NEURAL SUBSTRATES OF PSYCHOSOCIAL STRESS AND STRESS HABITUATION IN HUMANS

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1. INTRODUCTION

Known for its integrative role in the bodies stress response and its widespread physiological functions when it comes to maintaining the equilibrium of homoeostasis, the hypothalamus-pituitary-adrenal axis (HPA axis) has been the target of a longstanding scientific interest (Pacak & Palkovits, 2001; Selye, 1946; Tsigos & Chrousos, 2002). There is now sufficient evidence to support the notion that dysregulation of the HPA axis represents a crucial factor in the pathogenesis of a wide series of mental (Arborelius et al., 1999; McEwen, 2003), neurological (A. D. Smith et al., 2002), metabolic (Dallman et al., 2004) and immunological (Segerstrom & Miller, 2004) disorders. And while there is reason to believe that an exaggerated response or a lack of response habituation of this endocrine axis can promote various disease states (McEwen, 1998; McEwen & Seeman, 1999), neural substrates of the HPA axis regulation are still not entirely understood.

Based on animal models, it is well documented that states of stress and anxiety cause activation in limbic and prefrontal brain areas (Figueiredo et al., 2003b; Kollack-Walker et al., 1997; Rauch et al., 1996; Roozendaal et al., 1997; Rosen & Schulkin, 1998) as well as several distinct neurotransmitter systems (Carrasco & Van de Kar, 2003; Herman & Cullinan, 1997; Herman et al., 2004; Van de Kar et al., 2001). Moreover, recent receptor mapping studies in rodents (Cintra et al., 1994; McEwen et al., 1986) and non-human primates (Sanchez et al., 2000) further indicate, that glucocorticoid receptors are densely expressed not only in limbic but also in cortical and here especially in prefrontal areas. While limbic regions like the amygdala and the hippocampal formation have long been thought be involved in HPA axis regulation (Feldman et al., 1983; Jacobson & Sapolsky, 1991), these recent receptor mapping findings give rise to the notion that not only these prominent limbic areas but also prefrontal regions might act as major regulatory feedback sites when it comes to HPA axis functioning (R. M. Sullivan & Gratton, 2002b).

However, while the neural substrates of HPA axis regulation have been considerably studied in various non-human species, to date hardly any studies focused on the identification of neural substrates of HPA axis regulation and habituation in humans (Pruessner et al., 2004; Wang et al., 2005).

In the work presented here, two different functional brain imaging techniques were utilized and a preliminary attempt to study aspects of HPA axis regulation in the human brain was made. Based on what is currently known about HPA axis regulation in rodents and non-human primates, limbic areas such as the hippocampus and the amygdala as well as prefrontal regions were considered as the main regions of interest. However, it should be emphasized that due to the lack of pre-existing hypothesis generating findings from human studies, the work presented here refrains from elaborate á priori hypothesis and rather reflects a preliminary, hypothesis

generating work in this young scientific field.

The work presented here is introduced by a theoretical framework on the neuroendocrine stress response. This section starts with brief description of the early stress models of Selye, Cannon and Mason and closes with a summary on more integrative and recent concepts like the *allostatic load model*.

In a next step, the biological stress response is highlighted. Accordingly, an attempt is made to summarize what is currently known about neural substrates of HPA axis functioning and to give an overview over the several brain regions currently thought to be involved during states of HPA axis activation and habituation.

In the subsequent method section, the reader is familiarized with the physiological and methodological aspects of human brain imaging and the two imaging techniques used in the work presented here are briefly introduced.

The method section is followed by the description of two empirical studies on the neural substrates of stress in humans. The principal idea underlying the work presented here, was to make a first attempt in relating recent animal findings on HPA axis regulation to the human neurobiology of stress and to test for the role of limbic and prefrontal regions in human HPA axis functioning. For this reason, two different strategies along with the application of two distinct brain imaging techniques were chosen. In a first approach, positron emission tomography (PET) was the method of choice and 14 healthy males underwent a total of two PET scans. In order to assess brain activation during confrontation with a psychosocial stress test, participants were injected with fluoro-18-deoxyglucose (FDG) right before stressor confrontation or the matched, non-stressful control condition. In order to relate stress induced brain activation to HPA axis activity, cortisol was collected before and after the stress test or the control condition.

In a second approach, functional magnetic resonance imaging (fMRI) was chosen to investigate neural substrates of HPA axis activation and habituation in healthy human volunteers. This question seemed of special interest, as habituation of the HPA axis due to repeated confrontation with a homotypic stressor is a well established finding in various species. Moreover, inability to adapt to a frequently re-occurring stress has frequently been associated with various pathologies and states of allostatic load (McEwen, 1998). However, while little is known about the brain regions that are involved in regulation of acute HPA axis activity, currently almost no data exist on the possible neural mechanisms of HPA axis habituation. Based on the well established finding that HPA axis activity is common during states of fear and anxiety, it has been speculated that hyper-excitability in the brain's fear networks could be a state that causes hyper-frequent HPA axis activation that would be reflected in reduced HPA axis habituation. Hence, in the second study presented here, associations between excitability of the brain's fear circuitries and patterns of HPA axis activation and habituation were studied in a population of healthy young men. In a first step, an initial group of 90 participants was confronted with two almost identical versions of a psychosocial stress test, that were separated by exactly one week.

Spontaneous as well as habituated HPA axis responses were assessed by measuring saliva cortisol in each subject. In a subsequent follow up experiment, a total of 38 out the initial 90 subjects returned to the lab for functional magnetic resonance imaging brain scan. During the brain scans, participants were exposed to a paradigm known to induce states of anticipatory anxiety and hence activation of the brain's fear and anxiety circuitries. Subsequent data analysis focused on the question whether hyper-excitability in the brain's fear circuitries relates to spontaneous and/or habituated HPA axis response.

In a final section, results of the two empirical studies are summarized and some of the methodological issues are critically highlighted. The thesis finally ends with a series of remarks on possible implications of the here presented findings and a final outlook towards future trends.

2. THEORETICAL BACKGROUND

Chapter two is designed to provide the theoretical background and the scientific framework for the experiments described in chapter four. Initially, early and more recent stress concepts are introduced and the term stress is highlighted from an integrative perspective. Next, biological aspects of the stress response are elucidated with a special emphasize on the HPA axis and its hormones.

Thereafter, chapter two focuses on various aspects of HPA axis regulation and neural stress circuits involved in HPA axis function are outlined in more detail. Finally, the axis's role during acute and chronic stress is discussed and the concept of HPA axis habituation is introduced.

2.1 Stress Concepts

One might speculate that there is not a single one among us who does not experience the subjective feelings of stress from time to time. And although the term *"stress"* seems to be a common human experience, due to its subjective nature, it is not easy to define. A general definition of stress has not yet been established even among the various scientific disciplines (Levine & Ursin, 1991; Pacak & Palkovits, 2001). In the following sections an attempt is made to shed light on early as well as current stress concepts, presenting and when possible, integrating different perspectives from various disciplines.

2.1.1 Early Concepts - Bernard, Cannon and Selye

The beginnings of the current stress concepts can be traced back to the middle of the 19th century, when Claude Bernard proposed the idea of a *"milieu intérieur"*:

> *"It is the fixity of the 'milieu intérieur' which is the condition of free and independent life. All the vital mechanisms, varied as they are, have only one object, that of preserving constant the conditions of life in the internal environment."* (Bernard, 1878 cited from (D. Goldstein, 1995)).

It was roughly fifty years later that Walter Cannon shaped the term *"homeostasis"*, describing the organism's ability to maintain internal balance in the face of changing environments by generating a coordinated physiological response. As an example of such an orchestrated reaction, with the underlying activation of the autonomous nervous system, Cannon coined the phrase *"fight and flight"* response (Cannon, 1929, 1935).

In 1936, Hans Selye published his assay on the *"General Adaptation Syndrome"* (GAD) where he defined an organism's characteristic and unspecific response to a noxious stimulus and the possible implications of stress on an organism's health and survival. The GAD consists of 3 stages: a) *stage of general alarm*, b) *stage of resistance*, and c) *stage of exhaustion*.

The *stage of general alarm* typically sets in 6-48 hours after the onset of the noxious stimulus and is characterized by specific bodily changes such as adrenal hypertrophy, atrophy of the thymus and other lymphatic tissues and ulcers development in the digestive tract. After this initial stage and roughly 48 hours after the onset of the initial strain, the organism enters the *stage of resistance* that is characterized by remission of most of the symptoms observed during the alarm reaction. If the noxious stimulus vanishes or is rather mild in nature, the organism will have built up sufficient resistance and most of the bodily functions will have returned to a state of normalcy towards the end of this stage. If, on the other hand, the initial strain is very profound and continuous for an extended period of time, the organism will finally enter the lethal *stage of exhaustion* (Selye, 1936).

According to Selye, the word *"stress"* describes the unspecific bodily reaction to a noxious stimulus, whereas the stress-evoking stimulus is described as a *"stressor"* (Selye, 1974).

Selye's stress model acknowledges the fact that identical stressors can elicit distinct reactions in different individuals. According to his theory, this is due to *"conditioning factors"* (e.g. genetic disposition, age or gender). It is worth mentioning that Selye's doctrine of non-specificity does not deny the existence of stressor-specific response patterns. According to him, all these patterns have the same non-specific component accompanied by a stressor-specific aspect. Selye also argued for a strict distinction between stress and emotional arousal or tension as stress for example was observed in animals and humans under complete anesthesia (Selye, 1974, 1976, 1981).

2.1.2 Integrated Stress Concepts

The following two sections describe stress concepts that were highly inspired by Selye's early findings. However, while Selye focused on his doctrine of non-specificity, and stress experiments based on highly noxious stimuli which caused severe bodily reactions and damage, later stress concepts criticized and subsequently expanded these early narrow concepts and focused on the role of psychological factors on the organism's coordinated stress response.

2.1.2.a Mason

Selye's groundbreaking work was widely recognized in the scientific community but did not remain unchallenged. In 1968, John W. Mason (Mason, 1968) published a review dealing with aspects of psychoendocrine research and the pituitary-adrenal cortical system. In this article, he discussed Selye's doctrine on non-specificity and emphasized the role of emotional stimuli in consideration of the activation of the pituitary-adrenal system:

> *"No matter how seemingly threatening or drastic the life situation, it cannot be assumed that all, or even most, subjects will experience substantial emotional arousal or distress. The relevant question is "Do those individuals who become emotionally upset in this*

situation show significant hormonal response?"" (Mason, 1968, page 594).

Thus, Selye's work was challenged by Mason's introduction of psychological factors, such as emotional stimuli:

> *"While it was generally assumed 15 years ago that adrenal cortical response to any given stressful situation were caused by physical factors unless proved otherwise, it now appears increasingly important to take the position that concomitant emotional stimuli must be ruled out before it can be concluded that a physical stimulus is capable, by it self, of eliciting increased adrenal cortical activity. Selye's stress concepts, in fact, may well bear re-evaluation in this light."* (Mason, 1968; page 595)

According to Mason, factors like novelty, unpredictability, suspenseful anticipation, and social factors, as well as involvement and effort were potential elicitors of HPA axis activation. He even argued that psychological factors are amongst the most potent natural stimuli known to affect HPA axis activity (Mason, 1968) .
Masons ideas have inspired psychoendocrine stress research for more then 30 years and in a very recent meta-analysis it was shown that laboratory stress tests characterized by uncontrollability and social-evaluative threat were associated with the most pronounced stress hormone increases and the longest time to recovery, in comparison to other laboratory stress tests, not comprising these factors (Dickerson & Kemeny, 2004).
In light of these results, Kemeny and colleagues proposed a stress model called the *"Social Self Preservation Theory"* (Dickerson et al., 2004).
According to this theory, situations which are a threat to one's social image or standing, provoke a defined set of psychological and physiological reactions such as feelings of low social worth (e.g. shame, humiliation), drop in social self-esteem, and increased activity of the HPA axis. Hereby, the authors define threats to one's social self as situations that potentially devalue one's social self by questioning abilities, competencies, or traits on which a favorable self-image is based. The theory further states that achieving and maintaining a positive social self-perception is a primary human goal (Gruenewald et al., 2004).

2.1.2.b Levine and Ursin

One of the most comprehensive stress concepts, accounting for the psychological, physiological and behavioral dimensions of stress, comes from Levine and Ursin (Levine & Ursin, 1991). Their concept focuses on the existence of 3 main subclasses of stress: a) the *input* (stress stimuli), b) the *processing systems*, including the subjective experience of stress, and c) the *output* (stress responses) as well as the interaction between these subclasses.

When it comes to the input stimuli, Levine and Ursin prefer the term *"loads"* rather than stressors. Loads can occur in the form of physical loads (e.g. heat, light, muscle load) and psychological loads, which can either be a form of mental (e.g. task characteristics, degree of difficulty) or emotional load (e.g. threats, ambition, fear of failure). According to the authors, stress-inducing stimuli most likely represent the absence of a critical feature in the environment. An example would be a lack of information about possible outcomes of a situation (e.g. cancer diagnosis) that would result in feelings of uncertainty and unpredictability and a subsequent stress response. Similar to Mason, they emphasize the importance of emotional loads in current stress concepts:

> *"Psychological emotional "loads" are the most frequently reported stress stimuli, and are given as a reason for most stress responses for most individuals in most situations."* (Levine & Ursin, 1991; page 5)

The term load instead of stressor should be used because it is the subjective evaluation of each individual which determines whether a given load will be experienced as a stressor. According to Levin and Ursin, this evaluation takes place in the central nervous system (CNS), where the input stimuli is normally filtered before it gains access to a response system. The way in which the input is filtered, is very dependant upon previous experiences and the learning processes of the organism, which can for example result in defense and denial and possibly dysfunctional adaptation in a given situation. Previous experience and learning can also manifest in certain response outcome expectancies: Three principle expectancies hereby determine whether an individual will or will not show a stress response: coping, helplessness, and hopelessness. For the authors, the term coping does not so much refer to the strategies used in the face of a challenge but rather defines the result of a learning process, which predicts that the individual expects a positive outcome with a high degree of probability. The concept of coping is therefore highly related to the concept of perceived control, which in turn, highly depends on the ability to undertake an active response (e.g. escape) in the face of a stressful situation. Coping and perceived control can only be established through learning processes that are characterized by rapid and clear feedback on one's action.

The feeling of helplessness arises when the individual cannot establish any contingencies between the response available and the likely outcomes. Hopelessness even goes a step further and describes circumstances in which the individual assumes that any available response will lead to aversive and negative events (e.g. punishment). The concepts of helplessness and hopelessness have both been frequently associated with states of depression (Maier & Seligman, 1976).

With their filter theory, Levine and Ursin show parallels to another widely recognized stress concept: The transactional stress theory by Lazarus and Folkman (Lazarus & Folkman, 1984).

According to this concept, the subjective experience of stress depends on two appraisal steps: During the primary appraisal the individual evaluates whether a stimuli or situation actually reflects a threat or not. During the secondary appraisal, a decision is made whether one can meet the recourse necessary to overcome the threat. Stress is perceived when the individual feels that the resources are not adequate. However, other than Lazarus and Folkman, Levine and Ursin argue that a stimulus should be defined as stress stimulus based on its physiological and/or behavioral effects.

The authors acknowledge the fact that identical stressors can result in noticeably altered outputs. An effect that can be attributed to several factors such as prenatal stress, nutrition, disturbed mother-infant interactions, hormonal states during development and social as well as genetic factors.

Generally an association between physiological and behavioral outcomes is expected but discordance and dissociation of the outcome dimensions can occur under certain circumstances. Verbal reports, as part of the behavioral outcome, generally follow the physiological state according to the author's observation. However, limitations are present:

> *"In general, the subjective reports seem to follow the physiological state. However, as with all verbal statements from human subjects, there are reasons to question if they are always truthful, or if we hear what the subjects for some reason want us to hear. Verbal reports may be regarded as one aspect of the total number or responses available to a subject. The relationship between the internal state and overt behavior is not as simple as one might believe, even in animals."* (Levine & Ursin, 1991; page 10).

The physiological and behavioral factors of a stress response are described within the framework of the arousal and activation theory.

Arousal or activation is a process that takes place in the CNS, and it can manifest itself in vegetative, endocrine and immunological processes. Activations in these subsystems are part of the general response but non of these activations in any of these subsystems is regarded essential to the original reaction.

Arousal or activation in response to a possible challenge is regarded as the driving force that will initiate the solution of problems. In light of this thought, the authors emphasize the notion that a stress response has certain adaptive properties and should not be avoided by all costs:

> *"Feelings of stress, or related states such as anxiety, are not necessarily evils to be dampened by psychopharmacological interventions, even if they may appear to be unpleasant. They may be adequate responses to stimuli requiring full attention and integrated action for solution, and subsequent reduction of the source of the stress response. The purpose of the response is to eliminate not only itself but also the source of*

the state." (Levine & Ursin, 1991; page 13).

Activation will last until the CNS detects an agreement between the set value and the actual value or the system gives lower priority to the set value - a thesis that shows strong similarities to the principle idea of homeostasis.
The nature of the physiological and behavioral components of the stress response is further specified depending on the presence or absence of coping. If coping is present, the stress response is characterized by a short-lasting, phasic activation. If coping is absent, the stress response will manifest itself in a longer lasting or even chronic activation and might result in negative health outcomes.

2.1.2.c Allostatic Load Model

The implications of a chronically activated stress response and its influence on health and disease in light of various coping forms have recently been re-evaluated by Bruce McEwen within his *"allostatic load"* model (McEwen, 1998).
The term allostasis was first introduced by Sterling and Eyer (Sterling & Eyer, 1988) and describes how blood pressure and heart rate responses can vary with experiences or in response to other external cues. McEwen broadened the term to describe how bodily systems can vary on demand in order to maintain function of those systems that are truly homoeostatic. Strictly speaking, the term homoeostasis only refers to bodily systems that are truly essential for life and therefore change function only over a very narrow range (e.g. pH, body temperature, oxygen tension). Allostatic systems on the other hand are adaptive systems that change in response to demands and by doing so actively maintain function of homeostatic systems. McEwen refers to this mechanism as *"stability through change"*.
Instances, when allostatic systems are over-stimulated or do not perform normally such as a) frequent activation, b) failure to shut off allostatic activity after stress, c) inadequate response in one allostatic system that triggers compensatory increase in other systems, are characterized by the term allostatic load. Chronic states of allostatic load are regarded as health risks and can potentially lead to disease. Function or male function of allostatic systems is influenced by the way we experience and interpret adverse life events but also by the way we cope with these events (McEwen, 1998, 2000; McEwen & Seeman, 1999)

2.2 The Biological Stress Response

As described in the previous sections, confrontations with hostile instances, threats, or anticipation of negative events normally result in an orchestrated physiological and behavioral response to facilitate the organism's adaptation to the aversive circumstances.
This coordinated bodily response is often referred to as the biological stress response. It is characterized by various changes in physiological function such as a) increased energy

mobilization to guarantee brain and muscle function, b) enhanced focused attention, c) increased perfusion rates and local cerebral glucose utilization, d) acceleration of heart rate and respiratory rate and redistribution of blood flow to the brain and muscles, e) changes in immune function, f) suppression of reproductive hormones and sexual behavior, g) diminished appetite and feeding and in case of severe hemorrhage, h) water retention through renal and vascular mechanisms takes place as well (Carrasco & Van de Kar, 2003; Sapolsky et al., 2000).

These physiological changes are a result of the coordinated activation of stress sensitive neural circuits and the corresponding initiation of the two main effectors of these neural circuits: The hypothalamus-pituitary-adrenal (HPA) axis and the autonomic nervous system (ANS).

While both systems operate in a highly orchestrated way, the main focus of this work is on the neural regulation of the HPA axis. Subsequent sections highlight the anatomical basis, function and regulation of the axis and provide an overview over the various stress sensitive neural circuitries involved in the organism's stress response.

2.2.1 The Hypothalamus-Pituitary-Adrenal (HPA) Axis

Since the early beginnings of stress research, the HPA axis has been associated with the endocrine response to stress. Selye's early discoveries have stimulated numerous research projects investigating the role of the HPA axis during stress as well as under basal conditions (Chrousos, 1998; Harbuz & Lightman, 1992; Sapolsky et al., 2000).

The HPA axis is a hierarchical cascade comprised of 3 serially linked anatomical structures (hypothalamus, pituitary and adrenal cortex) as well as a series of endocrine signals allowing for the communication between the different structures:

In a first step, stress induced secretion of corticotropin releasing factor (CRF) takes place in parts of the hypothalamus. Via the infundibular stalk, CRF is then passed on to the pituitary, where it promotes synthesis of adrenocorticotropic hormone (ACTH), which is then passed on to the blood circulation. Finally ACTH reaches the adrenal cortex, where the release of the glucocorticoid (GC) cortisol is promoted (Kirschbaum & Hellhammer, 1999).

The individual stages and precise mechanisms of the HPA axis will now be described in further detail.

2.2.1.1 HPA Axis and Hypothalamic Control

The hypothalamus, with its discrete functions in thermoregulation, sleep-wakefulness, appetitive behavior, body weight and fluid control as well as endocrine and autonomic stress regulation, has a central role in maintaining the body's homeostatic state (Kupfermann, 1991; Martin, 2003).

The hypothalamus's distinct functions are organized in a series of discrete nuclei or small groups of nuclei that have numerous bi-directional connections to a variety of effector systems throughout the brain (Floyd et al., 2001; Ongur et al., 1998a; Sawchenko et al., 1996; Silverman et al., 1981; Swanson & Kuypers, 1980; Swanson & Sawchenko, 1980). The diencephalic

structure can be divided in 3 zones: the periventricular, the middle zone and the posterior zone. Within the periventricular zone, on the border to the third ventricle, lies the paraventricular nucleus (PVN) - a key structure in HPA axis regulation and autonomic outflow (Herman et al., 2003; Martin, 2003).

The PVN constitutes three divisions: a) a magnocellular zone with neurons containing vasopressin (VP) and oxytocin that project to posterior pituitary, b) an autonomic division, containing corticotropin releasing factor (CRF) neurosecretory cells that project to brainstem areas and c) a parvocellular division with CRF and VP positive neurons that are connected with local portal plexus (within the median eminence) (Kupfermann, 1991). Acute restraint stress leads to increased CRF mRNA levels in the hypothalamus (Imaki et al., 2001; Kiss et al., 1996) and the hormone is regarded as the main HPA axis stimulating agent. VP on the other hand, has comparably smaller effects on HPA axis activation by its own but seems to act synergistically with CRF (C. R. DeBold et al., 1984; Gillies et al., 1982; Lamberts et al., 1984). However, more recent data from Brattleboro rats, deficient in arginine vasopressin (AVP), did not indicate any significant effect of AVP on acute stress responses due to restraint stress. However, after a 10-minute swim stress ACTH rise in Brattleboro rats was significantly diminished compared to control rats indicating a stressor specific role of AVP in HPA axis regulation (Makara et al., 2004).

2.2.1.1.a CRF

CRF was first described in 1981 (Vale et al., 1981) and has ever since inspired a vast amount of research. Over the years it has been shown that CRF and its various ligands serve an important role in fear and anxiety, as well as in regulation of the endocrine and behavioral stress response, and are involved in numerous actions affecting the body's homeostatic balance (Bale & Vale, 2004). As central mediators of the biological stress response, CRF and its receptors have also been implicated in the etiology of stress-related disorders (Bale & Vale, 2003). In mammals CRF, urocortin-I (UcnI), UcnII and UcnIII have been detected and their individual distribution patterns and receptor pharmacology has been characterized (Bale & Vale, 2004).

CRF and its ligands exert their effects by binding to CRF receptors. CRF receptors belong to the B subtype of G protein-coupled receptors and are present in two subclasses: CRFR1 and CRFR2. CRFR1 exists in α and β isoforms. CRFR2 is present in 3 isoforms: α, β and γ with the CRFR2γ form only present in humans (Bale & Vale, 2004). CRFR1 is predominantly found in frontal cortical areas, the cholinergic basal forebrain (medial septum and diagonal band of Broca), the brainstem cholinergic nuclei (laterodorsal tegmental nucleus and probably the pedunculopontine tegmental nucleus), the superior collicullus, the basolateral nucleus of the amygdala (BLA), the cerebellum, the red nucleus, the trigeminal nuclei and the anterior pituitary (Steckler & Holsboer, 1999). CRFR2α is primarily expressed in the PVN, the ventromedial hypothalamus (VMH), the lateral septum, the cortical and medial nuclei of the amygdala and the

serotonergic raphé nuclei. Mixed distribution patterns are present in the olfactory bulb, the hippocampus, the entorhinal cortex, the bed nucleus of the stria terminalis (BNST), and in the periaqueductal gray (PGA) (Steckler & Holsboer, 1999). In non-human primates CRFR1 has also been detected in the locus coeruleus (LC) (Sanchez et al., 1999). Moreover, compared to rodent brains, both CRFR1 and CRFR2α were found throughout the monkey neocortex especially in limbic, prefrontal and cingulate cortices (Steckler & Holsboer, 1999).

The PVN is the major site for CRF containing cell bodies. Apart from that, CRF containing neurons are widely distributed throughout the neocortex (mostly layers II and III) with extremely high densities in the prefrontal and cingulate cortices in the rat (Swanson et al., 1983). CRF immunereactive cells have also been found in the central nucleus of the amygdala (CeA), the BNST, the hippocampus, the accumbens, in posteromedial thalamic nuclei, the substania nigra, the LC, the dorsal and medial raphé nuclei, the PGA, the olfactory bulbs, the parabrachial nuclei, the nucleus of the solitary tract (NST) and the cerebellum (Steckler & Holsboer, 1999).

In human brains Ucn is found in every region of the brain including hypothalamus, pons, cerebral cortex and cerebellum, with the highest concentrations in the frontal cortex, temporal cortex and the hypothalamus (Shimomura et al., 1998).

CRF has a 10-fold higher affinity for CRFR1 than for CRFR2 (Bale & Vale, 2004). Ucn on the other hand seems to be an endogenous ligand for CRFR2 as it binds with almost 40-fold higher affinity than does CRF (Vaughan et al., 1995).

A series of data from transgenetic animal models indicate a central role of CRF and Ucn when it comes to orchestrating the organism's endocrine and behavioral response to stress. Transgenetic mice over-expressing CRF are likely to develop HPA axis hyperactivity and Cushing like symptoms (Stenzel-Poore et al., 1992). Mice genetically lacking CRF show a drastically blunted HPA axis activity under basal as well as stimulated conditions (Bale & Vale, 2004). On a receptor level, mice deficient in CRFR1 show a blunted HPA axis response to restraint stress but normal basal corticosterone levels when compared to wild-type mice. Moreover the CRFR1 deficient mice showed markedly reduced anxiety (G. W. Smith et al., 1998).

Mice deficient in CRFR2 show normal basal ACTH and corticosterone levels as well as a normal circadian rhythm but an increased HPA axis response to stress (Bale et al., 2000). Interestingly, mice deficient in CRFR2 show prolonged elevated stress hormone levels, indicating a delayed recovery from stress (Coste et al., 2000).

Taken together, this data indicates that CRFR1 and CRFR2 serve distinct functions in modulation of the central stress response. Whereas CRF appears to play a stimulatory role in stress responsitivity through activation of CRFR1, actions mediated by CRFR2 may be important for dampening stress reactivity.

Ucn-deficient mice display normal anxiety-like behavior and do not show abnormalities in the autonomic regulation of the stress response which indicates that Ucn does not play an essential

role in stress-induced behavioral and autonomic response (X. Wang et al., 2002). On the other hand, Rainnie and co-workers showed that 5 daily injections of non-anxiety provoking doses of Ucn into the BLA resulted in long lasting anxiety-like responses (Rainnie et al., 2004).

Preliminary data gives evidence that administration of a CRFR1 antagonist may serve as a new treatment for depressive disorders. First clinical trials on depressed patients indicate good treatment response (e.g. reduction in depressive symptoms) without affecting HPA axis function (Kunzel et al., 2003; Zobel et al., 2000). In an animal study however, Gutman et al. showed that administration of the same CRF1R antagonist (RU 121919) prior to restraint stress resulted in a dose dependent attenuated peak plasma ACTH and corticosterone concentration (Gutman et al., 2003).

In a very recent investigation, a new transgenetic mice model with selective postnatal CRFR1 knockout in limbic and forebrain structures but intact CRFR1 receptor systems in the pituitary was introduced. It was shown that these animals have normal basal and circadian HPA axis activity. In response to restrain stress, the transgenetic mice showed similar initial ACTH response (5 minutes post stress). However, mutants displayed significantly elevated ACTH concentrations 30 and 90 minutes post immobilization stress indicating a role of limbic CRFR1 receptors in HPA axis adaptation. Analysis of hippocampal mineralocorticoid receptors mRNA after stress indicated a significant increase in wild-type mice but not in mutants. This leads to the hypothesis that mineralocorticoid receptors receptors within the hippocampus might server an important role in delayed HPA axis feedback. Interestingly, transgenetic animals showed significantly reduced anxiety-related behavior indicating that CRFR1 particularly in limbic and forebrain areas mediates anxiety related behavior without affecting basal, circadian or acute HPA axis response. It was further shown that anxiolytic tendencies previously observed in complete CRFR1 knockout mice was not due to a generally blunted HPA axis, but rather reflects an independent mechanism, most likely mediated via limbic and forebrain CRFR1 (M. B. Muller et al., 2003).

In summary, CRF and its receptors can be regarded as central agents orchestrating several aspects of the biological and behavioral stress response. Data on these phenomena are promising but still inconclusive and future models are needed to elucidate the exact underlying mechanisms.

2.2.1.2 HPA Axis – The Pituitary and ACTH

The pituitary is located ventrally to the hypothalamus and is regarded as the endocrine effector organ of the diencephalic structure. The pituitary is anatomically and functionally divided in two parts - the anterior (adenophypophysis) and posterior (neurohypophysis) pituitary. Magnocellular vasopressin and oxytocin secreting neurons within the PVN project to neurohypophysis form where the neurohormones are released into the systemic circulation (Martin, 2003; Trepel, 1999).

CRF and VP released from the parvocellular division within the PVN is passed on to the adenohypophysis, where the hormones bind to corticotroph cells and thereby promote the synthesis of adrenocorticotropic hormone (ACTH) from its precursor peptide proopiomelanocortin (POMC) (C. DeBold et al., 1987; Jia et al., 1991). From the anterior pituitary, ACTH is secreted into the blood stream with a (median) half-life time of about 19 minutes (Keenan et al., 2004).

2.2.1.3 HPA Axis – The Adrenals and Cortisol - Biosynthesis and Secretion

Biologically active ACTH reaching the adrenals binds to specific receptors and the GC cortisol is released (Faber & Haid, 1995).

The adrenals are located bi-laterally at the cranial end of the kidneys and consist of two, evolutionary, distinct structures - the adrenal medulla and the adrenal cortex. While the adrenal cortex is involved in storage and secretion of catecholamines (e.g. epinephrine and norepinephrine), the cortex is the primary production site for steroids. The adrenal cortex is divided in three layers - the outer layer zona glomerulosa, the medial layer zona fasciculata, and the inner layer zona reticularis (Faber & Haid, 1995; Kaplan, 1988).

GCs are primarily synthesized in the middle layer, the zona fasciculata (with a small contribution from the zona reticularis) and belong to the super-family of steroids, which are all cleaved from the precursor molecule cholesterol involving P450 enzymes (B. E. P. Murphy, 2000). Cortisol and corticosterone are the main GCs and whilst corticosterone secretion is more pronounced in animals, cortisol is the dominating GC in humans (ratio corticosterone : cortisol = 1 : 7) (Faber & Haid, 1995).

After ACTH binds to adrenal plasma membrane receptors, the biosynthesis of cortisol is initiated. In a first step, ACTH promotes a cAMP (cyclic adenosine monoaminephosphate) mediated conversion of cholesterol esters to free cholesterol, followed by the conversion to Δ5-pregnenolone which is then, in several steps, metabolized to corticosterone and cortisol (Kaplan, 1988; Murphy, 2000).

It should be mentioned that dissociations between systemic ACTH and cortisol concentrations have been frequently observed and there is considerable evidence that ACTH is not the exclusive glucocorticoid-stimulating agent (Bornstein & Chrousos, 1999).

Cortisol, once released into the systemic circulation, is bound to the binding globulin transcortin (CBG). Roughly 80% to 90% of cortisol is coupled to CBG and another 5% -10 % is bound to albumin. Only the remaining fraction circulates in a free and therefore biologically active state (Mendel, 1989) with a half-life of about 70-90 minutes (Kaplan, 1988; White et al., 1995). CBG is primarily synthesized in the liver and binds cortisol with high affinity and specificity. The average binding capacity of CBG is 20 μg/dL (White et al., 1995), but can be influenced by sex hormones and oral contraceptives (Wiegratz et al., 2003) as well as stress (Alexander & Irvine, 1998; Spencer et al., 1996). While other cortisol binding globulins are known (e.g. CBG-Lyon (Emptoz-

Bonneton et al., 2000), due to low affinity, they are of minor importance.
Cortisol is a small (MW 362) and highly lip-soluble compound, able to pass through lipid-bilayer membranes of nucleated cells. Due to this feature, free unbound cortisol can be detected not only in plasma, but in saliva as well. Concentrations of free cortisol in saliva and in the blood are in high correlation ($r \leq .90$). The concentration of cortisol in saliva, due to passive diffusion mechanisms, is not dependent on saliva flow rate or transport mechanisms (Vining & McGinley, 1986). Cortisol diffusion from plasma to saliva occurs rapidly with only a 2-3 minute time lag compared to plasma concentrations (Kirschbaum & Hellhammer, 1994, 2000).

2.2.1.4 Cortisol Effects

Unbound, biologically active cortisol exerts its various effects via special receptors present in virtually every nucleated cell type throughout the body (Munck et al., 1984). GCs bind to two different intracellular receptor types: Mineralocorticoid receptors (MR also called type-I receptor) and glucocorticoid receptors (GR also called type-II receptors). While MRs have a high affinity for endogenous GCs and are therefore readily occupied at lower GC levels, GRs only have a low affinity for endogenous GCs but a high affinity for synthetic GCs (e.g. dexamethasone), and seem to play a more important role during states of elevated GC concentrations (De Kloet et al., 1998; Joels, 1997; Reul & de Kloet, 1985). It has also been shown that type-I receptors bind corticosterone with a six-fold higher affinity than type-II receptors (Reul & de Kloet, 1985).
GRs and MRs are intracellular receptors forming a cytoplasmatic multiprotein complex consisting of a receptor molecule, an immunophilin and heat shock proteins (HsP 70 and HsP 90) (D. F. Smith & Toft, 1993). When GCs bind to their receptor, heat shock proteins are released and the receptor undergoes allosteric changes and dimerization – processes, needed for subsequent DNA binding. After several phosphorylation steps, the receptor is present in a transcriptionally active form. The activated receptor molecule then migrates to the cell nucleus, connects with DNA binding sites and initiates gene transcription and subsequent production of several intracellular and extracellular acting proteins. New protein synthesis normally takes 30 minutes or longer (Brann et al., 1995).
In the presence of a stressful stimulus, cortisol levels rise within 5 to 20 minutes and peak roughly 10 to 20 minutes after the stressor onset. (Kirschbaum & Hellhammer, 2000; Richter et al., 1996). Due to predominantly genomic transmission, most GC effects upon target tissue are not exerted until roughly an hour after the onset of the stressor (Sapolsky et al., 2000).
Fast, non-genomic, membrane dependent GC mechanisms have been described as well (Joels, 1997). Although the exact mechanisms are still poorly understood, it is currently assumed that GCs can exert rapid, non-genomic action via membrane-mediated mechanisms. It has been hypothesized that the lipophylic properties of GCs allow them to modulate membrane fluid properties in a non-specific manner and thus affecting membrane function and cellular response. Another possibility is the existence of a specific steroid membrane receptor. However, evidence

for this receptor is still inconclusive (Joels, 1997). It has also been speculated whether GCs interact with $GABA_A$ receptor structures, thereby affecting rapid cellular responses (Funder, 1997; Joels, 1997).

Although the release of GCs is regarded as a prototypical aspect of the stress response, the exact rational of GC actions remains obscure (Munck et al., 1984; Sapolsky et al., 2000).

While in early theories it was assumed that GCs enhance the organism's defense mechanisms in a phase of stress (Selye, 1946), Munck and colleagues (Munck et al., 1984) promoted the idea that elevated GCs levels during states of stress do not protect the organism from the stressor itself but prevent the body's defense reaction from overshooting and thereby potentially threatening homeostasis. The most recent and comprehensive view on this topic comes from Sapolsky et al. (Sapolsky et al., 2000), who make an attempt of a synthesis of early and more recent concepts, allowing for possible permissive, suppressive, stimulating and preparative GC effects. In section 2.2, a series of stress-related physiological changes have already been listed. Most of these changes are mediated or at least moderated by GC actions.

GCs have wide-ranging metabolic effects and promote increases in blood sugar, glucogenesis in liver and kidneys, hepatic glycogenesis, cellular insulin resistance as well as decreased tissue glucose up-take. GCs also affect lipid metabolism by causing lipolysis and thereby enhancing levels of free fatty acids (White et al., 1995).

Additionally, GCs have permissive effects on sympathetic nervous system function during stress by enhancing the cardiovascular stress response (Sapolsky et al., 2000).

This topic has attracted an enormous amount of attention over the course of the last 50 years, since the early findings of GC mediated immune suppressing effects. While for many decades it was believed that GC exerts mainly suppressive effects on immune function, recent findings point towards a more complex model allowing for suppressive as well as permissive effects on immune function (McEwen et al., 1997; Sapolsky et al., 2000).

Neurobiological effects of GC are very complex and of particular interest for this work and will therefore be described in more detail in the next section.

2.2.1.4.1 Cortisol and Neurobiological Effects

First reports on GC dependent neurobiological effects emerged almost 40 years ago (McEwen et al., 1968) when it was shown that GCs affect limbic structures. Over the course of the years these early results inspired a tremendous amount of investigations.

In more recent history, detailed maps on GR and MR brain distribution pattern have been established and a remarkable knowledge on how GCs influence neural structures has accumulated. GC mediated neurobiological effects on various brain functions have been described, such as: auditory brainstem responses (Born et al., 1989b), auditory evoked potentials (Born et al., 1989a), startle reflex (Buchanan et al., 2001), learned helplessness (Papolos et al., 1993) changes in taste detection acuity (Fehm-Wolfsdorf et al., 1989), pain

threshold (al'Absi et al., 2002) but also see (al'Absi & Petersen, 2003), learned helplessness behavior (Kademian et al., 2005), decision making (van Honk et al., 2003) and selective attention (van Honk et al., 2000). While there is only little research on most of these effects, possible GC mediated influence on mood and memory has received considerable scientific attention. The following sections will first highlight findings on brain MR and GR distribution patterns in various species. Genomic and non-genomic effects on brain function are described thereafter. Finally, an overview on GC related effects on memory and mood is presented.

2.2.1.4.1.a GR and MR Receptor Distribution in the Brain

As outlined in the previous section, GCs exert their effects via MRs and GRs. While both receptor types are widely distributed throughout the brain, the two receptor types do not share a common pattern of distribution. MR receptors are predominantly found in the hippocampal formation, lateral septum, medial and central amygdala, olfactory nucleus, layer II of the cortex, cerebellum and in brainstem sensory and motor neurons. MR mRNA has also been found in the anterior hypothalamus, subfornical organ and chorioid plexus (Joels & de Kloet, 1994; McEwen et al., 1986; Van Eekelen et al., 1988). GRs show a more widespread distribution patterns and high GR densities are found in the limbic system (hippocampus, septum), the medial prefrontal cortex (mPFC) and in the parvocellular nuclei of the PVN. High concentrations have also been found in the monoaminergic neurons in the brainstem, in a series of thalamic nuclei, the striatum and the central amygdaloid nucleus (Cintra et al., 1994; Diorio et al., 1993; Joels & de Kloet, 1994; Van Eekelen et al., 1988).

In the hippocampal formation GRs are primarily present in the CA1 and CA2 pyramidal cells. MRs were found in CA1-CA4. Co-expression of both receptor types within the identical neuron has been measured in CA1, CA2 and the granular cell layers of the dentate gyrus, however different effects on gene expression were assumed (Van Eekelen et al., 1988).

While these distribution patterns are primarily based on rodent brains, a recent GR mapping study performed on adult rhesus monkeys showed low GR mRNA densities only in the dentate gyrus and in the cornu ammonis, whereas GR mRNA concentrations were high in the pituitary, the cerebellum, the PVN and in parietal, cingulate and prefrontal cortices. Immunehistochemical staining performed in the same study also showed low densities of GR-like immunereactive cells within the hippocampal formation contrasted by high densities within the PVN, the prefrontal and entorhinal cortices and the cerebellum (Sanchez et al., 2000).

In a study on squirrel monkey brains, high levels of GR and MR mRNA were detected in the hippocampus, subnuclei of the amygdala, lateral geniculate nuclei, cerebellum, arcuate hypothalamus, pineal gland and periaqueductal regions of the brainstem (Patel et al., 2004). High MR and GR mRNA densities were also measured in the cerebral cortex. GR mRNA was evident throughout all cortical layers. MR mRNA was expressed in more superficial cortical layers and was less prominent in layers V and VI. Whereas GR mRNA was present in all

prefrontal cortical regions, MR mRNA was primarily restricted to orbitofrontal and dorsolateral prefrontal regions.

GR mRNA was absent in septal areas, putamen or caudate. High MR mRNA concentrations were detected in the lateral septum and, to a lesser extent, in caudate and putamen. In the amygdala, GR mRNA was strongly expressed in the central and lateral nuclei whereas MR mRNA was most pronounced in the corticomedial regions. Moreover, GR mRNA was expressed in the arcuate and paraventricular hypothalamus, hippocampal pyramidal cells fields CA1 and CA2 and the dentate gyrus granule cell layer. MR mRNA was expressed in all hippocampal pyramidal cell fields and dentate gyrus. Semi-quantitative analysis revealed that MR mRNA levels were 3-fold greater than GR mRNA levels in the dentate gyrus and 4.5-fold greater in hippocampal pyramidal cell fields. Within the amygdala, MR mRNA levels were nearly 2-fold greater in the medial amygdala. The reversed pattern was observed in the lateral amygdala. In the prefrontal cortex, GR mRNA levels were 2-fold greater than MR mRNA levels in the dorsomedial PFC (Patel et al., 2000).

While there are several reports on GR and MR distribution in rodent and monkey brains, data on GR and MR distribution patterns in humans are very rare. Watzka and colleagues (Watzka et al., 2000) looked at brain tissue from patients with epilepsy and found high concentration of MR and GR mRNA levels in the temporal lobe, the hippocampus, as well as the frontal lobe. In women, MR and GR mRNA expression was significantly lower in the hippocampus compared to frontal and temporal lobe areas. Women also had higher MR and GR mRNA in temporal lobe and frontal lobe cortex than men. While this data gives interesting insight into possible GR and MR distribution pattern in the human brain, results should be interpreted cautiously. As this data is based on brain tissue from patients with severe forms of epilepsy, disease specific pathological GR and MR distribution patterns cannot be ruled out.

2.2.1.4.1.b GC - Genomic and Non-Genomic Effects

As previously mentioned, GCs can act via a slow, long lasting, genomic or fast, non-genomic mechanisms. Both forms have been observed in the brain and it has been shown that both mechanisms influence properties of neuron membranes and as a consequence the neural firing patterns (for review see Joels, 1997).

In the hypothalamus, fast corticosteroid actions are well documented, predominantly showing a rapid inhibitory effect of GCs on neural firing rates (Saphier, 1987; Saphier & Feldman, 1990). Non-genomic effects have also been observed in prelimbic forebrain regions, where neurons in the lateral septum showed excitatory responses upon cortisol exposure (Saphier, 1987).

In the hippocampus, rapid effects seem to modulate voltage- and transmitter-induced processes. Interestingly, these effects occur roughly within a 10 to 20 minute time frame, which could point towards genomic effects. However, data does not indicated that these effects are mediated by gene transcription (Joels, 1997). Finally, rapid, most likely non-genomic effects have been

described in brainstem areas where neurons mainly show increased firing rates after iontophoretic application of corticosterone. Effects emerge within less than 2 minutes (Joels, 1997).

Genomic effects induce a series of cellular responses that can affect cellular structure, energy metabolism, or signal transduction. Changes in cellular structure are rather slow in nature and occur over the course of several days. Effects on energy metabolism and signal transduction usually become apparent within hours (De Kloet et al., 1998).

Genomic effects have been extensively studied in the hippocampus and GC effects on voltage-gated ion channels, ionotropic receptors and channels that are regulated via G-protein coupled receptors have been described. Genomic GC effects on excitatory amino acid (e.g. glutamate) and aminergic transmission (e.g. epinephrine, norepinephrine, acetylcholine, serotonin) and subsequent changes in neuron excitability in the hippocampus have also been well documented (for review see (De Kloet et al., 1998).

2.2.1.4.1.c GC and Memory

The hippocampus serves critical functions in memory and learning. In line with data showing that this brain structure, high in GR and MR densities, is a prime target for genomic as well as non-genomic GC actions, effects of stress hormones on memory and learning are well documented. Under acute exposure, GCs exert enhancing as well as impairing effects depending on the exact domain of memory (e.g. encoding versus retrieval; declarative versus procedural memory) and the circumstance during which learning occurs (McEwen & Sapolsky, 1995; Wolf, 2003).

There is now accumulative evidence showing that GCs can facilitate memory consolidation during stressful and emotionally loaded situations. In a series of elegant studies McGaugh and his group showed that these memory enhancing effects are not exclusively dependent on hippocampal structures but are also mediated by orchestrated GC and adrenergic actions in the in the basolateral amygdala (McGaugh, 2004; Quirarte et al., 1997).

Oitzl and De Kloet showed that GR antagonists given after a first learning experience in the Morris water maze resulted in impaired performance 24 h later. GR antagonists given either before the first or the second session in the maze had no effects. These results indicate GC mediated disruptions in encoding but not in either acquisition or retrieval of spatial information during a stressful situation (Oitzl & de Kloet, 1992; Oitzl et al., 1994) .

In humans, Buchanan and Lovallo showed that administration of 20 mg cortisol resulted in enhanced long-term recall performance on emotionally arousing versus neutral pictures (Buchanan & Lovallo, 2001). In a similar study Abercrombie et al. (H. C. Abercrombie et al., 2003) found that administration of 20 mg cortisol prior to presentation of emotionally arousing or neutral stimuli resulted in memory facilitation for both emotionally arousing as well as neutral stimuli Cahill and co-workers presented emotional and neutral slides and administered a cold pressure test (CPT) right after. CPT resulted in increases of cortisol. A memory test one week

later showed enhanced memory for emotional versus neutral slides indicating an interaction between arousal at initial encoding and subsequent memory consolidation (Cahill et al., 2003).

Conflicting data emerged from a recently published study: Takahashi and colleagues tested the impact of a psychosocial stress exposure along with increased cortisol levels on social memory. Pictures of faces along with corresponding names served as stimuli and were presented right before the confrontation with a psychosocial stress paradigm. A memory test immediately after the stress exposure revealed impaired social memory in a group of people with highest cortisol increases during the stress test (T. Takahashi et al., 2004).

GCs do not only exert effects on encoding. In rats, impaired retrieval of long-term spatial memory due to stress and GCs has also been reported (de Quervain et al., 1998). Others have shown GC mediated effects on retrieval (free recall) but not on recognition of verbal material (de Quervain et al., 2000). Testing whether emotional valence of stimulus material influences the effects of cortisol on retrieval, Kuhlmann and colleagues administered a single dose of 30 mg cortisol one hour prior to retrieval of negative and neutral verbal material. Learning took place exactly 4 hours prior to cortisol administration. The results of this study indicate that cortisol impaired retrieval for negative but not for neutral verbal material showing that GC not only exert valence specific effects on encoding but also on retrieval (Kuhlmann et al., 2005).

Putnam and colleagues tested the influence of basal cortisol levels on short and long-term memory in healthy young women. Facial pictures with happy, neutral or fearful expressions from the *Karolinska Directed Emotional Faces* set served as stimuli and were presented in a spatially coded manner. In the immediate recall condition, valence and identity memory was significantly better for happy versus neutral faces. There was a trend in the same direction for the fear versus neutral face comparison but the effect did not reach statistical significance. In the delayed recall condition (20 minutes after the initial learning) both happy as well as fearful faces were associated with better recall compared to neutral faces. Basal cortisol (median split high vs. low) only seemed to enhance recall of spatial information of emotional but not neutral faces (Putman et al., 2004).

In line with receptor distribution studies showing high densities of GRs in prefrontal lobe areas, acute GC effects on working memory have also been reported (Lupien et al., 1999).

Not only acute but also chronic exposure to GCs impact memory function. It has been shown that sustained elevated GC levels can cause atrophy in dendrites and soma shrinkage in the CA3/CA2 cell region of the hippocampus in primates (Sapolsky et al., 1990) and social stress has been shown to cause significant hippocampal cell loss and atrophy (Lucassen et al., 2001; Magarinos et al., 1996). Furthermore, chronic but not acute stress in rats induces significant inhibition in long-term potentiation (LTP)[1] in the medial perforant pathway input to the dentate

[1] Long-term potentiation (LTP) refers to the strengthening of connections between nerve cells that normally last for an extended period of time. LTP is regarded as the cellular basis of learning and memory.

gyrus and the commissural/associational input to CA3 of the hippocampal formation (Pavlides et al., 2002). Evidence that these effects are mediated by GCs comes from an earlier experiment performed by the same group: Chronic corticosterone administration (40 mg/kg/day over 21 days) resulted in notably decreased LTP. Testing for LTP was performed 48 h after the last corticosterone administration when GC levels already returned to baseline levels thereby excluding acute GC effects (Pavlides et al., 1993). Taken together these two studies give evidence that chronically elevated GCs mediate inhibitory effects on LTP in hippocampal areas and thereby exert impairing effects on memory.

Studying effects of chronically elevated GC levels in healthy human volunteers is ethically rather difficult (Wolf, 2003). However, findings from patient populations with chronically elevated cortisol levels give evidence that consistently elevated GC concentrations might have similar effects in animals and humans. Morbus Cushing for example is a disease characterized by adrenal hyperactivity, and if left untreated, chronically elevated cortisol levels. Memory deficits and hippocampal atrophy have been reported in this patient group, giving rise to the assumption that chronically elevated GCs might promote such pathologies (Forget et al., 2000; Starkman et al., 2001). Due to wide ranging effects (e.g. immune suppressive), GCs are often prescribed for treatment of various disorders. During long-term treatment with high doses of GCs, a pharmacological side effect called *"steroid dementia syndrome"* has been reported. Wolkowitz and others collected a series of case reports on this syndrome and listed the reported cognitive side effects. Common impairments were problems with encoding as well as recall of verbal information, word-finding difficulties, decreased implicit and explicit memory, difficulties with abstract reasoning, and impairment in working memory function (Wolkowitz et al., 2004). This data further supports the notion that constantly elevated GCs can exert negative effects on human memory function.

2.2.1.4.1.d GC and Negative Affect

A link between mood and cortisol levels has long been assumed, and increased negative mood scores have been reported during states of elevated cortisol levels:

A hyperactive HPA axis is a common symptom in depressive disorders, which are characterized by sever negative mood (Holsboer & Barden, 1996; Makino et al., 2002). Abnormal 24-hour secretory patterns along with increased cortisol levels have been reported in depressed patients (Linkowski et al., 1985; Maes et al., 1998). Dexamethasone, given to healthy subjects, normally results in a subsequent suppression of HPA axis activity. Diminished suppression patterns after dexamethasone suppression tests have frequently been reported in depressed patients and indicate disturbances in negative feedback regulation of the HPA axis in these patients (Gervasoni et al., 2004; Holsboer et al., 1987). Normalization of HPA axis dysregulation has been observed over the course of pharmacological treatment and seems to co-occur with mood improvement (Barden et al., 1995). It has been speculated that antidepressants might exert their

mood elevating effects by influencing brain GR expression and activity (Barden et al., 1995). A compelling hypothesis, which at first glance appears rather counterintuitive, comes from Pariante and colleagues. They argue that chronically elevated GC levels in the periphery reflect diminished HPA axis feedback due to restricted GC access to the brain (Pariante, 2004). So not a *"too much"* but rather a *"too less"* of GCs in the brain are associated with depressive symptoms. In line with this hypothesis, Pariante and his group showed that antidepressants can enhance GR function by inhibiting membrane steroid transporter and thereby increase access of GCs to the brain and possibly also increase intracellular GC concentration (Pariante, 2003, 2004; Pariante et al., 2001). In another investigation, Uhr et al. (Uhr et al., 2002) indeed showed that mice deficient in certain transporter proteins (mdr1a and mdr1b P-glycoproteins) involved in GC blood brain barrier passage, show enhanced cortisol and corticosterone concentration in the brain. Further support comes from studies showing that antidepressants mediate modification of GR mRNA in cultures of rat hypothalamic and amygdaloid neurons (Pepin et al., 1989) and enhanced, antidepressant mediated, GR gene transcription in mouse hippocampal cell lines (Herr et al., 2003).

Depressed mood has also been reported in patients suffering from Morbus Chushing. It has been speculated that these mood effects are mediated by elevated GC concentrations, as some studies reported reduction in depressive symptoms after normalization of GC levels (Dorn et al., 1997; Sonino et al., 1993; Sonino et al., 1998).

In line with the Cushing results, and seemingly in contrast to Pariante's theory, reduction in depressive symptoms after anti-glucocorticoid therapy was frequently reported in several patient groups (B. E. Murphy, 1997). However, because these studies were not performed in a double-blind or placebo- controlled fashion, these results are often difficult to interpret. Moreover, various forms of anti-glucocorticoid therapy were applied (e.g. GC synthesis inhibitor versus GC antagonists) and it cannot be ruled out that some of these drugs (e.g. GC antagonist RU 486) promote GR up-regulation in the brain. As a result, increases of GC – GR coupling in the brain would positively influence HPA axis feedback and function (B. E. Murphy, 1997).

It should be kept in mind, however, that not all depressed patients show hypercortisolemia and data on this phenomenon remains inconclusive (B. E. Murphy, 1997). Moreover, not only is there evidence that depressive symptom reduction can occur while GC levels remain high (Murphy et al., 1991) but cases of GC induced euphoria have also been described (B. E. Murphy, 1991).

Field studies assessing mood and cortisol in healthy volunteers are mostly correlational in nature and do not allow direct deductions about causality. Nonetheless studies on healthy populations give important hints on how mood and GCs are possibly interrelated.

Smyth et al. (Smyth et al., 1998) enrolled 120 participants in a study assessing stressors and affect at six fixed time points over the course of a day. Twenty minutes after each assessment, a saliva cortisol sample was collected. Subjective experience of a current stressor as well as anticipation of an up-coming stressful event was associated with higher cortisol levels. Negative

affect was more, positive affect was less pronounced in the presence of stressors and elevated cortisol levels. Furthermore, after controlling for negative affect, daily stressors no longer significantly predicted cortisol concentrations.

In a similar study, van Eck and co-workers assessed mood and perceived stress scores as well as salivary cortisol on ten fixed time points over the course of a day. In accordance with results from Smyth and co-workers, they also found a positive association between negative mood and cortisol levels (van Eck et al., 1996).

Prüssner and colleagues (M. Pruessner et al., 2003) investigated the relationship between self-reported depressive symptoms, acute feelings of distress and morning cortisol levels. Participants with high self-reported depression scores showed significantly higher morning cortisol responses after awakening. Momentary feelings of stress were also positively associated with elevated post-awakening cortisol levels.

While the majority of studies listed here so far support a positive link between peripheral GC concentrations and negative mood, there is evidence that this association might not hold true in all instances: Burnout syndrome is a serious psychopathological condition with a high prevalence in groups of chronic caregivers, teachers, social service employees and other professions potentially suffering from chronic emotional burden. The disorder is characterized by diffuse physical symptoms of fatigue, exhaustion and non-specific pain. Patients suffering from burnout syndrome also often report feelings of meaninglessness, apathy and negative affect (Pruessner et al., 1999). In a study on teachers, participants with high levels of burnout showed a blunted cortisol response during the first hour of awakening and increased suppression of cortisol levels in response to dexamethasone (Pruessner et al., 1999).

When one looks at associations between GC levels and mood, caution should be taken in interpreting the data. Apart from the above-discussed problem, in consideration of correlational data and conclusions of causality, in populations with psychiatric or somatic pathologies (e.g. depression, Morbus Cushing), a confounding role of the disease state cannot entirely be ruled out. Wolkowitz addressed these methodological issues in a double-blind study on healthy volunteers who were treated with high doses of GCs (prednisone 80 mg/day) for 5 consecutive days. Of the participants, 75% reported affective symptoms including depression, tearfulness, irritability, anger, insomnia, talkativeness, giddiness, confusion, racing thoughts and feelings of depersonalization but also mood elevation and increased energy (Wolkowitz, 1994). This study is a strong indicator that GCs, at least in high concentrations and over the course of several days, can influence mood states. That said - the neurobiological substrates of these effects are still poorly understood.

2.2.1.4.1.e GC and Fear and Anxiety

The general public as well as most scientists and clinicians tend to use the terms *“fear”* and *“anxiety”* quite interchangeably. However, according to various elaborate models on fear and

anxiety, these two terms seem to refer to two different constructs (Blanchard & Blanchard, 1990; J. A. Gray, 1982; J. A. Gray & McNaughton, 2000). It has been proposed, that fear has the function of moving the individual away from danger. Behavioral aspects of fear are fight, flight or freezing. On the other hand, when an approach-avoidance conflict is present, anxiety has the function of moving the animal towards the dangerous stimuli. Behaviorally, anxiety involves inhibition of prepotent behaviors, increased risk assessment and defensive quiescence (McNaughton & Corr, 2004). While the distinction between the two constructs will not be further discussed here, it seems noteworthy that according to this model on fear and anxiety, various brain structures that are also involved in the regulation of the HPA axis modulate functional and behavioral aspects of fear and anxiety (McNaughton & Corr, 2004). In line with this notion, GCs, at least in animal models, are well known to influence the brain's fear and anxiety circuitries (Korte, 2001). Receptor mapping studies have repeatedly shown that MRs and GRs are co-located in brain structures that are known to be involved in the regulation of fear and anxiety like the prefrontal cortex, the amygdala and the hippocampal formation (Cintra et al., 1994; McEwen et al., 1986; Reul & de Kloet, 1985; Sanchez et al., 2000). Based on animal data it has been assumed that corticosteroids not only help to re-establish homeostasis after a stressful and anxiety inducing incident, but also facilitate behavioral adaptation via their effects on the consolidation and potentiation of fear or the facilitation of extinction of avoidance (Korte, 2001).

In very young rats, removal of corticosteroids by removing the adrenals, significantly impairs the establishment of freezing behavior (L. K. Takahashi, 1994). Most interestingly, this effect could be reversed by administration of high doses of exogenous corticosterone, which indicates that GR-mechanisms are involved in mediating the ontogeny of freezing behavior (Levine, 1994; L. K. Takahashi, 1994). In adult rats, removal of the adrenals 1 h prior to the confrontation with a conditioned shock paradigm prevented freezing behavior, while administration of a low dose of corticosterone resulted in normalization of the conditioned freezing response (Bohus, 1987). In the elevated plus-maze, a well established animal model of fear, blockade of hippocampal MR receptors by an MR antagonist (RU28318), resulted in increased open arm explorations (Bitran et al., 1998), which indicates an anxiogenic effect of corticosteroids mediated via MRs. It is an established finding, that confrontation with an environment that has previously been associated with an inescapable stressor (e.g. shock) can induce fear potentiation in a subsequent fear inducing situation (Korte, 2001). It was shown in rats, that administration of a GR antagonist attenuates fear potentiation (Korte et al., 1995), which indicates that fear potentiation is mediated via GR mechanisms. In contrast, low levels of corticosterone seem to facilitate fear extinction via predominantly MR based mechanisms (Korte, 2001)

While these data highlight how corticosteroids influence and mediate fear related behavior via MR or GR based mechanisms, it should not be overlooked that states of fear and anxiety are themselves tightly associated with the activation of autonomic and endocrine systems (Davis & Whalen, 2001). In rats, unconditioned presentation of a fear inducing substance like predator

odor (2,5-dihydro-2,4,5-Trimethylthiazoline (TMT)) results in elevated levels of ACTH and corticosterone along with increased c-fos mRNA expression in several brain areas, including parts of the bed nucleus of the stria terminalis, the central nucleus of the amygdala, the paraventricular nucleus of the hypothalamus, the nucleus of the solitary tract and the locus coeruleus (Day et al., 2004) . Moreover, in infant rat pups, expression of fear to a predator odor and basolateral/lateral amygdala activity could be prematurely evoked with exogenous corticosterone (Moriceau et al., 2004). In infant rats, fear of novelty predicts magnitude in corticosterone response in middle age and early death (Cavigelli & McClintock, 2003). In monkeys, lesions of the CeA result in diminished fear-related behavior and decreases in CRH and ACTH in response to a fear-inducing stimuli (Kalin et al., 2004). Monkeys characterized by a fearful temperament and relative right asymmetric frontal brain activity showed higher basal cortisol concentrations as well as increased cerebrospinal fluid CRH concentrations (Kalin et al., 1998; Kalin et al., 2000).

In humans, children with an extremely inhibited behavioral style exhibit higher cortisol levels (Kagan et al., 1987; Schmidt et al., 1997) and exaggerated startle responses (Schmidt et al., 1997). Subjects characterized by high levels of trait anxiety or a repressive style show elevated basal cortisol levels (Brown et al., 1996). Moreover, trait negative affect also seems to be associated with higher total cortisol levels and increased morning rise in males (Polk et al., 2005). In patients with a diagnosis of social phobia, higher cortisol concentrations in response to a psychosocial stress encounter have been observed (Furlan et al., 2001; Martel et al., 1999). Patients with a diagnosis of PTSD, a HPA axis hyper-responsiveness to stressful challenges has been described (Yehuda, 1997).

In summary, a wealth of data indicate that states of fear and anxiety are highly associated with the activation of endocrine systems. Based on animal studies it has been shown that corticosteroids play an important role in mediating fear related mechanisms like freezing, fear potentiation and extinction via MR or GR based mechanisms. However, while states of fear and anxiety can be modulated by circulating GCs, the very same states are known to be prime activators of HPA axis activity. Hence, states of elevated levels of fear and anxiety have repeatedly shown to cause pronounced activation of the HPA axis.

2.3 HPA Axis & Central Regulation

The following sections will highlight the various modalities of HPA axis regulation. First, mechanisms of circadian rhythm are briefly outlined. Next, the classical negative feedback mechanisms are described. From here the concept of negative feedback is broadened and a spectrum of stress and negative feedback relevant brain areas are introduced and their potential influence on HPA axis activity will also be discussed.

2.3.1 HPA Axis and Circadian Rhythm

Similar to almost every other bodily function, the HPA axis is subject to a circadian rhythm.

Measured over the course of 24 hours (measurements taken every 15 minutes), 9 to 15 distinguishable secretion phases of CRF, ACTH and cortisol are detectable (Van Cauter, 1995; Weitzman et al., 1971).

In healthy humans with regular sleep-wake cycles, lowest concentrations of cortisol are measured during night times. In the early morning hours cortisol starts to rise and reaches an early morning peak about 30 minutes after awakening (awakening response; (Clow et al., 2004; Wust et al., 2000b). Over the course of the day, secretion pulse become less frequent and cortisol levels show a gradual decline towards the end of the day (Weitzman et al., 1971).

Circadian rhythms are influenced by various factors like sleep-wake and light-dark cycles (Van Cauter, 1995). The hypothalamic suprachiasmatic nucleus (SCN) is well known for its role in mediating circadian rhythm and light-induced activity (Shimomura et al., 1998; Yamazaki et al., 1998). It receives direct input from the retina and projects to various other brain regions amongst which the PVN (Martin, 2003). Bi-lateral destruction of the SCN has been shown to eliminate most circadian rhythms (Van Cauter, 1995). Moreover, the magnitude of the cortisol awakening response seems to be enhanced by light (800 lux) hitting the retina (Scheer & Buijs, 1999).

Changes in circadian rhythms have been associated with shift-work (Hennig et al., 1998), sleep deprivation (Ilias et al., 2002) as well as disease states (Ellenbogen et al., 2004).

2.3.2 HPA Axis and Feedback Control

As discussed in the previous sections, exogenous (stressors) as well as endogenous (circadian rhythm) factors can exert control over the functioning of the HPA axis (Chrousos, 1998). During states of basal or elevated demands, the CNS normally remains in control, and adequate up or down regulation of the axis is achieved via constant *"communication"* between control centers in the CNS and the periphery. This communication process is called *negative feedback*.

Negative feedback regulation is achieved via GCs binding to MR and GR receptors in the CNS and on a pituitary level. As previously outlined, MR receptors have a higher affinity for endogenous GCs and are therefore readily occupied at lower GC levels – thus, this receptor type plays a pivotal role in basal HPA axis regulation. GRs on the other side have a lower affinity for endogenous GCs and come into play during states of elevated cortisol (e.g. circadian peak, stress) (De Kloet et al., 1998; Reul & de Kloet, 1985).

That circulating GC levels and subsequent MR and GR binding are crucial in these feedback mechanisms was shown in a series of studies: Veldhuis et al. (Veldhuis et al., 2001) showed that under conditions of low glucocorticoid feedback, basal HPA axis regulation was affected in terms of a) repressing the mass of ACTH secretory bursts, b) reducing the orderliness of ACTH release, and c) modulation of the intrinsic diurnal rhythm of the hypothalamo-corticotrope unit.

Spencer and coworkers showed that administration of a selective MR antagonist (RU28318; 50

mg/kg sc) results in elevated basal corticosterone levels in the morning but not in the evening or during restraint stress. Administration of a selective GR antagonist (RU40555; 30 mg/kg sc) showed no effect on either basal or restraint stress conditions (independent of day time). Interestingly, combined treatment with RU 28318 and RU 40555 resulted in elevated basal evening corticosterone levels as well as increased GC concentration during and after restraint stress (Spencer et al., 1998). This data supports the notion that MR are not only important for basal regulation but also play a pivotal role in facilitating GR mediated feedback during states of elevated GC levels.

According to what is known today, at least three different feedback mechanisms exist: a) fast, b) intermediate, and c) slow feedback. These three mechanisms differ not only in their time domains of action but also show clear rate and location dependent distinctions (Keller-Wood & Dallman, 1984).

Fast feedback occurs within seconds to minutes, and is rate-dependent, meaning that the magnitude of inhibition depends on the rate of GC rise in plasma, and that fast feedback occurs only if GC levels are rising at a sufficient rate (1.3 μg/100 ml) (Keller-Wood & Dallman, 1984). Rapid feedback seems to exert its effects by CRF and ACTH inhibition. Due to the rapidness of the response, non-genomic signal transmission is most likely (Dallman et al., 2004; Keller-Wood & Dallman, 1984). More recent studies also support the notion that fast-feedback mediated HPA axis inhibition is highly dependent on stressor modality (e.g. hemorrhage (physical stressor) versus air puff startle (psychological stressor)) and that this effect might be due to different neural circuits involved in distinct stressors (Thrivikraman et al., 2000).

Intermediate feedback effects are relatively short in duration and are normally observed about 2-10 hours after exposure to elevated GC levels. In contrast to fast feedback effects, intermediate feedback seems to depend on the duration of exposure to increased levels of GCs rather than a rate-sensitive feedback mechanism. Intermediate feedback affects HPA axis function via a) inhibiting ACTH release but not synthesis, and b) inhibiting CRF synthesis as well as release (Keller-Wood & Dallman, 1984).

Slow feedback effects are only observed in situations of constantly prolonged elevated GC levels (more than 12 hours) and can cause inhibition of ACTH release and synthesis after exposure of 24 hours or more (Keller-Wood & Dallman, 1984).

It is argued that fast and intermediate effects are most relevant during states of stress-induced HPA axis activation. Stress-induced ACTH secretion is inhibited by fast feedback and occurs within seconds and ceases roughly 30 minutes after stressor onset. Further inhibition of ACTH secretion begins at about 90-120 minutes after the rise in GCs and is due to intermediate feedback effects. During the time between the two inhibition periods no inhibition takes place. This phase is called silent period. During conditions characterized by a stimulated HPA axis, GC mediated ACTH inhibition happens in a dose-dependent fashion (Keller-Wood & Dallman, 1984).

However, basal feedback regulation shows a different timing pattern and it is assumed that this

is due to different pathways stimulating basal versus stress-induced HPA axis function (Keller-Wood & Dallman, 1984).

In summary, fine tuned negative feedback control of the HPA axis seems to be crucial in order to prevent exaggerated response to stimulating agents. In this way, fast rate sensitive feedback seems to control the rate and magnitude of ACTH and GC response to stimulation. Intermediate feedback, on the other hand, may limit the HPA axis response to repeated stimulation within a short period of time (hours). Slow feedback seems to come intoplay when in limiting the response in cases of prolonged stress is required (Keller-Wood & Dallman, 1984).

While the feedback mechanisms described here mainly focus on feedback sites like the PVN and the pituitary, the input from other brain regions is another important factor when it comes HPA axis regulation. A graphical overview is given in figure 1 and detailed explanations on the various stress-sensitive neural circuits and mechanisms involved in HPA axis regulation will be given in the next section.

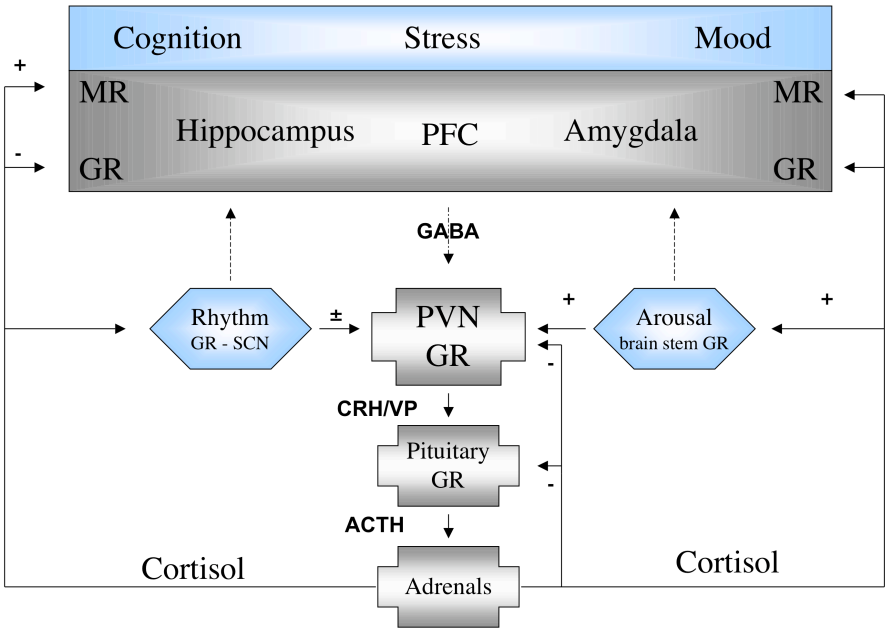

Figure 1: Mechanisms of HPA axis regulation (modified from de Kloet et al., 1998)

2.3.3 HPA Axis and Central Stress Circuits

According to some of the stress theories outlined in the first part of this chapter (e.g. Levine and Urisn; Mason; Folkman and Lazarus), the subjective perception of stress or threat and possible behavioral consequences highly depend on complex processing of the incoming stimuli. Therefore, activation of the HPA axis in situations of potential or actual threat can be regarded as a result of an orchestrated response of the CNS involving a series of brain regions that process the incoming stimuli along its various dimensions (e.g. threat). In line with this assumption, the PVN, a key region when it comes to HPA axis regulation, holds up a wide series of inhibitory as well as excitatory connections with other brain regions (Herman & Cullinan, 1997; Herman et al., 2003). An analysis of early gene expression (e.g. c-fos) after various stressors

confirmed wide spread activation in numerous neo- and allocortical brain regions as well as in several limbic (e.g. amygdaloid complex) and brainstem areas (Cullinan et al., 1995; Day et al., 2004). In the past few years, overwhelming evidence for stressor specific stress-regulatory circuits has accumulated (for review see Pacák & Palkovits, 2001). Activation of the HPA axis can be induced by either a actual stressor or by a predicated stressor. An actual stressor is regarded as a direct threat to homeostasis. Activation in such instances represents a response of the body to a real stimulus (e.g. hemorrhage). A predicted stressor occurs in the absence of an actual homeostatic or physiological challenge, and represents the effort of the organism to mount a GC response in anticipation of, rather in reaction to, homeostatic threats (Herman et al., 2003). There is now reasonable evidence (for review see Herman & Cullinan, 1997) that stressors, comprising an acute threat to homeostasis, activate the HPA axis via direct visceral efferent pathways, by way of brainstem circuitries that bypass the need for higher order processing (e.g. affective or cognitive) of the stimulus (Herman & Cullinan, 1997). Limbic pathways on the other hand seem to exert control over the PVN via indirect connections in the presence of stressors that require higher order sensory processing and integration of multiple sensory modalities as well as information about previous experiences (Herman & Cullinan, 1997).

In the following sections, some of these stress-sensitive neurocircuits are described in more detail and the impact of various brain regions on HPA axis regulation during states of stress will be highlighted.

2.3.3.1 The Amygdala

According to what is known today, the amygdala plays a central role in HPA axis regulation and seems to be crucially involved in a series of other complex information processing functions. This section begins with information on anatomical and functional aspects of the amygdala. In a second step, this information is integrated and the amygdala's role in regulating and modulating the stress response will be highlighted.

2.3.3.1.a Amygdala – Anatomy and Function

In 1819, the anatomist Burdach discovered an almond shaped mass of gray matter located near the temporal pole of the temporal cortex and in analogy to the shape, Burdach named this structure *"amygdala"* (Latin for almond) (Davis & Whalen, 2001). According to the Talairach atlas, the amygdala stretches from 17 – 30 mm lateral to the midline, 1 mm anterior to 11 mm posterior the anterior commissure (AC), and 7 – 21 mm below the intercommissural (AC-PC) line (Zald, 2003). Over the course of the years, microscopic examinations of the amygdala have revealed a high differentiation within the structure. In the rat, the amygdala constitutes 13 nuclei and cortical regions: basolateral (BLA) nucleus, the lateral (LA) nucleus, the accessory basal (AB) nucleus, anterior amygdaloid area (AAA), the periamygdaloid cortex (PAC),

amygdalohippocampal area (AHA), nucleus of the lateral olfactory tract (NLOT), medial (MEA), central (CEA), anterior cortical (aCOA), posterior cortical (pCOA), and intercalated nuclei (IA) (Aggleton, 2000).

Recent histochemical investigations showed that the different cell groups within the amygdala complex seem to be differentiated parts of the traditional cortex, the claustrum, or striatum. According to this concept, a major part of the amygdala is regarded as an integral part of the olfactory system with MEA, COA (posterior part medial zone), and posterior nucleus along with the olfactory bulb, constituting a sensory-motor system that might be involved in perception of pheromonal stimuli. The remaining aspects of the COA (nucleus of the lateral olfactory tract, piriform-amygdala area, postpiriform transition area, posterior parts of the basomedial nucleus, and posterior parts of the basolateral nucleus) reflect an integral of the main olfactory system. Based on neurotransmitter distribution patterns, there is evidence that the CEA can be regarded as a specialized region of the striatum highly involved in autonomic motor outflow. Finally, the LA and anterior parts of the BLA appear to constitute a ventromedial extension of the claustrum related most closely with temporal and frontal lobe structures (Swanson & Petrovich, 1998).

In line with these findings, several authors have questioned the term *"amygdala"* as being an arbitrarily unifying classification of an anatomically as well as functionally highly heterogeneous structure (Davis & Whalen, 2001; Swanson & Petrovich, 1998).

The different cell groups within the amygdala not only show numerous intra-amygdaloid connections but also have multiple descending and ascending projections to and from other parts of the brain (for an schematic overview see figure 2) (Aggleton, 2000).

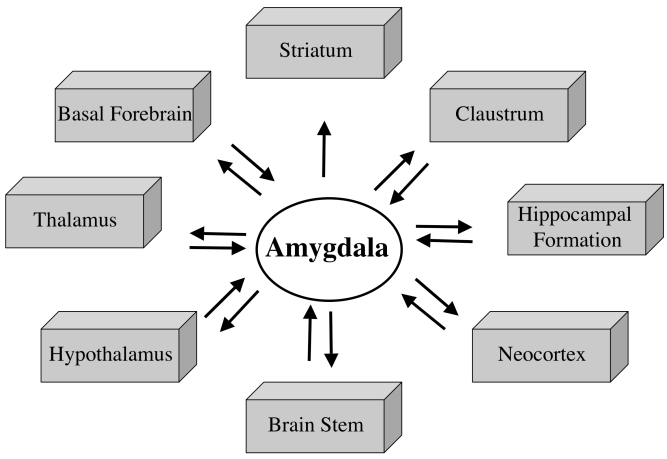

Figure 2: Overview over some of the primate amygdala connections (modified from Amaral, 2002).

Due to its high connectivity with sensory and higher order brain centers and brainstem regions, the amygdala complex takes over a pivotal role when it comes to monitoring environmental stimuli and the initiation of adequate behavioral interventions (Whalen, 1998). A somewhat related view comes from Amaral who believes that the amygdala is a protection device, which is

designed to detect and avoid danger. A primary function of the amygdala therefore is to evaluate objects or living beings prior to interaction with them (Amaral, 2002).

In a vast amount of experiments conducted in animals and humans, the amygdala has proven to be a crucial structure when it comes to the processing of emotional sensory stimuli (J. LeDoux, 2001; Zald, 2003). In this context, the role of the amygdala in detecting and generating fear related states as well as the modulation of fear related behavioral and autonomic output has attracted considerable attention over the course of the last decade. Sensory information from all modalities are passed on to the amygdala via the LA and BLA. From the LA and BLA information is then forwarded either to other brain regions directly involved in generating fear related output, (see figure 3) or the information is transmitted to the CEA or the BNST[2], which in turn project to a variety of brain regions mediating the behavioral, autonomic and endocrine signs of fear and anxiety (see figure 4) (Davis, 1998; Davis & Whalen, 2001).

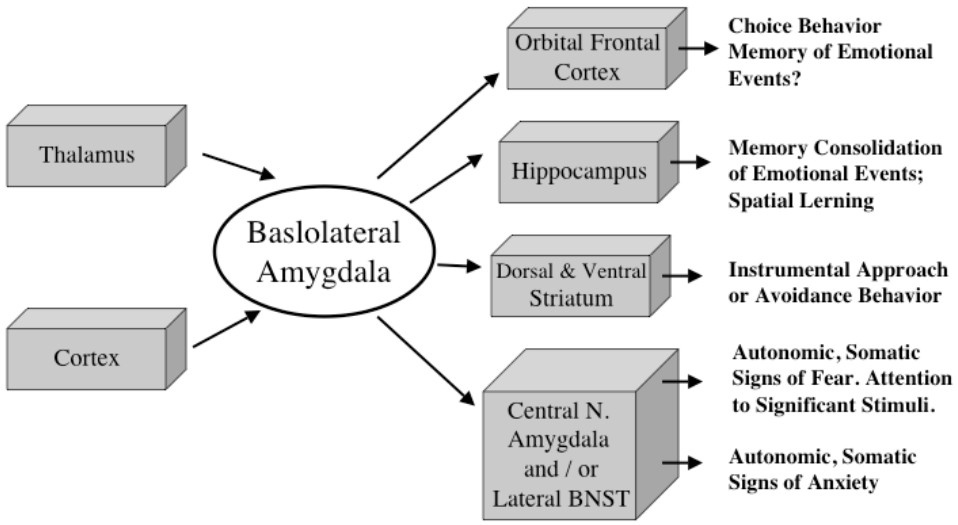

Figure 3: Schematic diagram of the outputs of the basolateral nucleus of the amygdala to various target structures and possible functions of these connections (modified from Davis & Whalen, 2001).

[2] The bed nucleus of the stria terminalis its, due to its connectivity with the amygdala complex, often referred to as the "extended amygdala" (see Davis & Whalen, 2001).

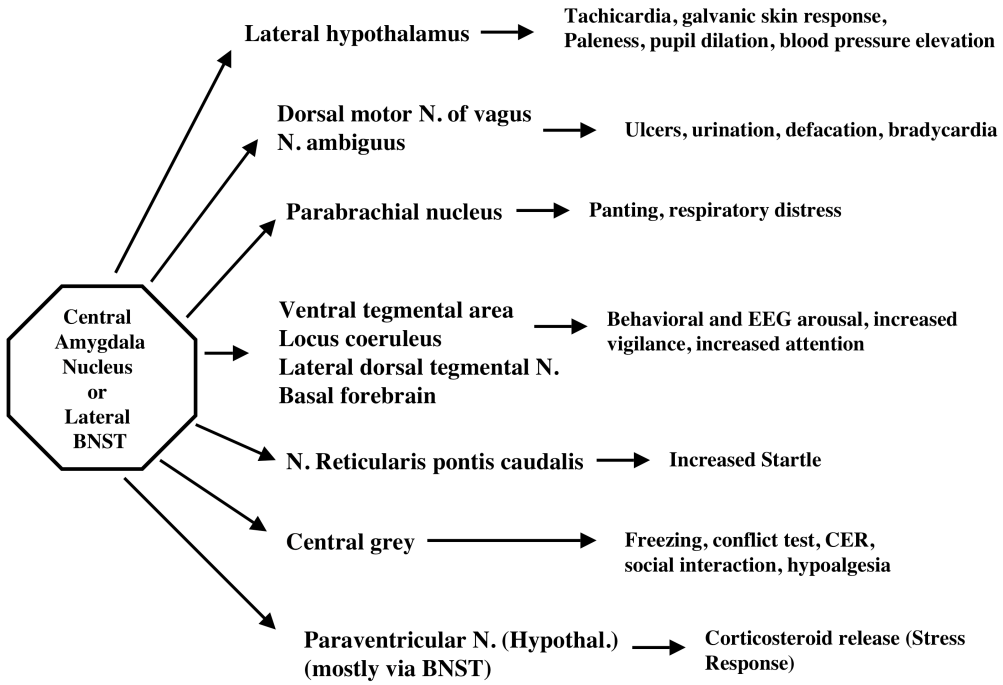

Figure 4: Schematic diagram of the outputs of the central nucleus of the amygdala or the lateral division of the bed nucleus of the stria terminalis (BNST) to various target structures and possible functions of these connections (modified from Davis & Whalen, 2001).

When it comes to the information flow to the amygdala, complex pictures have evolved over the course of the last few years: Using anterograde labeling techniques, Stefanacci and Amaral (Stefanacci & Amaral, 2002) showed that the inferotemporal cortex, portions of the superior temporal gyrus and the granular region of the insula, project primarily to the LA. Orbitofrontal, medial prefrontal and anterior cingulate cortex (ACC) regions (mainly Brodman area 10 and 25) project mainly to the basal and accessory basal nuclei. These areas, however, show little innervations of the LA. The orbitofrontal and medial frontal, but not the ACC, project to the medial and cortical nuclei and to the periamygdaloid cortex. Agranular and dysgranular insula and para-insula along with rostral portions of the superior temporal gyrus project to the LA, basal and accessory basal nuclei as well as to more medially located nuclei. Based on their data, Stefanacci and Amaral argue for a hierarchical information processing system within the amygdala complex, where the LA primarily receives sensory information (e.g. snake). This information is then passed on to the basal nuclei where sensory information is combined with context information from prefrontal areas and temporal cortices (e.g. in a zoo versus in wilderness). If sensory information along with the context information indicate a real threat, autonomic and behavioral effects are exerted via the central nucleus (Stefanacci & Amaral, 2002).

While the information presented in figures 3 and 4 reflects a summary of findings from more recent experiments, already in 1939 Kluver and Bucy (Kluver & Bucy, 1939) observed that bi-lateral removal of temporal lobes (including the amygdala, hippocampus and adjacent cortices)

in monkeys resulted in marked behavioral and emotional changes (*Kluver-Bucy-Syndrome*): Monkeys exhibited what Kluver and Bucy named *"psychic blindness"* where they would spontaneously and fearlessly approach animate and inanimate objects and examine these objects rather with the mouth than with the hand. Another very striking aspect of the syndrome was the absence of emotional motor and vocal reactions normally expressed in the presence of a fear eliciting stimuli or situations. Instead of showing a natural flight behavior or immobilization and the expression of facial and vocal signs of fear in the presence of an intruder, these monkeys showed a strong tendency to examine the fear related stimuli (e.g. stranger or other animal) (Davis & Whalen, 2001).

More recent data indicates that the amygdala might be a central fear system involved in the expression as well as acquisition of conditioned fear (Davis, 1998). In fear conditioning, a somewhat neutral stimulus (conditioned stimulus (CS) e.g. tone) is paired with an aversive event (unconditioned stimulus (US) e.g. shock). After a few pairings of the two stimuli, the CS enters fear networks and activates defense systems typically activated by natural threat (J. LeDoux, 1998). The responses observed tend to be hard-wired and species-typical expressions of fear. According to LeDoux, fear conditioning does not require response learning but instead involves the coupling of a new stimulus to a preexisting response (J. LeDoux, 1998). A well-studied paradigm is acoustic fear-conditioning. The acoustic CS travels through the auditory system to the auditory thalamus and from there it is transmitted to two target systems: the amygdala and the auditory cortex. The amygdala receives auditory information through the LA and information is transmitted to this structure within two time domains: A fast but less precise signal straight from the thalamus and a delayed but well processed signal from the auditory cortex (J. LeDoux, 1998). Information from the LA is then passed on to the CEA. Lesions of the LA have shown to inhibit fear conditioning of a tone (J. E. LeDoux et al., 1990). As outlined in figure 5, the CEA then sends information to the hypothalamus and the brainstem centers, which mediate the autonomic and behavioral signs of fear (J. E. LeDoux et al., 1988). Connections of the CEA with the hippocampus have been associated with context conditioning, which refers to the fact that environmental cues present during CS and US pairing are known to be able to elicit fear like behavior (e.g. the chamber in which the shocks were given). Damage to the hippocampus has been shown to interfere with context conditioning (Phillips & LeDoux, 1992).

A series of more recent investigations suggest that the mPFC plays a major role in the extinction of fear conditioning (Milad & Quirk, 2002; Sotres-Bayon et al., 2004) and that medial prefrontal cortex areas inhibit the fear conditioned responses by inhibiting CEA output (Quirk et al., 2003).[3]

The role of the amygdala in the processing of fear-related stimuli has also been convincingly described in patients with social phobia. Fredrikson's group uses ^{15}O labeled water to measure cerebral blood flow in social phobics undergoing a public speaking task. The results indicate that

[3] For a detailed discussion on prefrontal cortex and amygdala interaction please see section 2.3.3.4.

phobics when compared to healthy controls, show increase blood flow in the amygdaloid complex while performing a public speech (Tillfors et al., 2001) and during anticipation of a public speech (Tillfors et al., 2002). In both studies, tasks were also associated with increased levels of anxiety.

Studies on monkeys gave evidence for the existence of neurons within the amygdala that selectively respond to faces (Leonard et al., 1985). Studies on patients with selective amygdala lesions as well as functional neuroimaging studies in healthy subjects have indicated a pivotal role of the amygdala in processing emotional and social facial information.

Studies on patient SM046, a woman with bi-lateral calcification restricted to the amygdala (Urbach-Wiethe disease) showed that she could not identify the emotion of fear in a picture showing a human fearful face. Moreover, she was not able to draw a face showing a fearful expression. Facial expressions reflecting happiness, sadness, anger and disgust were identified and drawn within the normal range (Adolphs et al., 1994; Adolphs et al., 1995). In another study conducted on 3 patients with bi-lateral amygdala damage (patients SM, JM and RH), 7 with unilateral damage, some otherwise brain damaged patients and healthy controls showed that patients with bi-lateral amygdala damage rated pictures of unfamiliar faces more approachable and trustworthy compared to other patients or healthy controls. The effects were most striking for faces which normal subjects assigned the most negative ratings (unapproachable and untrustworthy looking individuals). This effect did not hold up when verbal descriptions of people were given. The authors therefore conclude that the amygdala seems to be an important brain region that helps to retrieve socially relevant knowledge on the basis of facial expression (Adolphs et al., 1998). This view was supported by a more recent study, showing that patients with uni- and bi-lateral amygdala damage when compared to control subjects showed impaired recognition of social emotions (e.g. arrogance, boredom, flirtatiousness) and that recognition of social emotions was even more strongly impaired than recognition of basic emotions (Adolphs et al., 2002).

In another study, Heberlein and Adolphs (Heberlein & Adolphs, 2004) showed that patients with bi-lateral amygdala lesions showed marked deficits in making social attributions to film with animated geometric shapes, whereas control subjects assigned the same film profound social meaning. Deficits could not be attributed to a bias in language use or the general inability to describe social stimuli. It has been assumed that the ability to anthropomorphize (imbue the world with social meaning) might be an adaptive response ((Guthrie, 1995) cited from Heberlein & Adolphs, 2004) and this study gives first evidence that the amygdala is not only involved in face perception but also plays an important role in assigning social meaning to the world around us.

The role of the amygdala in processing facial information has been confirmed by neuroimaging experiments performed with the help of healthy human subjects. Presentations of photographs showing human facial expressions have been shown to induce elevated amygdala activation,

(Blair et al., 1999; Morris et al., 1996; Whalen et al., 2001; Yang et al., 2002) and fearful expressions are often associated with stronger amygdala activation compared with neutral (Breiter et al., 1996; Morris et al., 1996) or happy facial expressions (Morris et al., 1996; Whalen et al., 1998).

In a very recent study, Whalen's group indicated that the amygdala also seems to respond to the eye whites (e.g. wide open eyes) of fearful faces. Fearful and happy eyes were showed in a backward masked fashion and amygdala response to fearful eye whites was significantly stronger compared to happy eye whites (Whalen et al., 2004).

Hariri and colleagues (Hariri et al., 2002) investigated the influence of functional polymorphism in the promotor region of the human serotonin transporter gene (5-HTT / SLC6A4) on amygdala function. The existence of one or two short alleles of this promoter polymorphism has been associated with reduced 5-HTT expression and increased fear and anxiety related behavior. Hariri and coworkers showed that participants with short alleles, when compared to subjects with long alleles, expressed stronger amygdala signal changes stimulated by fearful faces. Similar findings come from Heinz and colleagues (Heinz et al., 2005), who showed that carriers of a short allele of the 5-HTT showed stronger right amygdala signals in response to aversive versus pleasant stimuli than long allele carriers.

Studies in patients with social phobias have also frequently shown increased amygdala responses to faces (Birbaumer et al., 1998). Stein et al. (Stein et al., 2002) found that patients with social phobia when compared to healthy controls show stronger amygdala activation for contempt and angry faces (compared to happy expressions). No group differences were found for fearful or non-expressive faces (also compared to happy faces). Similarly, Straube et al. (Straube et al., 2004) found greater amygdala, parahippocampal, fusiform gyrus and superior temporal gyrus activation during implicit processing of angry (versus neutral) facial expression in social phobics compared to healthy controls.

Showing that fearful faces induce stronger signal intensities in the dorsal amygdala than angry faces (in healthy controls) (Whalen et al., 2001), Whalen argues for a revised perspective of the role of the amygdala: According to him, the amygdala is most prone to respond to stimuli that are highly ambiguous in nature, and the activation of the amygdala in the presence of ambiguous stimuli modulates vigilance in order to promote subsequent information processing throughout the brain. Vigilance therefore facilitates acquisition of additional information on the so far ambiguous stimulus. As soon as the stimulus contingencies are known (e.g. stimulus becomes predictable) amygdala activity will diminish (Whalen, 1998). In terms of angry versus fearful faces, Whalen argues that angry face are less ambiguous compared to fearful faces (e.g. an angry faces gives some information about the source of the threat whereas a fearful face does not) and therefore elicit less amygdala activation than fearful facial expressions (Whalen et al., 2001).

More recent work has suggested that not only ambiguity but also novelty and unfamiliarity might

be strong predictors of amygdala activation and a hyper-reactive amygdala in the face of novel or unfamiliar situations has been associated with being a risk factor for social phobia and the development of other anxiety related disorders (Schwartz et al., 2003).

There is ongoing discussion as to whether the amygdala is involved in experiencing negative affective states. While the amygdala has been shown to be activated during presentation of negative stimuli (Lane et al., 1997b), human subjects presented with fearful facial stimuli do not report being afraid, in spite of the fact that elevated amygdala is associated with such a manipulation (Davis & Whalen, 2001). Experiments actively inducing states of negative affect (Fredrikson et al., 1997; Kimbrell et al., 1999) have frequently failed to show pronounced amygdala activation. More support for the notion that the amygdala is not primarily involved in subjective feelings of emotions comes from a study where fearful and happy facial expressions were shown very briefly (33 ms) and were then immediately masked with neutral facial expressions. Although subjects only reported seeing neutral but no smiling or fearful faces, brain imaging data indicated stronger amygdala activation to masked fearful compared to masked happy face in the absence of noticeable changes in subjective emotional arousal (Whalen et al., 1998).

It has therefore been argued that the amygdala might rather represent an affective information processing system (Davis & Whalen, 2001) and that the subjective experience of emotion might be more dependent on other brain regions (e.g. insula region or medial frontal areas; for an overview see (Schulkin et al., 2003).

2.3.3.1.b Amygdala and the HPA Axis

As previously mentioned, the amygdala has a pivotal role in modulating behavioral, autonomic and endocrine aspects of fear and anxiety related states (Roozendaal et al., 1997).

When it comes to HPA axis regulation, the amygdala appears to have a primary excitatory function (Herman et al., 2003), and lesions of the amygdala have shown to decrease stress-induced ACTH and corticosterone secretion (Marcilhac & Siaud, 1996; Van de Kar et al., 1991). Direct anatomical interactions between the amygdala and the PVN however are few. Anterograde and retrograde tracer studies showed limited direct connections between the CEA, the MEA and the PVN (Herman et al., 2002; Prewitt & Herman, 1998). However, the MEA interacts with the peri-PVN region and heavily innervates the anterior hypothalamus, the medial preoptic hypothalamus as well as the posterior intermediate, anterior medial, ventral medial and ventral lateral regions of the BNST (Herman et al., 2002). The majority of medial amygdala neurons are GAD-positive[4] (Swanson & Petrovich, 1998) and there is evidence that the MEA exerts its excitatory effects on the HPA axis via multisynaptic GABA-GABA disinhibitory processes (Herman et al., 2002).

[4] GAD is a GABA synthesizing enzyme (Herman et al., 2002).

The CEA connects with the PVN predominantly trans-synaptically via the dorsomedial hypothalamic nucleus and a small groups of neurons in the fusiform subnucleus of the stria terminalis and similar to the MEA seems to exert its excitatory influence via GABA-GABA disinhibitory mechanisms (Herman et al., 2004).

When it comes to amygdala – PVN connections, the BNST can be regarded as one of the main extrahypothalamic relay stations connecting the two structures. Furthermore, Herman et al. (Herman et al., 1994) showed that selective lesions of the BNST resulted in marked decreases in CRF mRNA in the PVN, and data indicated that predominantly anterior and lateral regions of the BNST, which receive input from the CEA, seem to exert excitatory influence on HPA axis function. Dunn, (J. D. Dunn, 1987) on the other hand, found that stimulation of the lateral aspects of the BNST resulted in decreased plasma corticosterone levels whereas stimulation of the medial aspects of the BNST showed increased levels of corticosterone. Feldman et al. (Feldman et al., 1983) showed in a lesion study that the medial forebrain bundle (MFB) mediates the adrenocortical response to limbic stimulation, indicating that the MFB is crucially involved in the limbic-hypothalamic information flow.

It has also been shown that the CEA has descending projections to the nucleus tractus solitarius (NTS) (Schwaber et al., 1982), which in turn holds up dense connections with the PVN (Ter Horst et al., 1989). This pathway could reflect another circuitry through which the amygdala complex exerts control over HPA axis function. Moreover, the CEA contains numerous CRF positive cells and CRF induction in the CEA during stress has been reported (Hand et al., 2002; Hsu et al., 1998). The CEA projects to noradrenergic dendrites that extend into rostrolateral peri-coerulear LC areas and thereby modulates brain noradrenergic activity. This CEA – LC pathway may serve as a mechanism for the integration of emotional and cognitive responses to stress (Van Bockstaele et al., 2001; Van Bockstaele et al., 1998). In line with this hypothesis, norepinephrine released from various noradrenergic cell groups has shown to have excitatory effects on HPA axis function (Dayas et al., 2001b; Grossman & Costa, 1993) and LC activation seems to be important when it comes to overall arousal and vigilance (Aston-Jones et al., 1999).

Various stressors such as restraint and immobilization stress (Dayas et al., 2001a; Dayas et al., 1999; Ma & Morilak, 2004), cold stress (Baffi & Palkovits, 2000), swim stress (Dayas et al., 2001a), novelty (Emmert & Herman, 1999) and predator stress (Cook, 2002; Day et al., 2004; Figueiredo et al., 2003a) have proven to induce robust amygdala activation. As previously outlined, the amygdala also seems to be crucial in the processing of emotional and social information. In line with this notion, a series of experiments have shown a critical involvement of the amygdala in the face of social stressors (Martinez et al., 2002).

When macaque monkeys that do not know each other are brought together in a confined space, they normally take considerable time to evaluate each other. During this initial period, animals will normally act reserved, maintain distance in proximity and engage in limited social interaction.

As soon as social relationships are established, positive social interactions such as grooming and sexual behavior are initiated. This initial *"testing"* period is thought to be stressful and normally is associated with elevated levels of cortisol. However, mature monkeys with experimentally induced lesions of the amygdala do not show the initial inhibition, nor do they show significant increases in cortisol. (Amaral, 2002). However, it should be mentioned that when lesion are performed at an early age (2 weeks after birth), monkeys show a decreased fear of objects, but an enhanced fear in social interactions. This data evidences that amygdala lesions that took place early in development have different effects on social behavior than lesions that happened during adulthood (Prather et al., 2001)

A study performed on rats, confirmed amygdala involvement in aggressive social interactions. Rats, exposed to an acute defeat through a member of the same species, showed elevated c-fos expressions in the ventral aspect of the septum, lateral, dorsal and medial hypothalamic areas as well as the CEA and the MEA (Martinez et al., 1998). Additionally, when hamsters are confronted with an arranged social interaction situation, fighting between dominant and submissive males results in increased c-fos expression in the MEA in both groups. Dominant hamsters show elevated c-fos expression in the supraoptic nucleus of the hypothalamus whereas submissive animals exhibit increased c-fos expression in several limbic brain structures (e.g. cingulate cortex, lateral septum) as well as the CEO and the BNST. Submissive and defensive behavior has often been associated with increased HPA axis activity (Henry, 1992). In line with this observation, submissive hamsters in this experiment also showed significantly higher corticosterone concentrations when compared to the dominant animals (Kollack-Walker et al., 1997).

In the recent years an ongoing debate has emerged concerning the specific activation and involvement of the CEA and MEA in the face of stress. While numerous studies emphasized the role of the CEA in generating physiological responses in the face of emotional stressors (Kalin et al., 2004; Roozendaal et al., 1991b, 1997), more studies indicate a rather complex picture. In a study performed on rats, hemorrhage and immune challenge elicited c-fos expression throughout the CEA, whereas noise, restraint and forced swim stress primarily resulted in c-fos expression in the MEA (Dayas et al., 2001a). A similar pattern was observed in another study: In rats, a brief restraint stressor was applied and resulted in a stronger c-fos expression in the MEA compared to the CEA. Subsequent lesion experiments showed that ibotenic acid lesions in the MEA but not the CEA greatly reduced stress induced activation of cells located in the medial paraventricular nucleus of the hypothalamus (Dayas et al., 1999).

It has been argued that the discrepancy, when it comes to CEA involvement during states of stress, and the physiological response to stress might be partially explainable by methodological issues (e.g. electrolytical lesions do not only destroy the structure itself but also damage fibers crossing through the structure) (Davis & Whalen, 2001).

Another way to think about this is to assume that different parts of the amygdala are selectively

involved in mediating the neuroendocrine response to categorically distinct types of stressors (Dayas et al., 1999). Herman and colleagues (Herman et al., 2003; Prewitt & Herman, 1997) have proposed a model according to which the CEA is selectively involved in mediating the *"reactive"* stress response (e.g. threats to homeostasis), whereas the MEA seems to be predominantly active during states of *"anticipatory"* stress (e.g. novelty or restraint).
Another approach comes from Roozendaal et al. (Roozendaal et al., 1997) who emphasize the need to distinguish between the role of the CEA in conditioned and unconditioned stress situations, with the CEA being differently involved during these two manipulations: While CEA lesions before acute footshock only resulted in an attenuation of the corticosterone response (Roozendaal et al., 1991a), corticosterone response was completely abolished after CEA lesions in a conditioned stress response (Roozendaal, 1992). Moreover, CEA lesion had dramatic effects on coping behavior basically inhibiting passive coping behavior (e.g. immobilization) and related endocrine (e.g. elevated corticosterone) and autonomic (e.g. bradycardia) responses without affecting active coping strategies (Roozendaal et al., 1991b).
Finally, the amygdala has proven to be a critical structure when it comes to formation of emotional memories (McGaugh & Roozendaal, 2002). Humans with uni- (Adolphs et al., 2000) or bi-lateral amygdala lesions (Adolphs et al., 1997) have impaired memory for emotional material. Moreover, amygdala metabolism during encoding of emotional material is positively associated with long-term memory for the presented stimuli (Cahill et al., 1996). Similarly, amygdala BOLD signal during an event-related fMRI experiment involving an emotional memory paradigm predicted recall in an unexpected recall test 3 weeks after the initial learning (Canli et al., 2000). Data from McGaugh's lab indicate that the memory enhancing effects are mediated by joint action of GCs and ß-adrenergic activation within the BLA (Quirarte et al., 1997), which indicates an important role of stress hormones for emotional memory formation.

In summary – the amygdala complex plays a pivotal role in the processing of fear, threat and anxiety related stimuli. It further seems to be a crucial brain structure in dealing with social situations and social cues (e.g. facial expressions). Some authors have argued for a broader perspective, assigning the amygdala a central role in information processing in the face of novel and ambiguous stimuli. As a final note, the amygdala complex seems to be crucial in orchestrating the autonomic, behavioral and endocrine aspects of fear and anxiety related states and in line with this, the structure seems to impact coping behavior and HPA axis functioning during instances of perceived stress.

2.3.3.2 The Hippocampal Formation (HF)

The HF, with its high densities in GRs and MRs has long been known for its central role in HPA axis regulation and over the years, considerable research has accumulated on this topic (Jacobson & Sapolsky, 1991). The following section starts with some anatomical details.

Functional aspects in terms of HPA axis regulation, will be described thereafter.

2.3.3.2.a HF – Anatomy and Function

The HF is located in the medial aspects of the temporal lobe (Trepel, 1999) and consists of three components[5]: the *dentate gyrus*, the *hippocampus proper* and the *subiculum* (Martin, 2003). The hippocampus is divided into 4 distinct cell fields that are named CA1 – CA4 in direction from the subiculum to the dentate gyrus (Jacobson & Sapolsky, 1991). The three HF components form a cylindrical shaped structure that runs rostrocaudally within the temporal lobe (Martin, 2003). The HF receives its major input from the *entorhinal cortex* that is part of the so-called limbic association cortex. The entorhinal cortex is located on the *parahippocampal gyrus* adjacent to the HF. This structure gathers information from other parts of the *limbic association cortex* (*perirhinal* and *parahippocampal cortex*) as well as from other association areas and passes it on to the HF.

The output neurons of the HF are pyramidal neurons, which axon collaterals collect on the surface of the HF, and from there they form a compact fiber bundle called the *fornix* (Martin, 2003). There are two output systems that can be distinguished within the fornix, the subiculum and the hippocampus. From the subiculum, axons go to the mammillary bodies of the hypothalamus. This projection is part of a prominent anatomical loop: via the mamillothalamic tract, the mammillary body projects to the anterior nuclei of the thalamus, which project the cingulate gyrus. The cingulate gyrus then connects with the entorhinal cortex, which in turn projects to the HF. This loop was first described in 1937 by the anatomist Papez, who postulated this anatomical loop within an emotional information processing theory (Martin, 2003). From the hippocampus, axons synapse in the rostrally located septal nuclei. The lateral septum is connected with a series of limbic structures (e.g. amygdala, hypothalamus) and the mesocorticolimbic dopamine system and seems to have important functions in the regulation of mood and motivation (for an overview see (Sheehan et al., 2004)).

Moreover – the subiculum and the hippocampus project back to the entorhinal cortex, which has pronounced connections with the prefrontal and orbitofrontal areas, the cingulate cortex, the insular cortex and the parahippocampal gyrus (Carmichael & Price, 1995; Lavenex et al., 2004; Martin, 2003).

Its location as well its wide spread connections with different parts of the brain make the HF an ideal candidate for resembling information from different modalities, which is in line with the notion that the HF is most prominently involved in various forms of memory and learning, contextual fear conditioning (Phillips & LeDoux, 1992) and novelty detection (Ranganath & Rainer, 2003). Some of these aspects have already been described in previous sections and as

[5] The nomenclature of the HF is variable, and which components are considered to be part of this structure may differ, depending on the source (Martin, 2003).

complex details on the role of the HF in memory and learning would be beyond the scope of this work, some of these aspects will not be described in further detail.

Apart from the HF's prominent involvement in cognitive processing, there is growing evidence indicating an important role of this structure in affective disorders.

Several studies have shown reduced HF volume in patients with major depression (Bremner et al., 2000; P. J. Shah et al., 1998), whereas others could not find such differences (Posener et al., 2003; von Gunten et al., 2000). The HF is an important site for neurogenesis, and overexposure to GC has been suggested to be a limiting factor in hippocampal cell proliferation and neurogenesis. Long-term treatment with anti-depressants on the other hand seems to promote these processes (Campbell & MacQueen, 2004; Malberg, 2004). Inspired by these observations, it has been assumed that chronic overexposure to GCs as present in various affective disorders might modulate HF volume changes (Malberg, 2004).

Questions have been raised whether HF volume changes are necessarily a result or rather predisposing factor of affective disorders. In a study of Gilbertson et al. (Gilbertson et al., 2002) on monozygotic twins discordant in trauma exposure, it was shown that in twin pairs of which one of the twins was subject to severe PTSD, both twins had significantly smaller hippocampi compared to non-PTSD twin pairs. For the first time, these data indicate that smaller HF volume might indicate a pre-existing vulnerability factor for developing a stress-related affective disorder.

2.3.3.2.b HF and HPA Axis

Since the early beginnings of stress research, the role of the HF in HPA axis regulation has been a target of interest and recent anatomical tracing studies shed light on the functional connectivity between the HF and the PVN. Due to a lack of direct innervations, the HF exerts control over HPA axis functioning via intervening neurons (Herman et al., 2003). The main hypothalamic outflow exits the HF in the ventral subiculum and the ventral CA1 field (Cullinan et al., 1993; Herman et al., 2003) and involves the fimbria-fornix pathway as well as the stria terminalis via the amygdala (Cullinan et al., 1993; Feldman et al., 1987b; Herman et al., 1992). Rather than projecting straight to CRF neurons within the PVN, the HF sends its projections to other relay stations such as the BNST (anterior medial, posterior intermediate and ventrolateral) (Cullinan et al., 1993; Herman et al., 2002), the peri-PVN region (medial preoptic area) and the lateral septum (Herman et al., 2003; Sheehan et al., 2004), which in turn project to PVN regions (Feldman et al., 1987b; Herman et al., 2003).

Early lesion studies indicated an inhibitory role of the HF when it comes to basal HPA axis functioning (Fendler et al., 1961; Knigge, 1961). However, over the years, conflicting data has emerged and given rise to more complex perspective in considering the role of the HF in HPA axis regulation.

Several experiments give evidence that the HF influences basal and circadian functioning of the HPA axis. Lesions of the hippocampus or the fornix typically reduce circadian variations in

plasma corticosterone release (Jacobson & Sapolsky, 1991). In line with this observation, Sapolsky et al. (Sapolsky et al., 1991) showed a pronounced GC hyper-secretion throughout the day in primates with lesions of the HF. Interestingly, this pattern of hyper-secretion was transient and secretory activity returned to normal within 6 - 15 months. Another study indicated a negative correlation between basal cortisol levels and the volume and number of neurons in the left but not the right dentate gyrus in pigs (van der Beek et al., 2004). In a study on humans, increased basal 24 hours urinary cortisol values were inversely associated with hippocampal volumes (Wolf et al., 2002), whereas others did not find such an association (Maclullich et al., 2005).

Contradictory findings also come from Buchanan and colleagues who recently examined a group of patients with uni-lateral and bi-lateral lesions of the HF and found that these patients do not show the typical morning cortisol increase, while other circadian patterns were unaffected. Moreover, altered basal cortisol release persisted into a chronic epoch (> 1 year) (Buchanan et al., 2004). Similar findings were reported from Wolf and colleagues who showed that brain damaged patients[6] with severe amnesia also did not show the expected morning cortisol rise in the presence of otherwise unaffected circadian patterns (Wolf et al., 2005). These data give evidence that the HF seems to selectively influence the awakening cortisol response leaving other circadian characteristics intact. However, the exact nature of this effect remains speculative: The HF has been associated with corticosterone response to novel situations as lesions of the dentate gyrus resulted in significantly lower GC concentrations due to novelty stress (L. L. Johnson & Moberg, 1980). The physiological and psychological determinants of the cortisol awakening response are still not very well understood but some authors have argued that it could reflect a general novelty stress response (Buchanan et al., 2004) – a hypothesis that is speculative but would help to explain the present findings of Buchanan and colleagues as well as Wolf and colleagues.

The role of the HF in HPA regulation during states of stress has been a field of contradictory findings as well. Whereas some studies indicated no impact of the HF on HPA axis regulation (Tuvnes et al., 2003) others found evidence for an inhibitory (Herman et al., 1995; Herman et al., 1998) or excitatory (Feldman et al., 1987b) influence.

The reasons for these conflicting findings remain rather unclear, but it can be speculated that the different aspects of the HF serve different functions in HPA axis regulation (Feldman et al., 1987b) and that the opposing findings are due to differences in the exact location and extend of lesions (Tuvnes et al., 2003). Another compelling explanation comes for Mueller et al. (Mueller et al., 2004) who showed, that the ventral subiculum shows stressor specific effects (e.g. inhibitory versus excitatory) on HPA axis regulation. Keeping in mind that different laboratories not always

[6] Affected brain regions involved basal frontal and medial temporal lobes, extending to basal ganglia, thalamus or brainstem and were not selective to the HF.

used the same stress modalities, a stressor-specific role of the HF could also explain opposing results.

There is accumulating evidence that not only the HF but also the entorhinal cortex, located adjacent to the HF, plays an important role in HPA axis regulation. Umegaki et al. (Umegaki et al., 2003) showed pronounced increase in fos immunoreactivity in the entorhinal cortex after immobilization stress but not due to insulin-induced hypoglycemia. Lesions of this region resulted in an attenuated ACTH response to stress. These data indicate a stressor-specific excitatory role of the entorhinal cortex in HPA regulation.

Although there has been doubt as to whether hippocampal inhibition of HPA axis response to anticipatory stress involves GR activation and signaling (Herman et al., 2003), there has been considerable evidence that at least some of these effects are mediated by GRs: Sapolsky et al. (Sapolsky et al., 1984) found that hippocampal depletion of GRs without inducing cell loss resulted in corticosterone hyper-secretion and that reversal of the receptor deficit leads to a normalization in GC secretion. Stress-induced activation of the septo-hippocampal cholinergic system has been observed (Gilad et al., 1985), and it has been shown that HF mediated HPA axis function was also dependent on these septo-hippocampal cholinergic projections in so far as that removal of cholinergic innervation of the hippocampus resulted in significantly decreased hippocampal GR mRNA and a prolonged GC response to restraint stress (Han et al., 2002).

In summary, the HF is a crucial brain structure when it comes to various aspects of memory and learning and its high densities of MR and GR receptors make it an important brain region for HPA axis regulation. However, whether the HF plays a primary excitatory or inhibitory role in HPA axis functioning under stress and basal conditions remains unanswered at this point. Most recent data indicate a highly differentiated picture with different aspects of the HF supporting distinct functions in HPA axis regulation (e.g. excitatory versus inhibitory). Moreover, it seems very likely that the HF, similar to the amygdala complex, shows a stressor-specific involvement when it comes to HPA axis regulation.

2.3.3.3 The Brainstem and HPA axis

The midbrain and brainstem are home to two systems of diffuse-projecting cell groups that influence arousal, motivation, behavior, cognition and emotion in the most broad sense: a) the serotonergic system in the brainstem (raphé nuclei) and b) the mid brain and brainstem catecholaminergic cell groups (Martin, 2003). As these neurotransmitter systems are highly involved in the modulation of cognitive, emotional, motivational, behavioral, autonomic and endocrine aspects of the stress response (Carrasco & Van de Kar, 2003; Moghaddam, 2002), the individual impact of each system on HPA axis functioning will be briefly highlighted in the following sections.

2.3.3.3.a HPA Axis and the Serotonergic System

The serotonergic system and its neurotransmitter serotonin (5-HT) have a prominent role in modulating behavioral, autonomic and endocrine responses to stressful stimuli (Carrasco & Van de Kar, 2003; Lowry, 2002). Activation of the serotonergic system has been associated with behavioral arousal and motor activity (Lowry, 2002), uncontrollable stress (Grahn et al., 1999; Maswood et al., 1998), fear sensitization (Maier & Watkins, 1998) and learned helplessness (Dwivedi et al., 2005). Dysfunction of the serotonergic system seems to play an important role in affective disorders like depression and anxiety (Carrasco & Van de Kar, 2003). Moreover, the serotonergic system is well known for its influence on HPA axis activity (Carrasco & Van de Kar, 2003; Herman et al., 2003) and there is evidence that depending of the nature of the stressor, the system can either have excitatory or inhibitory effects on basal as well as stress-induced GC secretion (Lowry, 2002).

The serotonergic system is located within the brainstem. The majority of serotonergic neurons projecting to forebrain structures are located within the dorsal and median raphé nuclei (Carrasco & Van de Kar, 2003; Lowry, 2002). The raphé complex utilizes six different tracts to project to various sites throughout almost the entire brain, with the most prominent sites being the hippocampus, the amygdala, the prefrontal cortex (PFC), the cingulate cortex, the thalamus, the hypothalamus, the caudate putamen, the basal ganglia and the septum (Lowry, 2002). When it comes to these projections, the serotonergic system seems to be topographically organized, with different cell groups exerting distinct functional effects on the behavioral, autonomic and endocrine stress response (Abrams et al., 2004; Lowry, 2002).

Tracing studies support the notion that the middle and caudal portions of the dorsal raphé nucleus give rise to various limbic and autonomic areas like the CEA, the BLA, the LA, septo-hippocampal complex, the periventricular and periaqueductal gray matter and the parabrachial nucleus, which are thought to facilitate neuroendocrine, autonomic and behavioral responses to stress (Lowry, 2002).

In line with this topographical distinction it has been shown that swim stress induces robust c-fos expression in the dorsal raphé nucleus (Roche et al., 2003). Uncontrollable stress has also been associated with increases in extracellular 5-HT concentrations (Maswood et al., 1998) and activation of serotonin immunereactive cells (Grahn et al., 1999) in the dorsal raphé nucleus.

Moreover, uncontrollable stress or inescapable shocks have been shown to potentially induce a state of learned helplessness (Maier & Seligman, 1976) that is characterized by behavioral depression (e.g. lack of escape learning). Studies on uncontrollable stress indicate that inescapable but not escapable foot shocks result in selective activation of the caudal dorsal raphé nucleus (Grahn et al., 1999). It was also recently shown that CRF induces firing of serotonergic neurons in the caudal dorsal raphé nucleus (Lowry et al., 2000) and microinjections of CRF antagonists into the caudal aspects of the dorsal raphé nucleus have proven to block

behavioral changes to inescapable shocks via CRFR2 receptors (Hammack et al., 2002; Hammack et al., 2003). Accordingly, active coping (swimming) was negatively correlated with extracellular 5-HT in the lateral septum (Kirby & Lucki, 1997), which indicates a role of serotonergic projections on active versus passive coping and learned helplessness.

To this point, seven families of serotonin receptors (5-HT_{1-7}) have been identified and there is evidence that the receptor subtypes 5-HT_{2A} and probably also 5-HT_{1A}, which are found in the hypothalamus (Jorgensen et al., 1998) are primarily involved in HPA axis regulation by activating CRF cells in the PVN and stimulating the release of ACTH and corticosterone (Calogero et al., 1989; Mikkelsen et al., 2004; Van de Kar et al., 2001). Furthermore it was shown that hypothalamic depletion of 5-HT blocks GC rise after stimulation of frontal and extrahypothalamic limbic structures (Feldman et al., 1987a) and selective 5-HT_{2A} agonists stimulate CRF in an hypothalamic organ culture in a dose-dependent fashion, whereas 5-HT_{1A} and 5-HT_{2A} antagonists inhibit CRF secretion (Calogero et al., 1989). More support for a stimulating effect of 5-HT on HPA axis function comes from Bagdy et al. (Bagdy et al., 1989) who observed that 5-HT agonists injected in freely moving Sprague-Dawley rats lead to a pronounced and dose-dependent increase of ACTH. Epinephrine was also enhanced in a dose dependent fashion which indicates that 5-HT might also have stimulatory effects on the sympathoadrenomedullary system.

The work of Jorgensen and colleagues (Jorgensen et al., 1998) indicates a stressor specific involvement of the serotonergic system in HPA axis regulation. According to their findings restraint, but not ether, swim or endotoxin stress induced significant increases in raphé 5-HT concentration. All stressors applied induced marked increase in ACTH. For the ether and swim stress as well as the endotoxin exposure this effect was inhibited by various 5-HT antagonists whereas swim stress induced ACTH release was not affected by any of the antagonists used.

As direct serotonergic projections to the PVN are limited to a small number of fibers (Herman et al., 2003; Sawchenko et al., 1983) it has been argued that the serotonergic system influences HPA axis activity not only directly but also indirectly via other limbic structures. This assumption is supported by work of Feldman's group (Feldman et al., 1998), showing that infusion of a selective 5-HT_{2A} antagonist into the amygdala has an inhibitory effect on serum ACTH and corticosterone levels after stressful photic stimulation.

Taken together, these studies give evidence that anxiogenic or fear inducing stimuli increase serotonergic transmission in the dorsal raphé nucleus (in particular the caudal aspects) which then gives rise to a mesolimbocortical serotonergic innervations to forebrain structures that facilitate the various behavioral and endocrine responses to these stress associated stimuli. Additionally, an interaction between the serotonergic system and CRF seems to be crucially involved in mediating the behavioral aspects of the stress response. (Abrams et al., 2004; Hammack et al., 2002)

As initially indicated, there is evidence that the effects of the serotonergic system on HPA axis

activity are not exclusively excitatory in nature (Lowry, 2002). In his review on the serotonergic system and the HPA axis, Lowry (Lowry, 2002) describes a neural pathway that originates in the medial raphé nucleus and projects directly to the hypothalamus and the ventral subiculum which is known for its inhibitory function on HPA axis activity (Herman et al., 1995). According to Lowry it is very likely that the ventral subiculum exerts direct and indirect inhibitory effects on HPA axis activity and that the indirect influence is mediated via other brain structures that are thought to have an inhibitory influence on HPA axis activity (e.g. the PFC). He concludes that the projections arising from the medial raphé nucleus play an important role in resistance, tolerance, adaptation or adequate coping behavior during states of acute or chronic stress. Similarly, Graeff and others (Graeff et al., 1996) have postulated that a pathway connecting the median raphé nucleus and the dorsal hippocampus promotes resistance to chronic avoidable stress.

Support for this hypothesis comes from experiments on rats prone to hyper- or hypo-anxiety: In previous studies it was revealed that high-anxious rats preferably exhibit passive coping strategies (Liebsch et al., 1998) and show a strong HPA axis response in the face of mild emotional stress when compared to hypo-anxious rats (Landgraf et al., 1999). Exposure to emotional stress has been shown to increase 5-HT concentrations in the dorsal hippocampus (Umriukhin et al., 2002) and increase 5-HT_{1A} receptor mRNA in hypo-anxious but not in hyper-anxious rats. Moreover, 5-HT was increased in high-anxious rats after chronic paroxetine[7] administration (Keck et al., 2005). Similar results are presented by Veenema and colleagues who studied two mouse lines that differed in their attack latencies in response to environmental stimuli. Mice with a long attack latency (LAL) display low aggressive behavior and passive coping styles whereas short attack latency (SAL) mice are normally aggressive and display more active coping behavior. Exposure to an acute swim stressor resulted in a hyper-reactive HPA axis response and a reduced increase in 5-HT metabolism paralleled by a 50 % reduction in hippocampal cell proliferation in LAL mice compared with SAL animals (Veenema et al., 2004).

Taken together, there is rising evidence for the involvement of the medial raphé nucleus in stress resistance and active coping behaviors that goes along with elevated 5-HT concentrations in hippocampal regions. However – the exact projections (e.g. ventral vs. dorsal hippocampus) and mechanisms (e.g. how does it affect HPA axis activity) require further investigation.

In summary, the raphé system plays a complex role in the regulation of the HPA axis activity and stress related coping behavior. Whereas as a pathway originating in the dorsal raphé nucleus seems to have a primary excitatory effects on HPA axis activity, the medial raphé nucleus with its projections to hippocampal areas seems to be involved in inhibitory actions on HPA axis activity and active coping behavior. Much controversy remains on the exact effects of the

[7] Paroxetine is a clinical anti-depressant which acts as a selective serotonin re-uptake inhibitor (SSRI).

serotonergic system on stress related responses and its role in affective disorders but precise understanding of these dualistic properties might help promote further understanding.

2.3.3.3.b HPA Axis and the Catecholaminergic System

The noradrenergic system located in the midbrain and brainstem is part of a major stress sensitive neurocircuitry and plays an important role in HPA axis regulation and affective disorders (Calogero, 1995; A. J. Dunn et al., 2004; Koob, 1999; Lehnert et al., 1998). The noradrenergic system seems to be important when it comes to overall arousal and vigilance (Aston-Jones et al., 1999) as well as attentional modulation and regulation of goal-directed versus exploratory behaviors (Usher et al., 1999).

Axonal transport and immunohistochemical methods indicate that noradrenergic inputs to the PVN originate from the A1 cell group in the caudal ventrolateral medulla, the A2 region in the dosal vagal complex, and the A6 cell group (locus coeruleus) (Sawchenko & Swanson, 1982). Projections from the A1 cell group were detected primarily in the magnocellular division of the PVN, whereas the A2 region preferably projects to parvocellular aspects of the PVN. Projections to this part of the PVN were most dense in the dorsal medial part which is high CRF immunoreactive neuron density. Fibers rising from the LC were most pronounced in the periventricular zone of the PVN (Cunningham & Sawchenko, 1988). Projections from the ventro-lateral medulla, the NTS and the LC were also found in the CEA (Petrov et al., 1993). The medullary catecholamingeric cell groups also seem to project to the BNST, a major extrahypothalamic relay station connecting the amygdala and the HF with the hypothalamus (Ter Horst et al., 1989; Terenzi & Ingram, 1995).

The catecholaminergic system's role in stress related responses and HPA axis regulation is supported by the fact that various forms of stress (e.g. noise, restraint) have been shown to activate noradrenergic neurons (E. D. Abercrombie & Jacobs, 1987; Dayas et al., 2001b; Pardon et al., 2002) and that lesions of the LC have proven to reduce ACTH and corticosterone peak response to restraint stress (Ziegler et al., 1999).

As previously outlined, some authors suggested that direct brainstem pathways influence HPA axis response in the face of physical or real stressors (e.g. threat to homeostasis) but not in the face of emotional or anticipated stressors (Herman & Cullinan, 1997). Support for this notion comes from a study which has shown that either lesions of the NTS and the ventrolateral medulla or destruction of the catecholaminergic terminals within the mPVN resulted in inhibited activation of CRH neurons by systemic interleukin-1ß (a well known activator of the HPA axis) (Buller et al., 2001). This experiment confirms the hypothesis that a physical stressor such as interleukin-1ß activates the HPA axis via direct brainstem pathways. In an experiment, Dayas et al. (Dayas et al., 2001b) re-evaluated this concept in light of emotional stressors and found that restraint stress caused activation in A1 and A2 cell groups as well as the MEA and to a lesser extend the CEA. At the same time, destruction of catecholaminergic terminals in the PVN did not

influence CRF response in the PVN to restraint stress. However, lesion of the A1 and A2 cell groups resulted in a significantly decreased CRF response to restraint stress and also suppressed neuronal responses in the MEA. The authors therefore conclude that the medullary neurons influence HPA axis activity in the face of emotional stress through multisynaptic pathways most likely via noradrenergic input to the MEA. In turn, there is also evidence that the MEA activates medullary catecholaminergic neurons via the PVN during states of physiological stress (Dayas et al., 2001b) and MEA lesions completely abolish fos expression in the noradrenergic cells of the ventrolateral medulla and the NTS due to restraint (Dayas & Day, 2002). These findings give rise to the notion, that the amygdala acts as a threat detection system (see Amaral, 2002) and by activating the noradrenergic system puts the organism in a state of elevated arousal.
A series of experiments give evidence that the medullary catecholaminergic system also modulates behavioral aspects of the stress response and that most of these effects occur in concert with CRF (Koob, 1999). Stress increases CRF in the LC (Chappell et al., 1986) and direct injections of CRF into the LC have shown to increase the discharge rate of LC neurons (Curtis et al., 1997). The possible interaction between CRF and noradrenergic neurons in terms of the behavioral stress response was shown as infusions of a CRF antagonist in the LC region resulted in significant reduced freezing behavior after foot shock administration (Swiergiel, 2003).

In summary, the noradrenergic systems seems to influence the overall arousal state of an organism and is capable of modulating the HPA axis in an excitatory fashion. The exact mechanisms through with the noradrenergic system influence the HPA axis are not yet completely understood but there is evidence that the mechanisms vary depending on the exact nature of the stressor (e.g. threat to homeostasis versus psychological stress) and selectively involve other aspects of the limbic system.

Another member of the catecholamine group is dopamine. While this neurotransmitter is most prominently associated with reward learning and motivation (Wise, 2004), it also has a prominent role in the central stress response (R. M. Sullivan, 2004).
The majority of dopamine-containing cells are located in the substantia nigra and the ventral tegmental area from where they project to various regions throughout the brain (Martin, 2003). Based on different targets of these projections, three dopaminergic systems are differentiated: The *nigrostriatal system*, which originates in the zona compacta of the substantia nigra and projects primarily to the caudate-putamen. The *mesolimbic system*, which originates in the ventral tegmental area (VTA) and projects to the nucleus accumbens, the olfactory tubercle, the septum, the amygdala and the hippocampus. The *mesocortical system* originates in the medial VTA and sends its projections to the medial PFC (mPFC), the cingulate gyrus and the perirhinal

cortex (Wise, 2004). Several studies indicate that the mesocortical dopamine systems is readily activated by a series of stressors (Morrow et al., 2000; Redmond et al., 2002).

Although the activation of the dopaminergic system during stress is a well established finding, the exact purpose of this activation remains poorly understood. The main target for dopaminergic projections to the PFC are the perilimbic and infralimbic areas. As these areas have been associated with a stimulating function on endocrine output during stress (R. M. Sullivan & Gratton, 1999), it could be speculated that the dopamine response during stress is excitatory in nature. This notion would be in line with findings that show a positive correlation between stress related dopamine increases in the PFC and corticosterone response to stress (R. M. Sullivan & Gratton, 1998). However, dopamine depletion, especially in the right mPFC has been associated with increased anxiety during anxiety provoking situations (Espejo & Minano, 1999) and an elevated stress-induced ulcer incidence (R. M. Sullivan & Szechtman, 1995). In line with these findings, elevated dopamine metabolite (DOPAC) concentrations in the right but not in the mPFC were associated with anxiolytic behavioral effects in an anxiety provoking test (Thiel & Schwarting, 2001). Moreover, dopamine depletion in the ventromedial PFC has associated with delayed extinctions in fear conditioning paradigms (Morrow et al., 1999). Several studies indicate that normal dopamine turnover in the PFC seems to be important for appropriate coping behavior and adaptation to environmental stressors. In a learned helplessness paradigm, Carlson et al. (Carlson et al., 1993) showed that a well functioning dopamine turnover in the right mPFC was positively associated with successful escape behavior after uncontrollable footshock treatment. However, others have shown that right sided lesions of the ventromedial PFC result in anxiolytic behavioral effects in the elevated plus maze procedure (R. M. Sullivan & Gratton, 2002a). Similar findings come from Shah and colleagues (A. A. Shah & Treit, 2003) indicating strong anxiolytic effects in three different behavioral tests after bi-lateral lesions of the mPFC.

More puzzling results arise from studies showing that, while 15 minutes after restraint stress dopamine activity was most pronounced in the left mPFC, 45 minutes later this effect was shifted to the right PFC (Carlson et al., 1991). In an attempt to account for the diversity of these results, Sullivan (Sullivan, 2004) proposes the following model: Initial left-sided PFC activation in the face of mild stressors might reflect initial coping attempts by the more analytical, less emotional and motor dominant left hemisphere and activation of the left PFC during mild stressors might help to prevent small stressors becoming drastic stressors. If the individual however perceives that either coping has failed or is not accessible (as for prolonged or uncontrollable stress), activity in the right PFC would be the most likely outcome and would reflect a control mechanism to restrain excessive right PFC activity.

Only little is known about dopamine and the stress response in humans. In a recent PET study, Prüssner et al. (Pruessner et al., 2004) looked at a small group of healthy people who highly differed in their experience of early life parental care. This manipulation was of interest as

studies on animals and humans indicate that disturbances in mother-infant relationship can cause alterations in the dopamine system as well as HPA axis functioning. [^{11}C]raclopride, a dopamine receptor tracer was used to measure dopamine binding in the striatum during confrontation with a stressful task. Data indicated that participants with low parental care scores showed a significant dopamine release ventral striatum, indicated by a reduction in [^{11}C]raclopride binding in this region. Moreover, salivary cortisol response to the stressor was significantly correlated with the reduction in [^{11}C]raclopride in the same region ($r = 0.78$). This study, probably for the first time, showed an association between confrontation with a stress eliciting task and mesolimbic dopamine release in humans.

While there is no doubt that the cortical dopamine system is activated during confrontation with various stressors, little is known about the exact stress-related function of dopamine in the PFC. While there is accumulating evidence that dopaminergic activity in the PFC promotes coping behavior and dampens HPA axis response to stress, controversy remains when it comes to the exact location, the timing and the stressor specificity of these actions.

2.3.3.4 The Prefrontal Cortex (PFC)

The PFC is a highly complex brain area supposedly involved in a series of functions such as cognitive control and goal directed behavior (Miller, 1999; Miller & Cohen, 2001), stimulus-reward association (Price, 1999), encoding of abstract rules (Wallis et al., 2001), working memory (N. G. Muller et al., 2002), executive control of memory retrieval (Tomita et al., 1999), novelty detection (Dias & Honey, 2002), attention to threat related stimuli (Bishop et al., 2004), decision making (Bechara et al., 2000a; Bechara et al., 2000b), emotion regulation (Ochsner et al., 2002), social behavior and reasoning (Adolphs, 2003), self referential activity (Gusnard et al., 2001) and memory for the emotional self (Macrae et al., 2004). Additionally, it seems to be a site for integration of emotion and cognition (J. R. Gray et al., 2002) and functional abnormalities in prefrontal areas have been frequently associated with disturbances in mood as well as affective disorders (Davidson & Irwin, 1999; Davidson et al., 2002). Most important for this work, recent data also indicate an important role of the PFC in the regulation of the HPA axis function (R. M. Sullivan & Gratton, 2002b).

This list by far does not account for all the various cognitive and emotional domains sub-served by the PFC and as a detailed description of all the PFC related functions would be well beyond the scope of this work, the following section will only focus on the functional aspects of the PFC that seem to be relevant in the light of the PFC's role in HPA axis regulation. But prior, the section starts with some anatomical details reflecting the basis for understanding the various PFC functions that are described here. In the final part of this section, anatomical and functional data presented so far will be integrated and current knowledge on the PFC's role in HPA axis regulation will be highlighted.

2.3.3.4.a PFC – Anatomy and Function

From an anatomical perspective, the PFC in primates stretches from the frontal pole to the premotor cortex (Dombrowski et al., 2001) and according to Brodmann comprises the part of the frontal lobe located anterior to Brodmann's area (BA) 6 (Brodman, 1909). The PFC can be roughly divided into 3 broad areas: a) the ventromedial region, comprising the straight gyrus, orbitofrontal gyri and the medial frontal cortex (BA 10, 11, 12), b) the dorsolateral PFC involving the middle frontal gyrus and part of the superior frontal gyrus (BA 9, 46, 9/46), and c) the ventrolateral aspect including the inferior frontal gyrus (BA 44, 45, 47) (N. G. Muller et al., 2002).

In line with architectonic studies, lesion data and more recent neuroimaging results show convincing evidence for a functional segregation within the PFC. A recent meta-analysis indicates a distinction between the inferior medial areas and dorsolateral regions, with the inferior medial areas being involved in emotion related tasks and the dorsolateral aspect being primarily activated in cognitive based functions (Steele & Lawrie, 2004). According to Price (Price, 1999), at least twenty-two architectonic areas can be distinguished within the orbital and medial PFC (mPFC). These twenty-two regions differ when it comes to their structure as well as their connections with other brain areas. They hold up distinct patterns of cortico-cortical connections. While the orbital network shares inner-PFC connections primarily with regions on the orbital surface, the medial network is characterized by inner-PFC connections primarily restricted to the medial surface (Price, 1999).

Both the medial and orbital networks are connected with a variety of brain regions involved in emotion and stress related functions like the amygdala, HF, the entorhinal cortex, the perirhinal cortex, the temporal polar cortex, the hypothalamus (Carmichael & Price, 1995; Floyd et al., 2001; Kondo et al., 2003), the PAG (An et al., 1998), the dorsal raphé nucleus (Jankowski & Sesack, 2004) and the NTS (Terreberry & Neafsey, 1987).

The orbital network comprises most of the regions in the orbital cortex and receives input from various sensory domains (olfactory, gustatory, visceral afferents, somatic sensory and visual) (Price, 1999). Moreover, the orbital network is the target of numerous projections from the amygdala, the entorhinal and perirhinal cortex and the subiculum. Based on these connections it has been speculated that the orbital network might be involved in the integration of viscosensory information with affective signals (Price, 1999). Areas on the medial frontal surface along with a few areas on the orbital cortex comprise the medial network. Compared with the orbital network, the medial network receives only spares sensory input but also substantial projections from limbic areas. The medial network sends projections to the hypothalamus, the brainstem, the amygdala and the PAG and is therefore thought to influence the autonomic, endocrine and behavioral functions (Price, 1999).

As indicated in the beginning of this section, the PFC comprises a wide variety of functions and over the course of the last few decades a series of models and hypothesis on PFC function have

emerged:

In a recent model on the role of the PFC from Miller and Cohen (Miller & Cohen, 2001), it is stated that the PFC holds the representation of goals and the knowledge of how to achieve these goals. The role of the PFC is furthermore to guide behavior in situations that are ambiguous or during instances when task-appropriate responses need to be established in the face of competing alternatives. A prominent example used to illustrate this model is the idea of immediate versus delayed reward: In such an instance, the PFC would override the tendency to get an immediate reward (e.g. go to the football game) for the achievement of a goal that would be rewarding in the long run (e.g. do the homework in order to pass the class).

Davidson, in his model, strikes the importance of the PFC in approach- and withdrawal-related emotion and behavior (Davidson et al., 2003). According to this model, left-sided PFC regions play an important role in approach-related, appetitive goals. Right sided regions of the PFC on the other hand come into play when it comes to maintaining goals that require behavioral inhibition and withdrawal in situations that involve strong alternative response options to approach. Support for this model comes from a series of studies on depressed patients: Depression (Drevets, 1999) grief (Najib et al., 2004) and sadness (Mayberg et al., 1999) have frequently been associated with decreased metabolic activity in various prefrontal regions. In a study on treatment effects in major depression, 13 male patients with major depression underwent a PET scan before and after successful treatment with paroxetine. Data indicated that successful treatment was associated with enhanced glucose metabolism in dorsolateral, ventromedial and medial aspects of the PFC and changes were greater on the left side compared to the right (Kennedy et al., 2001). However, others reported reversed patterns: Holthoff and colleagues (Holthoff et al., 2004) for example compared glucose metabolism in patients with major depression during an acute and a remittent state. Comparison of the two PET scans (acute versus remittent) indicated a marked decrease in glucose metabolism in the left PFC.

However, in a study on the neuroanatomy of anxiety, Rauch and his group (Rauch et al., 1997) applied a symptom provocation paradigm in a group of participants with diagnosis for obsessive-compulsive disorder, simple phobia or posttraumatic anxiety disorder. Using positron emission tomography, Rauch observed pronounced activation in the right inferior frontal cortex and the right posterior medial orbitofrontal cortex in the anxiety patients compared with healthy controls. More support for Davidson's model comes from studies on monkeys showing that animals with pronounced right-sided prefrontal asymmetric brain electrical activity express high levels of trait-like fearful behavior and increased concentrations in plasma corticosterone and elevated cerebrospinal fluid CRF concentrations (Kalin et al., 1998; Kalin et al., 2000).

A different picture emerged in a study presented by Northoff et al. (Northoff et al., 2000) who tried to pin-point the exact spatiotemporal mechanisms of negative and positive emotional processing in medial and lateral orbitofrontal regions. According to their findings, strong

activations due to negative emotional processing was found in medial orbitofrontal activation whereas activation associated with positive emotional processing was located more lateral in the PFC.

More evidence for the PFC's general involvement in the processing of emotional information comes from a series of studies using various emotion induction paradigms which often show increased activity in areas throughout the mPFC (Lane et al., 1997b; Liberzon et al., 1999). In a study of Phan et al. (Phan et al., 2003) individual ratings of emotional paradigms presented during a fMRI scan were used as individual regressors and the corresponding correlation map showed robust activation in the mPFC (BA 9 / 10) and the amygdala region.

Latest electrophysiological and neuroimaging data indicate a prominent role of the PFC in the cognitive regulation of emotion (Jackson et al., 2000; Levesque et al., 2004; Ochsner et al., 2002). The term reappraisal refers to the cognitive transformation of emotional experience and the ability to cognitively regulate emotional responses to negative and adverse events seems to be important in the light of holding up mental and physical health (Ochsner et al., 2002). In the experiment designed by Ochsner and colleagues, participants were shown either neutral or negative pictures from the *International Affective Picture System* (Lang et al., 1993) and subjects were instructed to either attend or reappraise (e.g. interpret the pictures in a way that they no longer provoke a negative response) the emotions evoked by the various pictures. Compared with the *"attend"* condition, reappraisal was associated with lower negative affect as well as with increased activation in lateral and medial prefrontal regions and decreased activation in the amygdala and medial orbito-frontal cortex. Based on their findings, the authors conclude that the PFC is involved in mediating reappraisal strategies and possibly influences activity in multiple emotion-processing systems.

A historical tragic accident that took place more than 150 years ago gave first evidence that the PFC is involved in social and goal directed behavior. In summer 1848, the railway worker Phineas Gage was dealing with explosive material when in an unattended moment, a massive explosion went off. The explosion was so strong, that the solid iron that Phineas was holding in his hand was driven through his cheek and his frontal brain and skull. Surprisingly, Phineas remained fully conscious during and after the accident and managed to survive the terrible event. Before the incident he was described as a pleasant, successful and highly responsible person. However, after the accident he seemed changed and showed a highly socially unacceptable behavior that ranged from compulsive swearing, to heavy drinking, to frequent personal insults (A. Damasio, 1995). Inspired by this historical case, the group of Hanna and Antonio Damasio reconstructed Phineas' brain lesions (H. Damasio et al., 1994) and investigated the effects in patients with similar brain insults. They found that damage to the ventromedial PFC results in marked disruptions of social behavior in a way that previously well-adapted people become severely handicapped in attending to social conventions and making

appropriate decisions about their own life's (Bechara et al., 2000a). It was also shown that these patients have difficulties anticipating future beneficial or unbeneficial consequences that might arise based on their decisions (Bechara et al., 1994) and patients failed to have anticipatory electrodermal responses in the face of a risky choice (Bechara et al., 1996). Most strikingly, patients with ventromedial brain damage preserve their intellectual abilities such as learning, memory, language and attention. Remarkably they even perform within the normal range on tasks requiring executive function (e.g. Wisconsin Card Sorting Test). On the other hand, these patients show marked disturbances when they have to engage in emotions in the context of complex situations and circumstances (e.g. feelings of embarrassment) (Bechara et al., 2000a; Bechara et al., 2000b).

In line with these observations is Damasio's *"somatic marker hypothesis"*. According to this model, impairments in emotions and feelings play an important role in decision making (Damasio, 1995). Somatic markers describe physiological changes (e.g. increase in heart rate) that have previously been associated with significant events and provide a signal to guide behavior in corresponding situations. Somatic markers comprise a kind of a guideline in situations where decisions cannot be made on pure logical considerations. According to Damasio, the integration of knowledge about previous learning experiences and the existence of somatic markers take place in the ventromedial PFC and damage to this regions leads to severe impairment in daily decision making (Damasio, 1995). In light of Damasio's somatic marker model it would be interesting to investigate PFC function in patients with spinal cord injury and see whether the lack of viscerosensory feedback in these patients has influence on PFC function during decision making.

Dysfunction in prefrontal areas is also supposed to be involved in psychopathy, a disorder characterized by antisocial behavior and emotional impairment (e.g. lack of guilt) (Blair, 2003). Support for this hypothesis comes from Anderson et al. (Anderson et al., 1999) who examined two patients with early damage to ventromedial PFC areas and found that these people showed marked disturbances in social behavior and social and moral reasoning. On the basis of their observations the authors concluded that early onset of prefrontal damage could result in symptoms of psychopathy.

Recent data on non-brain damaged individuals further support the notion that the PFC plays a crucial role in social behavior and social thoughts. The *"Theory of Mind"* (TOM) is a classical model of social reasoning referring to the ability to attribute independent mental states to self and others in order to explain and predict behavior (Castelli et al., 2000). In the recent past, this model has been operationalized in order to investigate human social reasoning: In their experiment, Castelli and colleagues (Castelli et al., 2000) performed an fMRI scan while subjects were viewing simple, animated geometric forms whose movement patterns evoked mental state attributions (e.g. the two geometric shapes were interpreted as *"mother"* and *"child"* interacting with each other) or simple action descriptions (e.g. form is moving around). During states of

mental state attribution, marked activation was observed in the mPFC (BA 9), the temporoparietal junction (superior temporal sulcus), basal temporal areas (fusiform gyrus and the temporal pole adjacent to the amygdala) and extrastriate cortex (occipital gyrus). According to the authors, these four regions comprise the basis for a network that is involved in processing information about possible intentions and it seems to be relevant in order to make assumptions about what other people feel or intend to do. Interestingly, a very similar network was described by Haxby et al. (Haxby et al., 2000) outlining the neural basis for face perception. It can therefore be speculated that these two functions (face perception and theory of mind) are two highly related concepts maybe sharing an overlapping neural substrate.

In another study on healthy participants, people's brains were scanned while they were shown video clips of social interaction scenes with other human beings. The control condition consisted of scenes with just one actor who did not interact with any other person. Passively watching social interactions on a video screen (compared to the control condition) resulted (amongst other areas) in marked activation in the dorsomedial PFC, the precuneus., the superior temporal sulcus and the fusiform gyrus (Iacoboni et al., 2004). Based on their data, the authors argue for the existence of a neural network comprising the inferior frontal gyrus, superior temporal cortices and anterior superior temporal sulcus that seems to be involved in interpretation of social significance of actions. Further, they assume that the dorsomedial and medial parietal cortex (precuneus) have an important role when it comes to analyzing social relationships and consideration of their possible implications.

In line with these findings, Mah and colleagues (Mah et al., 2004) recently showed that patients with lesions in the orbitofrontal cortex had marked impairment in social perception and judgments.

These data nicely line up with another strain of evidence arguing for a mPFC substrate of the self. In a series of recent studies, several groups have shown that the medial PFC (BA 9, 10) is associated with self-reflection (S. C. Johnson et al., 2002), memory for the self (Macrae et al., 2004) and distinction between self and other (Ruby & Decety, 2003). A lot of these experiments were inspired by the work of Marcus Raichle's group who observed that certain brain areas like the mPFC, temporal lobe areas, posterior cingulate as well as the medial parietal areas (precuneus) frequently show a marked decrease in activation during attention-demanding cognitive tasks (Raichle et al., 2001). Based on these observations, Raichle and his group postulated a hypothesis according to which activity in the mPFC along with the posterior cingulate and the precuneus comprises a default state of the brain during states when the individual is awake and alert but does not engage in attention-demanding tasks. They further assume that these structures are highly involved in monitoring information from the internal and external milieu and that activity in these structures is decreased during states that require focused attention especially in the face of novel tasks. Gusnard and colleagues (Gusnard et al., 2001) published a study that gives evidence that the brain areas associated with the brain's

default network is also associated with the neural substrate of the self and self referential processing: In their experiment they asked their participants to judge pictures on the basis of whether the scene takes place indoors or outdoors (eternally cued condition - ECC) or whether the scene evokes unpleasant, pleasant or neutral feeling (internally cued condition - ICC). The task was performed while participants underwent an fMRI scan. Comparison of the ICC versus ECC condition showed significant increases in the mPFC (BA 9 and 10) and Gusnard and colleagues conclude that activity in these areas is increased when attention is directed towards self-referential and introspectively oriented mental activity. These results were confirmed by an fMRI study from Fossati et al. (Fossati et al., 2003), who asked their participants to judge whether a given word describing either positive or negative personality traits represented them or not. In the control condition subjects had to judge whether the given term described a desirable or an undesirable trait. Comparison of the two conditions showed clear activation in the right dorsomedial PFC (BA 10).

The PFC is a complex structure comprising various functions that are cognitive and affective in nature, and there is reasonable evidence from anatomical and functional studies to assume that distinct networks within the PFC are involved in discrete functions. Especially the orbitofrontal and the medial networks seem to be involved in the processing of internal and external cues related to social and self related aspects. However, the exact role of the PFC in these information processes is still poorly understood and the question of how social and affective information is integrated by the PFC and how this translates into behavioral, autonomic or endocrine output still needs to be elucidated in greater detail.

2.3.3.4.b PFC and HPA Axis

Over the course of the last two decades compelling evidence emerged that the PFC, and especially the medial and orbitofrontal PFC plays an important role in HPA axis regulation (R. M. Sullivan & Gratton, 2002b).

From an anatomical perspective this should not be very surprising as the PFC is densely interconnected with almost every limbic structure (Carmichael & Price, 1995) and the hypothalamus in particular (Ongur et al., 1998a; Rempel-Clower & Barbas, 1998).

Within the PFC most connections with the hypothalamus arise from the medial prefrontal network and the medial wall areas 25 and 32 have proven to hold up densest projections to the hypothalamus in particular to the anterior and ventromedial aspects. The orbital areas 13a, 12o and Iai that are related to the medial network also project to the lateral hypothalamus. Only little evidence is found for projections to the PVN, the supraoptic, the suprachiasmatic, the arcuate and the mammillary nuclei but it is speculated that these areas are influenced by prefrontal axons that scratch the borders of these nuclei (Ongur et al., 1998). The fact that several PFC projections synapse in autonomic centers of the hypothalamus that in turn project to brainstem areas involved in autonomic control (e.g. PAG, nucleus parabrachialis) has led to the hypothesis

that the PFC influences autonomic function multi-synaptically via the hypothalamus (Barbas et al., 2003).

The PFC is also closely connected with a series of limbic and brainstem areas that have been shown to influence HPA axis function: The PFC and the amygdala are densely connected via reciprocal projections (McDonald et al., 1996). For example, cells labeled in the medial wall areas 32, 24b and 10m resulted in labeled cells within the BLA and the amygdala in turn projects heavily to various medial, lateral and orbital areas (Ongur et al., 1998). Apart from the amygdala, the HF is also strongly connected with the PFC. Tracers injected into the orbital and mPFC resulted in labeled cells within the subiculum and a few labeled cells in CA1 an CA2. Subicular projections to the orbital and mPFC on the other hand seem to be restricted to medial orbital cortex and the gyrus rectus. Moreover, reciprocal connections between the orbital and mPFC and areas like the temporal pole, the entorhinal cortex, the parahippocampal cortex, the perirhinal cortex and the cingulate cortex have been reported (Ongur et al., 1998).

In line with the hypothesis that the PFC seems to be involved in autonomic function and behavioral expression of emotion (Barbas et al., 2003; Bechara et al., 2000b), the PFC has shown to hold up dense connections with various mid brain and brainstem areas: Tracer injection in infralimbic and perilimbic areas of the mPFC result in labeling within the lateral dorsal tegmental nucleus of the dorsal pons as well as labeling within the NTS (Terreberry & Neafsey, 1987). Most interestingly, the PFC has pronounced connections with the PAG, a brain area involved in autonomic functions as well as distinct emotional coping strategies (Bandler et al., 2000a; Bandler et al., 2000b). In a tracing study, An et al. (An et al., 1998) showed that mPFC areas 10m, 25 and 32 predominantly connect with the dorsolateral columns of the PAG whereas orbital areas 13a, Iai, 12o and caudal 12l predominantly project to the ventrolateral column of the PAG. Moreover, the infralimbic region of the PFC sends projections to the dorsal raphé nucleus and thereby directly interacts with the serotonergic system (Peyron et al., 1998).

Support for the PFC's role in endocrine regulation comes from receptor mapping studies showing dense GRs in the mPFC (Cintra et al., 1994; Diorio et al., 1993; Sanchez et al., 2000) with GR density being four to five times higher than for MRs (Diorio et al., 1993). Interestingly, the GR density in primates seems to be higher than in the hippocampus (Sanchez et al., 2000), which points towards the possibility that in primates, the PFC plays a much more important role in HPA axis feedback than the hippocampal formation.

A series of recent lesion studies all point towards a primary inhibitory role of the dorsal mPFC on HPA axis function: Diorio and coworkers (Diorio et al., 1993) showed that lesions in the rat mPFC resulted in significantly increased plasma ACTH and corticosterone concentrations (compared to sham lesions) in response to a 20 minute restraint stress. Implants of crystalline cort cannulas into the same region resulted in a significant decrease in plasma ACTH and corticosterone after exposure to a restraint stress. Interestingly neither effect was observed after

ether stress, indicating a stressor-specific involvement of the mPFC.
Similar results come from Figueiredo et al. (Figueiredo et al., 2003b): In rats, bi-lateral lesions of the mPFC resulted in significantly enhanced c-fos mRNA in the PVN and an increase of plasma ACTH concentrations after restraint stress. Interestingly, bi-lateral lesion of the mPFC also resulted in increased c-fos induction in the MEA, the ventrolateral BNST, the CA3 hippocampal field, the piriform cortex and the endopririform cortex. C-fos induction in the CEA on the other hand was not observed. Similar to Diorio's findings, these effects were stressor specific and were not observed as a consequence of ether stress.
Brake and colleagues (Brake et al., 2000) report that ibotenate-induced mPFC lesions in neonatal rats caused greater increases in plasma corticosterone and an exaggerated dopamine increase in the PFC when compared to sham-lesioned rats.
However, in a study on rats, lesions in the ventral mPFC led to reduced corticosterone peak response due to restraint stress (R. M. Sullivan & Gratton, 1999). This gives evidence for region specific effects with the dorsal mPFC being involved in HPA axis inhibition and the ventral mPFC being associated with HPA axis excitation. Support for a dorsal-ventral distinction comes from early studies in patients undergoing leucotomy: In these patients, electrical stimulation in the ventral (orbital) but not cingulate region caused increases in ACTH concentrations (Frankel and Jenkins, 1975 cited from Sullivan & Gratton, 2002b). Apart from a dorsal-ventral distinction, Sullivan and Gratton's data also indicated a left-right pattern: It was shown that bi-lateral or right but not left sided lesions causes reduced peak corticosterone responses which indicates that at least in the rat, a right side PFC specialization in endocrine activation. This parallels previously mentioned findings from Kalin's group showing that in monkeys extreme right frontal electrical activity is associated with elevated cortisol concentrations and increased levels of cerebrospinal fluid CRF (Kalin et al., 1998; Kalin et al., 2000).
The importance of cortical HPA axis feedback mechanisms is further supported by data from patients with cortical brain damage, who show elevated morning cortisol levels when compared to patients with subcortical lesions or healthy controls. Moreover, anterior cortical damage had a greater impact on morning cortisol levels than did posterior lesions (Tchiteya et al., 2003).

The mPFC and especially ventral aspects of the mPFC seem to be involved in comprising the controllability of stressors. According to Mason (Mason, 1968) uncontrollability of a stimulus or a stressor is an important factor for HPA axis activation and learned helplessness, a state induced by uncontrollable stress exposure has been associated with elevated stress hormone levels (Maier et al., 1986) and increased 5-HT concentrations in the dorsal raphé nucleus (Grahn et al., 1999). In a very recent experiment Maier and his group (Amat et al., 2005) showed that a blockade of ventral mPFC function by infusion of muscimol eliminated the impact of behavioral control over a stressor. In previous studies the group showed that uncontrollable but not controllable shock exposure results in increased 5-HT concentrations in the dorsal raphé

nucleus. However, a blockade of the ventral mPFC resulted in 5-HT induced increases in the dorsal raphé nucleus in response to controllable shock exposure. Moreover, the observed concentrations reached levels that would normally be expected from uncontrollable shock exposure. These data quite nicely show that the ventral mPFC seems to be involved in mediating brainstem effects in dependence of stressor controllability. Whether the ventral mPFC is the actual site for controllability assessment or whether it rather serves as an output station for this information that is possibly assessed elsewhere in the PFC can not be answered by this experiment. Moreover, Maier does not report any data on HPA axis functioning, but it can be speculated that the ventral mPFC affects the HPA axis in the face of controllable and uncontrollable stressors either directly or indirectly via serotonergic projections arising from the dorsal raphé nucleus.

Recent anatomical studies suggest that the mPFC is highly involved in mediating the various coping behaviors in response to stressor exposure. It has been shown that medial wall areas 10, 32 and 25 project to the dorsolateral column and the areas 9 and 24b project to the lateral column of the PAG. The PAG seems to be a crucial brain structure in mediating various forms of coping behavior with the dorsal and lateral column predominantly involved in active coping (e.g. flight (caudal) and defense reactions (rostral)) and the ventrolateral column mainly associated with passive coping strategies (e.g. hypoactivity). Although little is known about this topic in humans, the anatomical data from animal models give evidence that the mPFC areas 9, 10, 24b and 32, are highly involved in modulating the PAG output and it can be speculated that these mPFC areas are involved in the assessment and selection of the appropriate coping strategy in the face of a specific stressor (An et al., 1998; Bandler et al., 2000b; Bernard & Bandler, 1998; Keay & Bandler, 2001). Different coping behaviors are not only characterized by a certain behavioral pattern but are normally also associated with distinct patterns of autonomic and endocrine outputs (e.g. flight: increases in heart rate and blood pressure) (Henry, 1992). However, whether the autonomic and endocrine reactions that go along with the various coping strategies are also regulated by these medial wall areas is not known at this point in time.

As already mentioned in one of the previous sections, the mPFC and especially the infralimbic areas seem to play an important role in extinction of fear conditioning. Recent studies indicate that the activation of this area is not necessary during either habituation, the conditioning phase or initial extinction training, but consolidation of extinction learning in a second extinction training session was associated with increased neural tone and the increase in neural activity from extinction training one and two was inversely correlated with freezing behavior during session two (Milad & Quirk, 2002). This data indicate that the mPFC is rather involved in retrieval or consolidation of extinction learning than in the initial learning process (Sotres-Bayon et al., 2004). This assumption has been supported by a recent experiment showing that rats with

mPFC infusions of a protein synthesis inhibitor[8], extinguished normally during a primary extinction procedure but were unable to retrieve this information in a second extinction session the following day. This effect was not observed after insula infusions. Furthermore, extinction training was associated with increased c-fos gene expression in the mPFC. Based on their observations, the authors concluded that the mPFC is a critical storage site for extinction memory (Santini et al., 2004).

It has been speculated that the infralimbic areas reduce fear related behavior (e.g. freezing) via inhibitory influence on amygdala neurons. This hypothesis was tested and data indicate that stimulation of the mPFC resulted in decreased responsiveness to CEA neurons to BLA and the insula input. As the CEA reflects the major amygdala output system for autonomic and behavioral responses during fear, it has been assumed that the activation in the mPFC reduces fear related behavior by inhibiting amygdala function in this area (Quirk et al., 2003). Non of these studies investigated the impact of mPFC activation on HPA axis function or autonomic output during fear extinction, but it can be speculated that the mPFC reduces endocrine and autonomic output either via similar inhibitory actions on amygdala function or direct connections with hypothalamic and brainstem areas. In line with the data from Millad and Quirk it has been proposed that the connections between the mPFC and the amygdala serve as an adaptive system allowing the organism to adjust emotional behavior in the face of changing environmental conditions. It has also been assumed that disturbed communication between these two structures and a loss of control of the mPFC over the amygdala might be an underlying pathology in anxiety disorders (Sotres-Bayon et al., 2004).

The increasing use of functional neuroimaging techniques in the recent past has allowed for further testing of this hypothesis and various studies in healthy participants or patients with affective disorders have in some, but not all cases, confirmed a close functional interaction between the amygdala and several PFC areas.

In a study performed by Weinberger's group (Hariri et al., 2003) subjects were requested to watch affectively loaded pictures from the *International Affective Picture System*. Processing of the pictures resulted in bi-lateral increased signal in the amygdala and cognitive processing of the pictures resulted in an increased signal in the ventral PFC and attenuated amygdala signal. However, as the study used a poorly matched control condition (geometric shapes) and stimuli were used repeatedly (although this is not clearly stated in the method section), results of this study should be interpreted with caution.

In a study on healthy controls, Kim and colleagues (Kim et al., 2003) performed fMRI scans while participants watched pictures showing human faces with surprised and neutral expressions. Subjects who reported more negative valence ratings on the faces showed greater signal changes in the right ventral amygdala. Subjects indicating more positive valence ratings

[8] Memory consolidation requires gene expression and protein synthesis (Santini et al., 2004)

showed greater signal changes in the ventral mPFC. Accordingly, the signal changes observed in these two brain areas showed a negative correlation.

A recently published study from Heinz et al. (Heinz et al., 2005) showed that a carrier of a short allele of the 5-HTT showed stronger right amygdala signals in response to aversive versus pleasant stimuli than a long allele carrier. Moreover, left sided amygdala activity was associated with increased activity in the left ventral mPFC indicating a positive coupling between these two regions. Similar results were found for the right hemisphere. Interestingly these associations was stronger for subjects lacking a short allele.

Posttraumatic stress disorder (PTSD) is an affective disorder caused by exposure to traumatic events and is characterized by frequent recurring memories and flashbacks of the traumatic event. The disorder has often been associated with mechanisms of fear conditioning and failure of extinction learning (e.g. Milad & Quirk, 2002). In line with this notion, anterior cingulate gray matter reduction has been observed in PTSD patients (Yamasue et al., 2003) and several neuroimaging studies showed that PTSD patients undergoing symptom provocation paradigms showed increased amygdala activity along with decreased activity in mPFC areas (Shin et al., 2004). However, not all studies confirm these patterns and a recent publication from Gilboa and colleagues (Gilboa et al., 2004) points out the heterogeneity in amygdala and mPFC findings in PTSD subjects showing that some studies reporting PTSD specific activation of the anterior cingulate gyrus (ACC) (Rauch et al., 1996), whereas others report no changes in ACC signal changes (Pissiota et al., 2002), or increased signal changes in both patients and controls (e.g. Liberzon et al., 1999) or even greater increase in controls compared to PTSD patients (Bremner et al., 1999; Shin et al., 1999). In terms of amygdala activation, Gilboa (Gilboa et al., 2004) points out the fact that several studies found increased amygdala activation in PTSD patients, but in only two cases were these signal changes shown to be PTSD-specific (Liberzon et al., 1999; Pissiota et al., 2002). In light of these conflicting data, Gilboa and colleagues (Gilboa et al., 2004) conducted a PET study using H_2O^{15} water and a symptom provocation paradigm in PTSD patients and healthy control. The paradigm involved exposure to a previous personal traumatic event (recorded and played over tape recorder) as well as a neutral event. A structural equation model was used to determine connectivity between prefrontal areas and the other parts of the brain. PTSD patients and healthy controls did not differ in the interaction between the amygdala and the anterior cingulate gyrus or the subcallosal gyrus (BA 25) during trauma exposure. However, groups differed in their connectivity patterns during the neutral condition. Controls showed a large negative influence of the subcallosal gyrus on amygdala function than did PTSD patients. No such pattern was found for the anterior cingulate gyrus.

Major depression has frequently been associated with a hyperactive HPA axis (Holsboer & Barden, 1996), elevated basal amygdala function (H. C. Abercrombie et al., 1998) as well as positive associations between basal amygdala activity and stress-induced GC concentrations (Drevets et al., 2002). More recent data indicates that patients with major depression show

reduced cortical thickness in the mPFC (BA 9) (Rajkowska et al., 1999) and reduced metabolism in this area has frequently been observed in patients with major depression (Drevets, 1999; Galynker et al., 1998). Reduced metabolism in mPFC areas has also been shown to normalize after successful antidepressant treatment (Kennedy et al., 2001). Moreover, activation in BA 9 has frequently been observed in tasks involving emotional components (Lane et al., 1997a; Lane et al., 1997b; Reiman et al., 1997). Furthermore, in a study on healthy controls and patients with major depression, including anger attacks, Dougherty et al. (Dougherty et al., 2004) reported, , more pronounced signal increases in the mPFC in controls compared to the patient group in response to a anger induction paradigm. In controls, an inverse relationship between activation in the left mPFC and activation in the left amygdala complex was observed during anger induction. In patients with major depression with anger attacks, a positive association between the two structures was detected. Although the exact mechanisms are still not well understood, the data reported here give rise to the speculation that dysfunction in dorsomedial PFC areas may be involved in altering stress and emotional responses (Drevets, 1999).

And while there is much focus on the prefrontal control over amygdala function, evidence exists that the amygdala also influences prefrontal mechanisms. Applying a shock conditioning stress paradigm, Goldstein et al. (L. E. Goldstein et al., 1996) showed that bi-lateral amygdala lesions performed after post-conditioning training blocked stress-induced dopamine, 5-HT and norepinephrine increases in the mPFC. While these data indicate influence of the amygdala on stress-induced monoaminergic input to the PFC, the exact mechanisms are still not well understood.

In summary, rodent data indicate both inhibitory and excitatory influences of medial PFC areas on HPA axis function. These effects seem to be location specific with dorsal aspects serving inhibitory functions. Ventral regions, on the other hand, serve more excitatory functions. Moreover, in rodents, the mPFC seems to be crucially involved in fear extinction and the modulation of the serotonergic system in the face of controllable versus uncontrollable stress. The mPFC's connection to the PAG also indicate its involvement in coping behavior modulation. If and how these functions relate to the mPFC's role in HPA axis regulation is, however, completely unknown at this point in time. While there is much anatomical evidence for a close connection between the amygdala and the PFC, much controversy exits about the exact nature of prefrontal-amygdala coupling in humans. While some studies indicate a positive, others point out negative associations. Furthermore, results differ very much when it comes to the exact location (e.g. ventral versus dorsal, medial versus lateral PFC) and task specific effects have not been studied in greater detail.

2.4 Modulation of HPA Axis Function

The previous sections focused on the neural stress circuits involved in HPA axis regulation and modulation. The various paradigms that were used to investigate these neural stress circuits comprised a series of very different stressors including shock exposure, predator confrontation, immune challenge, and ether exposure. This underlines the notion that the HPA axis can be activated by a series of very different stressors either being psychological, physiological, physical or pharmacological in nature. As previously outlined, Mason (Mason, 1968) argued that the effects of physical stress can be confounded by psychological stress and that the latter one represents one of the most potent factors when it comes to HPA axis activation.

As the concept of psychological stress is central to this work, the following section focuses on the effects of acute and chronic psychological stress on HPA axis functioning and a special emphasize on HPA axis habituation will be made. Finally, factors, capable of HPA axis modulation and methodological confounders will be briefly discussed.

2.4.1 HPA Axis and Acute Psychological Stress

In his 1968 publication, Mason (Mason, 1968) lists five factors that are potentially capable of promoting an HPA axis response: novelty, controllability, suspenseful anticipation, social factors and involvement. In animals, situations that are novel, uncontrollable or social in nature have been shown to be useful paradigms to study stress responses in animals. Most commonly restraint stress (Brake et al., 2000), swim stress (Rittenhouse et al., 2002), novelty (Plotsky & Meaney, 1993), shock exposure (Amat et al., 2005; Maier et al., 1986), predator stress (Cook, 2002; Figueiredo et al., 2003a), maternal separation (Suarez et al., 2001) or social defeat (Kollack-Walker et al., 1997) have been proven successful when it comes to inducing HPA axis responses in many different species. In humans, several stressors involving cognitive challenges, such as mental arithmetic and public speaking (Gerra et al., 2001; Kirschbaum et al., 1993), anticipation of a stressful event (Kirschbaum et al., 1992a) as well as exam stress (Lacey et al., 2000; Spangler, 1997) have been shown to elicit a more or less reliable activation of the HPA axis.

In a recent meta-analysis, Dickerson and Kemeny (Dickerson & Kemeny, 2004) analyzed a total of 208 laboratory studies involving acute psychological stressors in order to delineate factors capable to elicit cortisol responses in humans. Best predictors of cortisol response were the factors *“uncontrollability”* and *“social-evaluative threat”*. While both factors did not differ in their prediction of effect size, paradigms involving both factors resulted in effect sizes nearly 3 times the size of tasks with either component alone.

An example of a standardized laboratory stress protocol comprising the factors uncontrollability and social-evaluative threat is the *“Trier Social Stress Test”* (TSST) (Kirschbaum et al., 1993). The TSST normally consists of 5 minutes of free speech and 5 minutes of mental arithmetic.

Both tasks are requested to be performed in front of an audience. The 10 minute performance is preceded by a 3 minute anticipation period. The TSST has been proven to be reliable in inducing cortisol and ACTH increases. 2-4 fold elevations above baseline are observed in roughly 70 % of all participants (Kirschbaum et al., 1993). Peak cortisol responses are seen around 10 minutes after the TSST ends and hormone levels return to baseline on average about 90 minutes after the start of the procedure (Kirschbaum et al., 1993).

In order to test for the impact of social-evaluative threat on HPA axis activation in humans, Gruenewald and colleagues (Gruenewald et al., 2004) tested the effectiveness of the TSST setting with and without the presence of a social-evaluative threat. In the no-threat condition, participants had to perform the exact same task as during the threat condition (e.g. speech, mental arithmetic) but without the presence of an audience. Data indicated that the threat but not the no-threat condition resulted in increases in saliva cortisol. Also, participants who underwent the threat condition exhibited greater increases in shame and more pronounced decrements in social self-esteem. However, groups did not differ in their anxiety ratings.

In animal models, stressor controllability has frequently been studied (e.g. inescapable shocks) and while some authors find an inverse relationship between stressor controllability and HPA axis response (Sandi et al., 1992), others find no difference in corticosterone response due to controllable versus uncontrollable shocks (Maier et al., 1986). While there is some evidence that the feeling of uncontrollability is positively associated with stress responses in humans (Steptoe & Willemsen, 2004), this question has hardly been studied in an experimental fashion.

2.4.2 HPA Axis and Chronic and Repeated Psychological Stress

The effects of chronic and repeated psychological stress exposure is a field of conflicting findings. Several studies indicate the existence of a hyperactive HPA axis during states of chronic stress. In a study on the cortisol awakening response, Wüst and colleagues (Wust et al., 2000a) found positive associations between the awakening response and the several aspects of chronic stress like *“worries”*, *“social stress”* and *“lack of social recognition”*. Elevated levels of cortisol have also frequently been reported in groups exposed to highly stressful jobs like pilots (Samel et al., 2004) or people who score high on job over-commitment (Steptoe et al., 2004) . People exposed to states of chronic stress like caregivers to a chronically ill relative (Bauer et al., 2000; Vedhara et al., 1999), people suffering from financial strain due to unemployment (Grossi et al., 2001) and patients with major depression (Holsboer & Barden, 1996) have also been reported to exhibit elevated cortisol concentrations.

However, not all people undergoing phases of chronic stress show hypercortisolism: Patients with PTSD (Yehuda, 2001; Yehuda et al., 1995)(but also see (Young et al., 2004)) seem to have a hypoactive HPA axis and in a study on patients with burnout syndrome, Prüssner and colleagues (Pruessner et al., 1999) found a blunted awakening cortisol response. Hypocortisolism has also been observed in a series of other chronic disorders like chronic

fatigue (Cleare et al., 2001), fibromyalgia (Gur et al., 2004), and chronic pelvic pain (Heim et al., 1998) (for an overview see (Heim et al., 2000).

While little is know about the mechanisms of hypocortisolism in humans, animal studies indicate that early environmental events can influence the HPA axis responsiveness later in life (Levine, 1957; Levine et al., 1967)(cited from (Levine, 2000). Adult rats that were exposed to handling during the first weeks of life show attenuated fearfulness in novel environments and a blunted HPA axis activation in response to a wide series of stressors. Moreover, handled rats also show faster recovery (hormone levels return to baseline) after stress exposure (Meaney et al., 1996). Handled rats do not differ in adrenal sensitivity to ACTH, pituitary sensitivity to CRF or CBG levels nor do they differ in basal HPA axis functioning when compared to non-handled rats (Meaney et al., 1992; Meaney et al., 1989). However, handled rats seem to have higher GR binding capacity in the hippocampus and the frontal cortex (Bhatnagar & Meaney, 1995) (but not in the hypothalamus or pituitary, the septum or the amygdala), and data indicate that this is not due to increased receptor affinity but to an increased number of receptors (Meaney et al., 1996). Chronic administration of corticosterone has been associated with GR down-regulation in the hippocampal formation (Sapolsky & McEwen, 1985). When handled rats were treated with corticosterone doses for 5 consecutive days, hippocampal GR densities were indistinguishable from non-handled rats. Moreover, corticosterone treatment resulted in comparable corticosterone response to restraint stress in groups of handled and non-handled animals (Meaney et al., 1996). This experiment gives evidence that GR receptor changes in the hippocampal formation and possibly in the mPFC that are present in neonatally handled rats are directly involved in increased HPA axis feedback and reduced stress responses later in life.

Handling normally involves separating pups from the mother for roughly 15 minutes (Meaney et al., 1996). While this form of maternal separation results in HPA axis hypo-responsitivity in later life, separation that lasts for example 180 – 360 minutes a day has been associated with an increased HPA axis response to stress and decreased GR binding in the hypothalamus and the HF (Meaney et al., 1996). In a recent study, Plotsky's group (Ladd et al., 2004) confirmed these earlier findings. Rats either underwent daily separations of 15 (HMS15) or 180 (HMS180) minutes on postnatal days 2 –14. HMS180 showed twofold higher stress responses to acute airpuff startle and hormone levels took three times as long to return to baseline when compared to HSM15. HSM180 also escaped dexamethasone suppression faster than HSM15 and expressed decreased cortical and hippocampal GRs when compared to HSM15. Other studies also indicate that prenatal stress can cause reduced GR densities in the septum, frontal cortex and amygdala as well as increased HPA axis responses to stress in the offspring (McCormick et al., 1995). Moreover, chronic stress (daily restraint stress for 21 days) also induces significant apical dendritic reorganization in the mPFC (Radley et al., 2004) and chronic stress (2 hours per day for four weeks) also caused decreased GR levels in the mPFC and the hippocampus (Mizoguchi et al., 2003). These data indicate that chronic stress affects brain regions that are

involved in HPA axis regulation. GR decrease in these areas after chronic stress could explain a hyperactive HPA axis due to a lack of negative feedback from these sites. Empirical data support the notion that these early life events can influence not only acute stress responses later in life but also impact responses to chronic stress: Bhatnagar and Meaney (Bhatnagar & Meaney, 1995) found that handled but not non-handled rats show patterns of ACTH habituation to a chronic, intermittent cold stressors and reduced HPA axis response to a novel stressor present after a period of chronic stress. In a more recent study, Bhatnagar (Bhatnagar et al., 2005) found that prenatal stress prevented habituation to repeated restraint stress in male rats. No such pattern was observed in female rats exposed to prenatal stress.

2.4.3 HPA Axis and Habituation

The term habituation describes the phenomena of an attenuated response that is associated with a repeated presentation of a stimulus (Groves & Thompson, 1970; Horn, 1967). Habituation has been discussed as a simple form of learning and seems to play an important role in the adaptation of an organism to its environment (Groves & Thompson, 1970).

In agreement with this notion, recent models on the impact of stress on health emphasize the idea that the inability to adapt or habituate to a frequently re-occurring stressor could be regarded as a possible pathogenic mechanism (McEwen, 1998; 2000).

Sokolov's work on the "*orienting response"* (OR) (Sokolov, 1963) and Groves and Thompson's *"Dual-Process-Theory on Habituation"* can be regarded as the empirically best supported theoretical frameworks on habituation.

According to Sokolov, the orienting response is characterized by a series of somatic (e.g. head turn, gaze shift) and physiological (e.g. cardiovascular, respiratory, electrodermal) changes, which reflect an orchestrated response in order to enhance the organism's information up-take and processing in the presence of a new or unfamiliar stimulus. The OR can be elicited at low stimulus intensities and is subject to habituation in the face of identical stimulation. According to Sokolov, an OR is elicited if the organism detects a discrepancy between an incoming stimulus and an existing neuronal model, with the size of the mismatch determining the magnitude of the OR. Every time the stimulus is presented, the neuronal model gets updated. After repeated presentation, the neuronal model matches the stimulus qualities and discrepancy is no longer elicited. As a result of this, the OR is prevented. If a new, unfamiliar stimulus is presented, brain areas involved in novelty detection (e.g. hippocampus) send discrepancy impulses to brain areas that are involved in overall activation and arousal (e.g. thalamus and formatio reticularis) and an orchestrated OR is elicited. If the presented stimulus is high in intensity or principally aversive in nature, the organism responds with a defensive reaction (DR) that is characterized by fight or flight behavioral components and the corresponding physiological changes (increase in heart rate, blood pressure etc.). According to Sokolov, the DR only shows limited or no habituation (Schandry, 1996). While Sokolov's model was intended to explain habituation of the

OR, Groves and Thompson's *Dual-Process-Theory* is regarded as an overall model on habituation. According to the authors, habituation involves two neural systems: One is the habituation system that represents the most direct route through the nervous system from stimulus to response and is responsible for response habituation. The state system comprises pathways, systems and regions that determine the general responsiveness of the organism. This system seems to be involved in stimulus response sensitization. Although the two systems are sub-served by separate neuronal mechanisms, the interaction between the two systems yield in the final behavioral outcome (Groves & Thompson, 1970).

Habituation has been observed in all living beings and in a wide series of physiological systems (e.g. skin conductance, peripheral pulse amplitude, cerebral evoked potentials) (Schandry, 1977). HPA axis habituation has been reported in some cases (Bhatnagar et al., 2005; Dal-Zotto et al., 2000; Spencer & McEwen, 1990), whereas others report no change in HPA axis response to repeated stress exposure (Bhatnagar et al., 2005; Dal-Zotto et al., 2000; Kant et al., 1983; Kant et al., 1985) while a few experiments even indicated response sensitization (Pitman et al., 1990).

The variability observed in these results can be partially explained by going back to the early theories on habituation. According to Thompson and Spencer (Thompson & Spencer, 1966), the habituation to a stimulus follows a negative exponential function of the number of stimulus presentations. If a stimulus is withheld, the response usually recovers over time. In the case of a homotypic stressor, habituation is positively associated with stimulation frequency. Thompson and Spencer also postulate that habituation is more rapid and more pronounced in response to weak stimuli. Strong stimuli might even prevent habituation. Habituation can generalize to other stimuli and the presentation of another stimulus can result in recovery of the habituation response (dis-habituation). Interruption in stimulus presentation can also cause dis-habituation (Groves and Thompson, 1970).

Several studies investigated the impact of stressor intensity and stimulus frequency as well as duration on HPA axis habituation.

Natelson et al. (Natelson et al., 1988) looked at the impact of stressor intensity on habituation patterns in rats. Rats were exposed to either handling (mild), restraint stress in prone position (medium) or restraint stress in supine position (high). Stress exposure took place once a week for four weeks. Across groups, a positive correlation between the corticosterone response during the first and the last stress exposure ($r = 0.66$, 0.51, and 0.81 for control, prone and supine) was observed, indicating that rats that showed a high response to the first stress encounter also tended to show higher responses during the final exposure. Based on these findings, rats were grouped in high and low responder. Low responder across the three groups did not significantly differ from one another at any sampling point and did not change over time, thus showed no clear pattern of habituation.

Rats in the high responder group showed a similar initial response across the three stress conditions. At the second week, rats in the supine group showed greater corticosterone responses when compared to the high responder in the other two groups (handling and prone). Also, high responder in the supine group showed a delayed pattern of habituation when compared to the other two groups. This study gives evidence that factors like stress intensity but also the magnitude of the initial stress response crucially influence subsequent habituation.

Fischer rats (F344), Lewis rats (LEW) and Spargue-Dawley rats (SD) significantly differ in HPA axis response to stress with F344 normally showing strongest HPA axis activation while LEW are well characterized in terms of a blunted HPA axis response to stress. Dhabhar and colleagues (Dhabhar et al., 1997) exposed rats from these three strains to a repeated immobilization paradigm in order to investigate the impact of the initial stress response on subsequent habituation patterns. Four hours of immobilization resulted in elevated ACTH and corticosterone levels and a significant pattern of habituation in each group. However, F344 rats not only showed the most pronounced HPA axis activation but also exhibited a significantly weaker habituation pattern when compared to the other two groups. Similarly, during an experiment involving one hour of immobilization over the course of 10 days, F344 again showed significantly higher corticosterone concentrations for each day and a significantly diminished habituation pattern of only 10 % compared to about 50 % in the other groups.

Recently, Armario and his group (Marquez et al., 2004) published data showing that rats exposed to one hour of immobilization for 13 consecutive days exhibited marked differences in the initial stress response and animals were accordingly characterized as high, intermediate and low responders. The results however indicated that individual differences more or less vanish with repeated stimulation (day 13). Careful data analysis also indicated that corticosterone was a better indicator of these individual differences compared to ACTH. The study also indicated that habituation occurs in two phases, an early phase with marked decreases in ACTH over the first 3 days and late phase (day 4-13) with only modest further response reductions.

While these studies give evidence that the magnitude of the initial response to a stressor impacts subsequent habituation, Dal-Zotto and coworkers (Dal-Zotto et al., 2002) found that basal corticosterone levels can be regarded as another important factor. They exposed adrenalectomized (ADX) and sham operated rats to either 9 consecutive days of immobilization stress or a single exposure of immobilization on day one. ADX rats were substituted with corticosterone in their drinking water in order to mimic normal basal hormone levels. Data indicate the sham rats expressed decreased ACTH response to 9 consecutive days of immobilization stress. Sham rats that underwent a single stress exposure on day one also showed a similarly decreased response to the same stress 8 days later. ADX rats also showed a decreased ACTH response over the course of the 9 days but no habituation was observed in rats that just underwent a single exposure on day one. These data indicate that a single exposure to a stressor can induce a similar habituation response as the same stressor that has

been presented in a repeated fashion. Moreover, stress-induced GC increases are not mandatory for subsequent habituation to repeated stimulus presentation. However, the initial GC increase at the first stress exposure seems to modulate habituation to a single stress exposure.
In a recently published follow-up experiment, the authors (Dal-Zotto et al., 2003) found that corticosterone synthesis blockade by metyrapone only partially replicated the findings in ADX rats (habituation inhibition after a single immobilization exposure). Administration of the GR antagonist RU468 yielded even weaker effects. It was concluded that GC influence habituation to a single stress exposure probably not via classical corticosteroid receptors but rather via the concerted action of several endocrine (or neurochemical) mechanisms.
In an early habituation experiment, Mason (Mason et al., 1968) exposed monkeys to 72 hour shock avoidance paradigm. On day two, significantly reduced GC concentrations were observed and continuation of the paradigm did not result in further HPA axis activation. However, increased stressor intensity resulted in increased GC levels. Hence, Mason could show that a) habituation is sensitive to stressor intensities, and b) habituation is not a result of adrenal depletion but rather represents a form of adaptation.
In line with findings on response sensitization and in line with Groves and Thompson, repeated presentation of stimuli can activate the state system which can lead to response sensitization.
Vogel and coworkers (Vogel et al., 1988) showed that repeated presentation of various stressors resulted in a sensitized GC response and Van Dijken et al. (van Dijken et al., 1993) found that rats undergoing a single exposure to foot shocks express elevated fear and anxiety related behavior along with elevated ACTH levels in novel environments. This effect was even detectable two weeks after the initial shock exposure.
In line with Thompson and Spencer (Thompson and Spencer, 1966), introduction of a new stimulus after habituation occurred to another stimulus should result in dis-habituation. While some authors report increased GC response to a novel stress after long-term immobilization (Harris et al., 2004) other find decreased HPA axis function in a similar paradigm (Gadek-Michalska & Bugajski, 2003; Sanchez et al., 1998). However, studies differ in their definition of long-term stress (ranging from 3 hours for 3 days to 2 hours for 41 days) as well as nature of stressors (e.g. social isolation and cold stress versus 2-deoxy glucose injection) which could at least partly explain the contradictory findings.

Habituation effects have also been described on a cellular level in the central nervous system. Melia et al. (Melia et al., 1994) found that acute restraint stress increased c-fos mRNA in the cortex, the hippocampus, the hypothalamus, the septum and the brainstem. In animals that were exposed to restraint stress daily for four days, the c-fos mRNA levels in these areas was significantly smaller. Increased c-fos mRNA expression in the same areas was non-existent in rats exposed to the same protocol for nine days. Amygdala c-fos mRNA levels were not statistically different in non-stressed, acutely restraint or four days restraint animals. However,

amygdala c-fos levels in these animals were significantly higher than in animals restrained for nine consecutive days. Animals adapted to restraint stress showed similar c-fos mRNA when confronted with swim stress as no-habituated animals facing swim stress for the first time.

In an experiment from Martinez and colleagues (Martinez et al., 1998) a single social defeat in male rats caused increased c-fos expression in the lateral septum, the BNST, the lateral preoptic area, the lateral hypothalamic area, the PVN, the CEA and MEA and brainstem nuclei (central grey, dorsal and median raphé nuclei, LC, NTS). However, after the tenth defeat only the BNST, the PVN and the MEA continued to expressed increased c-fos levels. On the brainstem level only the central gray and the raphé nuclei continued to show elevated levels of c-fos.

Campeau et al. (Campeau et al., 2002) reports decreased c-fos mRNA in the lateral septum, the BNST, some preoptic areas and the PVN due to repeated audiogenic stress when compared to acute audiogenic stress. In contrast, c-fos RNA in the orbitofrontal cortex was increased in the chronic compared to the acute condition. The authors speculate that this pattern could indicate an inhibitory role of orbitofrontal cortex in stress regulation. Mohammad et al. (Mohammad et al., 2000) found adaptation of fos expression to acute restraint stress in the hippocampus, the amygdala and cerebral cortex but not in the LC or the PVN. Fos expression was more pronounced in the PVN, the LC and the amygdala after sever immobilization stress when compared to mild restraint stress. The opposite pattern was observed in cingulate cortex.

In sheep, confrontation with a predator stress causes CRF increases in the PVN and the amygdala. CRF increases in these two structures precede increases in cortisol. Administration of an CRF antagonist in the amygdala immediately prior to stress caused no reduction in a subsequent GC stress response. However, CRF antagonist administration significantly reduced the stress response to a repeated stress administered two days later (Cook et al., 2004).

Stamp and Herbert (Stamp & Herbert, 2001) exposed three groups of rats (sham operated, ADX with low corticosterone substitution and ADX with high corticosterone substitution) to a nine day repeated restraint paradigm. Low dose ADX rats showed diminished heart rate habituation. Further, in comparison with the high dose ADX and control rats, low dose ADX still showed elevated fos-b expression in the lateral septum, the dorsal and medial parts of the PVN after nine days of repeated stress. These data are in line with previously mentioned results, indicating that a low GC response to stress is associated with less habituation.

Another study performed on sheep indicates that cellular responses to repeated stress might also be influenced by coping behavior. Cook (Cook, 2002) observed that confrontation with a predator results in marked initial CRF increases in the sheep's amygdala followed by a systemically as well as intra-amygdala specific cortisol response. The peak of this response co-occurred with a second CRF peak in the amygdala. Repeated exposure to the stressor resulted in a decreased first CRF peak response and an increased second CRF peak response within the amygdala. Both peaks were even more pronounced when sheep were confronted with a novel

foot shock stressor. However, sheep that had an escape route from the repeated predator stress showed smaller CRF responses to the novel stress.

In line with Sokolov's model, the thalamus also seems to be involved in habituation. Bhatnagar et al. (Bhatnagar et al., 2002) showed that lesions of the posterior division of the paraventricular nucleus of the thalamus prevents HPA axis habituation in chronically restraint stress and Hsu et al. (Hsu et al., 2001) observed that acute restraint stress increases CRF in the posterior nuclear group of the thalamus and a region at the interface of the central medial and ventral posteromedial nucleus. Repeated restraint stress did not alter CRF baseline levels in both regions. However, exposure to repeated stress prevented the effects normally seen during acute stress.

The exact mechanism through which the thalamus influences HPA axis activity and habituation are not clear. However, Bhatnagar et al. (Bhatnagar et al., 2002) point out that the thalamus receives afferent input from serotonergic and catecholaminergic brainstem afferents and in turn projects to the basolateral and basomedial aspects of the amygdala.

Another interesting finding on HPA axis habituation comes from Cole and colleagues (Cole et al., 2000). They exposed male rats to a repeated restraint stress paradigm (one hour per day for six consecutive days). Repeated stress exposure resulted in HPA axis habituation. However, on day 6, one hour prior to stress exposure, rats were injected with either vehicle or three different corticosteroid receptor antagonists: a selective MR antagonist (RU28318), a selective GR antagonist (RU40555) or a combined MR/GR antagonist (RU28318 & RU40555). Data indicated that the combined MR/GR antagonist as well as the MR antagonist alone prevented habituation in previously restraint rats. The GR antagonist had no such effect. Neither antagonist altered the stress response in rats exposed to restraint stress the first time. As a CRF challenge test in rats treated with either GR, MR or combined MR/GR antagonists did not affect HPA axis response, the authors conclude the effects observed in the first experiment don't take place on a hypothalamic-pituitary level. The explanation for the MR effects on HPA axis habituation remain speculative but Cole and colleagues hypothesize that selective MR based habituation inhibition after repeated stress could reflect an increased sensitivity of the HPA axis to MR mediated negative feedback. Support for this idea comes from Reul's group (Gesing et al., 2001) who observed a significant increase in hippocampal MRs after a single acute swim stress (but not cold stress). Administration of CRF antagonists prior to stress block the previously observed effect. Gesing et al. (Gesing et al., 2001) conclude, that this CRF-MR interplay could reflect a novel mechanism involved in the brain's adaptation to psychologically stressful events.

In humans, HPA axis habituation has also been described. In newborns, repeated discharge examination results in marked HPA axis habituation. While the first encounter with the procedure caused pronounced increases in saliva and plasma cortisol, the second examination did not elicit such a response (Gunnar, 1989).

Deinzer and colleagues (Deinzer et al., 1997) reported strong cortisol responses to a parachute jump in people novice to this discipline. A decrease in cortisol levels was observed from the first to the second jump but endocrine levels did not differ significantly. However, cortisol levels between the first and the third occasion differed significantly and a clear pattern of habituation became obvious. Another interesting finding of this study was that not all participants showed a decrease in cortisol response over the course of the three jumps. While some subjects remained at a more or less constant level, others even showed increased responses over the course of the experiment. In line with previously mentioned animal data, participants showing a habituation pattern had stronger initial cortisol responses when compared to participants who showed a sensitization pattern.

Kirschbaum and colleagues (Kirschbaum et al., 1995) confronted a group of healthy males to a series of five psychosocial stress encounters (TSST) over the course of 5 consecutive days. For the total group, significantly elevated cortisol levels were observed at all five days. However, cluster analysis revealed the existence of two responder types with completely dissimilar response kinetics. Based on their profile, the first group was called *"low-responder"* and participants in this group only showed elevated cortisol levels on day one. Cortisol levels from day two through day five were unaltered. However, *"high-responders"* were characterized by marked increases over the course of the five days and only a marginal response difference between day one and day five. High and low responders not only differed in their habituation pattern but also showed differences in the magnitude of the initial response with high responders showing almost twice the increase of low responders.

Similar findings come from Schommer et al. (N. C. Schommer et al., 2003). Sixty five healthy subjects (male and female) were confronted with 3 psychosocial stress encounters that were separated by 4 weeks in between. For the overall sample, all three encounters resulted in marked cortisol and ACTH increases. Saliva and plasma cortisol increases on stress test one were significantly higher than on stress test two and three. However, no differences were observed between stress test two and three. Similar to Kirschbaum et al. (Kirschbaum et al., 1995) a cluster analysis revealed the existence of a high (N = 30) and a low (N = 35) responder group. High responders showed significantly higher cortisol and ACTH responses to all three TSSTs when compared to low responders. Epinephrine and norepinephrine, two markers of sympathetic nervous system activity, showed marked increases at each stress encounter for the overall group. However, no pattern of habituation was observed in these two hormones. Moreover, high and low responders did not differ in their epinephrine and norepinephrine levels. These data give evidence that the same stressor causes habituation in one (HPA axis) but not in another (sympathetic nervous system) stress system. Moreover, high and low responders as identified on the basis of their HPA axis response are indistinguishable on the basis of sympathetic nervous system activity.

Gerra and colleagues (Gerra et al., 2001) exposed 20 healthy males to two subsequent psychosocial stress paradigms and found pronounced habituation of HPA axis activity but no meaningful habituation of epinephrine and norepinephrine levels. A cluster analysis revealed the existence of two responder groups: while one group (n = 12) showed marked habituation effects from stress test 1 to stress test 2, the other group (n = 8) even showed sensitized response patterns.

Wüst and colleagues (Wust et al., 2005) investigated HPA axis habituation in 54 male twin pairs. Participants were exposed to three stress encounters (TSST) that were each separated by one week. Saliva and plasma cortisol as well as ACTH showed marked increases for all three stress tests. A pattern of habituation across the three testings for all three markers was observed. An overall change score across the three sessions was calculated for each participant. Based on this change score, participants were characterized on whether they habituated (HAB), sensitized (SENSI) or did not show any change (NO-CHANGE) over time. 52 % were assigned to the HAB group, 16 % to the SENSI group and 32 % were assigned to the NO-CHANGE group. In line with previously listed findings (e.g. Deintzer et al., 1997), Wüst et al. found evidence that a strong initial HPA axis activation at the first stress test was associated with better habituation. Data indicated that the initial area under curve for cortisol during the first TSST accounts for 72 % of the variance in the change score. In a subsequent ACTH challenge test, participants with a low change score (e.g. HAB) showed higher cortisol responses to the challenge. No such differences were observed in a dexamethasone suppression test. Lower cortisol responses to the ACTH challenge in the SENSI and NO-CHANGE group could be an indicator for less sensitive adrenal cortex in these two groups.

Intra-pair correlation for the change score were ri = 0.33 for the dizygotic and ri = 0.25 for the monozygotic twin pairs, indicating no heritability for the HPA axis habituation response in this sample. However, due to a small sample size, heritability can not be excluded.

Little is now about the neural substrate of HPA axis habituation in humans. In an early fMRI study, Breiter and colleagues (Breiter et al., 1996) showed signal habituation in the right posterior amygdala for repeated presentation of fearful faces. Bi-lateral amygdala signal habituation was observed for repeated presentation of neutral faces. Wright et al. (Wright et al., 2001) found decreased activity in the left dorsolateral PFC, the premotor cortex and the right amygdala due to repeated stimulation with emotional facial expressions. In the left PFC more pronounced habituation was observed in happy versus fearful faces. The right amygdala showed greater habituation than the left. However, fearful versus happy faces elicited a stronger signal in the right amygdala than in the left. The authors argued that while the left amygdala might be specialized in sustained stimulus evaluations, the right might rather represent a dynamic emotional stimulus detection system. In a study from Fischer et al. (Fischer et al., 2003) habituation in the right amygdala and hippocampus and bi-laterally in the medial/inferior

temporal cortex was measured after repeated stimulation with emotional facial expression. The analyzed measure represented an overall signal for fearful and neutral faces. The authors therefore argued that signal habituation occurs in these brain regions independently from stimulus valence and might therefore represent signal decrement due to a decrease in novelty.
In a recent study on PTSD patients, Shin et al. (Shin et al., 2005) found inverse coupling between the amygdala signal change und mPFC signal change in PTSD patients but not in healthy controls. Moreover, PTSD patients showed diminished amygdala habituation in the right amygdala for fear versus happy faces.
While the above studies clearly show that neural activity in the limbic system is subject to habituation in the face of repeated homotypic stimulation, non of the above cited studies report any markers of HPA axis activity. Only the study from Shin et al. (Shin et al., 2005) gives way to speculate how these findings could relate to HPA axis function, as a hypoactive HPA axis has frequently been found in this patient group.

In summary, while the vast amount of studies indicate that the HPA axis is subject to habituation, the exact mechanisms remain poorly understood. In line with early theoretical frameworks on habituation, recent human and animal data indicate that HPA axis habituation is influenced by the type of stimulus and the stimulus intensity. There is also increasing evidence that the initial HPA axis response at the first stress exposure might influence the subsequent habituation pattern in re-occurring homotypic stress encounters. In animals, decreased cellular activity in many limbic structures has been associated with HPA axis habituation. In humans, few studies report signal habituation in limbic structures due to repeated presentation of social and emotional stimuli. However, if and how these pattern impact HPA axis habituation is completely unknown.

2.4.4 HPA Axis and Intervening Factors

The HPA axis responsitivity is known to be influenced by a wide series of factors. The factors most relevant for this work, are described in some detail here after.
According to a recent meta-analysis (Dickerson and Kemeny, 2004), time of day significantly predicts cortisol response effect size, with studies performed in the morning having lower effect sizes than studies performed in the afternoon. This effect is probably due to cortisol's circadian rhythm. Elevated morning levels, as seen during circadian rhythm, might influence cortisol increases caused through external stimulation during this time of the day.
Wüst and colleagues (Wust et al., 2000a) found indicators for a genetic influence on the awakening cortisol response whereas no heritability was observed in habituation patterns (Wust et al., 2005).
Sex differences in HPA axis response have also frequently been reported. In an early investigation, Kirschbaum and colleagues (Kirschbaum et al., 1992a) observed that salivary-free

cortisol increases due to the TSST were 1.5 – 2-fold higher in men compared to women. In a later study (Kirschbaum et al., 1999) it was shown that men, in response to the TSST, showed larger ACTH increases when compared to women. Women taking part in this study were characterized on the basis of their menstrual cycle phase (luteal versus follicular phase) and intake of oral contraceptives (OC). In response to the TSST, men and women in the luteal phase showed the largest salivary-free cortisol response. Men and women in the luteal phase did not significantly differ in their salivary-free cortisol response. Responses in these two groups were significantly higher when compared to women in the follicular phase. However, no such differences were detected in plasma cortisol, reflecting the total cortisol fraction (free/biologically active salivary cortisol versus bound/biologically inactive plasma cortisol). These data indicate a higher sensitivity to ACTH in the female compared to the male adrenal cortex. In women using OCs, comparable total cortisol responses were seen when compared to the other groups. However, salivary-free cortisol concentrations were significantly lower in the OC women when compared to men or women in the luteal phase. As OCs are known to have CBG enhancing properties, the differences seen in OC users might be due to this effect.

Smoking seems to influence HPA axis activity under basal (Kirschbaum et al., 1992b) as well as stress conditions (Kirschbaum et al., 1994) with smokers showing elevated basal levels (Kirschbaum et al., 1992b) but a smaller HPA axis response to psychosocial stress when compared to non-smokers (Kirschbaum et al., 1994). In terms of habituation, Wüst et al. (Wust et al., 2005) found diminished HPA axis habituation over the course of three stress encounters in smokers compared to non-smokers. Kudielka and Kirschbaum (Kudielka & Kirschbaum, 2003) analyzed awakening cortisol levels of 179 community based subjects and found no significant difference in awakening response between habitual smokers and non-smokers.

Kirschbaum et al. (Kirschbaum et al., 1997) reported the impact of glucose load on HPA axis responsitivity. Subjects given 100 g of glucose prior to confrontation with the TSST showed a marked cortisol response to the TSST, while fasted subjects didn't express a pronounced cortisol increases in response to the identical procedure.

Conflicting data on the association between personality traits on HPA axis activity exist. In a study from Schommer et al. (N. C. Schommer et al., 1999) circadian salivary-free cortisol patterns nor cortisol responses to a single psychosocial stress exposure (TSST) did not distinguish between subjects scoring high or low on either extraversion, neuroticism or psychoticism. Prüssner and colleagues (Pruessner et al., 1997) showed that while no correlation were found between questionnaire measures and the cortisol response to a single stress test, data aggregation over the course of four consecutive stress exposures leads to increased correlations between the observed cortisol stress response and personality traits (e.g. social dominance, locus of control). In another study (Kirschbaum et al., 1995), a combination of five personality scales (self-concept of own competence, internality, social resonance, prevailing mood, trustfulness) and scores from a symptom checklist (physical symptoms) significantly

discriminated between high and low cortisol responders in a habituation paradigm involving five consecutive encounters with psychosocial stress test (TSST). In general, high responders tended to show low self-esteem and a negative self concept.

HPA axis dysregulation has been reported in a series of affective disorders (Arborelius et al., 1999) and the association between affective traits and HPA axis function has attracted considerable interest. In monkeys, elevated basal cortisol levels and increased cerebrospinal CRF levels have been associated with fearful temperament (Kalin et al., 1998; Kalin et al., 2000). In two year old human infants, fearfulness in combination with insecure attachment style was associated with higher cortisol responses to a clinical exam situation as well as a *"strange situation"* (Gunnar et al., 1996)

Polk and colleagues (Polk et al., 2005) measured affect in a total of 334 healthy adults. Affect was assessed on every day of the week for a total of three consecutive weeks. On the last day, 14 saliva samples were collected over the entire course of the day. In men, negative trait affect was associated with higher total cortisol concentrations and greater morning rise. Men low in positive trait affect exhibited a diminished circadian decrease in the afternoon hours resulting in a flattened rhythm. In women, high positive trait affect was associated with low morning cortisol concentrations also resulting in flattened rhythms.

Interestingly, in a study on male subjects with high and low trait anxiety, the high anxious group exhibited lower cortisol and ACTH concentrations due to confrontation with a psychosocial stress test in comparison to the low anxious group. The authors conclude that high anxious individuals show an impaired ability to respond with adequate hormone levels to acute stressful situations (Jezova et al., 2004).

Another factor that is supposed to influence HPA axis activity is coping behavior. Brown et al. (Brown et al., 1996) found that a repressive-defensive coping style was associated with elevated basal cortisol levels. In women with breast cancer, a repressive coping style and high anxiety were also associated with a flatter diurnal cortisol slope (Giese-Davis et al., 2004).

In a recently published study, Gaab and colleagues (Gaab et al., 2005) introduced a new questionnaire assessing subjects primary and secondary appraisals in the face of an upcoming stressful task. The measure was designed analogous to Lazarus and Folkman's stress concept of primary and secondary appraisals (Lazarus & Folkman, 1984) and first data acquired with this questionnaire indicated that up to 35 % of the variance in HPA axis stress response can be explained by the primary appraisal scales (Threat and Challenge) while secondary appraisals were not found to be significant predictors.

2.5 Conclusion

In the previous sections the HPA axis as one of the organism's major stress system was introduced and anatomical as well as functional aspects of the axis were highlighted. In a subsequent step some of the various brain structures involved in HPA axis regulation were

described and the existence of highly complex stress sensitive neural circuits was emphasized. Based on animal data it was shown that brain areas such as the amygdala, the hippocampal formation, brainstem centers as well as the prefrontal cortex are frequently activated during stressful experience and seem to play a major role in HPA axis regulation. Finally, HPA axis function under instances of acute and chronic psychological stress in humans was outlined and the concept of stress habituation was introduced.

The data reported in this section were almost exclusively based on animal testing, and while there is an increasing understanding of the neural substrates of stress and stress habituation in animals, little is known about processes in humans.

3. METHODS – FUNCTIONAL NEUROIMAGING

Functional magnetic resonance imaging and positron emission tomography are the most widely used imaging techniques in the field of neuroscience. Due to the minimal risks involved in these techniques, both methods have been frequently utilized to study neural substrates of cognitive and affective processes in humans. However, neither PET nor fMRI actually measure neural activity directly. Instead, the signals underlying both methods depend on hemodynamic and metabolic changes associated with neural activation. And although fMRI and PET have been in use for a few decades, many questions about the exact nature of these associations and the measured signal remain only partially understood.

For the two empirical studies presented in this work, fMRI and PET imaging were the methods of choice. In order to allow for better understanding of the results and interpretations provided in chapter 4, the next section describes the neurophysiologic underpinnings of both methods as well as details about data acquisition and signal processing.

In a first step, this section highlights the neurophysiologic basis of fMRI and PET imaging data. In a second step, the physical principles of both methods are described. A third section explains the various processing steps involved in functional brain imaging data generation. Finally, the statistical models underlying fMRI and PET data analysis techniques are briefly delineated.

3.1. Neurophysiologic Coupling and Neuroimaging Signals

PET and fMRI are the most commonly used imaging techniques in measuring brain function in humans. However, neither methods are direct measures of neural activation. On the basis of hemodynamic and metabolic changes like cerebral blood flow (CBF), glucose (CMRglc) and oxygen metabolism (CMRO2), assumptions are made about the underlying neural activation. Therefore, in order to accurately interpret the PET and fMRI signal, one needs to understand how it relates to the underlying neural processes. And although there is no doubt about the existence of a regional coupling between neural activation, metabolism and hemodynamic fluctuations, the exact nature of these processes is still rather unclear.

Neural activity is metabolically demanding. This demand is reflected in an increase in oxygen and glucose consumption. In early experiments an almost linear relationship between glucose utilization and frequency of stimulation was observed (Yarowski et al., 1983, 1985), and there is much evidence that the sites of glucose utilization are synapses rather then cell bodies (see Jueptner and Weiller, 1995). Fox et al. (1986, 1989) showed that stimulation of the visual or somatosensory cortex in normal, awake human subjects, results in pronounced increases in blood flow and glucose utilization, but minimal increases in oxygen consumption. This observation gave rise to the idea that metabolic processes associated with neural activation might rely in part on glycolysis, a non-oxidative form of glucose metabolism: Neural activity involves synaptic signal transmission with release of neurotransmitters from the pre-synaptic

neuron. The main excitatory neurotransmitter is glutamate, which after detected by the postsynaptic neuron, needs to be removed from the synapse. This happens via up-take in nearby non-neural cells such as astrocytes. Once inside the astrocyte, glutamate is metabolized to glutamine and passed back on to the neuron. The metabolism of glutamate is energy consuming, and based on glycolysis, a non-oxidative process (Shulman & Rothman, 1998).

The increased need for glucose during neural activation is met by regional increases in CBF. There is now evidence that potassium ions, nitric oxides, neurotransmitters like adenosine, norepinephrine, acetylcholine and Vasoactive Intestinal Peptide (VIP), which are, among others, released by active neurons, can regulate CBF (Magistretti & Pellerin, 1997). However, the exact nature of this process is still rather poorly understood.

Neural activity is a complex process involving afferent inputs and sub-threshold integrative processes (pre- postsynaptic actions) as well as a neural output (action potentials). The afferent input is often assed as a measure of local field potentials (LFP), whereas the neural output is reflected in the spike rate.

There is little evidence for a proportional relationship between rCBF and spike rate on a cerebral cortex level (Mathiesen et al., 2000). This result was confirmed by Logothetis and colleagues (Logothetis et al., 2001): They observed that the BOLD contrast in fMRI, which depends on rCBF, blood volume, and oxygen consumption, instead reflects the input and intracortical processing of a given neural unit rather than its spiking output. It also seems that at very low as well as very high levels of synaptic activity no corresponding increase in CBF could be observed. However, in a medium activity range, CBF and postsynaptic activity was linearly coupled (Mathiesen et al., 1998; Norup Nielsen & Lauritzen, 2001).

At this point, there is much discussion about the exact coupling of hemodynamic and metabolic changes to excitatory versus inhibitory neural activity. Effective synaptic inhibition is energy demanding (Ackermann et al., 1984). However, the question remains whether this is due to excitation of inhibitory interneurons or the inhibitory synaptic activity per se.

Studies on this topic report conflicting results (Lauritzen & Gold, 2003) but in a very recent experiment a negative BOLD response was observed in the presence of neural inhibition (Stefanovic et al., 2004).

3.2. PET

Positron emission tomography uses radioactive labeled molecules to detect changes in physiological processes. Positron emitting radioisotopes commonly used are ^{15}O (oxygen / half-life 2 min), ^{13}N (nitrogen / half-life 10 min), ^{11}C (carbon / half-life 20 min) and ^{18}F (flourine / half-life 110 min) (Reiman et al., 2000).

As oxygen, nitrogen and carbon are present in many biological molecules, the corresponding isotopes can fairly easily be integrated into physiological or pharmacological compounds. Flourine is suitable for hydrogen substitutions in some cases. The important point is, that the

isotopes can be incorporated into these biological molecules without affecting their behavior in the body.

A typical example is ^{15}O-water that is commonly used to monitor CBF. ^{11}C-raclopride serves as a dopamine receptor (D2) ligand and ^{18}F-flourodeoxyglucose (FDG) is commonly used to measure cerebral glucose utilization (CMRglc).

Because of the excessive number of protons, isotopes are unstable. In order to reach a stable energy state, isotopes emit positrons and thereby convert the additional proton into a neutron. Positrons are positively charged electrons. Radioisotopes used in PET imaging decay by positron emission. Once the positron is emitted, the element is typically stable and no longer radioactive. Positrons emitted by isotopes normally travel a short distance (0.4 - 1.4 mm) before they annihilate with a nearby electron. Annihilation results in emission of two 511 keV gamma-rays that are emitted at 180-degree to each other. The photons easily penetrate the surrounding tissue (e.g. scalp) and can be recorded by external detectors. Detectors are normally arranged in rings and contain scintillation crystals composed of bismuth germanate (BGO), which convert light photons into electrical signals. Signals are only recorded when an opposite pair of detectors registers a simultaneous hit and the line between the two detectors goes through the emitting volume (e.g. head). Source localization is achieved by application of a computer based mathematical reconstruction algorithm.

Spatial resolution in PET is limited by several factors: a) the distance positrons travel before they annihilate, b) deviation from the 180-degree angle, c) the hardware itself and d) data smoothing prior to statistical analysis. For the study presented in chapter 4, a reconstructed resolution of 8-10 mm full-width-half-maximum (FWHM) was achieved.

Temporal resolution of PET imaging depends on the tracer and its half-life as well as the physiological process studied. FDG, the radiotracer used in the study described in chapter 4, has a fairly long half-life (110 minutes) and therefore has a very poor temporal resolution. The main tracer up-take into the brain happens during the first 15-30 minutes after tracer injection. Radiotracer up-take during this period is an index of regional brain activity. Glucose is delivered to the brain by the insulin dependent carrier GLUT1, which is expressed in brain capillary endothelial cells and in cells of the choroids plexus, ependymia and glia. GLUT1 can transport two to three times the amount of glucose that is needed by the brain and its expression is increased by hypoglycemia. Once FDG is delivered to the brain, it is transported into tissue and phosphorylated to FDG phosphate. Other than glucose, it does not undergo significant further metabolism. Thus once FDG is metabolized to FDG phosphate, it accumulates in the cell in proportion to local CMRglc. The normal CMRglc brain average is around 23-30 μmol glucose 100g per minute (gray matter: 40-60 μmol glucose 100g per minute; white matter: 15 μmol glucose 100g per minute). In healthy human subjects, CMRglc differs in respect to brain areas with highest values in the striatum and parietal cortex close to the eto-occipital sulcus and below

average rates in the temporal cortex and cerebellum. Age related decreases in CMRglc, predominantly in the PFC, have also been reported (Herholz et al., 2004).

One big advantage of PET imaging is the fact that physiological processes can be quantified. Quantification of PET data requires specific kinetic models describing the tracer transfer from one compartment to the other (e.g. capillaries, extracellular and intracellular tissue) or changes in chemical state (e.g. FDG glucose vs. FDG phosphate). Most of these models (e.g. Sokoloff et al., 1977) require information about the arterial tracer concentration (input function) that is available for brain up-take. Arterial blood sampling is an invasive procedure and therefore often replaced by sampling from a heated dorsal hand vein (hot venous sampling). Placing the hand in a hand warmer mimics tracer concentrations normally found in arterial blood. Still, certain experimental designs do not allow frequent arterial or venous blood sampling after tracer injection and alternative approaches regarding this problem haven been recently tested (grand mean scaling, application of a population curve) (Aine, 1995; Herholz et al., 2004).

3.3 MRI Signal and BOLD Response

The MRI signal is based on *"spins"*, a physical property of atoms with uneven numbers of protons and neutrons. Nuclei of hydrogen atoms in water spin and are regarded as the main source of the MRI signal. In the absence of an external magnetic field, hydrogen atoms (often referred to as spins) are randomly aligned in space. When an external magnetic field (B0) is induced, spins have the tendency to align parallel as well as anti-parallel to the direction of the magnetic field. As the anti-parallel alignment represents a higher energy state than the parallel alignment (at physiological temperatures), slightly more spins align parallel to the direction of the field. This results in a small net magnetization along the direction of the magnetic field applied. When spins align in the magnetic field, they precess around the field direction with a certain frequency. This frequency is called *"Lamor frequency"* and it is determined by the element's individual gyromagnetic rate as well as the strength of the applied magnetic field.

The induction of a radio-frequency (RF) pulse that matches the Lamor frequency of the spins, causes the spins to flip from the z-direction (B0) into the xy plane (B1), thereby inducing a small net magnetization in B1. As the RF pulse enhances the spin's energy state, this process is called *"excitation"*. After excitation, spins have the tendency to flip back to their low energy state in the z direction (relaxation), thereby emitting radiofrequency energy. This radiofrequency energy, when measured in a receiver coil, constitutes the MR signal.

The time constant for relaxation is called T1 and depends on the strength of B0 as well as the molecular motion of the molecules involved. Spins with a fast T1 constant relax very quickly, which means that full magnetization in B0 is re-established within a short period of time. The opposite is true for spins with a slow T1 constant. When two RF pulses are given within a short period of time, spins with a short T1 will contribute to the resulting MR signal more strongly. The time between two excitations is called *"time of repetition"* (TR). T1 weighted images have a

relatively short TR and tissue with a fast T1 constant appears in light colors. Tissue with a long T1 constant appears in dark colors.

Right after excitation, when spins flip in the xy plane, a coherent transverse magnetization is present. Due to interactions between the spins, a loss of transverse magnetization occurs. This loss of transverse magnetization is called T2. Fluctuations in the local magnetic field also result in loss of transverse magnetization. The time constant for this process is called T2*.

In blood, oxygen is carried via coupling to hemoglobin. Deoxygenated hemoglobin is paramagnetic, thus causes local inhomogeneties in the magnetic field. This leads to a decrease in the T2* weighted MRI signal (Ogawa et al., 1990). As described in the previous section, increases in neural activity are accompanied by increases in blood flow but only minor increase in oxygen utilization. Thus, a surplus of oxygenate hemoglobin can be observed in regions of neural activity. A surplus of oxygenated hemoglobin goes along with a decrease in local field inhomogeneties and therefore leads to a stronger T2* based MR signal. This signal is called the Blood-Oxygen-Level-Dependent (BOLD) signal.

Hemodynamic changes are tightly coupled to neural activity but appear delayed in time. Therefore, the BOLD signal starts to rise about 1-2 seconds after stimulus onset. Depending on stimulus characteristics (duration, type of stimulus), the BOLD signal takes 1-8 seconds to reach the peak and considerable time to return to baseline (Menon & Goodyear, 2001). The BOLD signal therefore has a decent temporal resolution but is not able to monitor processes on a neural timescale.

Hemodynamic changes normally extend to a slightly larger area than the actual site of neural activation, which determines spatial limitations that come with the BOLD signal. Artifacts due to large nearby vessels have also frequently been reported. Further limitations in spatial resolution arise from the magnitude of the signal: The BOLD signal is a rather weak signal and improvement of the signal to noise ration is achieved, amongst other techniques, with increased voxel size, and hence, a lower spatial resolution. However, by using scanners with high field strengths and stimuli short in duration, a very reasonable spatial resolution (1-2 mm) can be achieved (Bandettini et al., 2000).

3.4 Processing of Functional Neuroimaging Data

This section highlights the procedures used to analyze PET and fMRI data. Some of the procedures are similar for both techniques, some are quite unique to the individual approach. Therefore this section will describe the procedures for each imaging technique individually.

3.4.1 PET Data Processing

Typical FDG PET scans are acquired over a period of 30 to 50 minutes and the scan is often split up in two or more consecutive scans (frames). The first step in PET data analysis is therefore the combination of the different frames into one single image. Next, a smoothing

algorithm is applied. Smoothing enhances the signal to noise ratio in an image. The decision about the smoothing kernel depends on the region of interest. If small regions (e.g. amygdala) are of interest, a small kernel should be applied. Smoothing also depends on the tracer that is used in the particular study. Tracers with a higher temporal resolution require that scans are acquired in a relatively short period of time. Thus, resulting in low PET counts. Tracer like FDG with a slow time course and long scan times have higher PET counts (higher signal to noise ratio) and hence require less smoothing (Reiman et al., 2000).

3.4.2 fMRI Data Processing

The raw data obtained from a MRI scanner does not resemble a real image but instead is present in *"k-space"*, which reflects a spatial frequency transformation of real-space. K-space data are transformed into real space by applying a *"Fourier transformation"*.

Once a real image is obtained, data need to be *"slice time corrected".* This technique accounts for the fact that fMRI data are acquired over a certain period of time. Each volume, collected at a certain time point during the experiment, is acquired in a slice-by-slice fashion. If for example a coronal acquisition is used, the slices at the anterior part of the brain are acquired slightly earlier than the slices at the posterior part of the brain. This is problematic, as the statistical methods applied at a later stage, will assume that each voxel in a given volume was acquired at the exact same time. Slice timing correction is normally achieved by phase shifting the time series of values at each voxel (S. M. Smith, 2001).

Before the beginning of each scan, subjects' heads are aligned and fixated within the scanner's head coil. Even when subjects are fixed securely (e.g. bitebars, vacuum pillows) slight movement during the scans normally occurs.

This is regarded as a major problem when it comes to statistical analysis as even small movements on a millimeter scale can be regarded as major confounders (Hajnal et al., 1994). Data analysis takes place on a voxel level – e.g. the change of signal intensity within a certain voxel over time. A critical assumption hereby is that one looks at the exact same voxel over time. However, if head movement occurred, this assumption might be violated.

Most motion correction algorithm compute and apply the image transformation (the set of translations in x, y and z direction) that will match a reference image. This reference image is often chosen to be the first volume of an fMRI series (Friston et al., 1996). Extra caution should be spent when paradigms are used that are likely to induce certain stimulus correlated motion artifacts (e.g. threat of shock) and if possible, occurring artifacts need be adequately controlled for (e.g. in a statistical way).

The signal form each voxel constitutes of two factors: systematic signal and noise. Changes in intensity due to experimental stimulation normally range around 0.5 to 5 percent and the noise level is about 0.5 to 1 percent (S. M. Smith, 2001). Spatial filtering (blurring / smoothing) is therefore applied in order to reduce the noise level while retaining the underlying signal. The

spatial filtering function applied causes a local averaging which has the effect of noise values in the local neighborhood tending to cancel each other out. If the blurring stretches over a region that is larger than the activated region, the underlying signal will be diminished as well (S. M. Smith, 2001).

Smoothing should also be applied because some statistical theories (e.g. correction for multiple comparison) require data to be statistically smooth (Friston et al., 1994a; S. M. Smith, 2001).

Spatial filtering is achieved by applying a Gausian profile filter. The width of the filter (expressed in mm FWHM) determines the regional extent of blurring. Filters usually used in fMRI range from 3 to 10 mm, depending on the anatomical structures of interest.

In a final preprocessing step, temporal filtering is applied. During temporal filtering high-pass filters are used in order to remove all unwanted signals in each voxel's time series without affecting the actual signal. High-pass filters are normally set at 1.5 times the duration of the stimulus and are able to remove low frequency physiological noise (e.g. heartbeat, slow motion-related drifts) and scanner related noise (e.g. scanner related drifts) (S. M. Smith, 2001).

3.4.3 Statistical processing

The purpose of statistical analysis is to test for voxels that are activated by the stimulation. The most widely used technique is *"general linear modeling"* (General Linear Model – GLM), which defines a model of what the data, according to the experimental manipulation, should look like and tries to fit the data to this specific model.

A GLM on a single voxel level would be:

$$y(t) = ß^* x(t) + c + e(t)$$

Hereby, y(t) stands for the data in form of a 1D vector of intensity at a certain time point. X(t) refers to the model which is also a 1D vector with one value for each time point. For a square wave block design (e.g. stimulus on versus stimulus off) this would be a series of 0s and 1s (e.g. 111110000..). ß stands for the parameter estimate (PE) for x(t) and determines the factor by which the standard wave form (of height 1) needs to be multiplied in order to fit the data. The constant c, in this example, refers to the baseline intensity at rest. Finally, the error in the model fitting is reflected by e (S. M. Smith, 2001). While the stimulus function (e.g. stimulus on for 5 sec. – stimulus off for 5 sec.) describes a sharp on of waveform, the actual haemodynamic response underlying the signal is more blurred and delayed (S. M. Smith, 2001). The BOLD signal for example usually occurs roughly between 3 and 10 seconds after the stimulus onset and peaks at about 6 seconds (Worsley, 2001). In order to allow for a better model fit, x(t) is mathematically transformed in a haemodynamic response function (HRF) –convolved model (S. M. Smith, 2001) with the HRF often modeled as a simple gamma function (Worsley, 2001).

For the statistical maps, the parameter estimate (ß) is transformed in a T value (T = PE /

standard error (PE) which than can be used to define whether the activation in the given voxel is significantly different from zero.

Finally, the statistical map, based on T values (or Z values) needs to be thresholded to a certain level of significance. Most statistical packages offer a variety of ways to do the thresholding and little agreement exist on the most appropriate way of doing it.

However, a common approach is to select a significance threshold and to apply it to every voxel in the statistical map. This method, however, might be overly conservative: With 20 0000 voxels being tested, one needs to account for the problem of multiple comparison. With a significance level of p = 0.01, a Bonferoni correction would therefore result in a significance level of 0.01 / 20 0000 = 0.0000005.

A less conservative approach is the use of the *"Gausian Random Field Theory"*. Due to the fact that data have been smoothed during pre-processing, voxels are no longer regarded as statistically independent which reflects a smaller number than the original 20 000. The *Gausian Random Field Theory* estimates the amount of statistically independent voxels which results in a reduced correction of P values (by a factor of 2-20).

Another approach for statistical thresholding is to define an a-prior region of interest (ROI) and to assign a threshold for this pre-selected region. This approach is often used when strong a-priori hypothesis exist (e.g. amygdala activation due to face stimulation) and signal changes in a small region have to be detected.

4. EMPIRICAL STUDIES

The two empirical studies presented in this work were both designed to enhance the understanding of the neural substrates of stress in humans. While much research on the neural substrates of stress in rodents and primates has accumulated over the past years (Herman & Cullinan, 1997; Herman et al., 2003), still surprisingly little is known about the neural stress regulation in humans. In the absence of pre-existing data, the studies presented here therefore reflect a hypothesis generating framework in this young scientific field. However, to what is known from animal research, *a priori* hypothesis focused on limbic and prefrontal brain regions and it was believed that limbic areas like the amygdala complex would play an excitatory role, while the PFC would, depending on the exact anatomical location, exerts inhibitory (regulatory) or excitatory influence over HPA axis functioning during instances of stressful experiences.

In the first study, PET imaging was used to investigate the neural substrates of HPA axis function. In a total of 14 healthy male volunteers, joint measurement of brain glucose utilization and salivary cortisol was undertaken to elucidate specific involvement of various brain regions in HPA regulation during a psychosocial stress experience (TSST).

For the second study, fMRI was the imaging technique of choice in linking brain activation during affective processing with HPA axis activation and habituation patterns in the same subjects.

For this purpose, 90 healthy male subjects were exposed to two psychosocial stress tests (TSST) and salivary cortisol was obtained during each of the two sessions. Subsequently, a subset of these participants were invited back for an fMRI scan and brain activation was measured while participants underwent a threat inducing task.

The two studies are presented in separate sections followed by a final general discussion in chapter 5.

4.1 Neural Substrates of Psychosocial Stress – a PET Study

4.1.1 Abstract

Background: In humans, cortisol is the output of a cascade of changes produced by the hypothalamus-pituitary-adrenal axis (HPA axis) and as such, has been extensively used as a marker of stress. The concept of stress has frequently been discussed as a major risk factor in several psychopathologies (Arborelius et al., 1999) and there is now substantial evidence for the existence of various forms of HPA axis dysfunction in several affective disorders (McEwen, 2003). However, the neural substrates of stress and concomitant activation of the HPA axis have not been systematically studied in humans, largely due to the difficulty of measuring brain function during psychosocial stressors known to provoke HPA activation.

Methods: In this study, neural circuitry activated during a psychosocial stress exposure in humans, were identified using positron emission tomography (PET) and injection of fluoro-18-

deoxyglucose (FDG). In order to relate stress induced brain activation to HPA axis activity, cortisol was collected before and after the stress test. The endocrine stress marker was then later used as a covariate in a voxel by voxel based whole brain correlation.

Results: In a sample of 14 healthy male volunteers, increased glucose metabolic rate in the medial prefrontal cortex (mPFC) was associated with lower salivary cortisol concentrations during a psychosocial stress condition. Moreover, increased glucose metabolism in the mPFC was inversely related to metabolic rate in the amygdala/hippocampal region, the precuneus, the medial frontal gyrus, the inferior orbitofrontal gyrus, the fusiform gyrus and negative affective tendencies.

Conclusions: Data presented here give evidence that the prefrontal cortex, and in particular the mPFC is involved in HPA axis regulation in humans and that this influence is inhibitory in nature.

4.1.2 Introduction

The HPA axis is an hierarchically organized stress system, involved in the organism's adaptation to aversive conditions. Activation of the HPA axis results in secretion of glucocorticoids, which are known to have far reaching adaptive effects on the organism's metabolism, immune and central nervous system (Sapolsky et al., 2000). Central stress circuits orchestrate the activation of the HPA axis (Herman & Cullinan, 1997), though the precise details about the circuitries and brain regions involved in this regulatory process are not completely known.

In rats (Diorio et al., 1993), and especially in primates (Sanchez et al., 2000) there is a high density of glucocorticoid receptors in medial prefrontal areas (mPFC). In these same regions of the mPFC, stress-induced increases in immediate early gene expression (Figueiredo et al., 2003b), and dopamine concentration (Sullivan & Gratton, 1998), support the notion that the mPFC, with its distinct functions in higher order processing and its various ascending and descending projections (Carmichael and Price, 1995), plays a crucial role in HPA axis regulation.

Lesions in the mPFC of rats significantly increase ACTH and corticosterone secretion due to restraint stress (Diorio et al., 1993; Figueiredo et al., 2003b). Implants of crystalline corticosterone in the same region result in significantly decreased levels of ACTH and corticosterone due to restraint stress (Diorio et al., 1993). However, whereas dorsal regions of the mPFC seem to have an inhibitory influence on HPA axis function, there is evidence that ventral parts of the mPFC might have a rather excitatory impact on the axis (R. M. Sullivan & Gratton, 1999). More support for the regulatory role of the mPFC during stress exposure emerges from recent rodent data indicating that the mPFC is involved in mediating effects of uncontrollable and controllable stress, whereby the ventral mPFC seems to inhibit serotonergic activation in the dorsal raphé nucleus in face of controllable stressors (Amat et al., 2005). Other rodent data implicate a central role of the mPFC in extinction of aversive learning with lesions of this region resulting in impairments of extinction (Milad & Quirk, 2002). Quirk and his colleagues

have also demonstrated that stimulation of mPFC in rats results in decreased responsiveness of output neurons in the central nucleus of the amygdala (Quirk et al., 2003).
However, all the data presented here are exclusively based on rodent models and little is known about the mPFC's role during stress exposure and its possible inhibitory or excitatory impact on HPA axis regulation in the primate brain.
There are only few studies directly investigating neural circuits of stress in humans (Critchley et al., 2000; Pruessner et al., 2004; Soufer et al., 1998), but so far, only one specifically focused on examining patterns of neural activation in response to stressors known to activate the HPA axis (J. Wang et al., 2005). The here presented experiment was designed to specifically activate the HPA axis in order to identify the neural circuitry involved in the regulation of the axis with a specific focus on the prefrontal cortex. Stressors that include components of social threat and/or uncontrollability are most potent when it comes to HPA axis activation (Dickerson & Kemeny, 2004). A well-validated psychosocial stress test incorporating these components was therefore chosen. In order to evaluate the effectiveness of the stress versus the control condition, salivary cortisol samples were collected throughout the entire experiment. A control condition was devised that required the same amount of talking as the stress condition but did not reliably activate the HPA axis. Psychometric measures assessing subjective ratings on the stress and the control situation, affective tendencies and coping behavior were administered in order to gain further understanding of the observed physiological findings. Subjects performed these tasks on two separate days during which fluoro-18-deoxyglucose (FDG) was injected for subsequent measurement of regional glucose metabolism with positron emission tomography (PET). Using this paradigm we tried to adjudicate between the competing hypotheses regarding PFC function in response to stress. The hypothesis that holds that such changes are excitatory and part of the stress generation process itself would lead to the prediction of positive correlations between prefrontal function and salivary cortisol. The alternative hypothesis that increased PFC function is regulatory leads to the prediction that increased activation should be negatively associated with salivary cortisol. Following the idea that the PFC plays an integrative role in cognitive and affective processing (e.g. emotion regulation) (Ochsner et al., 2002), psychometric measures assessing subjective ratings on the perceived task stressfulness, affective tendencies and coping strategies were administered to gain further insight into how neural substrates of stress relate to psychological domains.

4.1.3 Methods

Participants

Fourteen male human subjects, recruited by posting flyers at university buildings, participated in the study. Participants were between 18 and 23 years old with a mean age of 20.5 years (SD ± 1.91 years).

All participants were screened on the phone and reported to be right handed (Chapman & Chapman, 1987) and non-smokers. People who reported a history of psychoactive substance use, head trauma, neurological, psychiatric, allergic, metabolic or cardiovascular disorder were excluded. People with previous experience of claustrophobia or fear of needles or blood were excluded, too. If eligible, participants were invited for their first session.

Experimental procedure

Each participant reported to the lab for a total of three sessions. The first appointment was a simulation session. Written informed consent for participation in the study was obtained from each subject prior to the beginning of this session. The study was reviewed and approved by the University of Wisconsin-Madison Human Subject Committee.

After written informed consent for participation was obtained, the study procedure was explained in full detail in order to acquainting participants with the experimental environment. At the end of the session, participants were asked to fill out two questionnaires: The *Beck Depression Inventory* (BDI) (Beck et al., 1961) and the *Mood and Anxiety Symptom Questionnaire* (MASQ) (D. B. Watson & Clark, 1991).

If the participant fulfilled all criteria in session one and agreed to take part in the study, he was scheduled for a total of two PET scans which were separated by exactly one week. PET scans for each participant were conducted at the exact same time of day. All scans were performed in the afternoon between 12.00 pm and 4.30 pm.

Participants were requested to fast for 4-5 hours prior to the experiment in order to facilitate later tracer up-take into the brain.

Following a cross over design, half of the subjects were randomly assigned to have the stress procedure at their first PET scan and the control procedure at their second PET scan. The other 7 participants had the reversed order.

PET scans

After arrival at the lab, participants were seated in a quiet, dim, preparation room and an intravenous catheter was inserted into each of the cubital veins of the left and the right arm respectively. After successful insertion of the catheters, participants were allowed to rest for 60 minutes. Questionnaires were given during this time. After the resting period, a baseline saliva sample was collected, followed by an injection of 5 mCi of FDG. Each saliva sample was accompanied by a blood sample (data to be reported elsewhere). Immediately after FDG injection, the subject was guided to a nearby room where either the stress test or the control condition was performed. After completion of the experimental condition, participants were guided back to the PET preparation room and the first of four post-treatment saliva samples was collected (30 minutes post injection). Subjects then filled out an in-house questionnaire assessing the stressfulness of the situation and were encouraged to void their bladder

thereafter. Two more samples were collected 40 and 50 minutes post injection. After the third post-treatment sample was collected (50 minutes after FDG injection), subjects were positioned on the scanner bed and the scan was initiated.

After the scan, subjects were guided back to the PET preparation room. A final saliva sample was collected (110 minutes post injection) and the catheters were removed. All participants were supplied with a light snack at the end of the experiment.

The second PET scan took place exactly one week after the first scan. The procedure for PET scan two mimicked the procedure for scan one. The only manipulation that occurred was the nature of the experimental condition: If the stress condition was administered at the first scan, participants had the control condition at their second scan (or visa versa). At the end of session 3, after removal of the catheters and after a light snack was served, participants were guided to the local MRI scanner and a high resolution anatomical scan was performed.

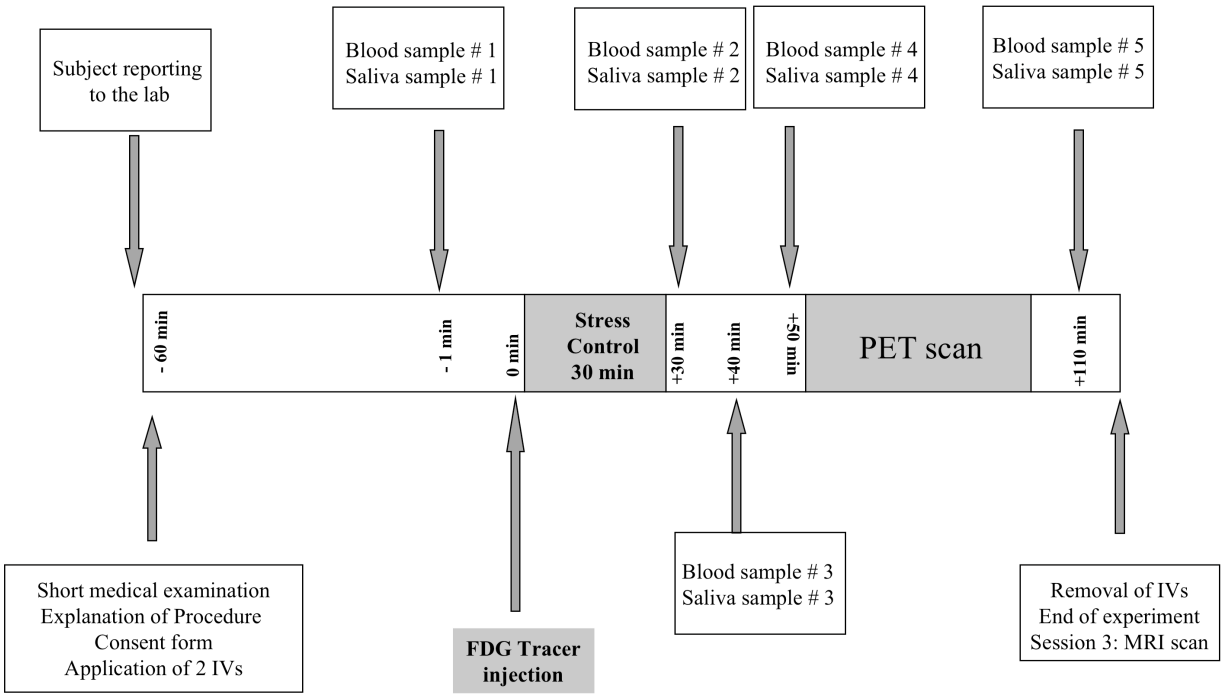

Figure 5: Study design. Blood draws, injection and scanning times are indicated.

PET scan acquisition

Fluoro-18-deoxyglucose (FDG) (Eastern Isotopes, Milwaukee) was used as a tracer and a dose of 5 mCi per scan was injected.

PET data were acquired using a General Electric/Advance PET scanner (DeGrado et al., 1994). This scanner has an intrinsic resolution of 5-6 mm full-width at half-maximum (FWHM), and a reconstructed resolution of 8-10mm FWHM for a brain positioned near the centre of the field of view. The scan started approximately 50 minutes after injection, and consisted of a set of 3, 10

minute emission scans followed by a 15 minute transmission scan. Images were reconstructed to 128 x 128 x 35 pixels using the manufacturer's software and incorporating corrections for deadtime, random events, detector normalization, scatter, and attenuation. The transmission scan was used as the input for an automatic segmented attenuation correction (1.75 x 1.75 x 4.25mm voxels) using the scanner software.

MRI scan acquisition

MRI structural images were acquired for anatomical localization of functional activity. For this purpose an axial 3D SPGR (TE = 1.8 ms, TR = 8.9 ms, flip angle = 10°, FOV = 256 mm x 256 mm, 124 slices, slice thickness = 1.2 mm) were acquired on a 3 Tesla GE Sigma MRI.

PET data processing and analysis

PET data were analyzed using the SPM2 software package (Wellcome Department of Cognitive Neurology, London) and the in-house software Spamalize. PET frames were corrected for innerframe motion, summed, and coregistered into standard MNI space. After co-registration, PET data were smoothed with a Gausian kernel (FWHM = 8 mm) and, to control for variation in mean glucose utilization, each voxel was globally normalized by the mean voxel value. All sum-images were then scaled to the group mean value (grand mean scaled). Glucose metabolism was not further quantified as repeated arterial blood sampling right after tracer injection, would have interfered with the study design.

PET data from the stress and control condition were statistically compared using a paired t-test (population main effect) via SPM2 (Friston et al., 1991).

Globally normalized, grand mean scaled and thresholded difference images (stress – control) were created for each subject. This difference image along with the difference score for the maximum increase during the stress and the control condition ($max_{increase\_delta}$) variable was entered in a single subject-single covariate voxel-wise whole brain correlation (SPM2).

A ROI mask for the significant mPFC clusters was created and values for the maximum grand mean scaled FDG concentrations (referred to as *glucose metabolic rate*) within these clusters were extracted for each subject in order to examine the data for outliers. Spamlize's BrainMaker ROI tool was used for this step.

All brain data are shown at p = .001 uncorrected for multiple comparison.

Salivary cortisol sampling and analysis

A total of 5 saliva samples were collected. Collection times were 1 minute prior to FDG injection as well as 30 minutes, 40 minutes, 50 minutes and 110 minutes post injection. Salivettes® (Saarstedt, Germany) were used for specimen collection. Salivettes® were stored at – 20 ° degree Celsius until samples were analyzed for salivary cortisol (nmol/L) using a luminocent

immuno assay (IBL, Hamburg, Germany). The intra-assay and inter-assay variability have been shown to be < 8 % respectively.

A score of maximum salivary cortisol increase ($max_{increase}$) was calculated for each subject and for each condition by subtracting the baseline sample from the maximum post-treatment sample (*sample 1* minus *sample 4*). The difference between $max_{increase}$ for the stress condition and $max_{increase}$ for the control condition was then calculated ($max_{increase\_delta}$) and used as a covariate in a voxel-wise whole brain correlation. Salivary cortisol levels for the two scan sessions were compared using analysis of variance (ANOVA) for repeated measures. All data were reported Greenhouse-Geisser corrected if sphericity assumptions were violated. $Max_{increase}$ scores for the two scan sessions were compared with a paired t-test. Associations between psychometric measures and cortisol data were tested by using two-tailed Pearson correlations. A statistical significance level of p = 0.05 was set for data reported here and all data are presented as standard errors of means (SEM). The entire statistical analysis were performed using SPSS 11 for Macintosh OS X.

Stress Condition

The stress condition used in this experiment was a modified version of the *Trier Social Stress Test* (TSST) (Kirschbaum et al., 1993). The TSST is a psychosocial stress test consisting of 3 minutes of preparation time, 5 minutes of free speech and 5 minutes of mental arithmetic in front of two panel members and a camera. The TSST was chosen as it has been shown to promote very robust activation of the HPA axis and to induce stronger increases in salivary cortisol than any other laboratory stress test known at this point (Dickerson & Kemeny, 2004).

In order to occupy the bulk of the initial FDG up-take, another 5-minute speech task (word definition) as well as another 5 minutes of mental arithmetic had been added to the original TSST design. For the speech task, participants were requested to describe their qualification on a given job position. The word definition task requested verbal definitions for words read out loud by the panel members (e.g. Tell us the meaning of *"opaque"*). The first math task required counting backwards loudly from 2043 in steps of 17. For the final math task the subject had to start with the number 5, add 3 and then multiply the result with 2. Each task lasted exactly 5 minutes and was performed in front of the two panel members. After the stress procedure was over, participants were guided back to the PET preparation room.

Control Condition

The control condition was designed to match the stress condition without inducing stress. In order to reduce the stressfulness of the situation, we removed the camera as well as the presence of the panel from the original design. In an initial pilot study on 10 subjects, it was shown that removal of the panel as well as the camera from the TSST setting prevented an activation of the HPA axis (data not published).

The control condition involved the following tasks: Participants had to give a free speech (about a movie, a trip or a book), followed by a word definition task (e.g. Tell us the meaning of *"happy"*.). Words were recorded on tape and played to the participant. Next, each participant was asked to count backwards from 5000 in steps of 7. Finally each subject was requested to start with the number 1, add 1 and then multiply the result by 2. Each task lasted for exactly 5 minutes. Participants were alone in the room for the tasks. The investigator entered the room only to give instructions between the tasks. Participants were told that the purpose of this session was to test whether speaking and mental arithmetic per se influences hormone concentration. A hidden camera in the shape of a radio clock was placed in the room in order to verify that each participant followed the instructions while being alone in the room.

At the end of the study each participant was debriefed about the existence of the hidden camera and written consent for usage of the tapes was obtained. Tapes were not inspected prior to when written consent was given. All 14 participants agreed on usage of the tapes.

Post-experiment inspections of the tapes showed that all subjects followed the given instructions.

Psychological Assessment

Each participant filled out several questionnaires. The *Beck Depression Inventory* (BDI) (Beck et al., 1961) and the *Mood and Anxiety Symptom Questionnaire* (MASQ) (D. B. Watson & Clark, 1991) were given at the end of the simulation session in order to exclude subjects with high depression scores or suicidal tendencies. The MASQ assesses affective tendencies on 5 subscales which are: *general distress*, *anxious symptoms*, *anhedonic depression*, *depressive symptoms* and *anxious arousal*.

During the rest period, prior to scan one and scan three more questionnaires were given: A coping questionnaire assessing problem-focused coping and emotion-focused coping (Carver et al., 1989). The *Marlowe Crowne Social Desirability Scale*, which assesses social desirability (Crowne & Marlowe, 1960) and the *State-Trait Anxiety Questionnaire* (STAI) (Spielberger, 1983).

After the end of either the control or the stress condition an in-house questionnaire was given to assess subjective feelings of stress. Ratings were given on a visual analog scale (0 – 100).

4.1.4 Results

Salivary cortisol

Salivary cortisol concentrations were significantly different between the stress and control condition (two factor (condition by sample) repeated measure ANOVA analysis: *main effect condition*: F (1,13) = 6.36, p = 0.026; *interaction of condition x sample*: F (4,52) = 6.35 p = 0.011).

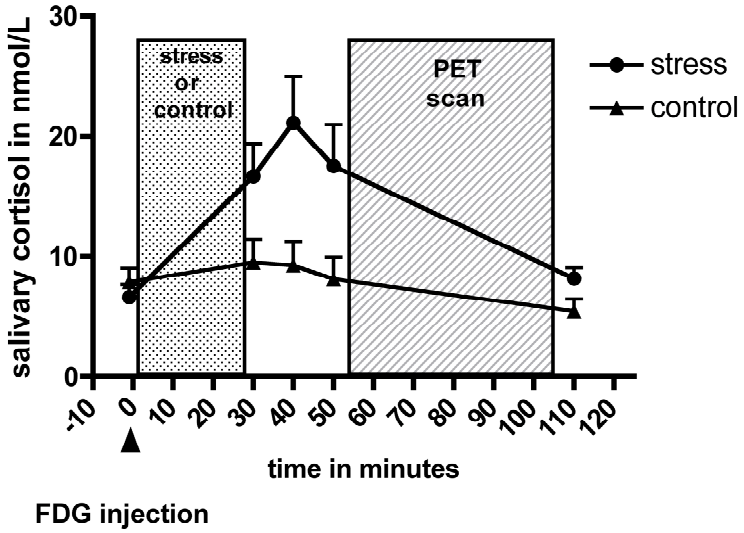

Figure 6: Salivary cortisol for the control and the stress condition. Error bars reflect standard errors of the mean (SEM).

Maximum salivary cortisol increase ($max_{increase}$) during the stress condition was significantly higher than during the control condition (paired t-test: mean $max_{increase}$ stress = 15.25 nmol/L ± 16.33; mean $max_{increase}$ control = 2.53 nmol/L ± 5.59; t (13) = -2.80; p = 0.015).

Whereas the order of the condition (control condition first vs. stress condition first) had no significant effect on maximum salivary cortisol increase during either condition (two-factor (order by condition) repeated measure ANOVA analysis: *interaction of order x condition*: F(1,12) = 0.18, p = 0.68).

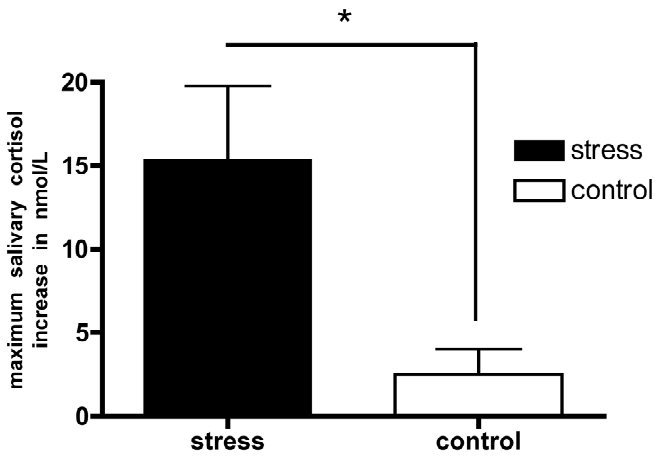

Figure 7: Maximum increase ($max_{increase}$) in salivary cortisol (nmol/L) in response to the stress and the control condition. Error bars reflect standard errors of the mean (SEM).

Salivary cortisol and psychometric measures

First, subjective ratings for the control and the stress situation were compared with a paired t-test. Pronounced differences in mean ratings for the *stressfulness of the task (stress > control)*, *perceived control (control > stress)*, and *satisfaction with the overall performance (control > stress)* were found. Differences in the *importance to perform well (stress > control)*, *perceived threat (stress > control)*, *perceived failure (stress > control)*, the *sense of making a foolish performance (stress > control)* were also observed but did not reach the critical significance level after alpha error adjustment. No meaningful difference was found for *stressfulness ratings of another stress exposure*.

Table 1: Subjective ratings for the control and stress condition on a visual analog scale (VAS). Ratings were compared with a paired t-test.

Item	Mean difference / SD	t-value	Significance (adjusted for multiple comparisons p = 0.0063)
I experienced the interview /speech to be stressful.	3.97 / 4.45	3.34	0.005
It was important for me to perform well.	2,14 / 3.30	2.42	0.031 / n.s.
During the interview I had total control over the situation.	- 3.89 /3.46	-4.21	0.001
The situation was very threatening.	2.36 / 2.96	2.98	0.011 / n.s.
I felt stressed because of my failure.	2.63 / 3.36	2.94	0.011 / n.s.
I was stressed because I felt I made a fool out of my self.	2.44 / 3.32	2.66	0.021 / n.s.
I was satisfied with my overall performance.	-2.70 / 2.78	- 3.63	0.008
Going through this again would be even more stressful.	1.35 / 4.39	1.15	0.27 / n.s.

For the stress condition, a negative association between *perceived threat* and *perceived control* was observed (r = - 0.61; p = 0.02; R = 0.37). The subjective feeling of *making a fool out of themselves* also negatively correlated with the perceived feeling of control (r = - 0.61; p = 0.021; R = 0.37). The importance to perform well correlated positively with the perceived stress due to *subjective failure* (r = 0.58; p = 0.03; R = 0.34).

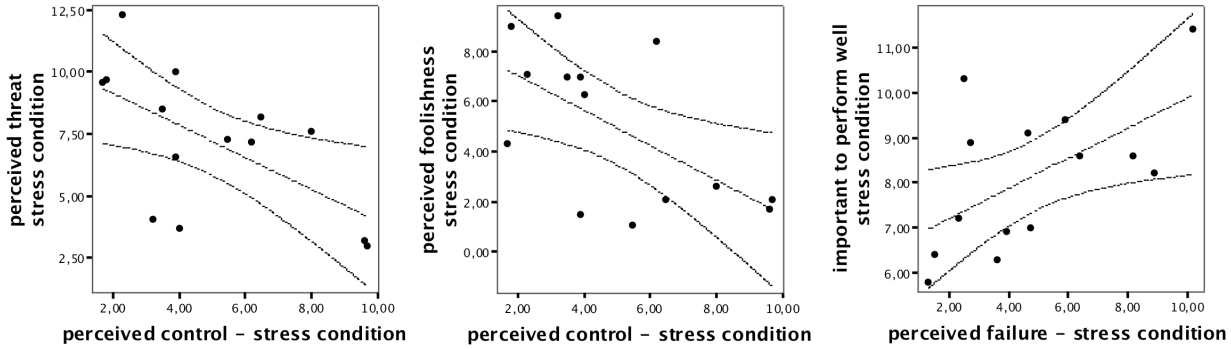

Figure 8: Subjective ratings (VAS) for the stress condition.

Perceived control (during the stress and the control condition respectively) was negatively associated with the maximum cortisol increase during the stress situation (r = - 0.56; p = 0.037; R = 0.31), but not the control condition (r = - 0.26; n.s.).

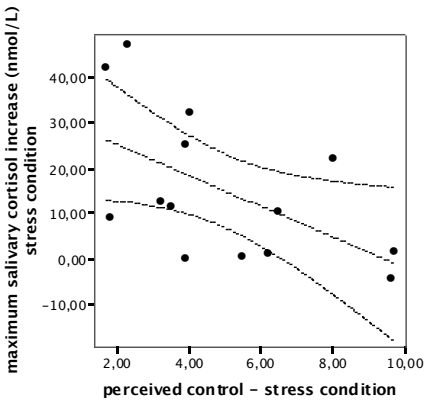

Figure 9: Perceived control and maximum increase during the stress situation.

After the stress condition, subjects were asked about their experience with the TSST committee (VAS):

- I felt that the committee liked me.
- I felt that the committee was satisfied with my performance.
- I liked the committee.

A positive association between $max_{increase}$ (r = 0.52; p = 0.056; n.s.) and $max_{increase\_delta}$ (r = 0.56; p = 0.039; R = 0.31) and the subjective ratings for *I liked the committee* were found.

Pearson correlations were calculated to test for associations in affective tendencies measured with the MASQ and the cortisol response during the control and the stress conditions. For the MASQ subscale *general distress*, positive associations were found with the maximum cortisol increase ($max_{increase}$) during the stress condition as well as with the difference in maximum increase between the two conditions ($max_{increase\text{-}delta}$). None of the other MASQ subscales showed any significant association with the cortisol response during either the control or the stress condition.

Table 2: Correlations (Pearson) for MASQ subscales and cortisol measures for the stress and the control condition.

MASQ	$max_{increase}$ stress	$max_{increase}$ control	$max_{increase\_delta}$
General distress	**r = 0.59** **p = 0.026** **N= 14** **R = 0.35**	r = - 0.48 N.S. N= 14	**r = 0.72** **p = 0.003** **N= 14** **R = 0.52**
Anxious symptoms	r = 0.15 N.S. N= 14	r = - 0.21 N.S. N= 14	r = 0.22 N.S. N= 14
Anhedonic depression	r = 0.33 N.S. N= 14	r = - 0.05 N.S. N= 14	r = 0.33 N.S. N= 14
Depressive symptoms	r = 0.38 N.S. N= 14	r = - 0.51 N.S. N= 14	r = 0.53 N.S. N= 14
Anxious arousal	r = - 0.14 N.S. N= 14	r = 0.21 N.S. N= 14	r = - 0.20 N.S. N= 14

To test for effects of different coping strategies on the cortisol response during the stress and the control condition, Pearson correlations for cortisol measures and scores on the *Carver Coping Questionnaire* subscales were calculated. The following subscales did not show statistically significant associations with cortisol responses during the stress or the control condition: active coping (e.g. $max_{increase}$ stress: r = - 0.17; n.s.; $max_{increase}$ control: r = 0.35; n.s.), planning (e.g. $max_{increase}$ stress: r = - 0.21; n.s.; $max_{increase}$ control: r = 0.51; n.s.), suppression of competing activities (e.g. $max_{increase}$ stress: r = 0.09; n.s.; $max_{increase}$ control: r = 0.27; n.s.), seeking social support for instrumental reasons (e.g. $max_{increase}$ stress: r = 0.29; n.s.; $max_{increase}$ control: r = 0.07; n.s.), positive re-interpretation & growth (e.g. $max_{increase}$ stress: r = 0.33; n.s.; $max_{increase}$ control: r

= 0.47; n.s.), acceptance (e.g. $max_{increase}$ stress: r = 0.44; n.s.; $max_{increase}$ control: r = 0.06; n.s.), turning to religion (e.g. $max_{increase}$ stress: r = 0.25; n.s.; $max_{increase}$ control: r = 0.17; n.s.), denial (e.g. $max_{increase}$ stress: r = 0.21; n.s.; $max_{increase}$ control: r = 0.09; n.s), behavioral disengagement (e.g. $max_{increase}$ stress: r = 0.27; n.s.; $max_{increase}$ control: r = - 0.52; n.s), and mental disengagement (e.g. $max_{increase}$ stress: r = - 0.45; n.s.; $max_{increase}$ control: r = - 0.38; n.s).

A strong positive association was found for maximum increase during the stress condition and scores on the *restraint coping* subscale (r = 0.65; p = 0.025). The tendency to *seek social support for emotional reasons* was negatively associated with cortisol responses during the control condition ($max_{increase}$: r = - 0.69; p = 0.01). For the stress condition, scores *on the focus and venting of emotions* subscale showed a negative association with $max_{increase}$ (r = - 0.66; p = 0.013).

Neither the *Marlowe Crowne Social Desirability Scale* ($max_{increase}$ stress: r = 0.21; n.s.; $max_{increase}$ control: r = 0.52; n.s.) nor the trait aspect of the *STAI* ($max_{increase}$ stress: r = - 0.09; n.s.; $max_{increase}$ control: r = 0.09; n.s.) were significantly associated with the cortisol response during the control or the stress condition.

PET activation data

The stress condition was associated with significantly elevated glucose metabolic rate in the mPFC (stress – control). No such mPFC activation was observed during the control condition (control – stress).

Table 3: MNI coordinates and maximum Z scores for brain areas activated for the *stress – control* and the *control – stress* contrast (paired t-test). Data are shown for p =.001 uncorrected.

Brain area	MNI coordinates (x,y,z)	Z score	Brain area	MNI coordinates (x,y,z)	Z score
Stress – Control			**Control - Stress**		
PFC			*PFC*		
M. sup. frontal gyrus / anterior cingulum	-12, 52, 8	4.22	Superior frontal gyrus	28, -2, 70	3.95
M. sup. frontal gyrus	8, 30, 56	4.21	Inf. frontal gyrus	50, 28, 22	4.09
M. sup. frontal gyrus	-2, 56, 26	3.89	M. frontal gyrus	-30, 40, 44	3.48
Sup. frontal gyrus	14, 48, 32	3.32	*Temporal*		
Sup. frontal gyrus	18, 34, 38	3.51	Sup. temporal gyrus	64, -16, 2	4.12
Insula	34, 28, -2	4.93	Sup. temporal gyrus	-64, -8, 4	3.90
Temporal			Sup. temporal gyrus	-46, -12, -8	3.68
Inf. temporal gyrus	-34, 4, -36	4.62	Sup. temporal gyrus	-58, -16, 10	3.63
M. temporal gyrus	48, -20, -16	4.45	Rolandic operculum	48, -20, 14	3.89
M. temporal gyrus	-46, 4, -32	3.63	*Parietal*		
Sup. temporal gyrus	64, -46, 16	3.53	Postcentral gyrus	12, -34, 80	4.81
Temporal pole	50, 10, -32	3.44	Postcentral gyrus	-64, -10, 36	4.21
Cerebellum			Postcentral gyrus	38, -38, 70	3.40
Cerebellum	-30, -78, -28	4.83	Postcentral gyrus	-50, -14, 34	3.39
Cerebellum	28, -44, -40	4.15	Precentral gyrus	46, 2, 34	4.10
Cerebellum	-10, -78, -26	4.11	Precentral gyrus	48, -12, 60	3.99
Cerebellum	42, -46, -28	4.11	Inf. parietal gyrus	-54, -28, 50	3.70
Cerebellum	20, -80, -30	3.61	Inf. parietal gyrus	-38, -50, 44	3.71
Cerebellum	-54, -58, -32	3.59	Inf. parietal gyrus	52, -36, 56	3.66
Vermis	4, -70, -16	3.57	*Occipital*		
			Angular gyrus	-42, -78, 44	4.47
			Angular gyrus	40, -76, 46	3.71
			Occipital gyrus	8, -60, 12	3.88
			Occipital gyrus	4, -88, 10	3.73
			Occipital gyrus	-16, -98, 10	3.69
			Occipital gyrus	-10, -96, -8	3.52
			Putamen	-30, 4, 0	3.89

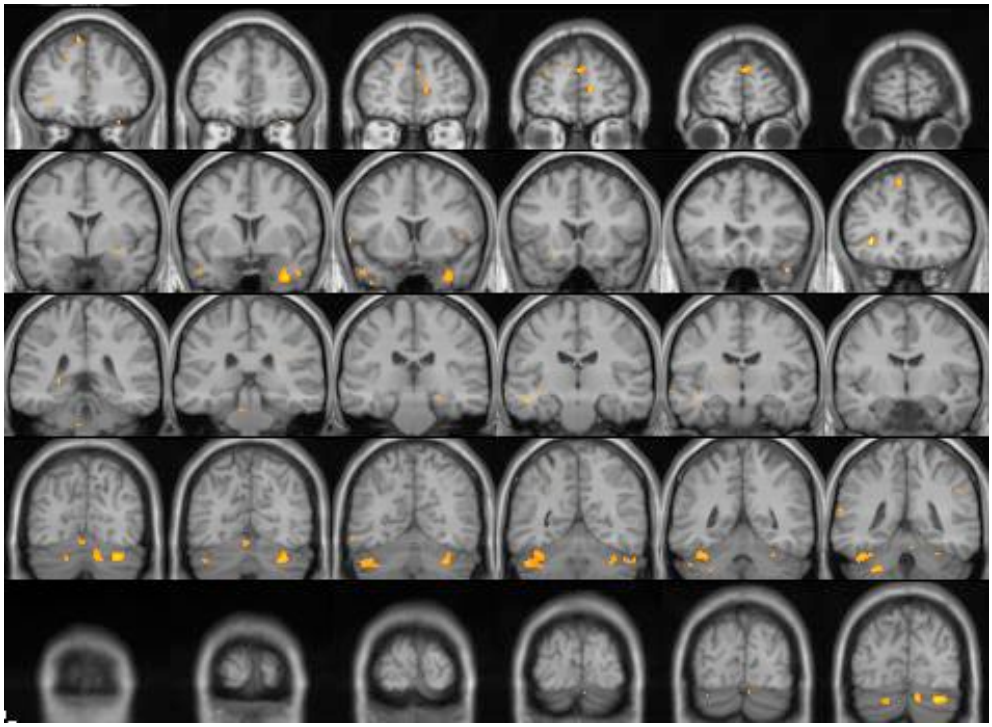

Figure 10: Brain activation (metabolic glucose rate) for the *stress* – *control condition* (paired t-test). Data are presented in a coronal view for p = 0.001 uncorrected for multiple comparisons.

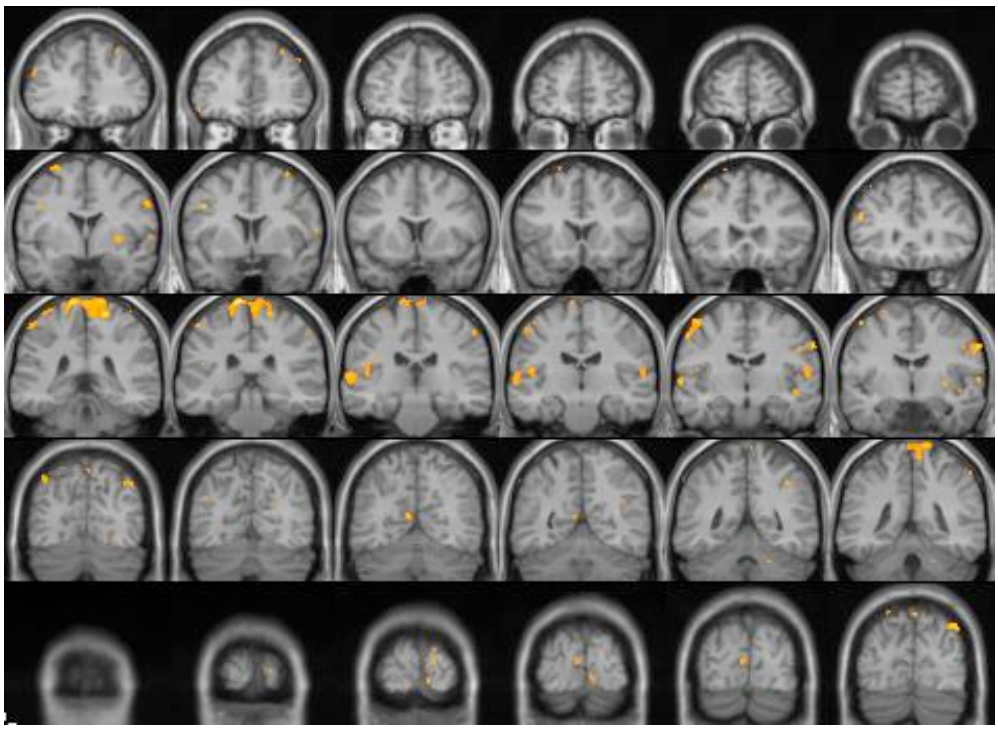

Figure 11: Brain activation (metabolic glucose rate) for the *control* – *stress condition* (paired t-test). Data are presented in a coronal view for p = 0.001 uncorrected for multiple comparisons.

PET activation and cortisol measures

Next, the association between the regional difference in glucose metabolic rate between the stress and control conditions and the difference in maximum salivary cortisol increase ($max_{increase\_delta}$) between these conditions were examined on a voxel-wise basis. In the mPFC, strong negative correlations were found for $max_{increase\_delta}$ and the difference in glucose metabolic rate between the stress and control condition.

Table 4: Correlation between *stress* – *control* difference images and $max_{increase\_delta}$. Clusters are reported for a minimum cluster size of k = 10 and for p = 0.001 uncorrected for multiple comparisons.

Brain area	MNI coordinates (x,y,z)	Z score	Brain area	MNI coordinates (x,y,z)	Z score
Stress – Control & $max_{increase\_delta}$ positive correlation			**Stress – Control & $max_{increase\_delta}$ negative correlation**		
Sup. frontal gyrus	24, 58, 4	3.88	M. sup. frontal gyrus	6, 72, 6	4.01
M. frontal gyrus	-32, 10, 50	3.99	M. sup. frontal gyrus	6, 58, 36	3.80
Fusiform gyrus	-42, -58, -14	4.34	Sup. frontal gyrus	-14, 14, 70	3.46
Fusiform gyrus	-30, -10, -32	3.60	Insula	34, 8, 8	4.14
Precuneus	-6, -54, 50	3.72	Precentral gyrus	48, 2, 50	4.31
Lingual gyrus	-2, -76, 4	3.59	Posterior cingulum	-14, -48, 32	3.71
			Supramarginal gyrus	-62, -44, 26	3.65

These associations were detected in two specific regions of the right medial superior frontal gyrus Brodman area [BA] 9 (x,y,z: 6, 58, 36, Z = 3.80) and BA 10 (x,y,z: 6, 72, 6, Z = 4.01). The direction of these correlations indicates that those individuals with greater increases in glucose metabolic rate in the mPFC in response to the stress versus control conditions showed the lowest levels of salivary cortisol increase during the stress versus control condition, consistent with the mPFC exerting inhibitory control on the HPA axis. In order to test for outliers, based on the clusters in BA 9 and 10 from this analysis, regions of interest (ROI) were defined and maximum glucose metabolic rate for these clusters were extracted for each participant and correlated with the individual salivary cortisol difference scores between conditions ($r = - 0.81$; $p < 0.000$ for BA 9; $r = - 0.89$; $p < 0.0001$ for BA 10).

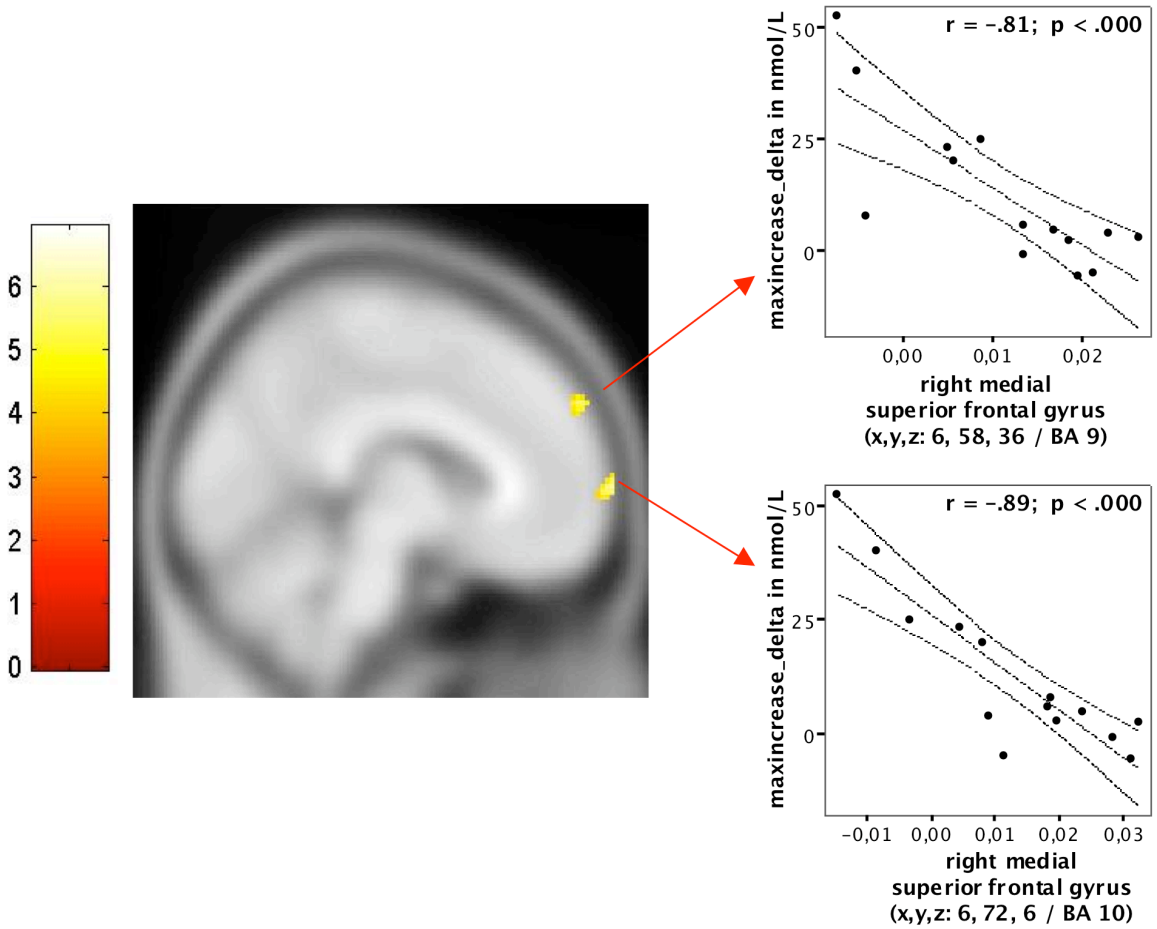

Figure 12: Voxel-wise whole brain correlation between $max_{increase\_delta}$ and the corresponding *stress – control* difference image. Brain data are shown for p = 0.001 uncorrected. Glucose metabolic rates for BA 9 and BA 10 were extracted for each participant and plotted against each participant's $max_{increase\_delta}$ score.

The next analysis step was intended to identify other components of the circuit with which the mPFC regions were functionally coupled. On the basis of the difference image between the stress and control condition, separate voxel-wise whole brain correlation analyses were performed with the maximum metabolic rate within each of the two stress-control mPFC clusters (BA 9 and BA 10 clusters). Area 9 showed a negative correlation with the following areas:

Table 5: Voxel-wise whole brain correlation for maximum metabolic glucose rate in BA 9.

Medial superior frontal gyrus (BA 9)				
Brain area	**MNI coordinates (x, y, z)**	**Z-score**	**Correlation coefficient**	**Significance**
left amygdala / hippocampal area	-30, -10, -32	4.69	r = - 0.87	p < 0.0001
right pallidum	20, -4, 4	4.01	r = - 0.78	p = 0.001
left inferior orbitofrontal gyrus	-50, 42, -8	3.80	r = - 0.83	p < 0.0001
left precuneus	-6, -50, 48	3.79	r = - 0.82	p < 0.0001

Area 10 showed negative correlations with the following areas:

Table 6: Voxel-wise whole brain correlation for maximum metabolic glucose rate in BA 10.

Medial superior frontal gyrus (BA 10)				
Brain area	**MNI coordinates (x, y, z)**	**Z-score**	**Correlation coefficient**	**Significance**
left fusiform gyrus	-42, -58, -14	4.33	r = - 0.92	p < 0.0001
left precuneus	-2, -56, 44	3.72	r = - 0.79	p = 0.001
left medial temporal gyrus	-68, -4, -16	3.59	r = - 0.75	p = 0.002

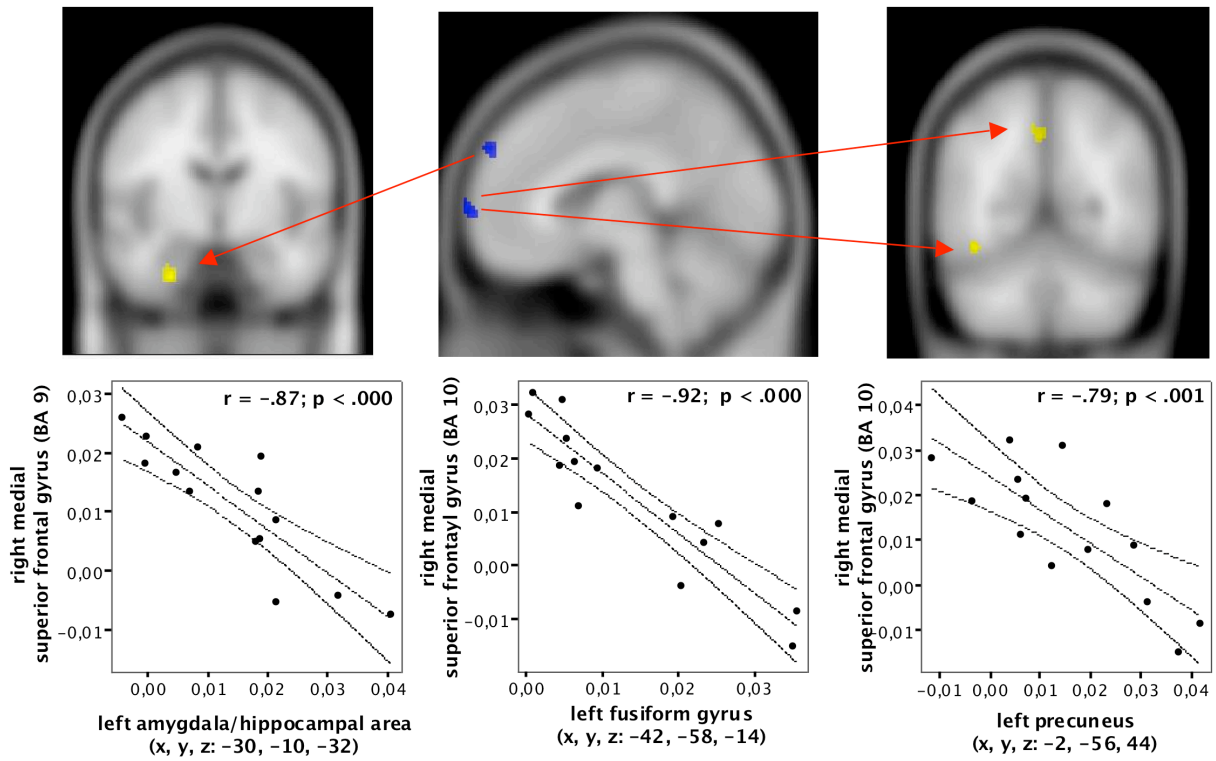

Figure 13: Glucose metabolic rate for BA 9 and BA 10 were entered as covariates in a voxel wise whole brain correlation. The metabolic rate in BA 9 showed an inverse relationship with the metabolic rate in the left amygdala/hippocampal area. Glucose metabolic rate in for BA 10 showed an inverse relationship with the metabolic rate in the left precuneus as well as the left fusiform gyrus. Brain data are shown for p = 0.001 uncorrected.

The direction of all of these effects indicates that individuals who exhibit larger increases in glucose metabolism in mPFC regions during stress compared to control conditions exhibit smaller increases in glucose metabolism during these conditions in the stated regions. Thus, for example, subjects with the largest increases in BA9 during stress versus control conditions show the smallest increases during these conditions in the amygdala/hippocampal region.

PET activation and psychometric measures

In a first step, correlation between affective tendencies (MASQ) and cortisol associated brain activation was tested.

The metabolic glucose rate in BA 10 was negatively correlated with subject's scores on the *general distress* subscale (r = - 0.61; p = 0.021).

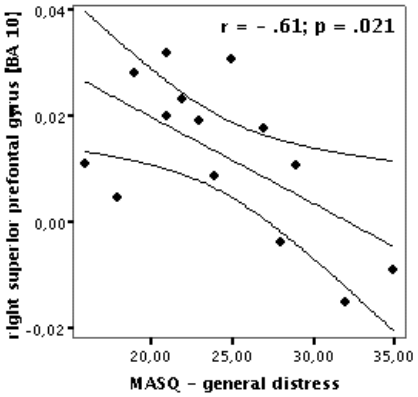

Figure 14: Pearson correlation between MASQ *general distress* scores and maximum metabolic glucose rate in BA 10.

Glucose metabolic rates in the precuneus (x,y,z: -2, -56, 42: r = 0.84; p < 0.000; R = 0.71) and the fusiform gyrus (r = 0.73; p = 0.003; R = 0.53) were positively associated with *general distress*. Glucose metabolic rate in the amygdala/hippocampal region also showed a positive associated with *general distress* but did not reach significance (r = 0.50; p = 0.07). Furthermore, glucose metabolic rate in the precuneus (x,y,z: -2, -56, 42: r = 0.77; p = 0.001; R = 0.59) and the fusiform gyrus (r = 0.67; p = 0.009; R = .45) significantly correlated with scores on the MASQ subscale *depressive symptoms*.

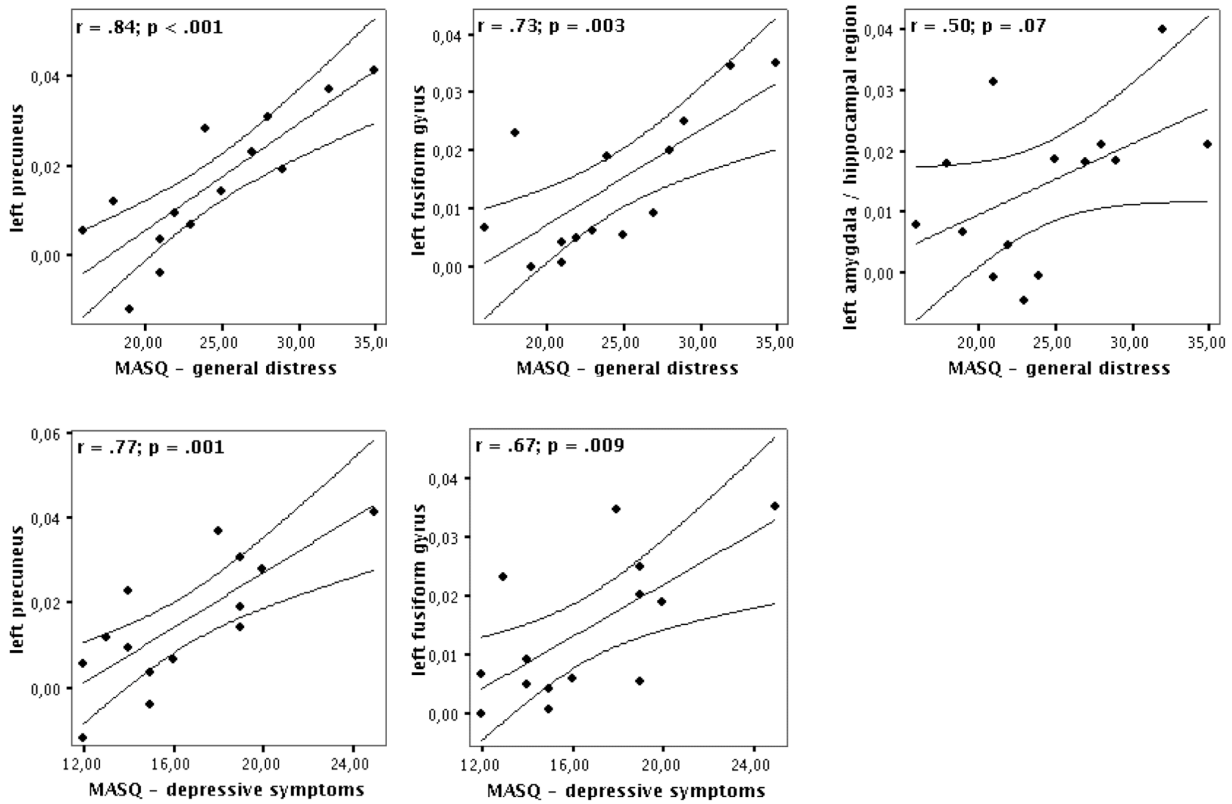

Figure 15: Pearson correlation for the MASQ subscale general distress and depressive symptoms and maximum metabolic glucose rate in the left precuneus, the left fusiform gyrus and the left amygdala / hippocampal region.

In order to test how subjective interpretations during the stress and the control condition related to the observed brain activation, associations between subjective ratings on the stress versus the control condition (VAS) and cortisol associated brain activation were investigated.

Therefore, a difference score between the stress and the control condition for the following VAS items was computed:

- I experienced the interview/speech to be stressful.
- It was important to me to perform well.
- During the interview I had total control over the situation.
- The situation was very threatening.
- I felt stress because of my failure.
- I was stressed because I made a fool out of my self.
- I was satisfied with my overall performance.
-

The difference scores for these VAS ratings as well as ratings on the committee (stress condition only) were entered in a correlation analysis (Pearson) along with cluster information from BA 9 and BA 10 as well as brain clusters identified in the previous connectivity analysis. The results from this analysis are listed in the below tables and graphs.

Table 7: Ratings for perceived control (stress versus control) and brain activation (Pearson correlation).

During the interview I had total control over the situation.				
Brain area	**N**	**Correlation coefficient**	**Significance**	**Effect size**
Right medial superior frontal gyrus (BA 9)	14	r = 0.60	p = 0.022	R = 0.36
Left inferior orbitofrontal gyrus	14	r = - 0.61	p = 0.019	R = 0.37

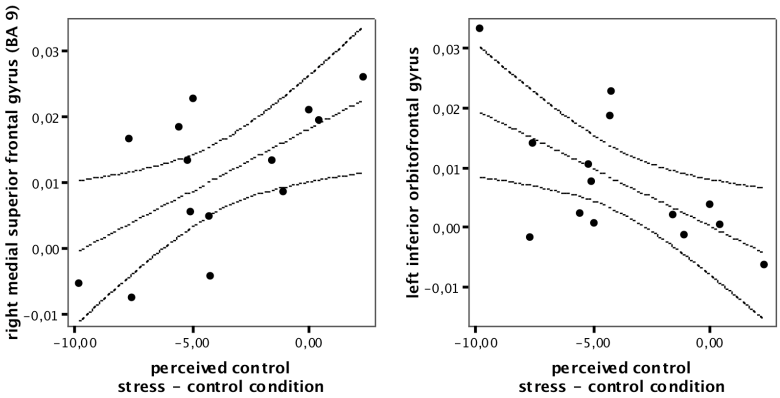

Figure 16: Ratings for perceived control (stress versus control) and brain activation (Pearson correlation).

Table 8: Ratings on performance (stress versus control) and brain activation (Pearson correlation).

It was important to me to perform well.				
Brain area	**N**	**Correlation coefficient**	**Significance**	**Effect size**
Left precuneus	14	r = 0.55	p = 0.043	R = 0.30

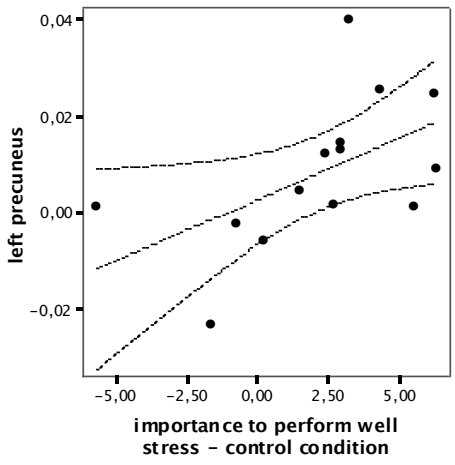

Figure 17: Ratings on performance (stress versus control) and brain activation (Pearson correlation).

Table 9: Ratings on the committee (liked me) (stress condition) and brain activation (Pearson correlation).

I felt that the committee liked me.				
Brain area	**N**	**Correlation coefficient**	**Significance**	**Effect size**
Right medial superior frontal gyrus (BA 9)	14	R = - 0.56	P = 0.036	R = 0.31
Left amygdala / hippocampal area	14	R = 0.56	P = 0.037	R = 0.37

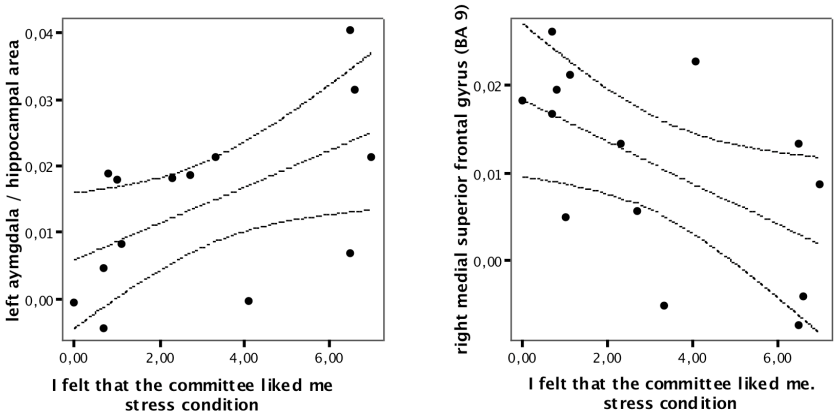

Figure 18: Ratings on the committee (liked me) (stress condition) and brain activation (Pearson correlation).

Table 10: Ratings on the committee (was satisfied with my performance) (stress condition) and brain activation (Pearson correlation).

I felt that the committee was satisfied with my performance.				
Brain area	**N**	**Correlation coefficient**	**Significance**	**Effect size**
Right medial superior frontal gyrus (BA 9)	14	r = - 0.62	p = 0.017	R = 0.38
Left amygdala / hippocampal area	14	r = 0.60	p = 0.024	R = 0.36
Left pallidum	14	r = 0.57	p = 0.035	R = 0.33
Left inferior orbitofrontal gyrus	14	r = 0.63	p = 0.016	R = 0.28

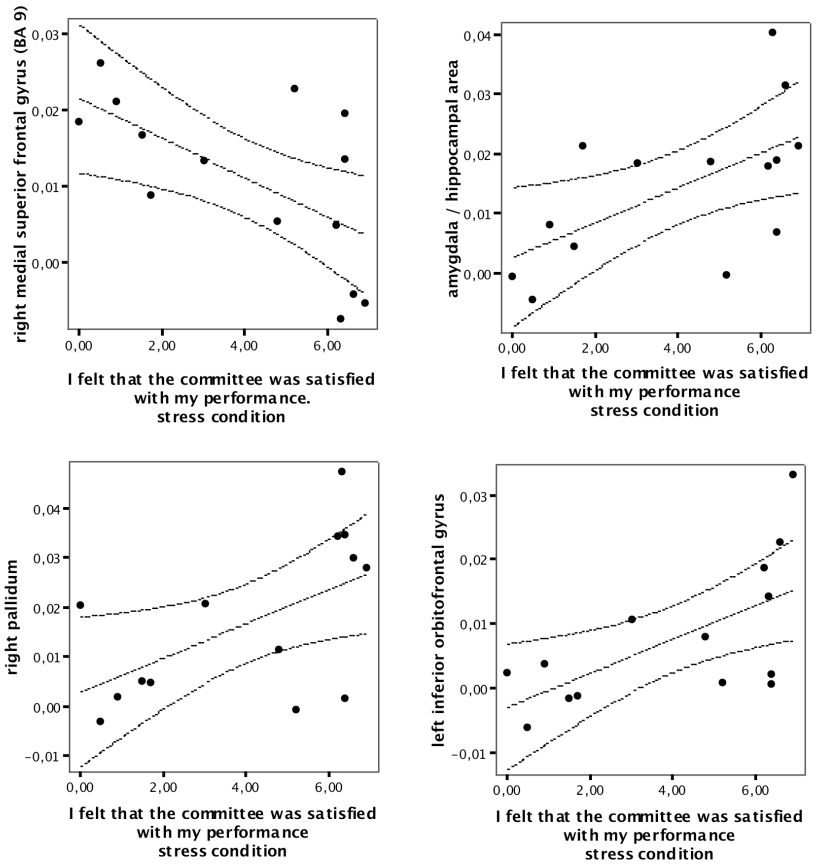

Figure 19: Ratings on the committee (was satisfied with my performance) (stress condition) and brain activation (Pearson correlation).

Table 11: Ratings on the committee (I liked the committee) (stress condition) and brain activation (Pearson correlation).

I liked the committee.				
Brain area	**N**	**Correlation coefficient**	**Significance**	**Effect size**
Right medial superior frontal gyrus (BA 9)	14	r = - 0.71	p = 0.004	R = 0.50
Left amygdala / hippocampal area	14	r = 0.73	p = 0.003	R = 0.53
Left fusiform gyrus	14	r = 0.55	p = 0.043	R = 0.30

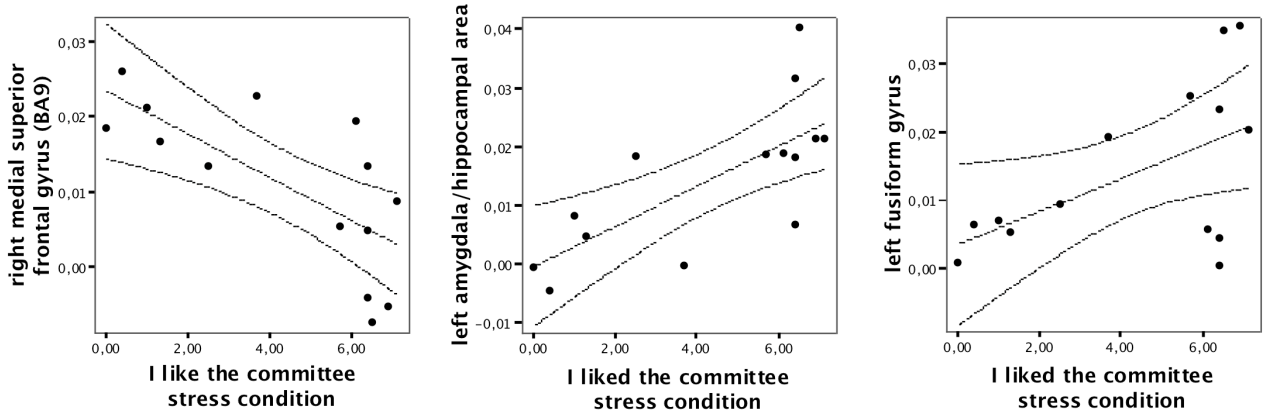

Figure 20: Ratings on the committee (I liked the committee) (stress condition) and brain activation (Pearson correlation).

Finally, association between stress-related brain activation and scores on the *Carver Coping Questionnaire* were analyzed.

Scores on the subscale *suppression of competing activities* were positively associated with the metabolic rate in the amygdala/hippocampal area (r = 0.58; p = 0.04; R = 0.34).

Scores on the *restraint coping* subscale were negatively associated with activation in BA 10 (r = 0.58; p = 0.049; R = 0.34). A positive association was found for scores on the *acceptance* subscale and the right pallidum (r = 0.57; p = 0.04; R = 0.33). Scores on the subscale assessing *focus & venting of emotion* were positively associated with activation in BA 9 (r = 0.68; p = 0.01; R = 0.46) and negative correlations were found with the metabolic rate in inferior orbitofrontal gyrus (r = - 0.74; p = 0.004; R = 0.55) and the mediotemporal gyrus (r = - 0.67; p = 0.012; R = 0.45). Scores on the subscale assessing *behavioral disengagement* showed negative associations with the metabolic rate in the precuneus (r = 0.68; p = 0.01; R = 0.46).

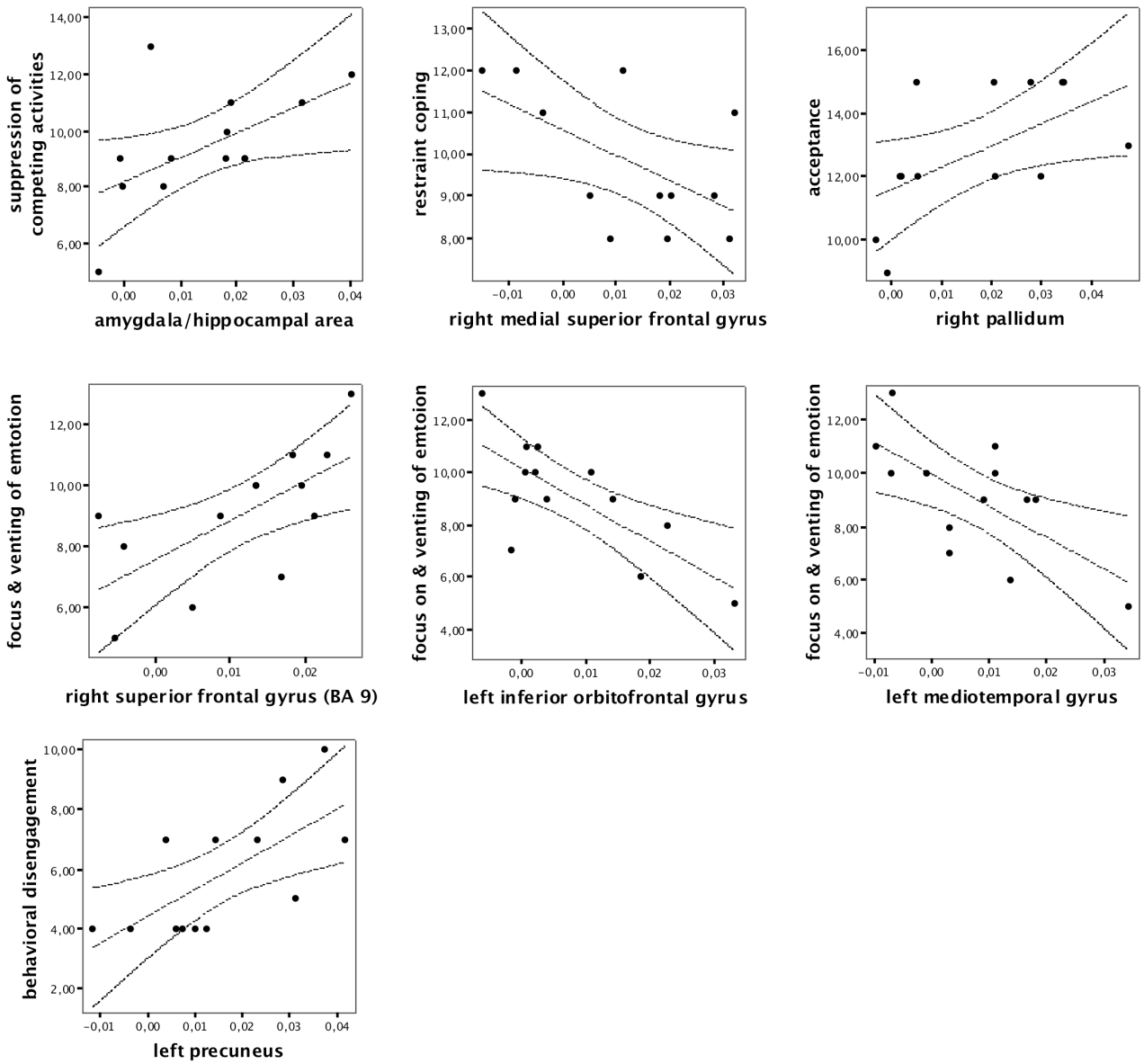

Figure 21: Coping strategies (*Carver Coping Questionnaire*) and stress associated brain activation (Pearson correlation).

4.1.5 Discussion

While much is known about the neural circuits of stress in rodents (R. M. Sullivan & Gratton, 2002b) and non-human primates (Kalin et al., 1998; Kalin et al., 2000), still little is known about brain areas involved in HPA axis regulation in humans and data reported here are among the first to give insights into the neural substrates of stress in humans.

Salivary cortisol concentrations for the stress and the control condition showed marked differences. While the stress condition was associated with the expected HPA axis activation and subsequent increases in salivary cortisol, the control condition did not provoke any meaningful salivary cortisol increases.

These data are in line with a pilot study on 10 subjects, showing that removal of the TSST panel but otherwise almost identical task requirements prevented HPA axis activation. In a very recent publication, Gruenewald and colleagues (Gruenewald et al., 2004) report similar results: A total of 81 participants underwent the TSST procedure either with (n = 41) or without (n = 40) the presence of the TSST committee. Similar to our findings, salivary cortisol increased in the condition with a social evaluative component. No such changes were observed in the non-evaluative condition. Participants who underwent the TSST with the panel present also showed greater increases in self-reported shame and more pronounced decreases in social self-esteem compared to participants in the non-evaluative condition.

These data are in line with the *Social Self Preservation Theory* proposed by Kemeny and colleagues (Dickerson et al., 2004) which states that situations which are a threat to one's social image or standing, provoke a defined set of psychological and physiological reactions such as feelings of low social worth (e.g. shame, humiliation), drop in social self-esteem and increased activity of the HPA axis.

Furthermore, these findings are in agreement with Mason (Mason, 1968), who stated that social factors, along with involvement and trying, are potential elicitors of HPA axis activation. Moreover, Mason argued that the perception of control during potentially stressful situations also influences HPA axis activation. This notion was recently supported by Dickerson and Kemeny's meta-analysis (Dickerson et al., 2004), which showed that controllability is a main predictor of HPA axis activation.

In line with these considerations, participants in this study reported the stress condition to be more stressful compared to ratings on the control condition. During the stress condition participants also reported significantly less perceived control over the situation and claimed to be less satisfied with their performance. There were also clear tendencies to perceive the stress condition more threatening and the presence of the TSST panel caused more feelings of failure and embarrassment.

Furthermore, the perception of threat was negatively associated with experienced controllability. The feelings of giving a foolish performance also showed a marked negative association with

perceived controllability. The importance to perform well was positively correlated with the subjective feelings of stress due to perceived failure.
Most strikingly, subjects reporting high levels of perceived controllability showed a clear tendency for lower salivary cortisol increases during the stress condition.
Interestingly, high ratings for *"I liked the committee"* were positively associated with the salivary cortisol response during the stress condition. This finding seems somewhat counter-intuitive as one might speculate that an atmosphere of *"liking"* would be associated with less stress. Alternatively it can be speculated that this statement does not reflect whether the participant actually liked the committee – as a matter of fact, the TSST is designed in a way that the committee acts in a distant and not very likable way - but rather points to an underlying motive characterized by a need for positive social interaction and affiliation. Although a few studies addressed the question of social motives to investigate sex differences in HPA axis response (Stroud et al., 2002; S. E. Taylor et al., 2000), data remain inconclusive and still little is known about the impact of social motives on HPA axis functioning. Moreover, concepts about these underlying motives are still poorly defined, and accurate and reliable measures are not easy to obtain. For example, in our data, high scores on the *Marlowe Crowne Social Desirability Scale* were not significantly associated with either the salivary cortisol stress response nor ratings on the committee (data not reported) and Weinberger and colleagues (Weinberger et al., 1979) point out that that the scale might actually be unrelated to the construct of social desirability but measures affect inhibition, defensiveness and protection of self-esteem.
However, an exaggerated need for social approval along with an outrageous fear of social rejection are key symptoms of patients with social phobia (Juster et al., 2000). Not surprisingly, a hyper-responsive HPA axis as been observed in this patient group in response to confrontation with a psychosocial stress test (Condren et al., 2002; Furlan et al., 2001). These findings give further evidence that certain social motives and schemata might influence HPA axis functioning in psychosocial settings. However, better methods to assess these motives as well as specifically designed experiments are needed to promote understanding in this promising field.

Confrontation with a psychosocial stress was associated with increased activity in the prefrontal cortex. When brain data were combined with endocrine measures collected right before and after the stress and the control condition, findings indicated that in response to a psychosocial stressor, a high glucose metabolic rate in mPFC areas BA 9 and 10 was inversely associated with salivary cortisol concentrations. This finding reflects that increased activation in these regions during the stress compared with the control condition was associated with significantly lower levels of salivary cortisol, suggesting that the mPFC is engaged as part of regulatory circuitry to modulate the response to a stressful stimulus. Based on the finding that the mPFC is high in GC receptor density (Sanchez et al., 2000) and corticosteroid implants in mPFC regions cause a blunted HPA axis response to stress in rats (Diorio et al., 1993), it can be speculated

that the inhibiting effects seen in the study presented here are mediated, at least in part, by GC receptors. If this hypothesis holds true, administration of a GC receptor antagonist right before the stress exposure should prevent the effects seen in our study. Consistent with this interpretation is the finding that the larger the increase in mPFC activation during the stress compared with the control condition, the less the overall general distress reported by these subjects on a questionnaire measure. Moreover, when functional connectivity analyses were conducted, it was observed that individuals with the greatest increases during the stress versus control conditions in these mPFC regions had the smallest increases during these conditions in the amygdala/hippocampus, the precuneus, the fusiform gyrus, the inferior orbitofrontal gyrus and the medial temporal gyrus. These findings thus further imply that activity in the mPFC may exert effects on HPA function through its modulation of activation elsewhere in the brain, particularly in the amygdala/hippocampus, in light of what is known about the participation of these regions in anxiety and stress (McEwen, 2003). In line with this notion, it was also found that brain regions inversely associated with BA 9 and 10 were positively associated with overall ratings of general distress und depressive symptoms.

Moreover, the level of perceived controllability was positively associated with activation in BA 9 whereas a negative association was found for activation in the inferior orbitofrontal gyrus. High scores on statements expressing a positive attitude towards the TSST committee (e.g. I like the committee. / I felt that the committee was satisfied with my performance.) were inversely correlated with the metabolic rate in BA 9 but showed positive associations with areas that were inversely correlated with BA 9 (e.g. amygdala/ hippocampal area). These data nicely line up with the finding that positive ratings in relation to the TSST committee were associated with higher cortisol levels during the stress condition. Taken together, these data give first hints that BA 9 is involved in processing information of perceived controllability and social motives.

To date, little is know about neural substrates of perceived controllability in humans, but recent rodent data indicate that medial aspects of the PFC are involved in mediating effects of controllability on the stress-induced activation of the serotonergic system (Amat et al., 2005). Although the study presented here was not specifically designed to answer questions about neural substrates of controllability, the findings are in line with animal data and give evidence that in humans, the medial prefrontal area BA 9 is involved in processing information about controllability during a psychosocial stress encounter.

Area 9 and 10 as well as the precuneus have frequently been associated with the brain's *"default network"* which has recently been linked to emotional, self-related and social processing and cognition in humans (Gusnard et al., 2001; Iacoboni et al., 2004; Raichle et al., 2001) Activation in the amygdala has been linked to the processing of socially cued information (e.g. facial expression) and somatic, autonomic and endocrine signs of fear (Davis & Whalen, 2001). These findings seem relevant in the context of this study, as the stress test chosen here reflects a psychosocial interaction situation with components of social evaluation and potential threat to

the social self. Although this experiment was not designed to specifically test for these hypothesis, data presented here, for the first time, give evidence that areas of the human brain that have frequently been associated with assessment and interpretation of social and self-related information, might actually be involved in regulating the HPA axis in situations of social evaluation and potential threat to the social self.

The influence of coping style on HPA axis activity has been frequently reported (Brown et al., 1996) although little is yet known about stressor specific effects of various coping strategies (Meichenbaum, 2003). Here, restraint coping was positively associated with the salivary cortisol increase during the stress condition, while the tendency to *focus on emotions and venting of emotions* was negatively associated with the observed stress response.
In line with these findings, the metabolic rate in BA 10 was negatively associated with scores on the restrain coping subscale, whereas the tendency to focus on and venting of emotions was positively associated with BA 10. These findings seem extremely interesting, in light of the fact that animal data indicate that medial wall areas of the PFC hold up dense projections to the periaqueductal gray (PAG) which seems to be highly involved in mediating coping behavior. In this way, the PAG is topographically organized and activation in the dorsal and lateral columns has been associated with active coping styles whereas the ventrolateral aspect seems to be involved in passive coping behavior. Anatomical data indicate BA 10 projects to the dorsolateral column and therefore seems to be involved in active coping strategies (An et al., 1998; Bandler et al., 2000b; Keay and Bandler, 2001). These animal findings are in line with data from this study showing that a tendency for a rather passive coping strategy such as restraint coping (e.g. I restrain myself from doing anything too quickly.) was negatively associated with activation in BA 10 whereas a rather active coping strategy such as *focus on and venting of emotions* (e.g. I get upset and let me feelings out.) was positively associated with activation in BA 10.

In summary, this study's findings strongly support the hypothesis that activation of the mPFC in response to stress is regulatory. Activation in mPFC regions showed a marked positive association with perceived stressor controllability and was strongly reciprocally related to salivary cortisol in response to the stressor, to limbic regions known to be involved in the HPA cascade (amygdala and hippocampus) and to a self-report measure of general distress. The direction of the association in each case supports the notion that mPFC activation in response to a stressor plays a regulatory role.
The findings presented here also highlight the fact that in response to a psychosocial stressor, large individual differences are present and such individual differences are associated with lawful patterns of neural activity. Future studies might target these individual differences as risk factors for the development of stress-related physical and psychiatric disorders.

4.2 Neural Substrates of Stress and Stress Habituation – a fMRI Study

4.2.1 Abstract

Background: The concept of stress has frequently been linked to the pathophysiology and pathogenesis of mood and anxiety disorders (Arborelius et al., 1999; Rosen & Schulkin, 1998). From a neurobiological perspective, neural structures involved in regulation of the HPA axis seem to be largely overlapping with the brain's neural fear and anxiety circuits (Coplan & Lydiard, 1998; Davis & Whalen, 2001; J. A. Gray & McNaughton, 2000; J. LeDoux, 1998; McNaughton & Corr, 2004) and glucocorticoids seem to play an important role in the expression of fear and anxiety related behavior (Korte, 2001), the consolidation of fear related memory (McGaugh, 2004; McGaugh & Roozendaal, 2002) and contextual fear conditioning (Pugh et al., 1997). In line with this notion, activation of the HPA axis and the autonomic nervous system have been regarded as a physiological response to fear and anxiety inducing stimuli (Davis & Whalen, 2001). However, the associations between the human neurobiology of fear and endocrine response patterns to stress and fear inducing states has not actually been studied. The experiment presented here therefore investigates the association between the HPA axis response magnitude to a single as well as repeated confrontation with a psychosocial stress encounter and reactivity patterns in brain areas known to be involvemed in fear responses and HPA axis regulation.

Methods: For this purpose, response patterns to two homotypic psychosocial stress exposures (Trier Social Stress Test; TSST) were investigated in a total of 90 healthy human male participants. In a follow-up experiment, brain responses (fMRI) to a threatening stimuli known to activate limbic structures involved in HPA axis regulation, were recorded in a sub-sample of the initial 90 subjects. Subsequently, associations between endocrine activation and habituation measures and threat induced brain activation were tested in the same subject.

Results: Anticipation of threatful stimuli, caused pronounced activation in areas of the brain's fear and anxiety circuitry. Most pronounced activation was seen in the ventro-medial prefrontal cortex (vmPFC), the lateral and dorsolateral PFC, the cingulate gyrus (anterior rostral and dorsal), the amygdala, striatum and brainstem areas. Data reported here indicate that excitability within the ventro-medial prefrontal cortex (vmPFC) during this stimulation was associated with the previously observed HPA axis habituation in the very same subjects. Moreover, we found evidence that activity in more lateral and dorsal aspects of the PFC were associated with affective processing of trait and state related measures of fear and anxiety.

Conclusion: Data reported here give evidence that these prefrontal areas play an important role in fear, anxiety and HPA axis habituation.

4.2.2 Introduction

In human and non-human primates as well as in other species, the perception of threat causes a orchestrated psychophysiological stress response. Key features of this response include activation of autonomic function, increased HPA axis response and subsequent release of endocrine stress markers. This coordinated psychophysiological response is a result of an orchestrated activation of the brain's fear and anxiety circuitries which involve several brain structures located in the limbic system. Inspired by the early findings of Kluver and Bucy (Kluver & Bucy, 1939), the amygdala has long been thought to play a crucial role in mediating fear and anxiety related responses. Accordingly, experiments utilizing fear conditioning paradigms have repeatedly shown that lesions to the lateral or basolateral amygdala prevent classical fear conditioning whereas lesion of the central nucleus interfere with the behavioral, endocrine and autonomic fear response (J. LeDoux, 1998; Roozendaal et al., 1991b; G. M. Sullivan et al., 2004). Based on a conceptual distinction between the construct of stimulus-specific fear and less stimulus-specific anxiety, it has further been proposed that activation of the CeA is involved in mediating stimulus-specific fear whereas activation of the bed nucleus of the stria terminalis is subject to less specific situations that are not necessarily based on conditioned but longer lasting threatening situations (Davis, 1998). It is worth mentioning that in monkeys, lesions of the amygdala result in a diminished acute unconditioned fear response but leave a trait-like anxiety-fear response unaffected (Kalin et al., 2001).

Another important brain region involved in fear and anxiety, as well as HPA axis regulation, is the mPFC. This brain region has recently been shown to inhibit HPA axis activity (Diorio et al., 1993; Figueiredo et al., 2003a) and to exert an inhibitory tone over amygdala and insula function (Quirk et al., 2003). In rats there is also evidence that this brain region is crucial for consolidation and storage of fear extinction memory (Milad & Quirk, 2002; Santini et al., 2004; Sotres-Bayon et al., 2004).

When it comes to models on the neurobiology of fear and anxiety in humans, a hyperactive amygdala along with a diminished prefrontal control has frequently been discussed (Bremner et al., 1999; Gorman et al., 2000; Hariri et al., 2003; Rosen & Schulkin, 1998). However, in PTSD patients, confrontation with trauma-related stimuli was associated with increased amygdala activation (Liberzon et al., 1999; Shin et al., 2004), no change in amygdala activity (Lanius et al., 2002; Shin et al., 1999) or a decreased amygdala activation (Britton et al., 2005). In the PFC, decreased (Britton et al., 2005; Shin et al., 1999) and increased (Lanius et al., 2002; Shin et al., 1999) activation in medial prefrontal areas has been observed. While there is some evidence for an inverse relationship between decreased mPFC activation and a hyper-reactive amygdala (Shin et al., 2004), others could not find evidence for this association in PTSD patients (Britton et al., 2005; Gilboa et al., 2004). In patients with social phobia, confrontation with, or anticipation of, phobia specific or fear inducing stimuli have been associated with increased amygdala activation (Birbaumer et al., 1998; Stein et al., 2002; Tillfors et al., 2002; Veit et al., 2002). Cortically,

decreases in brain activity and blood flow have been observed in orbitofrontal and insula cortices in this patient group (Tillfors et al., 2001). In patients suffering from panic disorder, confrontation with directed imagery of anxiety inducing situations caused increased activity in the inferior frontal cortex, the hippocampus and the anterior and posterior cingulate cortex (Bystritsky et al., 2001). Symptom provocation in patients meeting criteria for either PTSD, obsessive-compulsive disorder or simple phobia, resulted in increased activation in the inferior frontal cortex, right posterior medial orbitofrontal cortex, bilateral insular cortex and bilateral brainstem foci. Activation in the brainstem foci was positively associated with subjective anxiety scores (Rauch et al., 1997).

In healthy participants, anticipation of aversive stimuli (e.g. mild electrical shocks) has been associated with decreased activity in the medial PFC (BA 10/32 and 24/25) (Simpson et al., 2001). Others found increased activity bilaterally in the insula, the anterior cingulate cortex (Chua et al., 1999) and the ventral striatum (Jensen et al., 2003).

States of fear and anxiety have often not only been associated with activity of fear related neural brain circuitries, but also with excessive activation of autonomic and endocrine systems (Davis & Whalen, 2001). In rats, unconditioned presentation of a fear inducing substance like predator odor (2,5-dihydro-2,4,5-Trimethylthiazoline (TMT)) results in elevated levels of ACTH and corticosterone, along with increased c-fos mRNA expression in several brain areas, including parts of the bed nucleus of the stria terminalis, the central nucleus of the amygdala, the paraventricular nucleus of the hypothalamus, the nucleus of the solitary tract and the locus coeruleus (Day et al., 2004) . Moreover, in infant rat pups expression of fear to a predator odor and basolateral/lateral amygdala activity could be prematurely evoked with exogenous corticosterone (Moriceau et al., 2004). In infant rats, fear of novelty predicts magnitude in corticosterone response in middle age and early death (Cavigelli & McClintock, 2003). In monkeys, lesions of the CeA result in diminished fear-related behavior and decreases in CRH and ACTH in response to a fear-inducing stimuli (Kalin et al., 2004). Monkeys characterized by a fearful temperament and relative right asymmetric frontal brain activity showed higher basal cortisol concentrations as well as increased cerebrospinal fluid CRH concentrations (Kalin et al., 1998; Kalin et al., 2000).

In humans, children with an extremely inhibited behavioral style exhibit higher cortisol levels (Kagan et al., 1987; Schmidt et al., 1997) and exaggerated startle responses (Schmidt et al., 1997). Subjects characterized by high levels of trait anxiety or a repressive style show elevated basal cortisol levels (Brown et al., 1996). Moreover, trait negative affect also seems to be associated with higher total cortisol levels and increased morning rise in males (Polk et al., 2005). In patients with a diagnosis of social phobia, higher cortisol concentrations in response to a psychosocial stress encounter have been observed (Furlan et al., 2001; Martel et al., 1999). There is also evidence, that patients with a diagnosis of PTSD, express a HPA axis hyper-responsiveness to stressful challenges (Yehuda, 1997). In anticipation of a stressful situation,

anticipatory appraisal of threat and challenge were significant predictors of HPA axis response during the stressor in healthy men (Gaab et al., 2005).

While these findings show that there is much evidence for a close relationship between fearful/anxious affect and a hyper-reactive HPA axis, little is known about the underlying neurobiological correlates in humans.

It has been hypothesized that fear can be regarded as the major normal precursor for the development of pathological anxiety, which is conceptualized as an exaggerated fear state in which hyper-excitability of fear circuits that include the amygdala and extended amygdala is expressed as hyper-vigilance and increased behavioral responsiveness to fearful stimuli (Rosen & Schulkin, 1998). It has further been argued that chronic elevation of corticosterone and central CRH, due to constant states of anticipatory anxiety, represents a condition of allostatic load (Schulkin et al., 1998b), and promotes hyper-excitability in the brain's stress and fear circuitries.

In the light of these theoretical models, we investigated whether hyper-vigilance in the brain's fear circuit during a threatening confrontation is associated with hyper-responsitivity in HPA axis activation due to a single or repeated confrontation with a psychosocial stressor. Within the fear circuitry, brain regions that are known to influence HPA axis activity like the prefrontal cortex, the amygdala region and brainstem areas are of major interest. The hypothesis holds, that participants with a pronounced HPA axis activation during both stress tests do show a pattern of hyper-activity in these a fore mentioned brain structures during the threat inducing scanning paradigm when compared to participants with a less pronounced HPA axis response pattern. It is further assumed, that individuals with high cortisol profiles in response to a psychosocial stress paradigm show elevated levels of trait anxiety and fearfulness.

4.2.3 Methods

Study 1 - Participants

One hundred human male subjects, recruited by posting flyers at university buildings, participated in the study. All participants were screened on the phone and reported to be right handed (Chapman & Chapman, 1987) and non-smokers. All participants invited to the lab reported having any history of psychoactive substance use, head trauma, neurological, psychiatric, allergic, metabolic or cardiovascular disorder were excluded. People with previous experience of claustrophobia or any sort of metallic implants were excluded as well.

Out of the 100 participants taking part in the first stress test, one had to be excluded due to high scores on the BDI and MASQ items on suicidal tendencies. Out of the remaining 99, 90 returned for a second stress exposure and completed a total of two stress sessions.

One subject had to be excluded from further data analysis because of one missing post stress saliva sample. Another subject was excluded because he had major difficulties following the experimental instructions. Two more subjects were identified as outliers in their cortisol response

and were therefore excluded from data analysis. A final subset of 86 subjects went into data analysis. Subjects were on average 22.22 years (SD ± 4.31) of age and had a mean BMI of 23.42 (SD ± 2.83).

Study 1 - Experimental Procedure

All participants were screened on the phone for eligibility. Participants who meet all inclusion criteria were then invited for the first part of the study. Prior to their first experimental session, participants received instructions to refrain from extended physical exercise and large meals in the hours before taking part in the study. Participants were also instructed not to consume any alcoholic or caffeinated beverages 2 hours prior to participation in the study. These instructions were given as it was assumed that the mentioned activities could cause elevated endocrine baseline values.

This first part of the study involved participation in two stress tests that were scheduled exactly one week apart. All test were performed in the afternoon hours between 12.30 pm and 6.00 pm. In order to control for diurnal variation, each participant was exposed to the stress test at the same time of the day.

When participants entered the lab, prior to the application of any experimental procedure, written informed consent was obtained and any remaining questions were answered. The study protocol was reviewed and approved by the University of Wisconsin – Madison Human Subject Committee.

After written informed consent was obtained, the experiment began, and the first out of six saliva samples was collected. Next, participants were requested to sit and rest for a total of 20 minutes. During this time, questionnaires were filled out and grape juice was offered. Twenty minutes after the first saliva sample, a second baseline sample was taken and participants were then guided to a nearby room where the stress test was performed. After the stress test was over, participants were guided back to their room and the first out of 4 post-treatment samples was obtained. Next, an in-house questionnaire assessing subjective ratings on the stress situation was given. Three more post-treatment samples were collected in intervals of 10 minutes. During this time, more questionnaires were given.

At the end of the first session, people were reminded of their second appointment. A debriefing was offered but, participants were instructed that a debriefing at this point would prevent participation in the second part of the study.

The experimental designs of the two sessions were matched. Only the nature of the stress test was slightly manipulated and different questionnaires were given during the second session. At the end of session 2, subjects were debriefed about the nature of the stress tests. Each participant received a total of 50 US-$ for participation in both sessions.

Study 1 - Stress test

The Trier Social Stress Test (TSST) was used as experimental stress protocol (Kirschbaum et al., 1993). Prior to the experiment, subjects were told that they would have to undergo a short stress test but no further information was given. The stress test consisted of 3 minutes of preparation, 5 minutes of free speech and 5 minutes of mental arithmetic. All tasks had to be performed in the presence of an audience (one male and one female person) and a running video camera. Participants were also requested to speak into a stand-up microphone.
Instructions for the two TSSTs were slightly varied in order to prevent learning effects. For TSST1 subjects were asked to apply for their dream job and counting backwards from 2043 in steps of 17 was requested. For TSST2 subjects were asked to apply for participation in a game show and had to count backwards from 2077 in steps of 14.

Study 1 - Salivary cortisol sampling and analysis

A total of 6 saliva samples were collected. Sampling times were 20 minutes and one minute prior to the stress test. Post treatment samples were collected right after and 10, 20 and 30 minutes after the TSST was over (see figure 22).

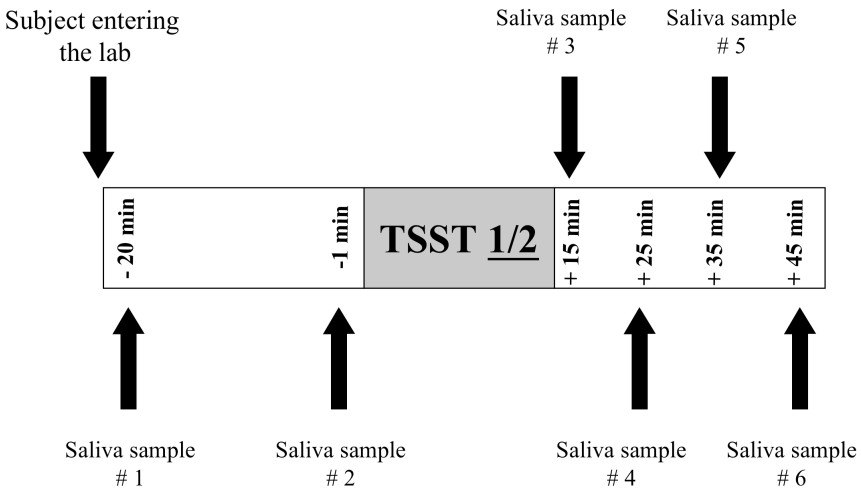

Figure 22: Timing for cortisol sampling.

Salivettes® (Saarstedt, Germany) were used for specimen collection. Salivettes® were stored at – 20 ° degree Celsius until samples were analyzed for salivary cortisol (nmol/L) using a luminocent immuno assay (IBL, Hamburg, Germany). The intra-assay and inter-assay variability have been shown to be < 8 % respectively.
A score of maximum salivary cortisol increase ($max_{increase}$) was calculated for each subject and for each stress test (TSST1 & TSST2) by subtracting the lowest baseline sample (sample 1 or sample 2) from the maximum post-treatment sample (sample 3 – 6). Additionally, an index for the magnitude of change between TSST1 and TSST2 was calculated (*Maximum increase*

change ($max_{inc\text{-}change}$) = ($max_{increaseTSST2} - max_{increaseTSST1}$) / $max_{increaseTSST1}$). Low $max_{inc\text{-}change}$ scores (below 0) indicate that habituation from TSST1 to TSST2 took place. High $max_{inc\text{-}change}$ (0 and above) indicate that no habituation took place. Responder groups were determined by applying a mean split to the $max_{inc\text{-}change}$ variable. All subjects located below the $max_{inc\text{-}change}$ mean were grouped as habituators (HAB) whereas all subjects located above the $max_{inc\text{-}change}$ mean were grouped as no-habituators (No-HAB).

Salivary cortisol levels for the two stress sessions were compared using analysis of variance (ANOVA) for repeated measures. All data were reported Greenhouse-Geisser corrected if sphericity assumptions were violated. $Max_{increase}$ scores for the two stress sessions were compared *with a paired t-test.* Associations between psychometric measures and cortisol data were tested by using two-tailed Spearman correlations. Effects at a statistical significance level of $p = 0.05$ were considered meaningful and all data are presented as standard errors of means (SEM). The entire statistical analysis were performed using SPSS 11 for Macintosh OS X.

Study 1 - Psychological Assessment

Each participant filled out questionnaires. The *Beck Depression Inventory* (BDI) (Beck et al., 1961) and the *Mood and Anxiety Symptom Questionnaire* (MASQ) (D. B. Watson & Clark, 1991) were given at the beginning of session one in order to exclude subjects with suicidal tendencies prior to confrontation with the stress test.

More questionnaires were given after the first and prior as well as after the second stress test. Amongst others, each participant filled out the following questionnaires: The *Marlowe Crowne Social Desirability Scale* assessing social desirability and protection and prevention of threat to social self-esteem from anticipated social rejection (Crowne & Marlowe, 1960; Pauls & Stemmler, 2003). The *Fear Survey Schedule* (FSS) (Wolpe & Lang, 2003) measuring fear tendencies and the *Taylor Manifest Anxiety Scale* (TMAS) (J. A. Taylor, 1953) assessing trait anxiety.

Study 2 - Participants

After data collection for study one was complete, all subjects were contacted for participation in a follow-up experiment. On average 12 months after confrontation with two stress test, a total of 38 subjects were brought back to the lab for an fMRI brain scan.

From this initial sample, one subject was excluded from further data analysis as he frequently had his eyes closed during the scan session. Two more subjects had to be excluded due to severe motion artifacts. Another participant had to be excluded due to problems during data acquisition. One more subject was identified as an outlier after ROI data inspection. A total of 33 subjects went into the final data analysis. These subjects were on average 21.73 years old (SD ± 4.45) and had an average BMI of 23.01 (SD ± 2.28).

Study 2 - Psychological Assessment

Prior to the scan procedure, each participant filled out the *Spielberger Trait and State Anxiety Inventory* (Spielberger, 1983) assessing state as well as trait anxiety levels.

The state version of the *Positive and Negative Affect Schedule* (PANAS) (D. Watson et al., 1988) assessing the momentary affective state was also given right before as well as right after the scanning procedure.

Also right after the scan, anxiety ratings for the first and the last shock were assessed on a visual rating scale (0 – 100).

Study 2 - Experimental Procedure

Each participant taking part in the follow-up experiment reported to the laboratory for a total of two sessions. The first visit was scheduled at least one week prior to the actual scan session with a main purpose of informing each participant about the experimental procedure. At the beginning of this session, written informed consent was obtained from each participant. The study was approved by the Human Subjects Committee of University of Wisconsin – Madison.

In order to test for claustrophobic reactions, each participant was then introduced to a mock scanner and a scan simulation was performed. None of the 38 participants showed any signs of claustrophobia. At this stage, each participant was also carefully screened for not having any metal in or on their body and MRI security procedures were explained in detail.

Session two comprised the actual fMRI scan. Each participant reported to the lab a total of 30 minutes prior to the actual scan and was seated in a quite room. MRI security procedures were performed one more time and questionnaires (STAI and PANAS state) were given.

The participant was then introduced to the MRI environment and was positioned on the MRI bed. An fiber optic visual system (Avotec, Stuart, Florida) for stimulus presentation was positioned and adjusted above each eye. Electrodes for skin conductance recording (data presented elsewhere) and shock administration were attached on the left and right hand. Shock electrodes (Coulborn Instruments, L.L.C., Allentown, Pennsylvania) were always attached to the small finger and the ring finger of the right hand. Shocks were administered via an isolated physiological stimulator (Coulborn Instruments, L.L.C., Allentown, Pennsylvania).

Subjects where then positioned in the scanner and the anatomical scanning procedure was performed followed by a first fMRI paradigm (data shown elsewhere) which was then followed by the actual scanning paradigm called *“threat of shock”.*

Study 2 - Scan Paradigm

The *threat of shock paradigm* lasted for a total duration of 10.36 minutes. The paradigm involved repeated, randomized presentation of two different male facial pictures. Presentation time was 3 seconds. One of the faces indicated a threat condition while the other face indicated a safe condition. The threat face was labeled by an orange background color whereas the non-threat

face was labeled by a green background color. Faces were presented in form of a black and white photograph which was each adjusted for brightness and luminosity. Each face was either used as a threat or a non-threat face and conditions were randomized between participants.

Each face presentation was followed by blank screen with a white fixation cross in the middle for a random duration ranging between 8 – 12 seconds. If a face indicating a threat condition was shown prior to the fixation screen, shocks were likely to happen any time during the 8 – 12 minute period. If a face indicating a non-threat condition was shown prior to the fixation screen no shocks were given during this period of time (figure 23). Each *face – fixation screen sequences* were separated by a black slide that was presented for a total of 2 seconds. A total of 20 threat and 20 non-threat sequences where shown. Participants were told that during the threat conditions, shocks would be given with a chance of roughly 20 %. During the actual experiment, each participant only received a total of 3 shocks. Shocks were given after the 3rd, the 10th and 15th threat face was presented. Shocks were administered with a duration of 20 ms at 4 mA causing a feeling of mild unpleasantness.

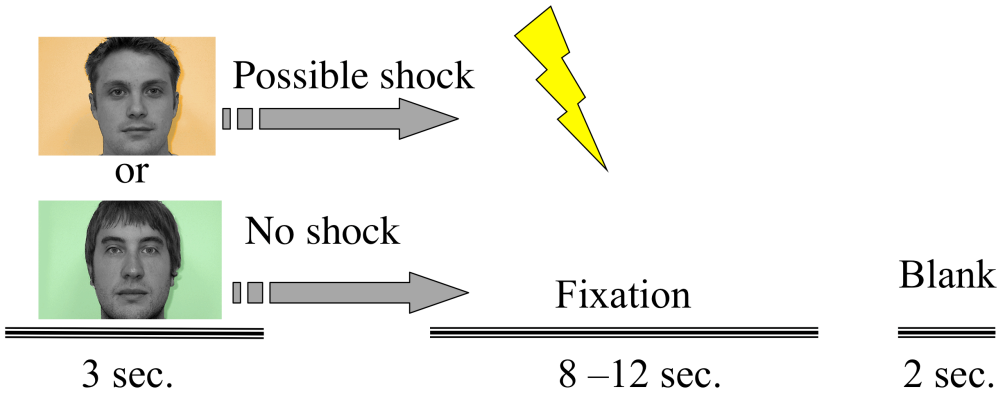

Figure 23: Threat of shock scan paradigm design.

Study 2 - MRI data acquisition and analysis

Anatomical and functional imaging was performed using a 3 Tesla GE Sigma MRI. T1-weighted anatomicals for superposition on activation images were collected by using a whole brain axial 3D SPGR (TE 1.8 ms, TR 8.9 ms, flip angle = 10 °; slice thickness = 1.2 mm, gap = 0) with a total of 124 slices and a field of view of 256 x 256 mm. A coronal 2D spin echo co-planar (TE 18 ms, TR 500 ms, flip angle = 90 °, slice thickness = 4 mm, gap = 1 mm) with a total of 30 slices and a field of view of 256 x 128 was also collected in a coronal direction (anterior – posterior). Functional images were collected using a multi slice echo planar coronal (anterior-posterior) imaging technique (TE = 60 ms, TR = 2000 ms, slice thickness = 4 mm, gap = 1 mm) with a total of 30 slices (partial brain acquisition) and a field of view of 64 x 64 mm.

Head movement was tried to control for by using a vacuum pillow which fixated the subjects head within the head coil.

Analysis was carried out using FEAT (FMRI Expert Analysis Tool) Version 5.1, part of FSL (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl). Higher-level analysis was carried out using FLAME (*FMRIB's Local Analysis of Mixed Effects*) (Behrens et al., 2003). Z (Gaussianised T/F) statistic images were thresholded using clusters determined by Z > 2.3 and a corrected cluster significance threshold of P = 0.01 (Forman et al., 1995; Friston et al., 1994b; Worsley et al., 1992). Cluster information (maximum and mean values) were extracted using *avwmath* and *avwstats* tools from the FSL package. For cluster extraction, the maximum activated voxel within each cluster of interest was selected and mean as well as maximum values (Z-scores) within a radius of 2 voxels were extracted for each participant. Extracted cluster information was further analyzed in SPSS11 for Macintosh OS X. Correlations between brain activation and physiological measures of cortisol and psychometric measures of fear and anxiety were performed using two-tailed Spearman correlations.

4.2.4 Results

Study 1 - Results

Study 1 - Saliva Cortisol

Both TSSTs resulted in significant saliva cortisol increases (*TSST1: F (5, 430) = 98.11, p = < 0.001; TSST2: F (5, 430) = 43.96, p = < 0.001*) with stronger increases at TSST1 (*F (5, 430) = 12.72, p = < 0.001*).

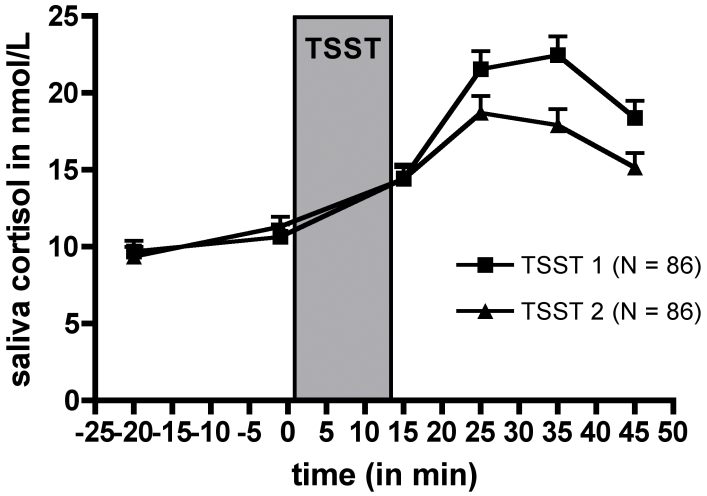

Figure 24: Saliva cortisol response for TSST1 and TSST2.

The mean $max_{increase}$, an index for the individual maximum HPA axis response, was significantly lager at TSST1 than at TSST2. The absolute difference in $max_{increase}$ between TSST1 and TSST2 was 4.22 nmol/L, which reflected a decrease of 26.47 %. Statistical data for this comparison is presented in table 12.

Table 12: Maximum salivary cortisol increase at TSST1 and TSST2.

Study 1 – total sample	**N**	**Mean**	**SE**	**t-value**	**df**	**p-value**
$Max_{increaseTSST1}$	86	15.94	1.01	4.72	85	< 0.001
$Max_{increaseTSST2}$	86	11.72	1.11			

Maximum increases at TSST1 and TSST2 were positively associated (*r = 0.65, p = < 0.001, R = 0.42*), while no statistically meaningful association between $max_{increaseTSST1}$ and $max_{inc\text{-}change}$ was observed (r = - 0.11; p = n.s.).

Study 1 - Saliva cortisol and psychometric measures of fear and anxiety

No meaningful associations were found for either *maximum increase (max$_{increase}$)* or *habituation* (max$_{inc\text{-}change}$) and overall trait anxiety (*Taylor Manifest Anxiety Inventory*), overall fear tendencies (*Fear Survey Schedule-III*), general distress (*MASQ*), anxious arousal (*MASQ*), anxious symptoms (*MASQ*), and *perceived threat* at TSST1 or TSST2 (*VAS*).

However, high scores on the the *Marlowe Crowne Social Desirability Scale* corresponded to high max$_{inc\text{-}change}$ scores, reflecting that participants with high scores on this scale showed poorer habituation. Corresponding Spearman correlation coefficients are reported in table 13.

Table 13: Saliva cortisol at TSST1 and TSST2 and psychometric measures of fear and anxiety (Spearman correlation).

Study 1 – total sample	**Maximum increase TSST1**	**Maximum increase TSST2**	**Maximum increase change**
MASQ anxious arousal	r = -0.06 n.s. N = 86	r = -0.08 n.s. N = 86	r = -0.06 n.s. N = 86
MASQ anxious symptoms	r = -0.19 n.s. N = 86	r = -0.06 n.s. N = 86	r = -0.07 n.s. N = 86
MASQ general distress	r = -0.13 n.s. N = 86	r = -0.11 n.s. N = 86	r = -0.07 n.s. N = 86
Fear Survey Schedule-III	r = - 0.002 n.s. N = 86	r = -0.01 n.s. N = 86	r = -0.14 n.s. N = 86
Taylor Manifest Anxiety Scale	r = - 0.03 n.s. N = 86	r = - 0.12 n.s. N = 86	r = - 0.08 n.s. N = 86
Marlowe Crowne Social Desirability	r = 0.11 n.s. N = 86	r = 0.14 n.s. N = 86	**r = 0.28** **p = 0.009** **N = 86** **R = 0.08**
Perceived Threat TSST1 (VAS)	r = 0.05 n.s. N = 86	r = - 0.05 n.s. N = 86	r = - 0.12 n.s. N = 86
Perceived Threat TSST2 (VAS)	r = - 0.01 n.s. N = 86	r = - 0.02 n.s. N = 86	r = 0.21 n.s. N = 86

Study 1 - Responder groups

Responder groups were defined by a mean split on the $max_{inc\text{-}change}$ variable. The mean for $max_{inc\text{-}change}$ was – 0.21. Subjects located above the mean were grouped as no-*habituators* (No-HAB; n = 35). Subjects located below the mean were grouped as *habituators* (HAB; n = 51).

Both groups (HAB and No-HAB) showed significant cortisol increases to both TSSTs (repeated measures ANOVA main effect of time HAB TSST1: F(5, 250) = 64.66, p = < 0.001; No-HAB TSST1: F(5, 170) = 35.98, p = < 0.001; HAB TSST2: F(5, 250) = 14.00, p = < 0.001; No-HAB TSST2: F(5, 170) = 37.99, p = < 0.001).

Groups did not differ in their response to the first TSST (*repeated measures ANOVA group x time TSST1: F (5, 420) = 0.36, p = n.s.*). The No-HAB group had a significantly higher response at TSST2 (*repeated measures ANOVA group x time TSST2: F (5, 420) = 14.61, p = < 0.001*).

Response curves for the HAB and No-HAB group are shown in figure 25. Mean values for each sample are listed in table 14.

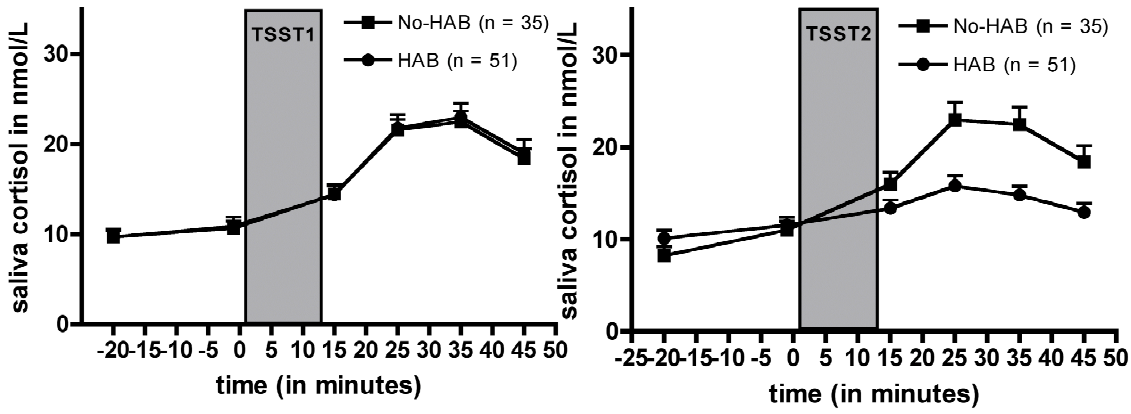

Figure 25: Saliva cortisol for the HAB (n = 51) and No-HAB (n = 35) group for TSST1 and TSST2.

Table 14: Mean saliva cortisol values in nmol/L for each sample and each TSST for the HAB and No-HAB group respectively. Data are shown for the total sample of 86 subjects.

Study 1 – total sample	**Mean**	**SE**	**Sample size**	**Mean**	**SE**	**Sample size**
	HAB			**No-HAB**		
TSST1						
Sample 1	9.70	0.87	51	9.70	1.14	35
Sample 2	10.89	1.03	51	10.33	1.21	35
Sample 3	14.38	1.15	51	14.55	1.47	35
Sample 4	21.76	1.50	51	21.26	1.89	35
Sample 5	22.98	1.57	51	21.73	1.88	35
Sample 6	19.00	1.52	51	17.49	1.53	35
TSST 2						
Sample 1	10.12	0.83	51	8.25	0.95	35
Sample 2	11.54	0.84	51	11.00	0.93	35
Sample 3	13.36	0.89	51	16.00	1.24	35
Sample 4	15.80	1.11	51	22.97	1.93	35
Sample 5	14.81	0.92	51	22.43	1.91	35
Sample 6	12.95	0.91	51	18.39	1.74	35

Groups did not significantly differ in $max_{increase}$ at TSST1 but at TSST2. For the HAB group, absolute difference in $max_{increase}$ between TSST1 and TSST2 was 8.90 nmol/L, which reflected a decrease of 54.01 %. For the No-HAB group, absolute difference in $max_{increase}$ between TSST1 and TSST2 was – 2.60 nmol/L which reflected an increase of 17.16 %. Statistical data for this comparison is presented in table 15.

Table 15: Difference in maximum cortisol increase for HAB and No-HAB group for TSST1 and TSST2 (independent t-test).

Study 1 – total sample	**HAB Maximum salivary cortisol increase**	**No-HAB Maximum salivary cortisol increase**
TSST1		
N	51	35
Mean	16.48	15.15
SE	1.31	1.61
t-value	-0.643	
df	84	
p-value	n.s.	
TSST2		
N	51	35
Mean	7.58	17.75
SE	1.05	1.86
t-value	4.76	
df	84	
p-value	0.001	

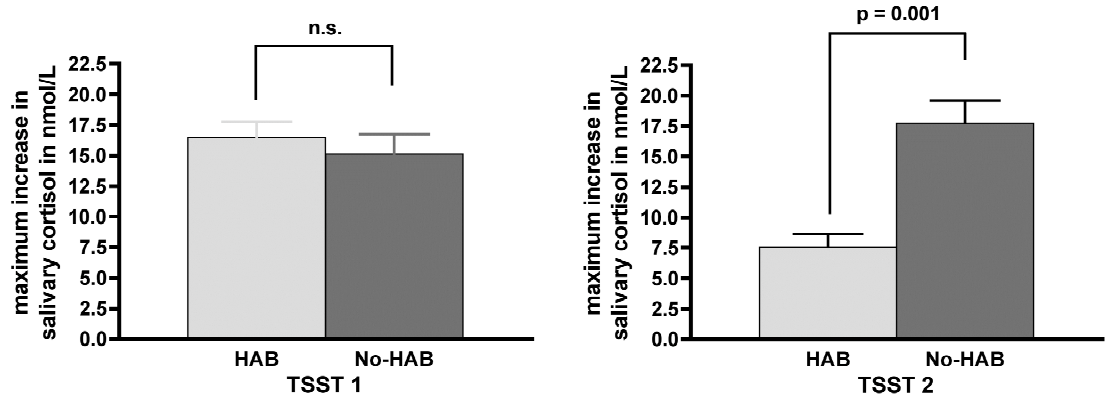

Figure 26: Maximum cortisol increase for the HAB (n= 51) and No-HAB (n= 35) group at TSST1 and TSST2.

Study 1 - Responder groups and psychometric measures of fear and anxiety

The two responder groups (HAB & No-HAB) did not differ in any psychometric measure of fear and anxiety. Groups also did not differ in ratings on perceived threat (*VAS*) during either of the two stress exposures. Results are stated in table 16:

Table 16: Responder groups (HAB - No-HAB) and psychometric measures of fear and anxiety (independent sample t-test).

Study 1 – total sample	**Mean difference**	**SE**	**N**	**t-value**	**df**	**p-value**
MASQ anxious symptoms	-0.53	1.07	86 HAB n = 51 / No-HAB n = 35	- 0.50	84	n.s.
MASQ anxious arousal	0.11	0.93	86 HAB n = 51 / No-HAB n = 35	0.12	84	n.s.
MASQ general distress	- 1.60	1.81	86 HAB n = 51 / No-HAB n = 35	- 0.88	84	n.s.
Fear Survey Schedule-III	- 1.77	3.96	86 HAB n = 51 / No-HAB n = 35	- 0.45	84	n.s.
Marlowe Crowne Social Desirability	1.88	1.02	86 HAB n = 51 / No-HAB n = 35	1.84	84	n.s.
Taylor Manifest Anxiety	- 2.63	1.58	86 HAB n = 51 / No-HAB n = 35	-1.66	84	n.s.
Threat TSST1 (VAS)	0.45	0.65	86 HAB n = 51 / No-HAB n = 35	0.69	84	n.s.
Threat TSST2 (VAS)	0.29	0.57	86 HAB n = 51 / No-HAB n = 35	0.50	84	n.s.

Study 2 - Results

Study 2 – Participants

Participants who returned for study 2 versus participants who decided not to take part in the follow-up experiment did not differ in (independent sample t-test) *age* (t (87) = - 0.79; p = n.s.); *BMI* (t (87) = - 1.14; p = n.s.), $max_{increaseTSST1}$ (t (87) = -0.43; p = n.s.), $max_{increaseTSST2}$ (t (87) = - 0.46; p = n.s.), $max_{inc\text{-}change}$ (t (87) = 1.67; p = n.s.) or any psychometric measure of fear and anxiety (*MASQ*: *anxious arousal* (t = - 0.71; p = n.s.); *anxious symptoms* (t (87) = - 0.49; p = n.s.); *general distress* (t (87) = - 0.31; p = n.s.); *Taylor Manifest Anxiety Scale* (t (87) = - 1.35; p = n.s.); *Marlowe Crowne Social Desirability Scale* (t (87) = 0.83; p = n.s.); *Fear Survey Schedule-III* (t (87) = 0.38; p = n.s.); *perceived threat at TSST1* (t (87) = 0.14; p = n.s.); *perceived threat at TSST2* (t (87) = 0.98; p = n.s.).

Study 2 - Saliva Cortisol

For the sub-sample of 33 subjects undergoing a follow-up fMRI scan, both TSSTs resulted in significant saliva cortisol increases (*TSST1: F (5, 160) = 27.49, p = < 0.001; TSST2: F (5, 160) = 4.41, p = < 0.001*) with a more pronounced response at TSST1 (*F (5, 160) = 4.41, p = 0.005*).

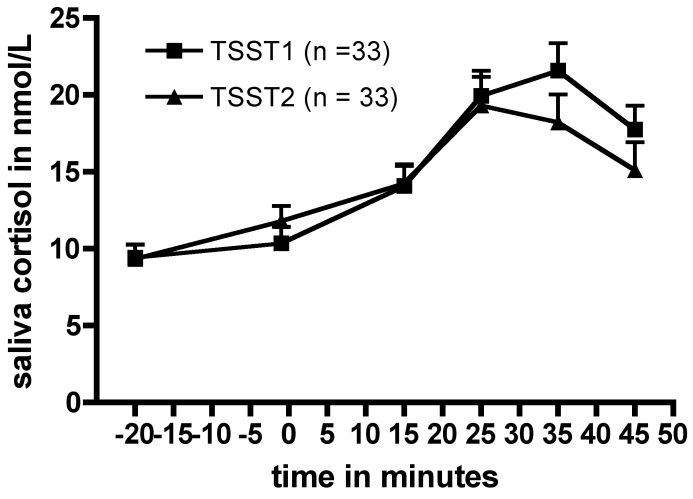

Figure 27: Saliva cortisol response for TSST1 and TSST2 for a subset of 33 subjects undergoing a follow-up fMRI scan.

For the scan sub-sample, the mean maximum salivary cortisol increase at TSST1 was significantly larger than at TSST2. The absolute difference in $max_{increase}$ between TSST1 and TSST2 was 3.33 nmol/L, which reflected a decrease of 21.36 %. Statistical data for this comparison is presented in table 17.

Table 17: Maximum salivary cortisol increase at TSST1 and TSST2.

Study 2 – Scan sub-sample	**N**	**Mean**	**SE**	**t-value**	**df**	**p-value**
$Max_{increaseTSST1}$	33	15.59	1.67	2.89	32	0.007
$Max_{increaseTSST2}$	33	12.26	2.08			

$Max_{increase}$ at TSST1 and TSST2 were positively associated (*r = 0.83, p = < 0.001, R = 0.69*), while no statistically meaningful association between $max_{increaseTSST1}$ and $max_{inc\text{-}change}$ was observed (r = 0.14; p = n.s.).

Study 2 - Saliva cortisol and psychometric measures of fear and anxiety

High scores on the *MASQ* subscale *anxious arousal* were negatively associated with $max_{increaseTSST1}$. Scores on the *Marlowe Crowne Social Desirability Scale* were positively associated with $max_{increaseTSST2}$. No meaningful associations between anxiety ratings on the *MASQ* subscales *anxious symptoms* and *general distress* and cortisol were found. Ratings on *perceived threat* (*VAS*) during TSST1 but not TSST2 were associated with max_{inc_change}. Corresponding Spearman correlation coefficients are shown in table 18.

Table 18: Saliva cortisol at TSST1 and TSST2 and psychometric measures of fear and anxiety (Spearman correlation).

Study 2 – scan sub-sample	**Maximum increase TSST1**	**Maximum increase TSST2**	**Maximum increase change**
MASQ anxious arousal	**r = - 0.41** **p = 0.017** **N = 33** **R = 0.17**	r = - 0.25 n.s. N = 33	r = - 0.15 n.s. N = 33
MASQ anxious symptoms	r = - 0.21 n.s. N = 33	r = - 0.25 n.s. N = 33	r = - 0.25 n.s. N = 33
MASQ general distress	r = - 0.19 n.s. N = 33	r = - 0.18 n.s. N = 33	r = - 0.11 n.s. N = 33
Fear Survey Schedule-III	r = 0.19 n.s. N = 33	r = 0.01 n.s. N = 33	r = - 0.21 n.s. N = 33
Taylor Manifest Anxiety Scale	r = - 0.11 n.s. N = 33	r = - 0.20 n.s. N = 33	r = - 0.20 n.s. N = 33
Marlowe Crowne Social Desirability	r = 0.32 n.s. N = 33	**r = 0.42** **p = 0.015** **N = 33** **R = 0.18**	r = 0.13 n.s. N = 33
Perceived Threat TSST1 (VAS)	r = 0.20 n.s. N = 33	r = 0.30 n.s. N = 33	**r = 0.39** **p = 0.026** **N = 33** **R = 0.15**
Perceived Threat TSST2 (VAS)	r = - 0.01 n.s. N = 33	r = - 0.04 n.s. N = 33	r = 0.24 n.s. N = 33

Study 2 - Responder groups

Based on the initial grouping performed for the entire sample of 86 subjects, 19 out of the 33 follow-up participants belonged to the No-HAB group. The remaining 14 belonged to the HAB group.

Both groups (HAB and No-HAB) showed significant cortisol increases at TSST1 (*repeated measures ANOVA main effect of time HAB TSST1: F(5, 65) = 9.72, p = 0.001; No-HAB TSST1: F(5, 90) = 18.38, p < 0.001*). At TSST2 only the No-HAB group showed a significant cortisol increase (*repeated measures ANOVA main effect of time HAB TSST2: F(5, 65) = 1.22, p = n.s.; No-HAB TSST2: F(5, 90) = 19.36, p < 0.001*).

Groups did not differ in their response to the first TSST (*repeated measures ANOVA group x time TSST1: F (5, 155) = 1.09, p = n.s.*). The No-HAB group had a significantly higher response at TSST2 when compared to the HAB group (*repeated measures ANOVA group x time TSST2: F (5, 155) = 7.59, p < 0.001*).

Response curves for the HAB and the No-HAB groups for both TSSTs are shown in figure 28 The mean saliva cortisol values in nmol/L for each time point of measurement are listed in table 19.

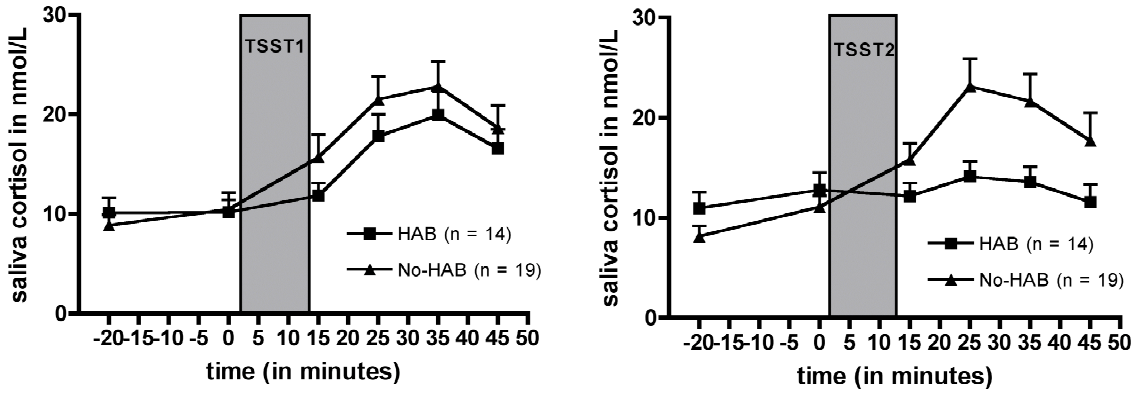

Figure 28: Saliva cortisol for the HAB (n= 14) and No-HAB (n = 19) group for TSST1 and TSST2.

Table 19: Mean saliva cortisol values in nmol/L for each sample and each TSST for the HAB and No-HAB group respectively. Data are shown for the scan sub-sample of 33 subjects.

Study 1 – total sample	**Mean**	**SE**	**Sample size**	**Mean**	**SE**	**Sample size**
	HAB			**No-HAB**		
TSST1						
Sample 1	10.13	1.52	14	8.89	1.02	19
Sample 2	10.19	1.21	14	10.48	1.66	19
Sample 3	11.85	1.27	14	15.75	2.20	19
Sample 4	17.82	2.18	14	21.52	2.32	19
Sample 5	19.93	2.48	14	22.81	2.50	19
Sample 6	16.58	1.94	14	18.65	2.27	19
TSST 2						
Sample 1	10.97	1.55	14	8.15	1.03	19
Sample 2	12.78	1.73	14	11.10	1.09	19
Sample 3	12.17	1.29	14	15.80	1.66	19
Sample 4	14.13	1.48	14	23.15	2.74	19
Sample 5	13.59	1.47	14	21.64	2.71	19
Sample 6	11.57	1.78	14	17.71	2.76	19

Groups did not significantly differ in $max_{increase}$ at TSST1 but at TSST2. For the HAB group, absolute difference in $max_{increase}$ between TSST1 and TSST2 was 8.65 nmol/L, which reflected a decrease of 63.88 %. For the No-HAB group, absolute difference in $max_{increase}$ between TSST1 and TSST2 was – 0.59 nmol/L, which reflected an increase of 3.45 %. Statistical data for this comparison is presented in table 20.

Table 20: Difference in maximum cortisol increase for HAB (n = 14) and No-HAB (n = 19) group for TSST1 and TSST2 (independent t-test).

Study 2 – scan sub-sample	**HAB Maximum salivary cortisol increase**	**No-HAB Maximum salivary cortisol increase**
TSST1		
N	14	19
Mean	13.54	17.10
SE	2.25	2.37
t-value	1.06	
df	31	
p-value	n.s.	
TSST2		
N	14	19
Mean	4.89	17.69
SE	1.87	2.76
t-value	3.55	
df	31	
p-value	0.001	

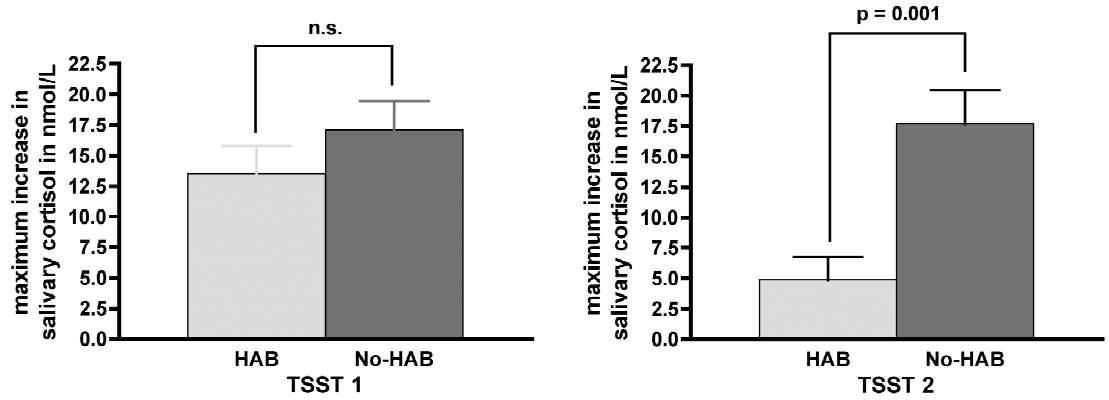

Figure 29: Maximum cortisol increase for HAB (n = 14) and No-HAB (n = 19) group at TSST1 and TSST2.

Study 2 - Responder groups and psychometric measures of fear and anxiety

After controling for alpha error accumulation (adjusted p value of p = 0.0063), no statistically meaningful group difference were found for any other psychometric measure of fear and anxiety applied. Groups also didn't differ in ratings on the perceived threat (*VAS*) during either of the two stress exposures.

Table 21: Responder groups (No-HAB (n = 19) & HAB (n = 14)) and psychometric measures of fear and anxiety (independent sample t-test: *HAB – No-HAB*).

Study 2 – scan sub-sample	**Mean difference**	**SE**	**N**	**t-value**	**df**	**p-value**
MASQ anxious symptoms	-0.95	1.37	33 HAB n = 14 /No-HAB n = 19	- 0.70	31	n.s.
MASQ anxious arousal	0.99	1.22	33 HAB n = 14 /No-HAB n = 19	0.81	31	n.s.
MASQ general distress	- 0.24	2.84	33 HAB n = 14 /No-HAB n = 19	- 0.08	31	n.s.
Fear Survey Schedule-III	- 6.91	5.89	33 HAB n = 14 /No-HAB n = 19	- 1.17	31	n.s.
Marlowe Crowne Social Desirability	3.26	1.59	33 HAB n = 14 /No-HAB n = 19	2.05	31	n.s.
Taylor Manifest Anxiety	- 2.50	1.90	33 HAB n = 14 /No-HAB n = 19	- 1.31	31	n.s.
Threat TSST1 (VAS)	0.95	0.93	33 HAB n = 14 /No-HAB n = 19	1.02	31	n.s.
Threat TSST2 (VAS)	0.75	1.08	33 HAB n = 14 /No-HAB n = 19	0.70	31	n.s.

Study 2 - Threat of shock fMRI data

Threat runs, when compared to no-threat runs, elicited pronounced activation in areas of the insula cortex, the prefrontal cortex, the hypothalamus, the amygdala, the brainstem, the striatum and cortical areas. A list of activated clusters along with Z scores for the most activated voxel within each cluster is presented in table 22 An axial view of the thresholded Z-score map is shown in figure 30.

Table 22: MNI coordinates and maximum Z scores for brain areas activated for the threat – no-threat contrast (paired t-test). Data are shown for a cluster Z threshold of Z = 2.3 and a cluster P threshold for P = 0.01.

Brain region	MNI co-ordinates (x, y, z)	Z-score
right ventromedial PFC	22, 34, -10	3.42
left ventromedial PFC	-14, 44, -16	3.50
right rostral anterior cingulate cortex (acc)	14, 30, -4	3.21
left rostral anterior cingulate cortex (acc)	-6, 26, -6	3.00
right lateral PFC	42, 52, 4	4.53
left lateral PFC	-34, 54, 12	4.12
right dorsolateral PFC	46, 34, 28	3.87
dorsal cingulate cortex	4, 6, 22	5.60
left insula	-30, 24, -2	6.30
right insula	36, 22, 2	6.95
left hypothalamus	-2, -18, -16	5.80
right amygdala complex	22, -6, -22	3.05
Right striatal – anterior thalamic region	12, 2, -4	6.13
Left striatal – anterior thalamic region	-6, 2, -2	6.63
brainstem	12, -28, -24	4.98
posterior cingulate cortex	2, -24, 22	5.02
precuneus	2, -56, 62	3.95
right inferior frontal gyrus opercular part	60, 6, 2	5.93
left inferior frontal gyrus opercular part	-54, 0, 6	4.36
right mediofrontal gyrus	40, -12, 60	5.80
left mediofrontal gyrus	-30, -14, 60	3.95
left superior frontal gyrus	18, -8, 70	4.94

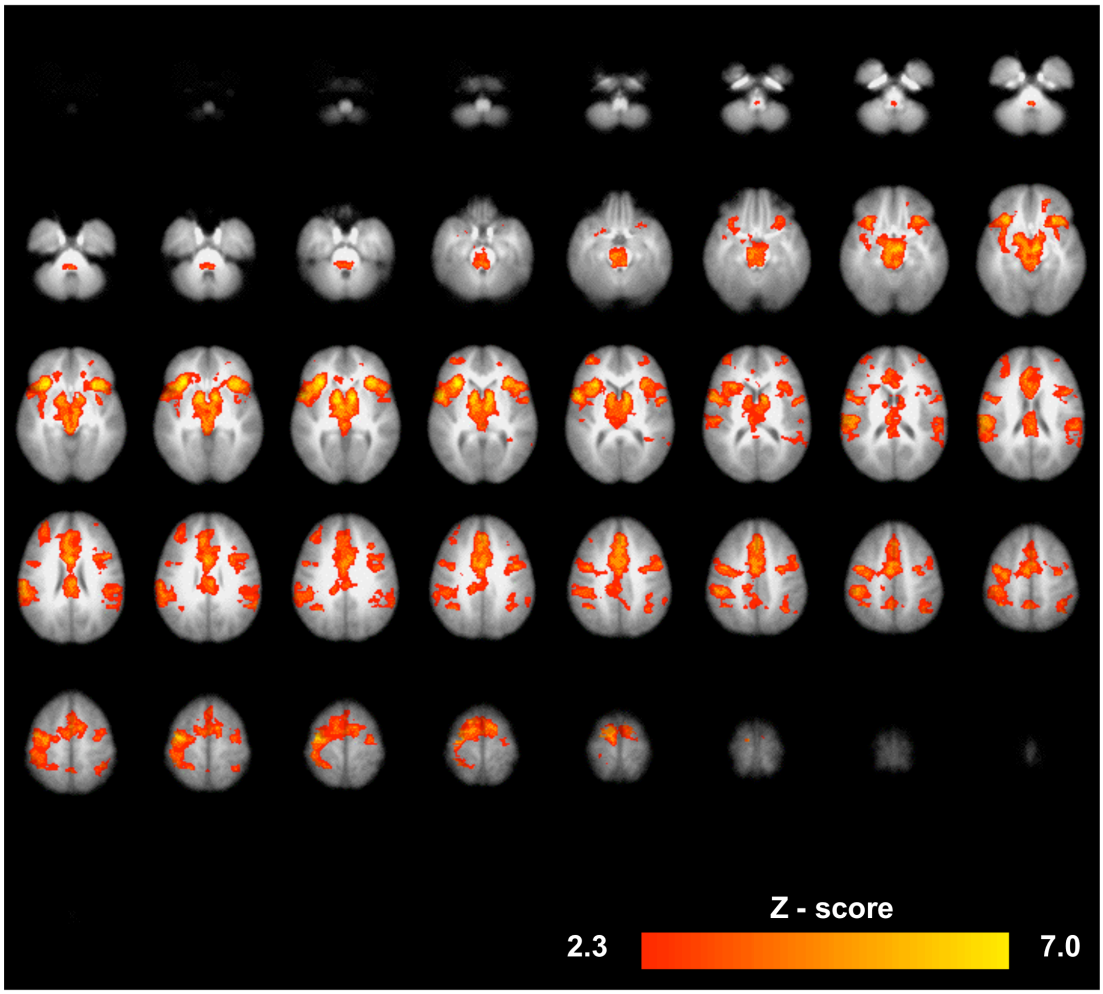

Figure 30: Statistical image for the threat – no-threat contrast (paired t-test) shown for an axial view (ventral to dorsal). Statistic images were thresholded using clusters determined by Z > 2.3 and a corrected cluster significance threshold of P = 0.01.

Guided by the *a-priori* hypothesis, only activation clusters that were associated with brain areas known to be involved in HPA axis regulation like the hypothalamus, the PFC, the amygdaloid area and brainstem foci were considered for a subsequent ROI analysis. Brain activation maps for the afore mentioned clusters are presented in figure 31 – 38.

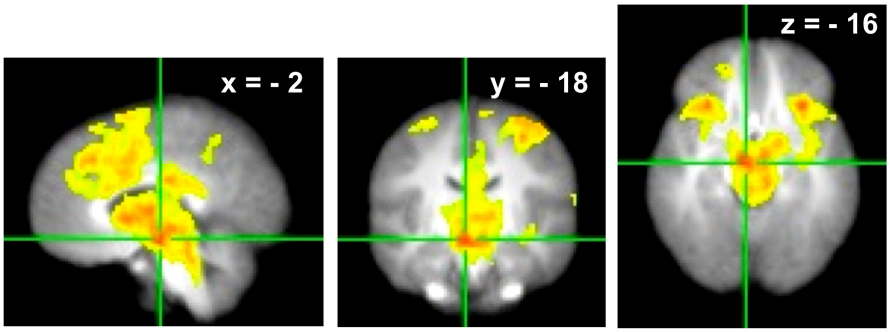

Figure 31: Activation for the left hypothalamus (x = - 2, y = -18, z = -16; max. Z = 5.80) for the threat – no-threat activation (paired t-test).

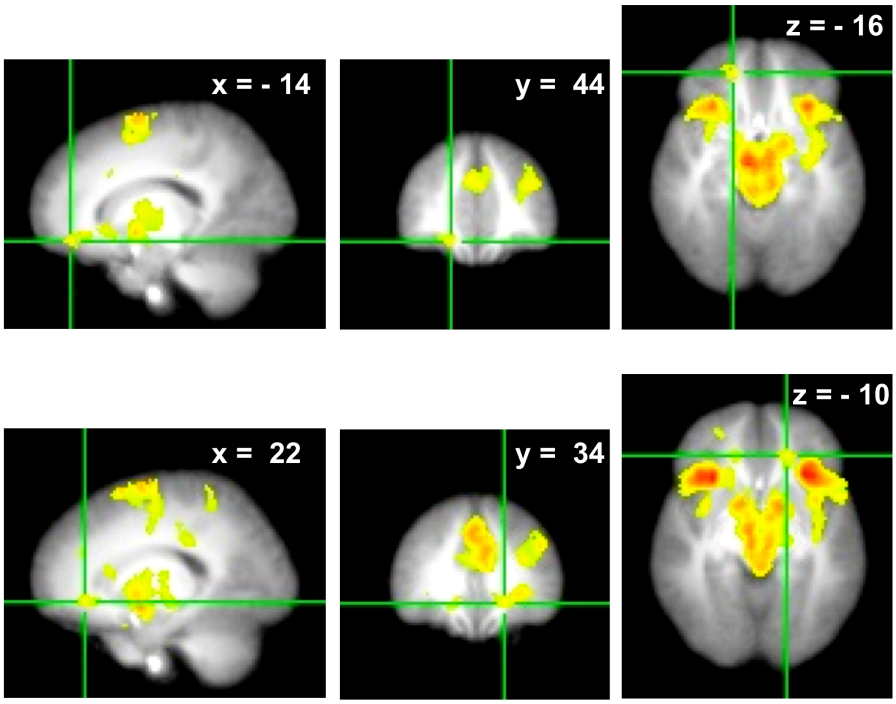

Figure 32: Activation for the left (top) (x = -14, y = 44, z = -16; max. Z = 3.50) and right (bottom) *ventromedial PFC* (*vmPFC*) (x = 22, y = 34, z = -10; max. Z = 3.42) for the threat – no-threat activation (paired t-test).

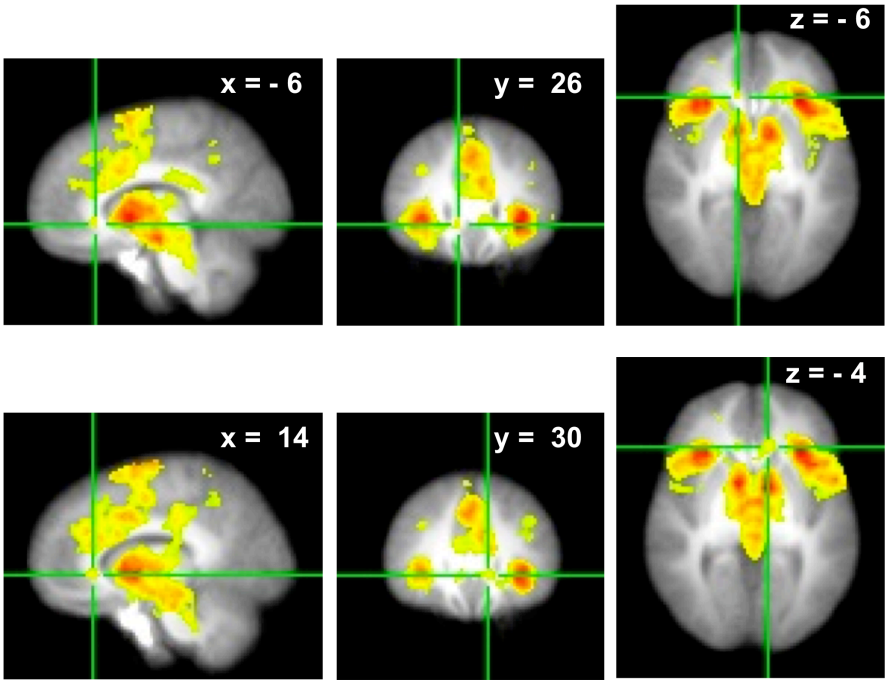

Figure 33: Activation for the left (top) (x = -6, y = 26, z = -6; max. Z = 3.00) and right (x = 14, y = 30, z = -4; max. Z = 3.21) (bottom) *rostral anterior cingulate cortex (rostral acc)* for the threat – no-threat contrast (paired t-test).

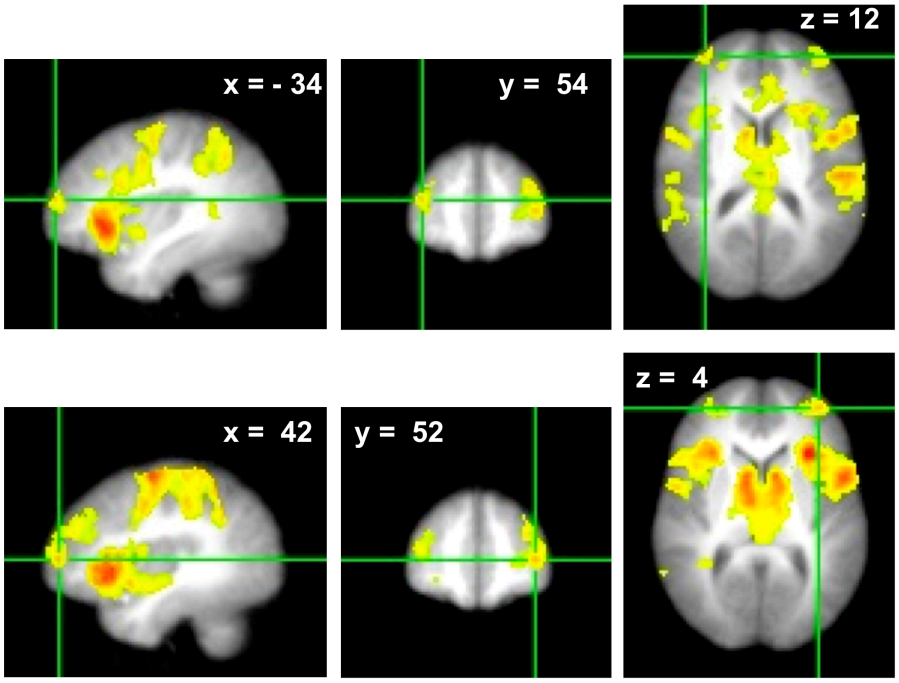

Figure 34: Activation for the left (top) (x = -34, y = 54, z = 12; max. Z = 4.12) and right (bottom) (x = 42, y = 52, z = 4; max. Z = 4.53) *lateral PFC* for the threat – no-threat contrast (paired t-test).

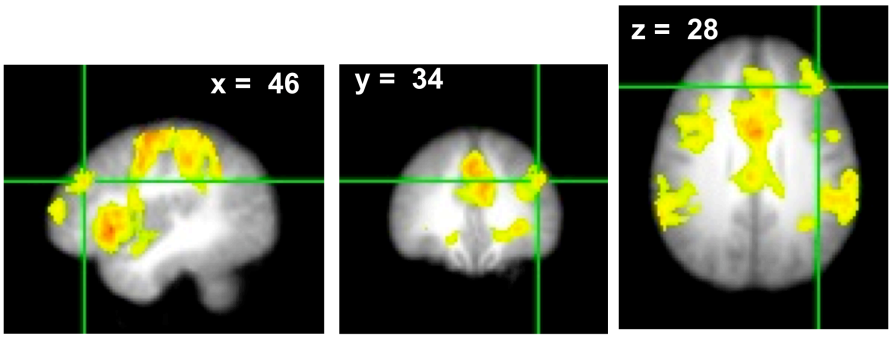

Figure 35: Activation for *right dorsolateral PFC* (x = 46, y = 34, z = 28; max. Z = 3.87) for the threat – no-threat contrast (paired t-test).

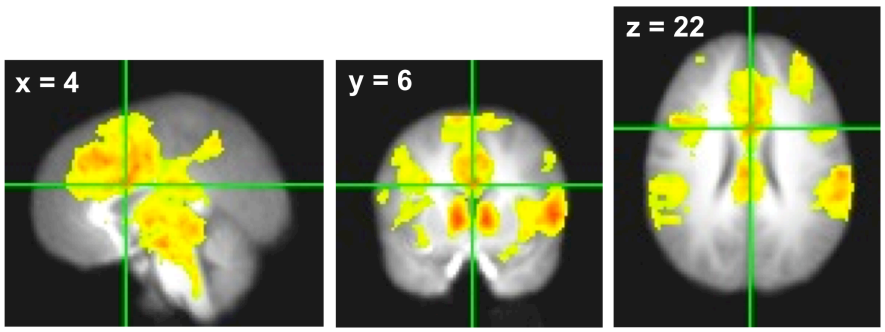

Figure 36: Activation for dorsal anterior cingulate cortex (dorsal acc) (x = 4, y = 6, z = 22; max. Z = 5.60) for the threat – no-threat contrast (paired t-test).

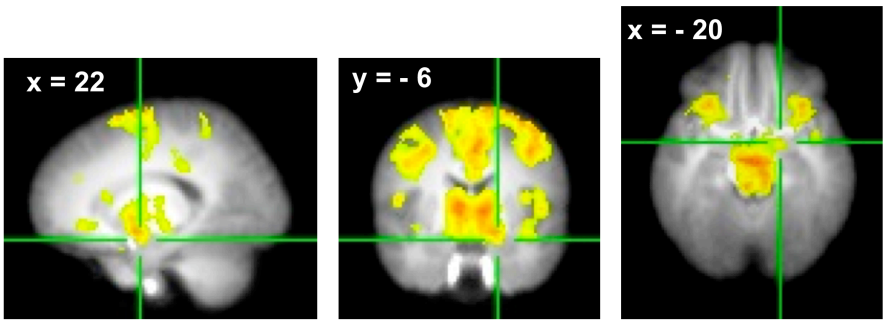

Figure 37: Activation for the right *amygdaloid complex* (x = 22, y = -6, z = -20; max. Z = 3.05) for the threat – no-threat contrast (paired t-test).

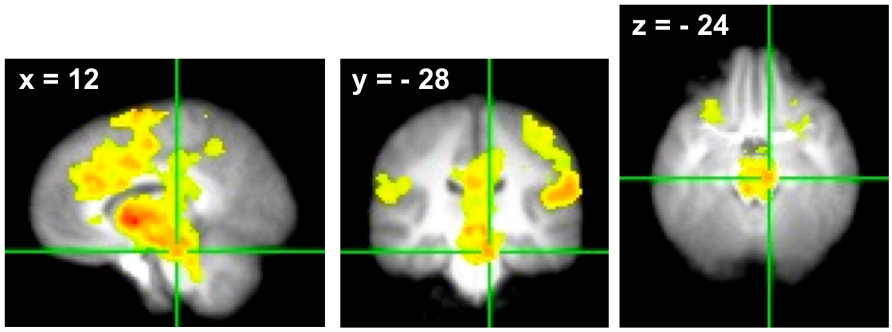

Figure 38: Activation for the *brainstem cluster* (x = 12, y = -28, z = -24; max. Z = 4.98) for the threat – no-threat activation (paired t-test).

Study 2 - Threat of shock and cortisol

Extracted mean values for the clusters in these areas were compared for the HAB and the No-HAB group. The No-HAB group showed significantly stronger activation in the right *vmPFC* compared to HAB group. The HAB group on the other side had significantly stronger activation on a brainstem level when compared to the No-HAB group. However, statistical significance was no longer given after alpha error correction (Bonferoni adjusted p value of p = 0.0045). A list of all comparisons with mean difference scores in mean cluster Z- values and corresponding statistical scores for the non alpha error adjusted comparisons are presented in table 23.

Table 23: Mean difference in mean cluster Z-score for clusters in brain areas associated with HPA axis regulation. Statistical data are shown for group comparison for the No-HAB - HAB group (independent t-test).

Study 2 – scan sub-sample	Mean difference	SE	N	t-value	df	p-value
Left hypothalamus	- 0.39	0.31	33 HAB = 14 / No-HAB = 19	- 1.24	31	n.s.
Right amygdala	-0.23	0.28	33 HAB = 14 / No-HAB = 19	- 0.76	31	n.s.
Right vmPFC	0.57	0.22	33 HAB = 14 / No-HAB = 19	2.60	31	0.014
Left vmPFC	0.19	0.30	33 HAB = 14 / No-HAB = 19	0.62	31	n.s.
Right lateral PFC	- 0.25	0.37	33 HAB = 14 / No-HAB = 19	- 0.68	31	n.s.
Left lateral PFC	0.26	0.26	33 HAB = 14 / No-HAB = 19	1.01	31	n.s.
Right dorsolateral PFC	0.09	0.35	33 HAB = 14 / No-HAB = 19	0.26	31	n.s.
Dorsal acc	- 0.28	0.28	33 HAB = 14 / No-HAB = 19	-1.01	31	n.s.
Right rostral acc	0.21	0.20	33 HAB = 14 / No-HAB = 19	1.06	31	n.s.
Left rostral acc	0.46	0.27	33 HAB = 14 / No-HAB = 19	1.72	31	n.s.
Brainstem	- 0.79	0.27	33 HAB = 14 / No-HAB = 19	- 2.94	31	0.006

Next, a correlation was performed to look for associations between saliva cortisol data and mean cluster Z-scores independently of group membership. For the entire group of subjects (N = 33) mean cluster Z scores in the *right vmPFC* were positively associated with $max_{increaseTSST2}$ and $max_{inc\text{-}change.}$

The activation within the *amygdala* cluster was not significantly associated with any cortisol increase or habituation measure. Mean Z-score values for the left and *right lateral PFC* cluster, the *left vmPFC* cluster, the *dorsolateral PFC* cluster, the *hypothalamic* cluster, the *acc* cluster and the *left* and *right rostral acc* cluster were also not significantly associated with any saliva cortisol measure.

The mean Z-score value for the *brainstem* cluster was negatively associated with $max_{inc\text{-}change}$, which indicates that participants with a strong habituation pattern tended to have higher *brainstem* activation for the threat – no-threat contrast.

Correlation coefficients along with statistical determinants for the above mentioned associations are listed in table 24.

Table 24: Mean cluster Z-score for clusters in brain areas associated with HPA axis regulation and saliva cortisol data for TSST1 and TSST2 (Spearman correlation).

Study 2 – scan sub-sample	**Maximum increase TSST1**	**Maximum increase TSST2**	**Maximum increase change**
Left hypothalamus	r = 0.06 n.s. N = 33	r = - 0.02 n.s. N = 33	r = - 0.11 n.s. N = 33
Right amygdala	r = - 0.01 n.s. N = 33	r = - 0.04 n.s. N = 33	r = - 0.002 n.s. N = 33
Right vmPFC	r = 0.20 n.s. N = 33	**r = 0.38** **p = 0.028** ***N = 33*** ***R = 0.14***	**r = 0.45** **p = 0.009** **N = 33** **R = 0.20**
Left vmPFC	r = 0.31 n.s N = 33	r = 0.29 p = n.s. N = 33	r = 0.11 n.s. N = 33
Right lateral PFC	r = - 0.25 n.s. N = 33	r = - 0.19 n.s. N = 33	r = - 0.07 n.s. N = 33
Left lateral PFC	r = 0.02 n.s. N = 33	r = 0.11 n.s. N = 33	r = 0.09 n.s. N = 33
Right dorsolateral PFC	r = 0.05 n.s. N = 33	r = - 0.10 n.s. N = 33	r = 0.09 n.s. N = 33
Dorsal acc	r = - 0.18 n.s. N = 33	r = - 0.30 n.s. N = 33	r = - 0.23 n.s. N = 33
Right rostral acc	r = 0.089 n.s. N = 33	r = 0.17 n.s. N = 33	r = 0.25 n.s. N = 33
Left rostral acc	r = 0.15 n.s. N = 33	r = 0.33 ns. N = 33	r = 0.31 n.s. N = 33
Brainstem	r = 0.05 n.s. N = 33	r = - 0.26 n.s. N = 33	**r = - 0.45** **p = 0.008** **N = 33** **R = 0.20**

Scatter plots for the statistically most meaningful associations are shown in figure 39:

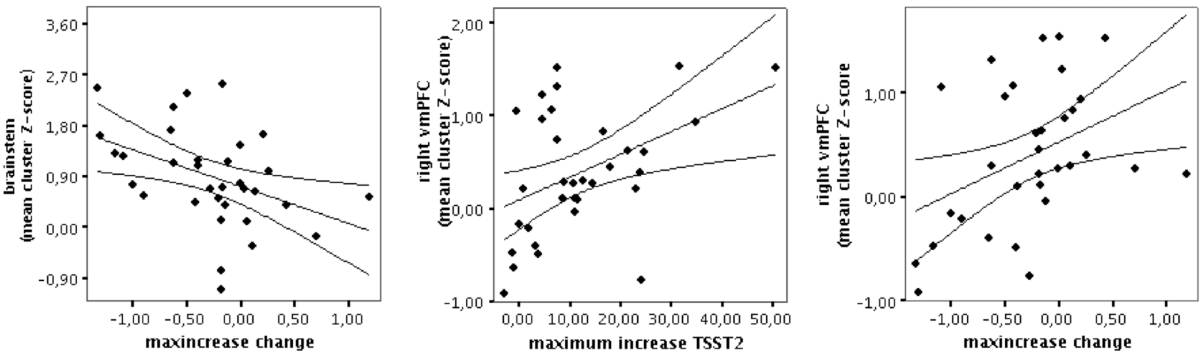

Figure 39: Associations between mean cluster Z-score and saliva cortisol measures.

Study 2 - Threat of shock and psychometric measures of fear and anxiety

High scores on the *MASQ general distress* subscale were positively associated with mean cluster Z-scores in the *dorsal acc* (*r = 0.37; p = 0.035; R = 0.14*). High scores on the MASQ subscale anxious arousal was positively associated with pronounced activation in the right dorsolateral PFC (r = 0.55; p = 0.001; R = 0.30). High scores on the *Fear Survey Schedule* where also positively associated with mean cluster Z-values of the *dorsal* acc (*r = 0.40; p = 0.02; R = 0.16*) and the mean left vmPFC (r = 0.37; p = 0.03; R = 0.14). *State anxiety* scores measured with the *STAI* prior to scanning were negatively associated with mean cluster Z-scores values of the *right lateral PFC* (*r = - 0.35; p = 0.047; R = 0.12*).

Anxiety ratings for the first shock were not significantly associated with any mean cluster activation, while *anxiety ratings* for the last shock were positively associated mean *dorsal acc* cluster Z-score (*r = 0.46; p = 0.007; R = 21*).

Negative affect assessed by the *PANAS* prior to scanning was positively associated with mean activation in the *right dorsolateral PFC* cluster (*r = 0.40; p = 0.022; R = 0.16*).

4.2.5 Discussion

It has been argued that fear can be regarded as the major normal precursor for the development of pathological anxiety, which is conceptualized as an exaggerated fear state in which hyper-excitability of fear circuits that include the amygdala is expressed as hyper-vigilance and increased behavioral responsivity to fearful stimuli (Rosen & Schulkin, 1998). It has further been assumed that psychosocial stress and the subsequent release of neuroendocrine hormones can cause over-activity in these fear circuits which can, along with a combination of behavioral and biological processes, lead to the development of hyper-excitable fear circuits that promote the turning of normal emotion of fear into pathological anxiety (Rosen & Schulkin, 1998). The aim of this study was to test whether exaggerated HPA axis response patterns to a single as well as a repeated psychosocial stressor are associated with hyper-excitability in the brain's fear circuit.

Cortisol response and habituation

In a sample of healthy male volunteers, confrontation with the TSST resulted in marked HPA axis activation on both days. During the first stress encounter, cortisol increased on average 3 fold above baseline, which is in line with previous reports on the TSST (Kirschbaum et al., 1999; Kirschbaum et al., 1993). Repeated confrontation with the almost identical protocol resulted in pronounced HPA axis habituation, which is also in line with earlier findings (Gerra et al., 2001; Kirschbaum et al., 1995; N. C. Schommer et al., 2003). While previous studies on HPA axis habituation found decreases in cortisol response of 35 % or more (Kirschbaum et al., 1995; N. Schommer, 2001; N. C. Schommer et al., 2003), the response decrease observed here was on average only 26 % in the total sample (N = 86) and 21 % in the sub-sample (N = 33), which returned for the follow-up fMRI study. According to studies on rats (Dhabhar et al., 1997) and humans (Deinzer et al., 1997; Wust et al., 2005), the initial cortisol response to a homotypic stress influences subsequent habituation patterns. However, in the study presented here, no such effect could be observed for either the total nor the sub-sample. This discrepancy could be explained by the way habituation was calculated. While in some protocols habituation was defined as a simple difference in HPA axis activation markers (e.g. difference in area under the curve between two or more stress exposures) (e.g. Wust et al., 2005), for the habituation data presented here, the magnitude of the initial response was taken into account and habituation was calculated in relation to the initial response to the first stress encounter.

Previous reports on HPA axis habituation in humans have repeatedly shown the existence of a sub-group of people characterized by marked differences in response magnitude and a diminished pattern of HPA axis habituation in response to repeated stressor confrontation (Gerra et al., 2001; Kirschbaum et al., 1995; N. C. Schommer et al., 2003). Here we identified two responder groups that do not differ in their HPA axis response to the first stress encounter but show pronounced response discrepancies after the second stress exposure in a way that some participants showed the expected HPA axis habituation patterns at the second stress test (*habituators*), while others did not demonstrate any meaningful signs of HPA axis habituation (*no-habituators*). In the initial sample of 86 healthy males, on average 60 % were characterized as habituators. In the sub-sample of 33 participants who returned for the follow-up experiment, roughly 42 % were identified as habituators. These data are in line previous findings showing a habituators rate of 50 – 60 % in humans (Gerra et al., 2001; Kirschbaum et al., 1995; N. C. Schommer et al., 2003; Wust et al., 2005). However, in sharp contrast to the previously reported habituation experiments (Gerra et al., 2001; Kirschbaum et al., 1995; N. C. Schommer et al., 2003; Wust et al., 2005), the two responder groups found here, did not show any statistically meaningful difference in HPA axis response to the first stress encounter. This effect is most likely explained by the statistical method by which the groups were defined. In previous reports on habituation, responder groups were determined by cluster analysis which were often based

on measures of area under the curve for each of the stress exposures (Gerra et al., 2004; Kirschbaum et al., 1995; N. C. Schommer et al., 2003). While the area under the curve can be regarded as an useful index of HPA axis activity over time (J. C. Pruessner et al., 2003), here the maximum increase as the most proximate measure of HPA axis activity was chosen. Furthermore, groups were not determined on the basis of their response to either of the two stress encounters, but rather by a mean split on the basis of each participants individual habituation index. This technique was chosen, as it is believed to discriminate best between habituators and no-habituators independently of response magnitude. A mean split was preferred over a median split technique as it was assumed that habituators and no-habituators are not equally distributed (N. C. Schommer et al., 2003).

HPA axis response and psychometric measures of fear and anxiety

In this study, little evidence was found for a strong relationship between self reported levels of fear and anxiety and HPA axis response to a repeated homotypic stress exposure. In the initial sample of 86 subjects no differences in psychometric measures of fear and anxiety were observed between habituator and no-habituator. However, correlations on the total sample revealed that high scores on the *Marlowe Crowne Social Desirability Scale,* which was used as an index of social desirability and protection and prevention of threat to social self-esteem from anticipated social rejection (Crowne & Marlowe, 1960; Pauls & Stemmler, 2003), were associated with a tendency for less pronounced habituation.

In the sub-sample of 33 participants returning for the follow-up fMRI scan, the habituator and no-habituator group did also not differ in any psychometric measure of fear and anxiety collected. However, correlational analysis independent of group membership indicated a negative association between the maximum cortisol increase to TSST1 and self reported anxious arousal (*MASQ*). Scores on the *Marlowe Crowne Social Desirability Scale* were positively linked to maximum cortisol increase at TSST2 and pronounced perceived threat at the first stress encounter was associated with a tendency for poorer habituation patterns.

Overall, these findings seem to be partly at odds with studies on animals and humans showing a close link between anxious and fearful temperament and HPA axis activity. According to Kalin and colleagues (Kalin et al., 1998; Kalin et al., 2000), monkeys characterized by a fearful temperament and relative right sided asymmetric frontal brain activity showed higher basal cortisol concentrations as well as increased levels of cerebrospinal fluid CRH concentrations. Children with an inhibited behavioral style express higher basal cortisol levels (Kagan et al., 1987; Schmidt et al., 1997) and individuals characterized by high trait negative affect also tend to have an elevated basal HPA axis activity (Brown et al., 1996). Furthermore, patients with a diagnosis of social phobia show higher cortisol levels to a psychosocial stress encounter when compared to healthy controls (Furlan et al., 2001; Martel et al., 1999). However, while many findings point towards a positive association between states of fear, anxiety and HPA activity,

others report no associations (Gerra et al., 2000) or even pronounced negative associations between reported anxiety and physiological signs of stress (Jezova et al., 2004).

Profound discrepancies between self reported anxiety and actual behavioral and physiological responding in humans have been a matter of debate for many years (Weinberger et al., 1979). Applying the *Marlowe Crowne Social Desirability Scale* along with a psychometric measure of trait anxiety, Weinberger and colleagues were able to identify a group of people claiming to be extremely low in trait anxiety but at the same time scoring very high on the *Marlowe Crowne Social Desirability Scale*. Based on the high scores of the latter scale, it was believed that these individuals were actually characterized by high levels of trait anxiety and were therefore called *repressors* (Weinberger et al., 1979). Participants characterized by a repressive coping style have shown to express elevated levels of basal cortisol (Brown et al., 1996), and tend to have higher resting systolic blood pressure as well as greater systolic blood pressure reactivity in response to a mental challenge (King et al., 1990). While it has been assumed that an excess of glucocorticoids and states of anticipatory anxiety contribute to states of allostatic load (Schulkin et al., 1998a), to the best of our knowledge no data have ever been reported on associations between levels of self reported anxiety and HPA axis habituation in humans. The data presented here do not strongly indicate such a link. However, based on our findings on the *Marlowe Crowne Social Desirability Scale,* there is evidence that such effects were masked by the existence of a sub-group of subjects characterized by a predominantly repressive coping style, with extreme repressors demonstrating a diminished pattern of HPA axis habituation when compared to a group of truly high anxious participants (data not shown) (Kern et al., 2005).

Threat of shock and brain activation

Anticipation of an unpleasant shock resulted in pronounced activation in brain areas that have previously been associated with the neural fear network (J. LeDoux, 1998, 2001; J. E. LeDoux et al., 1988; Quirk et al., 2003; Rosen & Schulkin, 1998). In this study, most striking patterns of activation were observed in areas of the ventromedial, lateral and dorsolateral PFC, the rostral anterior, anterior dorsal and posterior cingulate cortex, the insula (bi-lateral), the right amygdala region, the hypothalamus, widespread brainstem areas, the striatum (bi-lateral), inferior, medial and superior frontal gyrus as well as in parts of the parietal cortex. Observed activation in these areas are mostly in accordance with previous findings on painful shock anticipation and fear induction in humans (Benkelfat et al., 1995; Bystritsky et al., 2001; Chua et al., 1999; M. Gray et al., 2003; Jensen et al., 2003; Rauch et al., 1997; Shin et al., 2004). Jensen and colleagues (Jensen et al., 2003) found marked activation in the ventral striatum in response to anticipatory painful dermal stimulation and argued that the ventral striatum plays an important role in anticipation of aversive stimuli. Also applying an anticipatory anxiety inducing paradigm, Chua and colleagues (Chua et al., 1999) found strong activation in the left as well as the right insula region, the left anterior cingulate cortex as well as activation in the left fusiform gyrus and

superior temporal sulcus. Increased cerebral blood flow in areas comprising the acc in response to CCK4 anxiety provocation has also previously been reported in healthy volunteers (Benkelfat et al., 1995).

Our finding of increased amygdala activation in response to the anticipation of a dermal shock seems to be in line with a series of animal findings, according to which the amygdala is regarded a crucial brain region within the fear and anxiety network (Kalin et al., 2004; Kalin et al., 2001; J. E. LeDoux et al., 1990; J. E. LeDoux et al., 1988; Roozendaal et al., 1991a). Furthermore, amygdala activation in response to passive viewing of aversive facial expressions (Blair et al., 1999; Morris et al., 1996; Whalen et al., 2001) seems to be a well established finding in humans. When it comes to active fear induction, an increase in cerebral blood flow in areas of the claustrum-insular-amygdala region in response to an anxiety inducing CCK4 injection has been reported in healthy participants (Benkelfat et al., 1995). In patients with social phobia, symptom provocation has frequently been associated with increased activity in the amygdaloid region (Birbaumer et al., 1998; Tillfors et al., 2001; Tillfors et al., 2002). However, in contrast to the here reported findings, amygdala activation has been absent in a series of fear and anxiety inducing experiments in healthy volunteers (Chua et al., 1999; Jensen et al., 2003) and patients with anxiety disorders (Fredrikson et al., 1997; Rauch et al., 1997; Shin et al., 1999; Shin et al., 2001). Interestingly, even diminished amygdala activation has been reported in trauma exposed subjects (Britton et al., 2005) and patients with obsessive-compulsive disorder (Cannistraro et al., 2004). Selective reductions in amygdala volume in pediatric anxiety disorders has also recently been reported (Milham et al., 2005). In light of these findings, it should be mentioned that Benkelfat and colleagues (Benkelfat et al., 1995) interpret their claustrum-insular-amygdala finding rather cautiously, as they cannot rule out artifacts resulting from cerebral blood volumes changes from nearby larger vessels. When it comes to the amygdala finding reported here, similar effects cannot definitively be excluded as the observed activation could be a result of the wide spread activation in the striatum and could even represent an outflow effect of activation in the region of the bed nucleus of the stria terminalis.

Brain activation and HPA axis activation to a single and repeated stress exposure

In experiments involving rodents, stress frequently causes pronounced activation in areas associated with the brain's fear and anxiety circuitries (Day et al., 2004; Dayas et al., 2001a; Kollack-Walker et al., 1997). While acute stress exposure goes along with increased activation in areas like the hypothalamus, the amygdaloid-complex, the septum, the striatum, the PFC and brainstem foci, repeated confrontation with a homotypic stressor has repeatedly shown to result in diminished activation in many of these regions (Campeau et al., 2002; Martinez et al., 1998; Melia et al., 1994). Moreover, reduced activation in these areas has been associated with HPA axis habituation (Melia et al., 1994), which leads us to the assumption that reactivity of the brain's fear and anxiety networks could be associated with HPA axis habituation in situations

associated with stress and anxiety. While neural substrates of HPA axis habituation have considerably been studied in rodents, little is known about this subject in human and non-human primates.

Data reported here, indicate that there was a tendency for the habituator group to show less pronounced activation in the right vmPFC when compared to the no-habituator group. Looking at the same contrast, brainstem activation on the other hand was stronger in the habituator group than in the no-habituator group. Independently of group membership, HPA axis activation to the second stress test seemed to be positively associated with increased activity in the right vmPFC. Brainstem activation was positively associated with HPA axis habituation from TSST1 to TSST2, while activation in the right vmPFC was negatively related to the observed adaptation. No association between HPA axis response to the first stress encounter and brain activity to the threat paradigm was observed.

At a first glance, our finding of a positive association between right vmPFC activation and cortisol response to the second stress test seems to be contradictory to a series of recent findings indicating an inhibitory role of the mPFC on parts of the brain's fear circuitry (Amat et al., 2005; Figueiredo et al., 2003b). In brain imaging studies on humans, the question, whether prefrontal areas elicit an inhibitory tone over the amygdala has been a matter of debate: While some groups present findings supporting this hypothesis (Hariri et al., 2003) others do not (Gilboa et al., 2004). What we know from rodent data, these contradictory findings could be a result of looking at slightly different areas of the mPFC. There is reasonable evidence that activation within more dorsal regions of the mPFC normally act to inhibit HPA axis function, while the ventral and infralimbic areas of the mPFC are involved in facilitating HPA axis activation. Furthermore, it has been assumed that the latter two regions play an important role when it comes to mounting an appropriate response to a previously experienced stressor (R. M. Sullivan & Gratton, 2002b). In a study on rats, it was shown that lesions in the right vmPFC promoted HPA axis habituation (R. M. Sullivan & Gratton, 1999), which is in line with our findings, showing higher right mPFC activation in the no-habituator group and a positive association between activity in this same area and maximum cortisol increases to the second stress test.

The brainstem, and here the formatio reticularis, is home to a series of nuclei known to be critically involved in endocrine, autonomic and behavioral aspects of stress, fear and anxiety (Bandler et al., 2000a; Bandler et al., 2000b; Herman & Cullinan, 1997; G. M. Sullivan et al., 1999). Here we found that increased activation in parts of the formatio reticularis was associated with more pronounced habituation. This finding seems to be rather counterintuitive, as it is well known that several brainstem areas exert an excitatory tone over HPA axis function (Herman & Cullinan, 1997). However, activation in the brainstem area in response to anticipation of an aversive shock was widespread, and due fMRI related resolution restrictions, exact anatomical localization of the most activated voxel seemed rather difficult. Hence, at this point, it cannot be

ruled out that the activation reported here reflects brain areas that are involved in functions that facilitate HPA axis habituation (e.g. coping; periaqueductal grey; (Bandler et al., 2000a).

Surprisingly, amygdala activity was unrelated to any measure of HPA axis activity and habituation. This finding seems to be at odds with a wide series of experiments on rodents and non-human primates that emphasize this brain structure's role during states of fear and distress and concomitant endocrine reactions (Day et al., 2004; Kalin et al., 2004). Little is known about whether these associations can be projected on to human conditions. In a study on patients with a diagnosis of major depression or bi-polar disorder, glucose metabolism in the right amygdala was positively associated with stressed plasma cortisol levels in both patient groups when compared to healthy controls (Drevets et al., 2002). As to the best of our knowledge, little is known about the amygdala's role in HPA axis habituation in humans. Here, amygdala activity in response to threat of a dermal shock was also not linked to HPA axis habituation, which is in line with findings on rodents according to which amygdala lesions did not significantly affect HPA axis habituation in a response to confrontation with a homotypic stressor (Carter et al., 2004).

While still very little is known about how amygdala function is related to HPA axis activity in humans, this study on healthy male volunteers, indicates no link between increased amygdala function and HPA axis activity and habituation due to two threat inducing paradigms. Nonetheless, data should be interpreted with caution. In previous brain imaging studies on human amygdala function, evidence was found that the amygdala is subject to signal habituation (Breiter et al., 1996; Fischer et al., 2003). It therefore can not be ruled out that the non existing association between HPA axis activity and amygdala activation in this study could be attributed to amygdala signal habituation that happened during an early phase of stimulus presentation.

Brain activation and psychometric measures of fear and anxiety

In accordance to the data presented here, self reported measures of fear and anxiety were associated with increased activity in the brain's fear and anxiety networks. Stable negative affective states like anxious arousal as well as state negative affect were positively correlated with activation in the right dorsolateral PFC. Dorsal acc activation was related to high ratings on the *Fear Survey Schedule*, high general distress, and high anxiety ratings for the last out of three dermal shocks.

These data are generally in line with previous brain imaging studies on human volunteers: Using state STAI scores as a covariate in a anxiety inducing paradigm, Chua and co-workers (Chua et al., 1999) found associated brain activation in the left orbitofrontal cortex, the left insula and the left anterior cingulate cortex. Simpson and colleagues (Simpson et al., 2001) found that decreases in the mPFC (BA 10/32 and BA 24/25) blood flow were inversely correlated with anxiety self ratings such that the least anxious subjects exhibited the largest reductions in blood flow, whereas the most anxious participants exhibited no significant reduction or even a slight increase in blood flow. Here, we did not observe any association between brainstem activation

and self reported fear and anxiety. However, in a symptom provocation paradigm which was applied to a pooled sample comprising patients with three different anxiety disorders, Rauch and co-workers found positive associations between left sided brainstem activation and subjective anxiety ratings (Rauch et al., 1997).

In accordance with the notion that the PFC plays a critical role in cognitive control (Miller & Cohen, 2001) and emotion regulation (Ochsner et al., 2002), here, integration of affective states like the tendency to acutely or steadily experience fear and anxiety took place in areas of the PFC. The acc is thought to be involved in cognitive as well as emotional processing (Bush et al., 2000; Salomons et al., 2004) such that the dorsal aspects are predominantly involved in cognitive tasks, whereas the rostral subdivision is most likely involved in emotional processing (Bush et al., 2000). There is also evidence that the acc acts in concert with lateral and dorsolateral PFC areas in situation when cognitive control in form of top-down support for task-relevant processes is put forward (MacDonald et al., 2000). Further, there is support for the hypothesis, that the acc is primarily involved processing of conflict information, whereas the lateral PFC regions are important in order to establish control in situations of high conflict. (Botvinick et al., 2001). Dorsolateral areas are thought to regulate behavior and to control responses to environmental stimuli (Wood & Grafman, 2003). While an elaborate model on the integration of affective and cognitive processing within the PFC would be well beyond the scope of this work, it could hypothesized that being trapped in a scanner and expecting dermal shocks could represent such states of high conflict. Hence, information about the affective state could have played a role in processing the conflict information and establishing cognitive and behavioral control in the very situation. However, no evidence was found, that these functions are related to HPA axis activity or habituation.

Conclusion

In conclusion, the present fMRI study gives evidence that excitability in parts of the brain's fear and anxiety circuitries is associated with the ability to adapt to a re-occurring homotypic stressor. Hereby, the prefrontal cortex not only seems to be closely linked to endocrine markers of habituation but also seems to be a prominent region when it comes to incorporating subjective affective states. However, future studies are needed in order to determine how brain areas involved in affective processing interact with brain regions involved in HPA axis activation.

5. GENERAL DISCUSSION

In the final section, findings from the previously presented empirical studies on the neural substrates of stress and stress habituation will be summarized and critically discussed. Also, results will again be placed in the context of relevant previous findings on the neurobiology of stress and anxiety and possible mechanisms and explanations will be highlighted. Finally, this thesis closes with some remarks on the implications of the here presented findings on the current understanding of the neural substrates of stress in humans.

5.1 Aims

Research performed over the course of the past decades has accumulated a wealth of new information on the neurobiology of stress and anxiety. Moreover, based on a series of more recent findings, the concept of stress has frequently been discussed as a major risk factor in several psychopathologies (Arborelius et al., 1999) and there is now substantial evidence for the existence of various forms of HPA axis dysfunction in several affective disorders (McEwen, 2003). However, the neural substrates of stress, anxiety and concomitant activation and habituation of the HPA axis have not been systematically studied in humans, largely due to the difficulty of measuring brain function during psychosocial stressors known to provoke HPA activation.

Hence, the two studies presented here were designed to provide new insights into how brain regions such as the prefrontal cortex and the amygdaloid complex are involved in regulation of the HPA axis in humans. Most recent data from experiments on rodent and non-human primates provide considerable evidence for a primary role of the prefrontal cortex in HPA axis regulation. Data from studies on rats showed that lesions of medial prefrontal areas resulted in either pronounced facilitation or inhibition of HPA axis activation (Diorio et al., 1993; Figueiredo et al., 2003b; R. M. Sullivan & Gratton, 1999), while the exact nature of the PFC's influence on HPA axis activity seems to be dependent on the exact location of the lesion (R. M. Sullivan & Gratton, 2002b). Additionally, a series of receptor mapping studies indicate high GR densities in the prefrontal cortex of rodents (Cintra et al., 1994; Diorio et al., 1993; Meaney & Aitken, 1985) and non human primates (Sanchez et al., 2000). While in rodents GR and MR seem to be prominent within the hippocampal formation (De Kloet et al., 1998; McEwen et al., 1986), non-human primates seem to express more pronounced GR densities in prefrontal regions when compared to the hippocampal area (Sanchez et al., 2000). This finding indicates that in primates, the PFC might even play a more important role than the hippocampal formation when it comes to GR mediated negative HPA axis feedback mechanisms.

Interestingly, the PFC's role in HPA axis regulation seems to be restricted to stressors that are psychological in nature (Figueiredo et al., 2003b) – a finding, which is in accordance with the notion of the existence of stressor specific neural circuitries (Herman & Cullinan, 1997; Herman

et al., 2003), with stressors that are not acutely life threatening in nature influence HPA axis activity via cortical and limbic areas, while stressors that reflect an acute threat to survival influence HPA axis activity via direct brainstem based circuits. The principal idea underlying the work presented here, was to make a first attempt to relate these highly promising animal findings to the human neurobiology of stress and to test for the role of limbic and prefrontal regions in human HPA axis regulation. In order to do so, two different strategies along with the application of two distinct brain imaging techniques were chosen. In a first approach, positron emission tomography (PET) was the method of choice and 14 healthy males underwent a total of two PET scans. In order to assess brain activation during confrontation with a psychosocial stress test, participants were injected with fluoro-18-deoxyglucose (FDG) right before stressor confrontation or the matched, non-stressful control condition. In order to related stress induced brain activation to HPA axis activity, cortisol was collected before and after stress test or the control condition.

In animals and humans, habituation of the HPA axis due to repeated confrontation with a homotypic stressor is a well established finding. However, while little is known about the brain regions that are involved in regulation of acute HPA axis activity, currently almost no data exist on the possible neural mechanisms of HPA axis habituation. Based on the well established finding that HPA axis activity is common to states of fear and anxiety, it has been speculated that hyper-excitability in the brain's fear networks could be a state that causes hyper-frequent HPA axis activation that would be reflected in reduced HPA axis habituation. Accordingly, in the second study presented here, associations with excitability of the brain's fear circuitries along with patterns of HPA axis activation and habituation were studied in a population of healthy young men. In a first step, an initial group of 90 participants were confronted with two almost identical versions of a psychosocial stress test, that were separated by exactly one week. That way, not only spontaneous HPA axis activation but also HPA axis habituation could be assessed. In order to test for HPA axis activation, saliva cortisol was collected in each participant before and after each stress test. In a subsequent follow up experiment, a sub-sample of the initial 90 subjects returned to the lab for functional magnetic resonance imaging brain scan. During the brain scans, participants were exposed to a paradigm known to induce states of anticipatory anxiety and activation of the brain's fear and anxiety circuitries was elicited. Subsequent data analysis focused on the question whether hyper-excitability in the brain's fear circuitries relates to spontaneous and/or habituated HPA axis response.

5.2 Summary

This section is dedicated to summarize the main findings from the two studies presented here. Although both studies focused on neural regulation of the HPA axis, for reasons of clarity and in order to account for the different methods used (e.g. PET vs. fMRI), results will be summarized separately.

5.2.1 Results - Neural Substrates of Psychosocial Stress – a PET Study

In order to study the neural substrates on psychosocial stress in humans, a total of 14 healthy volunteers underwent two subsequent PET scans. Scans were performed exactly one week apart and each participant was scanned under a stress as well as a control condition. The control condition was designed to match the requirements of the stress condition but was supposed to leave HPA axis activity mostly unaffected. A randomized cross-over design was chosen and half of the participants were confronted with a psychosocial stress test (Trier Social Stress Test) at their first and a control condition at their second PET scan. The remaining seven subjects were exposed to the reversed order. In order to assess HPA axis activity, saliva cortisol samples were assessed in each participant before and after the stress and the control condition.

Confrontation with the TSST resulted in pronounced salivary cortisol increases while the control condition left the HPA axis activity mostly unaffected. Maximum cortisol increases in response to the stress condition were negatively associated with participant's perceived controllability during the stress situation. On the other hand, ratings of general distress were positively associated with HPA axis activity during the stress condition. PET data analysis revealed that confrontation with the stress or the control condition resulted in pronounced glucose metabolism in prefrontal, temporal, parietal and occipital brain areas and the cerebellum. Comparison between the two conditions revealed that the stress condition was characterized by a pronounced increase in glucose metabolism in medial prefrontal areas, while no such changes where observed during the control condition. Subsequent region of interest analysis revealed that glucose metabolism in the prefrontal areas (BA 9 and 10) was negatively associated with glucose metabolism in the amygdaloid region. Moreover, glucose metabolism in the medial prefrontal cortex (BA 9 and 10) was negatively associated with cortisol concentrations. When psychometric measures and brain data were linked, evidence was found that participants who reported high levels of general distress showed a tendency for lower glucose metabolism in brain areas known to inhibit HPA axis activity (BA 10). On the other hand, participants who perceived the stress condition rather controllable, tended to have higher glucose metabolism in brain areas known to inhibit the axis (BA 9).

Taken together, data presented here support the notion that the human prefrontal cortex is activated during exposure to a psychosocial stressor. Moreover, by linking brain data to endocrine markers of HPA axis activity, strong evidence was found, that the activated regions exert an inhibitory tone over HPA axis activation. In line with the notion that perceived uncontrollability is a strong predictor of HPA axis activation (Dickerson et al., 2004; Mason, 1968), glucose metabolism in inhibitory prefrontal brain regions was positively associated with subjective feelings of controllability.

5.2.2 Results - Neural Substrates of Stress and Stress Habituation – a fMRI Study

In order to assess associations between excitability of the brain's fear circuitries and patterns of HPA axis activation and habituation, a total of 90 healthy male participants were exposed to two homotypic stressors (two slightly different versions of the TSST). HPA axis activity was assessed by repeated collection of saliva samples before and after each stress test. In a subsequent follow up-experiment, a total of 38 participants returned to the laboratory for an fMRI scan. During the scanning procedure, each participant was confronted with a so called *"threat of shock"* paradigm, which was thought to result in pronounced activation of the brain's fear circuitries.

In the initial sample, confrontation with the first stress test resulted in pronounced activation of the HPA axis, while repeated exposure to the almost identical procedure resulted in a marked patterns of habituation in the overall group. Based on the response decrease from stress test one to stress test two, an index of HPA axis habituation was calculated for each individual. In order to distinguish habituators from no-habituators, a mean split was performed based on this index in a subsequent step.

Data indicated that the two groups did not differ in HPA axis response during their first stress exposure, while marked differences were apparent during the second exposure. Data clearly showed that habituators exhibited a much lower response to the second stress test when compared to the initial response at the first stress test. Also, at stress test two, the no-habituator group showed significantly higher cortisol results when compared to the habituator group.

In the overall group, high scores on the *Marlowe Crowne Social Desirability Scale* were associated with a poor habituation pattern. No other associations between any psychometric measures of fear and anxiety were found in the overall group. Also, habituators and no-habituators did not differ in any psychometric measure of fear and anxiety.

Out of the 38 participants who returned for the follow-up fMRI experiment, a total 33 went into the final data analysis. Confrontation with the *"threat of shock"* paradigm resulted in a marked increase in BOLD responses in prefrontal, limbic and brainstem areas (threat – no threat contrast). In a subsequent region of interest analysis, cluster information was extracted for brain areas known to be involved in HPA axis regulation. Hereby it was shown, that no-habituators expressed a tendency for elevated BOLD signal responses in the right ventromedial PFC, whereas habituators showed increases BOLD signals on a brainstem level. Individual self reported levels of fear and anxiety mainly correlated with BOLD signal increases in the lateral and dorsolateral PFC as well as dorsal anterior cingulate cortex.

Taken together, the study gives evidence that excitability in parts of the brain's fear and anxiety circuitries is associated with the ability to adapt to a re-occurring homotypic stressor. Hereby, the prefrontal cortex not only seems to be closely linked to endocrine markers of habituation but also seems to be a prominent region when it comes to incorporating subjective affective states.

5.3 Methodological issues

In regard to the experiments described here, several possible confounders should be addressed. First of all, it needs be emphasized that the population studied in both experiments consisted of healthy male individuals exclusively. Moreover, most participants were college students and accordingly young of age (< 25 years). A male population was considered on purpose, as it is well documented that HPA axis functioning is affected by menstrual cycle and the use of oral contraceptives (Kirschbaum et al., 1999). One way to control for this possible confounder would have been to study women in the luteal phase of their menstrual cycle (Kirschbaum et al., 1999) and to control for the usage of oral contraceptives (Kirschbaum et al., 1999). However, incorporating a female population in the studies presented here, would have implied a much larger sample size which would have been beyond the scope of what was manageable within the setting that was given. However, by focusing on one gender, the data presented here should be interpreted cautiously. Receptor mapping studies focusing on gonadal steroid receptors (e.g. estrogen) indicate, that brain areas involved in HPA axis regulation like limbic and brainstem areas are prominent receptor sites (McEwen, 2001). While pronounced gender effects in affective disorders are well documented (Hankin & Abramson, 2001) and the impact of gonadal steroids on mood and cognitive function have repeatedly been reported (McEwen, 2001), the exact mechanisms are still poorly understood and little is known how gonadal steroids effect and modulate neural stress circuits. However, recent studies indicate, that in female ovari-ectomized and estradiol-treated rats, intracerebroventricular infusions of oxytocin, a neuropeptide involved behavioral regulation (e.g. pair bonding, maternal behavior, formation of attachment, lactation), attenuates stress-induced HPA axis response and anxiety behavior (Windle et al., 2004). Hence, as the data reported here are exclusively based on a male study sample, caution should be spent when it comes to generalization of results across genders and similar studies on a female based study population are needed.

As mentioned previously, the study sample consisted of healthy volunteers. In order to control for health related confounders, only volunteers that reported no history of any endocrine, metabolic, allergic or mental disorders were included in the study. In order to screen for health problems, an elaborate phone screening was performed with each volunteer. However, data on the participant's health status were exclusively based on self-report and were not further evaluated by any health care professional. Hence, while it was much emphasize was spent on controlling for health problems, possible health related confounders cannot be ruled out for sure.

5.3.1 Methodological issues - Neural Substrates of Psychosocial Stress – a PET Study

The study presented here is amongst the first experiments using PET imaging technique for identification of neural substrates of HPA axis regulation in humans and thus represents a preliminary study that is, by its nature, not free of methodological issues.

A critical note should be spent of the fact that participants who undergo a glucose PET scan have to have sufficiently low pre-scanning glucose levels and are therefore requested to fast 4 – 5 hours prior to scanning This reflects a necessary procedure, which guarantees that enough radioactive labeled glucose makes its way to the brain. However, it has been shown, that sufficient blood glucose levels are requisite for adequate cortisol changes in response to the TSST (Kirschbaum et al., 1997). Based on these findings, it has been assumed that ready access to energy is prerequisite for HPA axis responses to stressful encounters (Kirschbaum et al., 1997). For the study presented here, fasting glucose levels were tested for all 14 participants on both days and data indicated the glucose levels were sufficiently low prior to scanning (all within a range of 70 – 100 mg/dL; data not shown). However, for the overall group, confrontation with the TSST resulted in pronounced overall cortisol changes. It should be mentioned that maximum cortisol increase scores in response to the TSST ranged from 0.3 – 47.40 nmol/L for the individual subjects. While glucose levels prior to the TSST were not significantly associated with the maximum cortisol increase in response to the stress test, a clear tendency for positive a associations between glucose levels and maximum cortisol increase was observed (data not shown). Accordingly, glucose levels were negatively associated with activity in brain areas previously shown to inhibit the axis (e.g. BA 9: r = -.51; n.s.). This finding seems to be in line with data, indicating that the medial PFC (BA 24 and 32) participates in the autonomic responses to hypoglycemia in humans (Teves et al., 2004). To this point, it remains speculative, why in the face of low glucose levels some participants show pronounced cortisol changes to the TSST whereas others do not. Moreover, the question to which extend these discrepancies might have affected the here presented findings, is subject to possible future studies.

Another issue worth mentioning is the nature of the stress versus the control condition. The latter one was designed to match the TSST in most aspects but was supposed to leave the HPA axis unaffected. However, creating a suitable control condition was one of the major challenges in preparation of the here reported study and at that point in time, little was known about the factors that would ultimatively influence the HPA axis response. Factors like presence of a committee as well as task difficulty were supposed to affect the axis. Therefore, during the control condition, similar verbal and math tasks were chosen and presented for the identical duration and in the same order as during the TSST but without the presence of the committee. However, for reasons of habituation and practice effects, different tasks were chosen for the two conditions (e.g. speech about a wanted job versus a recent book read by the participant) and arguably, objective task difficulty was slightly less pronounced during the control condition. For reasons of not adding additional noise to the already noisy PET paradigm, tasks were not counterbalanced between the two conditions, which could be regarded as a major drawback of this study. However, the study presented here should be looked at as one of the first one in this field of research. Hence, replication of the here presented findings are urged and given that this

preliminary design proofed to be successful, larger sample sizes, and hence less noisy data sets which give the option for a counter balanced task design, should be targeted in future studies.
The request for larger samples sizes in future studies leads to the final methodological issue that will be discussed in this section: As previously described, order of the tasks (e.g. TSST first versus control condition first) was randomized and counter balanced. Hence, half the subjects were confronted with the TSST at their first and with the control condition at their second scan. The other half received the reversed order. However, while within-subjects design have certain advantages, certain drawbacks should be considered: As reported earlier on, task order did not significantly affect the cortisol response during either condition. However, specific carry over effects cannot definitively be ruled out and ideally, group comparison should be the preferred method of choice. However, group comparisons would have requested a much larger sample size (e.g. 20 / 20) which would have been well beyond the financial scope of the project presented here.

5.3.2 Methodological issues - Neural Substrates of Stress and Stress Habituation – a fMRI Study

The fMRI data reported here are amongst the first one to show associations between HPA axis response to a psychosocial stress test and BOLD responses in the brain's fear and anxiety networks in the same pool of subjects. While this approach seems to be a promising way to study the neural substrates of stress in humans, the experiment presented here comes with some drawbacks that are worth to be mentioned.
First of all, the fact that the two stress tests were performed on average 12 month prior to scanning should be critically highlighted. Given the duration of time between the two experiments, one must assume that the two response patterns – namely the HPA axis activation to a psychosocial stress encounter and the BOLD signal changes in the brain's fear networks - are stable tendencies over time. In animals, there is evidence that a fearful temperamental style is associated with heightened HPA axis activity under basal (Kalin et al., 2000) and stress conditions (Cavigelli & McClintock, 2003). Moreover, a hyperactive HPA axis seems to be a stable trait over the life span in these animals (Cavigelli & McClintock, 2003; Kalin et al., 2000). However, while this trait aspect has been shown in animal models, little is known whether HPA axis responsitivity also reflects a trait like pattern in humans. Interestingly, it has even been speculated that a chronic hyperactive HPA axis could lead to states of hypocortisolism as a result of reduced biosynthesis or depletion at several levels of the HPA axis or as a consequence of an increased feedback sensitivity (Heim et al., 2000). However, these ideas seem to be speculative at this point and little is known about the time kinetics of such changes in HPA axis responsitivity.
Moreover, while it has been assumed that an excess of glucocorticoids due to various stressor promotes excitability of the brain's fear circuits (Rosen & Schulkin, 1998), based upon the data

presented here, no conclusions about the causality of these effects can be drawn. While it could be the case that an excess of glucocorticoids leads to a hyper-excitability in the brain's fear circuitries, at the same time it could be speculated that a hyper-responsiveness in the same areas makes an individual more prone to massively respond to stressful and potentially fear-inducing stimuli. In order to address this question, carefully designed animal studies are necessary and longitudinal studies in humans are of need.

Finally, three methodological issues arise from the brain imaging technique chosen here. First of all, fMRI data acquisition and analysis is very sensitive to movement artifacts, as the statistical procedures on hand make strict assumptions about a stable voxel location over the entire scanning period. Hence, applying a shock paradigm, which naturally comes with certain movement tendencies, seems to be rather critical. In order to control for this issue, subjects' heads were restraint by a special vacuum pillow that prevented major head movement. Additionally, a motion correction algorithm (AFNI) was applied and each data set was visually inspected thereafter. However, confounding effects by remaining motion artifacts cannot definitively be ruled.

Another remark should be made about the nature of the fMRI signal: In the past, several brain imaging groups have reported that the BOLD signal is prone to rapid habituation (Fischer et al., 2003; Wright et al., 2001). Habituation effects have been shown for various stimuli (e.g. faces, phobic stimuli) (Veltman et al., 2004; Wright et al., 2001) and in different brain regions (e.g. amygdala, prefrontal cortex, temporal lobe) (Fischer et al., 2003; Veltman et al., 2004; Wright et al., 2001). Given the duration of the paradigm used in the here presented experiment, habituation effects cannot be excluded. In a follow up data analysis it therefore might be worth to look at this possible confounder with a different data analysis approach (e.g. regression models; data file splitting).

A final note should be spent on the problem of geometric and magnetic susceptibility variations within the human head – a problem which can be regarded as one of the major down-sides of fMRI scanning. Magnetic susceptibility variations with the human head are most common superior to sphenoid / ethmoid sinus and the mastoid air cells and are known to cause local field distortions in the B_0 field (Wilson et al., 2002), which quite often causes major signal drop out in these regions. As the orbitofrontal and prefrontal area has been shown to be involved in the brain's fear network (Shin et al., 2004) as well as HPA axis regulation (R. M. Sullivan & Gratton, 2002b), these regions were of special interest for the here presented experiment. In order to address a possible signal drop out in these regions, a special local shimming technique similar to the one described by Wilson and colleagues (Wilson et al., 2002) was tested and evaluated prior to the actual experiment (data not shown). Visual inspection of the data based on this preliminary test indicated significant improvement of signal quality in prefrontal regions and was therefore applied for each subjects' scan sequence. While the application of this technique reflects a major improvement in signal quality in the above mentioned regions, it should be noted

that signal quality was still not perfect and selective drop outs could probably not have been entirely prevented. Whether and how these selective drop outs affected the individual scans and the overall result remains speculative at this point.

5.4 Implications

Data of the two empirical studies presented here give reason to believe, that in humans, the HPA axis is modulated by neural activation in limbic and most importantly prefrontal areas. In light of this, the here presented data are in line with findings from animal models, indicating that limbic regions (Fendler et al., 1961; Herman et al., 1998; Sapolsky et al., 1991) (Herman et al., 1994; Kalin et al., 2004; Roozendaal, 1992; Roozendaal et al., 1991a; Van de Kar et al., 1991) and the prefrontal cortex (Campeau et al., 2002; Cullinan et al., 1995; Diorio et al., 1993; Figueiredo et al., 2003b) play a regulative role during states of psychological stress.

The prefrontal cortex's role in HPA axis regulation

From an anatomical perspective, the prefrontal cortex holds a series of projections that make this brain region a prime candidate for the regulation of affective, endocrine and autonomic functions. Based on distinct properties and connections, an orbital network and a medial network has been defined (Price, 1999). The orbital network receives several sensory inputs (e.g. olfactory, gustatory, visceral afferent, somatic sensory and visual) as well as afferent inputs from limbic regions (e.g. amygdala, entorhinal and perirhinal cortex, subiculum) and may therefore serve as a station for integration of viscerosensory information along with affective signals (Price, 1999). The medial network on the other side provides the major output of the orbital and medial prefrontal cortex to the hypothalamus, brain stem areas, the amygdaloid complex and the hippocampal formation (Carmichael & Price, 1995; Floyd et al., 2001) and exerts cortical influence over autonomic and endocrine function (Price, 1999). These anatomical data are in accordance with recent neuroimaging findings showing that activation in medial prefrontal areas is associated with the induction of affective states in healthy volunteers (Lane et al., 1997a; Lane et al., 1997b; Reiman et al., 1997), emotion regulation (Levesque et al., 2004; Ochsner et al., 2002), and processing of self-referential information (Fossati et al., 2003; Gusnard et al., 2001; Schmitz et al., 2004). The notion that the PFC integrates affective information and promotes corresponding peripheral responses has been supported by lesion (Bechara et al., 1996) and brain imaging data (Nagai et al., 2004; Teves et al., 2004), which give evidence for the PFC's role in autonomic regulation.

Here, data are presented that the human prefrontal cortex also modulates endocrine function and the notion that cortical regions are crucially involved in HPA axis regulation is in accordance with recent receptor mapping studies in non-human primates, which indicate high GR densities in the prefrontal cortex of rodents (Cintra et al., 1994; Diorio et al., 1993; Meaney & Aitken, 1985) and non-human primates (Sanchez et al., 2000). While in rodents GRs and MRs seem to

be prominent within the hippocampal formation (De Kloet et al., 1998; McEwen et al., 1986), non-human primates seem to express more pronounced GR densities in prefrontal regions when compared to the hippocampal area (Sanchez et al., 2000), which indicates that in primates, the PFC might even play a more important role than the hippocampal formation when it comes to GR mediated negative HPA axis feedback mechanisms. More support for the notion that the prefrontal cortex exerts regulative control over endocrine functioning comes from studies showing pronounced neuronal and genomic activation especially in medial prefrontal areas during confrontation with several different stressors. Lesions of the anterior cingulate/prelimbic cortex are associated with increased corticosterone response to stress, which indicates that this region, at least in rodents, acts as HPA axis negative feedback site. In line with this notion, corticosteroid implants in the very same region resulted in a significantly blunted stress induced response of ACTH and corticosterone in rodents (Diorio et al., 1993). However, lesions of more ventral regions of the medial PFC (e.g. infralimbic cortex) have shown to significantly suppress stress induced corticosterone responses. Interestingly, this effect was most pronounced after repeated confrontation with a homotypic stressor (R. M. Sullivan & Gratton, 1999), which indicates that this brain region is involved in the facilitation of adequate endocrine responses to already familiar stressors. Based on this finding it should be emphasized that depending on the exact location, the prefrontal cortex not only exerts an inhibitory tone over endocrine functioning (Diorio et al., 1993), but also seems to promote HPA axis responses under conditions of a repeated homotypic stress exposure (R. M. Sullivan & Gratton, 1999).

This latter finding seems to be in accordance with here presented data, which indicate that the human ventral prefrontal cortex in contrast to more dorsal/rostral areas seems to be involved in facilitation of the HPA axis.

Prefrontal endocrine control and psychopathology

In rodents, chronic stress has been shown to attenuate glucocorticoid negative feedback due to changes in GR structure within the prefrontal cortex (Mizoguchi et al., 2003). Further, chronic behavioral stress has been associated with reorganization of apical dendrites in pyramidal neurons of the medial prefrontal cortex (Radley et al., 2004) and an acquired deficit of forebrain glucocorticoid receptors has been shown to produce depression-like changes in HPA axis regulation and behavior in mice (Boyle et al., 2005). In light of the fact that major depression in humans has frequently been associated with a hyperactive HPA axis (Barden et al., 1995; Maes et al., 1998), decreased metabolism in dorsolateral and dorsomedial prefrontal regions (Drevets, 1999) and pronounced decreased volume and neuronal and glia cell pathology in the very same or nearby regions (Cotter et al., 2002; Ongur et al., 1998b; Rajkowska et al., 1999), these recent animal findings seem to be of special interest in terms of prefrontal functioning in humans with major depressive disorder. The findings also seem to nicely fit the here reported data according to which, activation in medial rostro/dorsal medial prefrontal areas is associated with a less

pronounced HPA axis response in the face of a psychosocial stress encounter. Moreover, activation in the very same areas was positively associated with perceived control and negatively associated with rating of general distress - two affective states that have frequently been associated with the pathology of depressive disorders. Hence, it could be speculated that rostro/dorsal areas play an important role during affective and endocrine regulation during states of stressful experience and might also be involved in the pathology of major depressive disorders. Further, given that chronic stress affects GR structure and cell morphology in these areas, the here presented findings along with data from animal studies (Boyle et al., 2005; Mizoguchi et al., 2003; Radley et al., 2004) point towards a model on how stress could affect the pathogenesis of mood disorders.

Prefrontal – limbic communication and endocrine control

As mentioned previously, the prefrontal cortex is extensively connected with various limbic structures (Carmichael & Price, 1995; Floyd et al., 2001). Thereby, the prefrontal cortex, and here predominantly the medial network (Price, 1999), exerts endocrine and autonomic control via numerous connections with amygdaloid (Carmichael & Price, 1995; McDonald et al., 1996), hypothalamic (Floyd et al., 2001) and brain stem autonomic regions (Terreberry & Neafsey, 1987).

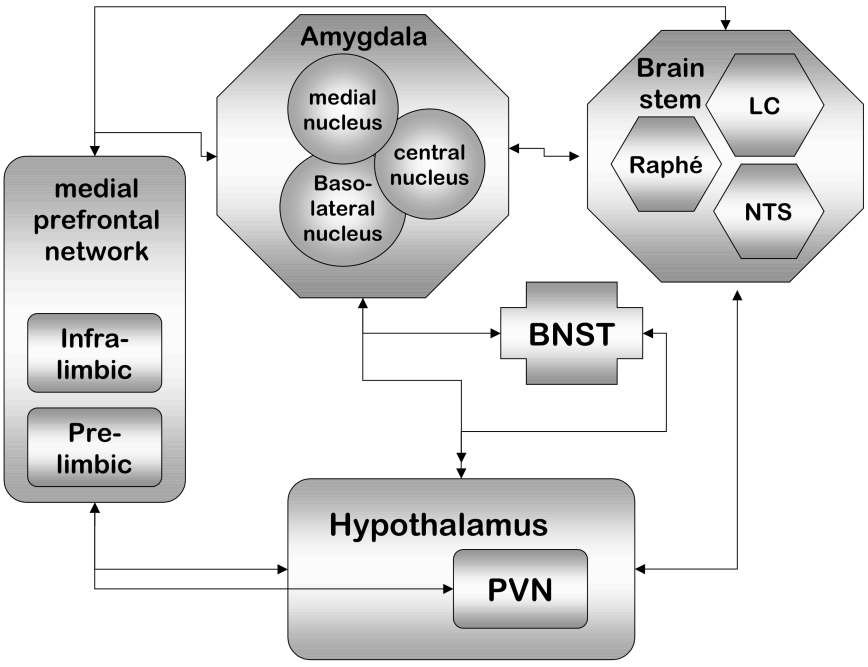

Figure 40: Overview: prefrontal projections to limbic and autonomic brain regions.

In the past recent years, functional connectivity between neocortical and limbic areas has attracted increased attention and there is now considerable evidence that a balanced interplay between prefrontal and limbic regions is requisite in terms of mental well being.

Strong evidence for this notion comes from a series of well conducted studies implicate that the medial prefrontal cortex plays an important role during fear extinction (Milad & Quirk, 2002) and inhibition of protein synthesis in the same region blocks extinction recall (Santini et al., 2004). Moreover, stimulation of medial prefrontal areas has been shown to cause profound decreases in amygdaloid output neurons (Quirk et al., 2003).

In line with these animal findings, neuroimaging studies in patients with anxiety disorders have frequently reported prefrontal hypoactivity during anxiety induction (Bremner et al., 1999). However, while some groups report an inverse functional connectivity between various prefrontal areas and amygdaloid activity (Hariri et al., 2003) others do not (Gilboa et al., 2004).

Data presented here indicated that activation within prefrontal rostro/dorsal brain regions (BA 9 & 10) was negatively associated with stress induced cortisol increases. Moreover, activity in these prefrontal areas was also negatively associated with activity in the amygdaloid complex. Although the exact mechanisms through which the PFC influence amygdala function and how this would translate into autonomic and endocrine control are still not sufficiently understood (Likhtik et al., 2005), data presented here at least indicates that such functional connections exists and might serve as a pathway on how the PFC influences HPA axis activity.

5.5 Outlook

The two empirical studies presented here, for the first time give straight evidence for the notion that the HPA axis in humans is regulated by limbic and most importantly prefrontal areas. However, further studies are needed in order to replicated these very preliminary findings in a broader study population (e.g. female participants).

Data from the here presented PET study clearly indicate that activation in rostro/dorsal medial prefrontal areas is associated with a less pronounced cortisol increase in response to a psychosocial stressor. However, whether these effects are based on GR mediated fast negative feedback effects remains rather speculative at this point in time. Hence, the application of either a cortisol synthesis inhibitor (e.g. metyrapone) or a GR antagonist could help to clarify this question in a way that both substance would prevent the here observed effects given the existence of a GR mediated mechanism.

Data presented here also seem to be of relevance when it comes to recent models on the psychopathology of mood and anxiety disorders. In a next step, it might be interesting to test whether depressed patients with a clear pattern of HPA axis hyperactivity show reduced basal glucose metabolism in here identified inhibitory prefrontal areas when compared to depressed patients that do not show any relevant changes in the axis' activity.

Also, in the fMRI study, an association between increased activity within the ventromedial PFC due to an anxiety inducing paradigm and a lack of HPA axis adaptation due to two homotypic psychosocial stress has been demonstrated. While the findings here did not indicate a clear association between anxiety ratings and patterns of HPA axis habituation, currently nothing is

known about HPA axis habituation in patients with anxiety disorders. If the assumption that a chronic excess of glucocorticoids promotes hyper-excitability in the brain's fear and anxiety circuitries holds true, anxiety patients would be expected to express low patterns of HPA axis habituation and presumably also express elevated activation in the ventromedial PFC in response to an according stimulation.

Based on the here demonstrated findings, further efforts should be undertaken to link neural substrates of stress and emotions with peripheral markers of the same states (e.g. skin conductance, heart rate variability, pro-inflammatory markers, etc.) in order to gain a better understanding of brain-periphery interactions. In summary, the two paradigms introduced here have proven to be useful tools when it comes to study neural substrates of stress in humans and have helped to promote a broader view on the fascinating interplay of the emotional brain.

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Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 03.04.2006 _____

Simone Maria Kern