to brain stem areas that permit fast bodily reactions in times of stress by directly innervating the viscera: the autonomic nervous system. For example, the PVN is linked to autonomic output nuclei in the brain stem that play a role in the stress response (Buijs and van Eden 2000). The autonomic nervous system can induce rapid changes in terms of blood pressure, heart rate, transpiration and many other bodily processes associated with negative emotions through descending connections with peripheral muscles and organs. In addition, it can control relatively simple motor behaviour associated with emotion, for example reflex-like eye movements and emotional facial expressions. A group of neurons in the brain stem termed the periaqueductal gray (PAG) is thought to play a role in the integration and coordination of the brain stem nuclei at the lowest level (Bandler and Shipley 1994; Tucker et al 2000). Moreover, in fear and stress this brain area has a critical role in the initiation of fast emotional responses (McNaughton and Corr 2004; Mobbs et al 2010). While the amygdala and hypothalamus are also thought to be involved in higher level orchestration of the stress response (Buijs and van Eden 2000), the PAG is mainly involved in the direct instantiation of the emotional reactions. In addition, it is a key relay station for the descending pathways involved in the central modulation of pain

related activity in the spinal cord (Benedetti et al 2005; Colloca and Benedetti 2005). In this way, it further prepares the body for situations that require resistance.

BOX 3. Key neurotransmitters and drugs in anxiety

Adaptive reactions to threats are dependent on multiple neurotransmitter systems. One important clue regarding the neurotransmitter systems that most principally underlie anxiety can be derived from the substances that are most efficacious in the treatment of anxiety disorders. Guidelines for pharmacotherapy consistently subscribe to the use of selective serotonin reuptake inhibitors (SSRI's) as the primary drug of choice across a range of different anxiety disorders (Baldwin et al 2005; Bandelow et al 2002; Dutch Multidisciplinary Directive Anxiety Disorders). For several anxiety disorders, other forms of medication, including tricyclic anti-depressants and benzodiazepines, can be equally effective but have a more aversive side effect profile. Pharmacological blockage of serotonin reuptake by the serotonin transporter (SERT) into presynaptic neurons by SSRIs therefore generally appears the most efficient pharmacotherapy for anxiety. Originating from the raphe nuclei in the brainstem, the serotonergic neurotransmitter system is therefore hypothesized to be a key modulator of anxiety. In recent years, this hypothesis has been supported by work on the genetics of the SERT. Common genetic variation in the SERT has been linked to trait anxiety levels in the general population and has been found to be a risk factor for stress-related disease. The neural and somatic consequences of inter-individual variance in SERT gene function are further investigated in **chapters 3 and 4** of this thesis.

Besides serotonin, the major inhibitory neurotransmitter gamma-aminobutyric acid (GABA) is another crucial moderator of anxiety. Substances that work on the GABAergic system, such as the benzodiazepines and alcohol, are known for their anxiolytic effects. Particularly binding to the GABA(A) receptor alpha 2 and 3 subunits is thought to mediate anxiolysis (Heldt and Ressler; McKernan et al 2000; Rowlett et al 2005).

Besides these traditional neurotransmitter systems, exciting new findings suggest that the anxiolytic drugs of the future could work by time-limited targeting of fear learning processes. The NMDA agonist D-cycloserine and cannabinoid substances are among the promising candidate drugs that could be used to improve fear extinction (see box 4). Moreover, modulation of noradrenergic activity by administration of the beta-blocker propanolol has been shown to disrupt consolidation of conditioned fear memories (Kindt et al 2009; Muravieva and Alberini 2010).

Summary and conclusion

The amygdala is heavily involved in initiating fear responses. A large body of evidence now points to the amygdala as a key structure for the detection of stimuli and events that are important to us. Depending on the context, emotional responses can involve fear, stress, aggression and other phenomena related to negative emotion. The basolateral nuclei, by input from sensory areas, play an important role in determining whether stimuli are important and can then, by activation of the centromedial nuclei, initiate the bodily responses that make the organism attend closely to this stimulus and act on it when needed. However, basic expression of negative emotion is not solely dependent on the amygdala. Human imaging data support a crucial role for the anterior insula and dorsal ACC in the expression and regulation of fear and anxiety. Moreover, the lower level output areas of the amygdala that are involved in the instantiation of bodily reactions (the HPA axis and brain stem nuclei) are not necessarily dependent on the amygdala for activation. Regulation of negative emotion in the brain could therefore occur by shifts in activity in each of these regions. As we will see in the next chapter, the prefrontal cortex plays a role in the regulation of the systems described above.

PREFRONTAL REGULATION OF FEAR AND ANXIETY: THE ROLE OF THE PREFRONTAL CORTEX IN INHIBITING THREAT-RELATED EMOTIONS

At some point reactions to threat should stop and the body must return to a more relaxed state. Inhibition of threat-related emotions can refer to a broad collection of phenomena that can be categorized in different ways. One dimension in which actions of emotion regulation differ is the extent to which they rely on diverting attention away from the emotion inducing event or stimulus (Ochsner and Gross 2005b). More specifically, the instructions for subjects in emotion regulation experiments can range from selectively ignoring emotion inducing stimuli to attending these stimuli but trying to change their meaning through cognitive strategies. Attentional control over emotional processes is outside the scope of this introduction but interested readers are referred to reviews by Pessoa et al. (2002) and Ochsner & Gross (2005b).

Extinction is a form of stimulus-response reversal learning in which animals inhibit a previously learned (emotional) response that is no longer adaptive. The extinction of conditioned fear is a well validated model for the regulation of negative emotions. Below, an overview of fear extinction studies in rodents and humans will give insight into how emotion regulation (in this basic form) requires adequate prefrontal cortex (PFC) function. After this, cognitive (re)

appraisal studies will be discussed that have used neuroimaging techniques to investigate how this more complex form of emotion regulation is instantiated in the human PFC. These experiments typically involve regulating reactions to aversive pictures or (the anticipation of) pain stimuli. As emotion regulation involves regulation of the bodily processes that accompany emotions, the role of the PFC in the control of the neuroendocrine and autonomic changes will also be investigated. Finally, findings on the role of the PFC in emotion regulation will be integrated with our current knowledge of patients with deficits in emotion regulation.

Extinction of fear as a model to study emotional regulation

Fear extinction provides researchers with a relatively simple model to study how previously acquired fear can be inhibited in both animals and humans. The process of fear extinction allows the reduction of previously learned emotional responses that are no longer adaptive. In this way, this process is important for regulating emotional associations that were acquired in the past. It can be considered a more gradual form of emotion regulation, in which emotion is reduced step-by-step by learning from previous experience. In fear extinction, after acquisition of a conditioned fear response, the conditioned stimulus is repeatedly presented without the reinforcing unconditioned stimulus. In this way, the organism learns that the CS (e.g. a tone) no longer predicts the aversive stimulus (e.g. an electric shock) and the fear response to the tone gradually diminishes over time. Recently, the extinction process has received a great deal of attention and this has resulted in an enormous increase in the understanding of the neurobiology of extinction (see Myers and Davis 2007 for review). Excitingly, this has opened up possibilities to pharmacologically improve extinction (see box 4).

It is now widely accepted that fear extinction cannot be completely explained in terms of erasure of a no longer adaptive memory trace because the original conditioned response can return and cause relapse (Milad et al 2006; Myers and Davis 2007). Therefore extinction must involve learning a new association, which then competes with the old association for control over behaviour. Interestingly, this implies that there must be structures that through their activation inhibit expression of fear. For this reason, extinction of fear is an interesting paradigm to investigate the neural pathways involved in the inhibition of fear.

Fear conditioning studies with rodents have pointed to an important role for the rodent PFC in extinction. First evidence came from lesion studies, in which large lesions of medial areas of the prefrontal cortex in rats led to a resistance to fear extinction (Morgan et al 1993). Later studies employed more selective lesions and found that lesions of the ventromedial prefrontal cortex (vmPFC), in the infralimbic area (IL), were sufficient to cause deficits in fear extinction (Sotres-Bayon et al 2006). Currently, the role of the vmPFC in extinction has

been supported by many studies with different experimental methods. These include single unit recording, metabolic mapping and molecular studies (Milad et al 2006; Quirk and Beer 2006). Interestingly, increasing activity of the vmPFC has been shown to improve extinction recall, i.e. rodents show less fear on a retention test after the extinction session when during extinction the vmPFC is stimulated (Quirk et al., 2006). This is direct evidence for the role

BOX 4. The pharmacology of fear extinction

Fear extinction can be used to study how previously acquired fear memories can be suppressed by subsequent experience indicating danger has subsided. A similar procedure is used in exposure-based psychotherapy, one of the most effective treatments applied for various anxiety disorders. After preclinical findings in animal fear conditioning suggesting that the partial NMDA-agonist d-cycloserine improved extinction of conditioned fear (Walker et al 2002), recent studies have indicated that this drug improves efficacy of exposure based psychotherapy in human anxiety disorder patients (Norberg et al 2008). This illustrates the potential predictive validity of the conditioning paradigm in modeling anxious behavior in patients. Research in animals has suggested that facilitation of the cannabinoid receptor 1 (CB1) system is another promising avenue to facilitate fear extinction (Chhatwal et al 2005; Marsicano et al 2002; Pamplona et al 2006). In **chapter 5** of this thesis, we investigated whether activation of CB1 receptors through administration of Tetra-Hydro-Cannabinol, the psychoactive constituent of cannabis, would facilitate human fear extinction. Besides the cannabinoid and NMDA pathways, a variety of other drugs have been studied (Myers & Davis 2007). Recently, cortisol was added to the list of promising agents after it had been shown to increase efficacy of exposure therapy for acrophobia (de Quervain 2010).

of this area in the learned inhibition of fear. Nevertheless, it has to be noted here that there are also studies in which no effects of vmPFC lesions (in the orbitfrontal and medial PFC) on extinction were found and that some studies point to involvement of additional, more dorsal medial prefrontal areas (Barrett et al 2003; Gewirtz et al 1997; Morgan and LeDoux 1995).

In humans, studies that have investigated the involvement of the PFC in extinction using fMRI have supported the animal data. Also in humans there is evidence for involvement of the vmPFC, which can be defined as the orbitofrtonal cortex and the more caudal medial prefrontal areas, in particular the ventral ACC. Phelps et al. (2004) showed that during acquisition there is deactivation in the PFC in response to stimuli that were previously coupled to a shock (CS+) compared to stimuli that were not coupled to shock (CS-). This can be interpreted as a cessation of PFC inhibition of fear when a threat is detected. In accordance with the results from animal studies, this was in medial prefrontal areas (BA 10, 24, 25 & 32). Furthermore,

extinction success during a subsequent extinction training phase was positively correlated to subgenual anterior cingulate (BA25) activation during an early phase of a recall session the day after. Gottfried & Dolan (2004) similarly found changes in prefrontal cortex activation during acquisition and extinction of conditioned fear although in more ventral areas (around BA 14). Furthermore, the authors compared activity during acquisition and extinction and reported activity in the medial OFC (BA 11, 12) that was specifically related to extinction. Using a similar analysis, Molchan et al. (1994) showed extinction-related activity in ventrolateral areas of the PFC (BA 44, 45). In recent fMRI work by Milad et al., the involvement of the ventromedial PFC (BA 25,32) in extinction learning and recall was confirmed (Milad et al 2007b) and evidence was presented for reduced recruitment of the vmPFC during extinction recall in PTSD (Milad et al 2009), while structural MRI analysis also implicated these areas are important for extinction (Milad et al 2005). Taken together, extinction research in both humans and animals has provided evidence for a role of the ventral and medial PFC in the regulation of negative emotions.

Reappraisal of emotional picture stimuli

Compared to extinction learning, a more direct way in humans to investigate the brain substrates of negative emotion suppression is to ask subjects to engage in emotion regulation while in an fMRI or PET scanner. In order to control some of the inherent inter-individual variation in strategies used for emotion regulation, subjects are often explicitly instructed and trained to use reappraisal strategies. However, reappraisal can involve different strategies itself. The two most commonly used strategies to suppress negative emotions are (1) reinterpreting a negative situation in a more positive way and (2) becoming a "detached observer" to reduce emotional impact. To induce negative emotion, most studies present subjects with negative pictures and as a control condition subjects just view or attend these stimuli. As can be expected, during the "attend" condition brain areas implicated in emotional expression are found to be active. Frequently found areas are temporal lobe structures, such as the amygdala (e.g. Goldin et al., 2008 ; Beauregard et al., 2006; Eippert et al., 2006; Ohira et al., 2006 ; Urry et al., 2006 ; Phan et al., 2005; Ochsner et al., 2004; Levesque et al., 2003; Ochsner et al., 2002), the insula (e.g. Goldin et al., 2008 ; Beauregard et al., 2006; Levesque et al., 2003 ; Ochsner et al., 2002) and the temporal pole (e.g Ochsner et al., 2002; Ohira et al., 2006; Levesque et al., 2003). Conversely, during the "reappraise" condition prefrontal areas are recruited. As can be expected in such a complex task as reappraising emotional pictures, a broad array of PFC areas have been linked to reappraisal. Most frequently reported however, are orbitofrontal (BA 11, 12, 13, 47; Beauregard et al., 2006; Eippert et al., 2006; Ohira et al., 2006; Urry et al., 2006; Phan et al., 2005; Ochsner et al., 2004; Levesque et al., 2003) and

medial prefrontal (BA 10, 24, 25, 32) areas (Beauregard et al., 2006; Eippert et al., 2006; Urry et al., 2006; Kalisch et al., 2005; Phan et al., 2005; Ochsner et al., 2004). More dorsal lateral FPC areas have also repeatedly been found in reappraisal studies (Eippert et al., 2006; Phan et al., 2005; Ochsner, 2004; Ochsner et al., 2002) and have been suggested to reflect general cognitive processing that is needed to carry out this task. In sum, findings from reappraisal studies confirm that both lateral and medial prefrontal structures seem to be involved in decreasing negative affect (Kalisch 2009; Ochsner and Gross 2008).

Autonomic and neuroendocrine control

Since the peripheral, bodily processes that occur during emotions can be considered core features of the emotional experience, direct or indirect control of these processes by the prefrontal cortex would be a necessary condition for the prefrontal cortex to be involved in emotion regulation.

In rodents, especially the medial area of the PFC has been implicated in the control of autonomic and endocrine changes in response to stress (Cechetto and Saper 1990; Sullivan 2004). More specifically, lesions of the medial PFC (IL, the more dorsal prelimbic cortex (PL) and anterior cingulate) have been reported to result in increased HPA responses to stress, i.e. increased ACTH and corticosterone plasma levels (Diorio et al 1993). Stimulation, ablation and physiological studies have also shown the importance of the mPFC in responses that are controlled by the autonomic nervous system such as heart rate and blood pressure (Cechetto and Saper 1990; Sullivan 2004). Finally, mPFC lesions result in a variety of effects on stress related behaviour in rodents (Sullivan, 2004). Reviewing these results, although the areas most consistently found to relate to autonomic and endocrine reactions are the more ventral regions (PL and IL), it is difficult to extract which areas of the mPFC are most important. Furthermore, there have been studies that find no effect of mPFC lesions on responses to stressors (Crane et al 2003; Diorio et al 1993) or even decreased responses after mPFC lesions (Morgan and LeDoux 1995; Sullivan and Gratton 1999).

In humans, stimulation of the ACC has been shown to stimulate the electrodermal response (SCR) (Mangina and Beuzeron-Mangina 1996). Furthermore, in neuroimaging experiments a variety of other autonomic measures has been related to activity in the ACC (BA 24) including pupillary, cardiovascular and electrodermal measures (Critchley 2005). Other prefrontal areas that have been related to autonomic (SCR) activity are ventromedial (BA 10, 32) and orbitofrontal areas (BA 11, 47) (Critchley 2005; Patterson et al 2002; Williams et al 2000). Finally, the vmPFC has also been linked to the control of heart rate by the vagal nerve (Wong

et al 2007). Thus, ventral PFC areas and the ACC may play an important role in the control of bodily emotional responses. Intriguingly, the available evidence on autonomic and endocrine control by the PFC points to the same medial areas of the PFC that were found in extinction studies.

Neurological and psychiatric patient studies and emotion regulation

Evidence from neurological patients also points to involvement of the vmPFC in emotion regulation. One of the typical symptoms after PFC damage is a tendency to act disinhibited. Patients with prefrontal lesions may act out their impulses, regardless of whether they are appropriate or advantageous in the future. Specifically, damage to the vmPFC is linked to abnormalities in emotional and social conduct. This can lead to increased irritability and aggression (Grafman et al 1996), irrational decision making (Bechara 2004; Koenigs and Tranel 2007) and inappropriate or blunted affect (Anderson et al 2006; Barrash et al 2000) with a sparing of basic intellectual capabilities. This is in contrast with patients that have lesions to more dorsal areas of the PFC, who are more cognitively impaired (Fuster 2001). On the basis of these findings, several theorists have tried to characterise the role of the vmPFC in social and emotional behaviour. There have been several suggestions about the underlying processes that are damaged in these patients. For example, the vmPFC may play a role in coping with changing stimulus-reinforcement associations. Specifically, the orbitofrontal cortex (OFC) is thought to play an important role in this due to its connections with sensory areas (Rolls 2004). The OFC seems to be able to signal and store contingencies between different stimuli and in that way can steer behaviour to advantageous choices. Note that this is related to functions of the amygdala. Indeed, the amygdala and OFC receive very similar information and are strongly interconnected (Barbas 2000). However, the fact that OFC damage results in more severe effects on behaviour may indicate that the OFC is in a hierarchically higher position than the amygdala, providing the organism with increased flexibility to learn important associations faster (Rolls 2004).

LeDoux and colleagues have conceptualized the symptoms after vmPFC lesions in humans and animals as “emotional perseveration” (Sotres-Bayon et al 2006). Perseveration refers to a failure to change behaviour in response to changing circumstances resulting in repetition of no longer adaptive behaviour. Moreover, vmPFC damage would leave the organism without updates on emotionally significant information, leading to a perseveration in responses that are no longer adaptive when the emotional information changes. In essence, this is the same as a deficit in emotional extinction and these theories are therefore consistent with the fact that

the vmPFC plays an important role in the extinction of fear. Interestingly, patients with damage to the vmPFC have also been reported to show a blunted emotional response as measured by autonomic measures (Tranel 2000). This finding has lead to Damasio's somatic marker theory in which the medial part of the OFC contributes to emotional decision making by providing information about lower level bodily reactions to different response options (Damasio 1995). By reinstatement of the bodily consequences of earlier situations that were similar, the OFC can provide rapid information about the possible consequences of specific choices. In this way it facilitates decision making. Integrating these different theories on vmPFC function, the vmPFC may thus function as an interface between typical cognitive functions localised in the dorsal and rostral PFC areas and subcortical areas that are important for emotion. Therefore, the vmPFC is bound to play an important role in the regulation of emotional processes by the PFC.

A final line of evidence that implicates the PFC in emotional processing comes from psychiatric patients who show impaired fear regulation. For example, patients with Post Traumatic Stress Disorder (PTSD) suffer from recurrent, uncontrollable attacks of anxiety in response to non threatening stimuli and situations after experiencing a traumatic event (e.g. fighting in war or being the victim of rape). Because these associations between non-harmful stimuli and the traumatic event can persist long after the traumatic episode, it has been suggested that these patients suffer from a deficit in extinction (Milad et al 2006). In accordance with this, these patients show consistent deficits in ventromedial prefrontal regions when investigated with neuroimaging. In general, patients demonstrate decreased activity in the vmPFC compared to controls (Milad et al 2006; Phan et al 2006). Similarly, decreased activity in vmPFC has been related to emotion regulation defects in other affective disorders like depression (Drevets 2007), bipolar disorder (Green et al 2007) and in violent offenders (Soderstrom et al 2000). This further adds to the hypothesis that certain areas in the PFC inhibit the areas related to emotional expression to control emotion.

Summary and conclusion

The PFC provides us with the flexibility to cope with changing circumstances and serves as a point of integration for emotional and cognitive information. In rodents, specifically mPFC areas (IL and PL) have been implicated in the regulation of defensive emotions. In humans, the most consistently found areas are OFC (BA 11, 12, 13, 14, 47) and mPFC (BA 10, 25, 32) and in more complex reappraisal also more lateral lateral PFC. Some of these areas that have been implicated may be involved in the detection of bodily changes, others more in the control of these changes.

WORKING MECHANISMS FOR EMOTION REGULATION IN THE BRAIN: CORTICO-SUBCORTICAL INTERACTIONS

Inhibiting negative emotions logically involves inhibition of the areas that mediate the expression of negative emotions (outlined above). Specifically, PFC inhibition of the amygdala has been put forward by several authors as a key mechanism for emotion regulation (Davidson et al 2000; Quirk and Beer 2006). However, as noted above the role of the amygdala in defensive emotion may be limited to the perception of salient events and the initial response orchestration. Many other areas are involved in more prolonged anxiety states. Inhibition of these other areas involved in the expression of defensive emotions may be of similar importance. Below, relevant anatomical connections will be discussed. Next, evidence for functional interactions between prefrontal and limbic areas in emotion regulation will be outlined. Although recent functional imaging studies have provided some insight into these interactions, more direct evidence comes from rodent and primate studies. Along the way, several previously proposed models of cortico-limbic interactions in the regulation of fear, stress and aggression will be discussed. Finally, a model will be proposed that integrates the available evidence to highlight pathways for the regulation of negative emotion in the human brain.

Cortico-subcortical connections for the regulation of negative emotion

Connections from the PFC to areas important for emotion are essential for emotion modulation by the PFC. Anatomical studies conducted in primates can shed light on the PFC areas that are involved in the regulation of negative emotions in humans. The main connections between each of the three main functional areas of the primate PFC and the main areas involved in the expression of emotion are summarized in Figure 1. The mPFC and OFC have direct connections to areas involved in negative emotion and are therefore able to modulate the expression of negative emotions. Anatomically closest to the limbic system, the caudal regions of these areas have been termed “limbic cortex” on the basis of their cellular architecture and close connections with subcortical structures (Barbas 2000; Heimer and Van Hoesen 2006). Conversely, the more rostral PFC areas and dlPFC are not in the most suitable position for regulation of activity related to negative emotions. Although the mPFC and OFC are similar in their connections, there are also some differences. For example, there are stronger connections between the mPFC and the hypothalamus and PAG (Barbas and Zikopoulos 2006). Notably, the mPFC and OFC do not always have direct connections with the output

regions within emotion areas (i.e. the PVN of the hypothalamus and the CE of the amygdala). Instead, they synapse to inhibitory interneurons in other regions nearby (Herman et al 2005). This increased complexity in connections provides the system with more flexibility.

Functional connectivity analyses in neuroimaging studies with humans

There is a growing tendency for neuroimaging studies to focus on interactions between different brain areas instead of looking at isolated activity in a predefined region. Usually, this entails performing correlational analyses in which the changes in BOLD signal (in fMRI) or regional cerebral blood flow (rCBF; in PET) in different brain areas are compared to reveal functional connectivity. Several of the neuroimaging studies on emotion regulation that were discussed in the previous chapter also performed these analyses. Generally, they have focussed on correlations between the PFC and the amygdala. For example, Ochsner et al. (2002) found that activity in a functionally defined region of interest in the vlPFC (BA 46/10) was inversely related to emotion related activity in the amygdala and (to a lesserextent) in the mOFC. In another study, a similar relation was reported between the amygdala and a slightly more ventral PFC area (BA 47; Phan et al 2005). Moreover, Urry et al. (2006) found that those subjects who were able to reduce amygdala activation while suppressing their emotion showed more activation in the vmPFC (BA 11, 32). In addition, the efficacy of functional connectivity between amygdala and mPFC has been directly related to trait anxiety, with decreased connectivity leading to higher anxiety (Kim et al 2003; Kim et al 2011). Also the structural integrity of the vmPFC and the white matter tract connecting the amygdala to the prefrontal cortex have been reported to affect anxiety (Pezawas et al 2005), which further supports the hypothesis that prefrontal-limbic interactions are a key mechanism of anxiety regulation. Interestingly, this interaction may be moderated by innate variability in serotonin transporter function (Heinz et al 2005; Pacheco et al 2009; Pezawas et al 2005; see box 5).

Also functional imaging studies with psychiatric patients have investigated whether the connectivity between the amygdala and prefrontal cortex is altered in patients compared to controls. There is evidence for deficits in cortico-subcortical communication in patients with PTSD and depression (Anand et al 2005; Rauch et al 2003). However, evidence that emotional disturbances in these patients also result from decreased inhibition of the amygdala by the PFC has been mixed (Gilboa et al., 2004; Anand et al., 2005b; Phan et al., 2006) and more research is needed to characterize the nature of possible deficits in cortico-subcortical interactions in these groups.

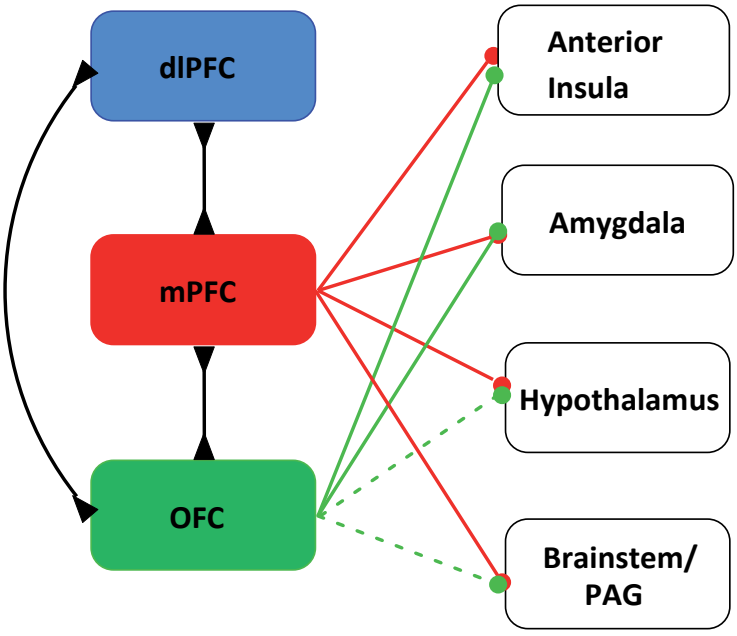

Figure 1. Connections of the primate PFC with brain areas important for negative emotion. Note that both the medial and orbital areas of the prefrontal cortex (mPFC & OFC) seem well suited to regulate activation of key areas in emotional expression. For the sake of simplicity there is no differentiation made between different regions within the areas shown (e.g. basolateral and central nuclei of the amygdala). dlPFC= dorsolateral prefrontal cortex. (Data from Augustine 1996; Barbas et al 2003; Barbas and Zikopoulos 2006; Ghashghaei et al 2007; McDonald 1998; Mufson and Mesulam 1982; Price 2006; Selemon and Goldman-Rakic 1988). Dotted lines signify weaker connections.

Taken together, functional connectivity studies in humans have confirmed the role of cortico-subcortical interactions in anxiety regulation. Most specifically, inhibition of the amygdala has emerged as an important working mechanism. However, these studies cannot provide direct evidence and typically rely on correlations with "activity" in the amygdala without distinguishing between different functional areas within this structure. Furthermore, other areas important for the expression of negative emotion (e.g. insula, hypothalamus and brain stem) are typically not included in the analyses. To further characterize inhibition of the emotion by the PFC we will return to evidence from animal studies.

BOX 5. Candidate gene studies in anxiety

Recent years have seen exciting findings concerning the influence of common genetic polymorphisms on anxiety. In the current thesis, we attempted to replicate and extend findings concerning a candidate polymorphisms related to serotonin, to further refine the associated phenotype. Similar to pharmacological manipulation studies, candidate gene studies hold the potential to elucidate key neurotransmitter systems involved in anxiety. A potential advantage is that this approach does not require the administration of exogenous substances which always creates an artificial, i.e. unnatural situation by flooding the body with a particular drug. In contrast, this approach allows for the assessment of existing individual differences in neurotransmission. A principal disadvantage is the fact that many polymorphisms are not yet characterized in how they affect functionality of transmitter systems. Particularly interesting is the serotonin transporter polymorphism that has been linked repeatedly to anxiety (the so-called 5-HTTLPR). The effects of this polymorphism on reactivity to threats is assessed in **chapters 3 and 4** of this thesis.

Pathways of PFC-amygdala inhibition

Prefrontal regulation of threat-related emotions in rodents seems to arise from the infralimbic and prelimbic cortices (IL & PL). If the amygdala and other subcortical areas would be under inhibitory control by these areas, stimulation or lack of stimulation (by lesions) can logically be expected to have measurable effects on amygdala activation in response to stress. Indeed, stimulation of IL and PL has been reported to decrease neuronal activation and plasticity in the basolateral amygdala (BLA) in response to fear (Rosenkranz et al 2003). However, results from another study seem to indicate the mPFC can also increase activity in the BLA (Likhtik et al 2005). The mPFC may thus be able to change activity in the basolateral amygdala bidirectionally but the question remains how this influences the centromedial output regions of the amygdala. In the end, suppression of negative emotion can be expected to result from inhibition of the centromedial nuclei. Indeed, Quirk et al. (2003) found that stimulation of the mPFC (IL/PL) decreases the responsiveness to inputs from the insula and BLA of centromedial amygdalar neurons that project to the brain stem. Similarly, Correll et al. (2005) found that lesioning the connections between the amygdala and PFC results in increased neuronal responses to footshock in the centromedial amygdala. The mPFC in rodents thus seems to control the output of the amygdala. Together with the anatomical data on amygdala-PFC connections, these findings have resulted in detailed models of how the prefrontal cortex may control amygdala excitability (Sotres-Bayon et al 2004). In these models, activation of the mPFC, more specifically IL, inhibits amygdala output from the centromedial nuclei to

the brain stem and in this way decreases emotional expression. This is thought to involve inhibitory interneurons located in nearby nuclei, such as the BLA and intercalated cell masses, since direct connections from the PFC to the centromedial nuclei are scarce (McDonald 1998). Indeed, in an elegant study it was recently shown that neurons in the BLA that facilitate extinction are coupled to the mPFC (Herry et al 2008).

It remains to be seen how these models of emotion regulation in rodents can best be translated to humans although area IL and PL have been suggested to be similar to the human mPFC (Price 2006). Additionally, orbital areas of the PFC also may contribute to amygdala inhibition in humans. Recently, the connections of different areas of the prefrontal cortex to the amygdala in primates have been studied in more detail (Ghashghaei et al 2007). The authors injected a neural tracer in different areas of the amygdala to see which areas were connected with the different areas of the prefrontal cortex. The results confirm previous findings that the caudal medial and caudal orbitofrontal regions of the PFC send most connections to the amygdala. Furthermore, they indicate that while the mPFC sends its output mainly to the basolateral amygdala, the OFC also sends outputs to the ITC and CE (see also Price 2006). Therefore, in primates the OFC more directly controls the output of the amygdala while the mPFC controls emotional processing at an earlier phase.

Prefrontal cortex inhibition of lower level emotion centres in the brain

The mPFC and OFC also have direct connections with lower level brain systems involved in the generation of endocrine and autonomic responses related to negative emotions. These can provide alternative ways for the prefrontal cortex to inhibit the emotional response, next to inhibiting amygdala output. The hypothalamus plays a central role in negative emotional responses by stimulating secretion of hormones. Furthermore, this structure also has access to the autonomic control centres in the brain stem. Control of the hypothalamus would therefore be of paramount importance in controlling emotions, particularly for more long lasting emotional states as increases of stress hormones released from the PVN is typically, but not exclusively, relatively slow (Joels and Baram 2009; Joels et al 2011). As discussed above, the PVN is the hypothalamic nucleus that functions as the main output region for neuroendocrine stress responses. In rodents, the prefrontal cortex does not have direct access to the PVN (Floyd et al 2001; Vertes 2004). Therefore, similar as for the amygdala, regulation of these areas by the PFC is mediated by connections to closely surrounding nuclei like the dorsomedial hypothalamus (DMH) and extended amygdala (BNST) (Buijs and van

Eden 2000). These areas contain a high density of GABAergic interneurons that connect to the PVN (Herman and Cullinan 1997). Activation of these areas by the PFC would therefore result in inhibition of the PVN and may provide an important pathway for prefrontal control of the stress response (Buijs and van Eden 2000; Crane et al 2003; Spencer et al 2005)

In primates, there are also no direct prefrontal connections to the PVN of the hypothalamus. However, there are connections between the medial and orbital prefrontal cortices to many other areas in the hypothalamus, including the DMH (Ongur et al 1998). In addition, there also seem to be connections with the BNST in primates (McDonald 1998). Prefrontal control of stress hormone release in primates could therefore very well involve similar pathways as in rodents. Furthermore, the vmPFC can also inhibit the medial hypothalamus directly. Besides the medial hypothalamus, the PFC is also connected with the lateral hypothalamus (Barbas et al 2003). This area in the hypothalamus contains autonomic neurons and is directly connected to the brain stem and spinal cord autonomic neurons that control emotional behaviour (Barbas et al 2003). In sum, the PFC can inhibit different hypothalamic nuclei to control negative emotions.

Direct control of the brain stem is yet another option for controlling negative emotions. In primates ventromedial areas in the PFC have direct connections to the brain stem autonomic cells in the PAG (Hadjipavlou et al 2006; Price 1999). As mentioned before, next to the autonomic brain stem nuclei the monoaminergic nuclei in the brain stem also play a role in negative emotion. One of these is the dorsal raphe nucleus, which is important for serotoninergic (5-HT) modulation and directly connected to the mPFC (Vertes 2004). Amat et al. (2006) investigated the effects of mPFC lesions on 5-HT activity in the dorsal raphe nucleus in rats. Normally, perceived control over a stressor is known to reduce the stress response compared to the response to an uncontrollable stressor. This reduction was absent in rats that received temporary lesions of the area around the junction of the prelimbic and infralimbic regions (by means of muscimol injections). This indicates the mPFC plays a role in complex computations to determine whether a stress response should be inhibited given a certain context. This control may arise from the direct connections but also from the connections to the amygdala and hypothalamus.

In conclusion, the PFC is equipped with several pathways that could contribute to the regulation of emotion. Through its connections, the prefrontal cortex exerts inhibitory control over areas with different positions in the hierarchy of emotional expression. These range from areas that are able to mobilize the response (the amygdala) to those that are at the start of the actual response (PVN and brain stem autonomic nuclei and neuro-modulatory aminergic

nuclei). In rodents, the most important sources of negative emotion regulation in the PFC seem to be IL and to a lesser extent PL. In humans, similar connections seem to arise from the orbital and medial prefrontal cortex.

SYNOPSIS

Given the findings from human neuroimaging studies and primate anatomical studies we can now build a model how the prefrontal cortex inhibits different areas underlying the expression of emotion (Figure 2). The areas of the prefrontal cortex primarily involved are the medial prefrontal cortex (BA 24, 25, 32) and orbitofrontal cortex (11, 12, 13, 47) since these are the areas that have been found to be active during functional neuroimaging investigations of emotion regulation and have been shown to have more direct connections to the brain centres underlying emotional expression. The mPFC and OFC directly connect to areas in the amygdala, insula, hypothalamus and brain stem to control autonomic functioning, neuromodulation by brain stem monoaminergic cells and neuroendocrine regulation. This control may involve several relay stations like the anterior insula, intercalated mass cells in the amygdala, the extended amygdala and dorsomedial hypothalamus. Although current knowledge does not permit to characterize interactions between these areas in detail (e.g. whether connections are excitatory or inhibitory), they may well be similar to those occurring in the rodent brain described in the previous sections of this chapter. Finally, optimal control almost certainly also depends on interactions of these areas with the dorsolateral PFC and frontopolar regions that can be thought to be involved in more abstract aspects of emotion regulation.

Summary and conclusion

The prefrontal cortex plays an important role in controlling reactions to aversive stimuli in mammals. Evidence from rodent, non-human primate and human studies together indicate that amygdala output can be inhibited by the PFC. In a similar way, the PFC also regulates the hypothalamic and brain stem nuclei that are important for negative emotion. Anatomical studies indicate that ventromedial areas of the PFC in primates and medial areas in rodents are best suited for the job given their connections to each of these areas. Through these connections, self-regulation of negative emotions can occur by inhibition of areas generating defensive responses.

THE CURRENT THESIS

This introductory chapter set out to investigate possible pathways for the inhibition of negative emotion in the brain by the prefrontal cortex. Converging evidence from animal and human studies has by now clearly indicated that the prefrontal cortex plays an important role in controlling the subjective, behavioural and physiological changes that correspond to negative emotions. These changes are for an important part mediated by the amygdala, anterior insula and dorsal ACC, the hypothalamus and the brain stem. Although optimal emotion regulation may rely on the PFC as a whole, the caudal ventromedial areas of the PFC seem most directly involved. A review of the relevant anatomical evidence has led to proposition of a preliminary model for pathways of prefrontal regulation of negative emotions in the brain. This was used as a basis for the studies described in this thesis, in which we further explored the neuroanatomical and neuropharmacological fundaments of human defensive behaviour.

In chapter 2, we further investigated the functional neuroanatomy of human anxiety by presenting subjects with cues that signalled shock threat while measuring indexes of brain activity through functional magnetic resonance imaging (fMRI). Moreover, we also investigated neural mechanisms of the inhibition of anxiety by assessing which brain areas responded to the termination of the threat. Brain measures were supplemented by measures of the startle reflex, assessed in a separate session. A more recently developed research strategy is to study the effects of variance in human genetics on anxiety. In chapters 3 and 4, we employed a larger study sample to replicate the findings from chapter 2 and assess whether individual differences in defensive processing might be related to common genetic variations in the serotonin transporter gene. In chapter 3 we describe the results for fear-potentiation of the startle reflex as read-out measure of defensive states and in chapter 4 we again used fMRI. In chapter 5, two selected drugs were tested for their effects on the extinction of fear on the basis of promising animal data. We assessed fear extinction in a healthy human sample, again using the startle measure and also skin conductance. Before the extinction training session, subjects received capsules containing a placebo or a dose of either the CB1 agonist tetra-hydrocannabinol or the partial NMDA agonist d-cycloserine in an attempt to improve fear extinction. Because fear conditioning and extinction are learning processes, it was not possible to investigate the effects of each substance in the same subjects. However, in chapter 6 we assessed several measures of anxiety for their potential in the use of within-subjects studies that can assess anxiety on different occasions within the same subjects. To this end, we evaluated whether anxiety as measured by indices of fear-potentiated startle and skin conductance but also increases in state anxiety due to the application of stressful tasks would show stability across repeated measurements. Finally, chapters 7 and 8 describe

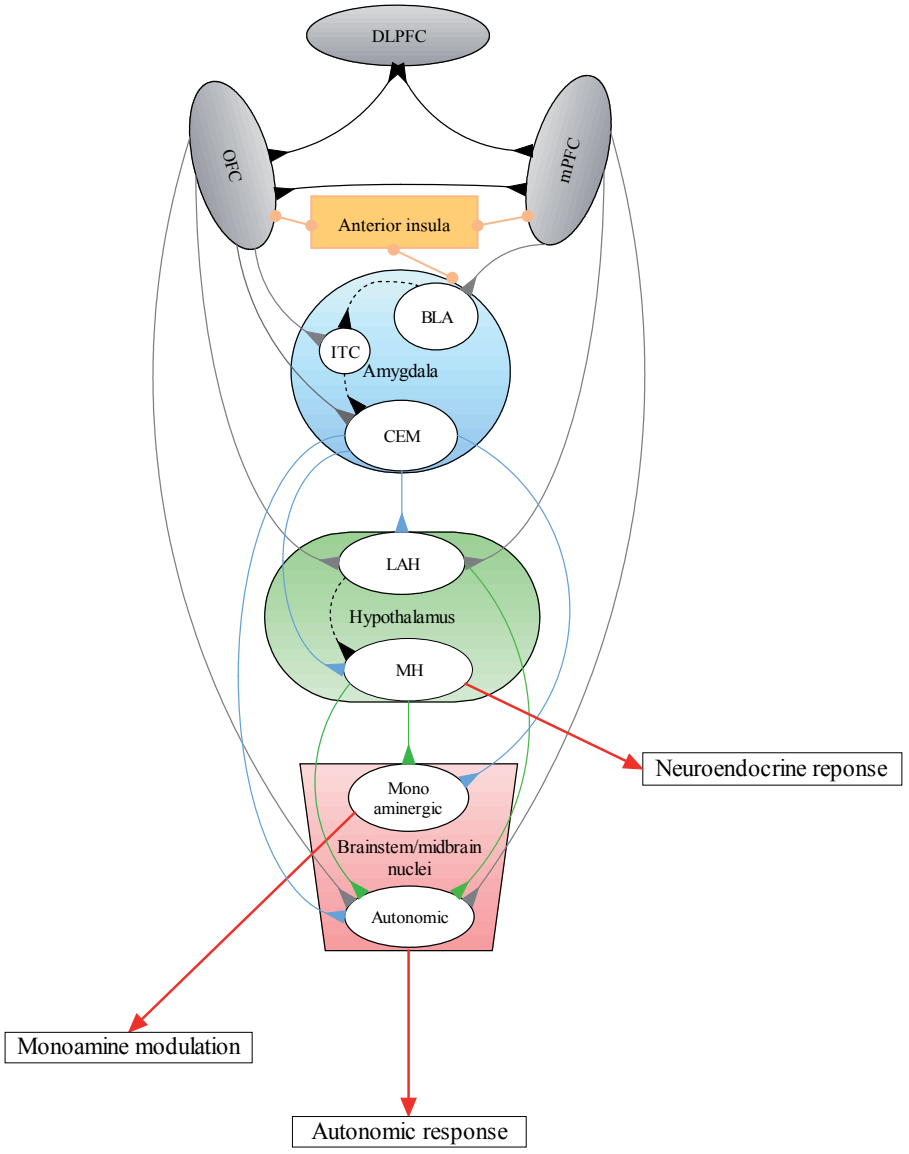

Figure 2. Medial and orbital prefrontal cortex (mPFC and OFC) can inhibit emotional behaviour on different levels. First, both have connections with the anterior insula and with the amygdala which itself modulates hypothalamic and brain stem output nuclei. On a lower level, mPFC and OFC also connect to the hypothalamus and brain stem to modulate emotion related processes. The dorsolateral prefrontal cortex (DLPFC) is not directly connected to these areas but may interact with these areas through the mPFC and OFC.

Abbreviations: BLA=Basolateral amygdala; ITC= Intercalated mass cells; CEM= centromedial nuclei (including the extended amygdala); LAH= lateral hypothalamus; MH= Medial hypothalamus (including the paraventricular nucleus).

data from two extraordinary clinical samples. In chapter 7 the role of the amygdala in fear conditioning was investigated by investigating patients with rare selective lesions in the basolateral amygdala. In chapter 8, patients that were treated with deep brain stimulation in the nucleus accumbens for severe treatment-refractory obsessive compulsive disorder were tested in a fear-potentiated startle paradigm. A unique study design allowed to test patients' defensive reactions during active stimulation and when the stimulator was switched off.

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CHAPTER 2

Prefrontal mechanisms of fear reduction after threat offset

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ABSTRACT

Reducing fear when a threat has disappeared protects against a continuously elevated anxiety state. In this study, we investigated the brain mechanism involved in this process. The threat paradigm consisted of discrete cues that signaled either threat of shock or safety. Healthy participants were tested in two sessions in which eyeblink startle (n =26) and blood oxygen level dependence (n =23) were measured to index subjects' defensive state and brain responses respectively. Startle results indicated that subjects could rapidly decrease their defensive state after the offset of shock threat. Functional magnetic resonance imaging data indicated that the termination of threat was associated with the recruitment of lateral and ventromedial prefrontal cortices. An exploratory connectivity analysis showed that activity in these prefrontal regions was linked and was also associated with activity in brain regions typically responding to threat, the right anterior insula and amygdala. These results provide first evidence for a prefrontal mechanism that functions to control anxiety after threat offset, which may be dysfunctional in patients who suffer from excessive sustained anxiety. Moreover, the results support a model in which the lateral prefrontal cortex controls anxiety related limbic activity through connections with ventromedial prefrontal cortex.

INTRODUCTION

When facing threat, the brain rapidly engages the neuroanatomic systems that support an adaptive response. Under normal circumstances, when danger is no longer perceived to be imminent, these systems are switched off to prevent unnecessary energy expenditure and negative side effects of prolonged stress. However, a sustained state of anxiety may emerge in individuals who fail to reduce fear adaptively after a threat signal has disappeared. To investigate the neural systems involved in this control process, this study explored neural correlates of the transition from a threatening situation to a resting phase using functional magnetic resonance imaging (fMRI).

It is widely acknowledged that deficits in neurobiological mechanisms of fear reduction may play an important role in some forms of anxiety disorders (Etkin & Wager, 2007; Shin & Liberzon, 2009). Excessive sustained anxiety appears to be a hallmark of some disorders of anxiety—for example, generalized anxiety disorder (Davis et al, 2010; Grillon et al, 2008). However, many previous studies on fear regulation have focused on control mechanisms operating during presentation of discrete threatening stimuli (Hartley & Phelps, 2010; Ochsner & Gross, 2005; Ochsner & Gross, 2008) and have not investigated the brain's natural control mechanisms that terminate the anxious state in the period following threat offset (Hartley & Phelps, 2010).

Previous neuroimaging studies that have investigated the neural substrate of top-down fear modulation have consistently found evidence for involvement of the prefrontal cortex (PFC) (Ochsner & Gross, 2005; Quirk & Beer, 2006) in this process. Extinction of a conditioned fear response, an increasingly well-investigated fear inhibition paradigm, is associated with activity changes in ventromedial PFC regions (Gottfried & Dolan, 2004; Phelps et al, 2004). In addition, neuroimaging research with cognitive appraisal paradigms has shown that areas of dorsolateral PFC are being recruited during more deliberate emotional regulation (Barbas et al, 2003; Bocker et al, 2004; Delgado et al, 2008; Eippert et al, 2007; Grillon & Baas, 2003; McDonald, 1998; Ochsner et al, 2002; Phan et al, 2005). Given the relative lack of connections between the limbic system and dorsolateral PFC (Barbas et al, 2003; McDonald, 1998), the PFC may inhibit fear through connections of the ventromedial PFC with the amygdala in both forms of fear regulation (Delgado et al, 2008). We hypothesized that the PFC may orchestrate reductions in fear that occur after the offset of a danger cue in a manner similar to the effects observed during fear extinction and cognitive regulation. In this way, the PFC may play an important role in the control of sustained anxiety.

To study brain areas associated with threat offset, subjects were presented with a cue signaling threat of receiving an electric shock, and another stimulus that indicated safety from shock. Fear was indexed in a session separate from the fMRI by measuring potentiation of the startle reflex, an objective and robust method for measuring defensive states in humans (Bocker et al, 2004; Bradley et al, 2005; Grillon & Baas, 2003). Startle probes were presented at different time points after threat onset and offset to capture the time course of the defensive response (Dichter et al, 2002; Germans & Kring, 2007; Grillon et al, 1993; Jackson et al, 2003; Larson et al, 2005; Larson et al, 2010). This also yielded a valid assessment of fear levels during the fMRI session, because fear-potentiated startle remains at similar levels across repeated tests in our instructed fear paradigm (Klumpers et al, 2010). Specifically, we tested the hypotheses that the offset of threatening stimuli is associated with 1) a significant reduction in fear indexed by startle amplitude and 2) with concurrent ventromedial and lateral PFC activation.

METHODS AND MATERIALS

Subjects

Subjects included 26 healthy participants aged 18–30 (mean 22.1 years) who gave written informed consent before participation.Three subjects were not scanned because of technical issues with the MRI scanner after already participating in the startle session. The final sample therefore consisted of 23 subjects (11 female) for the imaging data and 26 (12 female) for the startle session.

Procedure and Task

During a screening visit, subjects filled in a battery of personality and medical questionnaires and underwent a shock workup procedure (described more in the Supplemental Material) to set the shock intensity individually for the first session. After subjects had been admitted to the study, they were invited for two sessions separated by 2 to 14 days. We randomly assigned subjects to participate first in either the fMRI (n = 12) or startle (n = 14) session. The experimental paradigm for both sessions was identical. Before each session, subjects again underwent a shock workup procedure to reset the shock intensity. After the workup, instructions consisted of the sequential presentation of two pictures of faces with on-screen nstructions that subjects could receive shocks at any time during presentation of Picture 1 and never during Picture 2. Pictures were photos of two male faces selected from the Psychological Image Collection at Stirling (http://pics.psych.stir.ac.uk) that each received neutral ratings in a pilot study. One of the pictures was presented in blue and the other in

yellow to increase salience and distinctiveness of the cues. Association between the pictures and either threat or safety was counterbalanced across subjects. The subjects were explicitly instructed to rest when there was no face on the screen. Accordingly, the word *RUST* ("rest" in Dutch) was presented on the screen during the intertrial interval.

The experiment consisted of three 10-min runs that contained 14 presentations of each face. Pictures were presented in a semirandom order, counterbalanced over subjects and sessions, with no more than three consecutive repetitions of the same condition. Picture duration varied between 6 and 12 sec (9.3 sec average). The rest period between pictures ranged between 8 and 12 sec (9.9 sec average). Shocks were presented at varying time points during the threat condition. Two or three shocks were administered per run, with a total of six shocks for the first session and seven shocks for the second session. After each run, subjects retrospectively rated their anxiety during and immediately after the offset of the threat and safe cues on a scale from 0 (*not anxious / nervous*) to 10 (*very anxious / nervous*).

Electromyographic recording of the eyeblink startle reflex, startle probe presentation, and shock administration were carried out using previously published procedures (Klumpers et al, 2010). Briefly, startle probes were 50-msec, 106-dB(A) white-noise bursts, and the eyeblink startle reflex was measured from the orbicularis oculi with two Ag-AgCl electrodes placed under the right eye during the startle session. In the startle session, startle probes could be presented either between 1.5 and 3.5 sec following picture onset (early probes) or 5.5 to 11.5 sec post-onset (late probes). Similarly, probes were also presented either 1.5 to 3.5 or 5.5 to 11.5 sec after the offset of the threat and safe cues. For each condition, three probes were presented per run at each latency summing to a total of 72 (three runs X two conditions X four probe latencies X three) probes. The mean interval between probes was 21.6 sec, with a minimum interval of 14 sec after a shock reinforcement or previous probe. To avoid distortion of the magnetic resonance signal by shock stimulation, a nonmagnetic high-pass filter was placed between the stimulator and the MRI-compatible, carbon-wired silver electrodes used for shock administration. The same filter and electrodes were also used during the startle session to keep the shock intensity comparable. The intensity of the electrical stimulation varied between subjects and over sessions from .7 to 7.6 mA.

Imaging

Imaging was performed on a Philips 3T Achieva MRI scanner (Philips Medical Systems, Best, the Netherlands), 2250 T2*-weighted volumes of .813 sec obtained in three runs (echo time = 23 msec, repetition time = 15.6 msec, field of view = 224 X 224 X 136.5 mm, flip angle =8.85°, matrix =64 X 64, voxel size = 3.5mm isotropic). Each volume consisted of 39 sagittal slices.

The 3D PRESTO sequence (Neggers et al, 2008; Ramsey et al, 1998) was used combined with echo shifting and parallel imaging acceleration (Pruessman et al, 1999), which allows fast whole brain scanning (Neggers et al, 2008). Furthermore, we used in-plane segmentation (van Gelderen et al, 1995), allowing a shorter echo-train length and lower echo time to reduce distortion artefacts and to reduce signal dropout in regions susceptible for artefacts (e.g., ventral prefrontal areas, amygdalae). After the functional runs, a T1-weighted anatomic image of 175 sagittal slices was obtained (echo time = 3.8 msec, repetition time = 8.4 msec, field of view = 288 X 288 X 175 mm, flip angle = 17°, voxel size = 1 mm isotropic).

Data Processing and Analysis

Startle data were preprocessed and checked for artifacts according to previously published procedures and guidelines (Blumenthal et al, 2005; Klumpers et al, 2010; van Boxtel et al, 1998), described in more detail in the Supplementary Material. Startle magnitudes were transformed to *t* scores per subject, and trials were averaged according to condition and probe time (threat/safe, onset/offset, early/late). Statistical analyses for the startle data and subjective anxiety ratings consisted of repeated-measures analyses of variance (ANOVA) carried out in SPSS 16 (SPSS, Chicago, Illinois). For the anxiety ratings, threat condition (threat, safe) and time (cue, offset) were entered as within subjects factors. For the startle data, probe latency (early, late) was also included to create a four-level latency factor (cue early, cue late, offset early, offset late). A between subjects factor "order" was added to verify that there was no influence of previous exposure to the task.

The fMRI data were processed using SPM5 (Welcome Department of Imaging Neuroscience, University College London, United Kingdom; http://www.fil.ion.ucl.ac.uk/spm/). Functional scans were realigned, coregistered to the anatomic scan, and spatially normalized to the Montreal Neurological Institute T1 template image to a normalized resolution of 3.5 mm isotropic. The normalized images were then smoothed with a kernel with a full width at half maximum of 8 X 8 X 8 mm. Subsequently a general linear model was composed to relate blood oxygen level–dependent (BOLD) signal variation to the task conditions. The predictors in this model were the threat conditions, safe conditions, and shocks. For the threat and safe conditions, the onset and offset responses were modeled using a delta function. In addition, a sustained response to the cues was modeled with a boxcar that lasted throughout the threat and safe conditions. These regressors were convolved with the canonical hemodynamic response function. Multiple correlations between factors in the design were calculated to assess multicollinearity. All were around .40. Realignment parameters were included in the model to reduce movement-related artefacts. Visual inspection of the scans around shock

administration indicated extremely large and fast signal fluctuations. Therefore, each scan that contained these excessive fluctuations was modeled using a separate regressor. In this way, we removed any variance caused by these artifacts that could reduce statistical power for detecting our effects of interest. This was the case for 11 subjects, with, on average, around seven scans per subject removed because of artifacts.

One-sample *t*-tests were used to test for significant group effects for the contrasts comparing the threat and safe condition and their offsets. For the assessment of the impact of the threat cues, we only report the results from the sustained contrasts here; the results that compared threat and safe onset overlapped and did not give any additional activation in our areas of interest. Results were thresholdedat $p = .001$ (uncorrected for multiple comparisons). To minimize the chance of false positives, this voxelwise threshold was combined with a cluster threshold, and only clusters that survived a threshold of .05 (family-wise error corrected using random field theory) are reported.

Because of our a priori expectations on activations in limbic areas and prefrontal areas, we selected the clusters of activity in the insula and anterior cingulate cortex (ACC; threat vs. safe sustained contrast) (Etkin & Wager, 2007, Chua et al, 1999; Kumari et al, 2007; Nitschke et al, 2006; Phelps et al, 2001; Seeley et al, 2007; Simmons et al, 2006) and prefrontal areas (offset contrast) (Delgado et al, 2008; Eippert et al, 2007; Ochsner et al, 2002; Ochsner & Gross, 2005; Ochsner & Gross, 2008; Phelps et al, 2004) for further analyses on the basis of the whole-brain analyses. Mean signal change, relative to the mean signal in the cluster, was extracted from these areas using the MARSBAR toolbox (Brett et al, 2002) in SPM. Besides these functional regions of interest (ROIs), we also included the amygdala as an anatomic ROI because of extensive evidence for a role of the amygdala in fear. To this end, we extracted the average signal change from the bilateral amygdalae using an anatomic mask from the automated anatomical labeling atlas (Tzourio-Mazoyer et al, 2002). Between-subjects correlations were then calculated between mean signal change in our ROIs and startle potentiation. All correlations reported remained significant when removing outlier data points with absolute *z* scores > 2.5 (*z* scores calculated over subjects for each variable). To visualize the individual contribution of activity in the safe and threat condition to the offset contrast, we selected active ROI voxels based on the data from the first run using a threshold of $p = .001$ uncorrected and plotted mean signal change in these voxels for each condition based on the data from Runs 2 and 3 to prevent overestimation of the effect size (Kriegeskorte et al, 2009). Finally, we undertook an exploratory connectivity analysis to investigate the relationship between the activations in our ROIs. For this, we extracted the time course of activation in the ventromedial (vm)PFC ROI, calculated an interaction term for this time course and the contrast of interest,

and added both the time course and the psychophysiologic interaction (PPI) term to our regression model adjusted for general effects of the task (Friston et al, 1997; Gitelman et al, 2003).

RESULTS

Subjective Ratings

During both the MRI and startle session, subjects retrospectively reported more anxiety after the threat than after the safe cue [MRI $F(1,25) = 83.1$, $p < .001$; startle $F(1,22) = 74.3$, $p < .001$] and this difference was reduced significantly after cue offset [MRI $F(1,25) = 21.0$, $p < .001$; startle $F(1,22) = 38.7$, $p < .001$; see Table S1 in Supplementary Material for mean ratings per session]. For subjects that participated in both sessions, there was no difference in anxiety reported during the fMRI session and anxiety during the startle session [Fs$(1,22) < 1$, ns].

Startle Data

After threat onset, startle was increased compared with the safe condition [$F(1,25) = 40.5$, $p < .001$]. Fear-potentiated startle (FPS, the difference in startle magnitude between threat and safe) fluctuated significantly across time points [threat condition X latency: $F(3,75) = 21.9$, $p < .001$], reflecting a reduction of startle magnitude to levels of the safe stimulus early after threat offset (see Figure 1). Repeated contrasts indicated that there was no difference in startle potentiation for probes presented early and late after cue onset [early cue vs. late cue: $F(1,25) = 2.4$, $p < .13$]. After threat offset, potentiation was quickly reduced [late cue vs. early offset: $F(1,25) = 46.2$, $p < .001$] and then stable again [early offset vs. late offset: $F(1,25) = 1$, ns]. Finally, the startle results were not influenced by previous experience with the task [order X threat X condition and order X threat condition X time, Fs < 2.3, ns].

Imaging Data

Group Analyses

The group analysis focusing on brain areas that responded more during the threat cues than during the safe cues yielded activation in limbic areas. The active areas included the anterior insula/inferior frontal gyrus (IFG, BA 13/47 bilateral) and the dorsal ACC (BA 24/32; Figure 2, Table 1 for the whole brain group results). There was no activation in the amygdalae, also not when applying alternative analyses and thresholds (see Supplementary Material).

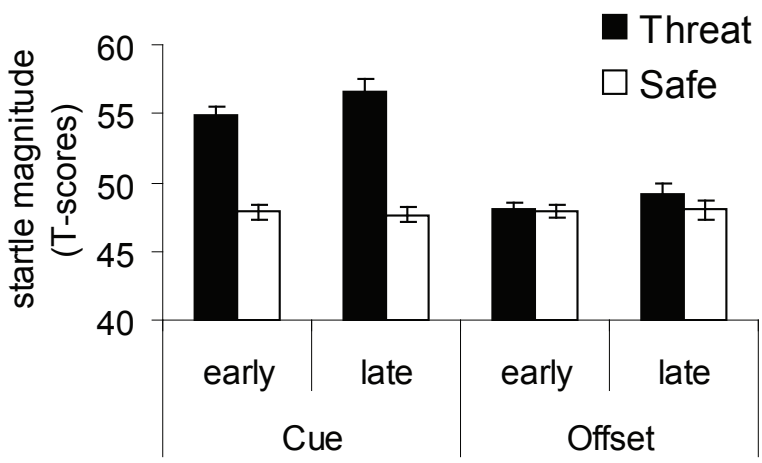

Figure 1. Mean startle magnitude during the threat and safe cues and after cue offset. Early probes were presented 1.5 to 3.5 sec after cue onset or offset and late cues after 5.5–11.5 sec. Error bars represent standard error of the mean.

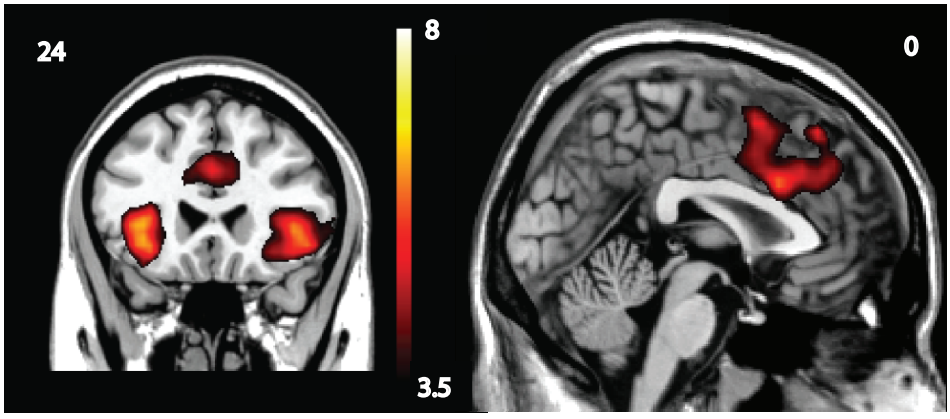

Figure 2. Functional magnetic resonance group results for the threat versus safe (sustained) contrast showing activations in bilateral insula/inferior frontal gyrus and anterior cingulate cortex during threat. Results are visualized on an anatomic template image; and the number in the top right corner represents the Montreal Neurological Institute coordinate for the selected slice, and the scale is in t scores.

The critical contrast that compared threat offset to the offset of the safe condition indicated a significantly increased response in the right anterior lateral PFC (lPFC; BA 46/10, Figure 3, Table 1) to threat offset. In addition, there was a significant decrease in BOLD signal in the subgenual vmPFC after the offset of threat (BA 11, Figure 3, Table 1). Additional analyses involving the alternative contrast threat versus threat offset indicated that these prefrontal areas also responded more to threat offset than to the threat condition (see Figure 4; see Supplementary Material for the full results of these analyses)

Region (of peak activation)	MNI coordinates			Z-score	voxels
	X	Y	Z		
Threat > Safe[1]					
Right anterior insula / inferior frontal gyrus	49	21	-7	4.97	273
Left anterior insula / inferior frontal gyrus	-35	21	-7	4.96	291
Posterior cingulate gyrus	4	-24	28	4.93	65
Anterior cingulate gyrus (ACC)	0	18	28	4.71	463
Right supramarginal gyrus	49	-42	32	4.65	209
Right medial frontal gyrus	46	4	42	4.51	67
Right thalamus	7	-14	10	4.32	187
Cerebellum	0	-56	-24	4.29	145
Left cerebellum	-38	-60	-32	4.15	153
Right medial frontal gyrus	46	38	32	4.09	144
Left lingual gyrus	-21	-102	-14	3.77	54
Left middle frontal gyrus	-46	49	21	3.54	53
Left supramarginal gyrus	-56	-49	26	3.46	46
Safe > Threat[1]					
Right superior parietal lobule	24	-66	60	5.22	62
Left precuneus	-35	-88	35	5.22	233
Medial frontal gyrus	0	60	-14	5.19	99
Precuneus	0	-56	38	4.91	452
Right postcentral gyrus	66	-18	38	4.39	102
Right parahippocampal gyrus	18	-14	24	4.37	74
Left parahippocampal gyrus	-21	-21	-18	4.30	52
Left superior frontal gyrus	-28	21	56	4.28	54
Right superior occipital gyrus	42	-88	28	4.14	130
Left postcentral gyrus	-63	-18	49	4.09	70
Left postcentral gyrus	-32	-35	77	3.95	64
Threat offset > Safe offset[2]					
Right middle frontal gyrus (lPFC)	35	66	4	4.11	53
Safe offset > Threat offset[2]					
Medial Frontal gyrus (subgenual vmPFC)	0	35	-14	3.59	42

Table 1. FMRI group results

[1] Statistic images assessed for cluster-wise significance; with a cluster defining threshold of P = 0.001 uncorrected, the 0.05 FWE-corrected critical cluster size was 38 voxels

[2] Statistic images assessed for cluster-wise significance; with a cluster-defining threshold of P = 0.001 uncorrected, the 0.05 FWE-corrected critical cluster size was 37 voxels

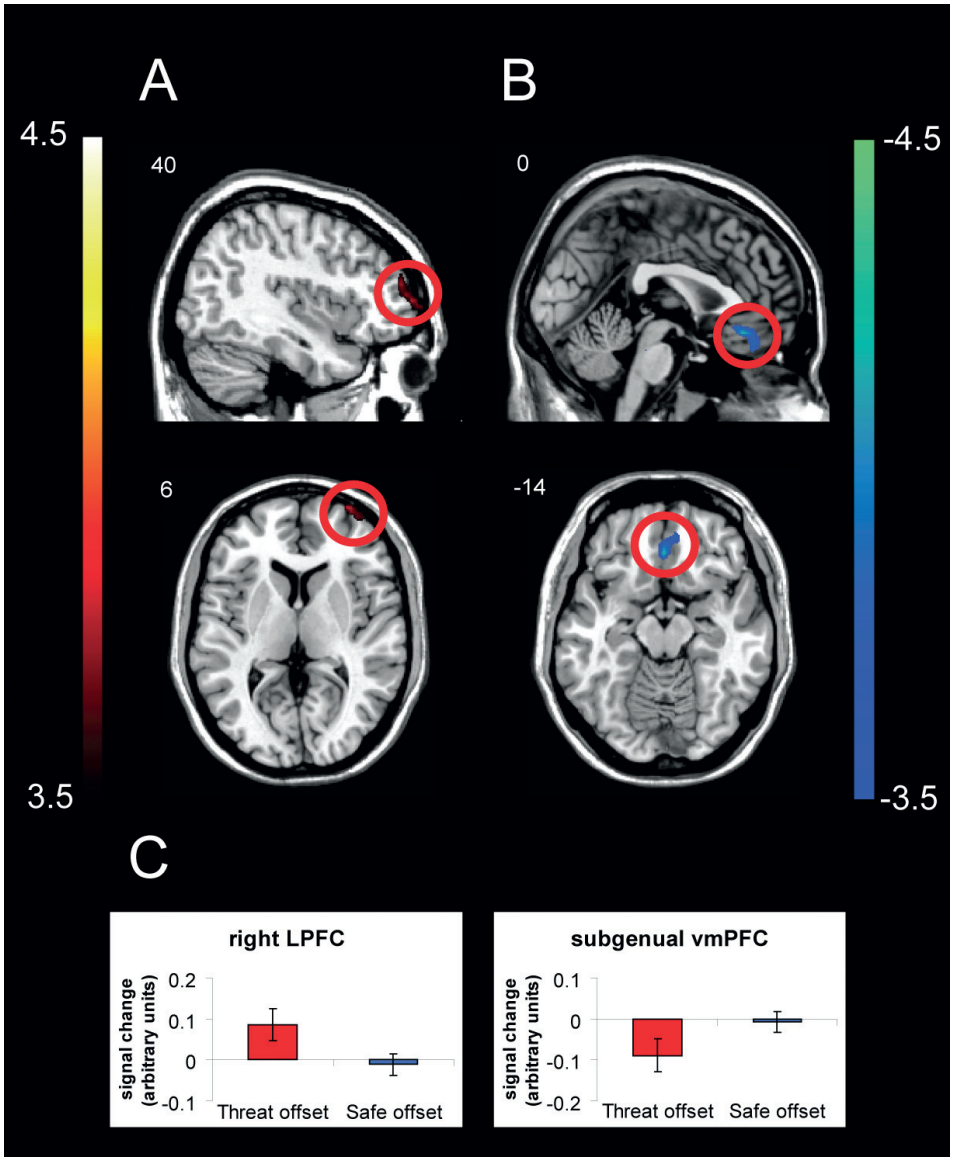

Figure 3. FMRI group results for the Threat vs. Safe offset contrast. (A) Loci of activation in the lPFC and (B) deactivation in the subgenual vmPFC in reaction to threat offset. Results are visualized on an anatomical template image, the number in the top right corner represents the MNI coordinate for the selected slice and the scale is in T-scores. (C) Mean percent signal change for each ROI for the Threat and Safe conditions separately, error bars represent standard error of the mean.

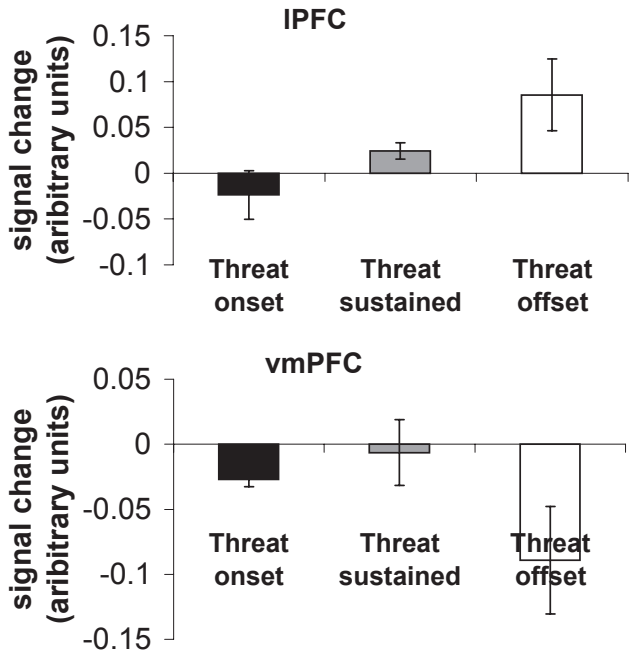

Figure 4. Percent signal change for each prefrontal region of interest for the threat onset, sustained, and threat offset conditions separately. These are again corrected for potential overestimation by using only the data from the last two runs from the region of interest that was determined based on the first run; the uncorrected data showed a similar pattern with stronger differentiation between the offset and the threat responses. Error bars represent standard error of the mean. lPFC, lateral prefrontal cortex; vmPFC, ventromedial prefrontal cortex.

Between Subject Correlations

Next, we tested whether individual differences in FPS were associated with 1) recruitment of limbic regions of interest (insula, ACC, and amygdala) during threat and 2) recruitment of prefrontal ROIs (right lateral PFC and vmPFC) after threat offset. FPS during cue presentation, averaged across early and late probes, showed a marginally significant correlation with activation of the amygdalae during the fMRI session (r = .38, p = .07; see Figure 5a). This effect was most pronounced for the early probes for which the effect reached significance (r = .47, p < .02 for the early probe condition; r = .17, ns for late probes). Those subjects with a higher startle potentiation showed more amygdala activation to the threat vs. safe stimulus. No correlations were observed for the functional ROIs that showed increased activity to threat (insula, ACC), and there were also no correlations between FPS after offset and vmPFC activity after offset. However, those subjects with greater mean startle potentiation after the offset of the threat versus safe cue (early and late probes averaged) showed more mean activation in

the right lPFC ROI after threat versus safe offset (r = .54, p < .01; see Figure 5b). This effect was significant for the late probes after threat offset (r = .52, p < .01) but not for the early probes (r = .32, ns).

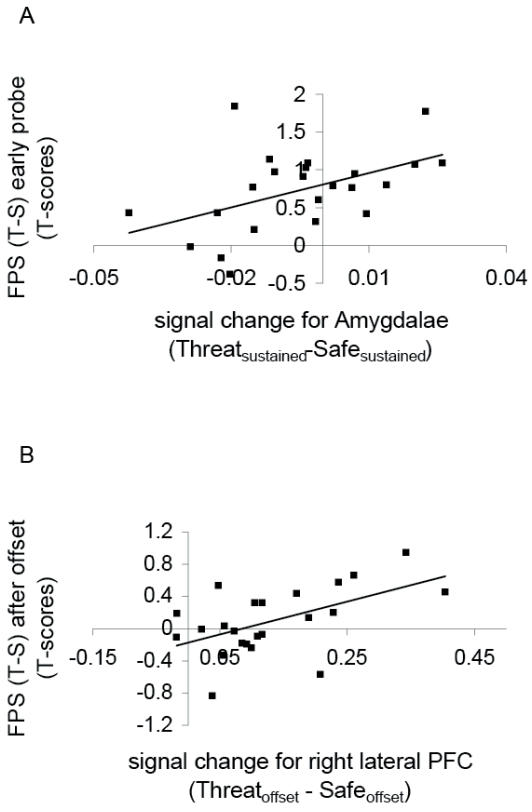

Figure 5. Correlations in signal change between functional magnetic resonance imaging data and the startle data. (A) Scatterplot of early startle potentiation (1.5–3.5 sec after onset) during the threat cue and differential blood oxygen level–dependent signal change in the amygdalae during threat.
(B) Scatterplot of residual mean startle potentiation after threat offset and differential blood oxygen level–dependent signal change in the right lateral prefrontal cortex (PFC) to threat offset. FPS, fear-potentiated startle

Functional Connectivity

Finally, we investigated the hypothesis that the lPFC modulated limbic activity through the vmPFC. To this end, we examined correlations between activity in the right lateral prefrontal ROI and the subgenual vmPFC and also between the activity in the vmPFC and our limbic ROIs. This connectivity analysis showed a negative correlation between activity in the subgenual vmPFC and activity in a region that overlapped with the right lPFC region activating in

response to threat offset (again, all analyses $p = .001$ and familywise error corrected at the cluster level $p < .05$). Moreover, there was also a negative correlation between vmPFC activity and activity in a right anterior limbic region encompassing the amygdala, anterior insula, and ventral striatum (Figure S1, Table S2 in Supplemental Material). This indicates that stronger deactivations in the vmPFC co-occurred with more lPFC activity and more activity in these limbic regions. These correlations were based on the entire fMRI time courses recorded during the task.We found no evidence of a psychophysiologic interaction in response to threat offset, that is, we found no indications of significant changes in the correlations in response to the offset of the threat condition compared with the safe condition.

DISCUSSION

The instructed fear paradigm increased both subjective anxiety and startle potentiation under conditions of threat. More important, the startle data also indicated that this fear response was quickly reduced to baseline levels across subjects within 3.5 sec. Together with previous research (Baas et al, 2002; Böcker et al, 2004; Grillon et al, 1993; Mol et al, 2007), this suggests that healthy subjects are able to adjust their defensive states rapidly in response to instructed conditions of threat and safety. Data from the fMRI session showed an increase in BOLD in the anterior insulae and dorsal ACC during the threat condition. These areas have been implicated in human anticipatory anxiety in many previous reports (Chua et al, 1999; Kumari et al, 2007; Nitschke et al, 2006; Phelps et al, 2001; Seeley et al, 2007; Simmons et al, 2006; Straube et al, 2007), and patients with anxiety disorders show hyperactivation in this circuit (Etkin & Wager, 2007), suggesting that these structures play a central role in human anxiety (Milad et al, 2007; Paulus & Stein, 2006). Perhaps surprising but in line with previous reports, our group analyses did not reveal discriminative activity in the amygdala in response to threat of shock (Chua et al, 1999; Kumari et al, 2007; Phelps et al, 2001; Mechias et al, 2010). The amygdala response to threat may have habituated quickly while the defensive state remained (Phelps et al, 2001; Marschner et al, 2008; Zald, 2003). Our data do indicate that amygdala activations are found more consistently in subjects with higher startle potentiation early after threat onset. This is in line with animal work that has suggested a direct role for the amygdala in fear potentiation of the startle reflex (recently reviewed by Davis et al, 2010).

After offset of threat, the reduction in subjects' defensive state was accompanied by activity in the anterior part of the right lPFC. Lateral PFC activation has repeatedly been found to be associated with cognitive regulation of fear (Delgado et al, 2008; Eippert et al, 2007; Ochsner et al, 2002; Ochsner & Gross, 2005; Phan et al, 2005). These studies use more

explicit regulation instructions, for example, to reappraise negative stimuli or feelings and find activations typically more dorsal than the area we found in this study (although see Phan et al, 2005). This could be due to higher demands in working memory in explicit regulation paradigms. Moreover, the finding that the activation at offset of the lPFC correlates positively with residual fear potentiation of the startle reflex after offset of the threat seems to indicate that the activation in this area does not automatically result in successful fear downregulation. Interestingly, Eippert et al. (2007) also reported an inverse relation between emotion regulation success and activity in a slightly more posterior right lateral prefrontal region.Wespeculate that activation in this area may reflect effort to downregulate, which logically would be higher in subjects who have difficulty controlling fear, although this post hoc interpretation clearly requires further study.

Besides lPFC activity, we found a deactivation in the subgenual vmPFC after threat offset. The vmPFC is also frequently reported as part of the default mode network (Gusnard & Raichle, 2001), signifying the potential importance of this area in returning the body to a homeostatic state. Studies on human fear extinction have also consistently reported involvement of the ventromedial PFC (Gottfried & Dolan, 2004; Kalisch et al, 2006; Milad et al, 2005; Milad et al, 2007 Phelps et al, 2004). The location of subgenual vmPFC activity in these studies corresponds well to the location of the deactivation observed at threat offset in our study, although these studies reported activation upon extinction relative to the prior fear state, rather than deactivation. With respect to this apparent controversy, it is interesting that in animals, deactivations of the vmPFC have been repeatedly linked to reductions in the expression of fear and stress (Radley et al, 2006; Sierra-Mercado et al, 2006; Sullivan, 2004), indicating that vmPFC activity is required for fear expression. However, vmPFC activity also appears required for reduction of fear through extinction (Quirk & Beer, 2006; Sotres-Bayon et al, 2004). In humans, changes in vmPFC activity may also either promote or reduce fear, perhaps dependent on the state of the fear-promoting regions (Sullivan, 2004) and the specific anatomic pathway involved (Quirk & Beer, 2006; Radley et al, 2006; Sierra-Mercado et al, 2006).

Because lPFC areas do not have strong direct connections to limbic areas such as the amygdala (Ghashghaei & Barbas, 2002; Ghashghaei et al, 2007), the lPFC may control limbic activity that drives the fear response indirectly through the vmPFC (Delgado et al, 2008). The results from our exploratory connectivity analyses support this idea by indicating that the lPFC and subgenual vmPFC interacted during our task. Although we found no direct evidence that this interaction became more pronounced around threat offset, the regions involved in this interaction matched the regions responding to threat offset well. Activity in the lPFC,

matching the area activated after threat offset, was correlated with decreased activity in the subgenual vmPFC seed region. Moreover, deactivations in this vmPFC region were related to activity increases in the right lateral amygdala and anterior insula. On the basis of these exploratory analyses, we hypothesize that the latter increases may reflect activity in local limbic inhibitory circuits that control the output of the limbic system areas that instantiate anxiety. Perhaps, deactivations in the subgenual vmPFC may thus reduce activity in limbic output centers by activating inhibitory circuits located nearby (Herman et al, 2005). In line with this idea and our results, animal work has indicated that the vmPFC modulates amygdala output through recruitment of an inhibitory circuit based in lateral areas of the amygdale (Sotres-Bayon et al, 2004; Rozenkranz et al, 2003). Clearly, it is necessary to confirm the results from our exploratory analyses and further investigate the connectivity between the neural components involved in fear offset.

In conclusion, our new approach to investigate brain mechanisms involved in fear reduction at threat offset indicates overlap with mechanisms recruited in extinction and cognitive regulation of fear. We found that healthy subjects can flexibly downregulate their defensive states within a few seconds while recruiting ventromedial and right lateral prefrontal areas. Moreover, our results provide support for the idea that these prefrontal areas form a circuit that interacts with the limbic system in fear regulation in healthy humans. Together with previous research, this suggests that these areas in the PFC are broadly involved in fear control, and effective recruitment of these areas may be instrumental in protecting individuals from chronic forms of anxiety.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTAL METHODS AND MATERIALS

Shock work-up procedure

The shock work-up procedure was introduced to the subject by informing the subjects that they would receive some shocks of varying intensity. No information was given to the subject regarding the goal of this procedure, i.e., that the subjects' responses determined the intensity used in the experiment. The work-up consisted of five sample shocks in total. Each shock was rated by the subject and shock intensity was subsequently adjusted in order to achieve an intensity that was rated as "quite annoying", a rating of 4 on a 5-point scale that ranged from "not painful/annoying" to "very painful/annoying". Starting with an intensity of 2.0 mA, shock intensities were adjusted step by step using a standardized procedure to a level that was rated "quite annoying". After the final shock, the intensity was adjusted once more to reach the intensity that would be used throughout the experiment.

Startle data recording, startle probe presentation and shock administration details

Recording and amplification of the EMG signal was carried out using the Biosemi ActiveTwo system (www.biosemi.nl) with matching FLAT Active Ag-AgCl electrodes at a sample frequency of 2048 Hz. The startle response was recorded from the orbicularis oculi muscle under the right eye. Electrodes were placed +/-15 mm apart. The electrodes were filled with standard electrolyte gel (Signa Gel; Parker Laboratories, Fairfield, NJ). Startle probes were delivered through earphones with foam earplugs (Earlink, Aearo Company Auditory Systems, Indianapolis, IN). Shocks were administered through tin cup electrodes on the left wrist, which were connected to a constant current stimulator (Digitimer DS7A, Digitimer Ltd., Hertfordshire, England). Shocks were administered at varying, semi-random time points for all participants to reduce predictability and to reinforce the instruction that shocks could really be administered at any time during the threat condition. For electrical stimulation, a train of 150 2-ms pulses was administered at a rate of 200 Hz.

Startle data processing

Startle data were processed with Vision Analyzer software (Brain Products, brainproducts.com). For the startle data, initially the signal was filtered using a 28-Hz, 12dB/oct high-pass, a 400-Hz, 24-dB/oct low-pass and a 50-Hz notch filter. The data were segmented into

epochs, starting 50 ms before onset of the startle probe and ending 200 ms after onset. Finally, the signal was baseline corrected, rectified and a low-pass filter (12 Hz, 12 dB/oct) was applied for smoothing. Startle magnitude was defined as the amplitude of the first peak in the resulting signal within a 25–100 ms latency window. Subsequently, an artefact rejection procedure was performed in which trials with baseline (-30 to 20 ms) activity greater than 2 standard deviations from the mean baseline activity were rejected. Null responses were defined as trials in which the standard deviation of the signal increased with less than 55% from baseline. Finally, startle data were z-transformed based on single-epoch data from all sessions per subject and converted to T-scores (T = z * 10 + 50). All subsequent analyses were conducted on the T-scores.

SUPPLEMENTAL RESULTS

Anxiety ratings

	During threat cue	During safe cue	After threat offset	After safe offset
Startle session	4.6 (1.7)	1.5 (1.1)	3.1 (1.6)	2.0 (1.3)
fMRI session	4.4 (2.0)	0.8 (0.9)	2.6 (1.5)	1.5 (1.6)

Table S1: Mean anxiety ratings (SD) on a scale from 0 (not anxious) to 10 (extremely anxious) for all conditions during the startle and fMRI sessions.
Subjects remained more anxious directly after threat offset than after safe offset (MRI $F(1,22) = 17.3$, $p < .001$; startle $F(1,25) = 12.3$, $p < .005$).

Imaging

Supplementary amygdala analyses

Given the extensive reports of amygdala involvement in activation of the defense system, we performed some supplementary analyses to further investigate the lack of amygdala activation to threat in our analysis. There were no significant amygdala activations comparing threat and safe onsets, also not when lowering our statistical threshold by doing a small volume correction for the bilateral amygdala using an anatomical mask (p-values > .1, FWE corrected for the bilateral amygdala). Given previous reports that the amygdala response may habituate quickly, we also analyzed the amygdala response in the first run only but again found no effects from either a whole brain or ROI analysis (p-values > .1). We did find significant right amygdala activation in response to the shock ($p < .001$, FWE corrected at the cluster level) and

there was also activation in the left amygdala to the shock at lower thresholds. This suggests that in our scans we had sufficient signal to noise ratio in ventral areas to be sensitive to bold changes in amygdala.

Threat offset vs. threat analysis

As a supplementary check to verify the specificity of the prefrontal responses to offset we investigated in a whole brain analysis whether the same prefrontal regions would be found in the comparison between threat offset to sustained threat and its onset. Importantly, we found clusters of deactivation in a very similar ventromedial prefrontal region, both when contrasting threat offset with threat onset (z = 4.83, $p < .001$ FWE corrected at the cluster, peak activation at x = 0, y = 32, z = -14) and threat offset versus threat sustained (z = 3.83, $p < .001$ FWE corrected at the cluster level, peak activation at x = 0, y = 32, z = -10). In this analysis we also observed activity in lateral prefrontal areas overlapping with the cluster that was found in the threat vs safe offset analysis, both for the threat offset vs onset comparison (z = 3.76, n.s. after whole brain FWE correction at the cluster level but $p < .01$ uncorrected at the cluster level and $p < .05$ FDR whole brain corrected at voxel level, peak activation at x = 38, y = 60, z = 10) and for the threat offset vs threat contrast (z = 4.37, n.s. after whole brain FWE correction at the cluster level but $p < .05$ uncorrected at the cluster level and $p = .001$ FDR whole brain corrected at voxel level, peak activation at x = 35, y = 66, z = 4).

Connectivity analyses

Region correlating with vmPFC seed	**MNI coordinates**			**Z-score**	**Voxels**
	X	Y	Z		
Negative correlations					
Left inferior frontal gyrus	-49	42	0	5.80	171
Left fusiform gyrus	-32	-42	-21	5.80	3172
Right inferior frontal gyrus	49	56	0	5.30	113
Right superior temporal gyrus / Amygdala / Insula	35	4	-32	4.57	153
Positive correlations					
Right superior temporal gyrus	66	-24	0	6.09	3190
Right precentral gyrus	52	-21	35	5.65	1941
Right cerebellum	32	-77	-24	5.30	416

Table S2. Results from the whole brain connectivity analyses using the vmPFC as the seed region. Statistic images assessed for cluster-wise significance; with a cluster defining threshold of $p = 0.001$ uncorrected, the 0.05 FWE-corrected critical cluster size was 37 voxels. MNI, Montreal Neurological Institute; vmPFC, ventromedial prefrontal cortex.

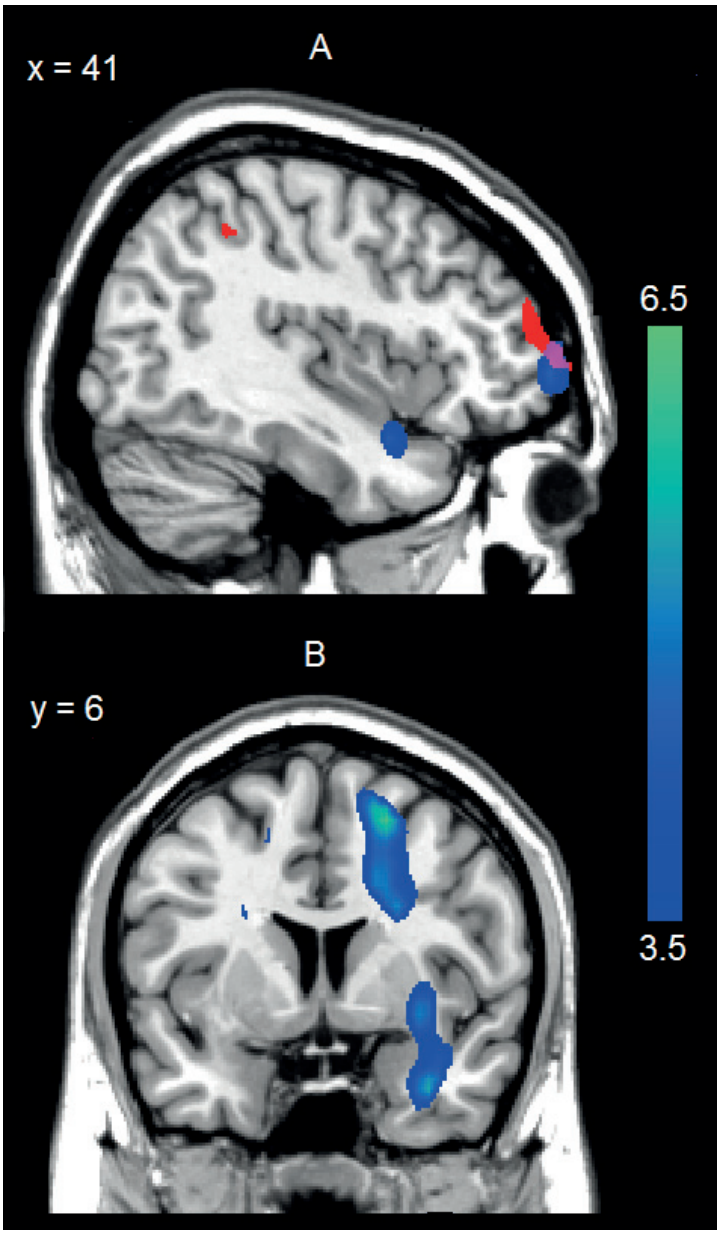

Figure S1. Areas of interest showing negative correlation with activity in subgenual vmPFC seed region. **A**) Region encompassing the amygdala and anterior insula (peak at x = 35, y = 4, z = -32). **B**) Right lateral prefrontal region (peak: x = 49, y = 56, z = 0) that overlaps with the region activated after threat offset (all active voxels from the Threat vs. Safe offset contrast displayed in red, overlap in purple). Results are visualized on an anatomical template image, the number in the top right corner represents the MNI coordinate for the selected slice and the scale is in T-scores.

CHAPTER 3

GENETIC VARIATION IN SEROTONIN TRANSPORTER FUNCTION AFFECTS HUMAN FEAR EXPRESSION INDEXED BY FEAR-POTENTIATED STARTLE

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ABSTRACT

The serotonin transporter (SERT) plays a crucial role in anxiety. Accordingly, variance in SERT functioning appears to constitute an important pathway to individual differences in anxiety. The current study tested the hypothesis that genetic variation in SERT function is associated with variability in the basic reflex physiology of defense. Healthy subjects (N = 82) were presented with clearly instructed cues of shock threat and safety to induce robust anxiety reactions. Subjects carrying at least one short allele for the 5-HTTLPR polymorphism showed stronger fear-potentiated startle compared to long allele homozygotes. However, short allele carriers showed no deficit in the downregulation of fear after the offset of threat. These results suggest that natural variation in SERT function affects the magnitude of defensive reactions while not affecting the capacity for fear regulation.

INTRODUCTION

The serotonin transporter plays a crucial role in anxiety. Accordingly, pharmacological adjustment of serotonin transporter (SERT) functioning by selective serotonin reuptake inhibitors (SSRIs) is among the most established clinical pharmacological strategies to control human anxiety disorders (Baldwin et al 2005; Bandelow et al 2002). It is therefore to be expected that genetic variation in SERT function underlies innate differences in individuals' fear reactivity. In the current study we investigated how genetic variance in the SERT gene affects the up and down regulation of basic fear responses.

To this end we studied the impact of a well-known polymorphism in the promoter region of the SERT gene (also called 5-HTTLPR), which influences anxiety-related personality (Lesch et al 1996). This common 5-HTTLPR polymorphism consists of an insertion/deletion of 43 bp in the 5' regulatory region of the gene, resulting in either a long or short allele. The short allele is associated with reduced SERT transcriptional activity in vitro (Heils et al 1996). Meta-analyses have more recently confirmed that carriers of the 5-HTTLPR short allele (S-carriers) report more anxiety related personality traits (Schinka et al 2004; Sen et al 2004). However effect sizes are small (Munafo et al 2009) and the relation between this polymorphism and anxiety- and stress related psychopathology remains a matter of debate (Karg et al 2011; Lonsdorf et al 2009a; Wankerl et al 2010).

A potentially fruitful approach to further investigate sequelae of genetic variability in serotonin transporter function is by establishing intermediate phenotypes associated with the 5-HTTLPR polymorphism (Canli 2008; Domschke and Dannlowski 2010). For example, imaging studies showed exacerbated amygdala activation towards fear cues in S-carriers (Hariri et al 2002; Munafo et al 2008). Another approach has been to compare 5-HTTLPR genotypes on peripheral psychophysiological measures such as skin conductance and startle during fear conditioning. In a classical conditioning study, S-carriers were more likely to show strong conditioned SCR responses than LL homozygotes who were found more often in a group of subjects that showed weak conditioning (Garpenstrand et al 2001). More recently, evidence was presented for stronger SCRs in S-carriers during vicarious conditioning (Crisan et al 2009). Finally, Lonsdorf et al. (2009b) reported that across the acquisition phase of a fear conditioning session, S-carriers showed stronger potentiation of the startle response. Potentiation of the startle reflex is a reliable index of the activation of the defensive system (Bradley et al 2005) and is widely used as an objective and rather specific measure of fearful responding (Grillon and Baas 2003; Hamm and Weike 2005). Taken together these results suggest that increased neural threat processing in S-carriers as witnessed by stronger

amygdala activations, may be reflected in autonomic measures of fear and defensive reflexes. Moreover, S-carriers may show altered amygdala regulation by the prefrontal cortex (PFC) (Heinz et al 2005; Pacheco et al 2009; Pezawas et al 2005). Since the interaction between these regions mediates fear regulation (Hartley and Phelps 2010; Quirk and Beer 2006), this implies that increased anxiety in S-carriers could stem from a genetic deficit in the capacity to suppress fear.

Here we investigated further how the 5-HTTLPR polymorphism affects fear reactions and the downregulation of fear responses. An instructed fear paradigm was employed, in which fear reactions were elicited in healthy volunteers by presenting cues that are identified before the experiment as signaling threat of electric shock (Bocker et al 2004; Grillon et al 1993; Grillon et al 1991). Moreover, startle was also measured after the termination of threat cues, during the transition from threat to a period of relative safety. In this way we could index how well subjects of each genotype were able to reduce fear when direct danger subsided. Previous work indicated that the capacity to return to a resting state after threat offset depends on prefrontal-limbic interactions (Klumpers et al 2010a). Based on the evidence that S-carriers display increased fear reactivity which perhaps relates to altered prefrontal control, we hypothesized that S-carriers would (a) demonstrate greater reactivity to threat cues by showing stronger startle potentiation and (b) would show a reduced capacity for fear regulation as reflected in a slower decline of startle potentiation after the offset of threat cues.

METHODS & MATERIALS

This study was approved by the medical ethical committee at the Utrecht University Medical Centre.

Subjects

Subjects were recruited through advertisements posted around the faculty and on the faculty website. A total of 95 subjects (aged 18-30) passed inclusion criteria and gave written informed consent. All subjects reported to be free of hearing problems, neurological conditions, cardiovascular disease and psychiatric diagnoses relevant to the current study, including mood and anxiety disorders. Moreover, included subjects reported no regular illicit drug use or use of psychoactive prescription drugs. Of these subjects, 94 completed the instructed fear paradigm. We excluded 11 subjects with missing data, corrupted data and/or minimal startle reactivity based on the criterion that each subject should have at least one artifact free, non-zero response for each condition in each task block (see task description

below). In the remaining 83 subjects, we could accurately determine 5-HTTLPR genotype for 82 subjects (60 female; see table 1). Of subjects these subjects, 76 were Caucasians of European descent. As an analysis excluding other ethnicities did not change the results, we decided to report on the full sample here.

Genotyping

DNA was collected with buccal swabs and isolated using a standardized kit (QiAmp DNA Mini Kit; Qiagen, Germany). 5-HTTLPR genotyping was performed using polymerase chain reaction (PCR) followed by gel electrophoresis as described by Lonsdorf et al. (2009a; see erratum). This procedure visualized for each subject either 2 short 486 bp DNA fragments (S/S), one short and one long (529 bp) fragment (S/L) or 2 copies of the long fragment (L/L). Out of the 83 subjects, we determined genotype successfully in duplicate for 82 subjects. Genotype percentages were: SS (17%), SL (57%), and LL (26%) and in accordance with the Hardy-Weinberg equilibrium ($p > .5$). Of note, three additional candidate polymorphisms (HTR1A C-1019G, COMT val158met, and DAT1 3' UTR VNTR) were assessed more exploratorily and did not affect fear-potentiated startle, the reduction in startle after threat offset and the anxiety ratings. Gene-gene interactions were not investigated given that for each interaction, smallest cells contained less than 5 subjects.

Stimuli & apparatus

Physiological recording and amplification was carried out using the BioSemi Active Two system with matching FLAT active Ag/AgCl electrodes (BioSemi, Amsterdam, The Netherlands). Startle probes were 50-ms white noise bursts with instantaneous rise time presented at 106 dBa through foam in-ear earplugs (Earlink, Aero Company auditory systems, IN, USA). Eye blink startle to these probes was measured by electromyographic (EMG) recordings from the orbicularis oculi. For EMG measurement, electrodes were placed under the right eye; one centralized under the pupil and the other 15 mm lateral towards the outer cantus of the eye. Shocks were administered through a constant current simulator (Digitimer DS7A, Digitimer Ltd., Letchworth Garden City, United Kingdom) with tin cup electrodes located over the upper, inner wrist of the left arm. Two photos of male faces with neutral emotional expression from the NIMSTIM database (Tottenham et al 2009; model 21 & 23) were used as cues to signal threat and safety in the instructed fear task. One of the photos was presented with blue background and the other with orange background to increase salience and distinctiveness of the cues.

Procedure

Subjects first completed a medical screening questionnaire and a Dutch translation of the trait portion of the Spielberger State/Trait Anxiety Inventory (STAI; (Defares et al 1980)). Electrodes for startle recording and shock administration were applied and the subjects underwent a standardized shock workup procedure consisting of 5 sample shocks to set the shock intensity individually for each subject at a level considered "quite annoying" (see Klumpers et al 2010a; Klumpers et al 2010b). This goal of this procedure was not revealed to subjects to assure an unbiased response. The final intensity of the electrical stimulation varied between subjects from 0.5 to 5.8 mA. After the workup, subjects received instructions about the task (see below). When the instructions were clear to subjects the earplugs were inserted. Twelve startle probes were presented for startle habituation. This initial series of probes was also used as a baseline startle measurement. Immediately after the last habituation probe the instructed fear task commenced.

Instructed fear task

The task was explained by showing the cue that signaled shock threat for that subject, with the instruction that "at any time during presentation of this cue shocks could be administered". Next subjects were presented with the second cue, and were instructed that they "would never receive shocks" when this cue was presented (Figure 1). Whether the orange or blue cue signaled shock threat was evenly distributed across subjects. During the task, cues were presented with a variable duration to make the offset of the conditions unpredictable (4-8 s, M = 5.6s). The word *"RUST"* ('rest' in Dutch) was presented on the screen during the intervals between cues. Subjects were instructed to relax during these periods. Rest periods lasted between 6 and 20.5 s (M= 11.1 s). Startle probes could be presented at three latencies: (a) during the cues, 3 s after cue onset ("cue probes"), (b) in the rest period following the cue, 1.5 s after cue offset ("offset – early probes") or (c) 5 s after cue offset ("offset – late probes"). Subjects were instructed to ignore the probes as much as possible. The startle probe timing is illustrated in Figure 1.

The full experiment consisted of 5 experimental blocks with brief breaks after blocks 2 and 4. During these breaks and at the end of the experiment, subjects retrospectively rated their state anxiety during each cue and immediately following the offset of each cue on a computerized scale from 0 (*not anxious/nervous*) to 10 (*very anxious/nervous*). After the breaks, instructions regarding the threat and safe cue were repeated and 4 startle probes were administered for startle habituation before continuation of the experiment.

Each experimental block contained 10 presentations of each cue. For each condition (threat / safe), 4 cue probes and 4 offset probes (2 X offset - early, 2 X offset - late) were presented per block. The mean interval with a previous startle probe was kept at 20 s for each of the 6 probe types (2 conditions X 3 probe latencies), with a minimum interval of 16 s after a previous probe or shock reinforcement. A semi-random event order was created, with no more than three consecutive repetitions of the same cue. To exclude order effects, half of the subjects received this event order while for the other half of the subjects threat and safe conditions were presented in the reversed order. This was distributed evenly across genotypes (χ^2 *p*-value > .6). A total of 9 shocks were administered at varying, semi-random time points to reduce predictability to reinforce the instruction that shocks could be administered at any time during the threat condition.

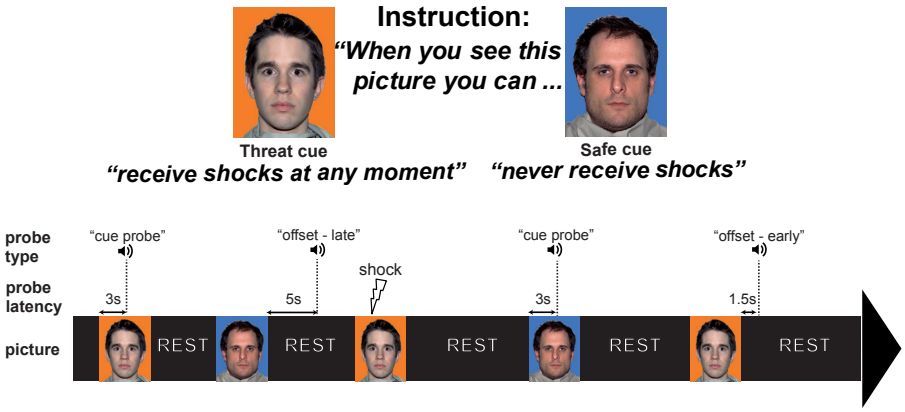

Figure 1. Conditions of shock threat and safety were cued through presentation of pictures of neutral faces. The bottom line illustrates how startle probes were presented at three latencies in an excerpt from an experimental block. Startle probes were presented during the cue ('cue probe'), in the rest period at 1.5 s after cue offset ('offset – early') or 5 s after cue offset ('offset – late'). Cues and rest periods varied in duration, details can be found in the text under "*instructed fear task*".

Data processing & statistical analysis

Startle data were pre-processed and checked for artifacts according to previously published guidelines (Blumenthal et al 2005) and procedures (Bocker et al 2004; Klumpers et al 2010a; Klumpers et al 2010b). To determine baseline startle, startle magnitudes from the habituation trials were log-transformed to correct deviations from normality. For the analysis of the instructed fear task, startle magnitudes for all experimental conditions were transformed to *z* scores per subject to simultaneously control for impact of baseline startle on fear-potentiated startle. As recommended (Grillon and Baas 2002), raw data are also reported. Finally, data

were averaged according to condition (threat, safe) and probe latency (cue, offset - early and offset- late). Similarly, the state anxiety ratings were averaged according to condition (threat, safe) and latency which only had 2 levels for the rating data (cue, offset).

All the subsequent statistical analyses were carried out in SPSS 17 (SPSS, Chicago, Illinois). Gender was not explicitly matched between genotypes and added as covariate for all genotype comparisons. Consistent with previous research (Brocke et al 2006; Lesch et al 1996; Lonsdorf et al 2009b), we grouped genotypes into S-allele carriers vs. L/L homozygotes to ascertain a minimum sample size of 20 subjects for each group (see Table 1). Genotype groups were compared on trait anxiety, final shock intensity, and baseline startle amplitude in univariate ANOVAs.

To test effects of our task on fear potentiation of startle, we performed a 2 X 3 repeated measures ANOVA with within factors Condition (Threat, Safe) and Probe Latency (cue, offset - early, offset - late). Fear potentiated startle (FPS) was defined as the difference between startle amplitude in threat and safe conditions and assessed through the Condition main effect. Regulation of defensive state after threat offset was assessed by the Condition X Probe Latency interaction. To evaluate the effect of the 5-HTTLPR polymorphism on our startle data, a between subjects factor 5-HTTLPR (S-carriers vs. LL) was added. Differences in FPS between genotypes were assessed by the 5-HTTLPR X Condition interaction and difference in regulation of defensive states by the 5-HTTLPR X Condition X Latency interaction. To explore genotype effects on FPS further, we assessed the 5-HTTLPR X Condition interactions for each Probe Latency separately.

A similar strategy was used to analyze the rating data. State anxiety ratings from one subject were missing due to technical error. In the remaining subjects, we verified that our task produced the intended effects by performing a 2 X 2 repeated measures ANOVA with within factors Condition (Threat vs. Safe) and Latency (cue, offset). Increases in state anxiety in Threat vs. Safe conditions were assessed through the Condition main effect. Fear regulation after threat offset was assessed through the Condition X Latency interaction. To evaluate whether the 5-HTTLPR polymorphism had an effect on anxiety induced by our task, a between subjects factor 5-HTTLPR (S-carriers vs. LL) was added. Differences between genotypes in task-related anxiety were assessed through the interaction between 5-HTTLPR and Condition and differences in the regulation of anxiety by the 5-HTTLPR X Condition X Latency interaction. Moreover, genotypes were compared on anxiety in each condition at each latency (Threat cue, Threat offset, Safe cue, Safe offset) in univariate ANOVAs.

RESULTS

Sample descriptives

Gender distribution, mean shock intensity, baseline startle amplitude and trait anxiety score for the 5-HTTLPR genotype groups are displayed in Table 1. 5-HTTLPR short allele carriers tended to report higher trait anxiety compared to L/L homozygotes, there were no other differences (see Table 1 for descriptives and statistics).

	S/S	S/L	L/L	S-carriers vs. LL
N	14	47	21	
% females[1]	79	77	62	$\chi^2 = 1.2$, $p = .18$
Trait anxiety[2] (STAI-t score)	35.4 (8.2)	37.6 (7.6)	32.9 (7.2)	$F_{(1,79)} = 3.6$, $p = .06$
Final shock intensity (μA)[2]	1.6 (0.8)	2.2 (1.0)	2.3 (1.2)	$F_{(1,79)} = 1.6$, $p = .21$
Baseline startle amplitude (μV)	70.4 (43.0)	90.3 (55.1)	78.0 (55.5)	$F_{(1,79)} < 1$

Table 1. Sample size and gender distribution as a function of 5-HTTLPR genotype as well as means (SD) for trait anxiety scores, shock intensity and baseline startle amplitude. Statistics are reported for the comparison between S-carriers and LL homozygotes. [1]Gender was used as a covariate in all analyses comparing genotypes. [2]Adding trait anxiety score and/or shock intensity as a covariate in the startle data did not change the results.

Instructed fear results

State anxiety

Across genotypes, subjects consistently reported higher state anxiety following threat vs. safe cues (Condition main effect: $F_{(1,81)} = 170.8$, $p < .001$, $\eta^2 = .69$). In the rest period following the offset of the cues, the difference in anxiety between Conditions was reduced as witnessed by a Condition X Latency interaction ($F_{(1,81)} = 123.7$, $p < .001$, $\eta^2 = .60$). The paradigm thus induced the expected effects on reported state anxiety with an increase during the threat cues that was regulated towards baseline after threat offset (Table 2). 5-HTTLPR genotypes did not differ in state anxiety (5-HTTLPR X Condition: $F_{(1,78)} < 1$; 5-HTTLPR X Condition X Latency: $F_{(1,78)} = 2.8$, $p = .10$, $\eta^2 = .04$; see Table 2).

	S/S	S/L	L/L	S-carriers vs LL
State anxiety Threat - cue	5.8 (2.5)	5.2 (2.3)	4.8 (2.7)	$F_{(1,78)} < 1$
State anxiety Threat - offset	4.1 (2.1)	2.7 (1.9)	3.1 (2.4)	$F_{(1,78)} < 1$
State anxiety Safe - cue	1.4 (1.2)	1.0 (0.8)	1.0 (1.1)	$F_{(1,78)} < 1$
State anxiety Safe - offset	2.6 (2.1)	2.3 (1.8)	1.7 (1.7)	$F_{(1,78)} = 1.6$, $p = .21$.

Table 2. Mean (SD) state anxiety ratings as a function of 5-HTTLPR genotype. State anxiety was rated on a scale from 0 (not at all anxious) to 10 (very anxious). Statistics are reported for the comparison between S-carriers and LL homozygotes.

Startle data

Mean startle data are presented in Figure 2 (top panel). Tested across probe latencies, threat cues induced strong increases in startle amplitude relative to the safe cues (Condition main effect: $F_{(1,82)} = 143.6$, $p < .001$, $\eta^2 = .64$). Fear potentiated startle (FPS), the difference between startle amplitude during threat and safe, diminished over probe latencies (Condition X Probe Latency: $F_{(2,164)} = 133.3$, $p < .001$, $\eta^2 = .62$). FPS was strong during the cues (Condition effect - cue probes only: $F_{(1,82)} = 250.0$, $p < .001$; $\eta^2 = .75$), weak 1.5 s after offset of the threat cue (Condition effect - offset-early probes: $F_{(1,82)} = 3.5$, $p = .08$; $\eta^2 = .04$) and absent 5 s past the offset of the threat cue (Condition effect - offset-late: $F_{(1,82)} < 1$). Subjects thus successfully regulated their defensive states after threat offset.

Mean FPS as a function of 5-HTTLPR genotype can be found in Figure 2 (bottom panel). Short allele carriers showed stronger FPS averaged across probe latencies than L/L subjects (5-HTTLPR X Condition: $F_{(1,79)} = 4.0$, $p = .048$; $\eta^2 = .05$). Further exploration revealed that S-carriers showed stronger FPS for cue probes (5-HTTLPR X Condition - cue probes only: $F_{(1,79)} = 4.24$, $p = .043$; $\eta^2 = .05$) but not for the probe latencies following cue offset (F's < 1). After threat cue presentation, S-carriers were equally efficient in reducing fear as L/L subjects (Figure 2, lower panel) which was also apparent from the non-significant 5-HTTLPR X Condition X Probe Latency interaction ($F_{(2,158)} = 1.3$, $p = .28$; $\eta^2 = .03$). For completeness raw data for each condition and probe latency are reported in Figure 3.

Finally, as a data quality check supplementary analyses were undertaken to confirm that FPS during the cue, where differences between genotypes occurred, provided a valid and reliable measure of individuals level of fear expression. Stronger FPS during cues correlated with greater increases in state anxiety during the threat condition (Spearman's $\rho = .39$, $p < .001$; Figure 4 top panel), indicating that basic defensive fear responses and subjective state anxiety were interlinked. Further, FPS during the first and second halves of the experiment showed a strong positive association (Spearman's $\rho = .58$, $p < .001$ for cue probes; see Figure 4 bottom panel). This confirmed that average FPS to the cues provided a reliable measure of subjects' tendency to express fear.

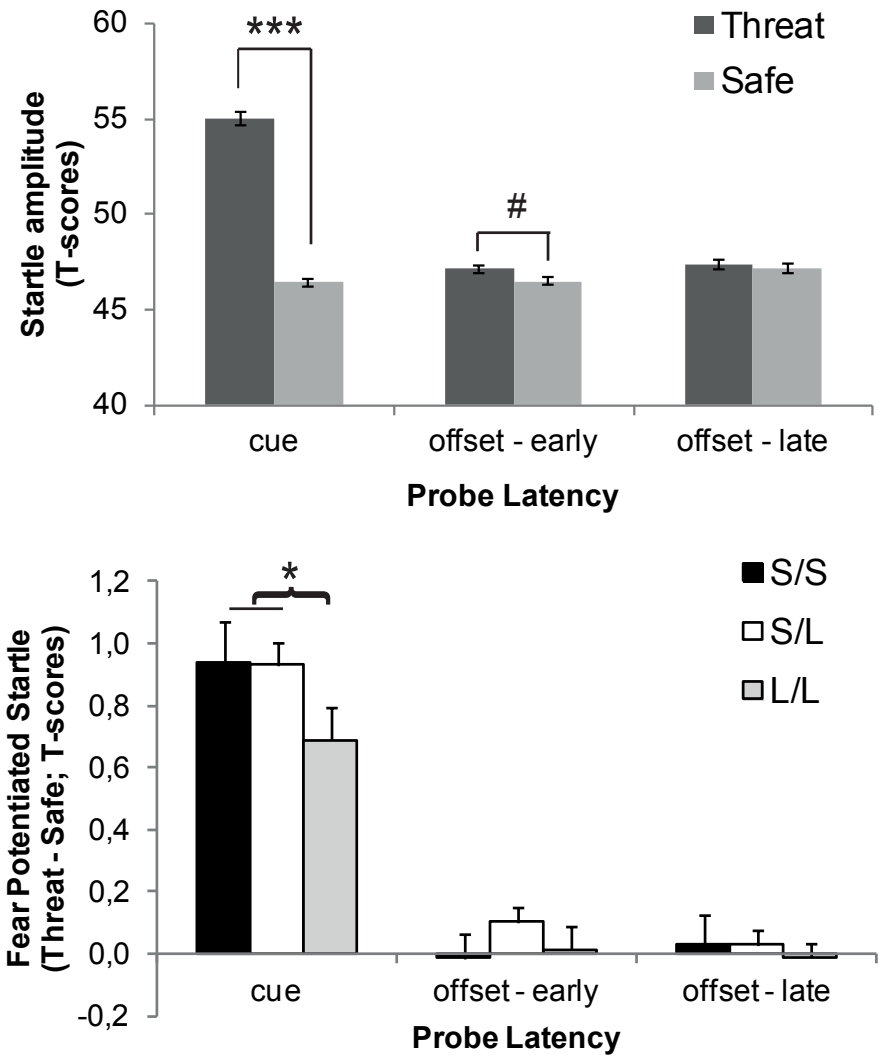

Figure 2. Mean startle amplitudes as a function of probe latency. The top panel depicts startle data averaged across all genotypes as a function of condition and probe latency. The bottom panel depicts fear-potentiated startle (Threat startle amplitude – Safe startle amplitude) for each 5-HTTLPR genotype as a function of probe latency, revealing stronger FPS in S-carriers during cue probes. Error bars represent standard error of the mean $^{***}p < .001$, $^{*}p < .05$, $^{\#}p < .1$

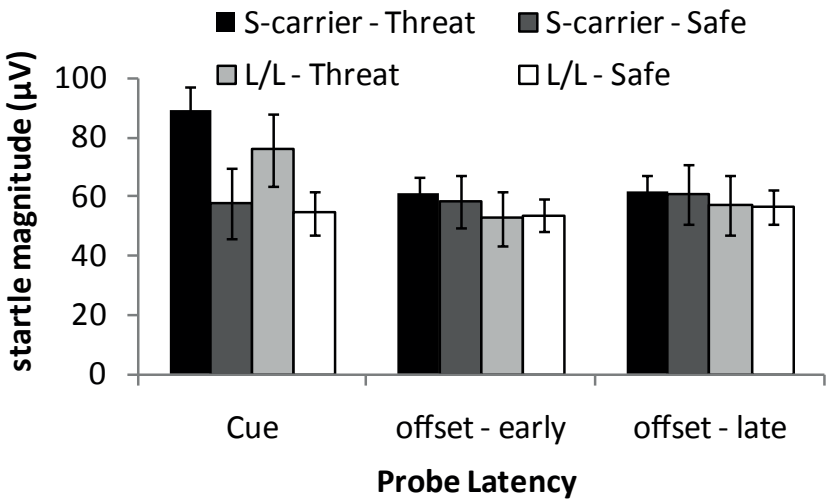

Figure 3. Mean raw startle EMG data in microvolt (µV) for the 5-HTTLPR groups as a function of probe latency and condition. SS and SL genotypes are combined into S-carriers for illustrative purposes. Error bars represent standard error of the mean

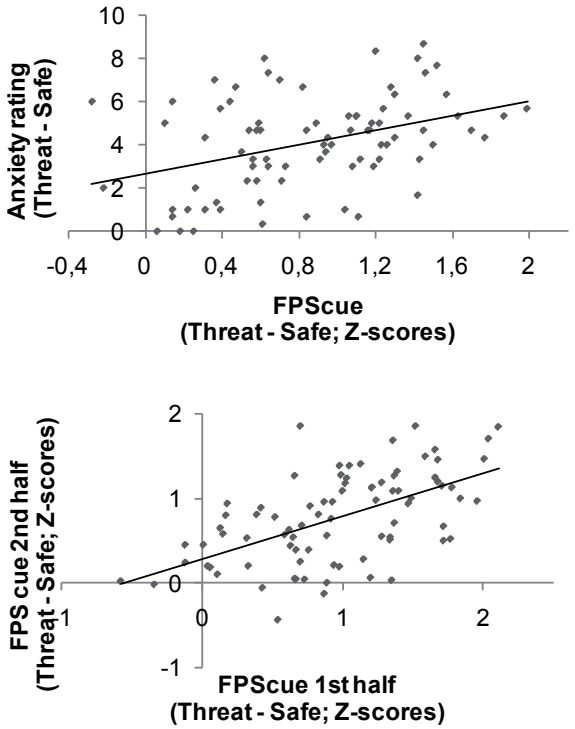

Figure 4. Scatter plots visualizing (top panel) the correlation across subjects between FPS and anxiety ratings during the cue and (bottom panel) FPS for the first 50% and last 50% of cue probes.

DISCUSSION

Our study tested the hypothesis that naturally occurring genetic variation in SERT function modulates human fear reactions. We elicited robust fear responses, measured by fear-potentiated startle (FPS), in a group of healthy subjects genotyped for the 5-HTTLPR polymorphism. Our data indicated that 5-HTTLPR short allele carriers show stronger physiological fear responses under threat compared to subjects carrying two long alleles. However, genotypes were equally efficient in the down regulation of their defensive state following threat termination.

Together with previous work (Lonsdorf et al 2009b), these results illustrate how the 5-HTTLPR polymorphism is associated with changes in basic defensive responding as indexed by potentiation of the startle reflex (Bradley et al 2005; Grillon and Baas 2003; Hamm and Vaitl 1996). As the amygdala plays an important role in startle potentiation (Anders et al 2004; see Davis 2006 for review), our results fit nicely with the evidence for 5-HTTLPR mediated amygdala hyperactivity (Dannlowski et al 2010; Munafo et al 2008). These changes in basic defensive reactivity may contribute to increases in (pathological) anxiety observed in S-carriers (e.g. see Lonsdorf et al 2009a; Schinka et al 2004). While 5-HTTLPR genotypes did not differ significantly in retrospectively rated state anxiety in our study, reports of higher subjective fear were associated with greater FPS. In line with previous work, we also demonstrated that our measurement of FPS was reliable (Klumpers et al 2010b) which is an important prerequisite for assessing trait-like individual differences (Larson et al 2000).

Previous studies suggested that 5-HTTLPR genotype may gate fear learning as S-carriers show enhanced responding in fear conditioning paradigms (Crisan et al 2009; Garpenstrand et al 2001; reviewed in Lonsdorf and Kalisch 2011; Lonsdorf et al 2009a). Here, we demonstrate that also when subjects are explicitly made aware beforehand which cue signals danger, S-carriers respond more strongly to threat cues. Our results therefore suggest that the 5-HTTLPR polymorphism more generally affects fear reactivity, and not specifically the acquisition of fear responses through classical conditioning. This is also consistent with the observation of enhanced amygdala responding to intrinsically affective fear stimuli, such as fearful and angry faces, in S-carriers (Domschke and Dannlowski 2010; see Munafo et al 2008 for overview).

It is noteworthy that several previous studies found no difference between 5-HTTLPR genotypes in potentiation of startle (Armbruster et al 2009; Brocke et al 2006; Larson et al 2010; Pauli et al 2010). In all these studies startle potentiation was induced through aversive picture presentation. Comparing the affective picture paradigm directly with a shock threat

paradigm similar to ours, Lissek et al. (2007) reported that the physical threat of electric shock provoked considerably higher anxiety states than presentation of aversive pictures. In line with this, several studies that assessed 5-HTTLPR effects (Armbruster et al 2009; Brocke et al 2006) did not find significant startle potentiation across all subjects. Perhaps also relevant is that while acute treatment with the SSRI citalopram exacerbates fear potentiated startle in the instructed fear paradigm in humans (Grillon et al 2007) there is no effect on picture-induced emotion modulated startle responses (Browning et al 2007). Therefore it is possible that SERT mediated effects on FPS come into play most clearly in the context of direct and relatively strong threats. Similarly, in a recent study genotype effects on amygdala responses emerged only under a heightened stress state (Cousijn et al 2010) illustrating that genetic effects on defensive systems may arise most clearly when sufficient levels of anxiety are reached.

In line with previous work (Klumpers et al 2010a), we again observed that healthy subjects regulated their defensive state quickly, but without significant differences between genotypes. Our data thus suggest that 5-HTTLPR genotype affects the generation of the fear response, but not its subsequent downregulation following threat offset. The 5-HTTLPR polymorphism also does not affect the magnitude of fear extinction (Lonsdorf et al 2009b) or the recovery from defensive states induced by aversive pictures (Larson et al 2010). Moreover, in a recent neuroimaging study S-carriers were able to down regulate their excessive amygdala reactivity to fear pictures through volitional reappraisal (Schardt et al 2010). These findings match our results and suggest that 5-HTTLPR genotype primarily influences fear reactivity and not the basic capacity to reduce fear after danger subsides. Importantly however, both in the current study and in previously mentioned work (Lonsdorf et al 2009b; Schardt et al 2010) primarily healthy volunteers were tested. Across healthy populations S-carriers may be able to overcome their innate tendency to react more strongly to threats when required, perhaps by recruiting additional prefrontal resources (Schardt et al 2010). However a different picture may emerge in those suffering from excessive anxiety or those exposed to severe stress (Caspi et al 2010; Karg et al 2011).

In conclusion, we report evidence that innate variability in SERT function modulates basic defensive reactivity indexed by FPS but does not affect the basic capacity for fear downregulation. These results yield further insight in the relation between genetically determined variability in serotonin transporter function and individual differences in anxiety.

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CHAPTER 4

INDIVIDUAL DIFFERENCES IN THE NEURAL MECHANISMS UNDERLYING ANXIOUS ANTICIPATION AND FEAR REGULATION: THE 5-HTTLPR POLYMORPHISM

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ABSTRACT

The up and down regulation of anxiety require distinct neural circuits. In the current study we delineated neural correlates for these processes in a comparatively large sample. Moreover, we investigated the possible influence of the 5-HTTLPR polymorphism on inter-individual variance in these neural processes. Sixty-nine subjects viewed instructed cues of shock threat and safety that were separated by resting phases. Functional magnetic resonance imaging was used to map neural responses to both the on- and offset of these cues. Results showed that across subjects, the onset of threat cues was associated with moderate increases in subjective state anxiety and with blood oxygen level dependent (BOLD) signal increases in a limbic network that included the anterior insula, dorsal anterior cingulate and midbrain. The offset of danger cues resulted in a reduction in state anxiety and was associated with signal increases in the lateral prefrontal cortex. Reactivity to the threat cue, specifically in the left anterior insula, was stronger in subjects carrying two short alleles for the 5-HTTLPR polymorphism than in long allele homozygotes. Moreover, stronger limbic reactions were associated with greater fear potentiated startle measured in a separate session. Short allele carriers also showed stronger lateral prefrontal reactivity at threat offset, which could explain how S-carriers were able to regulate their anxiety after threat termination equally well as LL subjects. Our results provide further insight into the neuroanatomical mechanisms that enable the flexible up and down regulation of defensive states. Moreover, they illustrate how genetic variability in serotonin transporter function may affect the generation and control of fear responses.

INTRODUCTION

A significant proportion of individual differences in anxiety in everyday life may be determined through genetic predispositions (Canli 2008; Hettema et al 2001). This suggests that in anxiety-prone individuals, genetic factors bias neuroanatomical defense systems to be more responsive to potential threats. The serotonin transporter (SERT) gene has emerged as an important candidate gene for anxiety. The common 5-HTTLPR polymorphism in this gene involves an insertion/deletion of a 43 base pair sequence in the promoter region, leading to a either a long or a short allele. The short allele is associated with reduced SERT expression in vitro (Heils et al 1996; Hu et al 2006). Crucially, short allele carriers (s-carriers) report more trait anxiety than subjects carrying two long alleles (L/L homozygotes) (Lesch et al 1996; Schinka et al 2004).

Functional neuroimaging research has demonstrated that s-carriers also exhibit elevated amygdala reactivity when viewing aversive pictures or pictures of faces expressing negative emotion (Dannlowski et al 2010; Hariri et al 2002; Munafo et al 2008). However, the effect of this polymorphism on neural correlates of anxiety remains untested. This is especially important given that anxiety is typically associated with other neural concomitants than the processing of negative images, particularly with activity in the anterior insula and dorsal anterior cingulate rather than in the amygdala (Drabant et al 2011; Klumpers et al 2010a; Mechias et al 2010; Straube et al 2007). Moreover, the anticipation of negative events, rather than the processing of inherently negative stimuli, is central to the concept of anxiety (Barlow 2000).

In the current study, the neural systems that allow a switch between calm states and anxious apprehension of a stressor were mapped. Moreover, we investigated the possible influence of common genetic variation in the serotonin transporter (SERT) gene on inter-individual variance in the reactivity of these systems. To this end, we employed a task in which two cues are associated either with the possibility of receiving an electric shock or with short-term safety (Grillon et al 1991; Phelps et al 2001). Using functional magnetic resonance imaging (fMRI), we tested the hypothesis that the presence of the 5-HTTLPR S-allele leads to extended reactivity to threat cues, as reflected in greater neural activity across threat responsive regions.

The 5-HTTLPR polymorphism has been associated with altered connectivity between prefrontal and limbic areas, with either reduced or enhanced connectivity depending on the specific prefrontal areas involved (Heinz et al 2005; Pezawas et al 2005). Because the interaction between prefrontal areas and limbic regions is considered of crucial importance

for the regulation of defensive states (Hartley and Phelps 2010; Quirk and Beer 2006), this suggests that increased anxiety in S-carriers could evolve out of a reduced capacity for prefrontal fear regulation. Previously we reported in a smaller subsample that in the instructed threat paradigm the transition from threat to safety resulted in fast reductions in state anxiety and startle potentiation in healthy subjects (Klumpers et al 2010a). Moreover, this transition was associated with activity changes in the lateral and ventromedial prefrontal cortex (Klumpers et al 2010a), in line with the presupposed roles for these areas in fear down regulation (Delgado et al 2008; Eippert et al 2007; Phan et al 2005). As an additional hypothesis we investigated the possibility that the S-allele would be associated with reduced prefrontal control of anxiety by comparing genotypes on prefrontal reactions to the offset of the threat cues.

Of note, recent work has found no evidence for a reduced capacity in S-carriers to downregulate fear. More specifically, three studies have shown that while S-carriers exhibit excessive defensive reactions compared to L/L homozygotes, there is no reduction in the capacity for fear regulation. This was true in a study that investigated the extinction of conditioned fear-potentiated startle (Lonsdorf et al 2009b), an investigation of reductions in amygdala reactivity following the reappraisal of images of negative valence (Schardt et al 2010), and in the reduction of fear-potentiated startle after the termination of instructed threat cues in a sample that overlapped with the current sample (Klumpers et al in press). Therefore we hypothesized that S-carriers may use additional recruitment of the prefrontal cortex to mitigate higher levels of fear.

METHODS

This study was approved by the medical ethical committee at the Utrecht University Medical Centre.

Subjects

All subjects were recruited through advertisements posted around the faculty and faculty website. A total of 73 subjects (aged 18-30) passed inclusion criteria and gave written informed consent. All subjects reported to be free of hearing problems, neurological conditions and cardiovascular disease and psychiatric diagnoses relevant to the current study, including mood and anxiety disorders. Moreover, included subjects reported no regular illicit drug use or use of psychoactive prescription drugs. MRI and startle data for 21 subjects reported on here were described earlier (Klumpers et al 2010a). Fifty-two subjects were

added to this sample to allow investigation of genotype effects. About 50% of these subjects previously participated in research in our laboratory and we specifically attempted to include as many 5-HTTLPR homozygotes (SS and LL) as possible. For the total sample of 73 subjects, 72 completed the instructed fear paradigm in the MRI scanner. We excluded 2 subjects with corrupted data due to a technical issue. In the remaining 70 subjects, for 69 subjects we could successfully determine 5-HTTLPR genotype in duplicate. Over 90% of these subjects were Caucasians of European descent. As an exploratory analysis excluding other ethnicities did not change the main findings, we report on the full sample. Further sample descriptives can be found in Table 1 of the results section.

Genotyping

DNA was collected with buccal swabs and isolated using a standardized kit (QiAmp DNA Mini Kit; Qiagen, Germany). 5-HTTLPR genotyping was performed using polymerase chain reaction (PCR) followed by gel electrophoresis (as described in Lonsdorf et al 2009a (see also erratum)). This procedure visualized for each subject either 2 short 486 bp DNA fragments (S/S), one short and one long (529 bp) fragment (S/L) or 2 copies of the long fragment (L/L). Genotype percentages in the final group were: SS (22%), SL (32%), and LL (46%).

Procedure

After subjects had been admitted to the study, they were invited for two sessions for fMRI and startle recording. Startle data are described elsewhere (Klumpers et al in press; Klumpers et al 2010a). Prior to the scan session, subjects filled in personality and medical questionnaires for screening. Subjects were installed in the scanner and electrodes for shock administration were applied. An anatomical scan was acquired for each subject. Then, subjects underwent a shock work up procedure to set the shock intensity for each subject individually (Klumpers et al 2010a; Klumpers et al 2010b). The shock work-up procedure was introduced by informing the subjects that they would receive several shocks of varying intensity. No information was given to the subject regarding the goal of this procedure, particularly that subjects' responses determined the intensity used in the experiment. The work-up consisted of five sample shocks in total, or three shocks for those subjects that participated earlier in the startle experiment. Starting intensity was always 2.0 mA or was based on the intensity used in the startle experiment (with a correction for the use of a high-pass filter in the fMRI session, which reduced aversiveness of the shocks). Each shock was rated by the subject and shock intensity was subsequently adjusted in order to achieve an intensity that was rated as "quite painful/annoying", a rating of 4 on a 5-point scale that ranged from "not painful/annoying" to "very painful/annoying". Shock intensities were adjusted step by step using a standardized

procedure to a level that was rated "quite painful/annoying". After the final shock, the intensity was adjusted once more and this final intensity was used throughout the experiment. After the work-up subjects received instructions on the instructed fear task (described below). Total duration of the task was approximately 30 minutes. Afterwards, subjects were debrieved, paid and thanked for their participation.

Stimuli

For electrical stimulation, a train of 150 2-ms pulses was administered at a rate of 200 Hz with a total duration of 750 ms. Shocks were administered through a constant current simulator (Digitimer DS7A, Digitimer Ltd., Letchworth Garden City, United Kingdom) with tin cup electrodes located over the upper, inner left wrist. To avoid distortion of the MR signal by shock stimulation, a non-magnetic high pass filter was placed between the stimulator and the MRI-compatible, carbon-wired silver electrodes used for shock administration. The intensity of the electrical stimulation varied between subjects from 1.0 to 7.8 mA. Periods during which subjects could (threat) and could not (safe) receive shock administration were cued through pictures of two male faces. Pictures were selected from the Psychological Image Collection at Stirling (PICS, http://pics.psych.stir.ac.uk/) and each received neutral ratings in a pilot study. One of the pictures was presented in blue and the other in yellow to increase salience and distinctiveness of the cues.

Instructed fear task

Subjects were instructed that the two cues signaled when a shock was to be expected. This was explained by showing the cue (face) that signaled shock threat for that subject, with the instruction that "at any time during presentation of this face shocks could be administered". Next they saw the second cue, and were instructed that they "would never receive shocks" when this face was presented. Whether the yellow or blue cue signaled shock threat was evenly distributed over subjects and genotypes ($\chi^2 = .01$, $p = .99$). The subjects were explicitly instructed to rest when there was no face on the screen. Accordingly, the word 'RUST" (rest in Dutch) was presented on the screen during the inter trial interval.

The experiment consisted of three 10-minute runs that contained 14 presentations of each face. During each run, pictures were presented in a semi-random order that was counterbalanced over subjects, with no more than 3 consecutive repetitions of the same condition. Picture duration varied between 6 and 12 seconds (9.3 seconds average) to allow more reliable separation of the on- and offset responses. The rest period between pictures

ranged between 8 and 12 seconds (9.9 seconds average). Shocks were presented at varying time points during the threat condition to reinforce the instruction that shocks could occur at any moment during the threat cue. Two or three shocks were administered per run, with a total of six or seven shocks depending on whether subjects already participated in the startle session. This variation in the number of shocks was introduced to avoid that subjects who participated in the startle session would receive the same number of shocks in the fMRI session and thus could predict whether shocks could be delivered. There was no association between SERT genotype and the number of shocks received in the MR scanner ($\chi^2 = 1.6$, $p = .46$). In previous work we showed that repeating the instructed fear paradigm in general (Klumpers et al 2010b) and this paradigm specifically (Klumpers et al 2010a) in the same subjects does not cause changes in subjective, startle or neural outcome measures. Immediately after the end of each run subjects retrospectively rated their state anxiety during the preceding run on a scale from 0 (not anxious/nervous) to 10 (very anxious/nervous). Subjects first rated anxiety during presentation of each cue and then rated their level of anxiety for the period (immediately) after the offset of the cues.

Imaging

Imaging was performed on a Philips 3T Achieva MRI scanner (Philips Medical Systems, Best, The Netherlands). 2250 T2*-weighted volumes of .813 seconds were obtained in three runs (TE = 23 ms, TR = 15.6 ms, FOV = 224 x 224 x 136.5 mm, flip angle = 8.85°, matrix = 64x64, voxel size = 3.5mm isotropic). Each volume consisted of 39 sagittal slices. The 3D PRESTO sequence (Neggers et al 2008; Ramsey et al 1998) was used combined with echo shifting and parallel imaging acceleration (Pruessmann et al 1999), which allows fast whole brain scanning (Neggers et al 2008). Furthermore, we used in-plane segmentation (van Gelderen et al 1995) allowing for a shorter echo-train length and lower echo time to reduce distortion artefacts and signal dropout in regions susceptible for artefacts (e.g. ventral prefrontal areas, amygdalae). A T1-weighted anatomical image of 175 sagittal slices was obtained for registration purposes (TR = 8.4 ms, TE = 3.8 ms, FOV = 288 x 288 x 175mm, flip angle = 17°, voxel size = 1mm isotropic).

Data processing and analysis

Previous studies investigating 5-HTTLPR genotype effects have frequently grouped SS and SL subjects into an S-carrier group. However, there is evidence that personality (Sen et al 2004), brain reactivity (Bertolino et al 2005), brain structure (Pacheco et al 2009), and gene expression (Hu et al 2006) varies more or less linearly with the number of S-alleles. As an overall strategy, we therefore report data for each genotype (SS, SL, LL) separately. Because of

the unequal sample sizes per genotype (see Table 1 in the results section) we checked whether there were significant inequalities in variance for the three groups through Levene's test in SPPS 17.0 (SPSS Inc., Chicago, Illinois) for each dependent variable. This was the case for none of the variables that are reported to show significant genotype effects. For each dependent variable, we first evaluated whether there was any difference between the three genotypes in an omnibus analysis of variance (ANOVA). In case this ANOVA indicated differences between groups, we compared the genotypes in two-sample t-tests. We repeated all the analyses for the contrast S-carriers (SL+SS) versus LL homozygotes. Results that reached significance are reported to facilitate comparison with previous work.

Genotype groups were compared on gender distribution in a Chi-square test and on trait anxiety and final shock intensity in univariate ANOVAs. State anxiety ratings were averaged across runs and we verified that our task produced the intended effects by performing a 2 X 2 repeated measures ANOVA with within-subject factors Condition (Threat vs. Safe) and Latency (cue, offset). Increases in state anxiety in Threat vs. Safe conditions were assessed by follow-up paired sample T-tests to compare state anxiety during threat and safe cues. To test whether there was a significant reduction in state anxiety following threat offset, state anxiety during the threat cue was contrasted with state anxiety after threat offset. Fear regulation after threat offset was assessed through the Condition X Latency interaction. To evaluate whether the 5-HTTLPR polymorphism had an effect on anxiety induced by our task, a between-subjects factor 5-HTTLPR (SS, SL, LL) was added. Differences between genotypes in task-related anxiety were assessed through the interaction between 5-HTTLPR and Condition and differences in the regulation of anxiety by the 5-HTTLPR X Condition X Latency interaction. For completeness, genotypes were compared on anxiety in each condition at each latency (Threat cue, Threat offset, Safe cue, Safe offset) in univariate ANOVAs.

The fMRI data were processed using SPM5 (Welcome Department of Imaging Neuroscience, University College London, UK: http://www.fil.ion.ucl.ac.uk/spm/). Functional scans were realigned, co-registered to the anatomical scan and spatially normalized to the MNI (Montreal Neurological Institute) T1 template image to a normalized resolution of 3.5mm isotropic. The normalized images were then smoothed with a kernel with a full width at half maximum of 8x8x8 mm. Visual inspection of the scans indicated extremely large and fast signal fluctuations around shock administration for some but not all subjects. Therefore, we used a custom in-house script to determine which scans showed clear deviations in mean signal intensity compared to other scans acquired in that run. Each scan that contained these excessive fluctuations was then replaced by the mean of the two surrounding scans that did not contain artefacts. In this way we removed any artefact-related variance that could reduce

statistical power for detecting our effects of interest. This was the case for 34 subjects, for those subjects on average 4 out of 2250 scans per subject were removed. Subsequently a general linear model was composed to relate BOLD signal variation to the task conditions. The predictors in this model were the threat conditions, safe conditions and shocks. For the threat and safe conditions, the onset and offset responses were modelled using a delta function with zero second duration. In addition, to preclude that regions that showed sustained activity to the cue would be associated with the offset of the cues a sustained response to the cues was modelled with a boxcar that lasted throughout the threat and safe conditions. The onset, offset, sustained and shock regressors were convolved with the canonical hemodynamic response function in SPM. Multiple correlations between factors in the design were calculated to assess multi-collinearity. All were around 0.40. Realignment parameters were included in the model as regressors of no interest to reduce movement related artefacts.

To examine which brain regions responded to the threat cues across all subjects, we used a one-sample T-test for the contrasts threat onset vs. safe onset and threat sustained vs. safe sustained. Prefrontal areas associated with the termination were examined with a one sample T-test of the threat offset vs. safe offset contrast. Results for the sustained regressors revealed brain activations across subjects very similar to the results that are reported below for the onset regressors (cf. Klumpers et al 2010a) and are therefore omitted for brevity. Whole brain results were thresholded at $p < .001$ uncorrected for multiple comparisons. To minimize the chance of false positives, this voxel-wise threshold was combined with a cluster threshold and we report only clusters that survived a threshold of $p < .05$ family wise error (FWE) corrected using random field theory as incorporated in SPM.

Because of our a priori expectations on activations in limbic areas (Etkin and Wager 2007; Klumpers et al 2010a; Mechias et al 2010; Nitschke et al 2006; Paulus and Stein 2006; Phelps et al 2001) and prefrontal areas (Eippert et al 2007; Ochsner et al 2002; Ochsner and Gross 2005; Phan et al 2005; Quirk and Beer 2006), we selected the clusters with significant activity in the bilateral insula, ACC and midbrain (threat vs. safe onset contrast) and all prefrontal areas (offset contrast) for further analyses. The clusters of activity were masked using anatomically defined masks from the Wake Forest University (WFU) pick atlas (Maldjian et al 2003) for the insula, anterior cingulate cortex, midbrain, inferior frontal gyrus (ventrolateral PFC), middle and superior frontal gyri (anterolateral PFC) and inferior, medial, middle, and superior orbitofrontal gyri, the gyrus rectus and BA 25 (vmPFC) to obtain anatomically confined functional regions of interest. Mean signal change, relative to the mean signal in the cluster, was then extracted from these areas using the MARSBAR toolbox (Brett et al 2002) in SPM. In addition to these functional regions of interest (ROIs) we also included the amygdala as an

ROI given the large body of evidence for an effect of 5-HTTLPR genotype on fear processing in the amygdala. Because our whole brain results did not reveal significant amygdala activations on which a functional ROI could be based, we extracted the average signal change from the bilateral amygdalae using an anatomical mask from the WFU pick atlas.

We explored whether 5-HTTLPR genotype moderated neural fear reactions and the neural mechanisms underlying the termination of fear responses. For these analyses we used the ROI signal changes derived with MARSBAR and imported these into SPSS 17.0 for further analyses. Signal changes in each of our ROIs were used as dependent variables in a general linear model with 5-HTTLPR genotype (SS, SL, LL) as a predictor. Specifically, we tested for the limbic areas whether there were differences in signal change following the onset of the threat cues and for the prefrontal ROIs we compared genotypes on signal changes to the offset of the threat cues.

Finally, we explored the relation between percent signal change in our ROIs and mean fear-potentiated startle (FPS) magnitudes during the threat cues that we observed in these subjects during the startle sessions (described in Klumpers et al in press; Klumpers et al 2010a). To this end, non-parametric correlations (Spearmans ρ) were calculated between mean FPS magnitudes and ROI signal change.

RESULTS

Sample

Sample descriptives as a function of 5-HTTLPR genotype are reported in Table 1. There were no significant differences between genotypes in gender distribution and trait anxiety scores (Table 1). There was a marginally significant difference between the genotypes in sensitivity to the shocks, as reflected in the final shock intensity that was selected after the shock work-up (Table 1). Post hoc testing revealed that there was a significant difference between the homozygote groups ($t_{(66)} = 2.0$, $p = .046$), the difference between SL and LL did not reach significance ($t_{(66)} = 1.6$, $p = .10$) and neither did the difference between SS and SL genotypes ($t < 1$). Also when testing S-allele carriers (SS + SL) versus LL homozygotes the final intensity for S-carriers was significantly lower than in LL homozygotes ($t_{(1, 67)} = 4.9$, $p = .03$).

	S/S	S/L	L/L	Statistic
N	15	22	32	n/a
% females	73	68	56	$\chi^2 = 1.6$, $p = .46$
Trait anxiety (STAI-t score)	33.5 (8.8)	35.2 (8.6)	31.5 (7.5)	$F = 1.8$, $p = .18$
Final shock intensity (μA)[1]	4.4 (1.1)	4.6 (1.2)	5.2 (1.4)	$F = 2.6$, $p = .09$

Table 1. Sample size, sex distribution and mean (SD) trait anxiety scores and shock intensity for each 5-HTTLPR genotype. The last column contains statistics for differences between the three genotype groups. STAI-t = trait portion of the Spielberger Trait Anxiety Inventory, n/a = not applicable.

State anxiety ratings

Across genotypes, subjects reported a moderate increase in state anxiety during presentation of the threat cue compared to the safe cue, and a reduction in anxiety after threat offset (Table 2). Our repeated measures analysis confirmed a significant Condition X Latency interaction ($F_{(1,66)} = 64.2$, $p < .001$). Anxiety was significantly higher during presentation of threat vs. safe cues ($t_{(68)} = 10.5$, $p < .001$), and was significantly reduced after threat offset (threat cue vs. threat offset $t_{(68)} = 9.8$, $p < .001$) .

Across conditions S/S subjects reported more state anxiety than the L-carriers (Table 2), but average anxiety ratings did not differ significantly (5-HTTLPR main effect: $F_{(2,65)} = 2.12$, $p = .13$). There was also no significant difference between genotypes in retrospectively reported anxiety during presentation of threat vs. safe cues ($F < 1$, n.s.) or in the reduction of anxiety following threat vs. safe offsets, as witnessed by the absence of a 5-HTTLPR X Condition X Latency interaction ($F < 1$, also when testing S-carriers vs. LL).

	S/S	S/L	L/L
State anxiety $Threat_{cue}$	5.5 (1.9)	4.4 (2.4)	4.3 (2.6)
State anxiety $Threat_{offset}$	3.4 (2.1)	2.6 (1.9)	2.6 (1.8)
State anxiety $Safe_{cue}$	1.5 (1.1)	1.0 (1.3)	0.9 (1.5)
State anxiety $Safe_{offset}$	2.9 (2.2)	1.9 (2.2)	1.5 (1.7)

Table 2. Means (SD) for state anxiety ratings as a function of 5-HTTLPR genotype. State anxiety was rated on a scale from 0 (not at all anxious) to 10 (very anxious).

fMRI results

Neural correlates of threat on- and offset

Across genotypes, threat cues elicited greater signal changes than safe cues in areas typically found to be involved in anticipatory anxiety. The anterior insula, dorsal anterior cingulate (dACC) and thalamus/midbrain showed greater BOLD signal increases for the Threat onset versus Safe onset contrast (Figure 1) among other areas (see Table 3 for whole brain results). All these areas were also identified in the contrast Threat sustained vs. Safe sustained – which gave very similar results (data not shown).

	peak coordinates (MNI)			Voxels	Z
	x	y	Z		
Ton > Son[1]					
L Superior frontal gyrus / bilateral anterior cingulate	-18	-4	70	994	6.31
L Insula / L Inferior frontal gyrus	-35	21	7	179	5.72
R Thalamus / midbrain	7	-14	0	134	4.60
R Inferior frontal gyrus / R insula	42	24	4	126	4.66
L Inferior parietal lobule	-63	-28	24	112	5.23
R/L Medial frontal gyrus	4	32	-18	61	4.93
L Caudate	-7	4	10	50	4.72
Son > Ton[2]					
R Precuneus / R Occipital lobe	42	-74	35	507	5.28
R Paracentral lobule	4	-32	63	299	6.72
R Precuneus / R Occipital lobe	35	-74	38	270	4.48
L Cerebellum	-35	-63	-42	242	4.87
R Posterior cingulate cortex	10	-52	21	177	4.87
R Middle frontal gyrus / R Superior frontal gyrus	28	28	49	175	4.61
R Cerrebellum	42	-74	-46	144	5.37
L Middle frontal gyrus / L Inferior frontal gyrus	-52	42	-4	134	5.07
R Precentral gyrus	70	-7	28	90	4.82
R Inferior frontal gyrus	42	38	14	77	4.32
R Inferior temporal gyrus	49	-56	-14	69	4.59
L Post central gyrus	-60	-14	49	59	4.59
R Superior temporal gyrus	60	-10	0	54	3.98

Table 3. Whole brain results for the threat onset (Ton) versus safe onset (Son) contrast. All results reported are FWE-corrected at the cluster level, $p < .05$. [1]with a cluster defining threshold of $p = .001$, uncorrected, the .05 family wise error–corrected critical cluster size was 50 voxels. [2]With a cluster defining threshold of $p = .001$, uncorrected, the .05 family wise error–corrected critical cluster size was 54 voxels.

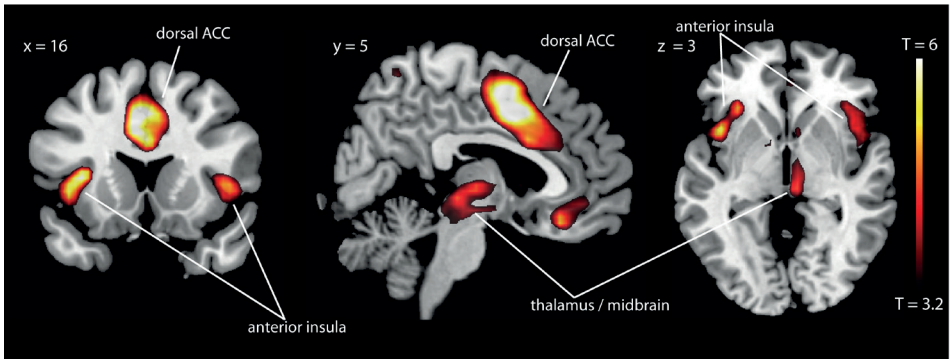

Figure 1. Results for the Threat onset – Safe onset contrast. Greater reactivity to threat cues was observed across limbic areas of interest including the anterior insula, dorsal acc and midbrain. Results are shown at an uncorrected voxel-wise threshold of $p < .001$. All the labeled clusters reached cluster-level significance (FWE corrected $p < .05$). MNI coordinates are indicated in the top left corner of each slice.

Next we examined reactivity of prefrontal areas to the termination of the threat cues. Relative to the offset of safe cues, the termination of threat was associated with signal increases in the bilateral caudal ventrolateral prefrontal cortex and in a more rostral area of the right lateral prefrontal cortex (Figure 2 in red). Moreover, the ventromedial PFC showed reduction in BOLD signal after threat cue offset relative to safe offset (Figure 2 in blue, see Table 4 for whole brain results). Of note, these same areas were also identified in an analysis comparing threat offset to threat onset (data not shown).

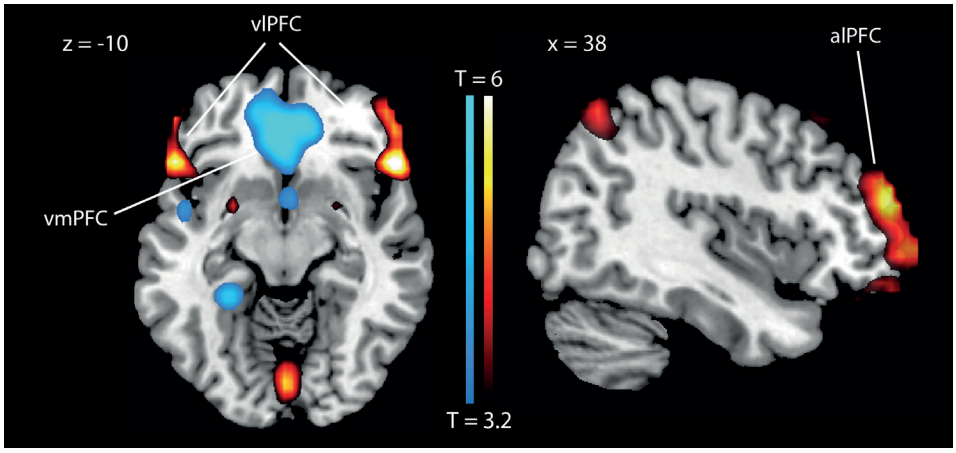

Figure 2. Results for the Threat offset – Safe offset contrast. Greater reactivity to the termination of threat cues (in red) was observed across prefrontal areas of interest including the bilateral ventrolateral prefrontal cortex (vlPFC) and right anterior lateral prefrontal cortex (alPFC). Reduced activity (in blue) was observed in the ventromedial prefrontal cortex (vmPFC). Results are shown at uncorrected voxel-wise threshold of $p < .001$. All the labeled clusters of interest reached cluster level significance (FWE corrected $p < .05$).

	peak coordinates (MNI)			Voxels	Z
	x	y	z		
Toff > Soff[1]					
R/L Cerebellum	-38	-80	-24	840	5.93
R Inferior frontal gyrus (R vlPFC / alPFC)	52	24	-7	738	6.28
R Post central gyrus / R Superior parietal lobule	60	-42	52	340	5.32
L Inferior frontal gyrus (L vlPFC)	-52	24	-7	142	5.48
R Superior frontal gyrus	4	24	70	78	5.00
L Superior temporal gyrus	-63	-60	7	52	3.92
Soff > Toff[2]					
R/L Medial frontal gyrus (vmPFC)	10	42	-14	1892	6.23
L Posterior cingulate cortex / L Precuneus	-14	-56	10	158	5.18
L Parahippocampal gyrus / L Fusifrom gyrus	-28	-38	-14	122	5.10
L Middle frontal gyrus	-38	-7	46	108	5.40
L Superior temporal gyrus	-35	10	-32	102	4.55
R Insula	38	-18	18	92	5.10
L Middle temporal gyrus	-42	-77	28	76	5.10
L Insula	-38	-28	21	68	4.61
R Parahippocampal gyrus / R Fusiform gyrus	32	-28	-21	54	4.78
R midbrain / Thalamus	4	-28	-4	52	4.41
L Superior parietal lobule / L precuneus	-14	-63	60	48	3.94

Table 4. Whole brain results for the threat onset (Ton) versus safe onset (Son) contrast. All results reported are FWE-corrected at the cluster level, $p < .05$. [1]with a cluster defining threshold of $p = .001$, uncorrected, the .05 family wise error–corrected critical cluster size was 52 voxels. [2]With a cluster defining threshold of $p = .001$, uncorrected, the .05 family wise error–corrected critical cluster size was 48 voxels.

5-HTTLPR genotype effects on the neural anxiety circuit

Comparing the genotype groups in a whole-brain GLM analysis did not reveal significant effects of 5-HTTLPR for the contrasts of interest (Threat-Safe, Threatoffset – Safeoffset all p-values > .1 FWE corrected at the cluster level for multiple comparisons). Subsequently we explored more directly whether 5-HTTLPR genotype affected reactivity to threat in the limbic ROIs specifically (the ROIs that showed signal increases during threat cue presentation together with an anatomical amygdala ROI). As can been seen in Figure 3 the pattern was similar across ROIs, with increasing activation to the threat condition with an increasing number of S-alleles. Comparing the three groups on responses to the threat cue in each ROI,

the effect of genotype reached significance for mean signal change in the left anterior insula ROI ($F_{(2,66)} = 3.5$, $p = .038$), while effects in the right insula and right ACC bordered significance (p-values < .1).

Post hoc tests confirmed a significant difference between the homozygote groups in the left and right insula ($t_{(66)} = 2.6$, $p = .011$ and $t_{(66)} = 2.3$, $p = .026$) and a trend for the right ACC ($p < .1$), while there were no differences between SL and LL subjects (p-values > .24). No significant differences were observed between genotypes in responses to threat in the amygdala and midbrain, although similar patterns were observed.

Genotypes were most pronounced in the onset responses. There were no significant differences between 5-HTTLPR genotypes in signal changes associated with the sustained regressor modelling the entire threat cue duration. In the left insula there was a marginally significant trend ($p = .07$, other ROI p-values > .17). However, further testing in the left insula ROI revealed no significant differences comparing S-carriers or SS homozygotes to LL homozygotes. Instead, heterozygotes showed greater signal changes than SS subjects ($t_{(35)} = 2.17$, $p = .037$)

Next, we explored whether 5-HTTLPR genotype affected signal changes in the prefrontal ROIs that responded to the termination of the threat cues. No differences between genotypes were observed in the left and right ventrolateral ROIs. However, the overall ANOVA revealed a marginally significant difference between the genotypes in the alPFC ROI ($F_{(2,66)} = 2.7$, $p = .074$). Follow up tests confirmed that the homozygote groups showed a significant difference in alPFC activation, with greater activations in the SS group ($t_{(45)} = 2.2$, $p = .032$); the difference between the SL and LL group bordered on statistical significance ($t_{(35)} = 1.9$, $p = .07$; Figure 4).

Exploring individual differences in the neural anxiety circuit

Subjects with stronger reactions to the onset of threat cues in the left anterior insula, where genotype effects were strongest, also showed stronger activations in other components of the threat circuit. Signal change increases to threat onset in the left anterior insular region were robustly correlated across genotypes to signal changes in the right insula, and bilateral dACC (all ρ's > .49, p-values < .001) and also with signal change in the left amygdala (ρ = .33, $p = .006$) and midbrain (ρ = .37, $p = .002$). This is consistent with our findings of similar patterns of genotype effects across limbic ROIs and supports the interpretation that the limbic ROIs functioned as a circuit. There were no significant correlations with activity to the onset of the threat cues in any of the offset ROIs. Signal change associated with the termination of threat cues in the right alPFC also correlated with signal change in the other lateral prefrontal areas

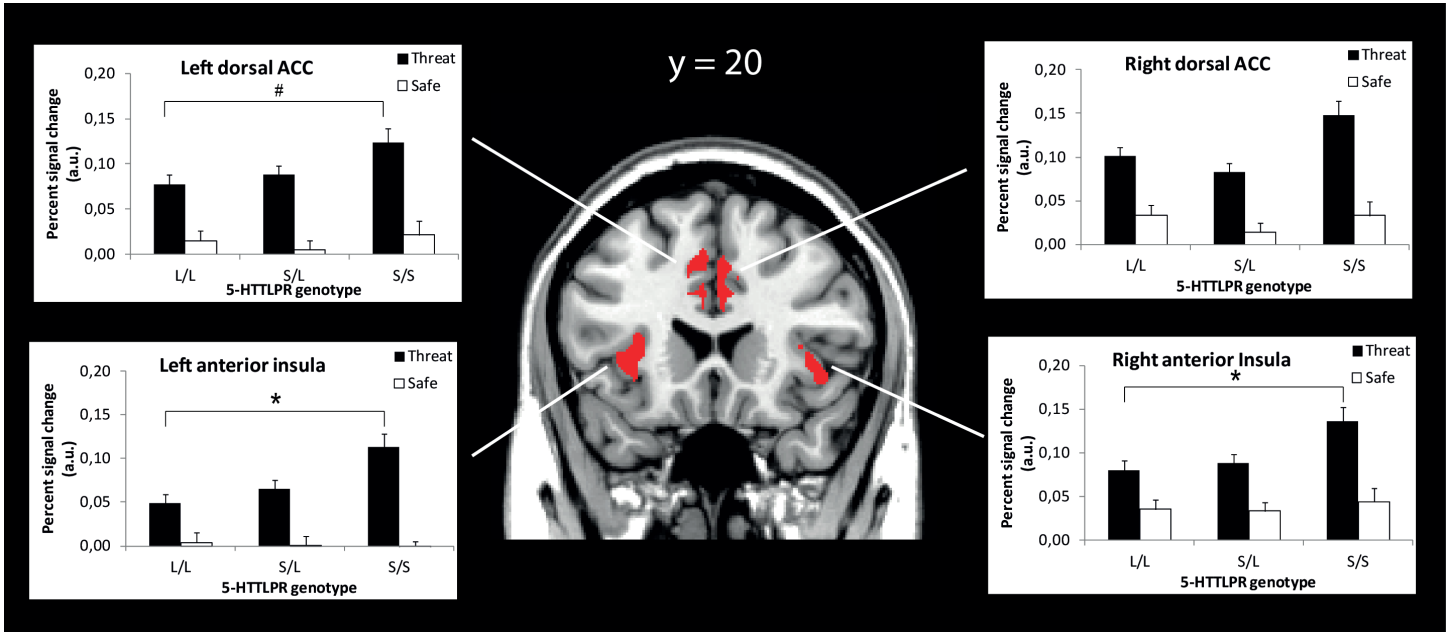

Figure 3. 5-HTTLPR genotype effects on signal change to the onset of threat (black bars) in insular and anterior cingulate (ACC) regions of interest (in red). Reactions to safe cues are also plotted for completeness (white bars). Significant differences were observed in signal change to the threat cues between SS and LL homozygotes in the right and left anterior insula ROIs. Similar patterns were observed in the anterior cingulate ROIs. $^{*}p < .05$, $^{\#}p < .1$

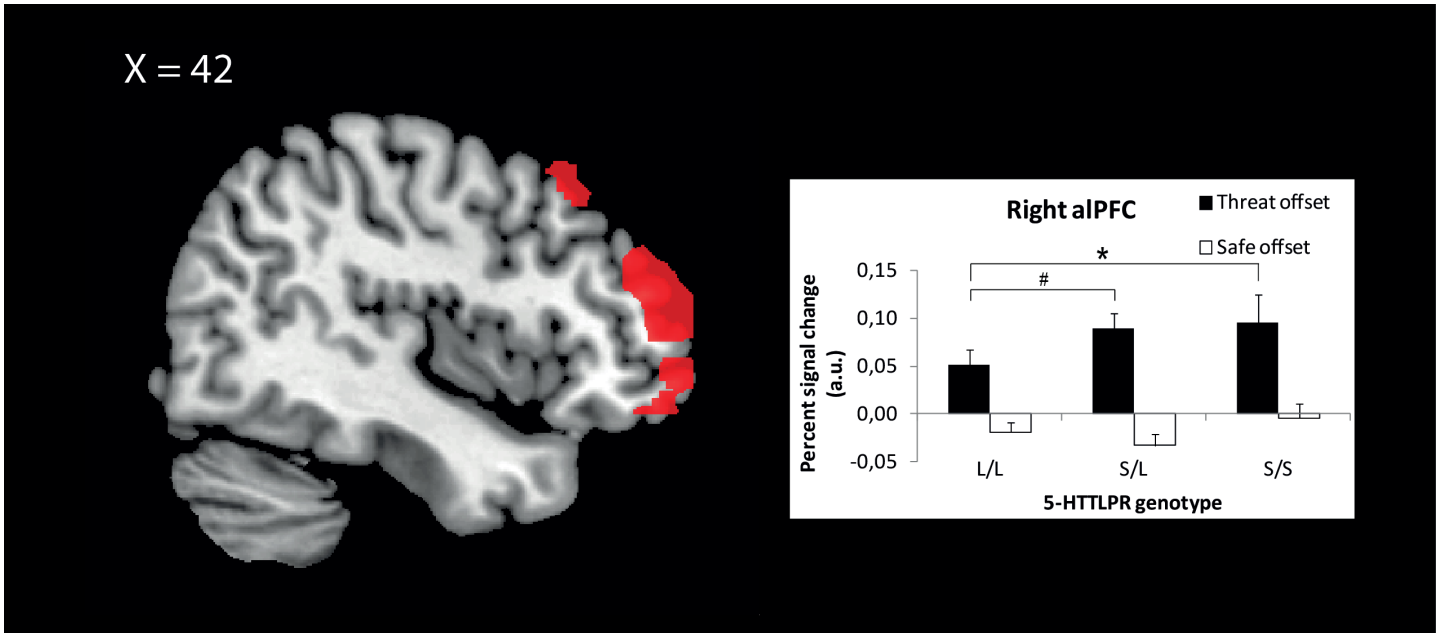

Figure 4. 5-HTTLPR genotype effects on signal change to the offset of threat (black bars) in the anterior lateral PFC ROI (in red). Reaction to safe cues are plotted for completeness (white bars). Significant differences were observed in signal change to the threat cues between SS and LL homozygotes. $^{*}p < .05$, $^{\#}p < .1$

around threat offset (p's < .002).

Finally we investigated how individual differences in the neuroanatomical systems responsive to threat on- and offset modulated defensive reactivity on the behavioural level. We explored the relation of signal changes in our limbic and prefrontal ROIs with mean fear-potentiated startle (FPS), an objective index of defensive responding measured in a previous study (Klumpers et al in press). Interestingly, larger signal change around threat cue onset in the left and right dACC was associated with greater FPS magnitude (ρ =.33, p = .005 and ρ =.35, p = .003 respectively). Positive correlations with other limbic areas (midbrain-onset, right and left insula and right dACC-sustained) bordered on significance (p-values < .1). FPS magnitude during the cue was not associated with reactions to threat offset or vice-versa, no significant correlations were observed. Moreover, residual FPS after the offset of threat did not correlate with reactions to the termination of threat cues in the prefrontal ROIs. Overall these exploratory analyses suggest that enlarged limbic reactivity is associated with more intense defensive responses during threat cue presentation.

DISCUSSION

Across this relatively large sample we observed responses to the threat of electric shock in a limbic circuit encompassing the anterior insula, dorsal ACC, thalamus and midbrain. Activation in these areas during conscious threat appraisal is in line with previous work (Drabant et al 2011; Mechias et al 2010). While this network may play a more general role in salience processing (Seeley et al 2007) these areas are core parts of a limbic anxiety circuit (Milad et al 2007; Paulus and Stein 2006) and show exaggerated reactivity in anxiety prone individuals (Etkin and Wager 2007; Simmons et al 2011; Straube et al 2007). In this expanded sample we again observed that the offset of threat cues is associated with a specific pattern of brain responses in prefrontal areas. The offset of threat was associated with reported reductions in state anxiety. Moreover the termination of threat cues was associated with activity changes in the (ventro-) lateral prefrontal cortex. The lateral orbitofrontal areas we observed have been widely implicated widely in inhibitory processes (Berkman et al 2009; Hooker and Knight 2006) and more specifically in emotion regulation (Wager et al 2008). In addition, more dorsal lateral prefrontal areas similar to the right alPFC area reactive to threat offset have been implicated previously in the regulation of anxiety (Eippert et al 2007; Ochsner and Gross 2005; Phan et al 2005). This suggests that these prefrontal areas play a crucial role in anxiety control.

To our knowledge, this is the first study to assess moderation of neural anxiety reactions by the 5-HTTLPR polymorphism by measuring reactions during anticipation of threat. Earlier studies have focussed on the amygdala, which reacts quite consistently during the processing of symbolic fear cues such as aversive pictures or fearful faces (Dannlowski et al 2010; Hariri et al 2002; Munafo et al 2008; Schardt et al 2010). The anticipatory fear states induced by instructed fear are generally not associated with strong amygdala reactions as measured by fMRI (Klumpers et al 2010a; Mechias et al 2010). Accordingly, we did not observe significant amygdala activations to the threat cues in this study. We did observe that carriers of the 5-HTTLPR short allele, particularly SS homozygotes, showed greater BOLD signal changes across threat reactive regions of the anterior insula and dorsal ACC as compared to LL homozygotes. This is striking given that shock intensities were actually lower in S-carriers.

The current study illustrates that other components of the defensive neural circuit, besides the amygdala, may also be involved in increases in basic defensive responding (Klumpers et al in press; Lonsdorf et al 2009b) and trait anxiety (Lesch et al 1996; Sen et al 2004) in S-carriers relative to LL-homozygotes. Of note, subjects who showed stronger activity in the left anterior insula were also likely to show stronger responses to threat in other regions of the defense circuit, including the amygdala and midbrain. Therefore our results may be taken to suggest that the 5-HTTLPR polymorphism more broadly increases sensitivity in this limbic circuit. Genotype effects were strongest in the anterior insula regions. This result seems to fit well with work on the anterior insula response during the anticipation of negative stimuli (Simmons et al 2009) showing an effect of pharmacological manipulation of SERT function, by sub-chronic treatment with a serotonin transporter reuptake inhibitor.

Finally, we observed stronger responses in s-carriers to the offset of threat stimuli in the right lateral prefrontal cortex. Previous work has indicated that while s-carriers may show enhanced fear reactions to threat, fear regulation may not be affected (Lonsdorf et al 2009b; Schardt et al 2010). Our results indicate that while the instruction to regulate fear may lead to comparable decreases in anxiety, s-carriers may require stronger activation of the lateral prefrontal cortex to achieve this regulation. This could imply that under circumstances when prefrontal fear regulation systems are out of order, for example when severe stressors disrupt normal prefrontal functioning, heightened fear states may perpetuate in s-carriers. This is in line with research demonstrating heightened vulnerability for emotional disorder in s-carriers faced with severe stress (Caspi et al 2010; Karg et al 2011). Moreover, it is also compatible with findings of disturbed prefrontal functioning in anxiety disordered patients, particularly those suffering from post-traumatic stress (Etkin and Wager 2007; Milad et al 2006).

In conclusion, we delineated in a large sample the neuroanatomical mechanisms that enable the up and down regulation of defensive states in healthy humans. Moreover, our findings illustrate how genetic variance in the serotonin transporter gene may lead to higher anxiety by biasing sensitivity of limbic regions involved in anxious anticipation.

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CHAPTER 5

TESTING THE EFFECTS OF Δ9-THC AND D-CYCLOSERINE ON EXTINCTION OF CONDITIONED FEAR IN HUMANS

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ABSTRACT

Preclinical evidence implicates several neurotransmitter systems in the extinction of conditioned fear. These results are of great interest, because the reduction of acquired fear associations is critical in therapies for anxiety disorders. We tested whether findings with respect to the N-Methyl-D-Aspartate (NMDA) and cannabinoid receptor (CB) systems in animals carry over to healthy human subjects. To that end, we administered selected doses of d-cycloserine (partial NMDA receptor agonist, 250 mg), Delta-9-tetrahydrocannabinol (THC, CB_1 receptor agonist, 10 mg), or placebo prior to the extinction session of a three-day conditioning protocol. D-cycloserine did not affect within session extinction, or the retention of extinction in healthy human participants, in contrast with patient data but in line with previous reports in healthy volunteers. During extinction training, Δ9-THC reduced conditioned skin conductance responses, but not fear-potentiated startle. This effect was not retained at the retention test two days later, suggesting it was dependent on acute effects of the drug. Our findings implicate that facilitation of the CB_1 or NMDA system with the substances used in this study does not affect conditioned fear extinction lastingly in healthy humans. The apparent discrepancy between these findings and the results from (pre-)clinical trials is discussed in terms of room for improvement in these systems in healthy volunteers, and the lack of specificity of THC as a CB_1 agonist.

INTRODUCTION

Fear conditioning is considered central to the etiology and maintenance of anxiety disorders (Mineka and Oehlberg 2008). Accordingly, an initially neutral conditional stimulus (CS) comes to elicit fear after being associated with an unconditioned aversive stimulus (US). Exposure therapy, one of the most effective treatments for anxiety disorders, attempts to counteract the CS–US association through repeated exposure to fear-provoking stimuli in absence of negative consequences. Repeated exposure to the CS in the absence of the US (extinction training) leads to extinction, a reduction in conditioned fear responses. Because suppressing the fear reaction provoked by emotional and traumatic memories appears crucial to successful treatment of anxiety disorders, there is growing interest in pharmacological treatments that may facilitate extinction. Recent findings have identified receptor systems that may constitute pharmacological targets for facilitation of the extinction process (reviewed by Myers and Davis 2007). While the ultimate test for treatment aimed at modulating extinction lies in clinical trials, screening substances in healthy human subjects can bridge the current gap between preclinical findings and application of these substances in humans. Tools with great potential for this cross-species translational research are the startle reflex and fear conditioning (Davis et al 1993; Grillon and Baas 2003).

Animal work has indicated that the NMDA receptor is a crucial link in fear extinction (Davis 2002; Falls et al 1992; see Myers et al 2011 for review). D-cycloserine (DCS), a partial agonist at the glycine modulatory site of the NMDA receptor enhances learning and memory in several animal models without producing obvious toxicity (Flood et al 1992; Monahan et al 1989; Steele et al 1996; Thompson and Disterhoft 1997). Moreover, DCS enhances extinction retention in rodents, both in fear-potentiated startle (Walker et al 2002), and in conditioned freezing. In addition, clinical trials have demonstrated that DCS administered during exposure therapy improves long-term extinction of pathological fear responses in patients with fear of heights (Ressler et al 2004b), social anxiety (Guastella et al 2008; Hofmann et al 2006), and obsessive compulsive disorder (Kushner et al 2007; Storch et al 2010; Wilhelm et al 2008); but see also (Storch et al 2007). In contrast, DCS did not affect extinction of fear measured with autonomic measures (skin conductance and heart rate) in healthy volunteers and subjects selected for subclinical phobic fear of spiders (Guastella et al 2007a; Guastella et al 2007c). This result calls for more experimental work to elucidate the working mechanism of DCS on extinction. We set out to further test effects of DCS in a human fear extinction paradigm, including fear-potentiated startle (FPS) as a physiological measure for fear (Lang et al 1990). FPS indexes fear at a basic physiological level that may reflect more implicit processing than skin conductance (Hamm and Vaitl 1996) and may be more sensitive to pharmacological

treatments (Graham et al 2005). Moreover, FPS is also used in the preclinical work (Davis et al 2006) making it a more direct translational tool.

Converging findings indicate that the CB_1 receptor is also crucial in the extinction of conditioned fear. This was demonstrated by blocking CB_1 transmission in mice either by gene knockout or administration of the CB_1 antagonist SR141716A (Marsicano et al 2002a). These findings were replicated and extended using an FPS protocol (Chhatwal et al 2005a) and contextual fear induced freezing in rats (Pamplona et al 2006). In addition, increasing availability of endogenous cannabinoids (eCB) by blocking eCB reuptake and breakdown (Chhatwal et al 2005a) or activating this receptor with an agonist (Pamplona et al 2006) both facilitate fear extinction retention in rodents. These findings suggest that enhancing CB_1 transmission may also facilitate fear extinction in humans. Nevertheless, up to now there has been no study published on the effect of cannabinoid agents on conditioned fear in humans. The non-selective CB1/CB2 ligand Delta-9-tetrahydrocannabinol (THC) exerts its central effects through agonism of the CB1 receptor. THC, the primary constituent of cannabis, produces cannabis-like subjective effects (Wachtel et al 2002) and is readily available for administration in humans; it is prescribed as an anti-emetic and appetite stimulant.

In the current study, three groups of healthy volunteers participated in a three-day conditioning protocol, in which on the second day, 2 hours before extinction training, a single dose of either 250 mg DCS, 10 mg of the CB_1 receptor agonist Δ9-THC or a placebo was administered. Two days later, extinction retention was tested without drug administration. We hypothesized that both DCS and Δ9-THC would increase retention of fear extinction as indexed by fear-potentiated startle.

MATERIALS AND METHODS

Subjects

Healthy volunteers age 18-30 years were recruited with flyers on university campus. Exclusion criteria were: never having been exposed to marijuana, current drug use, low startle reactivity, pregnancy, and first or second degree family members with a history of psychosis. Sixty-one subjects were included in the trial. A complete data set was aquired[1] for 18 subjects per group (total N = 54, 31 female, 23 male). See Table 1 for an overview of the gender distribution, mean age and mean Spielberger trait anxiety scores (Spielberger 1972) in each group.

[1] Two subjects dropped out because of side-effects of THC, one was removed after a positive drug test for cannabis. EMG data of four subjects was not usable due to equipment failure.

	DCS	Placebo	THC
N	18	18	18
Gender (m/f)	8/10	7/11	8/10
Age	21.9 (1.9)	21.7 (1.8)	21.0 (1.2)
STAI score	29.4 (5.9)	29.5 (5.6)	30.8 (5.3)

Table 1. Sample size (N), male/female distribution and mean (SD) age and STAI trait score for each of the drug groups.

Treatments

Treatments were administered in a double blind parallel placebo controlled design. Capsules containing either 10 mg of Delta-9-tetrahydrocannabinol (synthetic Δ9-THC, Marinol), 250 mg of D-cycloserine or placebo were administered orally, two hours before the extinction training session. Previous work showed efficacy of 50-500 mg of DCS (Norberg et al 2008b; Ressler et al 2004a), which lead us to select an intermediate dose. For THC, evidence from preclinical studies shows that relatively low doses of CB_1 agonists are most effective in facilitating extinction (Chhatwal et al 2005b; Pamplona et al 2006) and that high doses are anxiogenic (Moreira and Lutz 2008; Viveros et al 2005). Therefore we selected a relatively low dose of 10 mg which typically does not produce anxiety (Zuurman et al 2009) or strong negative effects on cognition (Curran et al 2002; McDonald et al 2003).

Procedure

This study was conducted in accordance with the Declaration of Helsinki and approved by the medical ethical committee of the University Medical Centre Utrecht. Following telephone screening eligible subjects were invited to the institute for informed consent, a thorough medical and psychiatric screening including the Dutch version of the Mini-International Neuropsychiatric Interview (MINI; (Van Vliet et al 2000)), a startle test and an initial acquisition session (details of conditioning sessions described below). Approximately 4 weeks later, day 1 of the protocol consisted of a final acquisition phase. On day 2 extinction training took place and the retention test was conducted two days later (day 4), separated into two parts (Retention 1 and Retention 2; see Figure 1 for overview). Retention sessions 1 and 2 were separated only by a 35 second interval during which 3 shocks were administered (details below).

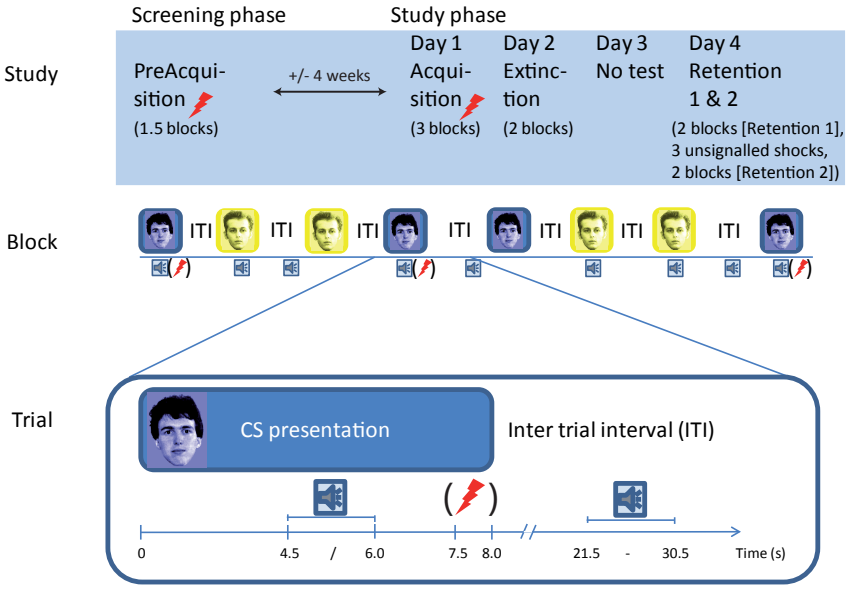

Figure 1. Illustration of the study set up. The top row illustrates the different study sessions, the middle row gives an example of one experimental block, and the bottom row exemplifies how a trial was construed. The flash of lightning represents administration of a shock (between brackets as shocks were only delivered during trials in the acquisition phase). The sound icon represents administration of a noise to elicit a startle reflex (startle probe).

Stimuli and apparatus

CS+ and CS- stimuli were two neutral faces taken from the Psychological Image Collection at Stirling (PICS, http://pics.stir.ac.uk/), one coloured blue and one coloured yellow to increase distinctiveness. Each was presented against a black background. A white fixation cross replaced the CS during the inter trial interval (ITI). A trial consisted of a CS presented for 8 seconds. In the reinforced CS+ trials, shocks were administered 7500 ms after CS onset on the right wrist by a constant current generator at 2.5 mA for 50 ms. Startle probes were presented at 4500 or 6000 ms after CS onset or at semi-random moments during the ITI. Inter startle intervals were 17, 20 or 23 seconds with a mean of 20 s for each of the conditions (CS+, CS– and ITI). Also, the minimum interval between a shock presentation and the next startle probe was 17 seconds (see Figure 1 for illustration of the startle timing). ITIs between CSs were varied in duration between 4 and 22.5 seconds. The startle probes consisted of a 95 dB(A) burst of white noise with near instantaneous rise time and 50-ms duration presented binaurally through foam earplugs by Ear Link headphones (Aero Company).

Physiological recording and amplification was carried out using the Biosemi Active Two system (www.biosemi.nl). Startle was measured with electromyography (EMG) from the orbicularis oculi muscle under the right eye. The skin conductance response (SCR) was recorded by two electrodes on the palm of the left hand.

Conditioning procedure

To ensure acquisition of conditioned responses, an initial training session was included in the screening visit (approximately 4 weeks before the experimental phase) comprised of 6 CS+ (all reinforced) and 6 CS– presentations. The sessions on experimental days 1, 2 and 4, started with a brief startle habituation phase consisting of 9 startle probes followed by a single test shock to confirm proper electrode fixation and equipment function to the subject[2]. Directly afterwards, subjects completed subjective fearfulness and shock expectancy ratings for each cue as supplementary measures of conditioning (these data matched the physiological data and are therefore described in the Supplemental Material). After the ratings, the experimental phase commenced. Each experimental phase consisted of blocks of 4 trials of each CS (4 CS+ & 4 CS–) with 3 startles probe per condition in each block (CS+, CS-, ITI). Unless stated otherwise, blocks were not separated by delays. Below the details for each phase are given, summarized in Figure 1.

Acquisition (day 1)

During day 1, subjects were presented with 3 blocks of 4 CS+ and 4 CS– presentations. 3 out of the 4 CS+ trials were reinforced with a shock. Following the first block of acquisition, subjects received a written on-screen instruction that they could only receive shocks during the previously reinforced CS.

Extinction (day 2)

Day 2 started with drug ingestion, after which subjects filled out paper-and-pencil visual analogue scales (VAS) on drug effects at time points 0, 1, 2, and 3 hrs post ingestion. The extinction phase commenced 2hrs post ingestion, consisting of 2 blocks (no pause) of 4 CS+ and 4 CS– trials all without shock reinforcement.

[2] Incorporated after a pilot in which subjects alerted the experimenter during the extinction session because they supposed that the shock administration was not functioning properly

Retention 1 & 2 (day 4)

The retention phases each comprised two blocks without shock reinforcement. After the first retention phase, a black screen with only a fixation cross was presented for 35 s during which subjects received 3 unsignalled shocks spaced 5 s apart. These isolated shocks were administered to assess differential impact on retention between substances.

Data processing and scoring

Startle and SCR data pre-processing is described in detail in the Supplementary Material. Startle amplitudes were quantified as the highest peak between 25 and 100 ms after probe onset and average amplitude was calculated for each block. First interval SCR responses (FIRs) were defined as the first peak with an onset 1-4 s after CS onset and second interval responses (SIRs) as peaks between 4-8 s. SCR data were normalized with a square-root transformation.

Statistical analyses

All analyses were SPSS 18 repeated measures ANOVAs. Physiological data were analysed with Phase (Acquisition, Extinction, Retention 1, Retention 2), CS (CS+, CS-), and Block (1, 2) as within subjects factors, and Drug as between-subjects factor. Conditioned responding was assessed by evaluating the difference in response to the CS+ and CS- for all measures, as indexed by the factor CS in our repeated measures model. To evaluate the development of the conditioned response across phases we compared the CS main effect in the extinction, retention 1 and retention 2 phases to the CS main effect during acquisition. Additional details are provided in the Supplemental Material. Finally, subjective effects of the drugs were assessed directly after drug administration and 1, 2 and 3 hrs after ingestion. These data were analysed with Time (0, 1, 2, 3 hr after ingestion) as within-subjects factor, and Drug as between-subjects factor.

RESULTS

Subjective drug effects

For all questions in Figure 2 there were significant Drug main effects ($F(2,51)$-values > 6.6, p-values < .005) and Time X Drug interactions ($F(6,153)$-values > 3.9, GG-ε corrected p-values < .005). THC, in comparison to placebo, resulted in significant increases in reportable effects of the drug (Time x Drug THC vs. placebo ($F(3,102)$-values > 4.8, all p-values < .01). Peak effects were reached at 2 hrs, immediately before the start of the extinction session (see Figure 2). DCS did not differ from placebo on any question (F values all < 1.1, n.s.).

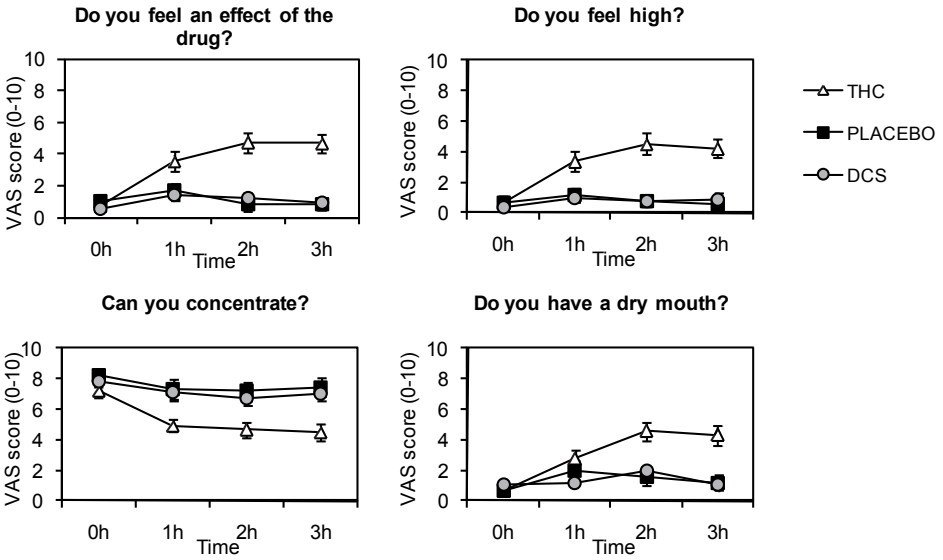

Figure 2. Mean subjective ratings of drug effects scored with visual analogue scales (VAS). Anchors were 'not at all' (0) and 'very much' (10). Error bars are standard error of the mean (SEM).

Fear-potentiated startle

See Figure 3 for startle responses during CS+ and CS- relative to responses during the inter trial interval (ITI) for each of the conditioning phases. A plot containing ITI data is available in the Supplementary Material (Figure S2). The conditioning procedure produced the intended results as witnessed by the development of conditioned responding across phases (Phase x CS $F(3,153; GG\varepsilon=.75) = 4.4$, $p = .01$). Compared to acquisition, the difference between CS+ and CS- (FPS) in the extinction phase was significantly reduced ($F(1,51) = 4.8$, $p = .03$). Compared to acquisition the first retention phase showed a marginally significant reduction in FPS ($F(1,51) = 3.6$, $p = .06$). In the second retention phase (retention 2), no evidence of a reinstatement effect due to the uncoupled shocks was found. Instead, the conditioned FPS response was completely extinguished, reflected in a highly significant difference with acquisition ($F(1,51) = 19.8$, $p < .001$). See Table S1 for CS effects per phase.

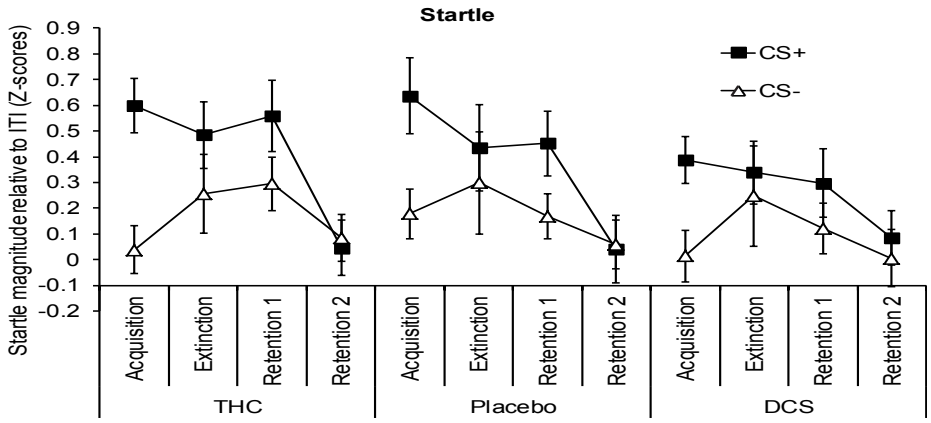

Figure 3. Mean startle magnitudes relative to ITI in the three drug groups during the different phases of conditioning. The difference between CS+ and CS- constitutes fear-potentiated startle (FPS). Error bars are SEM.

Neither drug affected baseline startle as indexed by the mean raw startle amplitude (not T-transformed) during the habituation phase preceding the extinction session (THC M = 97.2 μV SD = 73.7, Placebo M = 100.5 μV SD = 35.8, DCS M = 100.3 μV SD = 55.1; ANOVA F-value < 1). Moreover, although conditioning was successful, our analyses suggested no effect of either drug treatment on the extinction of fear measured with startle. None of the interactions with Drug reached significance in the overall analysis (all F's < 1.5, n.s.). More specific analyses also gave no significant results (for the specific contrasts including Drug and the different levels of the factors Phase and CS all F-values < 2.5, n.s.).

SCR

First interval responses (FIR; Figure 4a) showed sensitivity to the conditioning procedure as reflected in a Phase x CS interaction ($F(3,153; GG\varepsilon=.96) = 9.6$, $p < .001$). Compared to acquisition, specific contrasts indicated no significant extinction of CS differentiation in the SCR data across extinction ($F(1,51)= 1.7$, n.s.), or early retention ($F(1,51)= 0.5$, n.s.). During late retention (retention 2), the conditioned response was significantly reduced with respect to acquisition ($F(1,34)= 14.9$, $p < .001$). See Table S1 for CS effects per phase.

Neither interactions in the overall analysis with Drug nor any of the specific contrasts between levels of the factors Phase and CS with Drug were significant (all F's < 1.4, n.s.). One specific contrast, between CS+ and CS- in the difference between acquisition and extinction in interaction with drug, approached significance (CS x Drug on acquisition versus extinction,

F(1,34) = 2.7, p = .08). Testing per active drug condition against placebo revealed that this effect was caused by a CS (acquisition versus extinction) difference between THC and placebo (F(1,34) = 6.0, p = .02), while there was no difference in the comparison DCS versus placebo (F(1,34) = 0.7, n.s.). The THC group showed more evidence of extinction than the placebo group (see Figure 4). However, the effect did not carry over into the retention phase, as reflected in non-significant differences between drug groups in the comparisons of the CS effect between acquisition and both parts of the retention phase (Phase x CS x Drug interaction < 1.1, n.s.).

Similar conditioning effects were observed for the SIR (Figure 4b, omnibus statistics omitted for brevity). A comparable THC effect was observed in the comparison between acquisition and extinction, which again did not carry over to retention. Tested against placebo, the THC group differed from placebo in a marginally significant Phase (acquisition vs. extinction) x CS x Drug (F(1, 34) = 4.0, p = .05) interaction. There were again no effects in the corresponding specific comparisons between acquisition and the retention phases (all F-values < 2.7, p > .11).

DISCUSSION

The current study sought to evaluate the effect of selected doses of THC and DCS on the extinction of fear conditioning in humans. Fear conditioning was successful in that there was consistent and significant cued conditioned responding. A significant reduction of conditioned responses was observed only at the last retention phase for several of the measures, leaving room for drug induced extinction facilitation. However, neither DCS nor THC affected the retention of fear extinction.

This study may be seen as one of the initial steps in the development of paradigms that could be used for this purpose. Creating sufficient room for improvement in our dependent measures of fear extinction was crucial, as this could explain previous negative findings in healthy humans for DCS (Guastella et al 2007b; Vervliet 2008). In the current study we took several precautions to leave room for facilitation of extinction as extinction in healthy participants can be fast (Vansteenwegen et al 1998). The main measures that were taken to delay extinction were (I) acquisition training on two different occasions (screening and experimental day 1), (II) partial reinforcement during the latter part of acquisition and (III) a relatively short extinction training phase compared to other human extinction protocols (e.g. Alvarez et al 2007; Norrholm et al 2006). Accordingly, the average conditioned response during the extinction session was only significantly reduced compared to acquisition in the startle data. Also, the presence of a significant conditioned response in all of the measures

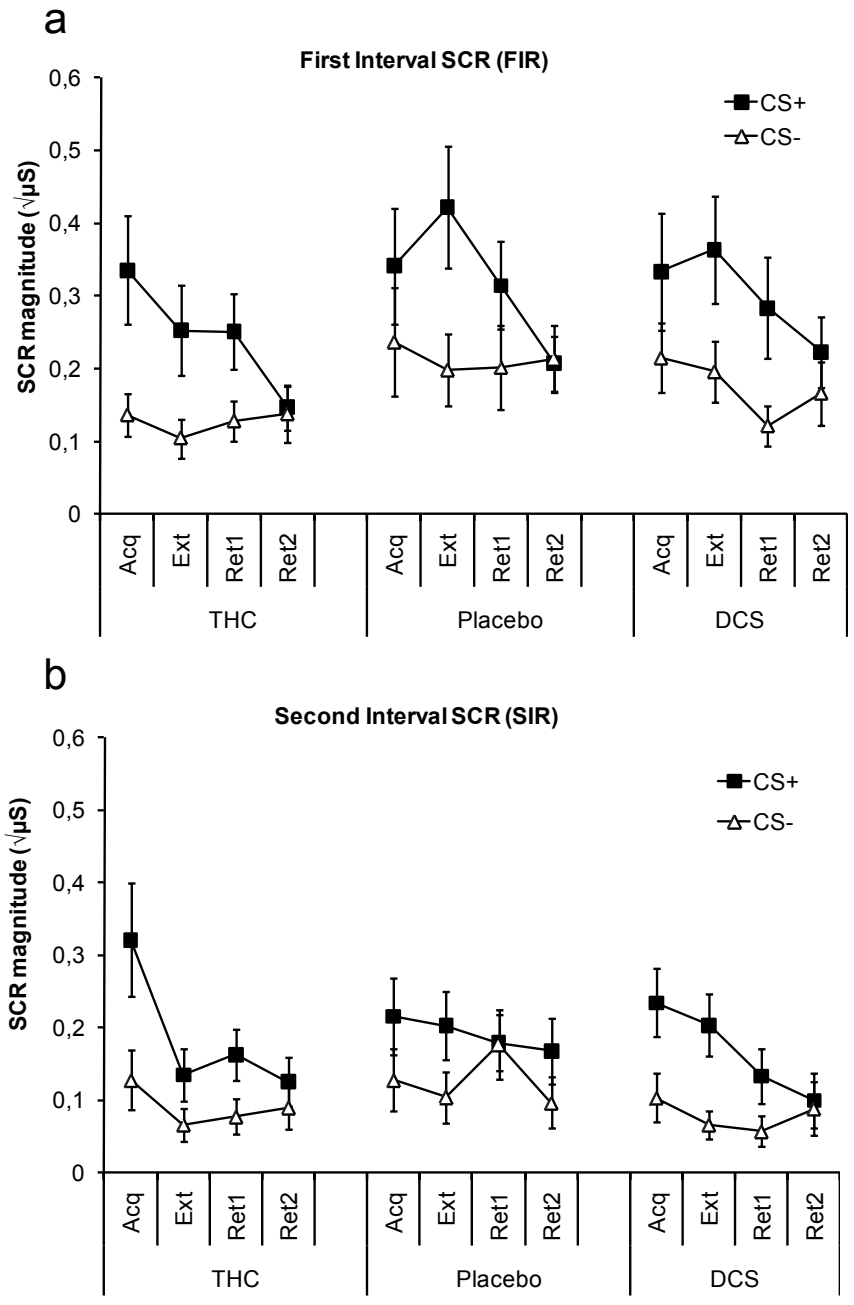

Figure 4. Mean skin conductance responses in the three drug groups during the different phases of conditioning. SCRs are divided into (a) early responses reflecting an orienting response to the CS (first interval responses) and (b) late responses reflecting anticipation of the US (second interval responses, panel B). Error bars are SEM. Acq = Acquisition, Ext = Extinction, Ret = Retention

in the first retention phase indicates there was room for drug induced improvement of extinction. Nevertheless, in contrast to our hypotheses, neither DCS nor THC convincingly affected any measure of fear extinction in this study. While reinstatement of conditioned responding has been demonstrated in humans after unsignalled US presentation following a single extinction session (Kindt et al 2009; Norrholm et al 2006), unsignalled shocks did not reinstate the conditioned response after 2 extinction training sessions, extinction and retention 1, that took place on separate days in our study. Therefore drug effects on reinstated fear could not be evaluated.

The absence of enhanced extinction following DCS administration could be explained in several ways. It has been suggested that effects of DCS on extinction training may only be effective if extinction is well under way (Bouton et al 2008; Weber et al 2007). In line with this, there is evidence that DCS may strengthen the reconsolidation of fear memories rather than facilitate extinction when few extinction trials are presented (Lee et al 2006). The relatively short extinction training phase and the limited reductions in conditioned responding that occurred as a result could thus be an explanation for the negative findings in this study. Nevertheless, alternative explanations should be considered given the consistency of our finding with other negative results in healthy humans in paradigms that produced more robust reductions in fear during extinction training (Guastella et al 2007b). While one explanation has been that DCS exerts its effects through lower level mechanisms than assessed previously (Grillon 2009), a lack of effects on our startle measure indicates that also when extinction is assessed using basic defensive measures that do not require explicit awareness (Weike et al 2005) DCS treatment has no effect in healthy humans. A variety of conditioning studies in healthy participants to date thus do not confirm effects of DCS on fear extinction observed in animal and patient work.

Another explanation for the negative findings is that DCS produces facilitating effects only when there is resistance to extinction. In both patients and experimental animals anxiety is expected to reach higher levels. Moreover because clinical anxiety typically does not extinguish naturally, patients may be characterized by a resistance to extinction. Rather than acting as cognitive enhancer, DCS may thus be better described as a substance that enables to overcome extinction resistance. For example, DCS may only exert an augmenting effect on glutamatergic NMDA receptors when the receptors are not already saturated (Davis et al 2006). In healthy individuals extinction, and thus the NMDA receptor activation necessary for this process, may already be optimal.

Following promising findings in the animal literature (Chhatwal et al 2005b; Marsicano et al 2002b) this was the first study to assess effects of a cannabinoid agonist on human fear extinction. Administration of THC resulted in less responsiveness to presentations of the CS+ in the skin conductance measure during the extinction phase. Although THC also has affinity for CB_2 receptors, these are localized predominantly outside the central nervous system. The effect of THC was primarily observed in responding to the CS+, indicating it most plausibly emerged from stimulation of central CB_1 receptor transmission. However, we could not confirm this pattern with our other measures of extinction, particularly startle, and therefore this result needs to be interpreted with caution. Moreover, no differences were observed compared to the placebo group during the retention phase, suggesting that THC only affected conditioned responding on the SCR measure acutely and not long term. Tentatively, this result seems consistent with the recently developed notion that CB_1 transmission may primarily affect within- session extinction (Plendl and Wotjak 2010) perhaps through habituation-like mechanisms (Kamprath et al 2006). One potentially powerful option to scrutinize this further in humans would be to utilize a CB_1 antagonist, as it may be easier to block rather than facilitate extinction in healthy humans already capable of fast fear extinction. Unfortunately, the CB_1 antagonist Rimonabant was recently taken off the market due to serious adverse events making its use in a human trial problematic. Use of a drug aimed at prolonging availability of endogenous cannabinoids would arguably be a more elegant approach than use of THC. These drugs may render more confined effects (Moreira and Lutz 2008) only acting when and where endocannabinoids are released. Moreover, depending on local endocannabinoid levels and CB1 receptor occupancy THC may exert antagonistic effects (Hoyer and Boddeke 1993; Laaris et al 2010). Unfortunately, substances that inhibit the reuptake or breakdown of endocannabinoids are currently not available for use in humans. An interesting alternative could be the use of cannabidiol (Bitencourt et al 2008).

Importantly, the lack of drug effects on extinction performance in this study is not likely the result of insufficient drug availability at the time of testing. For DCS plasma levels plateau for several hours after reaching a peak level approximately one hour after ingestion (van Berckel et al 1998; van Berckel et al 1997). Also, doses ranging from 50 to 500 mg have shown efficacy in human studies without clear evidence of dose dependency (Norberg et al 2008a) although one study did find stronger effects in 500 than 50 mg (Ressler et al 2004a). While pharmacokinetics of orally administered THC such as peak concentration and time until peak concentration vary across individuals (Grotenhermen 2003), subjective drug effects

after THC administration arrived at peak levels at the time of the extinction test (2 hours after ingestion) which is in line with previous work (Curran et al 2002; McDonald et al 2003; Phan et al 2008). Nevertheless, our use of single, preselected doses for both drugs should be considered a limitation to the current study.

In conclusion, we implemented a human conditioning paradigm with fear potentiated startle as primary outcome measure to test for substances facilitating extinction of conditioned fear. In line with previous work, DCS did not facilitate extinction of fear in healthy subjects. This suggests that DCS is not a cognitive enhancer per se, but may be most effective when there is resistance to extinction. Also, the cannabinoid agent THC did not facilitate extinction of fear lastingly. However, as this was only the first study exploring cannabinoid involvement in humans more work is necessary to elucidate the role of the CB_1 system in human fear extinction, preferably with more selective compounds.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTAL METHODS

Data processing and scoring

Startle data were filtered (28–500 Hz, 24 dB/oct) and rectified. The signal was smoothed with a low pass filter (16 Hz, 24 dB/oct). Artefact detection was performed as described by (Bocker et al 2004). Most subjects (51) displayed very few null responses (< 2%), although three subjects displayed more than 25% missing in at least one condition. Startle analyses were done with and without these three subjects. Also, analyses were performed on raw data and data that were z-transformed per subject to take away individual differences in overall startle amplitude. As there were no differences in conclusions based on these analyses, only the results from the analyses on all subjects and the z-transformed data are reported.

For skin conductance, trials with artefacts or with response peaks outside the defined windows were scored 'missing'. First interval responses could be scored according to the set criteria in 91.6% of the cases (8.4% of trials missing). For trials with a startle probe, SIR responses with onsets later than 0.5s after probe onset or with a maximum later than 1s after probe onset were set missing. These missing values were replaced by neighbouring values. Responses smaller than 0.10 μSiemens were scored as zero.

Subjective ratings of conditions during the experiment were acquired through computerized Visual Analogue Scales (VAS). Directly before and after the first and last experimental blocks of each phase, subjects rated how (un)pleasant they felt while viewing the CS pictures (0 = very unpleasant, 100 = very pleasant) and to what extent they expected a shock during presentation of the CSs (range 0-100%). For the acquisition session, ratings were also acquired after the first block; before subjects received instructions regarding the shock-CS contingencies.

Statistical analyses

To allow inclusion of acquisition (the only phase that contained 3 blocks of trials) in the omnibus analysis the first block of trials was omitted from the analyses, including only blocks 2 and 3. In addition to the overall main and interaction effects, specific contrasts were evaluated. In case of the factor Phase, extinction, retention 1 and retention 2 were contrasted directly with the acquisition phase that was conducted prior to drug treatment. Besides direct comparisons of CS+ versus CS- (fear-potentiated startle; FPS), differentiation between

CS- versus ITI was evaluated separately to preclude that changes in responses to the CS- confounded our analyses of FPS. For the factor Drug, the full model including all three drug conditions was complemented by direct comparisons of both active drug conditions against placebo. Block was included in the design to evaluate possible developments within sessions, but effects of Block that do not involve interactions with Phase, CS, and Drug are not reported. Graphs show data averaged across blocks.

VAS unpleasantness ratings were analysed with Phase (Acquisition, Extinction, Retention 1, Retention 2), CS (CS+, CS-), and Pre/Post (1, 2) as within subjects factors, and Drug as between-subjects factor. Shock expectancy was measured at all of the same time points as the unpleasantness rating, but not prior to extinction to assure this would not trigger suspicion that contingency would be altered compared to the acquisition phase. Therefore the analysis included the same factors as VAS unpleasantness, without the level extinction in the factor Phase.

Supplemental results

Subjective rating data

Ratings of how unpleasant subjects felt while viewing the CS+ or CS- face differentiated as a result of the conditioning procedure, as indicated by significant interactions Phase x CS ($F(3,153; GG\varepsilon=.81) = 3.1$, $p = .04$), CS x Pre/Post ($F(1,51) = 6.4$, $p = .02$) and Phase x CS x Pre_post ($F(3,153; GG\varepsilon=.95) = 15.5$, $p < .001$) (see Figure S1a).

All subsequent phases differed from the acquisition phase in the CS x Pre/Post interaction (all F-values > 25.6, $p < .001$), reflecting that the difference between CS+ and CS- acquired during acquisition diminished during the other sessions. Specific contrasts revealed that the Phase x CS interaction was due to differences in ratings between acquisition and late retention (retention 2; $F(1,51) = 7.2$, $p = .01$, other contrasts n.s.), which is in line with the physiological data showing delay of extinction of the conditioned response until the last part of the retention phase. The remaining CS effect in the last phase remained significant (see Table S1), indicating resistance to extinction of conditioning at a subjective level. In short, the pattern of results from the shock expectancy ratings matched results of the unpleasantness ratings (see Figure S1b & Table S1; omnibus statistics omitted for brevity). Also for the unpleasantness rating data, neither overall effects nor specific contrasts were significantly affected by Drug (all F's < 2.1, n.s.).

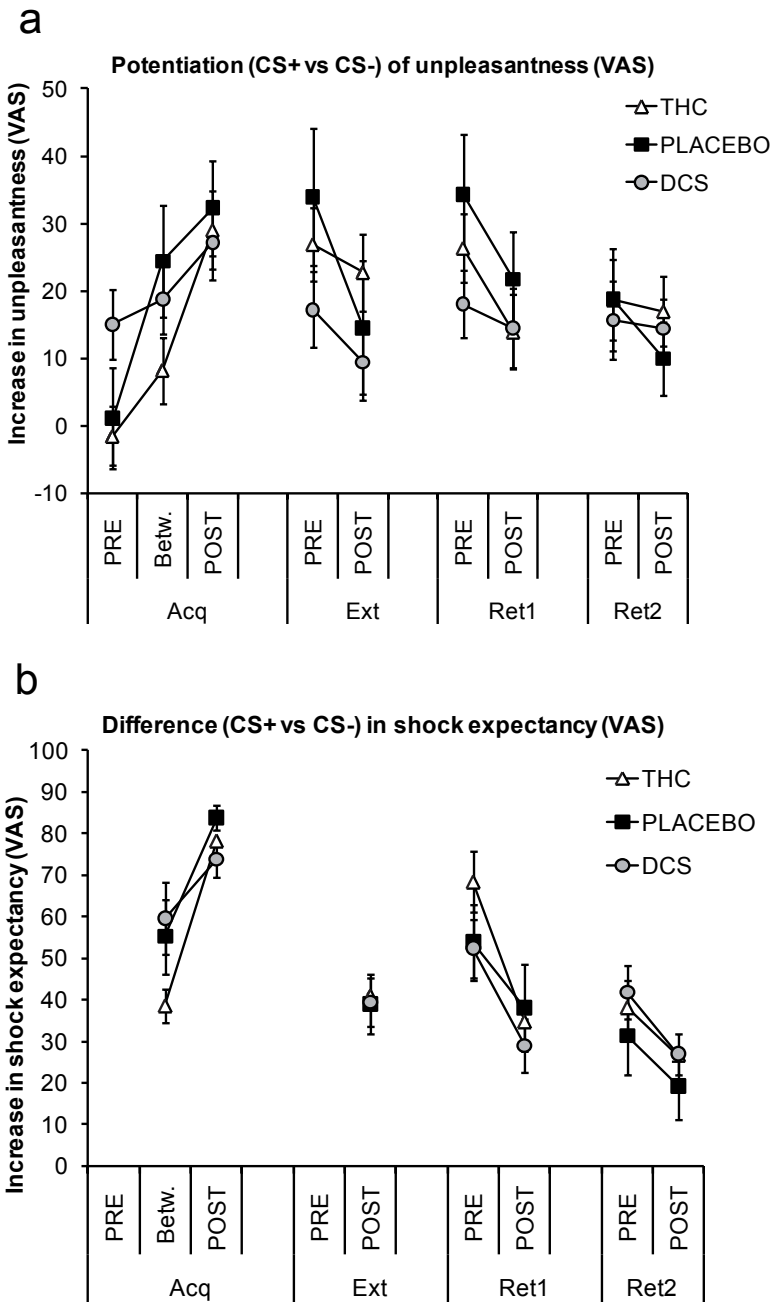

Figure S1. Visual analogue scale (VAS) ratings of subjective responses for the three drug groups. The difference in ratings for the CS+ and CS- is presented, measured before (pre) and after (post) the different phases of conditioning. Subjects rated (a) how unpleasant they felt while viewing both CSs, and (b) how likely they rated the chance of receiving a shock during that CS. Error bars are SEM. Acq = Acquisition, Ext = Extinction, Ret = Retention

			Startle Z		SCR FIR		SCR SIR		VAS unpleasant		Shock expectancy	
			F	p	F	p	F	p	F	P	F	p
Acq	CS		44.2	.000								
		CS+ - CS-	55.4	.000	18.7	.000	21.7	.000	47.3	.000	459.8	.000
		CS- - ITI	1.9	n.s.								
Ext	CS		7.8	0.002								
		CS+ - CS-	1.4	n.s.	29.1	.000	31.2	.000	26.3	.000	93.1	.000
		CS- - ITI	6.3	0.015								
Ret1	CS		16.5	.000								
		CS+ - CS-	7.0	0.011	19.7	.000	4.7	.036	40.5	.000	125.2	.000
		CS- - ITI	11.9	0.001								
Ret2	CS		0.4	n.s.								
		CS+ - CS-	0.0	n.s.	1.5	n.s.	4.4	.041	25.1	.000	54.7	.000
		CS- - ITI	0.7	n.s.								

Table S1. Overview of the differences between threat conditions per phase for all measures of conditioned responding. Startle has three levels of the factor CS (CS+, CS-, ITI), other measures only two (CS+, CS-). Df for test effect of CS on startle (2,102), for other tests (1, 51). Acq = acquisition, Ext = Extinction, Ret = Retention, n.s. = not significant.

Startle data

As can be observed in Figure S2 that includes startle responses during the inter trial interval (ITI), the startle results revealed a main effect of Phase ($F(3,153; GG\varepsilon=.72) = 32.7$, $p < .001$), reflecting an increase in average startle amplitude on the extinction day that was again reduced during the retention session. The increase in overall startle as measured during the extinction session was absent in analyses over startle data collected during the habituation phases preceding the experimental phases (data not shown). This suggests that the effect must be related to the extinction procedure, perhaps reflecting increased uncertainty due to the change in contingencies.

In supplementary analyses, we observed no significant interactions between the CS- versus ITI difference and Phase and more importantly no interaction with Drug. Across drug groups, there was a trend for increased responding to the CS– vs. ITI during extinction, when compared to the acquisition session ($F(1,51) = 2.9$, $p = .09$; see table 2 for CS- vs. ITI effects per phase). However, there were no differences between drug groups in responding to the CS- vs. ITI (for the specific contrasts including Drug and the different levels of the factors Phase all F-values < 2.6, n.s.; see Figure S2) illustrating that other contrasts (e.g. CS+ vs. ITI) did not lead to other outcomes for our analyses concerning the drug effects.

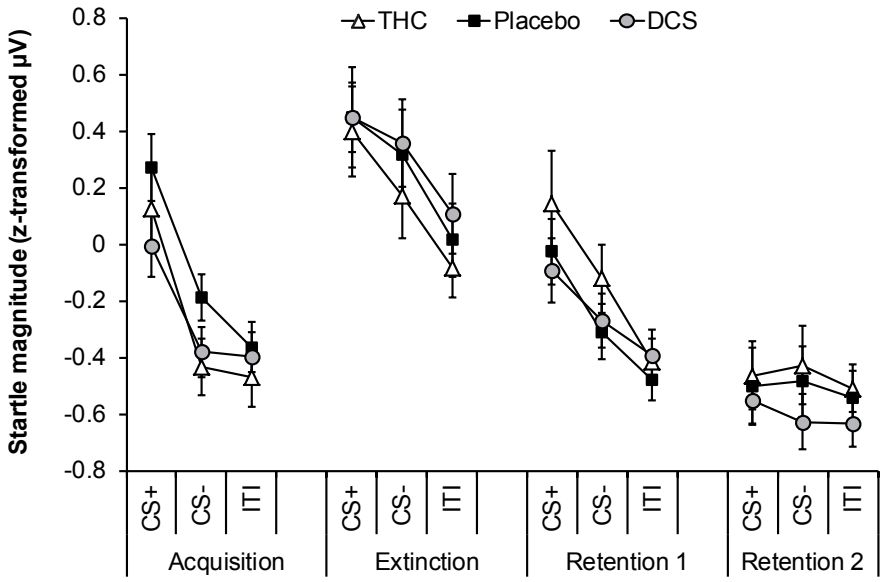

Figure S2. Mean startle amplitudes for each of the drug groups across the experiment, including amplitudes from responses during the Inter Trial Interval (ITI)

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CHAPTER 6

METHOD DEVELOPMENT STUDIES FOR REPEATEDLY MEASURING ANXIOLYTIC DRUG EFFECTS IN HEALTHY HUMANS

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Chapter 6

ABSTRACT

Human experimental models for anxiety may serve as translational tools for translating preclinical psychopharmacological investigations into human studies. For the evaluation of drugs of which pharmacokinetics and -dynamics are unidentified, repeating measurements after drug administration is necessary for characterizing the time course of drug effects. In experiment 1, a threat-of-shock paradigm and adaptations of the Trier mental arithmetic test and the Stroop colour naming test were repeated 4 times within a day to evaluate whether anxiety responses to this test battery remain stable after repeated testing. This procedure was repeated on 4 days in a second experiment to evaluate suitability of the paradigm for a crossover design with multiple sessions. Results indicate no reductions or changes in fear potentiated startle, the main outcome measure for the threat paradigm, over test sessions or days. Skin conductance responses and subjective ratings under threat of shock showed significant fluctuations, but also no systematic decline over time. Finally, the threat paradigm and Stroop test resulted in small increases in reported state anxiety while mental arithmetic produced larger effects that diminished after the first test day. It is concluded that especially the startle paradigm could be a useful new instrument for screening new anxiolytic drugs.

INTRODUCTION

Human experimental models for fear and anxiety have the potential to greatly facilitate the screening for new anxiolytic drugs. Because these models do not rely on patients they offer many practical advantages, most notably in finding study participants and creating more homogeneous study samples. Furthermore, human models are potentially more powerful in terms of predictive validity than animal models. One of the most widely used objective methods for measuring fear and anxiety experimentally is the fear potentiated startle (FPS) paradigm. The startle reflex, generally probed by presentation of a loud noise, increases in amplitude reliably both in humans, non-human primates and rodents during states of fear and anxiety (Falls et al 1997; Grillon 2007; Hamm et al 1991; Walker et al 2002; Winslow et al 2002). The fact that the FPS-technique is applied in a similar manner in a variety of species, including the use of threat of electric shock as a means of inducing anxiety, provides a considerable advantage in the translation of pre-clinical studies to the human realm (Grillon and Baas 2003). Animal work using this technique has created a comprehensive overview of the neurobiological and pharmacological systems that are involved in anxiety (Davis 2006), providing fertile ground for human research to build on. Recently, research using the FPS played an important role in the early evaluation of a promising anxiolytic by suggesting the use of d-cycloserine as a facilitator of fear extinction (Walker et al 2002) which was later validated in human trials (Hofmann et al 2006; Ressler et al 2004). Finally, fear potentiation of the startle reflex has been found to be increased in patients with anxiety disorders (Grillon 2007; Grillon et al 2008; Lissek et al 2008), further adding to its face validity. For these reasons, the FPS is a promising candidate method in the development of novel screening tools to investigate new, potentially anxiolytic compounds.

Validation of different FPS-paradigms with benzodiazepine administration in healthy subjects has been attempted by several laboratories over the past years (Baas et al 2002; Bitsios et al 1999; Graham et al 2005; Grillon et al 2006; Riba et al 2001; Scaife et al 2005). These studies have yielded mixed results (Baas et al 2002), possibly reflecting differences in design parameters. One important factor may be the extent to which anxiety is directly linked to a cue (Baas et al 2002). In a recent study, benzodiazepines were found to have an effect on contextual fear but not on cued fear (Grillon et al 2006). This differentiation is also supported by animal data showing that these two types of fear seem to have distinct neurobiological bases (Walker et al 2003). In humans, contextual anxiety has shown the highest sensitivity to benzodiazepines (Bitsios et al 1999; Graham et al 2005; Grillon et al 2006) and was recently found to specifically differentiate between patients with post traumatic stress disorder and

healthy controls (Grillon et al 2008). Therefore, the goal of this study was to further develop an FPS paradigm that is specifically designed for measuring anxiolytic drug effects and focuses on the manipulation of contextual fear.

If the FPS human paradigm is to be used in early drug development process it would be useful to have more flexibility in the method application. Up to date, there have been several reports showing that a paradigm that induces FPS by threat of shock is repeatable up to four days, separated by up to a week (Baas et al 2002; Bitsios et al 1999; Graham et al 2005; Grillon et al 2006), making it possible to test multiple doses or substances in a cross-over design. However, testing new molecules with unknown pharmacokinetic (PK) and pharmacodynamic (PD) characteristics involves uncertainty about the timing of tests relative to drug ingestion. This could be resolved by repeating the paradigm at different time points during one day and in this way covering a larger part of the pharmacokinetic and pharmacodynamic curves. Importantly, this approach demands a paradigm that can be repeated without alterations in the anxiety levels that are induced. To the authors' knowledge multiple repetitions of a threat-of-shock experiment within a day have not been reported so far and it is unknown whether in such a procedure the FPS and other outcome measures are affected by the repetitions. Two other stressor tasks, computerized adaptations of the mental arithmetic component of the Trier Social Stress Test (Kirschbaum et al 1993) and the video-recorded Stroop color naming task (Leite et al 1999; Teixeira-Silva et al 2004), were added to the threat-of-shock paradigm to create an anxiety battery that measures different anxiety facets. This study set out to test whether four repetitions of this battery within a day could be performed without reductions in induced anxiety. Subsequently, this procedure was repeated on four days to explore whether it would be feasible to use this method for investigating different doses or substances in a cross-over design.

METHODS

The study protocol was approved by the medical-ethical review committee of the University Medical Center Utrecht.

Subjects

Subjects were recruited by means of flyers on Utrecht University campus and an advertisement on the University website. A total of 10 subjects completed each experiment (study 1: 8 females, study 2: 10 females). All subjects were aged 18 to 40 and gave written informed

consent prior to participation. Average score on the trait portion of the Spielberger state-trait anxiety inventory (STAI; Spielberger et al 1970) for these subjects was 33.3 (study 1: 33.4, study 2: 33.2). Subjects satisfied inclusion criteria as per self-report, including no history of hearing problems, color blindness, cardiac disorders, neurological disease, psychiatric disorders and alcohol or drug dependence. Furthermore, subjects reported to not smoke regularly or use any illicit drugs or psychoactive medication. Two subjects were excluded after inclusion due to a lack of sufficient startle responses during the first test day; another subject was excluded after failure to comply with the instructions for the experiment.

Procedure

Each subject was first briefly screened by telephone before receiving an invitation for the screening session at the institute. Upon arrival for the screening the study procedures were explained to the subject, after which they signed the informed consent for participation in the study. Next, they were seated in the experimental room and filled in a computerized Dutch version of the trait portion of the STAI (Spielberger et al 1970; Van der Ploeg and Defares 1979) and a questionnaire regarding their medical condition and drug use (including tobacco and alcohol). After this, subjects completed a training session for the color-word Stroop task to familiarize them with the task and response mode to reduce practice effects. Next, electrodes were placed for startle EMG recording and subjects underwent a brief startle test to evaluate startle reactivity. Finally, shock electrodes were placed and a shock work-up procedure was performed to individually set the level of shock used during the first day of the study. The work-up consisted of 5 sample shocks rated by the subject, shock levels were adjusted in order to achieve an intensity that was rated as "quite annoying". When no impediments for participation were found during the screening, subjects were invited to participate in the experiment.

In study 1, the total experiment consisted of 4 sessions within one test day. At the start of the test day, subjects were seated in the experimental room and the shock workup procedure was repeated to assure that subjects again rated the previously determined intensity as "quite annoying". If necessary, the intensity was adjusted. Next, subjects completed a (non-stressful) practice run for the Stroop task and filled in the state anxiety inventory to measure anxiety immediately prior to the test battery. Subsequently, subjects were instructed that during the threat blocks they could receive shocks at any moment and that during safe blocks no shocks would be presented. To reinforce the instructions, before shock blocks shock electrodes were physically attached to the shock generator in front of the subjects and detached in a clearly visible manner prior to each safe block. Following startle habituation, subjects rated

their current subjective state of fearfulness ("how afraid do you feel?"), alertness ("how alert do you feel?") and feelings of unpleasantness ("how unpleasant do you feel?") using Visual Analogue Scales (VAS). They then were informed about the condition for the next block (safe/threat) by a text on the screen, and the shock electrodes were either attached or detached by the experimenter (depending on the condition of the first block) who thereafter left the test room. After each block this procedure was repeated, only now subjects also retrospectively rated their subjective state during the preceding block. The interval between blocks was 35 seconds. After the shock threat experiment, subjects again filled in the state anxiety inventory and received instructions for the Stroop test on the computer screen. Directly after the Stroop, subjects again filled in the state anxiety inventory. After receiving instructions, subjects did 5 minutes of mental arithmetic and filled in the state anxiety questionnaire for the final time that session. Total duration of each test session was approximately 1 hour, not including the shock workup and Stroop practice run which were only performed once prior to the first session of the test day. The interval between the start of test sessions was kept at 2 hours. Between test sessions subjects rested and were allowed to eat and drink, but not to drink caffeine-containing beverages or smoke. For study 2, the same procedure was followed and now repeated on 4 test days. The interval between consecutive test days was 3-7 days.

Tasks and stimuli

Threat of shock experiment

The experimental procedure for the shock threat experiment consisted of a habituation phase followed by 3 safe and 3 threat blocks of 2.5 minutes duration each; safe and threat conditions alternated. The habituation phase involved 12 startle probes presented with an interval that was randomly varied between 20 and 30 seconds. Startle probes were 50-ms bursts of white noise at 106 dB(A) with an instantaneous rise time. The order of threat and safe blocks was balanced across participants, half started with a safe block and the other half with threat. During the safe blocks an instruction on the screen read: "SAFE! Shock electrodes are disconnected". During the threat blocks the instruction was: "THREAT! Shock electrodes are connected". Each block contained 6 startle probes. The interval between probes varied between 20 and 30 seconds, with a mean interval of 25 seconds. Shocks were administered at fixed time points for all participants. These were placed according to a preset schedule to reduce predictability and to reinforce the instruction that shocks could really be administered at any time during the threat condition. For example, shocks during the first test day in both studies were administered at the end of the last threat block of test session 2 and at the beginning of the first threat block of test session 4. During the other test days in study 2

shocks were also placed at predefined moments (2 in test day 2 and 4, and 3 in test day 3). For electrical stimulation, a train of 150 2-ms pulses was administered at a rate of 200 Hz. Pulse intensities varied between 0.5 and 9 mA as a result of the workup procedure.

Stroop task

The color-word Stroop task that was used in this study was a computerized adaptation of the video-recorded anxiogenic Stroop color-word test (Leite et al 1999; Teixeira-Silva et al 2004). The task consisted of 20 color-word trials in experiment 1, this was extended to 40 trials in experiment 2. The trials started with 1 second of fixation (an asterisk presented in the screen center) followed by one of the color words "RED", "GREEN" and "BLUE" (Font size 45, type Arial), displayed in an incongruent color against a white background for 500 ms (e.g. the word "RED" was always displayed in either green or blue letters). Trials were presented in a semi-random order with the restriction that the same word color was never presented more than twice in a row. Before each run there was a brief refresher practice run of 20 trials. The practice sessions during the screening and at the start of each test day consisted of 60 trials. During practice, display duration of the color words and maximum response time were extended to 1 second. Also, there was no auditory feedback and color words were replaced by "XXXX".

Subjects were instructed that they had to respond as fast as possible to the color of the presented words without making any errors. Furthermore, they were told that they were videotaped during the test, and that the videos would be analyzed by a team of experts. In fact, although the video camera in the experiment room was indeed installed and on, no recordings were made. Subjects had to respond within 500 ms by pressing the key on a response box that corresponded with the color in which the letters where displayed. When subjects made an error or responded too late they received a feedback message ("Error!" or "Too late!") together with a strident sound. This visual feedback stayed on the screen for 1s after which the next trial began.

Mental arithmetic task

For the mental arithmetic task, subjects were instructed to perform a series of backward counting calculations at a pace indicated by a recurring 2000-Hz, 160-ms tone that was played every second. Subjects were told that the next answer had to be given before the next beep to conform to what was defined as the minimal performance criteria (going from a later defined starting number to zero for 4 times in 5 minutes), a criterion that was actually virtually impossible to satisfy. Subjects were again made to believe that they were recorded by video

camera even though the camera was not actually recording. When subjects were ready to start, the experimenter started the experiment and a starting number (a randomly selected number between 696 and 705) was presented for 5 seconds with the instruction to count backwards in 7-number steps. Then, "START" was presented and subjects were required to start counting at the set pace. When subjects were too slow, they were instructed by the experimenter to increase their speed. When subjects made a mistake, the experimenter, who was sitting next to the subject with a list of the correct answers, pressed a button and "ERROR!" appeared on the screen for 1 second together with a strident sound and the experimenter repeated the last correct number. After 1 second, "START" was again presented and subjects had to continue counting from the last correct number. After 5 minutes "STOP!" was presented and the subject completed the next state anxiety inventory.

Measurement and apparatus

All physiological recording and amplification was carried out using the Biosemi Active Two system (www.biosemi.nl) with matching FLAT Active Ag-AgCl electrodes at a sample frequency of 2048 Hz. The startle response was recorded using electromyography (EMG) from the orbicularis oculi muscle under the right eye. Electrodes were placed +/- 15 mm apart. The skin conductance response (SCR) was recorded by two electrodes placed on the thenar and hypothenar eminences of the palm of the left hand. All electrodes were filled with standard electrolyte gel (Signa Gel; Parker Laboratories). Subjective measurements during the threat experiment were performed using computerized Visual Analogue Scales (VAS) ranging from 0 (not at all fearful/unpleasant/alert) to 100 (very fearful/unpleasant/alert). Dutch versions of the Spielberger state and trait anxiety questionnaires were also presented on the computer. Startle probes were delivered through earphones with foam earplugs (Earlink, Aero Company auditory systems). Shocks were administered through tin cup electrodes on the left wrist, which were connected to a constant current stimulator (Digitimer DS7A, Digitimer Ltd).

Data reduction and statistical analysis

All psychophysiological data were processed with Vision Analyzer software (BrainProducts). For the startle data, initially the signal was filtered using a 28-Hz, 12-dB/oct high-pass and a 500-Hz, 24-dB/oct low-pass filter (Blumenthal et al 2005; van Boxtel et al 1998). The data were segmented into epochs, starting 50 ms before onset of the startle probe and ending 200 ms after onset. Finally, the signal was baseline corrected and rectified and a low-pass filter (14 Hz, 24 dB/oct) was applied for smoothing. Startle magnitude was defined as the amplitude of the first peak in the resulting signal within a 25–100 ms latency window. Subsequently, an artefact rejection procedure was performed in which trials with baseline (-30 to 20 ms)

activity greater than 2 standard deviations from the mean baseline activity were rejected. Null responses were defined as trials in which the standard deviation of the signal increased with less than 55% from baseline. Finally, startle data were z-transformed based on single-epoch data from all sessions per subject and converted to T-scores (T = z * 10 + 50). All subsequent analyses were conducted on the T-scores.

Skin conductance responses were quantified by scoring the peak response between 0.5s and 5s after condition onset and computing the difference between the peak response and the mean skin conductance level during a period from 5 to 0s prior to block onset. Missing data were replaced by the mean of responses to the other two blocks of the same condition for that test session, or when an entire test session was missing by the mean of two nearest test sessions (this occurred only once). Before statistical analysis, SCR data were normalized by taking the natural logarithm (ln (SCR + 1).

The first part of the analysis concerned repeating the threat-of-shock paradigm 4 times within a day (study 1). Because for the first day of study 2 the exact same procedures were followed, these data were also used for this analysis creating a final sample of 20 subjects for study 1. FPS, SCR and VAS data were averaged per condition for each test session and analyzed with SPSS 12 for Windows using analyses of variance with repeated measures for startle and SCR magnitudes, and for subjective ratings in the threat and safe conditions. Green-house Geisser epsilon corrections for violations of the assumption of sphericity were used if appropriate. To determine whether the difference between threat and safe conditions varied significantly between test sessions a 2 (threat, safe) X 4 (test session 1- 4) repeated measures ANOVA was carried out. For the second experiment, Test day was added as a factor creating a 2 (Threat, Safe) X 4 (Test session) X 4 (Test day) repeated measures ANOVA. In case the repeated measures ANOVA yielded a significant effect, Student's t-tests and Univariate ANOVAs were used to characterize differences between conditions. For experiment 1, anxiety potentiation during threat was also tested for each test session using t-tests on the difference between safe and threat conditions for each test session. Subsidiary analyses consisted of planned t-tests to compare differences between threat and safe conditions on different test sessions to further confirm results from the ANOVA. For experiment 2, univariate ANOVAs to compare differences in potentiation between test sessions were run for day 2,3 and 4 separately (day 1 results are reported under experiment 1). Main effects of Threat and interactions with Test session and Test day (for study 2) are always reported, main effects of Test day and Test session and three-way interactions (Threat X Test session X Test day) are only reported when significant.

To investigate stability of FPS across test sessions within subjects, an additional analysis was conducted in which Pearson correlations between mean startle potentiation for test sessions within a day (study 1) and for different test days (study 2) were calculated. All correlations (6 for each study) were then averaged to one average correlation for each study. Because of the relatively small N, especially in study 2, only averaged correlations are reported. No correlations were calculated for skin conductance and VAS scores because of the limited amount of data that was acquired per test session (three trials per condition) in addition to the small N.

To determine state anxiety fluctuations during the anxiety battery a repeated measures ANOVA was carried out with Time (Pre, After FPS, after Stroop, After Arithmetic) and Test session (1-4) as within subjects factors. For experiment 2, Test day (1-4) was again added to the model. Main effects of Test day and Test session are only reported when significant. In case of a significant Time effect, repeated contrasts were used to evaluate effects of each task on state anxiety level by comparing the measurements before and after each test.

RESULTS EXPERIMENT 1

Threat experiment

Startle

Startle data[1] for threat experiment 1 are displayed in Figure 1. The repeated measurements analysis revealed a highly significant main effect of Threat indicating robust startle potentiation during threat blocks ($F(1,19) = 45.19$, $p < .001$). Planned t-tests revealed significant potentiation of the startle response during all test sessions (all p-values < .001). Furthermore, a significant main effect of Test session indicated that the overall startle response was reduced over consecutive test sessions during the test day ($F(3,57$; $Gg\varepsilon = .73) = 10.83$, $p < .001$). Importantly, there was no indication for a systematic FPS reduction during the test day as the Test session X Threat interaction turned out to be non-significant ($F(3,57$; $Gg\varepsilon = .47) < 1$, n.s.). To further investigate possible differences in FPS between test sessions,

1 These analyses concern startle amplitude only, latency analyses are not reported. In this study, onset latency reductions in the threat condition were small. The difference between threat conditions only reached marginal significance ($p = .06$) over all sessions and was only significant during the first measurement ($p < .05$). However, there was no significant Test session X Threat interaction to support the hypothesis that the latency modulation was significantly reduced over sessions ($p = .45$). Because the difference between threat conditions was already small during the first measurement (+/- 1 ms) and given that emotional modulation of latencies may be less responsive to anxiolytic drugs than amplitudes (e.g. see Bitsios et al., 1998 and Graham et al., 2005), the authors decided not to report the full latency analyses.

exploratory t-tests were performed to compare threat-safe differences across test sessions. All these tests returned non-significant p-values, another indication for the absence of any trend in startle potentiation across the 4 measurements (all *p*-values > .15). The average correlation of FPS between test sessions was r = .55.

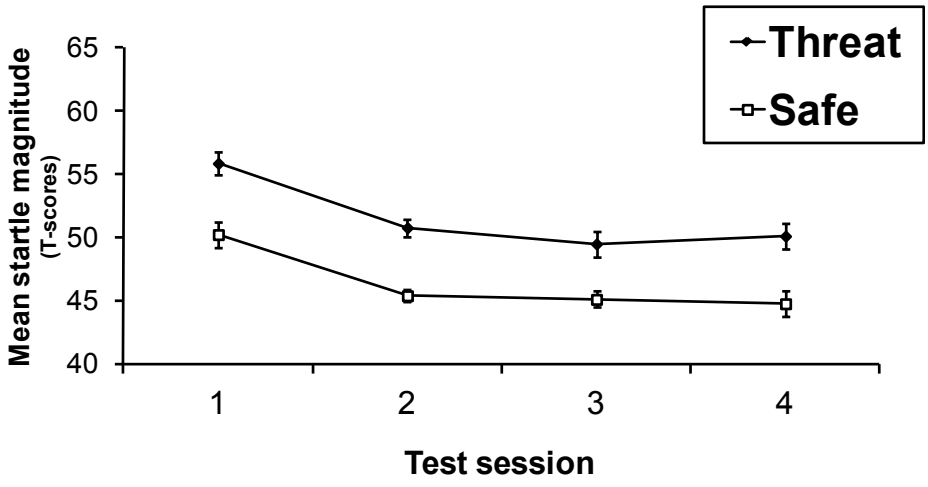

Figure 1. Mean startle magnitude during threat and safe conditions in the shock threat paradigm for each measurement in experiment 1. Error bars in all graphs represent standard error of the mean.

Skin conductance

Figure 2 shows the skin conductance responses (SCRs) for the first threat experiment. SCRs were larger in threat vs. safe blocks ($F(1,19) = 50.09$, $p < .001$). The planned t-tests showed significant potentiation of SCR during all test sessions (all *p*-values < .05). Average SCR magnitude differed significantly between test sessions ($F(3,57; Gg\varepsilon = .78) = 3.63$, $p < .05$) and the difference between threat conditions in average SCR also differed between sessions ($F(3,57; Gg\varepsilon = .95) = 2.94$, $p < .05$). SCR potentiation during the first test session was larger compared to all other test sessions (All *p*-values < .05) while there were no differences between test session 2, 3, and 4 (*p*-values > .6).

Subjective measures

Subjects reported increases in fearfulness, alertness and feelings of unpleasantness during threat blocks compared to safe blocks ($F(1,19) = 36.6$, $p < .001$; $F(1,19) = 43.3$, $p < .001$; and $F(1,19) = 26.8$, $p < .001$ respectively, see Figure 3). Planned t-tests confirmed potentiation was present for all test sessions (All *p*-values < .005). Fearfulness and alertness irrespective of threat condition varied over test sessions (fearfulness $F(3,57; Gg\varepsilon = .58) = 5.62$, $p < .05$;

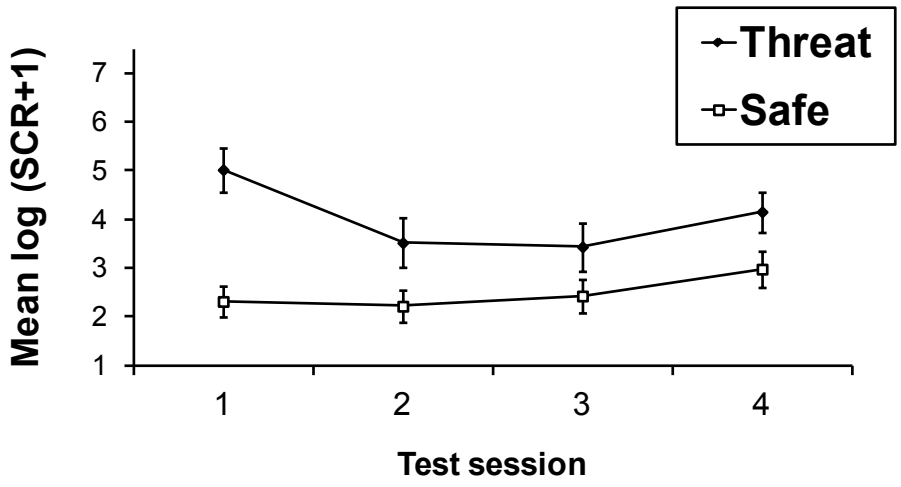

Figure 2. Mean skin conductance response during threat and safe conditions in the shock threat paradigm for each measurement in experiment 1.

alertness F(3,57; Ggε = .71) = 3.25, $p < .05$; unpleasantness F(3,57; Ggε = .63) < 1, n.s.). Importantly however, increases in fearfulness and unpleasantness in threat compared to safe blocks did not significantly differ between test sessions (F(3,57; Ggε = .68) > 1, n.s. and F(3,57; Ggε = .66) > 1, n.s. respectively). The interaction Test session X Threat reached trend-level significance for alertness (F(3,57; Ggε = .72) = 2.56, $p = .06$). This was confirmed by the t-tests comparing differences between threat conditions for each test session. For fearfulness and unpleasantness no differences between test sessions were observed (all *p*-values > .15). For alertness there were marginally significant differences between test session 4 and the other test sessions, indicating a larger difference between threat conditions in alertness in the final test session (.05 > *p*-values ≤ .10) While alertness during the safe condition diminished over consecutive test sessions, alertness during the threat blocks of the final test session increased to a level similar as during the first test session (only difference scores plotted in Figure 3).

Anxiety battery

State anxiety scores significantly increased during the test battery (F(3,27; Ggε = .68) = 15.61, $p < .001$; see Figure 4). This increase was not modulated by Test session (F(9,81; Ggε = .42) = 1.11, $p = .37$). State anxiety significantly increased during the threat-of-shock experiment (F(1,9) = 5.38, $p < .05$) and mental arithmetic task (F(1,9) = 12.70, $p < .01$) but not significantly so during the Stroop task (F(1,9) = 3.82, $p = .08$). Initially, none of these increases seemed significantly modulated by Test session as witnessed by non-significant interaction effects

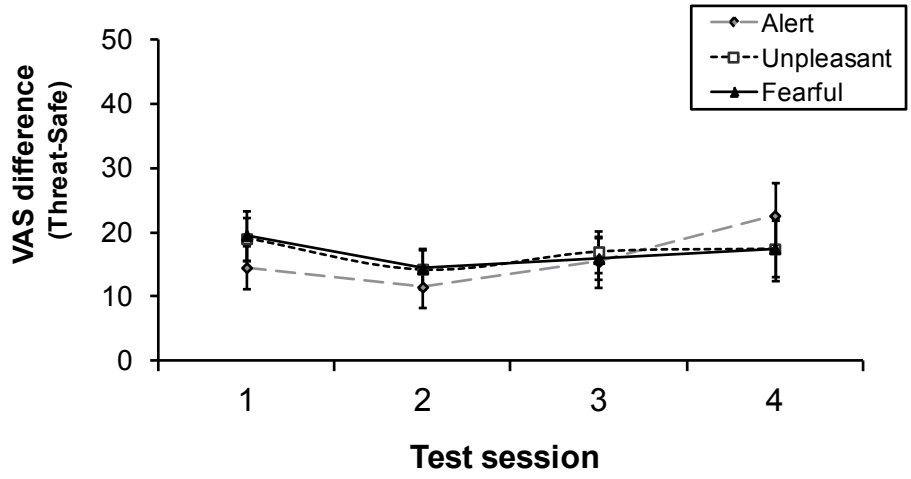

Figure 3. Mean difference between subjective VAS ratings (ranging from 0-100) during threat and safe conditions in the shock threat paradigm for each measurement in experiment 1.

($F(3,27; Gg\varepsilon = .76) > 1$, n.s.; $F(3,27; Gg\varepsilon = .72) = 2.25$, $p = .13$ and $F(3,27; Gg\varepsilon = .75) = 1.8$, $p = .19$ respectively). However, the planned t-tests indicated there were significant differences between anxiety increases by the Stroop test in test session 2 and 4 compared to test session 3 (p-values < .05). Furthermore, state anxiety induced by the Trier was significantly reduced in the third test session compared to the first measurement ($p < .05$).

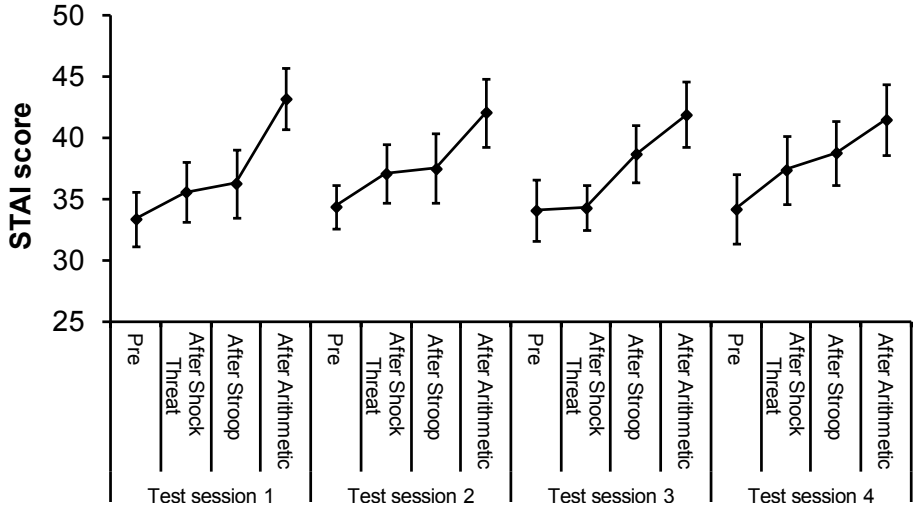

Figure 4. Mean Spielberger state anxiety scores for each measurement in experiment 1.

RESULTS EXPERIMENT 2

Threat experiment

Startle

The startle results for the second threat experiment (see Figure 5) again indicate significant potentiation of the startle response under threat of shock ($F(1,9) = 19.96$, $p < .01$). More important, there was no indication of systematic fluctuations in startle potentiation over test days ($F(3,27; Gg\varepsilon = .86) < 1$, n.s.). Further exploration of potential differences between test sessions indicated that for none of the test days there were differences in FPS between test sessions (All p-values > .15). The average correlation between the mean startle potentiation on different test days was r = .54.

Skin conductance

SCR data for the second threat experiment are illustrated in Figure 6. Subjects responded with larger electrodermal activity to threat vs. safe blocks ($F(1,9) = 15.99$, $p < .01$). This increase did not change systematically over test sessions as evidenced by the non-significance of interactions with test session ($F(3,27; Gg\varepsilon = .73) = 1.815$, $p = .187$) and test day ($F(3,27; Gg\varepsilon = .58) < 1$, n.s.). Separate comparisons of the 4 test sessions on day 2,3 and 4 reveal a marginally significant Test session X Threat interaction for test day 2 ($F(3,27; Gg\varepsilon = .59) = 2.902$, $p = .089$; all other test days: $p > .58$). On this test day, the increase in SCR during threat blocks was larger during the first test session than during test session 2 and 4 (p-values < .05).

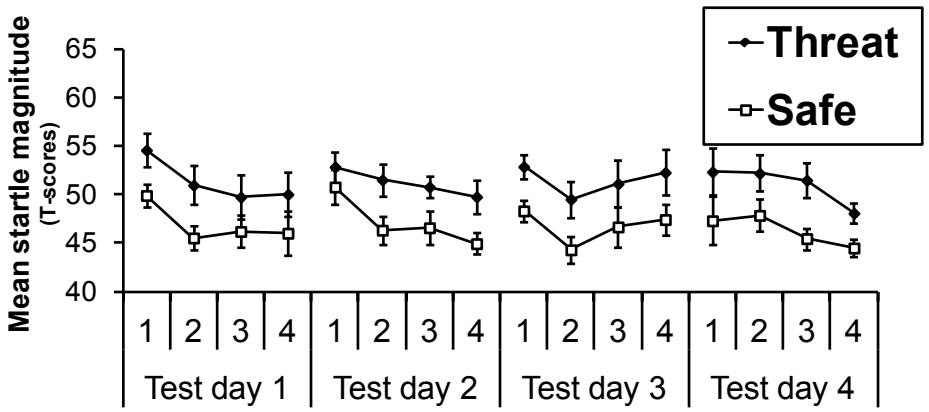

Figure 5. Mean startle magnitude during threat and safe conditions in the shock threat paradigm for each test session in experiment 2.

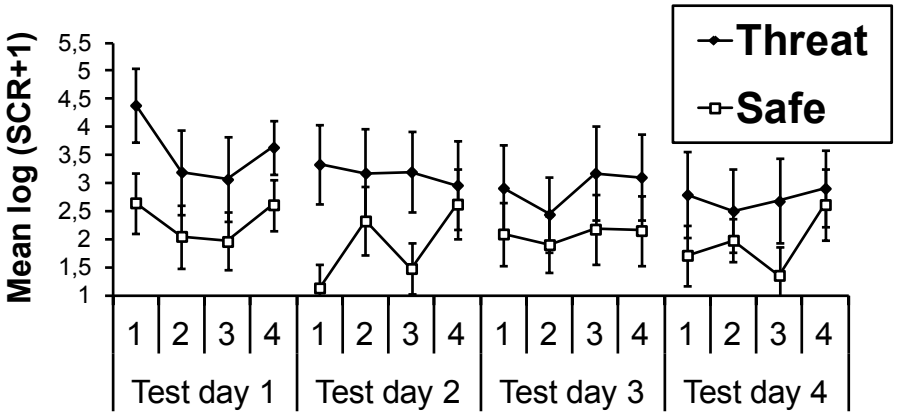

Figure 6. Mean skin conductance response during threat and safe conditions in the shock threat paradigm for each test session in experiment 2.

Subjective measures

Subjective questionnaire data for experiment 2 as expected indicate clear potentiation of fearfulness, alertness and feelings of unpleasantness during threat vs. safe blocks ($F(1,9) = 13.45$, $p < .01$; $F(1,9) = 11.93$, $p < .01$; and $F(1,9) = 22.01$, $p < .01$ respectively, see Figure 7). Increases in arousal and unpleasantness ratings during threat blocks did not significantly change over repeated test sessions ($F(3,27; Gg\varepsilon = .45) = 1.22$, $p = .31$; and $F(3,27; Gg\varepsilon = .56) = 2.07$, $p = .16$ respectively) and also not over test days (Threat X Test day $F(3,27; Gg\varepsilon = .78) = 1.76$, $p = .20$ and $F(3,27; Gg\varepsilon = .51) = 1.15$, $p = .33$ respectively). More elaborate testing of differences between test sessions on test day 2,3 and 4 again revealed no differences (p-values > .3).

Increases in fearfulness appeared to vary somewhat between test sessions (Test session X Threat, $F(3,27; Gg\varepsilon = .72) = 3.77$, $p = .04$) but not between test days (Threat X Test day, $F(3,27; Gg\varepsilon = .74) < 1$, n.s.). Post-hoc t-tests indicated that across test days, ratings were significantly lower during test session 2 and 3 than during test session 4 (p-values < .05). Although the Test session X Threat X Test day interaction did not reach significance ($F(9,81; Gg\varepsilon = .34) = 2.85$, $p = .10$), further inspection of the data per test day indicated that during the first test day there was a significant decrease in fearfulness during the second test session compared to all other test sessions and that during the third test day ratings during the first test session were lower than during the fourth test session (p-values < .05).

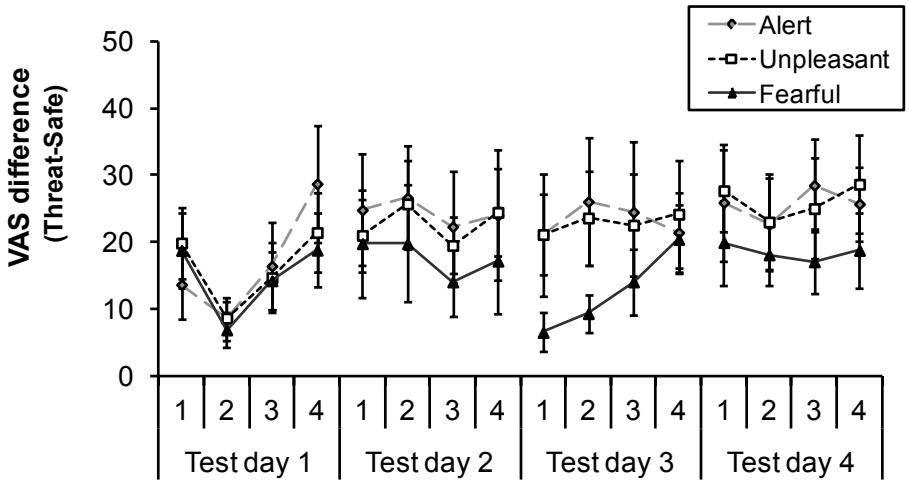

Figure 7. Mean difference between subjective VAS ratings (ranging from 0-100) during threat and safe conditions in the shock threat paradigm for each test session in experiment 2.

Anxiety battery

State anxiety scores in study 2 were again found to increase during the test battery ($F(3,27; G_{G\varepsilon} = .57) = 8.79$, $p < .01$). This increase was not significantly different for different test sessions ($F(9,81; G_{G\varepsilon} = .44) = 1.02$, $p = .41$) but differed between test days ($F(9,81; G_{G\varepsilon} = .27) = 3.88$, $p < .05$; see Figure 8). In the test days after test day 1, increases in anxiety were less pronounced (see figure 4) and did not reach significance on the second and fourth day ($F(3,27; G_{G\varepsilon} = .42) = 2.14$, $p = .17$; $F(3,27; G_{G\varepsilon} = .92) = 5.63$, $p < .01$ and $F(3,27; G_{G\varepsilon} = .49) = 2.13$, $p = .17$ for test day 2,3 and 4 respectively).

Looking more specifically at the effects of separate tasks, significant increases after the threat of shock experiment ($F(1,9) = 6.54$, $p < .05$) and Stroop task ($F(1,9) = 9.54$, $p < .05$) were found, indicating task related increases in state anxiety for these tasks but not for the mental arithmetic task ($F(1,9) = 3.47$, $p = .10$). Further analyses showed that only state anxiety increases by mental arithmetic showed an interaction with test day ($F(3,27) = 5.83$, $p < .05$), caused by a reduction in state anxiety increases by this test after the day 1 (see figure 8).

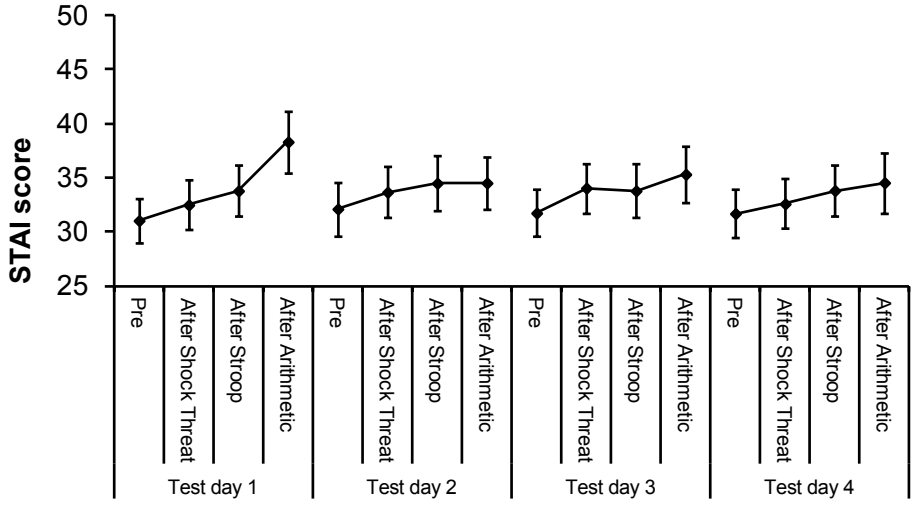

Figure 8. Mean state anxiety scores for each test day in experiment 2 collapsed over test sessions.

DISCUSSION

This study set out to test a battery developed to evaluate anxiolytic drug effects at multiple time points within a day and across multiple days using fear potentiated startle as a main readout measure. Results of the first experiment show that when the battery is repeated four times within a day, potentiation of the startle response in the shock threat experiment remains at similar levels while other measures all show some systematic variation. In the second experiment, it was shown that repeating the paradigm four times at four different test days again does not result in significant changes in startle potentiation. Of all measures, the skin conductance response during the threat paradigm and state anxiety increases by mental arithmetic are the only measures that show systematic reductions after repeated measurements.

Previous placebo controlled, cross-over studies have looked at effects of well known anxiolytics on at a single time point (Baas et al 2002; Bitsios et al 1999; Graham et al 2005; Grillon et al 2006). In this study we sought to extend the FPS paradigm to potentially allow the measurement of the time course of anxiolytic effects. This may especially be useful when the exact onset of anxiolysis cannot be predicted, as can be the case in early drug development. To the authors' knowledge, no previous studies have been reported that assessed FPS under threat of shock on multiple occasions without interventions or substantial changes in the procedure between test sessions. The results of this study indicate that fear potentiation

of the startle response does not show systematic habituation in the current paradigm and that therefore consecutive test sessions can be compared validly. In contrast, general startle amplitude (irrespective of condition) was found to habituate during the test day in study 1. Habituation in baseline startle when measurements are repeated is a common observation (e.g. see Quednow et al 2006), but at the same time FPS is not reduced (Bradley et al 1993; Grillon and Baas 2002) suggesting that these processes are independent to a certain extent. Habituation in baseline startle calls for the inclusion of an extensive habituation procedure in the threat paradigm to reduce the extra variance caused by the fact that subjects start with either the threat or safe condition.

In contrast to the startle results, in both experiments potentiation of the SCR to the onset of threat conditions relative to safe was found to be reduced after the first measurement. Further inspection of the SCR data showed that the main part of this reduction occurred already during the first block of the first measurement (data not shown). In a previous study that used affective picture stimuli to induce emotion, the habituation of the SCR resulted in a loss in differentiation of responses to emotional and neutral pictures (Bradley et al., 1993). In the current paradigm, although increases in SCR were reduced after the first test session, the SCR continued to differentiate between conditions during the 4 repetitions of this paradigm in study 1 and there were no significant differences in differentiation between the test sessions on each test day in study 2. However, visual inspection of the data indicates that potentiation almost disappeared during the last test sessions of day 2 and 4 supporting the notion that potentiation of the SCR may be more vulnerable to habituation than startle potentiation (Bradley et al., 1993).

In both study 1 and 2, subjective ratings showed significant variability between test sessions. These variations are likely to be related to the fixed shock reinforcement schedule that was used in this study. The first shock during study 1 and the first test day of study 2 was not administered until the end of the final threat block of the second test session. Subjects may have stopped anticipating the shock after the first test session resulting in the apparent "dip" for all the subjective measures for the second test session. The administration of the second shock (at the start of the first threat block of the fourth test session on day 1) probably resulted in the elevated alertness that subjects reported during threat blocks throughout the fourth test session. Similarly, reductions in fearfulness during the first test session of test day 3 could be caused by the fact that subjects did not receive any shocks during the first test sessions of day 1 & 2 and may not have expected a shock during the first session of day 3. Even though these fluctuations in conscious expectations did not affect the physiological anxiety

response level, varying the shock reinforcement schedule per subject is required to eliminate this systematic variation in future studies.

Both the threat-of-shock paradigm and the adapted Stroop task resulted in small increases in reported state anxiety. Furthermore, these effects were not systematically reduced after repetitions of the paradigms within the same test day or on different test days. However, because the paradigms resulted in increases of only a few points on the state anxiety questionnaire that showed some apparently random fluctuations these challenges in their current form seem not especially suited for manipulation with anxiolytic drugs. In previous studies that used the video recorded Stroop in populations with medium trait anxiety, effects were larger (e.g. Leite et al 1999; Silva and Leite 2000; Teixeira-Silva et al 2004) probably owing to the fact that these studies included simultaneous exhibition of subject's performance on a screen and a video recorded instruction (Teixeira-Silva et al 2004) to make it more plausible that subjects are being monitored by a panel. Also, these studies showed that state anxiety increases are largest when measured during the task instead of after completion of the task. Because other studies that used a digital version of the video-recorded Stroop test also found more substantial effects in a normal population (Hainaut and Bolmont 2006), it is unlikely that the low increases in state anxiety found in this study are related to the fact that the task was computerized.

The effects on state anxiety of the mental arithmetic task that was employed in this study appeared to be more substantial than those of the other tests and initially are even comparable to those found on in social anxiety patients (Soravia et al 2006) and healthy subjects (Singh et al 1999) after completion of the complete Trier Social Stress Test. It therefore seems to be a good alternative to the original version that costs less time and personnel. However, the effects diminished (non-significantly) after the first test session and significantly after the first test day. Gradually increasing difficulty of this task may counteract some of these decreases, although none of the subjects reached the preset performance criterion for this task at any test session. For that reason, it is more likely that decreases in experienced anxiety are the result of habituation to the testing procedure. Because an important source of stress in this task (and even more so in the original version) is social-evaluative threat, one may speculate that habituation to the experienced feelings of shame and habituation to the experimenter played an important role. Switching experimenters each test day or even session may be required to keep anxiety responses more stable but can also introduce more variability and is practically demanding.

A general limitation of the measures that were included in this study is that they all have relatively high within-subjects variability when compared to more established parameters for analyses of concentration-effect relationships such as eye movements. Correlations between mean FPS during different test sessions or test days were found to be only in the moderate range (around 0.55 for the z-transformed data). This is similar to findings in other studies that correlated startle potentiation during aversive picture viewing (Larson et al 2000; Larson et al 2005) while another study reported lower consistency in responses to emotional pictures (Manber et al 2000). Even though multiple repetitions apparently leave the validity of this paradigm intact since startle potentiation is not reduced systematically over time, random variability in startle potentiation within subjects may limit statistical power for detecting drug related differences between test sessions. On the other hand, the measures used in this study have high face validity for predicting effects on anxiety while more reliable measures primarily give information on sedation. Therefore, incorporation of these measures at an early stage of drug development could potentially make the screening for therapeutic efficacy of anxiolytic compounds more efficient. These exploratory studies suggest that repeated testing using these measures is feasible, although the within-subject variability needs to be addressed further. Although the suitability of repeated FPS-measurements for PK/PD analysis therefore remains to be established, repeated testing offers the additional advantage that it is likely to provide more robust results than a single test at a predefined time point after drug administration.

In conclusion, this study shows that FPS measurements in a threat-of-shock experiment are resistant to systematic habituation effects when repeated within and across days. This indicates that repeated test sessions within a day can be used to characterize drug effects on anxiety at different time points after administration. The other tests in the test battery that was used in this study require further adaptation to increase stability of induced anxiety. Validation of this procedure with a compound with known PK/PD characteristics will be the next step in further refining the paradigms for screening of new anxiolytic compounds.

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CHAPTER 7

THE NEUROANATOMY OF HUMAN FEAR CONDITIONING: NEW EVIDENCE FROM PATIENTS WITH BILATERAL BASOLATERAL AMYGDALA LESIONS

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Manuscript in preparation

ABSTRACT

Fear conditioning is successfully used as a model for the acquisition of fear. Patient data have pointed towards a crucial role of the amygdala in fear learning. Animal work has suggested that particularly the lateral amygdala is of fundamental importance. Here we describe fear conditioning data of a group of patients (N = 4) with bilateral selective lesions of the basolateral amygdala. Relative to a group of matched controls, this rare group of patients showed reduced startle magnitudes to the threat predicting stimulus (CS+). These results provide first evidence that in also humans lesions of the basolateral amygdala disrupt normal fear learning.

INTRODUCTION

Fear conditioning is the best established model to study the acquisition of fear across species. Moreover, fear conditioning is thought to have considerable merit as a model for pathological anxiety (Lissek et al 2005; Mineka and Oehlberg 2008). Animal work has indicated that the amygdala is a crucial cogwheel in the mechanics of fear conditioning (Antoniadis et al 2009; Davis and Whalen 2001; Maren and Quirk 2004). Moreover, functional neuroimaging has generally supported a role of the amygdala in fear learning (Buchel et al 1998; Indovina et al 2011; Sehlmeyer et al 2009; Tabbert et al 2011).

Previous work in patients with lesions that among other areas encompass the amygdala has supported the animal and imaging data by showing reduced fear conditioning in patients. In a first study, unilateral temporal lobectomy patients with amygdala and hippocampal damage showed reduced conditioned anticipatory skin conductance responding to cues signaling an aversive noise (LaBar et al 1995). More recently these findings were extended. Weike et al. (2005) showed that unilateral temporal lobectomy patients also show reduced potentiation of the startle response, a more specific measure of fear that reflects basic defensive responding (Hamm and Weike 2005), to a cue coupled to aversive shock administration. However, data from patients with more specified, bilateral lesions could provide more definite answers to the question of the involvement of the amygdala in human fear conditioning.

Here we report new fear conditioning data of subjects with Urbach-Wiethe disease (UWD), a rare genetic disorder that leads to calcification of the amygdala (Thornton et al 2008). Seminal work on fear conditioning in an UWD-subject has previously indicated that complete bilateral damage to the amygdala is associated with a lack of fear conditioned skin conductance responses (Bechara et al 1995). In the current study we were able to investigate a set of 4 UWD-subjects who showed less extensive damage. Moreover, damage in these patients was restricted to basolateral amygdala regions, providing a unique opportunity to more specifically test the contribution of basolateral nuclei of the amygdala in human fear conditioning. On the basis of animal work particularly the lateral amygdala is considered a crucial hub in fear learning (Davis and Whalen 2001; Maren and Quirk 2004), as it receives perceptual information needed for the acquisition and expression of conditioned fear (Herry et al 2008; Jimenez and Maren 2009; Johansen et al 2010; Sierra-Mercado et al 2011). We used a classical fear-conditioning paradigm, in which patients viewed 2 pictures of which one was associated with an unpleasant loud scream. Eye-blink startle was measured through electromyography to quantify conditioned fear-potentiation of startle in patients, compared to a group of matched controls.

METHODS

Subjects

Patients were 4 female subjects from the Northern Cape in South Africa, part of a UWD cohort described earlier (Thornton et al 2008). A group of 4 age and IQ matched healthy subjects that lived in the same area served as comparison subjects. None of the patients or controls had a history of secondary neurological or psychiatric disease. Demographic data is presented in Table 1.

	UWD	Controls
N	4	4
Age	38.5 (7.7)	32.0 (9.0)
VIQ	84.3 (6.3)	89.3 (2.5)
PIQ	82.0 (6.1)	89.0 (2.9)
FSIQ	81.5 (5.6)	88.0 (2.9)

Table 1. Demographic data for the patients and controls showing mean age and intelligence scores from the Wechsler Abbreviated Scale of Intelligence (WASI). VIQ = verbal IQ, PIQ = performance IQ, FSIQ = Full scale IQ. All participants were female.

Lesion extent is portrayed in Figure 1, showing primary damage in the posterior amygdala extending slightly into the anterior hippocampus while amygdala damage was restricted to basolateral regions. Importantly, the hippocampus is involved in contextual and trace conditioning but is not involved in cued fear conditioning as we assessed here (Antoniadis et al 2007; Bechara et al 1995; Marschner et al 2008).

Stimuli and apparatus

Conditioning stimuli were two neutral faces taken from the Psychological Image Collection at Stirling (PICS, http://pics.stir.ac.uk/), one coloured blue and one coloured yellow to increase distinctiveness. Each was presented against a black background. A black and white masking image of similar size replaced the conditioned stimulus (CS) during intervals between two CS presentations, to allow indexation of startle when no CS was presented (noise alone condition). Pictures were presented in a semi-random order that was designed to equally distribute conditions across each phase. See figure 2 for an overview of the design.

To rule out any confounding order effects, 2 balanced trial sequences were created which were equally distributed across controls and patients. Each CS was presented for 6 s. One CS (CS+) was always followed by an aversive 100 dBA female scream (Lissek et al 2005; Massar et al 2011). The scream was administered 5500 ms after CS onset and presented through

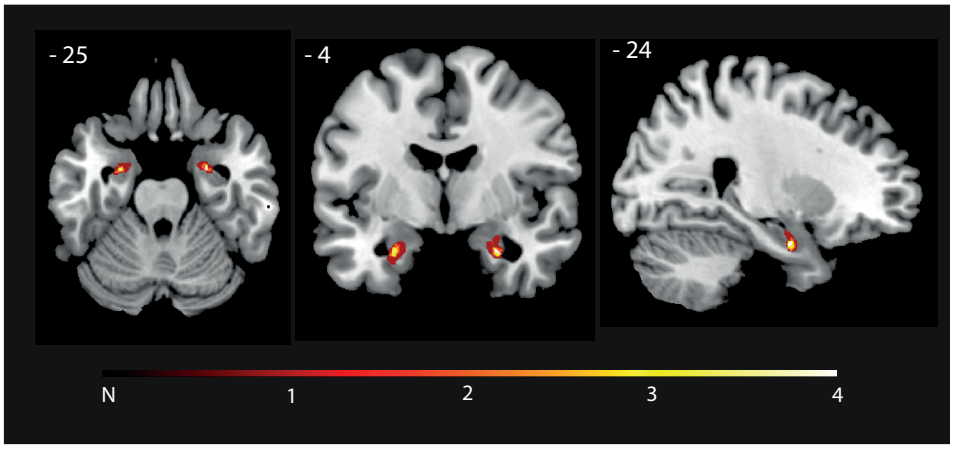

Figure 1. Lesion extent and overlap of the patient sample projected on a T1 template image. Colour coding reveals to what extent lesions overlapped across the 4 patients.

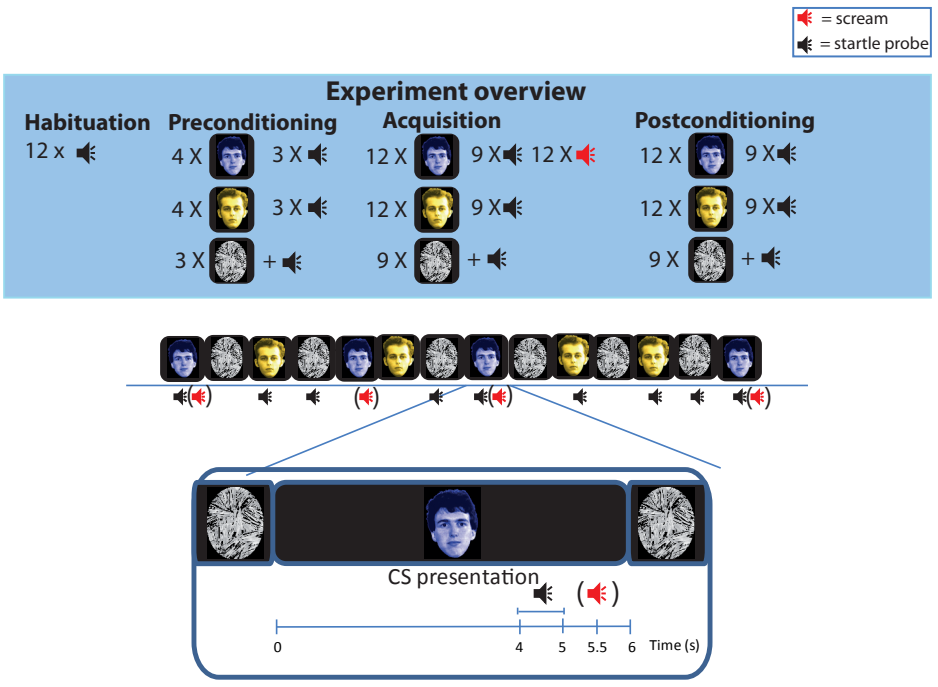

Figure 2. Illustration of the design. The top of the illustration (in blue) gives an overview of the number of trials, startle probes and aversive scream presentations during each experimental phase (habituation, preconditioning, acquisition and post conditioning). The bottom contains an excerpt of the task and shows the timing of startle probe and scream during CS presentations. Screams were only presented during presentation of the CS+ (the blue face in this illustration). Screams are presented in brackets because they were only delivered during the acquisition phase.

headphones (specs / manufacturer). The other CS (CS-) was never coupled to the scream. The startle probes consisted of a 105 dB(A) burst of white noise with near instantaneous rise time and 50-ms duration. Startle probes were presented at 4000 or 5000 ms after CS onset or at semi-random moments during the presentation of the mask (noise alone or NA trials). Inter startle intervals were programmed to be 17, 20 or 23 seconds with a mean of 20 s for each of the conditions (CS+, CS–, NA). To preclude confounding effects of the screams on startle reactions, screams were also counted as startling stimuli and the determination of minimal interval after a scream was the same as for startle probes (thus at least 17 seconds.). ITIs between CSs were varied in duration between 3.5 and 31 seconds.

Physiological recording and amplification was carried out using the Biosemi Active Two system (www.biosemi.nl). Startle was measured with electromyography (EMG) from the orbicularis oculi muscle under the right eye. One electrode was located under the pupil and the other +/- 15mm more towards the lateral cantus of the eye.

Procedure

General instructions

Before the procedure, subjects were instructed that during the task pictures and loud sounds would be presented. Subjects were instructed to refrain from large movements and to keep watching the pictures on the screen. No instructions were given as to the CS-US contingencies.

Habituation

A series of 12 startle probes were presented to induce startle habituation and index baseline startle levels.

Preconditioning

After the habituation phase, subjects were presented with 4 presentations of each conditioned stimulus. In this phase neither CS was followed by the scream. Startle probes were presented in 3 out of 4 CS presentations.

Conditioning

After the preconditioning phase, subjects were informed that screams could be presented in the following phase. The conditioning phase consisted of 12 presentations of each CS. Presentation of one of the CS pictures (the CS+) was always followed by the aversive scream. The other CS (CS-) was never followed by the scream. 9 startle probes were presented in each condition (CS+, CS-, NA).

Post conditioning

The post conditioning phase again consisted of 12 presentations of each CS. The scream was no longer presented. Again, 9 startle probes were presented in each condition (CS+, CS-, NA).

Awareness check

After the post-conditioning phase the subjects were asked whether they could predict the scream in any way. Following a positive response, the CS+, CS- and ITI pictures were presented and subjects were asked to indicate which of the pictures predicted presentation of the scream.

Data processing

Startle data were preprocessed according to previously published guidelines (Blumenthal et al 2005) and artifact rejection procedures (Bocker et al 2004). In brief, startle data were segmented, bandpass filtered (28–500 Hz, 24 dB/oct), rectified, smoothed and again baseline corrected in Analyzer (Brainproducts) and the highest peak in the resulting signal was taken as the amplitude of the response. Data were checked for artifacts such as spontaneous blinks and movement in the analysis window using in-house developed scripts and trials that showed less than 55% increase in standard deviation relative to the baseline immediately preceding the response were scored as null responses. In one patient, very small startle responses were recorded, possibly related to skin abnormalities commonly present in UWD (cf. Buchanan et al 2004; Thornton et al 2008). In this patient, artifact free, non-zero startle responses were present in 30 % of trials only. Therefore results are also reported excluding this patient from the analysis. In other subjects adequate responses were observed in at least 60 % of trials, and there were no significant differences between patients and controls in the number of adequate responses ($t < 1$).

Amplitudes from all artifact free epochs were averaged per condition and phase. Startle amplitudes during the habituation phase were averaged to characterize startle habituation and baseline startle amplitude. For the preconditioning and conditioning phase all amplitudes were converted into Z-scores per subject by using all startle trials, so that potential differences in baseline startle would not confound the results. Due to the small number of subjects and the resulting low signal to noise ratio, all analyses for the conditioning phases were conducted on the average of all startle amplitudes per condition (CS+, CS-, ITI) in each phase of the experiment.

Statistical analysis

We compared patients to controls in independent samples T-Tests on startle amplitudes during each condition (CS+, CS-, NA) in the preconditioning phase to rule out differences in general reactivity to the stimuli. In conditioning and post conditioning phase, we tested for differences in fear potentiation of startle (FPS) which was indexed by contrasting CS+ vs. NA. CS discrimination was assessed by comparing groups on the difference in startle amplitudes during CS+ and CS-. For further exploration, average startle amplitudes for each condition (CS+, CS-, NA) were compared between groups. Given the limited sample size, p-values smaller than .10 were considered significant when testing our main hypothesis of reduced fear conditioning in patients vs. controls. For the most significant results we added non-parametric statistics (Mann-Whitney U tests).

RESULTS

Subjective report data

3 of 4 controls reported awareness of the CS+-US contingency, while 2 of 4 UWD patients reported awareness. Of the subjects that reported awareness 1 subject (from the control group) did not accurately identify the CS+ in a multiple-choice question. The percentage of subject that could accurately predict the US on a cognitive level was therefore 50% in both groups.

Startle data

Habituation

During habituation, average baseline startle amplitude was at comparable levels for patients and controls ($t < 1.$)

Preconditioning

During the preconditioning phase, there was no difference between patients and controls in startle amplitudes during CS+, CS- and NA (all p-values > .5). There was also no difference in FPS or CS discrimination (t-values < 1).

Conditioning

During fear conditioning, controls showed the expected pattern of startle modulation while patients showed no modulation (Figure 3). In controls, startle amplitudes were largest during the CS+ and smallest when no face was on the screen (NA), with the amplitudes during the CS- in the middle. Exploratory testing revealed that in this small group of controls there was a non-significant startle potentiation during the CS+ compared to NA ($F = 4.8$, $p = .11$; CS+ vs. CS-). Contrarily, patients showed no sign of startle potentiation during the CS+ relative to ITI or CS - (Figure 3, F's < 1). Controls showed greater FPS than patients (CS+ versus NA; $p = .09$, non-parametric $p = .15$). Crucially, patients compared to controls showed reduced startle amplitudes during the CS+ ($t = 2.37$, $p = .06$, non-parametric $p = .08$). There were no differences between the groups in the other conditions (CS- and NA; p-values > .24) or in CS discrimination (T < 1).[1]

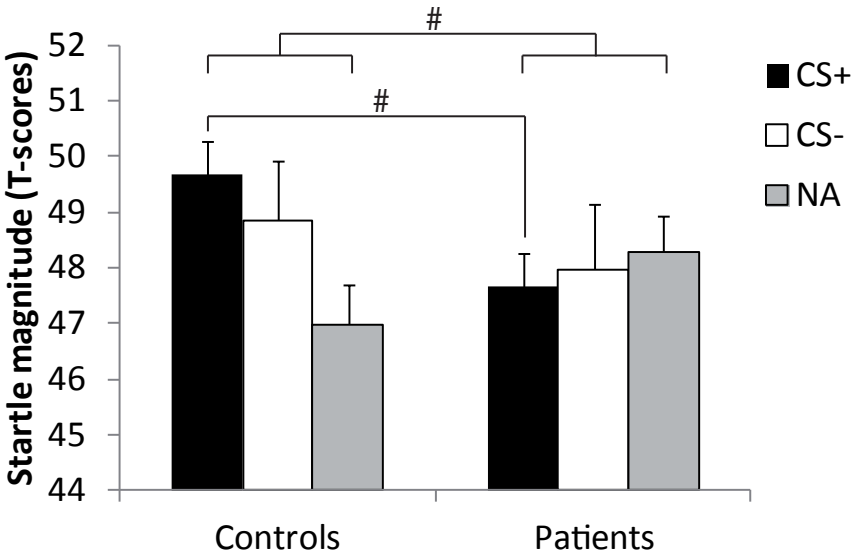

Figure 3. Mean startle amplitudes for the acquisition phase. Error bars represent standard error of the mean. $^{\#}p < .1$

1 When the patient who showed a large number of null responses was removed a similar trend of reduced responding to the CS+ was present although statistics were weaker (CS+ vs NA: T = 1.5, $p = .20$; CS+: T = 1.8, $p = .13$).

Post conditioning

In the post conditioning phase controls again showed greater FPS than patients (T = 1.9, p = .11; non-parametric p = .09; Figure 4). Again reduced responding to the CS+ was observed in patients, although statistics were weaker than in the conditioning phase (T = 1.5, p = .19; non-parametric p = .24). There were no differences in the other conditions (p-values > .51) and there was also no difference in CS discrimination (T < 1).[2]

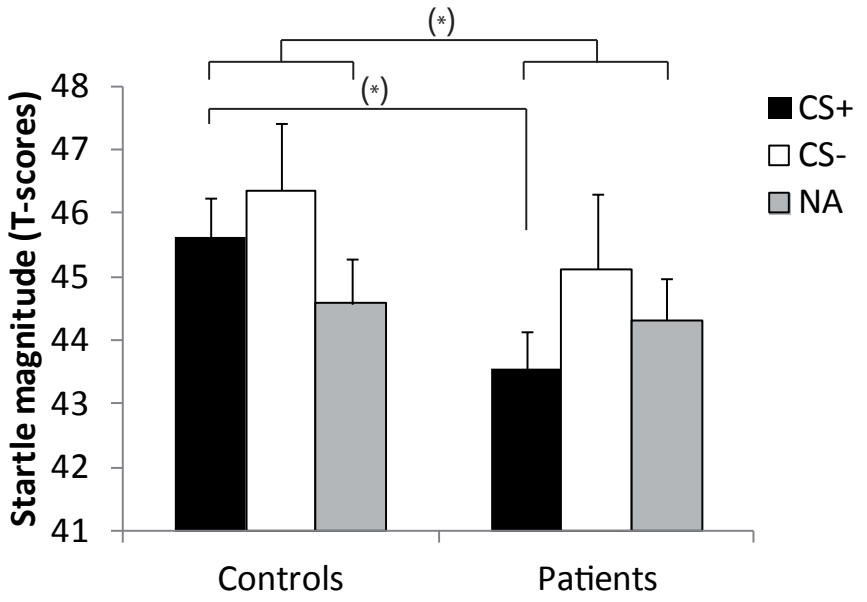

Figure 4. Mean startle amplitudes for the post conditioning phase. Error bars represent standard error of the mean. (*) p < .05 when excluding patient that showed excessive null responses.

DISCUSSION

Across this small sample of patients with rare selective lesions in the basolateral nuclei of the amygdala, we observed a lack of differential responding during fear conditioning. While controls showed an increase in startle potentiation during conditioning, patients showed no

2 When the subject with missing data was excluded, there was a significant difference between patients and controls in responding to the CS+ vs NA (F = 6.7, p = .05, non-parametric p = .03). Also responding to the CS+ was significantly higher in controls (T = 3.8, p = .01, non-parametric p = .03). There was no difference between patients and controls in the other conditions (p-values > .28).

evidence for startle modulation. In a post-conditioning phase, patients again showed reduced responding to the CS+ compared to controls.

Differences between conditions and groups did not always reach formal statistical significance in this small group. However, it is important to note that the pattern of results in the control group was identical to what has been observed in previous fear conditioned startle studies with similar designs (Klumpers et al in press; Weike et al 2005). In comparison to those studies, one difference is the scream that was used in the current study as unconditioned fear stimulus instead of electrical stimulation. Previous studies that have used this scream stimulus have found robust conditioned responses (Lissek et al 2005; Massar et al 2011). In a recent study, even low anxious participants showed an attentional bias towards a cue that was coupled with the aversive scream (Massar et al 2011). Nevertheless, patients with damage to the lateral amygdala showed not even a numerical increase in startle potentiation during the CS+, neither during nor following conditioning.

Given that UWD brain calcification progresses gradually, we cannot rule out that other brain areas also show abnormal function due to altered plasticity that may have developed in these subjects. This is however not likely to explain these findings for two reasons. First, the amygdala must be considered the most relevant structure for fear conditioning given the prior evidence (Antoniadis et al 2009; Bechara et al 1995; Davis and Whalen 2001; Jimenez and Maren 2009; LaBar et al 1995; Maren and Quirk 2004; Sierra-Mercado et al 2011; Weike et al 2005). This makes it unlikely that secondary plasticity in other regions is the driving force behind the difference between patients and controls. Second, in a sample that overlaps with the current sample it was recently found that patients with primary damage in the LA show relatively normal recruitment of the centromedial amygdala. More specifically, patients showed activation of the centromedial amygdala areas as measured with functional magnetic resonance imaging during emotional face matching (Terburg et al., in preparation), a task that is known to reliably activate the amygdala in healthy subjects (Hariri et al 2002; Manuck et al 2007). This suggests that even within the amygdala, other nuclei may still show adequate reactivity.

In conclusion, this study provides exciting new evidence for the role of the human amygdala, particularly the lateral amygdala, in the acquisition of fear. Our findings suggest that reduced function of the lateral amygdala leads to a reduced capacity to show automatic defensive responses to potentially threatening stimuli, replicating findings from animal studies (Davis and Whalen 2001; Maren and Quirk 2004; Sierra-Mercado et al 2011).

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CHAPTER 8

ANXIOLYSIS FOLLOWING DEEP BRAIN STIMULATION OF THE NUCLEUS ACCUMBENS DOES NOT AFFECT FEAR-POTENTIATED STARTLE

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ABSTRACT

Deep brain stimulation (DBS) targeted at the nucleus accumbens is effective in reducing severity of obsessive compulsive disorder (OCD). The site of stimulation near the bed nucleus of the stria terminalis (BNST) may imply that part of the therapeutic efficacy observed involves anxiolysis through modulation of contextual anxiety generated by the BNST. The hypothesis that DBS could affect contextual anxiety was tested with baseline startle and potentiated startle in an experimental threat of shock paradigm. Eight patients with DBS treatment for severe OCD were tested in a double-blind crossover design with randomly assigned two-week periods of active and sham stimulation.DBS resulted in significant treatment effects on clinical assessments of obsessive symptoms, anxiety and depression. The threat manipulation resulted in a clear context potentiated startle effect, but none of the parameters derived from the startle recordings was affected by the treatment. Also, no differences were observed with measurements in healthy controls taken from a previous study. The finding that baseline startle and context potentiated startle are not affected by DBS in this sample of OCD patients suggests that the efficacy of this treatment is through modification of brain systems unrelated to the basic defense circuits that modulate startle. These results are in line with the suggestion that fronto-striatal pathology observed in OCD can be dissociated from the altered reactivity of brain circuits involved in defensive states observed in other anxiety disorders.

INTRODUCTION

Obsessive Compulsive Disorder (OCD) is an anxiety disorder characterized by persistent thoughts (obsessions) and repetitive ritualistic behaviors (compulsions). Treatments for OCD consist of cognitive-behavioral therapy and pharmacotherapy with serotonin reuptake inhibitors (SRIs). Even when the best available treatments are applied approximately 10% of all patients remain severely affected and suffer from treatment-refractory OCD (Denys 2006). For a proportion of these patients, deep brain stimulation (DBS) may be a treatment option (Denys and Mantione 2009).

DBS is a neurosurgical treatment involving programmable electrical stimulation of brain tissue through electrodes implanted in specific locations of the brain. DBS is effective in patients with treatment-refractory OCD when aimed at several different targets, including the anterior limb of the internal capsule, the ventral striatum, the nucleus accumbens, or the subthalamic nucleus (Abelson et al 2005; Greenberg et al; Greenberg et al 2006; Mallet et al 2008; Nuttin et al 1999; Nuttin et al 2003). A placebo-controlled study showed that bilateral stimulation of the nucleus accumbens may be an effective and safe treatment in highly refractory OCD patients (Sturm et al 2003). In a second study, accumbens stimulation resulted in immediate and pronounced reduction of obsessive compulsive symptoms (Yale Brown Obsessive-Compulsive Scale, YBOCS 46%), depressive symptoms (Hamilton Depression Rating Scale, HAM-D 50%) and anxiety (Hamilton Anxiety Rating Scale, HAM-A 50%) (Denys et al 2010). This study also included a double blind cross-over phase, during which the current study was carried out in an attempt to clarify the underlying mechanism for the therapeutic effect of accumbens stimulation in OCD.

The stimulation site near to the nucleus accumbens includes many nuclei among which the bed nucleus of the stria terminalis (BNST). The BNST is considered a part of the extended amygdala, which 'appears as a large ring formation around the internal capsule' (de Olmos and Heimer 1999), and has its largest cell concentration surrounding the anterior commissure bilaterally (Mai et al 2003). Preclinical work has established that phasic fear responses to specific threats are orchestrated by the amygdala (Davis and Whalen 2001; LeDoux 2003). In contrast, contextual anxiety, a more sustained state in anticipation of potential threats not related to a specific stimulus (Bouton et al 2001; Grillon 2002; Grillon and Baas 2003), relies on the BNST and the hippocampus (Bouton et al 2001; Davis et al 2010; Duvarci et al 2009; Straube et al 2007; Sullivan et al 2004; Walker et al 2003). The involvement of the BNST in contextual anxiety may imply that the therapeutic efficacy observed in OCD with DBS of anatomical areas near the BNST may involve anxiolysis through modulation of contextual

anxiety generated by the BNST. The double blind cross over DBS protocol allowed a unique opportunity to assess acute effects of DBS on reactivity of human defense states. The aim of the current study was therefore to investigate whether anxiolysis through DBS involves changes in basic defense systems in the brain indexed by fear potentiated startle. We hypothesized that DBS may specifically affect anxiety in a threatening context that models the future-oriented anticipation of threats that is characteristic for OCD.

To this end, the current study employed an experimental threat of shock paradigm in combination with fear-potentiated startle (FPS) recordings. The design was developed to elicit defensive states at different levels. Cued fear was elicited by means of a predictable shock condition and contextual anxiety was evoked by the general experimental context (assessed with baseline startle recordings) and an unpredictable shock condition (Grillon et al 2004). Patients who were stabilized several months after having been implanted with a deep brain stimulator, were tested in a randomized on / off protocol, allowing each patient to serve as his or her own control. The specific hypotheses were that contextual anxiety would be elevated with the stimulator off with respect to stimulator on, as reflected in elevated baseline startle or shock sensitization after the shock workup in addition to increased context-potentiated startle. In contrast, we expected cued fear-potentiated startle to be unaffected. Because relatively few studies have assessed startle reactivity in patients with OCD, a comparison with a group of healthy control subjects who underwent the same startle modulation protocol was included.

METHODS AND MATERIALS

Patients

Patients were recruited from the clinic for anxiety disorders at our university hospital. All patients consented to participate in this study and signed an informed consent form. The Medical Ethical Review committee of our hospital approved the trial of which this experimental study was part. Eight patients from a clinical trial reported in reference (Denys et al 2010) participated in the startle protocol. See reference (Denys et al 2010) for patient details (case numbers 2, 4, 5, 8, 10-12, 15 participated in the startle protocol) and the report of the primary clinical endpoints. The trial was registered under trial number ISRCTN23255677 in the international controlled trial registry. One patient underwent only baseline startle measurements and due to logistical complications not all patients completed all sessions. Patient characteristics of this sample were: 4 male, 4 female, mean age 39.2 (S.D.

11.1), mean Y-BOCS scores prior to start of DBS treatment 34.0 (S.D.3.7) and after having been stabilized on DBS treatment 13.1 (S.D. 10.6), mean percentage improvement was 61% (range 7% - 100%).

Treatments

The study was part of a larger protocol in which the patients underwent surgery for implementation of the deep brain stimulator and follow up assessments (Denys et al 2010). The DBS electrodes were 4 direct-contact electrodes (Medtronic Inc, Minneapolis, Minnesota; contact points 1.5-mm long, separation 0.5 mm) implanted bilaterally. Target coordinates for the electrode tip were close to the anterior commissure (7 mm lateral to the midline, 3 mm anterior to the anterior border of the anterior commissure, and 4 mm inferior to the intercommissural line; see (Denys et al 2010) for more detail). The phase of the protocol in which this study took place was a one month, double-blind, sham-controlled phase that took place after patients had been stabilized on the DBS treatment for 8 months(see (Denys et al 2010) for more detail). Patients were randomly allocated to two periods of two weeks with the stimulators blindly turned on (active stimulation) in one period and turned off in the other. The laboratory personnel conducting the startle protocol was blind to stimulation conditions. Fear-potentiated startle measurements were planned at three time points surrounding the on-off phase of the protocol: (I) at baseline (i.e. after the 8 moth stabilization, stimulator on), (II) at the end of a two week period of active or sham stimulation (right before switching to the other condition) and (III) at the end of the second two week period of reversed active or sham stimulation.

Stimuli and apparatus

Shock reinforcements were delivered through two disk electrodes located on the inside of the subjects' forearms. Stimulation level was individually set with a standardized shock work-up procedure. The startle reflex was evoked by bursts of white noise presented through headphones with 50-ms duration and an intensity of 105 dB(A). Eye blink electromyography (EMG) was measured with 2 electrodes on the lower orbicularis oculi with a Biosemi system (www.biosemi.com). Task conditions and visual analog scales (VAS) were presented on a computer screen by automated scripts (Presentation, www.neurobs.com). Patients were tested using laboratory equipment set up in a dedicated room at the University Medical Center of Utrecht University or the Academic Medical Center of the University of Amsterdam, the Netherlands.

Experimental procedure

Clinical rating scales for Obsessive-Compulsive symptoms (YBOCS), depressive symptoms (HAM-D) and anxiety (HAM-A) were assessed prior to the FPS procedure. The FPS procedure started with placement of electrodes on the orbicularis oculi muscle for startle measurement. The design of the current study allowed assessment of several levels of contextual anxiety. First, baseline startle measurements were taken (2 series of 9 startle probes presented with average intervals of 16 sec). Then, the shock work up procedure to determine the shock levels for the FPS experiment was performed. Instructions concerning the FPS experiment were given, after which another series of 9 startle probes was presented. The FPS experiment followed immediately after the last habituation phase, and consisted of three contexts in which instructions regarding possible shock administration varied (cf. (Grillon et al 2009a)). The three contexts were signaled by written instructions displayed on a computer monitor. Instructions were: 'No shock' (Neutral context, N); 'Shock only during cue' (Predictable context, P); and 'Shock at any time' (Unpredictable context, U). In each context, cues were presented, e.g., red square for N, blue circle for P, and green triangle for U (counterbalanced between subjects). These cues were only predictive of a possible shock in the P context. Duration of contexts was 90-100 s, during which the written instructions concerning shock delivery remained on the screen and four cues were presented at regular time intervals with 8-sec duration. Startle responding was assessed during all experimental conditions, i.e., during the presentation of these cues and in their absence to measure responses to the context. After each block subjective ratings on levels of fear specified for all experimental conditions were collected using computerized VAS scales [anchors 0 = not fearful, 100 = very fearful], as well as ratings on subjective state at that time, including fearfulness [same anchors], calmness [anchors 0 = not at all calm, 100 = very calm], alertness [anchors 0 = not alert, 100 = very alert] and need for control [anchors 0 = no need, 100 = strong need].

Data analyses

Analysis of the EMG signal was carried out in Brain Vision Analyzer software (www.brainproducts.com) according to established procedures (Blumenthal et al 2005). All reported analyses were based on normalized data to reduce between-subjects variance in baseline startle. There was no difference in statistical results with the raw data analyses. Z-scores were calculated across all measurements per patient and converted to T-scores ([Z*10]+50). Averages per session and condition across trials were calculated. To use all available data, data from the two 'on' sessions were averaged together for patients who participated in a baseline assessment in addition to an 'on' session in the crossover phase (5 out of 8 patients). Pattern

of results from both the baseline and double blind 'on' sessions did not differ and results were the same for analyses in which only the blinded conditions were included. Order of on/off sessions was balanced across patients with 4 patients starting with 'on' and 4 patients starting with 'off'.

Statistical analysis

All statistical analyses were univariate repeated measures analyses of variance in SPSS 16.0 for Windows. Statistical tests for the habituation phase were aimed at the series before and after the shock workup separately with factors DBS (on, off) x Block. The factor Block consisted of averages of three subsequent startle trials, and contained 6 levels in the series before and 3 levels in the series after the shock workup. For threat modulation of startle, a DBS (on, off) x Context (neutral, predictable, unpredictable) x Cue (intertrial interval, cue) repeated measures analyses was conducted. In addition, specific tests were aimed at DBS on/off effects on FPS and subjective fear evoked by cued threat and unpredictable shock. These consisted of Cue (absent, present) x DBS (on, off) and Context (Neutral, Unpredictable) x DBS (on, off) repeated measures ANOVAs. An additional ANOVA tested for differences with normalized startle modulation data from a control group (placebo session taken from reference (Baas et al 2009)) with repeated measures for the task conditions and group as a between subject factor for the on and off sessions separately.

RESULTS

Effects of treatment on mood and anxiety

Switching the stimulator off during the cross over phase resulted in significant increases in OCD symptoms (Y-BOCS on 19.4 (S.D. 10.8), off 28.5 (S.D. 10.9), difference $t(7) = 2.7$, $p < .05$), anxiety (HAMA on 12.3 (S.D. 4.7), off 24.3 (S.D. 8.5), difference $t(7) = 4.4$, $p < .001$), and depressed mood (HAM-D on 11.6 (S.D. 5.5), off 23.8 (S.D. 9.6), difference $t(7) = 5.2$, $p < .001$).

Baseline startle

Baseline startle data is illustrated in Figure 1. The repeated measures ANOVA for the series before the shock workup included factors DBS (on, off) x Block (6). There was a significant main effect of Block, indicating habituation of the startle reflex across time (Block $F(5,35) = 8.2$, $p < .001$). Our hypothesis that baseline startle would be higher or more resistant to habituation with the stimulator turned off was not confirmed (main effect for DBS $F(1,7) = 1.1$, NS; DBS x Block $F(5,35) = 1.5$, NS). The series after the shock workup analyzed with

factors DBS (on, off) x Block (3) yielded comparable results: main effect of Block ($F(2,14) = 20.8$, $p < .001$), no effect of DBS (DBS $F(1,7) = 0.8$, NS; DBS x Block $F(2,14) = 0.2$, NS). To investigate the increases in startle after the shock workup, a specific comparison between the last block before and first block after the workup was made. The workup induced a significant increase in startle in the transition from series 2 to series 3 ($F(1,7) = 71.5$, $p < .001$). This effect did not differ between the DBS conditions ($F(1,7) = 0.6$, NS). This indicates that there were no effects of DBS in the startle magnitude data on baseline startle or due to shock sensitization following the workup.

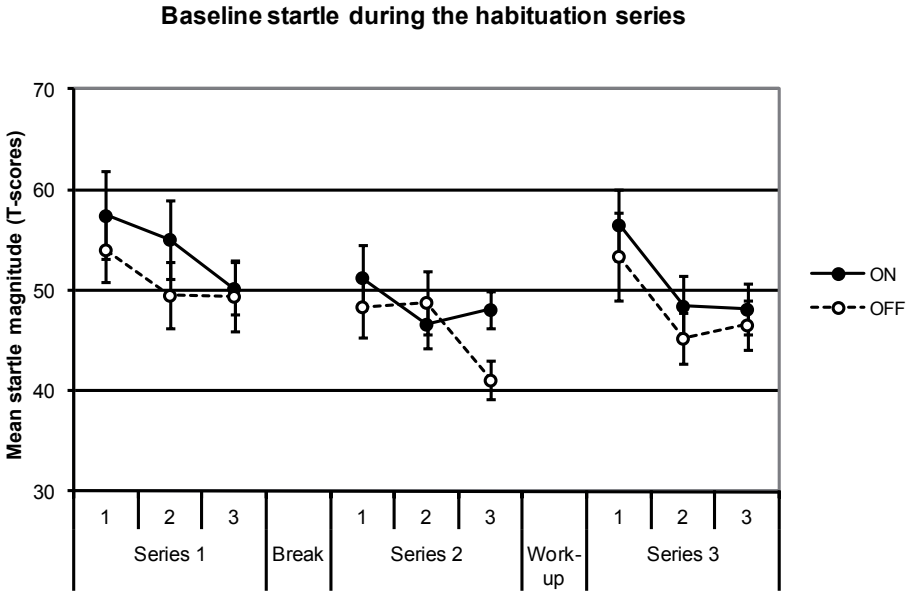

Figure 1. Baseline startle, data averaged across groups of three subsequent trials in each of the three habituation series. Displayed are the mean magnitudes with standard errors of the mean.

Fear-potentiated startle - Contexts and Cues

The overall analysis of the startle data revealed the expected effects of threat, see Figure 2. The main effects of Context ($F(2,12) = 9.1$; $p = .004$) and Cue ($F(1,6) = 5.7$; $p = .064$) were (trend level) significant, as well as their interaction ($F(2,12) = 7.7$; $p = .007$). DBS (on, off) did not interact with any of these factors (DBS x Context $F(2,12) = 0.4$, NS; DBS x Cue $F(2,12) = 0.1$, NS; DBS x Context x Cue $F(2,12) = 0.2$, NS). Also, as observed in the baseline analysis, the manipulation of switching the DBS on or off did not affect the overall level of startle ($F(1,6) = 0.3$, NS).

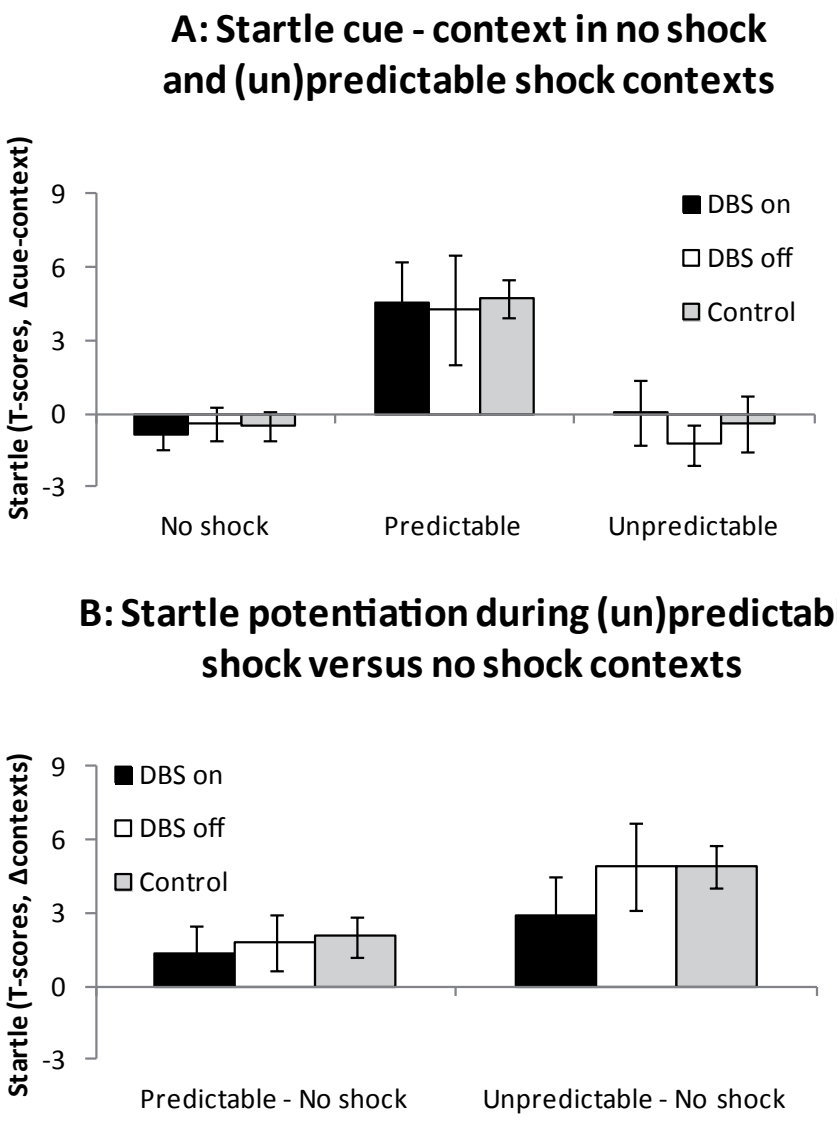

Figure 2. Significant fear potentiated startle effects as a result of the threat manipulation were apparent during the Predictable context (panel A, difference scores cue minus context), and in the comparison between the Neutral and Unpredictable contexts (panel B, difference scores (un)predictable minus neutral contexts). Displayed are the mean magnitudes with standard errors of the mean.

More specific contrasts to compare the cue effect in the predictable context (Figure 2, panel A), and the context effect across neutral and unpredictable contexts in absence of the cues (Figure 2, panel B) were performed. Effects of DBS on explicitly cued fear were tested within the predictable condition with factors DBS (on, off) and Cue (Absent, Present). This test again yielded a significant effect of Cue ($F(1,6) = 9.6$; $p = .021$) but no interaction DBS x Cue ($F(1,6) = 0.0$, NS), in line with our hypothesis. The specific test of differences in contextual anxiety effects on startle potentiation between DBS on and off included only the factors Session (DBS on, off) and Context (Neutral, Unpredictable). This test yielded again a significant effect of Context ($F(1,6) = 7.7$; $p = .033$) and a non-significant effect of DBS (DBS x Context $F(1,6) = 1.4$, NS). This is not due to lack of power, as the apparent DBS effect on context (U – N) in the figure disappeared almost completely after removing one patient with excessive artifacts, yet the pattern of statistical results remained the same. There were no differences between the patients and the control group taken from a previous study on between-subjects comparisons of cued or contextual startle potentiation (all t values < 1.4, NS).

Subjective reports – Threat manipulation

See Figure 3 for the fearfulness ratings during all conditions in the FPS experiment. In line with the startle results there were (trend level) significant effects of both the context and cue manipulation. The overall analysis with the factors DBS (on, off) x Context (Neutral, Predictable, Unpredictable) x Cue (Absent, Present) x Block (1,2) revealed (trend level) significant main effects of Context ($F(2,10) = 4.3$, $p = .045$) and Cue ($F(1,5) = 5.2$, $p = .071$). Their interaction was not significant ($F(2,10) = 2.4$, NS). DBS (on, off) did not interact with any of these factors (DBS x Context $F(2,10) = 0.1$, NS; DBS x Cue $F(2,10) = 2.4$, NS; DBS x Context x Cue $F(2,10) = 1.2$, NS). Specific tests of the cue effect in the predictable condition (Figure 3 panel A) and the context effect (unpredictable – neutral, Figure 3 panel B) between DBS conditions were also not significant ($F1,5) < 1.5$, NS). Also, in contrast to the baseline anxiety ratings, DBS on / off did not significantly affect the overall level of fearfulness ($F(1,5) = 1.6$, NS).

Subjective state

See Table 1 for the assessments of anxiety, need for control, calmness and alertness. Subjective reports were missing for one patient. Because of the small size of the remaining sample (n=6), effects of trend level significance ($p < .10$) are considered meaningful. Ratings of subjective states of anxiety, calmness, alertness and need for control were collected before the experiment and after each of the habituation blocks (3) and each of the FPS blocks (2).

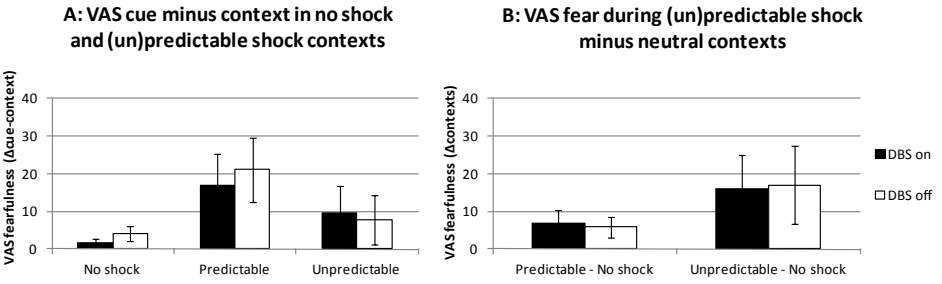

Figure 3. Ratings of fearfulness during threat manipulation. Effects of threat were found during the Predictable context (panel A, difference cue minus context), and in the comparison between the Neutral and Unpredictable contexts (panel B, difference scores (un)predictable minus neutral contexts). Displayed are the mean magnitudes with standard errors of the mean.

		Moment					
		Pre	Hab 1	Hab 2	Hab 3	FPS 1	FPS 2
Anxious	On	33.3 (9.2)	45.0 (12.3)	38.8 (12.4)	24.9 (10.4)	52.4 (7.4)	68.0 (11.3)
	Off	54.0 (9.6)	55.2 (13.0)	55.2 (12.0)	51.4 (16.6)	37.2 (10.1)	52.5 (16.0)
Need for control	On	37.4 (8.5)	42.0 (13.3)	34.5 (12.0)	38.8 (12.7)	35.0 (12.4)	24.8 (11.0)
	Off	47.0 (8.6)	49.8 (10.8)	43.0 (10.8)	41.0 (12.7)	49.3 (13.6)	45.0 (15.8)
Alert	On	63.7 (10.6)	53.8 (9.9)	51.7 (8.7)	50.9 (7.5)	35.8 (10.4)	34.0 (11.6)
	Off	62.8 (12.0)	56.8 (13.6)	42.8 (10.5)	53.0 (16.2)	56.5 (12.2)	34.0 (11.4)
Calm	On	62.0 (4.6)	48.4 (13.4)	56.4 (12.0)	57.0 (13.1)	36.6 (12.2)	26.5 (11.8)
	Off	40.0 (11.6)	42.8 (13.9)	39.0 (10.5)	67.6 (15.4)	50.0 (10.7)	44.0 (11.1)

Table 1. Subjective ratings taken at 6 moments in between the blocks of the habituation and fear-potentiated startle experiments. The moments were before start of the session (Pre), after each of the three habituation blocks (Hab 1,2,3), and after the two fear-potentiated startle blocks (FPS 1,2).

Only subjective anxiety differed between the DBS on and off conditions, with patients reporting more anxiety throughout the test session when the stimulator was switched off (see Table 1, trend level significant main effect of DBS, $F(1,5) = 4.9$, $p = .078$; F values for other measures < 1.8, NS). The need for control showed a similar sustained effect, with assessments taken during the stimulator off condition being higher, but this did not result in a (trend level) significant test. There was no interaction between DBS status and the different time points at which the measurements were taken (DBS x Moment for all measures, $F(1,5) < 1.8$, NS).

Chapter 8

DISCUSSION

The data of this study demonstrate clear effects of startle potentiation by threatening cues and contexts, even in this small sample of patients. Moreover, electrical stimulation of the nucleus accumbens (NAcc) had clear effects on subjects' mood and OCD symptoms. Yet, we found no effects of NAcc stimulation on any parameter of baseline startle or fear-potentiated startle. The threat manipulations also affected subjective fearfulness but again without an effect of DBS. Therefore, our hypothesis that patients would display increased sensitivity to contextual fear manipulation with the stimulator turned off was not confirmed. As hypothesized, there were also no effects of electrical stimulation on cued fear. Additionally, an exploratory comparison showed that the startle modulation data from the patients did not differ from the data from a group of healthy individuals assessed in a related study.

Even though most of our hypotheses could not be confirmed, these results are certainly meaningful. Though DBS had a pronounced effect on anxiety symptoms in these patients (see also (Denys et al 2010)), the basic startle and fear-potentiated startle effects remained unaffected. This suggests that DBS at the NAcc is effective in treating anxiety symptoms of obsessive compulsive disorder without modulating the startle circuitry. Effective reduction of symptoms without effects on startle in addition to no difference in startle modulation between patients and controls suggest that the core pathology in OCD may not involve alterations in a basic defensive state that results from the threat of electric shock. In interpreting these findings it may be of importance that OCD is not considered a typical anxiety disorder (Stein et al). This argument is supported by neurocognitive, neuroimaging and pharmacotherapy studies that differentiate OCD from other anxiety disorders. OCD patients typically show baseline hyperactivity and increased activity after symptom provocation within the corticostriatal circuitry, a profile that is absent in neuroimaging studies of patients with other anxiety disorders (Etkin and Wager 2007; Menzies et al 2008; Rotge et al 2008; van den Heuvel et al 2005; Whiteside et al 2004). Distinct neurocognitive deficits related to corticostriatal circuitry, e.g. impaired motor initiation and execution, have been found in patients with OCD but not in other anxiety disorders (Purcell et al 1998). Finally, though pharmacological treatment with benzodiazepines is successful in anxiety and related disorders, benzodiazepines do not show efficacy in patients with OCD (Crockett et al 2004; Hollander et al 2003; Koran et al 2005). Taken together, evidence of various nature distinguishes OCD from other anxiety disorders.

The unpredictable shock context that we used apparently does not reflect the form of anxiety present in OCD. The unpredictable shock context was designed to evoke a state of longer duration contextual threat generated by the BNST as opposed to 'fear' responses to short duration cued threats generated by the amygdala (recently reviewed in reference (Walker

et al 2009)). Based on this, DBS effects were expected on contextual threat. No effects were expected on cued threat, as abnormalities in amygdala activation have been observed (Simon et al; van den Heuvel et al 2004) but not consistently (Cannistraro et al 2004; Deckersbach et al 2006), suggesting that they are not at the core of OCD pathology. However, other authors have suggested an alternative distinction between fear and anxiety responses in immediate versus potential threats. Potential threats evoke a set of behaviors that is clearly distinct from immediate threats, including risk assessment (Blanchard et al 2011) and precautionary behavior (Eilam et al 2011). A failure to shut down precautionary behaviors has been suggested to underlie the pathology of OCD (Boyer and Lienard 2006; Szechtman and Woody 2004). Whereas threat detection systems including the amygdala and BNST are involved in the initiation of precautionary behaviors (Szechtman and Woody 2004), the failure to terminate these behaviors may be more related to dysfuntional cortico-striatal loops (Woody and Szechtman 2011).

The idea that the pathology in OCD involves a failure to terminate risk assessment behavior associated with potential threats rather than responding to threat itself concurs with the present finding that OCD patients did not differ from the healthy control group in startle responding to cued and contextual threat. Interestingly, patients with generalized anxiety disorder (GAD) did also not differ from controls in cued and contextual startle responses (Grillon et al 2009b). Both OCD and GAD have been associated with more psychic or higher cognitive aspects of anxiety such as worrying and rumination, while physiological reactivity to a general stressor is not enhanced (Craske et al 2009). No previous studies of cued or contextual fear-potentiated startle in OCD have been published to date. Startle studies in the absence of a stressor, e.g. in the context of a pre-pulse inhibition experiment, did not find a general increase in startle reactivity in OCD (Hoenig et al 2005; Swerdlow et al 1993). Two studies reported elevated baseline startle in OCD in the context of aversive emotion manipulations (Hoehn-Saric et al 1995), though in one of these studies this effect disappeared when excluding patients who were on psychotropic medication (Buhlmann et al 2007)]. Our study did not find evidence for stress induced hyper reactivity in OCD patients. Taken together, these results support the conclusion that baseline startle and startle increases after general aversive stimulation does not reflect the anxiety present in OCD. However, it cannot be ruled out that OCD patients will show differential modulation of startle when using threats specific to the patients' anxiety, e.g. pictures of dirty surroundings for patients suffering from fear of contamination.

Our findings are in line with an apparent dissociation between the brain circuitry involved in defensive states in humans (Mechias et al; Mobbs et al 2009) and the orbitofrontal-striatal

circuit primarily affected in OCD as referred to above (Whiteside et al 2004). Indeed, only the latter circuit was shown to be affected by DBS treatment of the ventral striatum (Rauch et al 2006). The nucleus accumbens is involved in reward processing and motivation. Recent neuroimaging research has demonstrated dysfunctional NAcc activation related to harm avoidance (Jung et al 2010) and reward anticipation (Figee et al 2011) in OCD. DBS at the NAcc may thus improve anxiety in OCD patients secondary to a motivational shift from harm avoidance towards more healthy reward-driven behavior.

A final issue pertaining to our hypothesis based on putative effects on the BNST with stimulation of the nearby NAcc is that stimulation effects are highly dependent upon the exact brain target. As discussed by Denys et al (Denys et al 2010), response differences of 60% -80% were observed depending on which of the four electrode contacts were stimulated, even while these lie as little as 1.5 mm apart. In this protocol, the site of stimulation that appeared most effective were the relatively dorsal electrode contacts most adjacent to the BNST (Denys et al 2010). Based on the hypothesized role of the BNST in anxiety, and the projections of the BNST that determine contextual startle modulation (Davis et al 2010; Walker et al 2003) an effect on startle parameters was expected in this study. Yet, the exact effect of stimulation on neuronal tissue remains to be clarified. Relatively little is known about the exact consequences of stimulating areas of the brain in which so many functional units are tightly packed. In order to delineate the exact mechanisms behind the treatment efficacy more detailed studies of effects of stimulation at different sites within the ventral striatum are needed. These types of procedures could greatly enhance the understanding of the role of different parts of the ventral striatum in anxiety and defense.

The first and foremost limitation of the study concerns the relatively small number of patients (n = 8). Yet, this did not prohibit replication of the basic effects of cued and contextual startle potentiation. The relatively small sample also precluded detailed analysis of potential confounds with depressive symptoms, since depression may have opposite effects on fear-potentiated startle than anxiety (Melzig et al 2007). Moreover, our study employed electric shocks as threatening stimuli. Future studies could use symptom relevant threats which may prove to elicit differential reactivity in OCD.

In conclusion, DBS at the NAcc, close to the BNST, clearly improves anxiety in OCD patients, but does not affect contextual anxiety or other startle parameters in an experimental fear-potentiated startle paradigm. Experimental studies assessing alterations in basic processes as a function of targeted brain stimulation in humans are highly valuable in determining how this treatment may alleviate disease. The present findings can be taken to conclude that it is not at a basic level of defense that DBS affects symptoms of OCD.

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FINANCIAL DISCLOSURE

This DBS intervention was supported by an unrestricted investigator-initiated research grant by Medtronic Inc to Drs Denys and Schuurman, which provided the devices used. Dr Schuurman is an independent consultant for Medtronic Inc on educational matters and received travel grants from the company.

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CHAPTER 9

GENERAL DISCUSSION

Chapter 9

SUMMARY OF MAIN FINDINGS & DISCUSSION

Introduction

This thesis contains a variety of studies on neurobiological correlates of human fear and anxiety. Many years of previous research gave a relatively good overview of the neurobiological mechanisms that jump into work when a threat is detected (reviewed in **chapter 1**). The amygdala, brainstem and hypothalamus but also paralimbic cortical regions such as the anterior insula (aIns) and dorsal anterior cingulate (dACC) form a circuit that supports threat detection and subsequent expression of defensive emotion to cope with danger. On the other hand, lateral prefrontal areas allow reappraisal of threatening situations to promote more relaxed states. Ventromedial prefrontal areas permit learning that a previously threatening situation should no longer be deemed threatening. In addition, it was outlined in chapter 1 that especially ventromedial parts of the prefrontal cortex (vmPFC) are anatomically well placed to substantiate control over the defensive brain systems. The vmPFC may therefore serve as a gateway for fear control by other prefrontal areas. In the present thesis we further evaluated which neuroanatomical and pharmacological pathways are crucial in the generation and downregulation of defensive states. I start this discussion section with a brief recap of the main findings in each chapter, followed by a discussion of these findings as they pertain to the neuroanatomical and neuropharmacological circuits for the generation and downregulation of defensive states.

Summary of main findings

In **chapter 2,** we investigated the somatic and neural concomitants of the offset of threat cues. Strikingly, we found that in healthy young volunteers the somatic defensive states that arise during cues that signal threat of shock level off surprisingly fast after threat termination. Within 1.5-3.5 seconds, the defensive startle response reached levels that are comparable to responses measured during safe cues. This implies that healthy humans can rapidly switch from a state of heightened defense to a relatively relaxed state. Threat cues were associated with increased reactions of the aIns, dACC and thalamus among other regions. Moreover, we found that activity in the right anterolateral and ventromedial prefrontal cortices was linked to the termination of the threat cues, but not the termination of safe cues. These prefrontal areas showed a negative correlation in activity across the task, and activity in these areas also correlated with signal changes in the right anterior insula and amygdala. This was taken to suggest that the anterolateral PFC inhibits fear responses by connectivity with the ventromedial PFC, an area well-connected with the limbic regions involved in the generation of defensive responses.

We replicated our finding that startle potentiation in healthy young adults is reduced fast after threat diminution in **chapter 3,** showing that almost no startle potentiation remains 1.5 s after threat cues disappeared. In this study we also examined the role of a common polymorphism in the serotonin transporter gene, which has been repeatedly linked to anxiety and neural processing of fearful faces and aversive pictures. We showed that subjects carrying the less active serotonin transporter gene (S-carriers) show enhanced fear expression as indexed by fear-potentiated startle. This fitted well with the pre-existing literature that showed enhanced amygdala reactivity and trait anxiety in subjects carrying the short allele for this polymorphism. Interestingly, we did not find a difference between genotypes in startle measured 1.5 seconds after threat termination. This suggests that these healthy subjects that show genetically driven increases in reactivity during the threat cue are not necessarily impaired in the downregulation of their stronger defensive state when direct threat has waned.

In **chapter 4**, we then re-examined the neural correlates of reactions to the on- and offset of threat. Importantly, we replicated our previous findings from chapter 2 on the neuroanatomical substrates of anxiety up- and downregulation in an extended sample. Further, we explored individual differences in neural responses and their relation with serotonin transporter genotype. As expected we found evidence for exaggerated neural responding in 5-HTTLPR SS homozygotes to threat cues in the bilateral insula and at trend-level in the dACC. Moreover, higher startle potentiation was linked to more reactivity in the dACC. Interestingly, S-carriers also showed enhanced reactions to threat offset. Together with the results from chapter 3 this indicates that healthy S-carriers can recruit additional resources to regulate their stronger initial anxiety reactions after threat termination.

The psychopharmacology of conditioned fear extinction, another paradigm to measure fear regulation, was investigated in **chapter 5**. We explored the potential therapeutic value of increasing neurotransmission in two transmitter pathways during extinction training. We chose to focus on the main excitatory neurotransmitter pathway (glutamate) and particularly the N-Methyl-D-Aspartate (NMDA) receptor which has been shown to play a critical role in memory. Moreover, for the first time we explored effects on human fear extinction of a partial agonist at the main target site of endocannabinoids in the brain, the cannabinoid receptor type 1 (CB1). Based on animal literature that demonstrated that the NMDA and CB1 receptors are crucially involved in fear extinction, we administered two substances that could facilitate activity in these systems and thereby boost fear extinction. This time-limited type of pharmacological treatment could be used to increase the efficacy of the most prominent behavioral therapy for anxiety: exposure. However, administration of the partial NMDA

agonist d-cycloserine or the CB1 agonist tetra-hydrocannabinol in a group of healthy subjects before extinction training did not facilitate the retention of fear extinction measured 2 days later with fear-potentiated startle, skin conductance and subjective ratings. These results were explained in terms of a possible lack of room for improvement in healthy subjects' extinction learning. Moreover, we argued that administration of tetra-hydrocannabinol may not be the best way to facilitate CB1 transmission and further research with more selective CB1 agents may give better results in this promising field of research.

In **chapter 6** we developed a paradigm that would aid in the early screening for new anxiolytic drugs. To this end, we constructed a broad anxiety test battery and investigated whether repeatedly applying this battery was feasible within the same subjects. Subjects that were repeatedly tested showed no systematic changes in a fear-potentiated startle in a shock threat paradigm, while increase in skin conductance during threat showed evidence of habituation. Also state anxiety increases after a stressful task were reduced when the test was repeated. This indicated that particularly fear-potentiated startle is suitable for measuring effects of drugs over time. Moreover our results showed that this procedure with 4 measurements on one day can be repeated on 4 different days in the same subjects, which opens up the possibility to acquire data from different drugs, doses or other experimental conditions in the same subjects.

The last two chapters were devoted to work in two rare patient populations. We again explored the neural basis of fear learning in **chapter 7**. Previous work showed that the human amygdala plays an essential role in facilitating the acquisition of fear memory. In this study we found that selective calcification of the basolateral amygdala (BLA) impairs development of a normal fear response to cues that signaled threat of an aversive scream. This is the first evidence that shows that circumscribed lesions to the basolateral amygdala as a result of a rare genetic disease, apparently not involving the centromedial amygdala, are sufficient to cause impairments in human fear learning. As such these results extend rodent data that have indicated that the BLA is essential for normal fear learning.

In contrast to the natural lesion approach taken in chapter 7, **in chapter 8** we assessed how brain stimulation affected anxiety responses. More specifically, it was tested whether a promising treatment for severe obsessive compulsive disorder, deep brain stimulation of the ventral striatum, would affect basic defensive responding measured in a threat of shock paradigm. The results showed a dissociation between OCD symptomatology and basic defensive fear and anxiety responses. Whereas the treatment had clear effects on OCD symptoms, there was no difference in startle potentiation when the stimulator was

switched on or off. Moreover, irrespective of stimulator status OCD patients showed normal startle potentiation as observed previously in healthy controls. This result is consistent with previous suggestions that OCD symptomatology, although historically considered as evolving out of anxiety, is to some extent independent of basic defensive brain circuits.

General Discussion

Pathways for the generation of defensive states: the role of the amygdala and other brain regions

Given our findings, one interesting point of discussion is the role of the amygdala versus the role of paralimbic cortices such as the insula and anterior cingulate in human anxiety. The amygdala is considered crucial for the generation of defensive states (Davis and Whalen 2001; Feinstein et al 2011; LeDoux 2003; McNaughton and Corr 2004). Indeed, neuroimaging research into the neurobiology of fear has been dominated by amygdala research for a decade or so, in most cases by utilizing emotional face presentation as a reliable activator of the amygdala (Costafreda et al 2008; Domschke and Dannlowski 2010; Hariri et al 2002) or by applying fear conditioning paradigms (Buchel et al 1998; Sehlmeyer et al 2009; Tabbert et al 2011). Nevertheless, our instructed fear paradigm that induces reliable increases in subjective anticipatory anxiety, startle amplitude, skin conductance and activity in a broad neural network does not produce robust amygdala activation as measured with fMRI. Importantly, other groups have had similar results with both PET and fMRI (Chua et al 1999; Drabant et al 2011; Kumari et al 2007; Mechias et al 2010). Our findings that the amygdala does not reliably respond to instructed threat, while we find robust effects in other brain regions, indicate that focussing purely on the amygdala in studies investigating defence may be unwise. This is also consistent with a stronger role for the amygdala in the acquisition of fear and the following initiation of responses than in the continued expression of anxiety, as described in **chapter 1**.

Our finding that immediate threat in the form of electric shock does produce a response in the amygdala (chapter 2 – supplementary material) seems to imply that the amygdala is strongly involved in the response to direct threat. While we found no amygdala activation to the threat cues across all subjects, we found that the amygdala is *relatively* more active in subjects that show stronger defensive startle potentiation (chapter 2). Therefore, the amygdala affected the magnitude of defensive responses to more distant threat in a more subtle way. This is in line with an expanding line of animal literature that suggest that while the amygdala is a crucial modulator of fear responses, anxiety-like responses for more distant threats are also dependent on other structures and a on different neuropharmacology (Baas et al 2002; Davis et al 2009; Walker et al 2003). With this in mind, the question arises whether we measured fear,

or anxiety in response to the threat cues in our instructed threat paradigm. As mentioned in the introduction, fear can be thought of as a transient reaction to clear and discrete threat cues while anxiety is characterized by more uncertainty and a longer duration. We used discrete cues that were of limited duration (6-12 s in our fMRI paradigm) and defensive responses to these cues were quickly downregulated after threat termination. These characteristics indicate that our paradigm predominantly measures acute fear, rather than sustained anxiety. Similar results have been found for rodents, who show a fast up and down regulation of the startle response when discrete cues predict shock threat, while other more contextual stressors such as being placed in a brightly lit cage produce a response that takes minutes to level off (de Jongh et al 2003). On the other hand the threat cues in our paradigm did not straightforwardly signal that a shock would be delivered soon. Rather, the cues conveyed a risk of receiving a shock due to the fact that shocks were only delivered in approximately 15 % of threat trials. This uncertainty may have produced an increase in the so-called defensive distance, which has been suggested to produce more anxiety-like reactions (McNaughton and Corr 2004). Also, fear reactions are typically measured on a shorter time-scale than 12 seconds (Alvarez et al 2011; Miles et al 2011; Chapter 8). Therefore, even though a discrete cue of limited duration signalled threat, the low shock rate was perhaps insufficiently powerful to engage the amygdala, which may react more strongly to innate cues such as fearful faces or cues that signal more imminent threat (Ohman 2005). Higher reinforcement rates, as typically used during fear conditioning which does produce more consistent amygdala activity (Buchel et al 1998; Tabbert et al 2011), may lead to more amygdala activation (Dunsmoor et al 2008) also in instructed fear (e.g. see Alvarez et al 2011) because amygdala activity may be reduced quickly when instructions are not reinforced (Phelps et al 2001).

As mentioned in chapter 1 & 7 the amygdala should however not be considered a unitary structure. Because fMRI is dependent on relatively coarse signals, that is slow BOLD responses that reflect the net output across local neuronal circuits (Logothetis 2008), it is possible that smaller activations in local circuits are left undetected. This could be an alternative explanation for the lack of activation observed in instructed threat studies, which perhaps produce a pattern of neural responses in the amygdala which are not easily detected with fMRI. Indeed, amygdala activation as measured with fMRI are most often observed in dorsal amygdala regions (Costafreda et al 2008) which encompass the centromedial portions of the amygdala (Amunts et al 2005; Bach et al 2011). The amygdala activation we observed in response to aversive electrical stimulation was also localized in the dorsal, centromedial areas of the amygdala. This is consistent with the assigned role for this area as the output region of the amygdala that is involved in the generation of fear responses by recruitment of

brainstem and hypothalamus (Davis and Whalen 2001). On the other hand, the basolateral amygdala seems also in humans involved in extended sensory processing to determine whether the centromedial areas should be switched on or off. Reduced conditioned responses after selective basolateral amygdala damage as reported in chapter 7 support this view. One could argue that the anxiety reactions produced in instructed fear are more likely lead to basolateral amygdala activation, which could be harder to detect with fMRI. Elegant recent animal experiments show that different neuronal populations within the basolateral amygdala are important for fear reductions (Tye et al 2011) and fear increases (Herry et al 2008). These populations may be intermingled in a pepper and salt like fashion (Herry et al 2008), which could explain why basolateral amygdala activity is hard to pick up in fMRI contrasts. Because inhibitory activity may cause either BOLD signal increases or decreases, the net result of simultaneous activity is hard to predict (Logothetis 2008) and may not always be detectable with fMRI. Similarly, small activations in the hypothalamus and brainstem may not always be picked up in the MRI scanner.

With the limitations of fMRI findings in mind, it nevertheless remains striking to see that other areas do respond more consistently during the anticipatory anxiety states that are induced with the instructed threat paradigm. As discussed before, our results and those of others point to a broad role of cortical regions like the dorsal anterior cingulate (dACC) and anterior insula (aIns) in threat appraisal and the generation of defensive responses (Etkin et al 2011; Milad et al 2007). We observed stronger responses in this circuit during threat in subjects carrying two copies of the short allele for the 5-HTTLPR polymorphism (chapter 4), who also showed increased startle reactions to threats (chapter 3). Moreover, activation in this circuit correlated with potentiation of startle (chapter 4). Previously we showed that startle responses during the *early* phase of threat presentation correlated with amygdala activation during threat cues (chapter 2). Not surprisingly, activity in these different regions of interest (ROIs) was correlated across subjects. Higher activations in the insular ROI coincided with greater BOLD signal change in other ROIs, including the dACC, amygdala and midbrain.

One interesting question that arises is how exactly then increases in somatic defensive behaviour in S-carriers of the 5-HTTLPR polymorphism, as measured with startle potentiation, arise out of the changes in this distributed network of brain regions. Due to the correlational nature of our findings it is not possible to determine whether one of the areas plays a more principal role than others, perhaps driving activity in other areas. One could speculate that the modulation of startle primarily evolves out of the amygdala brainstem circuit that was delineated in animal work (Davis 2006; Davis et al 1993). This would fit with the consistent findings of amygdala hyper-reactivity in S-carriers of the 5-HTTLPR (Hariri and Holmes

2006; Munafo et al 2008) who also show enhanced startle potentiation (Lonsdorf et al 2009 and chapter 3). Nevertheless, in our paradigm we did not observe any evidence for stronger amygdala activation in S-carriers or SS homozygotes compared to long allele homozygotes. This is perhaps not surprising given that we did not find robust signal increases after the threat vs. safe cues in the amygdala across genotypes.

An alternative explanation could be that stronger startle potentiation evolves out of hyper-reactivity in other nodes of the neural defence circuit. For example, SS homozygotes tended to show stronger activity in the dorsal anterior cingulate while activity in this area also correlated positively with startle potentiation (chapter 4). This interpretation is preliminary, given the small effect size, the absence of differences observed between SL and LL subjects in dACC activation and the lack of studies available currently to confirm this hypothesis. However, it fits well with the proposed role for the dACC area in fear expression (Etkin et al 2011; Milad et al 2007), sympathetic nervous system activity as indexed by heart rate and SCR (Critchley 2005; Milad et al 2007) and the strong connections of the ACC with brainstem regions (reviewed in the chapter 1).

It deserves mention that although previous research found specific evidence for 5-HTTLPR mediated differences in amygdala reactivity, this was mainly investigated with tasks that are tailored to probe amygdala function, particularly involving phasic presentation of emotional faces (Domschke and Dannlowski 2010; Munafo et al 2008). Analyses are often also specifically directed at the amygdala through region of interest (ROI) analysis. This amygdala-centred approach potentially obscures differences in other defensive brain regions, emphasizing the need for more widespread use of alternative paradigms and analysis strategies to characterize defensive behaviour. Moreover, amygdala activity to threatening stimuli can be observed in subjects unaware of threat (Ohman 2005; Tabbert et al 2011) while the conscious experience of anxiety during instructed threat is apparently not associated with strong amygdala activation. Indeed, conscious deliberation of emotional responses may reduce reactivity of the amygdala (Lieberman et al 2007). Related to this, amygdala-damaged patients show normal fear conditioning when they are forced to make continuous ratings of shock expectancies (Coppens et al 2009). These dissociations between subjective anxiety and amygdala activation indicate that amygdala activity is not likely to be the single cause of excessive fear experiences as observed in anxiety disorders (Fiddick 2010). In the study of anxiety, the amygdala should be considered as one node in a broad neural network of threat processing. Therefore, studies that specifically investigate the amygdala as a region of interest should also look at other nodes in this network, in particular the dACC and aIns regions (Fiddick 2010; Milad et al 2007; Paulus and Stein 2006).

Interestingly both aINS and dACC may be more strongly involved in sustained anxiety experience. Animal research showed a role of the dACC in the light enhanced startle anxiety model (Veening et al 2009) and human imaging research showed activation of these areas in contextual anxiety (Alvarez et al 2011). Moreover the aINS has close connections also with the bed nucleus of the stria terminalis (Davis et al 2009; McDonald 1998) and there is a growing body of preclinical work suggesting a specific role for this area in the expression of long lasting anxiety states (Davis et al 2009; Walker et al 2003), perhaps together with the OFC (Kalin et al 2007). Recent studies in humans seem to confirm a role for the BNST in anxiety, specifically in highly anxious subjects (Somerville et al 2011) and contextual anxiety (Alvarez et al 2011). Interestingly, the ventral striatum, dorsal anterior cingulate and OFC are also considered essential parts of the pathophysiology of obsessive compulsive disorder (Fiddick 2010; Milad and Rauch 2011). In line with this, in chapter 8 we replicated earlier findings that deep brain stimulation of the ventral striatum relieved OCD symptomatology in patients with severe OCD. Interestingly, the reduction in OCD symptoms after nucleus accumbens stimulation was not associated with any reductions in fear-potentiated startle nor did severe OCD patients show aberrant responding compared to healthy subjects. This double dissociation between OCD symptomatology and startle measurements provides new evidence that the pathophysiology of OCD does not primarily involve basic defensive responding in the amygdala-brainstem circuit (Fiddick 2010; Milad and Rauch 2011) that is thought to mediate startle (Davis 2006; Davis et al 1993).

The role of the ventral striatum in human anxiety is evident but currently remains enigmatic. Few studies have investigated the role of bed nucleus of the stria terminalis in human anxiety (Alvarez et al 2011; Somerville et al 2011), and even fewer have assessed the role of the nucleus accumbens. We did not see convincing evidence for ventral striatal involvement in anxiety regulation measured in chapters 2 and 4. However, in a study by Wager et al. (2008) a lateral OFC – nucleus accumbens pathway was delineated that may aid in successful emotion regulation independent of the OFC-amygdala pathway. This study entailed the presentation of negative emotional pictures that were to be reappraised. We did not observe any ventral striatal activations in our threat paradigm, which suggests that nucleus accumbens involvement is not a pathway of great importance in the control of anxiety, at least after threat offset. As suggested by Wager et al., the accumbens pathway may be predominantly involved in positive reappraisal of negative events and the associated increase in positive emotion that occurs with this. Certainly, increasing positive emotion as also occurs in counter conditioning procedures used in the treatment of anxiety (Davison 1968) is another option for anxiety control which may well depend on mechanisms different from those discussed in the chapters

2-4. Similarly, the finding that startle potentiation is unaffected while OCD symptoms are reduced illustrates that defensive states measured in our threat paradigm may not always reflect subjective anxiety. In all, further and deeper understanding of fear and anxiety calls for a comprehensive approach with a diversity of measures and study populations. This is further emphasized by results from chapter 6, that show that different measures may have a different pattern of habituation and thus sensitivity as an index of anxiety.

Loci of fear and anxiety downregulation: control over defensive responses by the prefrontal cortex and other areas.

Throughout this thesis, the locus of fear and anxiety downregulation has been hypothesized to lie in the PFC. At this point, it is not entirely clear how different areas within the PFC are involved in different aspects of emotion regulation. Previous overviews about contributions of different PFC areas to emotion regulation in humans generally have acknowledged the important role medial and orbital areas of the PFC play relative to dorsolateral areas (Davidson et al 2000; Ochsner and Gross 2005; Quirk and Beer 2006). It has been hypothesized that specifically the OFC plays an important role in the inhibition of the amygdala to reduce activation in response to events that can trigger an emotional response (Blair 2004; Quirk and Beer 2006). Although there is a indeed special relation between the OFC and amygdala in terms of the information processed (Barbas et al 2003; Rolls 2004), activity in the mPFC has also been linked to reduced amygdala activity (Kim et al 2003; Kim et al 2011; Urry et al 2006), and especially the caudal mPFC / ACC has strong anatomical connections to the amygdala (Ghashghaei et al 2007). Nevertheless, the connections of the OFC are more directly aimed at the centromedial output areas of the amygdala than those arising from the medial PFC (Ghasghaei et al., 2007) and therefore may be more influential. The medial PFC on the other hand may be implicated in more directly controlling physiological changes associated with negative emotion and stress through its stronger connections with the hypothalamus and brain stem (Quirk and Beer 2006). Indeed, more than the OFC the mPFC is related to autonomic changes as already alluded to above.

Both the OFC and mPFC have strong interconnections with the dorsolateral and anterolateral PFC (BA10) (Pandya and Yeterian 1996; Petrides 2005). It seems plausible that when the latter areas are found to be involved in emotion regulation in neuroimaging (e.g. Kalisch et al 2005; chapters 2 & 4; Phan et al 2005; Wager et al 2008) they instantiate their influence on subcortical areas by recruiting the OFC and mPFC. However, until recently there was little direct empirical support for this hypothesis (Lorenz et al 2003). In line with Delgado et al. (2008) we showed that BOLD signal fluctuations in the lateral prefrontal areas that appear to

be involved in fear regulation show a correlation with activity fluctuations in the subgenual vmFPC. This provides further support for an intermediate role of the vmPFC in the regulation of fear.

In general, separating source and target of emotion regulation is complicated. Emotion regulation may even be an inherent part of the emotional processing, making it difficult to distinguish between emotion generation and its simultaneous regulation. There is also evidence that the vmPFC regions involved in the inhibition of negative emotion also play a role in enhancing fear, stress and aggression (Blair 2004; Herman et al 2005; Radley et al 2006; Sullivan 2004; Vidal-Gonzalez et al 2006). This also fits well with diverse consequences of vmPFC lesions on emotional behaviour, which can involve both reduced emotionality and emotionally disinhibited behaviour. Interestingly, there is some evidence that the more dorsal mPFC areas in rodents (dorsal PL and anterior cingulate gyrus) may be involved in inhibiting stress, while more ventral areas (PL and IL) would activate the stress response system (Radley et al 2006; Sullivan 2004). These results seem to be difficult to reconcile with the robust findings that area IL also seems to be actively involved in the extinction of fear, and the role of the dorsal mPFC in the expression of fear (Etkin et al 2011; Milad et al 2007). Our findings primarily match the account by Etkin et al. (2011) suggesting that dorsal mPFC/ACC is primarily involved in fear expression, while the vmPFC is important in fear inhibition. Nevertheless, given the conflicting findings more complex models may be required and emotional activation and inhibition pathways could arise from overlapping neuronal populations within these PFC areas, just as overlapping populations of neurons in the basolateral amygdala can either drive or reduce fear response (Herry et al 2008). Another influential view is that lateral OFC areas are involved in monitoring punishment, while reward monitoring would be more localized in medial OFC areas (Kringelbach and Rolls 2004). Our findings support this model, when both reactions to threat and threat offset are considered as punishment monitoring. Indeed, we found lateral OFC activation to the threat cues, as well as a deactivation in anterior mOFC. After threat offset we again found activation of (more anterior) lateral OFC and deactivations in (more posterior) vmPFC.

Although throughout this thesis the source of fear downregulation is mainly regarded to be in the PFC, sources of emotion regulation can also be found at lower levels. Given the extensive role of the PFC in higher cognition it is plausible that goal oriented, cognitively driven emotion regulation originates mainly from the PFC. Alternatively, emotion regulation at lower levels may support more automatic and reflexive forms of regulation. Especially, the amygdala is in a good position to regulate emotion on a lower level by its connections to all main emotion

effectors and some of these regulatory processes may also come about independently from the PFC. In line with this, recent years of research have provided ample evidence that activity within the amygdala contributes to fear regulation. Especially the basolateral nuclei may inhibit output of the central amygdala nuclei (Tye et al 2011) and this inhibition appears to play an essential role in fear extinction (Herry et al 2011). Similar mechanisms may operate in the other brain regions. An example of the more automatic regulation at the lower level is the negative feedback loop in the HPA-axis that functions to restrain the stress response. The inhibition of defensive responses is a process that can occur at various levels in the neural defensive hierarchy. In sum, it seems that the up- and downregulation of defensive states are dependent on partially overlapping but distributed neural substrates

Implications for the pharmacology of anxiety and its investigation

The work in the current thesis provides new evidence for a role of innate individual variance in serotonin transporter (5-HTT) function in determining the intensity of defensive reactions. We observed stronger startle potentiation during verbally instructed threats in subjects carrying an S-allele for the 5-HTTLPR polymorphism, and in subjects carrying two S-alleles also observed stronger reactions to threat cues in the anterior insula and dorsal ACC. A question that arises from this combination of findings is to what extent the S-allele exerts functional dominance in anxiety. In other words: does a single S-allele predispose to anxiety or only when two such alleles are present? The fact our startle and fMRI data provide different answers indicates that in some cases carrying one S-allele is enough to differentiate from subjects carrying two LL alleles, but that two S-alleles can provide an even stronger predisposition. This is also consistent with findings from in vitro studies that have indicated that the effects of carrying two low-function alleles may be additive (Hu et al 2006). Alternatively, this divergence of findings could also reflect differences in signal-to-noise ratio of the different measurements: startle EMG may be a more sensitive indicator of threat reactivity than fMRI and thus be able to differentiate better between groups.

One important caveat to this work is that the exact impact of these genetic variations on the serotonergic system is unclear. For example, while the commonly studied 5-HTTLPR polymorphism in the serotonin transporter gene appears to have robust effects in vitro (Hu et al 2006; Lesch et al 1996), its effects on the human serotonin system in vivo remains unclear (Murthy et al 2010). It has been suggested that the effect of allelic variation is mainly on structural brain development, but also findings of structural effects have been inconsistent (Jedema et al 2010; Pacheco et al 2009; Selvaraj et al 2010). This missing link makes it difficult to propose mechanistic models to explain the effects of these polymorphisms on the macrolevel, i.e on behavior or macroscopic neural events as measured through neuroimaging.

Our findings are nevertheless consistent with an enormous amount of work showing involvement of the 5-HTT in anxiety. Chronic SSRI administration is effective in alleviating symptoms in almost all anxiety disorders (Baldwin et al 2005; Bandelow et al 2002). Moreover, recent research has also shown efficacy of these drugs after chronic administration in similar threat-of-shock anxiety models in both healthy humans (Grillon et al 2009) and rodents (Miles et al 2011). More complete mapping of the polymorphisms within the SERT gene and more clarification of their functional role is a necessary step to provide a clearer view of the manner in which natural innate fluctuations in this gene contribute to anxiety and its disorders. Moreover, effects of the innate variability in 5-HTT function would perhaps be stronger when the 5-HTT and/or anxiety systems are challenged (Caspi et al 2010). One interesting approach has been to combine pharmacological manipulations with genetic 5-HTT mapping (Markus and De Raedt 2011; Markus and Firk 2009), which show that differences between 5-HTTLPR genotypes can be exacerbated when the serotonin system is challenged by means of depletion of the serotonin precursor trypthophan. Another possibility would be to see how experience with severe stressors might further increase anxious responding in 5-HTTLPR short allele carriers (Armbruster et al 2009; Karg et al 2011).

In chapter 5, we observed no effect of the partial NMDA agonist on fear extinction. In relation to this negative finding, it is interesting to discuss a recent study by Kuriyama et al. (2011). In this study, no effects were found during extinction training or during extinction recall, which replicated our findings and the findings by Guastella et al. (Guastella et al 2007). However, with an elegant design Kuryama et al. show that in healthy humans DCS may particularly weaken the return of fear after a brief reacquisition training that follows extinction (Kuriyama et al 2011). This result requires replication because the drug effects were only found using an unusual measure for indexing conditioned skin conductance responses. Moreover previous animal work did not show dependence of DCS effects on re-acquisition (Langton and Richardson 2008; Ledgerwood et al 2005). In any case, this study illustrates the potential power of the approach to test potential anxiolytic substances in anxiety paradigms in healthy humans. Result from these studies can be used to generate more specific hypotheses about the exact conditions under which promising anxiolytic agents (such as DCS) exert their maximal effects. This finding also illustrates the potential importance of differences in the exact set-up for measuring fear conditioned responses. Currently, the full potential of this research line is probably not realized due to uncertainty about the best set-up. More basic experimental work is needed to further characterize the exact parameters for a fear extinction paradigm that provides the best predictive validity of clinical responses, just as has been done for the instructed fear paradigm (Baas et al 2002; chapter 6).

In chapter 5, we also found no support for a role the CB1 receptor system in the extinction of conditioned fear. However, we do not believe that the negative finding with our CB1/CB2 partial agonist THC rules out CB1 involvement in fear extinction. This was a first attempt, and THC's expected workings vary across brain regions and are hard to predict. Besides the wealth of animal data supporting a role for the CB1 receptor system in fear extinction, recent evidence suggests that also in humans the cannabinoid system is a crucial regulator fear. The cannabinoid cannabidiol has been shown to exert anxiolytic properties and was found to alleviate public speaking anxiety in social phobics (Bergamaschi et al 2011). Moreover, there is now interesting evidence that also innate genetic variability in the cannabinoid system may affect amygdala reactivity (Hariri et al 2009). The investigation of genetic variability in the CB1 system could be another interesting avenue to demonstrate a role for CB1 transmission in fear extinction, especially given the lack of specific pharmacological agents. In a recent study, we found that a common polymorphism in the CB1 gene dose-dependently affected the magnitude of fear extinction in healthy subjects as indexed by fear-potentiated startle (Heitland et al submitted). At the end of extinction training, subjects carrying a specific variant of the polymorphism showed no evidence of fear extinction and hence were left in a state of sustained anxiety. This highlights the potentially crucial role of the CB1 system in fear extinction.

Critical methodological considerations: the issue of replicability

Several limitations of the work in this thesis have already been mentioned above. Before bringing this thesis to a close, it is useful to discuss one especially important methodological issue: the issue of reliability of the results. It has been argued that, due to widespread problems in scientific data analysis (Simmons et al 2011), most research findings reported in medical science articles are false (Ioannidis 2005), i.e. they are not replicable and thus these findings have essentially little scientific merit. Also this thesis is situated at an intersection of research fields (affective neuroscience, neuroimaging, genetics) in which there is a paucity of replication of findings in the literature (Simmons et al, 2011; Sullivan et al, 2007; Vul et al, 2009). This issue could greatly slow scientific progress by burying true findings under a haystack of false ones (Jasny et al 2011).

In light of this serious issue, how should we view the results described in this thesis? In this thesis, analyses aimed at describing reactions *across* all participants typically produced robust results. For example, within this thesis we replicated results on startle potentiation and diminution after threat offset, and BOLD signal changes to threat cues and their offset. On the other hand, less robust results (i.e. statistical outcomes: p-values) were obtained from analyses on individual differences between subjects (for example differences between the

5-HTTLPR genotypes). Smaller effect sizes make the outcomes of these analyses crucially dependent on the reliability of the measurements. In other words, the analyses that made comparisons between subjects (or groups) are generally more dependent on the extent to which experimental variables are assessed reliably, so that they provide an accurate index of subjects' individual value on that measure.

While questionnaires are typically well validated in large samples to assure reliability, psychophysiological measures very often are not. Unfortunately in many papers describing the relation between experimental measures and genotype or other trait variables the assumption of reliability of the outcome measure (e.g. startle potentiation in fear conditioning) is not explicitly tested. More information on the reliability of our FPS measure in instructed fear was obtained from chapter 2 and 4. Unsurprisingly, the results show that there is variability *within* subjects in how they react on different occasions. FPS magnitude assessed at different time points showed moderate correlations (r-values around .5). This variability can be attributed to a combination of state-dependent fluctuations and measurement error, e.g due to differences in electrode application. Nevertheless, these significant correlations also indicate consistency in fear expression across measurements. This is suggestive of an underlying trait component or fixed property which may be related to personality or genetic factors. FPS measured in the instructed fear paradigm therefore show promise for investigating individual differences in defensive reactivity. Nevertheless, the unexplained variance will unavoidably limit power for analyses aimed at finding correlates of FPS. It is therefore not surprising that findings of individual differences between subjects (e.g. genetics) obtained in this thesis were not more robust statistically. Also for measures of brain activity obtained from fMRI, reliability can be expected to vary widely depending on task design and area of interest. Therefore also the power for detecting correlations with other outcome variables (e.g. startle or genotype) will be reduced (Johnstone et al 2005; Vul et al 2009). Our findings concerning differences between subjects and genotypes where obtained using state-of-the-art statistical procedures and robust measurement techniques. Moreover, we used relatively large subject samples. Nevertheless, it remains of crucial importance that these findings are replicated in unrelated and preferably larger subject samples.

Conclusion

In conclusion, the overview of data from different research methodologies presented in this thesis shed more light on fundamental neurobiological mechanisms of the up- and downregulation of defensive behaviour. The step towards clinical utility is still a large one. However, knowledge about these processes could ultimately be used in the development of new treatments for patients that have difficulties controlling their anxiety.

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SUMMARY IN DUTCH/NEDERLANDSE SAMENVATTING

INTRODUCTIE

Kort geleden, bij een oponthoud op een treinstation hoorde ik NS werknemers op nogal paniekerige wijze tegen elkaar zeggen dat het station zou moeten worden ontruimd. Direct ontwaakte ik uit mijn lusteloze staat - ontstaan bij het vooruitzicht op een lange avond met treinvertragingen. Meteen begon ik ook mijn omgeving nauwlettend in de gaten te houden en te denken over de snelste uitgang. Als plots iemand over het perron mijn richting uit komt rennen voel ik mijn spieren verstijven en mijn hart nadrukkelijk bonzen. Uiteindelijk begrijp ik van een NS medewerker dat het vals alarm is, en na een moment van opluchting verschuift mijn aandacht al snel naar andere belangrijke zaken: wat te doen met avondeten.

Deze situatie illustreert de psychische en fysieke verschijnselen en de gedragingen behorende bij de emotie angst. Iedereen is wel eens angstig. Angst is een over de hele wereld voorkomende, universele basis emotie (Ekman 1992; Frijda 1986) en ook in extreme vorm komt angst relatief vaak voor. Angststoornissen behoren zelfs tot de vaakst voorkomende psychische problemen (Kessler et al 2005). Tot 28% procent van de bevolking in westerse maatschappijen zou gedurende zijn of haar leven te maken krijgen met ofwel posttraumatische stress stoornis, obsessief compulsieve stoornis, gegeneraliseerde angst, paniek stoornis, specifieke fobie, agorafobie of sociale fobie (Kessler et al 2005). Angst is echter in de meeste gevallen een nuttige reactie die adaptief handelen in dreigende situaties vergemakkelijkt.

De neurobiologische mechanismen die behoren bij angst reacties in dieren zijn vrij gedetailleerd beschreven (Davis 2006; LeDoux 2003). Nieuwe onderzoekstechnieken maken het nu mogelijk om de neurobiologische basis van humane angst reacties steeds gedetailleerder in kaart te brengen. Dit proefschrift heeft als doel om de kennis te vergroten over basale angst reacties in mensen. Hiertoe is een verscheidenheid aan technieken gebruikt in zowel gezonde deelnemers als speciale patiëntenpopulaties om relevante hersengebieden, somatische reacties, en chemische en genetische achtergronden van angst aan het licht te brengen. Zoals hierboven bleek kan na afloop van een bedreigende situatie ook snel worden geschakeld naar een staat waarbij andere belangrijke zaken met betrekking op overleving weer meer aandacht krijgen (zoals eten). Hiertoe moet angst worden afgeremd op het moment dat het “sein veilig” wordt gegeven. Dit mechanisme helpt om de angst reactie functioneel en in toom te houden. Een tweede doel van dit proefschrift was om meer inzicht te krijgen in de rol van de hersenen, en specifiek de prefrontale cortex, bij het remmen van angst.

Voordat ik hieronder de bevindingen uit de afzonderlijke hoofdstukken samenvat, lijkt het goed om even kort in te gaan op de meetmethode die in dit proefschrift steeds is gebruikt

is om angst objectief te bepalen: de schrikreflex. Door herhaaldelijk een kort, hard geluid bij deelnemers aan te bieden werden tijdens de verschillende experimenten steeds oogknip reflexen opgewekt. Deze oogknip reflex wordt gemeten aan de hand van elektrische activiteit van de spier die het oog sluit, met behulp van electromyografie. Voor deze metingen werden daarom steeds 2 elektroden net onder het oog aangebracht om zo de schrikreacties te kunnen meten. Belangrijk is, dat de kracht waarmee het oog sluit bij het aanbieden van deze plotselinge geluiden afhangt van de emotionele staat waarin deelnemers zich bevinden. Er is veelvuldig aangetoond dat dit samenhangt met de mate waarin mensen zich in een defensieve staat bevinden. Hoe *groter* de dreiging, in experimenten bijvoorbeeld geïnduceerd door het aanbieden van schokerende plaatjes (bijvoorbeeld van mensen met verwondingen) of door het dreigen met een ongevaarlijke maar vervelende elektrische prikkel, hoe *groter* de schrikreflex is als deze plotseling opgewekt wordt door het harde geluid. Deze vergroting, of *potentiatie*, van de schrik reflex wordt aangeduid met de Engelse term *fear-potentiated startle*, afgekort FPS (Bradley et al 2005; Grillon et al 1991; Grillon and Baas 2003; Lang et al 1990). In dit proefschrift zijn FPS metingen steeds gebruikt in combinatie met andere technieken, zoals functionele magnetische resonantie imaging (fMRI) in een MRI scanner om activiteit in de hersenen te bepalen, wangslijmvlies afname voor bepaling van het genotype voor specifieke stukjes DNA, huidgeleidingsmetingen voor het meten van activiteit van de zweetklieren, en subjectieve metingen van angstigheid aan de hand van vragenlijsten. Met deze combinatie van technieken is geprobeerd angst in brede zin te onderzoeken, van veranderingen in basale reflexen, hersenactiviteit, en subjectieve beschrijvingen tot achterliggende neurochemische en genetische mechanismen.

SAMENVATTING VAN DE HOOFDSTUKKEN IN DIT PROEFSCHRIFT

In hoofdstuk 1 is allereerst een overzicht van de literatuur gepresenteerd om de huidige kennis over neurobiologische angst mechanismen samen te vatten. De generatie van angst lijkt afhankelijk van een verspreid netwerk in de hersenen. De amygdala, dorsaal anterior cingulate cortex (dACC) en anterior insula (aIns) lijken een rol te spelen in het aanzwengelen en in stand houden van angst door lager gelegen hersengebieden, zoals de hypothalamus en de hersenstam (vooral periacquaductaal), aan te zetten tot activiteit. Voor de *vermindering* van angst lijkt de prefrontale cortex een belangrijke bron. Zowel meer lateraal gelegen als ventromediale prefrontale gebieden lijken betrokken, waarbij opgemerkt kan worden dat vooral de ventromediale gebieden ook sterke verbindingen hebben met de gebieden die

betrokken zijn bij de generatie van angst. Dit laatste suggereert dat de ventromediale PFC mogelijk als een doorgeefluik functioneert om regulatie door andere prefrontale gebieden mogelijk te maken.

Hoofdstuk 2 beschrijft een studie waarbij we lichamelijke en neurale reacties in deelnemers hebben gemeten naar aanleiding van plaatjes die schokdreiging aangaven. Specifieke aandacht was hierbij voor wat er gebeurd bij de *beëindiging* van dreiging, een proces dat eerder nog weinig was onderzocht. De resultaten waren opvallend. Binnen 3.5 seconden bleken gezonde jonge vrijwilligers in staat om hun defensieve staat, zoals gemeten met FPS, te verminderen naar een nivo zoals tijdens veilige plaatjes werd gemeten. Blijkbaar zijn gezonde mensen in staat om snel te switchen tussen een defensieve staat van paraatheid tijdens dreiging en een relatief ontspannen toestand. Daarnaast werd volgens verwachting met behulp van fMRI gevonden dat schokdreiging ondermeer geassocieerd was met toenemende activiteit in de anterieure gedeeltes van de insula (aIns), de dorsale anterior cingulate cortex (dACC) en de thalamus. Belangrijker, de beëindiging van schokdreiging bleek gekoppeld aan veranderingen in activiteit in de rechter anterolaterale prefrontale cortex en ventromediale prefrontale cortex (PFC). De activiteit in deze prefrontale gebieden liet onderling een negatieve correlatie zien tijdens de taak en was ook gekoppeld aan veranderingen in activiteit in de rechter amygdala en anterieure insula. Dit ondersteund een model waarbij de rechter anterolaterale PFC via de ventromediale PFC angst reacties kan reguleren, in overeenstemming met de in de literatuur beschreven verbindingen van de ventromediale PFC met hersengebieden die betrokken zijn bij de totstandkoming van angst.

We repliceerden de bevindingen wat betreft de schrik reflex in een vervolgstudie, beschreven in hoofdstuk 3. In deze studie toonden we aan dat 1.5 seconden nadat schokdreiging beëindigd was, de schrikreflex niet langer meer vergroot was ten opzichte van periodes van relatieve rust. Daarnaast onderzochten we in deze studie de mogelijke invloed van een veelvoorkomend genetisch polymorfisme in het gen dat codeert voor de serotonine transporter (ook wel het 5-HTTLPR polymorfisme genoemd). Natuurlijke variatie in dit gen is herhaaldelijk gekoppeld aan de mate waarin mensen angstigheid rapporteren en hersenreacties vertonen bij het zien van angstige gezichten en onprettige plaatjes. Deelnemers met een minder actief serotonine transporter gen (de dragers van een kort allel voor dit polymorfisme) lieten een grotere toename in defensieve staat zien zoals bepaald met de schrik reflex tijdens de plaatjes die schokdreiging aangaven. Er was geen verschil tussen de genotypen na afloop van het bedreigende plaatje. In alle groepen zagen we een vergelijkbare afname in FPS. Dit wijst erop dat mensen die, een genetisch gestuurde, toename in basale angstreacties tijdens dreiging laten zien niet per definitie ook meer moeite hebben met het controleren van angst als directe dreiging afneemt.

In hoofdstuk 4 onderzochten we opnieuw de neurale reacties op het komen en gaan van dreigende situaties. We repliceerden in deze studie in een grotere groep proefpersonen onze eerdere bevindingen betreffende de hersengebieden die reageren op dreiging en de beëindiging van dreiging. Daarnaast bekeken we individuele verschillen in deze reacties en de relatie met het 5-HTTLPR polymorfisme. Zoals verwacht vonden we relatief sterkere reacties op dreiging in dragers van twee korte 5-HTTLPR allelen in de bilaterale aIns en (op statistisch trend nivo) in de dACC. Daarnaast vonden we dat proefpersonen die grotere FPS lieten zien tijdens dreiging ook relatief meer dACC activiteit lieten zien. Interessant was dat dragers van het minder actieve allel ook grotere reacties op de afloop van dreiging lieten zien. Samen met de resultaten van hoofdstuk 3 suggereert dit dat de groep mensen die een verhoogde reactie op dreiging laat zien ook in staat is om deze reactie na afloop van de dreiging te controleren door het sterker rekruteren van controlerende hersengebieden. Het is hierbij wel belangrijk om te benadrukken dat de deelnemers per definitie gezonde vrijwilligers waren, zoals bepaald met medische vragenlijsten, in de leeftijd van 18-40 jaar. Mogelijk dat genetisch vergrote angst in andere, meer kwetsbare groepen mensen tot meer problemen leidt.

De psychofarmacologische achtergrond van extinctie van angst, een ander paradigma om de mechanismen van angst remming te onderzoeken, werd onderzocht in hoofdstuk 5. Meer specifiek onderzochten we in dit experiment de therapeutische waarde van het faciliteren van neurotransmissie in twee geselecteerde neurotransmitter systemen door toediening van medicijnen voorafgaand aan extinctie training. Extinctie training is een proces dat kan worden vergeleken met exposure therapie, waarbij de vrijwilliger of patiënt steeds opnieuw aan een eerder bedreigende situatie wordt blootgesteld om zo te ervaren dat de dreiging niet langer realistisch is. Het doel van deze procedure is dat de angst reacties geleidelijk uitdoven. Er is nu duidelijk dat dit niet simpelweg een kwestie is van het vergeten van de dreiging, maar dat er een nieuw leerproces optreedt waarbij de koppeling aan de veilige situatie wordt aangeleerd. We richtten ons bij dit onderzoek op het wijdverspreide glutamaterge systeem en meer specifiek op de zogenaamde N-Methyl-D-Aspartate (NMDA) receptor, waarvan is aangetoond dat deze een belangrijke rol speelt in leren en geheugen. Daarnaast bekeken we voor het eerst effecten op de extinctie van angst van een stof die als partiële agonist fungeert voor de belangrijkste cannabinoïde receptor in het brein, de cannabinoïde receptor type 1 (CB1). Op basis van dierstudies die lieten zien dat de NMDA en CB1 receptoren een cruciale rol spelen in extinctie, verwachtten we dat deze twee stoffen de extinctie van angst mogelijk zou kunnen verbeteren. Op deze manier zou het toedienen van deze stoffen tijdens exposure therapie, een veelgebruikte therapievorm bij angststoornissen, mogelijk de effectiviteit van deze therapie kunnen verbeteren. Toediening van de NMDA agonist D-cycloserine

ofwel de CB1 agonist Tetra-Hydro-Cannabinol (THC) tijdens het aanbieden van extinctie training in gezonde mensen na angst conditionering bleek echter geen aantoonbaar effect te hebben op de mate van angst 2 dagen later, gemeten met de schrikreflex, huidgeleiding en subjectieve beoordelingen. Dit kan worden verklaard door een mogelijk gebrek aan ruimte voor verbetering van extinctie in deze gezonde groep deelnemers, bij wie extinctie mogelijk al bijna optimaal verloopt. Daarnaast is THC mogelijk niet de meest ideale stof om CB1 transmissie te faciliteren, meer selectieve stoffen zouden betere resultaten kunnen geven. De farmacologische verbetering van de efficiëntie van angst reductie tijdens therapieën blijft een potentieel interessante benadering, zeker vergeleken met het meer chronisch gebruik van angstremmende medicatie voor diegenen bij wie psychologische therapieën op zichzelf niet voldoende zijn.

In hoofdstuk 6 ontwikkelden we een methode die gebruikt kan worden om effecten in kaart te brengen van potentiële anxiolytica (angstremmende medicatie) die nog in ontwikkeling zijn. Hiervoor werd een brede angst en stress test batterij samengesteld waarmee we herhaaldelijk metingen in dezelfde personen konden uitvoeren. Dit laatste zou gebruikt kunnen worden om effecten van medicatie over de tijd in kaart te brengen, maar ook om in dezelfde proefpersonen meerdere metingen te kunnen herhalen waarbij de ene dag een actief medicijn wordt toegediend en op een andere dag een placebo. Allereerst moest echter aangetoond worden dat reacties niet al te veel veranderden als metingen herhaald worden bij de zelfde mensen. Deelnemers lieten bij herhaalde metingen geen systematische veranderingen zien in FPS zoals gemeten tijdens schokdreiging. Huidgeleidings reacties tijdens dreiging in ditzelfde paradigma namen gemiddeld wel af na een aantal herhalingen, en ook de subjectieve mate van stress werd na herhaling minder Dit laat zien dat vooral FPS een potentieel interessante maat is om effecten van medicatie te meten, zowel op verschillende momenten na toediening van de medicatie als in studies waarbij effecten van verschillende vormen van medicatie afgezet worden tegen placebo metingen in dezelfde proefpersonen.

De laatste twee hoofdstukken beschrijven onderzoek in twee uiterst bijzondere groepen. In hoofdstuk 7 bekeken we opnieuw de neurobiologische basis van angst conditionering. Eerder verschenen literatuur bewees al dat de amygdala een cruciale rol speelt bij het aanleren van geconditioneerde angst. In overeenstemming met deze literatuur lieten de resultaten van onze studie zien dat patiënten met selectieve schade aan de basolaterale regio's van de amygdala (BLA) geen normale angst reactie ontwikkelen bij het herhaaldelijk zien van een plaatje dat gevolgd werd door een vervelende, harde schreeuw. Dit is het eerste bewijs dat selectieve schade van de menselijke amygdala, ten gevolge van een zeldzame genetische aandoening, het aanleren van geconditioneerde angstreacties kan verslechteren.

In plaats van onderzoek met deelnemers met genetisch bepaald schade in specifieke delen van het brein, werd in hoofdstuk 8 gekeken naar de effecten van directe stimulatie van specifieke hersendelen. Hierbij werd bekeken hoe elektrische stimulatie van het ventraal striatum, een veelbelovende experimentele behandeling van ernstige vormen van de obsessief compulsieve stoornis (OCD), basale angst reacties beïnvloedt tijdens schokdreiging. De schrikreflex werd in patiënten in 2 situaties bekeken, te weten als de stimulator die in die na een chirurgische ingreep in de hersenen was geplaatst aan stond of als deze uit stond. De resultaten lieten een dissociatie zien tussen OCD symptomatologie enerzijds en basale angst reacties anderzijds. De elektrische stimulatie van de nucleus accumbens verminderde duidelijk subjectief gerapporteerde OCD symptomen maar of de stimulator aan dan wel uit stond had geen effect op FPS tijdens schokdreiging. Verder lieten de patiënten op het moment dat de stimulator uit stond een normaal patroon van resultaten zien, vergelijkbaar met reacties van gezonde vrijwilligers. De resultaten zijn in lijn met eerdere bevindingen dat OCD symptomen, hoewel historisch vaak bezien als voortkomend uit onderliggende angst, tot op zekere hoogte onafhankelijk zijn van de hersengebieden die basale defensieve staten aansturen.

DISCUSSIE

De bevindingen beschreven in dit proefschrift bevestigen de rol van structuren zoals de dACC en aIns in reacties op dreiging. Deze gebieden waren overduidelijk actief tijdens het zien van de plaatjes die schokdreiging impliceerden. Daarnaast zagen we ook dat mensen die meer angstig reageerden op schokdreiging zoals gemeten met de schrikreflex ook sterkere activiteit in dit aINS-dACC circuit lieten zien. Hierbij moet wel worden opgemerkt dat de analyses in dit proefschrift geen causaal verband toetsen, alleen een samenhang. Het kan dus theoretisch ook zo zijn dat activiteit in deze gebieden niet direct betrokken is bij de totstandkoming van angst maar een bijverschijnsel is. Dit laatste is echter onwaarschijnlijk omdat, in het bijzonder voor de dACC, er ook bewijs van dieronderzoek is dat een causale relatie suggereert (Etkin et al 2011). Het was opvallend om te zien dat de dreiging van elektrische prikkel toediening relatief sterke reacties in de schrikreflex en dACC/aINS activiteit te weeg bracht maar géén meetbare veranderingen in activiteit in de amygdala. Dit is opvallend omdat de amygdala vaak als kernstructuur wordt gezien als het gaat om angst. Omdat ook andere onderzoeken met soortgelijke paradigma's weinig amygdala activiteit laten zien (Chua et al 1999; Mechias et al 2010; Phelps et al 2001), duidt dit op een meer beperkte rol van de amygdala in de expressie van angst in situaties waarbij de dreiging duidelijk afgebakend is en de bron van dreiging vooraf expliciet is aangekondigd. Wij vonden in hoofdstuk 2 dat de amygdala vooral

betrokken is bij vroege vergroting van de schrikreflex, en in hoofdstuk 7 dat de basolaterale gedeelten van de amygdala betrokken zijn bij het aanleren van angstreacties. Dit schetst een beeld waarbij de amygdala vooral betrokken is bij identificatie van potentiële dreiging en het vervolgens opstarten van angst reacties. Structuren zoals de dACC en aINS zijn mogelijk meer direct betrokken bij de subjectieve beleving en het aanhouden van angst. De implicatie hiervan is dat in angstonderzoek de extreme belangstelling voor de amygdala in vergelijking tot andere structuren wellicht contraproductief zal blijken voor het ontwikkelen van een overkoepelend beeld van de hersenstructuren betrokken bij angst.

Buitengewoon interessant zijn ook de bevindingen in hoofdstuk 8 waarbij stimulatie van het ventraal striatum in OCD patiënten wél leidde tot vermindering van angst maar niet tot verandering in de grootte van de schrikreflex – een veel gebruikte en objectieve maat voor reacties op dreiging. Hiermee wordt aangetoond dat de subjectieve ervaring van angst in deze patiëntengroep tot op zeker hoogte onafhankelijk is van het neurale circuit dat betrokken is bij het aansturen van basale defensieve reacties zoals de schrikreflex. Hierbij moet ook opgemerkt worden dat OCD mogelijk een andere neurobiologische achtergrond kent dan andere angst stoornissen – iets wat ook gereflecteerd wordt in de huidige plannen in de klinische praktijk om OCD buiten de categorie angststoornissen te plaatsen in de nieuwe versie van de DSM (Diagnostic and Statistical manual). Verschillende hersencircuits, enerzijds ventraal striataal en aan de andere kant gebieden zoals de amygdala die de grootte van de schrikreflex bepalen, kunnen zo hun eigen weerslag hebben op angst als veelkoppig fenomeen. Dit pleit voor een gevarieerde benadering bij onderzoek van angst en angststoornissen, iets wat verder ondersteund wordt door uiteenlopende bevindingen met verschillende uitleesmaten voor angst in hoofdstukken 5 en 6.

Deze pluriformiteit geldt tevens de processen die kunnen leiden tot vermindering van angst. Hoewel de belangrijke rol van de laterale en ventromediale prefrontale cortex in het controleren van angst werd bevestigd in dit proefschrift lijken ook andere hersengebieden bij te dragen. De gebieden die belangrijk zijn in de generatie van angst lijken bijvoorbeeld ook interne controle mechanismen te bevatten. Hoewel deze gebieden aangestuurd kunnen worden door de prefrontale cortex zijn ze niet per definitie hiervan afhankelijk, er vindt ook interne controlering van angst plaats (Herman and Cullinan 1997; Herry et al 2008; Tye et al 2011). Aan de andere kant spelen de prefrontale gebieden die betrokken zijn bij de controle van angst ook weer een rol in de totstandkoming en het in stand houden van angst wanneer nodig. Deze verschillende functies worden waarschijnlijk ondersteund door verschillende hersenmechanismen maar deze zouden wel eens zeer moeilijk te scheiden kunnen blijken met fMRI, een relatief grove maat voor neurale activiteit (Logothetis 2008). Het is zelfs mogelijk

dat deze scheiding van functies alleen te traceren is naar groepen neuronen, waarvan de afzonderlijke neuronen uit beide groepen op een peper-en-zout-achtige wijze door elkaar heen liggen (Herry et al 2008).

De bevindingen in dit proefschrift ondersteunen verder de rol van de serotonine transporter in angst, en wijzen op een relatie tussen de genetische variatie in de activiteit van dit eiwit en de mate waarin mensen basale angst reacties vertonen. Dit vertoont een mooie overeenkomst met de effectiviteit van de zogenoemde serotonine heropname remmers (SSRI's) in bijna alle vormen van angst. Een belangrijke beperking van deze bevindingen is dat op dit moment onvoldoende duidelijk is hoe deze genetische variatie precies de activiteit van de serotonine transporter in het menselijk brein beïnvloedt. Tot nu toe zijn resultaten beperkt gebleven tot studies waarbij celkweken zijn bestudeerd (Heils et al 1996; Hu et al 2006; Lesch et al 1996), terwijl studies waarbij het levende menselijke brein werd onderzocht wisselende resultaten geven (Murthy et al 2010).

Naast de farmacologie van angst reacties, is in dit proefschrift ook gekeken naar specifieke farmacologische beïnvloeding van de processen die angst remmen. Helaas konden we extinctie van angst, de basis voor de effecten van exposure therapie, niet verbeteren met de NMDA agonist d-cycloserine. Interessant is dat een recent onderzoek uit Japan wel meer belovende resultaten laat zien, waarbij d-cycloserine met name het opnieuw tot stand komen van angst verminderd na afloop van de extinctie procedure (Kuriyama et al 2011). Dit geeft mooi de kracht aan van deze benadering waarbij met fundamenteel onderzoek in gezonde mensen, de precieze werking van medicatie kan worden bepaald. We vonden ook geen effecten van de partiële CB1 agonist THC, maar in meer recent onderzoek tonen wij aan dat genetische variatie in de CB1 receptor wel de mate van extinctie lijkt te beïnvloeden (Heitland et al submitted). Het cannabinoïde systeem blijft daarom een uiterst interessant neurotransmitter systeem als het gaat om angst en het afleren van angst.

Ten slotte nog een kritische methodologische noot. Door wijdverbreide problemen in veelgebruikte methoden voor data analyse (Simmons et al 2011) is gesuggereerd dat het merendeel van medisch wetenschappelijke artikelen niet repliceerbaar zou zijn en dus beperkte waarde heeft (Ioannidis 2005). Dit is in potentie een zeer ernstig probleem (Jasny et al 2011) en het is daarom goed om kort stil te staan bij de betrouwbaarheid van de resultaten in dit proefschrift. Zoals hierboven al genoemd, hebben we alle belangrijkste resultaten uit hoofdstuk 2 inmiddels zelf gerepliceerd in een grotere groep deelnemers, wat erg bemoedigend is. Mogelijk minder betrouwbaar, want statistisch minder robuust, zijn de resultaten waarbij we verschillende groepen deelnemers met elkaar vergeleken, zoals de bevindingen met het

serotonine transporter polymorfisme. Replicatie van deze bevindingen in onafhankelijke en grotere groepen deelnemers is daarom van zeer groot belang. Het is daarnaast belangrijk om experimentele opstellingen beter door te testen op de betrouwbaarheid van resultaten. Dit is bij uitstek belangrijk als er verschillende groepen deelnemers met elkaar worden vergeleken omdat dan de betrouwbare bepaling van individuele waardes cruciaal is voor de onderzoeksvraag. In hoofdstuk 3 & 6 hebben we bijvoorbeeld bepaald dat de intensiteit van de schrikreflex redelijk consistent is over verschillende metingen binnen dezelfde deelnemers. Deelnemers die op het ene moment relatief grote angst reflexen laten zien, scoren ook op het andere moment hoog. Deze betrouwbaarheid is een cruciale vereiste voor wetenschappelijk onderzoek waarbij verschillen tussen individuen centraal staat en wordt momenteel te weinig gerapporteerd in de literatuur.

CONCLUSIE

De resultaten beschreven in dit proefschrift dragen bij aan de kennis over de basale neurobiologische mechanismen die ten grondslag liggen aan de emotie angst. De stap naar klinische toepasbaarheid voor het verlichten van angst in angststoornissen is nog groot. Toch kan deze fundamentele kennis uiteindelijk bijdragen aan de behandeling van mensen die lijden aan extreme angstigheid.

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Floris Klumpers, februari 2012

CURRICULUM VITAE

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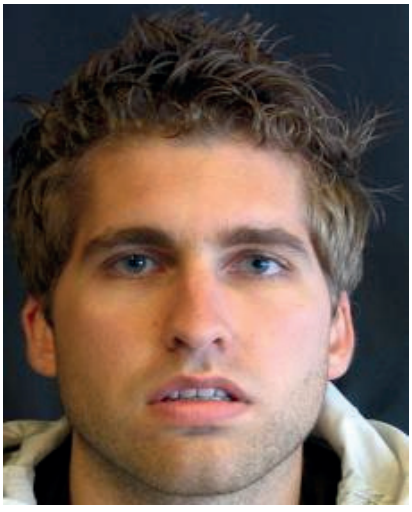
Floris Klumpers was born the 24th of February 1983 in Lisse, the Netherlands. In 2001 he started his studies in Psychology at Utrecht University, with the ambition to learn more about the foundations of human behaviour. His favourite approach was the study of neurobiological processes that underlie emotion and their relation with psychopathology. In 2004 he received a bachelor degree (cum laude), majoring in Biopsychology. He then chose to expand his knowledge with the interdisciplinary master program Neuroscience and Cognition in Utrecht. He worked as an intern at the University Medical Centre Utrecht (UMCU) where he investigated structural brain changes in schizophrenia as related to antipsychotic medication intake under the supervision of Dr. Neeltje van Haren. Afterwards, he went on to do an internship at het Experimental Psychology and Psychopharmacology departments at Utrecht University with his future co-promoter Dr. Johanna Baas and promoter Prof. Dr. Leon Kenemans. In this second internship the goal was to test new medication for its effects on psychophysiological correlates of fear extinction. In conclusion of his master program he wrote his master thesis under supervision of Dr. Dennis Schutter and Dr. Jack van Honk at Experimental Psychology on cortico-subcortical interactions and their involvement in emotion regulation. In 2007 he received his masters degree (cum laude). After working as a research assistant he started his PhD training in 2007, which ultimately led to the current thesis. As of December 2011, Floris works a post doctoral researcher in the Stress & Memory research group of Prof. Dr. Guillen Fernandez at the Donders Centre for Brain, Cognition and Behaviour.



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Heitland I, Klumpers F, Oosting RS, Evers D, Kenemans JL & Baas JMP (under review). Failure to extinguish fear is associated with a common polymorphism in the human cannabinoid receptor 1 gene.

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