

A large, stylized neuron in a light gray color serves as the background for the entire cover. The neuron has a central cell body with several branching processes extending outwards. One process extends horizontally to the left, another extends upwards and to the right, and a long, thin process extends downwards and to the right, ending in several smaller branches. The neuron is set against a white background, with a teal horizontal band across the middle.

# **IMAGING THE STRESSED BRAIN**

**Elucidating the time- and region-specific  
effects of stress hormones on brain function;  
a translational approach**

**Marloes J.A.G. Henckens**

**DONDERS**

series



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**Marloes J.A.G. Henckens**

The research presented in this thesis was carried out at the Donders Institute for Brain, Cognition and Behaviour, Centre for Cognitive Neuroimaging, Radboud University Nijmegen, the Netherlands, and the Rudolf Magnus Institute, Department of Neuroscience and Pharmacology, University Medical Center Utrecht, the Netherlands, with financial support from the Netherlands Organization for Scientific Research (NWO, Toptalent grant 021.002.053).

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# IMAGING THE STRESSED BRAIN

**Elucidating the time- and region-specific  
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a translational approach**

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door

**Marloes Jeronimus Anna Gertrudis Henckens**

geboren op 19 mei 1984  
te Beegden

Promotoren:

Prof. dr. G. Fernández

Prof. dr. M. Joëls (UMCU)

Prof. dr. D.G. Norris

Copromotor:

Dr. G.A. van Wingen (AMC)

Manuscriptcommissie:

Prof. dr. B. Roozendaal

Prof. dr. K. Roelofs

Prof. dr. O.T. Wolf (Ruhr-Universität Bochum)

voor mijn Vader



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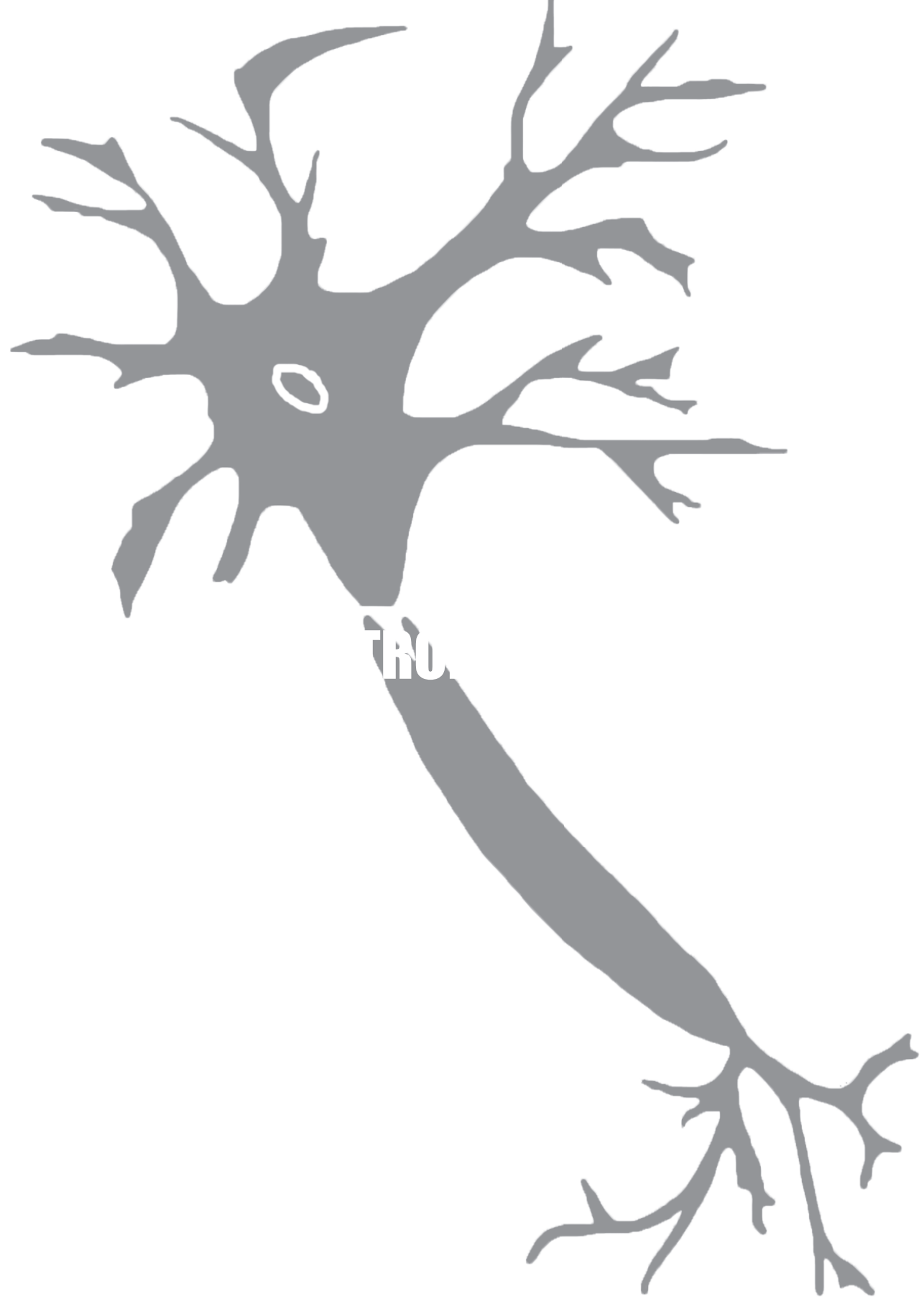
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**“It’s not stress that kills us, it is our reaction to it” Hans Selye**

Stress is often considered to be an abominable and destructive phenomenon, profoundly influencing one's quality of life. However, any threat to homeostasis can be considered a stressor (Levine 2005), which makes it part of daily life. Our body is equipped with several stress response systems that work in concert to orchestrate the most optimal response to this threat, both in terms of physiology and psychology. Thereby, the stress response first and foremost constitutes a highly adaptive response that is of vital importance to an organism's survival. However, when not properly regulated or with prolonged exposure to stress, the same response can cumulate in stress-related psychopathology. Therefore, it is of major importance to understand how brain processing is affected by stress and the accompanying hormones and how these hormones restore homeostasis in the aftermath of stress exposure.

Our physiological bodily state is obviously affected by stress exposure. We feel our heart bumping into our throat, start sweating, maybe freeze, or start trembling a bit. All these phenomena are the result of the actions of stress hormones in our body, providing it a sufficient amount of energy to deal with the stressor. However, the exact same hormones also affect our brain, inducing a state of highly alert, but rather unfocussed processing, in which we start scanning the environment for potential threats. In such a state, we do not think of anything else but the present; our retrieval of memories is impaired, as are many other complex thoughts. At the same time, the present stressful experience is strongly imprinted in our brain, and we will be very well able to remember it any later time point. It could be even remembered to such an extent that we cannot forget it anymore, even when wanted to.

Although stress hormones thus profoundly influence brain functioning and might be related to the development of psychopathology, remarkably little is known about how they exert their effects in the (human) brain. Animal studies have spent great effort investigating the mechanistic underpinnings of stress effects in the brain by studying stress hormone actions on neuronal function. These studies have pointed towards highly time-dependent and brain region-specific effects of stress hormones, especially of corticosteroids. The work presented in this thesis set out to increase our understanding of stress hormone actions in the brain – the human brain in particular – and thereby to contribute to our insight in stress-related mental disease. To do so, we investigated the time- and region-specific effects of stress and corticosteroid exposure on the neural correlates of human brain function by the use of functional MRI. We extended the knowledge on cellular actions by studying the region-specific effects of corticosteroid exposure in the rodent brain using electrophysiology. Moreover, we assessed stress and corticosteroid effects on connectivity patterns in the brain using fMRI in rodents and humans.

The introduction to this work starts by describing the neuroendocrine stress response and the hormones involved. It continues by introducing the effects of these hormones on the rodent brain and discusses the insights that were obtained so far. Subsequently, it provides an overview on our current understanding of the effects of stress hormone exposure on human brain function, and ends

with a discussion on the effects of prolonged exposure to stress (hormones) and the development of psychopathology. Our specific research questions are introduced from the open questions mentioned in these sections, and they are summarized in the final section of this introduction.

## THE NEUROENDOCRINE STRESS RESPONSE

The stress response is a highly adaptive response to help one cope with potential threats in the environment. Upon threat exposure, the incoming sensory information immediately triggers the activation of the autonomic nervous system (ANS), which through its sympathetic and parasympathetic arms provokes rapid alterations in the physiological states of organs throughout the entire body. The activation of the sympatho-adrenomedullary system (SAM) represents the classic ‘fight-or-flight’ response (Cannon 1929) which generally increases circulating levels of adrenalin (primarily from the adrenal medulla) and noradrenalin (primarily from sympathetic nerves), and elevates heart rate, blood pressure and energy mobilization (Iversen et al. 2000). Activation of the locus coeruleus (LC), either directly or indirectly via the actions of the catecholamines on the nucleus of the solitary tract (NTS) (Williams and Clayton 2001), increases LC’s tonic firing rate, and thereby elevates noradrenalin levels in the brain (Ashton-Jones and Cohen 2005; Valentino and Van Bockstaele 2008; Sara 2009). Moreover, activation of the noradrenergic cell groups in the NTS elevates noradrenalin in the brain directly by its (direct) projections to several brain regions. This rise in brain noradrenalin levels induces a surge of vigilance. Adaptive behavior is optimized by the reallocation of neural resources away from higher-order cognitive processes in order to promote vigilance, instinctive behavior and the encoding of the stressful experience into memory (Diamond et al. 2007). The detection and assessment of threats is optimized by the prioritization of sensory processing (de Kloet et al. 2005) and the activation of the key modulator of vigilance and emotional processing in the brain, the amygdala (Phelps and LeDoux 2005; van Marle et al. 2009), at the cost of complex higher-order cognitive function as performed by the prefrontal cortex (Qin et al. 2009; Arnsten 2009). Importantly, the excitation of the ANS wanes quickly, owing to reflex parasympathetic activation, resulting in rather short-lived responses.

The activation of the hypothalamic-pituitary-adrenocortical (HPA) axis ensures a longer-lasting response to stress. Stress exposure activates the paraventricular nucleus (PVN) of the hypothalamus to secrete corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the portal circulation of the median eminence. These releasing hormones act on the anterior pituitary to promote the secretion of adrenocorticotrophic hormone (ACTH), which in turn stimulates the adrenal cortex to initiate the synthesis and release of corticosteroids (corticosterone in rodents and cortisol in humans). This response is slower in onset than the SAM-system, and corticosteroid blood levels peak at approximately 20 min after the initiation of stress and stay elevated for > 1 hour (Droste et al. 2008). The HPA-axis is thought to provide the metabolic support for the stress response by mobilization of stored energy and to potentiate numerous sympathetically mediated effects, such as peripheral vasoconstriction (Ulrich-Lai and Herman 2009). Moreover, the adrenal cortex is directly innervated by the sympathetic nervous system, which can regulate corticosteroid

release (Ulrich-Lai and Engeland 2005), suggesting complementary actions of both systems. However, one of the critical roles of HPA-axis activation is the restoration of homeostasis in the aftermath of stress exposure by diverting energy supply to challenged tissues and suppressing the immune response (de Kloet et al. 1999).

In contrast to the catecholamines, the lipophilic corticosteroids easily cross the blood-brain-barrier to exert their effects on brain function (McEwen 1979). Their actions are known to be mediated by two receptors, the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). Glucocorticoid receptors are widely expressed throughout the brain, but are most abundant in the hypothalamic CRH neurons and pituitary corticotropes. MR expression is mainly restricted to the limbic areas, with highest expression levels found in the hippocampus (Sapolsky et al. 1983; Reul and de Kloet 1985; de Kloet 1991), an area involved in learning and memory processes. While both receptors display high homology in their DNA-binding domain, the two receptors markedly differ in their steroid-binding domain, which results in a differential affinity for their ligand. The MRs binds corticosteroids with much higher affinity than the GR (10-fold), meaning that while MRs are close to being saturated under basal (low corticosteroid) conditions, the GRs are only significantly occupied by relatively high levels of circulating corticosteroids, e.g. in situations of stress (Reul and de Kloet 1985; de Kloet et al. 1998).

Typically, MRs and GRs reside in the cytoplasm in a complex together with heat shock proteins. Upon ligand binding the complexes however dissociate and the receptors translocate to the nucleus, where they influence gene transcription both directly by the binding of either their homodimers or heterodimers to glucocorticoid responsive elements (GREs) in the DNA and the recruitment of co-repressors or co-activators, and indirectly by GR-interaction with other stress-induced transcription factors to dampen their activity (de Bosscher et al. 2003). Thereby, the receptors can either induce or repress the transcription of over 200 genes that are involved in a multitude of cellular processes such as energy expenditure, cellular metabolism, protein synthesis and turnover, signal transduction, neuronal connectivity, and neurotransmission (Datson et al. 2001).

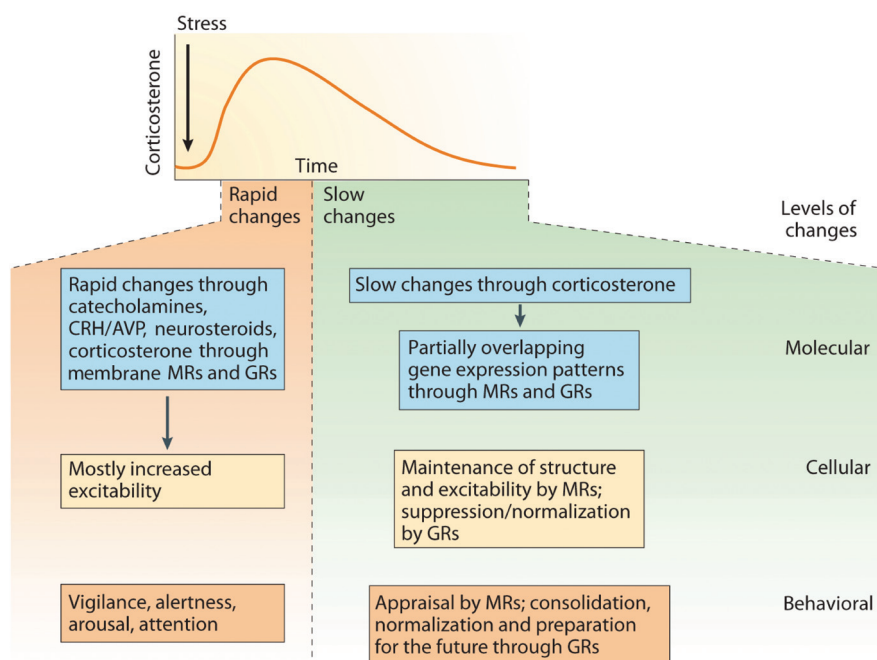
These findings on corticosteroid receptors have led to the hypothesis that the MRs are primarily involved in the ongoing transfer of information and stability of circuits. They would control the sensitivity or threshold of the system's response to stress, and promote the coordination of circadian events, such as the sleep/wake cycle and food intake (Joëls et al. 2008). The GRs on the other hand are thought to play a role in normalizing the activity after stress exposure, to help an organism to cope with, adapt to, and recover from stress. Activation of GRs in the hypothalamus and pituitary exerts a negative feedback action, thereby reducing the enhanced HPA-activity and terminating the stress response (de Kloet et al. 1993; Herman and Cullinan 1997).

However, next to these well-known slow, genomic effects of corticosteroids induced by their binding to the intracellular receptors, more recent work has indicated that the steroids also bind receptors presumably residing in the plasma membrane (Karst et al. 2005) and thereby affect neuronal function in a non-genomic fashion. The membrane MR displays a 10-fold lower affinity for its ligand (comparable to that of the intracellular GR) (Joëls et al. 2008), allowing it to play a

### Box 1. Corticosteroid availability

Under basal conditions corticosteroids are released from the adrenal cortex following a circadian rhythm, reaching peak levels at the start of the active phase (early morning in humans, evening in rodents), followed by a gradual decline over the course of the day. This circadian rhythm in fact overarches underlying series of ultradian pulses with a frequency of approximately one pulse per hour (Jasper and Engeland 1991). These rhythms are not only observed in plasma levels of free corticosteroids, but are also evident in subcutaneous tissue in a highly synchronized fashion (Qian et al. 2012). Besides this pulsatile release pattern, several other factors influence the amount of active corticosteroid available. Approximately 95% of the total corticosteroids in the blood is bound to carrier proteins, of which 80–90% to corticosteroid-binding globulin (CBG), and the rest to serum albumin (Lewis et al. 2005). Secondly, availability of corticosteroids is regulated by the multidrug resistance (MDR) P glycoprotein in the blood–brain barrier (BBB), which hampers the penetration of synthetic glucocorticoids and some naturally occurring glucocorticoids into the brain (Meijer et al. 1998). Thirdly, available corticosteroid levels are regulated by 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD), which catalyses the conversion of the active glucocorticoids corticosterone and cortisol to inert 11-keto-products (11-dehydrocorticosterone, cortisone) and vice versa. Two distinct isozymes occur, of which 11 $\beta$ -HSD-1 predominantly functions as an 11 $\beta$ -reductase (regenerating active glucocorticoids), and 11 $\beta$ -HSD-2 as an exclusive 11 $\beta$ -dehydrogenase (glucocorticoid inactivating enzyme). 11 $\beta$ -HSD-1 is widely expressed in the brain, with highest levels found in the cerebellum, hippocampus, cortex, and pituitary, where it reactivates inert corticosteroids (Seckl 1997). The availability of the corticosteroid-binding proteins and the efficacy of 11 $\beta$ -HSD are also subject to circadian variations (Hsu and Kuhn 1988; Lewis et al. 2006; Droste et al. 2009; Veniant et al. 2009), constituting a complex regulatory mechanism of corticosteroid availability.

prominent role in the behavioral stress response. Activation of this receptor quickly and reversibly enhances the frequency of spontaneous release of glutamate vesicles (Karst et al. 2005), raising neuronal excitability. These rapid actions of corticosteroids have been hypothesized to work in concert with the effects of catecholamines in boosting hypervigilance. More recently also a membrane-located GR has been discovered in the basolateral amygdala (BLA). This receptor was shown to rapidly decrease spontaneous glutamate release (Karst et al. 2010), and was hypothesized to play a role in the meta-plasticity observed in the amygdala (i.e. differential responding to corticosteroids depending on its recent history) in the adaptation to repeated stress exposure. By these mechanisms corticosteroids are capable of influencing neuronal processing in a time-dependent manner in order to produce the most adaptive response to stress (Fig. 1).

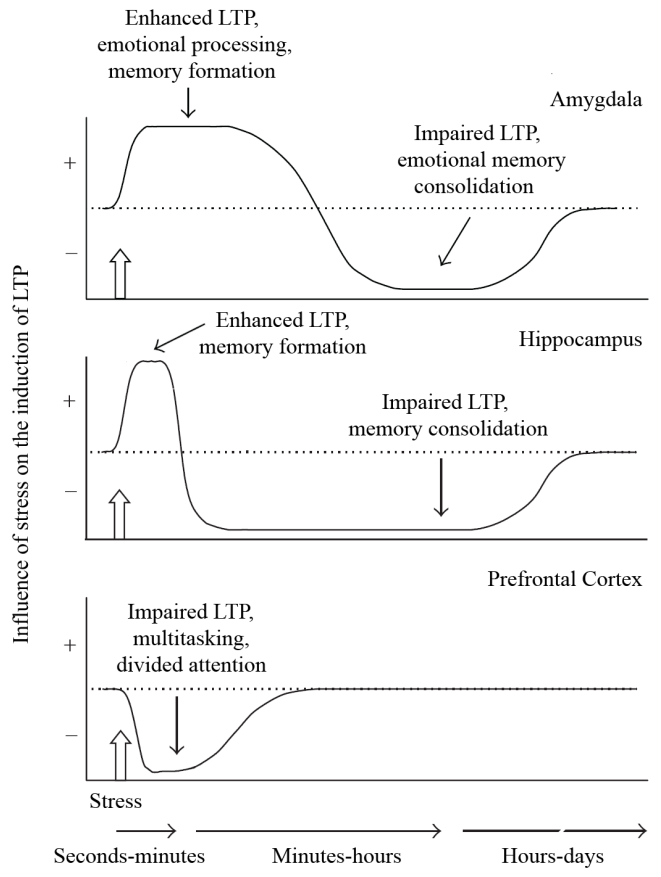


**Figure 1.** Time course of molecular, cellular, and behavioral responses to stress hormones. Activation of the hypothalamic-pituitary adrenal (HPA) axis by stress leads to a temporary rise in circulating corticosteroid levels. In the early phases of the stress response, when the corticosteroid levels rise, fast-acting agents (such as catecholamines, neuropeptides, and corticosteroids themselves by binding to mineralocorticoid (MR) and glucocorticoid receptors (GR) located in the plasma membrane) are thought to contribute to an adequate response to the stressor, which leads to enhanced vigilance, alertness, arousal and attention. Gradually, gene-mediated corticosteroid effects take over through the transcriptional regulation of specific sets of genes by the intracellular MRs and GRs, which affects the cellular function in cells that carry these receptors. Typically, the dose-dependence curve of these cells for the hormone is inverted U-shaped. The MR- and GR-mediated actions affect structural integrity and excitability, and proceed in a coordinated manner, which is linked in time to a particular stage of information processing. The MR is mostly responsible for the maintenance of the stress-related neural circuits, whereas the GR is important for the normalization of homeostasis and the stage of information in preparation for future use. AVP, vasopressin; CRH, corticotrophin-releasing hormone (adapted from de Kloet et al. 2005).

# STRESS AND CORTICOSTEROID EFFECTS IN THE RODENT BRAIN

One of the main questions in stress research is how stress affects (emotional) memory processing. Stressful events are much better remembered than every day, neutral events, and great effort has been taken to elucidate the mechanistic underpinnings of this evolutionary phenomenon. Animal research has largely focused on three distinct regions in the brain, all involved in emotional memory processing in their own unique way; the hippocampus, the amygdala (BLA), and the medial prefrontal cortex (mPFC). The temporal effects of stress exposure on the function of these regions have been extensively reviewed by Diamond and colleagues (2007) in their 'temporal dynamics model of emotional memory' (Fig. 2). This model summarizes the effects of stress on long-term-potential (LTP), the alleged neurobiological substrate of memory formation (Martin and Morris 2002), and states that, in order to promote memory formation for the stressful event, the brain is affected in a region- and time-specific manner. In brief, the model states that upon stress exposure, emotional memory encoding is boosted by an enhanced function of the amygdala and hippocampus, whereas prefrontal cortex function is suppressed. On the longer time-scale, prefrontal cortex function is restored, whereas both amygdala and hippocampal function are suppressed to optimize the consolidation of the emotional memory trace. The Diamond model was the starting point for most of the work presented in this thesis, which tested whether corticosteroids (in isolation) induce similar temporal effects to stress in the amygdala, hippocampus, and prefrontal cortex, and whether these effects translate to the human brain.

**The Hippocampus.** The hippocampus plays a prominent role in the encoding, consolidation, and the retrieval of memories, which makes it of main focus in memory research. Electrophysiology studies have shown that corticosteroids, similar to stress exposure, affect hippocampal signaling in a time-dependent manner. The rapid effects of corticosteroids were shown to quickly and reversibly enhance the frequency of miniature excitatory postsynaptic currents (mEPSCs), each of which reflects the spontaneous release of a glutamate-containing vesicle (Karst et al. 2005). This non-genomic effect critically depended on the presence of MRs residing in the presynaptic terminal membrane, and involved the activation of the ERK1/2 pathway (Olijslagers et al. 2008). Also hippocampal LTP was shown to be rapidly facilitated by corticosteroids (Korz and Frey 2003; Wiegert et al. 2006), but only when present around the time that LTP was induced. Gene-mediated GR actions on the other hand have been shown to slowly increase the amplitude of high-voltage-activated Ca currents (Kerr et al. 1992; Karst et al. 1994; Karst et al. 2000; Joëls et al. 2003), which is most likely caused by an increase in the number of available L-type Ca-channels in the plasma membrane (Chameau et al. 2007). Moreover, corticosteroids' slow actions increased firing frequency accommodation on depolarization, and enhanced the amplitude of the slow afterhyperpolarization (sAHP) that is seen when the depolarization is terminated (Joëls and de Kloet 1989; Kerr et al. 1989). Recently, it has become evident that slow GR-mediated actions enhance surface expression of GluA2 subunits, in association with an enhanced mEPSC



**Figure 2.** Temporal dynamics model of how stress affects memory-related processing in the amygdala, hippocampus, and prefrontal cortex. The initiation of a strong emotional experience activates memory-related neuroplasticity in the amygdala and hippocampus, and suppresses PFC functioning. The most rapid actions would involve increases in ACTH, CRH, noradrenalin, acetylcholine, dopamine, and changes in GABA receptor binding, followed within minutes by elevated levels of corticosteroids. The combination of the activation of the hippocampus by these neuromodulators with coincident tetanizing stimulation produces a great enhancement of LTP. Within minutes of the initiation phase, the hippocampus undergoes a reversal of its plasticity state, and tetanizing stimulation during this phase will result in an impairment of the induction of LTP. The amygdala continues in its potentiated form longer than the hippocampus, but eventually exhibits an inhibitory phase as well, potentially as it is involved in the consolidation of emotional memory. The PFC is only inhibited by stress, and the recovery from its suppression of functioning might depend on the nature and intensity of the stressor, interacting with the ability of the individual to cope with the experience (adapted from Diamond et al. 2007).

amplitude (Karst and Joëls 2005; Groc et al. 2008; Martin et al. 2009). All of these phenomena may contribute to the observation that the slow, genomic effects of corticosteroids typically suppress LTP (Pavlidis et al. 1995; Wiegert et al. 2005) and promote long-term depression with a delay of at least an hour (Kim and Diamond 2002). Moreover, genomic corticosteroid effects are known to suppress excitatory  $\beta$ -adrenergic actions and enhance inhibitory effects of serotonin

(reviewed in Joëls et al. 2006, Joëls and Krugers 2007). Overall, several hours after stress exposure, information flow through the CA1 hippocampal area is attenuated and thus earlier aroused activity normalized, through a GR-mediated mechanism (Joëls and Krugers 2007), while excitatory transmission in specific synapses is enhanced. This would promote ongoing activity, but elevate the threshold for synaptic strengthening of input from other sources, in a fashion known as ‘meta-plasticity’ (Abraham and Bear 1996). This action would enhance the signal-to-noise ratio of information attached to the stressful event, since information reaching the same circuit hours after the initial learning process must be salient enough to overcome this threshold and gain access to memory resources.

Thus, high levels of corticosteroids (as circulate after stress) quickly and reversibly enhance hippocampal glutamatergic transmission via non-genomic actions requiring mineralocorticoid receptors, followed by a suppression of hippocampal cell function by corticosteroids’ genomic actions via glucocorticoid receptors, in order to slowly and long-lastingly normalize its function. However, whether these effects translate to the human brain is currently unknown.

**The Amygdala.** The amygdala is the key regulator of emotional processing and vigilance in the brain, and thought to be involved in the initiation of the stress response by acting on the hypothalamus (de Kloet et al. 2005; Phelps and LeDoux 2005; van Marle et al. 2009). Its dense connectivity pattern places it at the center of the brain’s emotional processing network as a physical hub linking numerous distant regions, allowing emotions to influence brain processing from the first stages of perception (Vuilleumier and Driver 2007) to the regulation of social behavior (Adolphs 2010).

Previous work has indicated that stress increases the firing rate within the BLA; exposure to footshock increased and synchronized firing rate of BLA neurons (Pelletier et al. 2005), and conditioned stimuli induced a similar response (Maren and Quirk 2004). Moreover, restraint stress enhances LTP within the amygdala (Sarabdjitsingh et al. 2012), whereas stress-induced activation of adrenoceptors was shown to enhance long-term potentiation (LTP) of cortical inputs to lateral amygdala pyramidal neurons (Faber et al. 2005). However, also blunted amygdala LTP has been observed in response to stress (Kavushansky et al. 2006; Kavushansky and Richter-Levin 2006; Kohda et al. 2007), leaving this issue unresolved.

Recently, studies were conducted into the effects of corticosteroid administration on BLA neuronal properties. Corticosteroids were shown to rapidly increase glutamatergic transmission in BLA neurons (Karst et al. 2010). In contrast to the quickly reversible effects in the hippocampus, the rapid enhancement in glutamatergic transmission and increased excitability of BLA neurons appears to be long-lasting, and a subsequent exposure to corticosteroids rapidly reduced glutamatergic transmission (Karst et al. 2010). Thereby, the BLA seems to respond in a meta-plastic way to stress and corticosteroid exposure, depending on its recent history. At a more delayed time-scale, corticosterone was found to induce a depolarization of the resting potential, as well as an increase in input resistance, a dramatic decrease in spike-frequency adaptation (Duvarci and Paré 2007) and a reduction in GABA-mediated inhibitory potentials (Duvarci and

Paré 2007). Overall, these results, together with those of others (Kavushansky and Richter-Levin 2006), suggest that corticosteroids enhance the excitability of principal BLA cells by increasing their intrinsic excitability and decreasing the impact of inhibition by GABA.

Recent studies suggest that slow GR effects in the BLA also enhance rather than suppress excitatory transmission (Duvarci and Paré 2007; Liebmann et al. 2008), indicating that corticosterone via its genomic pathway affects hippocampal and BLA neurons differently. This is of interest, because chronic over-exposure to corticosteroids also affects neurons in these two regions in an opposite manner. While principal neurons in the hippocampal CA3 area show reduced apical dendritic complexity after 21 days of high levels of corticosterone, expanded apical trees were reported for BLA neurons (Vyas et al. 2002). This difference may be related to the divergence in response to acute exposure to corticosteroids. To provide more evidence for this assumption it would be of interest to also investigate acute responses to corticosterone in another brain area that, like the BLA, responds to long-term corticosteroid over-exposure with expansion of the dendritic tree. One such area is the orbitofrontal cortex (OFC) (Liston et al. 2006).

**We therefore examined whether regional differences can be observed in the response of pyramidal neurons in the hippocampus and OFC to acutely administered corticosterone (Part 3.4).**

## **BOX 2. Critical role for the amygdala in mediating stress and corticosteroid effects?**

Next to the direct effects corticosteroids exert on brain regions by binding to the locally expressed receptors, there is growing evidence that corticosteroid' actions on regional functioning are to a great extent indirectly established by the modulation of amygdala functioning and its interactions. Activation of the BLA is known to modulate LTP in other brain regions; its activation has been shown to greatly facilitate LTP induction at corticostriatal synapses (Popescu et al. 2007), the hippocampus (Ikegaya et al. 1995, 1997; Akirav and Richter-Levin 1999, 2002; Frey et al. 2001; Korz and Frey 2003; Nakao et al. 2004), and the prefrontal cortex (Richter-Levin and Maroun 2010). Next to this modulatory role of the amygdala under basal conditions, physiological studies into synaptic plasticity have indicated that the effects of stress and corticosteroids on other brain regions are modulated by the amygdala. Blocking the amygdala – either by electrolytic lesioning (Kim et al. 2001) or by microinfusions of the GABAA-receptor agonist muscimol (Kim et al. 2005) – prior to stress exposure, has been shown to prevent stress-induced impairment of hippocampal LTP to occur.

Also in behavioral studies, stress and corticosteroid effects seem to depend on an intact amygdala function. Neurotoxic lesions of the BLA were shown to block glucocorticoid-induced memory enhancement (Roozendaal and McGaugh 1996; Roozendaal et al. 1996), as did the administration of a GR-antagonist in the BLA (Roozendaal and McGaugh 1997;

Donley et al. 2005). These behavioral studies have been explained by the requirement of BLA noradrenergic activity for corticosteroids to establish their effects on memory performance (Quirarte et al. 1997; Roozendaal et al. 2002). Furthermore, specific administration of a GR-agonist in the BLA enhanced memory consolidation (Roozendaal and McGaugh 1997; Donley et al. 2005), and thereby thus mimicked the effect observed for systemic corticosteroid administration. These observations have led to the hypothesis that corticosteroids enhance memory consolidation by rapidly potentiating the noradrenalin signaling cascade in the BLA (Roozendaal et al. 2002). However, infusion of a GR-antagonist into the BLA was shown to attenuate the effects of a  $\beta$ -adrenoceptor agonist on memory retention (Roozendaal et al. 2002), pointing towards a mutual dependence. The current working model is that glucocorticoids affect the noradrenergic system first of all presynaptically in brainstem noradrenergic cell groups projecting to the BLA, and secondly by interacting with the  $\beta$ -adrenergic system postsynaptically in the BLA via coupling with  $\alpha$ -adrenoceptors. Recent evidence suggests that these rapid effects of glucocorticoids on the noradrenergic system may be mediated by membrane-bound receptors which activate a G-protein-coupled, non-genomic signaling cascade that leads to rapidly developing alterations in neuronal excitability (Karst et al. 2005, 2010; Barsegyan et al. 2010; Roozendaal et al. 2010).

However, not all corticosteroid-modulation of memory processes is mediated by corticosteroid effects on the amygdala. Corticosteroid-impairing effects on memory retrieval were shown to be mediated by their actions on the hippocampus (Roozendaal et al. 2003, 2004b), whereas the corticosteroid-impairment of working memory processing was depending on their effects on the medial prefrontal cortex (Roozendaal et al. 2004a). Although these studies also indicated the requirement of an intact noradrenergic function of the BLA (Roozendaal et al. 2003, 2004a), another recent study showed noradrenaline independent effects on memory retrieval (Segev et al. 2012). Overall, an intact amygdala may be necessary to establish the behavioral phenotype of corticosteroid administration, but the actual actions of corticosteroids might be elsewhere in the brain, depending on the process studied.

**The Prefrontal Cortex.** The prefrontal cortex (PFC) is known for its role in higher-order executive function. It is involved in a multitude of processes, such as planning complex cognitive behavior, decision making, and moderating social behavior. Its overall function is considered to be the orchestration of thoughts and actions in accordance with internal goals. Under conditions of acute stress, prefrontal LTP has been shown to be impaired (Maroun and Richter-Levin 2003, Rocher et al. 2004), as well as working memory performance (Birnbaum et al. 1999, Roozendaal et al. 2004a). These effects are at least partly caused by the stress-related hormones noradrenalin and dopamine, which are known to deteriorate prefrontal cortex function in higher doses (Arnsten 2009). However, GR blockade just after exposure to stress was shown to prevent the stress-induced impairment in prefrontal LTP, indicating that the rapid effects of corticosteroids are

involved as well (Mailliet et al. 2008). Moreover, systemic administration of corticosteroids or a local injection of a GR-agonist in the mPFC was sufficient to induce an impairment in working memory performance (Roosendaal et al. 2004a). These effects appeared to critically depend on the BLA, since lesioning the BLA prevented the corticosteroid-induced impairment to occur. These data suggest that the rapid effects of corticosteroids, in conjunction with the effects of other stress hormones, impair prefrontal cortex function.

Until recently, not much was known about the slow, genomic effects of corticosteroids on prefrontal cortex function. However, two recent rodent studies showed that the administration of corticosterone to pyramidal neurons in the ventromedial PFC enhances glutamatergic transmission (increased mEPSCs amplitude) by an increase in surface levels of NMDA- and AMPA-receptor subunits (Yuen et al. 2009, 2011). Moreover, the first study showed that stress improved performance on a WM-task 4 hours later, but not immediately, indicating that the slow, genomic effects of corticosteroids improve working memory performance. The timing of the observed effects of these studies, as well as their neural underpinnings, should be tested in future studies to obtain elusive evidence for the involvement of a genomic mechanism.

**Noradrenergic-corticosteroid Interactions.** Besides working as separate entities, the end products of the bodies' stress systems – noradrenalin and corticosterone/cortisol – seem to interact in establishing their effects (Krugers et al. 2012). Despite the different kinetics for adrenergic and steroid signaling, there is a time window during which the brain is simultaneously exposed to elevated levels of both catecholamines and corticosteroid hormones, allowing these neuromodulators to affect neuronal processes in concert (Joëls et al. 2011). Recent studies investigating the effects of combined administration of corticosteroids and  $\beta$ -adrenergic receptor agonists have shown increases in AMPAR phosphorylation, surface expression, and mEPSC frequency in the hippocampus (Zhou et al. 2012), and acceleration of LTP in the dentate gyrus (Pu et al. 2007), suggesting a rapid interactive effect of both hormones.

The slow genomic effects of corticosteroids however seem to suppress the effects of subsequent noradrenergic activation. Pretreatment with corticosteroids > 1 h prior to activating  $\beta$ -adrenergic receptors, prevented the noradrenergic boost in LTP in the DG to occur (Pu et al. 2007). Similarly, the efficacy of noradrenalin to reduce a calcium-dependent K-conductance in CA1 pyramidal neurons, causing cells to fire more action potentials during a depolarizing episode, was strongly attenuated by corticosterone pretreatment (Joëls and de Kloet 1989). In the BLA, pretreatment with corticosteroids reduced the noradrenergic-induced rapid enhancement of AMPAR-mediated synaptic responses (Liebmann et al. 2009), and corticosteroids gradually reversed the noradrenergic potentiation of LTP (Pu et al. 2009). These data all suggest that whereas the rapid effects of corticosteroids may work in concert with the actions of the catecholamines, their slow genomic effects might counteract them to restore normal functioning in the aftermath of stress exposure.

Conversely, initial noradrenergic activation might modulate genomic effects of corticosteroids later on. A recent study showed that gene binding of the GR is targeted to preexisting foci of

accessible chromatin (John et al. 2011) and that previous stress exposure or arousal induces alterations in chromatin structure modulating the GRs effect. Stressful challenges (e.g. forced swimming (Bilang-Bleuel et al. 2005), novelty (Chandramohan et al. 2007), and fear conditioning (Chwang et al. 2006; Gupta et al. 2010) were shown to evoke such post-translational changes. Moreover, the rapid effects of corticosteroids were shown to play a role in establishing the observed epigenetic modifications (histone modifications and DNA (de-)methylation) and conformational changes in the chromatin by GRs interacting with the NMDA-receptor activated ERK-MAPK pathway in a rapid, non-genomic fashion (Trollope et al. 2012). This suggests that corticosteroids' slow genomic effects might be modulated by earlier rapidly induced changes by corticosteroid signaling and concurrent noradrenergic activation.

**Behavioral Consequences.** The time-dependent effects of stress and corticosteroids on hippocampal, amygdala, and prefrontal cortex signaling have been shown to influence behavior. By far most research is performed into the effects of stress and corticosteroids on memory processing. Memory tasks used vary from emotional learning paradigms like inhibitory avoidance, fear conditioning or Morris water maze learning, to rather neutral learning settings as implemented in the object recognition task. Stress and corticosteroid administration clearly affect memory processing, but whether they enhance or impair performance in these tasks depends on the memory process affected. Memory retrieval is generally impaired under conditions of acute stress or elevated corticosteroid levels (de Quervain et al. 1998; Roozendaal et al. 2003, 2004b; Park et al. 2008; Li et al. 2012), whereas memory formation, consolidation, and reconsolidation seem to be improved by stress or corticosteroid exposure (Roozendaal and McGaugh 1996, 1997; Roozendaal et al. 1996, 2006; Pugh et al. 1997; Hui et al. 2004; Donley et al. 2005; Pitman et al. 2011; Zhou et al. 2012). Noradrenalin is known to influence memory formation by acting on primarily the  $\beta$ -adrenergic receptors (Hatfield and McGaugh 1999; Debiec and LeDoux 2004; Hu et al. 2007; Bush et al. 2010). Corticosteroids are thought to boost the appraisal and response selected during the learning process by acting on the MRs (Oitzl and de Kloet 1992; Sandi and Rose 1994), whereas they have been shown to promote long-term consolidation of information by acting on the GRs (de Kloet et al. 1999; Joëls et al. 2006; Roozendaal et al. 2009). Post-training application of GR agonists has been shown to promote the consolidation of information (Sandi and Rose 1994; Roozendaal 2000), which is in line with the finding that corticosteroids via GR-binding promote consolidation of information in a genomic fashion (Oitzl et al. 2001). However, a recent study suggested that membrane-associated GRs also promote long-term memory in an object recognition task via chromatin modification (Roozendaal et al. 2010). Thus, both the non-genomic and genomic actions of corticosteroid hormones on GRs might promote memory consolidation processes.

However, next to these rather independent effects of noradrenalin and corticosteroids in modulating memory processing, their interaction might affect (emotional) memory formation in particular (Roozendaal et al. 2009). The presence of noradrenalin has been shown to be critical for corticosteroid induced facilitation of memory consolidation (Quirarte et al. 1997; Roozendaal et

al. 2006a, 2006b) and impairment of retrieval (Roosendaal et al. 2004b), since the administration of a  $\beta$ -adrenoceptor antagonist prevented these effects to occur. Similarly, corticosterone was ineffective in rats with reduced training-associated emotional arousal due to prior habituation to the experimental context (Okuda et al. 2004), whereas it did enhance object recognition in naive rats. Conversely, emotional arousal effects were mimicked in well-habituated rats by releasing endogenous noradrenalin via administration of the  $\alpha$ 2-adrenoceptor antagonist yohimbine immediately after object recognition training (Roosendaal et al. 2006a). There is also evidence for the reverse interaction, i.e. corticosteroids influencing noradrenalin effects, since corticosteroids were shown to increase the availability of noradrenalin in the BLA (McReynolds et al. 2010).

The effects of stress and corticosteroids on purely emotional processing and anxiety have also been investigated. Stress and the administration of noradrenalin in rodents clearly cause an anxious phenotype (Gorman and Dunn 1993; Khoshbouei et al. 2002; Cecchi et al. 2002a, 2002b; Morilak et al. 2005). Administration of corticosteroids on the other hand, has been shown to induce anxiolytic effects. Corticosteroid administration resulted in more explorative and socially interactive behavior in rats, which was the exact opposite effect of acute stress (File et al. 1979; Andreatini and Leite 1994; Oitzl et al. 1994). Interestingly, chronic administration of an MR antagonist induced a similar anxiolytic effect (Hlavacova et al. 2010), whereas the MR agonist aldosterone induced an anxious phenotype (Hlavacova and Jezova 2008). Acute administration of the GR agonist dexamethasone on the other hand reduced anxiety when administered in moderate doses, but increased anxiety related behaviors at a high dose (Vafaei et al. 2008). These data suggest a critical balance in the MR/GR ratio for corticosteroid effects to induce anxiogenic or anxiolytic effects (de Kloet et al. 1998), but more research is needed to confirm this hypothesis.

Lastly, concerning prefrontal cortex functioning rather inconsistent behavioral effects of stress and corticosteroid exposure have been reported. In general, stress-induced release of noradrenalin is thought to impair prefrontal cortex function, as assessed by working memory performance (Arnsten 1999; Birnbaum et al. 1999) and response inhibition (Kobori et al. 2011). However, disruption of noradrenaline signaling in the PFC on the other hand has also been related to impaired cognitive function (Clinton et al. 2006; Tait et al. 2007; Milstein et al. 2007; Newman et al. 2008; Bari et al. 2011). Thus, noradrenalin seems to modulate PFC function in an inverted U-shaped manner, with both very low or very high levels impairing performance (Arnsten 2009). The effects of corticosteroids on PFC cognitive function are even less clear, since both enhancements (Yuen et al. 2009; Yuen et al. 2011) and impairments (Roosendaal et al. 2004a; Butts et al. 2011) in function have been reported. Like catecholamines, endogenous corticosteroids were shown to be essential for maintaining PFC function, since HPA disruption appeared to contribute to PFC cognitive deficits (Mizoguchi et al. 2004). However, the effects of an elevation in corticosteroid levels on PFC function are currently unknown, as are the time-dependent effects of corticosteroids.

# STRESS AND CORTICOSTEROID EFFECTS IN THE HUMAN BRAIN

Over the past years, many behavioral and neuroimaging studies have been executed to elucidate the effects of stress and corticosteroids on human cognitive function. The most commonly used and well-established method for stress-induction is the so-called Trier Social Stress Test (TSST) (Kirschbaum et al. 1993). In this task, participants are asked to prepare a job interview and present themselves to a very neutral, non-responsive committee wearing white lab coats. Meanwhile, participants are video-taped and told that they will be evaluated on both the content of their speech, as well as their body language. Following the interview, participants are asked to perform a mental arithmetic task, subtracting steps of 13 from 1022 (or in an adapted version 17 from 2041). Every single time they make a mistake they are instructed to start over again. A disadvantage of the traditional TSST is that it cannot be executed in an fMRI environment, making it impossible to test for the rapid effects of the stress induced by the task. However, a MRI-compatible version of the TSST was recently developed; the Montreal Imaging Stress task (MIST) (Dedovic et al. 2005). Similar to the TSST, participants are asked to perform a mental arithmetic task, but this time their performance is tweaked and they receive very negative feedback on their performance. An alternative method for stress-induction is the cold pressor task (CPT), which is more based on physical stress (Andreano and Cahill 2006). Participants are asked to hold their hand in a bucket of ice-cold water for as long as they can stand (maximally 3 min). During this period, the experimenter is sitting next to them, which ensures a social-evaluative component in this task as well. Although they differ in the basal component causing the stress (social evaluation versus physical pain), these stress induction procedures have been shown quite effective in inducing a reliable psychological and physiological stress response, increasing salivary cortisol levels and heart rate (Dedovic et al. 2005; Andreano and Cahill 2006; Foley and Kirschbaum 2010). Nevertheless, neither the MIST nor the cold pressor test are optimal tests to be used in the scanner. In the MIST, task difficulty is inherent to the stress manipulation, which causes the data to be confounded by this factor. The cold pressor task activates brain circuits that are unlikely to be involved in more psychological type of stressors, which prevail in normal life. We therefore developed a stress paradigm with optimal homology to real-life stress, which allowed us to investigate the neural correlates of stress exposure in an fMRI scanner (Part 2.1).

A more controlled way of increasing stress hormone levels is by merely administrating them, either by injection or oral intake. An important factor to take into account is the dose in which the hormones are administered, since next to the inverted U-shaped effect of noradrenalin on cognitive function, a similar relationship has been reported for corticosteroids, with both very low or very high levels impairing cognitive performance (Abercrombie et al. 2003; Andreano and Cahill 2006).

Although testing the effects of these stress procedures on cognitive performance has yielded many interesting results, these studies overall lack consistency in a multitude of experimental factors,

leaving many issues unresolved. Factors that emerged over the past years shown to influence the effects of stress (hormones) on cognitive function, are age (Wolf et al. 2001a; Lupien et al. 2002), gender (Wolf et al. 2001b), menstrual cycle and oral contraceptive use (Kuhlmann and Wolf 2005), time of day (Lupien et al. 2002; Maheu et al. 2005), order of testing (Wirth et al. 2011), personality traits (Abercrombie et al. 2012), exogenous dose (Abercrombie et al. 2003), and emotional arousal (Buchanan and Lovallo 2001; Abercrombie et al. 2006). Obviously, results also highly depend on the type of cognitive process, and thus involved brain region, targeted. Moreover, as has become evident from animal research, the relevance of the delay between stress/corticosteroid exposure and behavioral testing is also an important factor that deserves closer attention.

**Memory Processing.** The behavioral effects of stress or corticosteroid exposure on memory processing observed in animals, have been largely replicated in humans. Stress exposure or hydrocortisone administration prior to memory retrieval has been shown to impair the recall of previously learned material (de Quervain et al. 1998, 2000, 2003; Tops et al. 2003; Kuhlmann et al. 2005a, 2005b; Kuhlmann and Wolf 2006; Buchanan et al. 2006; Buchanan and Tranel 2008; Tollenaar et al. 2009), and was associated with reduced activation of the medial temporal lobe (de Quervain et al. 2003; Oei et al. 2007; Weerda et al. 2010). Many of these studies have pointed towards a corticosteroid-dependence on concurrent noradrenergic activation in mediating these effects, since the recall deficit was only observed for emotional words (Wolf et al. 2004; Kuhlmann et al. 2005a, 2005b), or when participants were emotionally aroused during retrieval (Kuhlmann and Wolf 2006). Moreover, one study showed that the administration of propranolol prevented the corticosteroid-induced impairment on retrieval to occur (de Quervain et al. 2007). However, other studies have indicated impaired retrieval regardless of the emotional valence of material studied (de Quervain et al. 2003; Tops et al. 2003; Tollenaar et al. 2009), leaving this issue unresolved.

Human studies on the effects of stress and corticosteroids on memory consolidation have also replicated the effects found in rodents; stress or high levels of cortisol during consolidation have been shown to improve long term memory performance (Cahill et al. 2003; Abercrombie et al. 2006; Andreano and Cahill 2006; Beckner et al. 2006; Preuss and Wolf 2009). Also for consolidation, emotion specific (Cahill et al. 2003) and non-specific (Abercrombie et al. 2006; Andreano and Cahill 2006; Beckner et al. 2006; Preuss and Wolf 2009) effects of corticosteroids have been reported.

Finally, the effects of stress (Maheu et al. 2005; Payne et al. 2006, 2007; Smeets et al. 2007; Schwabe et al. 2008, 2009; Cornelisse et al. 2011) and corticosteroid administration (Buchanan and Lovallo 2001; Abercrombie et al. 2003; Kuhlmann and Wolf 2006; van Stegeren et al. 2010) on memory encoding in humans have been studied. In general, memory boosting effects have been reported, either for emotionally arousing information specifically (Buchanan and Lovallo 2001; Kuhlmann and Wolf 2006; Smeets et al. 2007; Payne et al. 2006, 2007; Cornelisse et al. 2011), or in general (Abercrombie et al. 2003; Maheu et al. 2005; Schwabe et al. 2008; van

Stegeren et al. 2010). However, in order for stress-enhanced memory encoding to take place, a few conditions have to be met, which were recently reviewed by Joëls and colleagues (2006). This review states that enhanced memory encoding will only take place when the encoded material shows both overlap in time and space with the stressor. That is, only when the learning takes place closely in time and in the same context as the stressor, memory encoding will be enhanced. This hypothesis was recently supported by a study showing that stress-exposure enhanced the learning of stressor-related words specifically, whereas memory for arousing stressor-unrelated and neutral words was unaffected (Smeets et al. 2009). Moreover, it might explain why other studies have only reported emotionally arousing (i.e. relevant) information to be enhanced following stress or corticosteroid exposure. However, more evidence is needed to support this hypothesis.

Although some neuroimaging studies have been performed, relatively little is known about the neural underpinnings of the stress and corticosteroid effects on human memory processing. Studies investigating the effects of stress on brain function have examined neural activity during stressful compared to non-stressful arithmetic (e.g. the MIST) and have reported on deactivations of the limbic system (Pruessner et al. 2008), and on both activations (Wang et al. 2005; Dedovic et al. 2009a) and deactivations (Pruessner et al. 2008) of regions in the prefrontal cortex. Moreover, the deactivation of the hippocampus was shown to predict the subsequent cortisol release in response to the stressor (Pruessner et al. 2008). However, as mentioned before, task difficulty in these studies is inherent to the stress manipulation, which causes the data to be confounded by this factor.

**We here tested the effects of a stressor with high homology to real-life stress on the neural correlates of memory formation (Part 2.1).**

Studies investigating the effects of corticosteroids on memory encoding have reported on both increases (van Stegeren et al. 2010) and decreases (Kukolja et al. 2011) in hippocampal memory-related activity. For memory retrieval, MTL down-regulation has generally been reported for corticosteroids (de Quervain et al. 2003; Oei et al. 2007), which was also seen during rest (Lovallo et al. 2010) and fear conditioning in men (Merz et al. 2010). However, most of these studies neglected the time-dependency (non-genomic vs. genomic actions) of corticosteroid effects on brain functioning, leaving this issue unresolved.

**In this thesis (Part 2.2) we examined the relevance of the delay between corticosteroid exposure and testing its effect on memory formation.**

**Emotional Processing.** Stress and corticosteroids are also known to influence emotional processing in itself. Recent studies from our lab indicated that both acute (van Marle et al. 2009) and prolonged stress exposure (van Wingen et al. 2011a) increase amygdala responsivity to emotional input. This boost in amygdala processing has previously been attributed mainly to the actions of catecholamines on brain function (Arnsten and Li 2005; van Marle et al. 2009). Studies using pharmacological manipulations to either suppress or enhance noradrenergic activation, by

administering propranolol ( $\beta$ -adrenergic receptor antagonist), and yohimbine ( $\alpha$ -2 adrenoceptor antagonist) or reboxetine (selective noradrenalin reuptake inhibitor) respectively, confirmed this suggested noradrenergic modulation of amygdala activity (Strange et al. 2003; van Stegeren et al. 2005; Hurlemann et al. 2005, 2007). The exact effect of corticosteroids on amygdala functioning is less clear. Behavioral (though not electrophysiological) studies in animals have suggested a boosting effect of corticosteroids on noradrenergic signaling in the amygdala, but this has not consistently been replicated in humans. Some studies investigating the influence of endogenous cortisol levels on amygdala activity, have reported on a positive interaction of cortisol with noradrenergic activation of the amygdala; participants with relatively high basal cortisol levels displayed a larger emotion effect in the amygdala than those with relatively low levels (van Stegeren et al. 2007, 2008). However, other studies have related a higher amplitude of cortisol release to lower responsivity of limbic brain regions (amygdala, hippocampus and hypothalamus) towards emotional stimuli (Cunningham-Bussell et al. 2009), and an enhanced capability to regulate negative affect (Urry et al. 2006). Corticosteroid administration studies have also shown conflicting results, showing both reductions in amygdala activity (Lovallo et al. 2011) or increases (van Stegeren et al. 2010). Results on the combined administration of corticosteroids with either reboxetine or yohimbine has also produced confusing results, with studies reporting on both a negative (van Stegeren et al. 2010; Kukolja et al. 2011) and positive interaction (Kukolja et al. 2008; Hurlemann et al. 2007) between corticosteroids and the noradrenergic system. The dose and time of administration could play a major role in these inconsistencies, as could the differences in experimental tasks participants were subjected to.

It is remarkable to note that in contrast to the described models in animal literature so far, corticosteroids also seem to have a protective role in coping with stress. Next to the observations in animals, findings in humans have also indicated anxiolytic effects of corticosteroids. Corticosteroids were shown to reduce the anxiety-driven selective attention to threat (Putman et al. 2007; van Peer et al. 2009), attenuate fear responses (Soravia et al. 2006), and protect mood during exposure to stressful situations (Het and Wolf 2007, Het et al. 2012). A recent review suggested that the immediate effects of cortisol may facilitate stress-coping via the inhibition of automatic processing of goal-irrelevant threatening information and through increased automatic approach-avoidance responses in early emotional processing (Putman and Roelofs 2011). Moreover, a corticosteroid-induced tonic suppression of the acoustic startle reflex, thought to be modulated by the amygdala, was observed in humans, an effect that occurred independent of emotional modulation (Buchanan et al. 2001). All these data would suggest that corticosteroids suppress, rather than boost amygdala function.

In conclusion, the effects of corticosteroids on emotional processing in the amygdala are currently unclear. However, the time-dependency of corticosteroid effects has been neglected in the large majority of the aforementioned studies and might explain the contradictory results.

**We here addressed the question whether rapid effects of hydrocortisone in humans affect amygdala function differently than slow (presumably) genomic actions (Part 3.1 and 3.2).**

**Higher-order Cognitive Function.** As mentioned before, higher-order cognitive function as performed by the prefrontal cortex is generally impaired under conditions of acute stress (Arnsten 2009). Human PFC function is often assessed by studying working memory (WM). WM refers to a system which maintains relevant information in a temporary buffer that is constantly updated to guide behavior (Baddeley 2003). It is typically associated with the activation of the frontoparietal executive network, including the dorsolateral prefrontal cortex (DLPFC) (Baddeley 2003). Previous studies have shown that under conditions of acute stress, working memory performance is generally impaired (Elzinga et al. 2005; Oei et al. 2006; Luethi et al. 2008; Schoofs et al. 2008, 2009; Taverniers et al. 2010) and prefrontal cortex activity suppressed (Qin et al. 2009). These effects are at least partially caused by the release of noradrenaline and dopamine, which are known to suppress PFC function when present at high doses (Arnsten 2009). The role of corticosteroids in mediating these effects is however not completely clear, since previous reports on corticosteroid-modulation of working memory performance have reported rather conflicting findings. Some studies have shown no effects on WM-performance (Monk and Nelson 2002; Kumsta et al. 2010), others found corticosteroid-induced improvements (Oei et al. 2009), as well as impairments (Lupien et al. 1999; Wolf et al. 2001a) depending on concurrent sympathetic activation (Elzinga et al. 2005) or WM-load (Oei et al. 2006). The latter findings suggest that the effects of corticosteroids might have effects additive to noradrenergic activation impairment in WM. Rodent work has shown that concurrent noradrenergic activity of the amygdala is actually essential for corticosteroid-induced impaired WM to occur (Roosendaal et al. 2004a). In line with this, a recent human study into the effects of noradrenalin and corticosteroids on the neural correlates of memory formation, showed that specifically the combined administration of both hormones caused a strong deactivation in the prefrontal cortex, whereas no such effects were observed when corticosteroids were administered alone (van Stegeren et al. 2010). However, the neural underpinnings of the observed behavioral effects remain largely unknown, as well as the time-dependency of these effects of corticosteroids on WM-processing.

**To further elucidate the time-dependency and circuits involved in corticosteroid actions on higher-order cognitive function, we tested the rapid and delayed effects of hydrocortisone on working memory and the associated brain networks (Part 3.3).**

**Connectivity.** Besides influencing the activation pattern of specific brain regions, stress has also been shown to affect the functional connectivity between regions. Several functional connectivity networks can be distinguished in the human brain, with three networks of major importance to the stress response and its regulation: the so-called salience network, the executive control network, and the default-mode network (DMN) (Seeley et al. 2007). The salience network comprises the ventral emotional processing system, including the brain stem, amygdala, insula, ventral striatum, and ventral regions of the ACC and prefrontal cortex. It is known to be involved in the identification of the emotional significance of a stimulus and the production of an affective state (Phillips et al. 2003; Roy et al. 2009). It is reciprocally connected to the dorsal executive

control network which includes the hippocampus and dorsal regions of the anterior cingulate and prefrontal cortex. This network is responsible for the regulation of the affective state (Phillips et al. 2003), and enables an organism to sustain attention, and supports working memory (Curtis and D'Esposito 2003) and response selection (Lau et al. 2006). The DMN on the other hand is most active during passive resting conditions, and seems to be involved in task-independent introspection or self-referential thought processes, such as autobiographical memory, prospection, self, attention, and theory of mind (Mevel et al. 2010).

Activity and connectivity within these networks is known to be affected by stress exposure, and altered in stress-related psychopathology. Connectivity within and between the salience, executive control, and the DMN has been shown to be affected in stress-related mental disorders, such as PTSD (Gilboa et al. 2004; Daniels et al. 2010; Lanius et al. 2010; Patel et al. 2012; Sylvester et al. 2012) and depression (Hulvershorn et al. 2011; Sylvester et al. 2012; Whitfield-Gabrieli et al. 2012). Moreover, previous research has indicated that the connectivity of the amygdala within the salience network is boosted by both acute (van Marle et al. 2010, Hermans et al. 2012) and prolonged (van Wingen et al. 2011a, 2011b) exposure to stress, which might even last long after the stressor is gone (van Wingen et al. 2012). The exact role of corticosteroids in the modulation of this altered connectivity is currently unknown.

**Therefore, we examined the effects of corticosteroids on the functional amygdala network in humans (Part 4.1).**

## **CHRONIC STRESS AND THE DEVELOPMENT OF PSYCHOPATHOLOGY**

The acute response to a stressful situation, which allows an organism to respond optimally to the threats in the environment, is quite short lasting. Once the stressor is gone, SAM-activation is normalized and the negative feedback mechanism of the HPA-axis ensures the termination of its own activation (De Kloet and Reul 1987). However, chronic activation of the stress systems can have much longer lasting consequences to brain function. Animal research has indicated that prolonged periods of stress exposure affect both brain function and structure in a region-specific manner. Higher-order cognitive function, performed by the hippocampus and medial prefrontal cortex, generally deteriorates as a consequence of chronic stress. Chronic restraint, unpredictable, and psychosocial stress have been shown to impair memory performance (McEwen 2001), reduce hippocampal LTP (Pavlidis et al. 2002), and impair attentional set-shifting in rats (Liston et al. 2006). Structurally, this deterioration of function is associated with a reduced hippocampal volume (Lee et al. 2009), and dendritic atrophy in hippocampal (CA3) and medial prefrontal (prelimbic and cingulate cortex) cells. Pyramidal neurons in these regions show reduced branching of the apical dendritic tree and a decrease in apical dendritic length as a consequence of stress (Woolley et al. 1990; Watanabe et al. 1992; Magariños and McEwen 1995a; Cook and Wellman 2004; Radley et al. 2004; Liston et al. 2006). These deteriorating effects of chronic stress could be

mimicked by chronic corticosteroid treatment (Woolley et al. 1990), and blocking corticosteroid actions prevented the stress-induced alterations to occur (Magariños and McEwen 1995b), indicating that corticosteroids play a role in mediating these effects on higher-order cognitive function and morphological structure. The amygdala on the other hand, seems to enhance its function upon chronic stress exposure, displaying dendritic hypertrophy (increased dendritic length and larger amount of branch points; Vyas et al. 2002) and increased spine density (Mitra et al. 2005). Behaviorally, these structural changes translate into an increased anxiety phenotype (Vyas et al. 2002).

Human research into the effects of chronic stress exposure have largely been restricted to retrospective investigations because of obvious ethical reasons. Interestingly, these studies provide initial evidence for disturbed higher-order cognitive function (Liston et al. 2009) and increased emotional processing (van Wingen et al. 2011) as well. Moreover, reductions in mainly hippocampus (Papagni et al. 2011), medial PFC (Soares et al. 2012; Ansell et al. 2012), and anterior cingulate volumes have been found (Papagni et al. 2011; Ansell et al. 2012). These findings would relate the chronic stress induced morphological hypotrophy observed in the rodent brain to volume reductions in the human brain, but more research is necessary to actually link the two phenomena.

Cumulative adversity and stress exposure during life are associated with the risk of development of stress-related psychopathology, and much more research has been done into the diseased brain. Stress-related disorders such as depression and PTSD are generally characterized by abnormalities in HPA axis signaling. In general, depressive patients are thought to display hypercortisolism, i.e. elevated basal levels of cortisol, whereas PTSD patients seem to be characterized by lower basal cortisol levels (hypocortisolism) potentially caused by an increase in negative feedback sensitivity. Patients suffering from one of these illnesses are characterized by functional impairments and volumetric reductions in hippocampal, prefrontal cortex, and anterior cingulate volume (Drevets et al. 1998; Bremner et al. 1999; Shin et al. 2006; Lorenzetti et al. 2007), whereas the amygdala seems to be hyperresponsive, especially to negative emotional stimuli (Drevets 1999; Shin et al. 2006; Leppänen 2006). Importantly, as mentioned before, these psychiatric disorders are characterized by alterations in functional and structural connectivity patterns throughout the brain, which are suggested to be at least as important as the activational differences observed (Gilboa et al. 2004; Greicius 2008; Liberzon and Sripada 2008; Zeng et al. 2012; Whitfield-Gabrieli et al. 2012; Admon et al. 2012; Xu et al. 2012). Especially prefrontal-amygdala connectivity seems to be impaired in these stress-related diseases, which translates to generally impaired emotion regulation, and an attentional bias towards negative emotional information (Williams 1996; Leppänen 2006). In contrast to human research, animal studies afford an approach where the influence of chronic stress on brain connectivity can be studied under highly controlled circumstances.

**We have studied the effects of chronic stress on the structural integrity and functional connectivity patterns in the rodent brain (Part 4.2).**

## OUTLINE

This thesis describes the studies performed into the time- and region-specific effects of stress hormone exposure on both the human and rodent brain. This work was set out to provide a mechanistic account by which stress and corticosteroids affect brain function and establish their well-known effects on behavior. To do so, we investigated the effects of stress and corticosteroids on several distinct brain functions, known to be affected. Ultimately, newly gained insights into the underlying mechanisms of stress-induced alterations in brain function, will contribute to the understanding of the etiology of stress-related mental disorders and provide new handles for their treatment.

In Part 2 of this thesis, we investigated the effects of stress hormones on the neural correlates of human memory formation. As described before, stress is known to enhance memory formation, sometimes even to such an extent that the aversive memories cannot be forgotten, even when wanted to. Here, we tested the neural underpinnings of this stress-enhanced memory formation by integrating a stress induction procedure (using highly aversive movie clips) into a memory encoding paradigm during fMRI scanning (Part 2.1). Next, we wanted to assess the role of corticosteroids in establishing the observed effects, which made us repeat the exact same memory paradigm after the intake of a tablet of hydrocortisone. To investigate the time-dependency of the corticosteroid effects, hydrocortisone was administered at two different time points prior to fMRI scanning. The rapid, presumably non-genomic, effects of corticosteroids were assessed by administering hydrocortisone just prior to the task onset, whereas the slow, presumably genomic, effects were targeted by administering hydrocortisone a few hours earlier (Part 2.2).

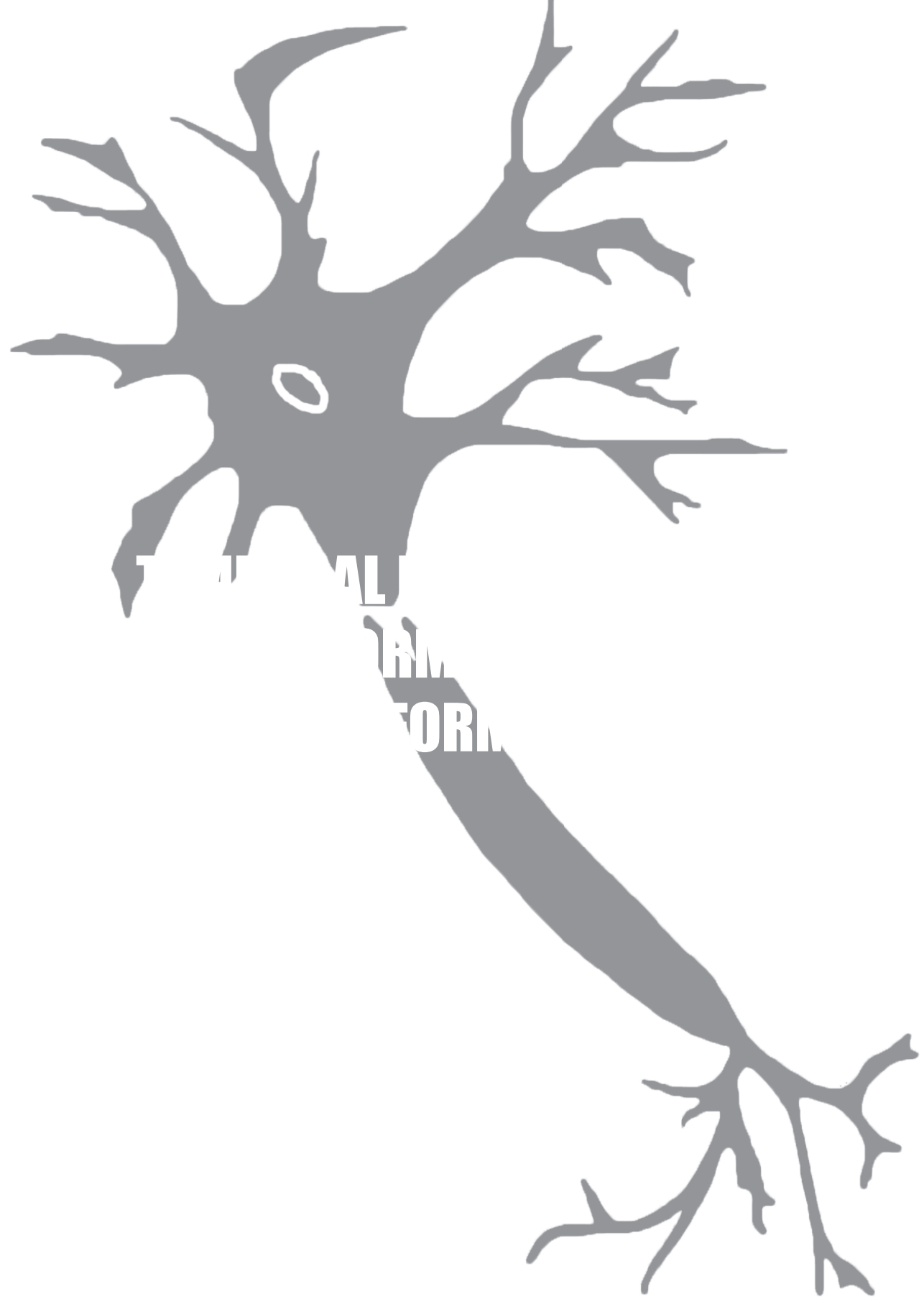
In Part 3 of this thesis we investigated the role of corticosteroids in the stress-induced alterations with regard to emotional and attentional processing and higher-order cognitive function. Acute stress is thought to induce a state of highly alert, but rather unfocused processing, together with a boost in emotional processing and impairment in higher-order cognitive function. Moreover, disturbed emotional processing and cognitive control are observed in stress-related psychopathology. Here, we addressed the contribution of corticosteroids in mediating these effects, and determined their neural underpinnings. Moreover, we investigated whether corticosteroids affect these processes in a time-dependent manner; a factor largely ignored in corticosteroid research in humans so far. In order to do so, we again combined carefully timed hydrocortisone administration with fMRI scanning, but this time participants were asked to complete several distinct tasks. They were first of all subjected to a passive emotional processing task to assess corticosteroid effects on pure emotional processing as occurring in the amygdala (Part 3.1). Furthermore, participants were asked to complete an emotional interference task in which they were instructed to suppress incoming emotional distracters. This task was used to assess corticosteroids' time-dependent effects on attentional processing and higher-order cognitive control over emotion (Part 3.2). Lastly, participants performed a working memory task, in order to assess the effects of corticosteroids

on PFC executive function (Part 3.3). Next, we switched to the rodent brain to investigate the neurobiological underpinnings of the region-specificity of corticosteroid effects in the brain. To tackle this issue, we compared the effects of corticosteroid exposure on neuronal functioning in two brain regions known to be affected by chronic stress exposure in an opposite manner; the orbitofrontal cortex and the CA1 region of the hippocampus (Part 3.4).

In the next section of this thesis, Part 4, we investigated whether stress and corticosteroid exposure, next to affecting neural activity, also influenced the connectivity patterns in the brain; known to be affected by stress exposure. Previous findings have indicated that acute or prolonged stress exposure strengthened amygdala resting-state connectivity in the human brain. Thereby, stress exposure seems to affect the basal state of brain processing, even in the absence of any external stimuli. Here, we extended these findings by testing the role of corticosteroids in establishing these effects on the amygdala-centered functional connectivity network in the human brain (Part 4.1). Moreover, we were interested in the effects of prolonged corticosteroid elevation on the functional connectivity networks in the brain, and to relate these to the effects observed in psychopathology. To test this under controlled conditions, we reverted again to an animal model. We combined a chronic stress-induction paradigm in rodents with functional and structural neuroimaging, and assessed chronic stress effects on dendritic morphology (Part 4.2).

The findings of all these studies are subsequently summarized, discussed, and integrated in the last section of this thesis (Part 5), followed by the description of the main conclusions and remaining open questions. Finally, the findings are interpreted into future and clinical perspectives on the potential treatment and prevention of stress-related mental disorders.





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# **Stressed memories: How acute stress affects memory formation in humans**

# 2.1

**Marloes J.A.G. Henckens, Erno J. Hermans, Zhenwei Pu,  
Marian Joëls, and Guillén Fernández**

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# 2.1

## ABSTRACT

Stressful, aversive events are extremely well remembered. Such a declarative memory enhancement is evidently beneficial for survival, but the same mechanism may become maladaptive and culminate in mental diseases such as post-traumatic stress disorder (PTSD). Stress hormones are known to enhance post-learning consolidation of aversive memories, but are also thought to have immediate effects on attentional, sensory, and mnemonic processes at memory formation. Despite their significance for our understanding of the etiology of stress-related mental disorders, effects of acute stress at memory formation, and their brain correlates at the system scale, remain elusive. Using an integrated experimental approach, we probed the neural correlates of memory formation while participants underwent a controlled stress induction procedure in a crossover design. Physiological (cortisol level, heart rate, and pupil dilation) and subjective measures confirmed acute stress. Remarkably, reduced hippocampal activation during encoding predicted stress-enhanced memory performance, both within and between participants. Stress, moreover, amplified early visual and inferior temporal responses, suggesting that hypervigilant processing goes along with enhanced inferior temporal information reduction to relay a higher proportion of task-relevant information to the hippocampus. Thus, acute stress affects neural correlates of memory formation in an unexpected manner, the understanding of which may elucidate mechanisms underlying psychological trauma etiology.

## INTRODUCTION

Information encoded into memory during stressful experiences is generally well-remembered (Kim and Diamond, 2002), especially if this information is relevant to the stressor (Joëls et al. 2006; Sandi and Pinelo-Nava 2007; Smeets et al. 2009). Although this phenomenon represents adaptive behavior, dysregulation of the underlying mechanism might result in psychological trauma and thus potentially mental disease (McEwen 2004; de Kloet et al. 2005). Past research has put strong emphasis on the mechanisms by which acute stress enhances memory consolidation (Roosendaal et al. 2006c). It is widely assumed that rapidly unfolding neurochemical events during the initial stress phase exert immediate effects on attentional, sensory, and mnemonic processes (de Kloet et al. 2005). However, such putative effects of acute stress have received little attention and remain poorly understood.

The effects of stress on memory are thought to be mediated through hormones and neurotransmitters released by two interacting effector systems: the (nor)epinephrine (NE) - sympathetic system and the hypothalamic-pituitary-adrenal (HPA) axis. Under stress, the sympathetic system, with the locus coeruleus (LC) at its core, shifts towards a tonically active state (Aston-Jones and Cohen 2005; Valentino and Van Bockstaele 2008). This shift causes an increase in NE-tone almost in the entire brain including the medial temporal lobe (MTL; Valentino and Van Bockstaele 2008; Sara 2009), the key-structure of the declarative memory system (Squire and Zola-Morgan 1991). This increased NE tone, which is associated with peripheral effects such as pupil dilation, supports neural plasticity that underlies memory formation (Roosendaal et al. 2006c), and causes a surge of arousal which is thought to lead to hypervigilance and prioritized processing of information relevant to the stressor (Aston-Jones and Bloom 1981; Ramos and Arnsten 2007). On a slightly longer time-scale, the HPA axis increases the release of glucocorticoids, which also modulate MTL plasticity (Lupien and Lepage 2001; de Kloet et al. 2005; Roosendaal et al. 2006c; McEwen 2007). Together, neuromodulators active during acute stress can therefore be hypothesized to induce a system level reorganization of mnemonic processes, geared towards more effective memory encoding.

To tackle this issue, we used functional Magnetic Resonance Imaging (fMRI) to probe effects of controlled stress induction on the neural substrates of memory formation. In order to satisfy the putative requirements for stress-enhanced memory to occur (Joëls et al. 2006; Sandi and Pinelo-Nava 2007), we maximized overlap between stressor and learning material by fully embedding a learning task in a stressful context created by strongly aversive movie clips (Qin et al. 2009; van Marle et al. 2009). In order to isolate neural activity related to successful memory encoding, we employed a well-established subsequent memory paradigm (cf. Dolcos et al. 2004). Crucially and in contrast to previous studies that looked into the effects of arousing items on memory formation (Cahill 2003; Richardson et al. 2003; Dolcos et al. 2004), we implemented a crossover design with separated stress and non-stress control sessions. Thus, the present study allowed us to assess prolonged modulations of mnemonic operations caused by a protracted state of acute stress.

# MATERIALS & METHODS

## Participants

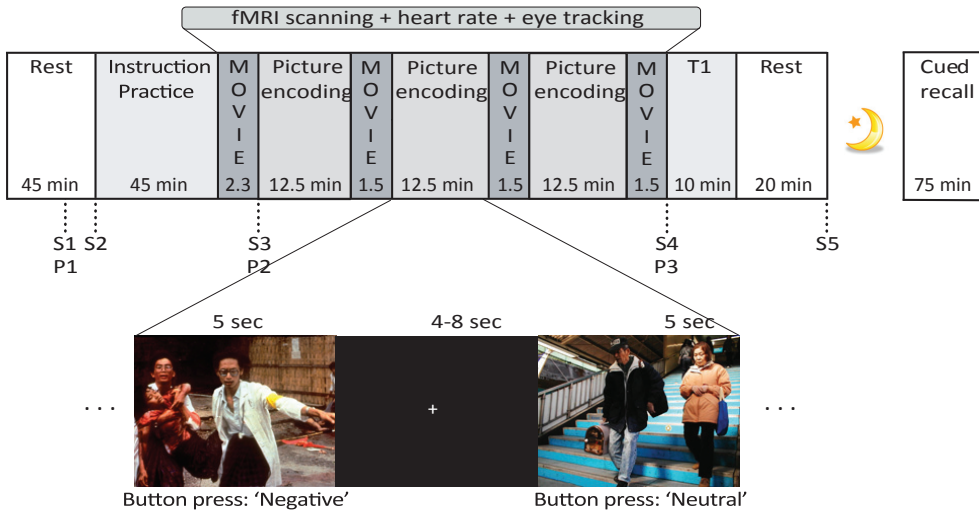
Eighteen young (ages 19-31, median 22), right-handed, healthy male volunteers gave informed consent to participate in the study. Individuals who met any of the following criteria were excluded from participation: history of head injury, treatment with psychotropic medications, narcotics, beta-blockers, steroids, or any other medication that affects central nervous system or endocrine systems, medical illness within the three weeks prior to testing, self reported mental or substance use disorder, daily tobacco use, regular night shift work, current stressful episode or major life event, previous exposure to slides used in the study (i.e. International Affective Picture System; Lang et al. 1999), and regularly viewing extremely violent movies or playing violent computer games. Moreover, volunteers with high scores on depression (score above 8 on the Beck Depression Inventory; Beck et al. 2002) were excluded from participation. The study was in accordance with institutional guidelines of the local ethics committee (CMO region Arnhem-Nijmegen, Netherlands) and the declaration of Helsinki.

## Study design

In a counter-balanced crossover design, eighteen young, healthy men underwent two sessions, separated by one month, of intentional episodic memory encoding during functional magnetic resonance imaging (fMRI). Memory was tested 24h after each fMRI session by cued recall. Both neutral and negative pictures were encoded, that were either embedded in a stressful or neutral control context created by short movie clips (Fig. 3). This allowed us to investigate brain activation during memory formation in a coherent stressful experience as function of later remembrance, both within (contrasting brain activation during the processing of subsequently remembered and forgotten items (Wagner et al. 1999) and between subjects (relating brain activation to memory performance across subjects). Physiological (cortisol level, heart rate, and pupil dilation), and psychological (negative affect) indices were measured to confirm successful stress induction. Data was analyzed with the factors stress (stress induction versus control context), subsequent memory (later remembered versus later forgotten items) and item valence (negative versus neutral pictures).

## Procedure

**Prior to arrival.** To minimize differences in baseline cortisol levels we instructed participants not to use any recreational drugs for three days and to refrain from drinking alcohol, exercising, and smoking for 24 h prior to each session. Furthermore, participants were requested not to brush their teeth, floss, or eat and drink anything but water for two hours prior to all sessions enabling adequate saliva sampling for cortisol assessment. To reduce the impact of diurnal variation in cortisol levels, all testing was performed in the afternoon, between 14:00 h and 18:00 h, when hormone levels are relatively stable.



**Figure 3.** *Experimental design. IAPS pictures (Lang 1999) were encoded during fMRI scanning in either a stressful or neutral control condition generated by short movie clips. Psychological and physiological measures were obtained to monitor the effectiveness of stress induction. Memory was tested 24h later in a cued recall test. S = saliva sample, P = Positive And Negative Affect State (PANAS) questionnaire (Watson et al. 1988).*

**Arrival.** On the first day, participants rested 30 min prior to taking the first saliva sample. To increase familiarity with the procedure and minimize task repetition effects, participants were explicitly informed about all details of the memory experiment. A financial reward was promised proportional to the participant's performance in the recall test to encourage encoding. Further, participants were asked to complete Spielberger's Trait Anxiety Inventory (van der Ploeg et al. 1980) and the NEO-FFI (Costa and McCrae 1992).

**Scanning.** Participants lay supine in the scanner and viewed the screen through a mirror positioned on the head coil. They were asked to lie as still as possible, keep their eyes open, and look directly and continuously at the center of the screen in front of them. Four movie fragments were used to create the appropriate context, shown prior to, in between and at the end of picture encoding (dividing the encoding session in three blocks, Fig. 3). Participants were instructed to view each movie clip and picture for the entire time that it was displayed. Pictures belonged to two categories, either with a neutral or negative picture valence. Participants were asked to memorize and rate the valence of each picture. Ratings were given with right-hand button presses, with the index finger for negative and the middle finger for neutral pictures. Pictures were shown in a pseudorandom order (no more than two pictures of the same valence consecutively), and all first slides were neutral to avoid ceiling effects in recall that might result from the combined effect of arousal and primacy on memory. Slides were presented for 5 s with a 4–8 s inter-trial interval (fixation cross). After completion of the encoding task, a structural scan was performed.

**Subsequent memory test.** Participants came back the subsequent day to perform a cued recall

test, lasting 75 minutes. One- or two-word written cues for each picture (with similar valence as the picture) were provided, describing the readily identifiable gist of the picture, which is the most salient feature of the scene depicted on the picture. Participants were asked to write down as many characteristics of all pictures as they possibly could remember, providing enough relevant characteristics so that an outsider could identify each picture and discriminate it from similar studied pictures (Dolcos et al. 2004). A short introduction was written to help the participants in listing characteristics. One rater evaluated initially the written descriptions provided by the participants and only pictures with a description that allowed both identification and discrimination were classified as remembered. Pictures with no recollection of characteristics were considered forgotten. Picture descriptions that could not clearly be linked to a particular picture were scored as a non-response and not included in the analyses. Subsequently, a second rater, blind to the study condition, independently re-rated all responses in the memory test to probe reliability. Inter-rater correspondence was very high (95.6%), and comparable to other studies using similar designs (Buchanan and Lovallo 2001; Payne et al. 2006, 2007).

## Stimulus materials

**Stressor.** Four short movie fragments were used to create the proper context, (1 x 140 s, 3 x 90 s). They were either selected from a distressing movie [*Irréversible* (2002), Gaspar Noé] or a neutral control movie [*Comment j'ai tué mon père* (2001), Anne Fontaine]. Selected fragments were comparable in amount of speech, human presence, luminance, and language. The stressful movie clips contained scenes with aggressive behavior and violence against men and women. Occasionally, people in the video could be heard shouting and crying out in anger, pain, or distress. Previous studies have confirmed the effectiveness of these movie clips in inducing stress (Qin et al. 2009; van Marle et al. 2009). Although considerably distressing, the film content was approved by the NICAM (Dutch Institute for Audiovisual Media) for viewers above 16 years. Participants were informed prior to the experiment that watching the film could be stressful and that they could terminate the experiment at any point. This stress induction method was chosen because it meets the criteria described by Joëls et al. (2006) for stress enhanced memory to occur, i.e. close spatio-temporal proximity and content overlap of stressor and task (the memory encoding was part of a continuous and coherent stressful episode experienced within an fMRI environment). This overlap in content was achieved by parallelizing studied pictures and movies based on content features; both depicting real-life, emotionally salient stimuli. To be more precise, the movies used in the stress condition contained, e.g. male to male and male to female violence, mutilations, and injuries, which were also present in many negative IAPS photographs. There was also considerable overlap between the neutral movie and neutral pictures. Examples of scenes shown in both are, e.g. people eating, talking, and walking.

**Pictures.** Three stimulus sets were created for picture encoding, two of which were used per participant. Each set consisted of 80 negative and 80 neutral pictures, supplemented with 41 null events (fixation). Pictures were selected from both a standard set of affective pictures (IAPS;

Lang et al. 1999) and an additional set of newly rated pictures. New pictures were downloaded from the internet and selected on the authors' assessment of emotionality and similarity to IAPS pictures. New pictures were rated on a scale from 1 to 9 on both arousal and valence using the Self-Assessment Manikin (SAM) scales (Bradley and Lang 1994) by an additional group of 20 male volunteers. To assure reliable rating that did not significantly differ from IAPS ratings, and to serve as a reference frame, positive and negative IAPS pictures were added to this test set. All selected negative slides were chosen for their moderate to high arousal quality (average arousal score 5.5, S.E.M. = 0.7), and negative valence (average valence score 3.1, S.E.M. = 0.7), rated on a 1-9 point rating scale as determined by the SAM (Bradley and Lang 1994). Neutral slides were selected for their relatively low arousal (average arousal score 2.5, S.E.M. = 0.7) and neutral valence (average valence score 5.3, S.E.M. = 0.3). Used picture sets contained about 50 percent newly rated neutral and 15 percent newly rated negative pictures and were matched on chromatic features and complexity, while overlap in content within one set was minimized. Used stimulus sets did not differ in mean arousal and valence ratings.

## Stress measures

**Saliva collection and analysis.** Cortisol levels were measured from saliva at five time points: baseline measurements at the beginning of the experiment (twice) ( $t = 30, 45$  min), immediately after the first movie clip ( $t = 90$  min), immediately after the last movie clip ( $t = 135$  min), and at the end of the experiment ( $t = 165$  min).

Saliva was collected using a commercially available collection device (Salivette®, Sarstedt, Germany). For each sample, the participant first placed the cotton swab provided in each Salivette tube in his mouth and chewed gently on it for 1 min to produce saliva. Third and fourth sample were taken in the scanner. Swabs were handed over to the participants and they were instructed not to move their head while chewing. The swab was then placed back in the salivette tube, and the samples were stored in a freezer at  $-25^{\circ}\text{C}$  until assayed. Laboratory analyses were performed at the Department of Biopsychology, TU Dresden, Germany. After thawing, salivettes were centrifuged at 3,000 rpm for 5 min, which resulted in a clear supernatant of low viscosity. Salivary free cortisol concentrations were subsequently measured using a commercially available chemiluminescence-immuno-assay (CLIA) with high sensitivity of 0.16 ng/mL (IBL, Hamburg, Germany). For analyses, area under the curve with respect to increase (AUC<sub>i</sub>) was calculated and analyzed for cortisol levels expressed as baseline percentage of each session (average level of measurements 1 and 2).

**Heart rate.** Cardiac rhythm of the participants was measured during scanning, using a pulse oximeter placed on their left index finger. Participants were instructed to keep their hands as still as possible during the measurement. Heart rate frequency was calculated using in-house software. Data of one subject was discarded from analyses, due to excessive artifacts in the recorded signal.

**Pupil diameter.** A commercial MR compatible eye-tracking device from SensoMotoric Instruments (S.M.I.) (MEyeTrack-LR) mounted on the scanner bed was used to measure eye

movements and pupil diameter at a sampling rate of 50 Hz. Moreover, eye-tracking confirmed attentive viewing of all slides and movie fragments.

Eye pupil data were analyzed using in-house software implemented in Matlab 7.5 (The Mathworks, Inc. Natick, MA, USA), which was based on methods previously described by others (Siegle et al. 2003). Eye blink artifacts were identified by differentiating the signal in order to detect eye pupil changes occurring too rapidly to represent actual dilation. Blinks were removed from the signal using linear interpolation. Scanner pulses recorded simultaneously enabled synchronization with stimulus presentation. Pupil diameter for each trial was normalized to the average 1 s pre-stimulus onset baseline. The averaged normalized pupil diameter during picture presentation was used as response measure. These were collapsed over trials within stress induction and picture valence conditions. Due to data loss or excessive artifacts in the recorded signal in either of the sessions, data of 5 subjects were not included into analyses. It is important to note that this method does not measure absolute pupil diameter.

**Psychological measures.** Mood state was assessed using the Positive and Negative Affect Schedule (PANAS) questionnaire (Watson et al. 1988) at three time points: at the beginning of the experiment ( $t = 30$  min), immediately after the first movie clip ( $t = 90$  min), and immediately after the last movie clip ( $t = 135$  min). Picture valence ratings (neutral or negative), which were obtained during picture encoding blocks, were scored as either corresponding ('correct') or not corresponding ('incorrect') with a priori categorizations. Furthermore, average reaction times were calculated for those items with 'correct' rating.

## Behavioral and physiological statistical analysis

Behavioral and physiological data were analyzed in SPSS 15.0 (SPSS, Inc. Chicago, IL, USA) using repeated measures ANOVAs and paired samples t-test statistics. Where no main effects or interactions involving the order factor were significant, this factor was omitted. Furthermore, in cortisol data analyses the difference in time of day between both sessions was entered as a covariate. Alpha was set at 0.05 throughout.

## MRI acquisition

Participants were scanned in a Siemens (Erlangen, Germany) TIM Trio 3.0 Tesla MRI scanner equipped with an 8 channel phased array head coil. Blood oxygenation level dependent (BOLD)  $T_2^*$ -weighted gradient echo EPI images were acquired with the following parameters: TR = 2.18 s, TE = 25 ms, FA =  $90^\circ$ , 37 axial slices approximately aligned with AC-PC plane, slice-matrix size =  $64 \times 64$ , slice thickness = 3.0 mm, slice gap = 0.3 mm, FOV =  $212 \times 212$  mm<sup>2</sup>. Because of its relatively short TE, this sequence yields optimal contrast-to-noise ratio in the medial temporal lobe.

A high-resolution anatomical image was acquired for each participant using a  $T_1$ -weighted 3D Magnetization-Prepared RAPid Gradient Echo (MP-RAGE) sequence combined with

GeneRalized Autocalibrating Partially Parallel Acquisitions (GRAPPA (Griswold et al. 2002)). The following parameters were used: TE/TR: 2.96/2300 ms, flip angle: 8°, FOV: 256 x 256 x 192 mm, voxel size: 1 mm isotropic, GRAPPA acceleration factor 2. The total duration of each MRI session was about 1 h.

## **fMRI data analysis**

Data were analyzed using Statistical Parametric Mapping software (SPM5; UCL, London) and in-house software. The first five EPI-volumes were discarded to allow for  $T_1$ -equilibration. Prior to analysis, the images of the three encoding blocks were separately motion corrected using rigid body transformations and least sum of squares minimization. Subsequently, they were temporally adjusted to account for differences in sampling times across different slices. All functional images were then co-registered with the high-resolution  $T_1$ -weighted structural image using normalized mutual information maximization. The anatomical image was subsequently used to normalize all scans into MNI152 (Montreal Neurological Institute) space. All functional images were resampled with a voxel size of 2 mm isotropic. Finally, all images were smoothed with an isotropic 8-mm full-width-at-half-maximum Gaussian kernel in order to accommodate residual functional/anatomical variance between subjects.

Data were analyzed using a general linear model, in which individual events were modeled based on stress, subsequent memory, and item valence. Regressors were temporally convolved with the canonical hemodynamic response function of SPM5. The six covariates corresponding to the movement parameters obtained from the realignment procedure were also included in the model. To reduce unspecific differences between scan sessions, global normalization using proportional scaling was applied. The single subject parameter estimates from each session and condition obtained from the first level analysis were included in subsequent random effects analyses. For the second level analysis a factorial ANOVA was used, with stress induction (stress vs. control context), picture valence (negative vs. neutral), and subsequent memory (remembered vs. forgotten) as within subject factors. Alpha for statistical tests was set at 0.05, family-wise error (FWE) rate corrected using Gaussian random field theory. Based on our *a priori* hypothesis about their involvement in memory and attention, data for the regions of interest – MTL and ventral visual stream – were corrected for a reduced search region (based on their size) and small volume corrected using a sphere with 15 mm radius. Statistical tests for all other regions corrected for a whole brain search region.

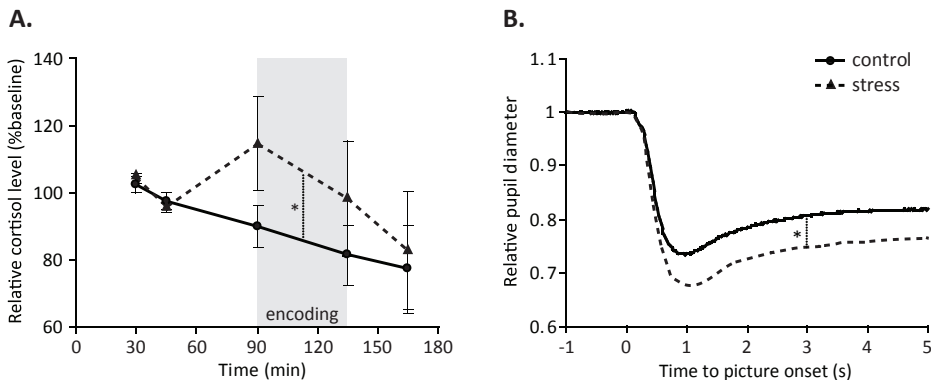
To test the regional overlap between the main effects of memory and stress, conjunction analyses were performed using the minimum statistic compared to the conjunction null (MS/CN) method as implemented within SPM5 (Nichols et al. 2005). We used a reduced search volume with a radius of 10 mm (approximating the underlying spatial resolution of the fMRI signal) centered on the maxima of the main contrasts as proposed by Friston et al. (2005). For purpose of visualization of the overlap of both contrasts, the less conservative minimum statistic compared to the global null method (MS/GN) with a threshold of  $p < 0.001$ , uncorrected, was used in Figure 6C.

To assess the relationship between neural activity and memory performance across subjects, mean activity of the anatomically defined hippocampus was extracted (using the Automated Anatomical Labeling of Activations; Tzourio-Mazoyer et al. 2002), and the differences in responses between the stress and control conditions were entered in regression analyses as a predictor for the difference in memory performance. Visualizations of activations were created using MRIcroN (<http://www.sph.sc.edu/comd/rorden/mricron/>) by superimposing statistical parametric maps thresholded at  $p < 0.001$ , uncorrected, onto a canonical  $T_1$ -weighted image in standard MNI152 space.

## RESULTS

### Effectiveness of stress induction: Physiological measures

Physiological measures confirmed successful stress induction. Area under the curve measures of salivary cortisol levels indicated that HPA axis activity was elevated throughout the picture encoding procedure in the stress condition ( $F(1,15) = 6.49$ ,  $p = 0.02$ , Fig. 4A). Moreover, heart rate frequency (mean  $\pm$  SD,  $HR(\text{control}) = 59.26 \pm 9.36$  bpm,  $HR(\text{stress}) = 65.95 \pm 9.69$  bpm), which is associated with elevated sympathetic tonus, was increased ( $F(1,16) = 12.34$ ,  $p = 0.003$ ). Finally, pupil dilation responses to pictures were decreased ( $F(1,11) = 4.90$ ,  $p = 0.05$ , Fig. 4B). Given the direct association of LC activity and pupil dilation, this finding is consistent with the notion that phasic LC responses diminish against a background of enhanced tonic activity (Aston-Jones and Cohen, 2005). Moreover, in agreement with previous literature (Bradley et al. 2008), a significant effect of item valence was observed in pupil dilation responses, with negative pictures causing more dilation, indicating stronger phasic sympathetic responses, than neutral ones ( $F(1,11) = 52.08$ ,  $p < 0.001$ , Fig. 5A). However, this measure did not yield any significant interaction effects between item valence and stress ( $F(1,12) < 1$ ).



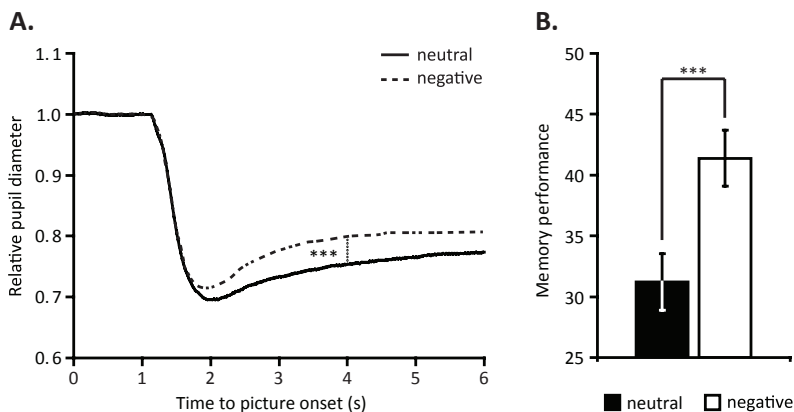
**Figure 4.** Physiological effects of stress. (A) The stress induction procedure increased (area under the curve) cortisol levels (expressed as percentage of baseline) (45–135 min) and (B) reduced mean phasic pupil dilation (expressed as ratio of baseline diameter) after the initial light reflex. Significance refers to the observed within subject effects, while the error bars represent S.E.M. of the between subject variance. \*:  $p < 0.05$

## Effectiveness of stress induction: Psychological measures

Stress induction led to an increase in subjective stress, as measured by elevated self-reported negative affect (PANAS questionnaire) measured just before the encoding blocks (mean  $\pm$  SD, NA(control) =  $13.97 \pm 4.62$ , NA(stress) =  $16.08 \pm 4.79$ ;  $F(1,17) = 7.21$ ,  $p = 0.02$ ). Picture ratings obtained during encoding blocks were highly consistent with pre-determined picture categories, with  $94.7 \pm 0.3$  % corresponding ('correct') responses. While reaction times for ('correct' only) picture rating were independent of picture valence ( $F(1,17) < 1$ ), stress induced a trend towards slower reaction times (mean  $\pm$  SD, RT(control) =  $1.39 \pm 0.33$  s, RT(stress) =  $1.51 \pm 0.34$  s) ( $F(1,17) = 3.57$ ,  $p = 0.08$ ).

## Effectiveness of stress induction: Memory enhancement

Memory was tested in a cued recall (CR) test (Dolcos et al. 2004) the subsequent day. Stress enhanced memory performance: pictures encoded during the stressful experience were more often remembered one day later than pictures encoded in the control condition (mean  $\pm$  SD, CR(control) =  $69.33 \pm 20.67$  pictures, CR(stress) =  $75.83 \pm 18.96$  pictures) ( $F(1,17) = 4.42$ ,  $p = 0.05$ ). This stress effect on picture encoding did not change over time during the encoding session (as evidenced by a non-significant stress by encoding block interaction,  $F(1,17) > 1$ ), indicating that this stress modulation was a rather stable state during the entire scanning session. As expected, memory performance was better for negative than for neutral pictures (mean  $\pm$  SD, CR(neutral) =  $31.19 \pm 10.88$  pictures, CR(negative) =  $41.39 \pm 10.17$  pictures) ( $F(1,17) = 51.41$ ,  $p < 0.001$ , Fig. 5B). However, this picture valence effect did not interact with stress induction ( $F(1,17) < 1$ ).

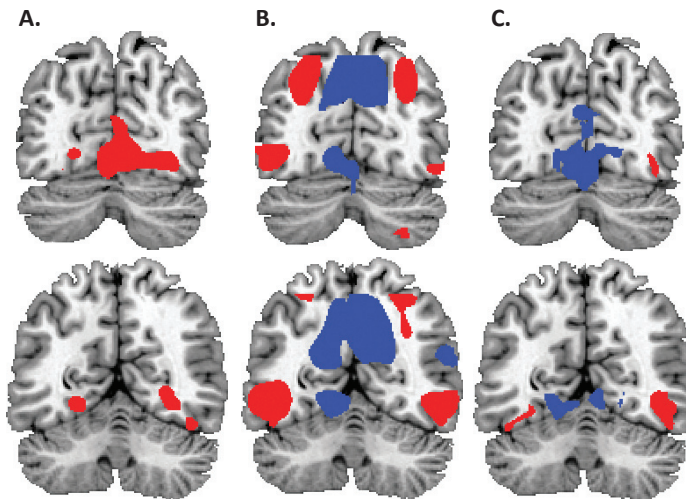


**Figure 5.** Picture valence effects. (A) Mean phasic pupil dilation (expressed as ratio of baseline diameter) after the initial light reflex was larger during the encoding of negatively arousing than neutral pictures. (B) Cued recall memory was better for negative than for neutral pictures. \*\*\*:  $p < 0.001$

## Brain activation maps: Main effects of stress, memory, and picture valence

Imaging data were analyzed using a random effects ANOVA with stress (stress induction vs. control context), subsequent memory (correct vs. incorrect subsequent recall), and item valence (negative vs. neutral pictures) as within subject factors. Given strong neurophysiological evidence for its involvement in memory formation and stress-memory interactions, the MTL, and more specifically, the hippocampus (Joëls et al. 2004), was our main region of interest. Furthermore, we focused on stress-induced changes in both lower- and higher-order visual processing regions, known to be modulated by vigilance (Munk et al. 1996). Therefore, data for the MTL structures and the ventral visual stream were thresholded at  $p < 0.05$ , small volume corrected (SVC) ( $r = 15$  mm). A threshold of  $p < 0.05$  whole brain corrected was applied to all other regions.

We first identified brain responses to pictures in general that were affected by stress induction. Larger responses to picture presentation for the stress induction than the control condition were found in visual areas: activation in regions of the primary visual cortex, right inferior temporal region, and fusiform gyrus, associated with higher-order visual processing and attention (Moran and Desimone, 1985; Heinze et al. 1994), was elevated by stress induction (Table 1, Fig. 6A). Second, regions supporting successful memory formation were identified. In line with previous literature of picture encoding (Brewer et al. 1998; Dolcos et al. 2004), regions displaying larger



**Figure 6.** Brain regions affected by stress induction and memory ( $y = -72, -59$ ). (A) Stress induction increased responsiveness within the primary visual cortex and right inferior temporal region, centered on the fusiform gyrus. (B) Positive (in red) subsequent memory effects (SME) in large inferior temporal and superior parietal regions and negative (in blue) SME in posterior midline structures comprising the cuneus and the lingual gyrus. (C) Conjunctions of positive effects of stress induction with positive (in red) or negative (in blue) SME. These figures show that enhanced recruitment of the primary visual cortex after stress induction was detrimental to memory formation. In contrast, stress-enhanced inferior temporal activation proved beneficial. All statistical parametric maps are thresholded at  $p < 0.001$ , uncorrected, employing minimum statistic/global null methods for conjunction effects, for visualization purposes. See Table 1 for formal statistical tests.

neural activity during encoding of subsequently remembered than subsequently forgotten pictures were the bilateral fusiform gyrus extending into the parahippocampal region, inferior temporal gyrus, inferior frontal cortex, inferior parietal gyrus, precentral gyrus, and the middle/superior occipital lobe. Negative effects of subsequent memory were found in the cuneus, precuneus, lingual gyrus, posterior cingulate cortex, and middle frontal cortex (Fig. 6B).

As expected, brain imaging results also revealed strong main effects of item valence (Table 2), with encoding activity being greater for negative than for neutral items in regions associated with visual processing (including the middle occipital and middle temporal gyri) (Lang et al. 1998; Wagner et al. 1998). Additional differences in activation were observed in the amygdala, fusiform gyrus, cerebellum, brainstem, thalamus, and inferior frontal cortex; regions typically activated in tasks involving emotional processing and arousal (Phan et al. 2002). Item valence and memory effects interacted in an extended medial temporal region, which showed larger subsequent memory effects for negative than for neutral pictures, reflecting better memory performance for these items (Table 2). These findings are consistent with other studies concerning emotional subsequent memory effects (Dolcos et al. 2004; Dougal et al. 2007). In line with behavioral and physiological measures, however, picture valence effects did not interact with stress induction.

**Table 1. Brain regions revealing significant main, interaction, or conjunction effects**

Region	MNI Coordinates			Peak
	x	y	z	T-value
Main effect of subsequent memory				
Remembered > Forgotten				
Middle occipital gyrus, L	-26	-68	36	6.64***
Middle occipital gyrus, R	30	-68	38	7.39***
Inferior temporal gyrus, L	-46	-62	-6	7.94***
Inferior temporal gyrus, R	54	-56	-10	8.69***
Fusiform gyrus, L	-34	-32	-20	4.46 <sup>++</sup>
Fusiform gyrus, R	34	-32	-22	4.15 <sup>++</sup>
Inferior parietal lobule, L	-44	-44	56	6.63***
Inferior parietal lobule, R	36	-52	56	5.31**
Inferior frontal gyrus, L	-50	34	6	8.47***
Inferior frontal gyrus, R	52	6	22	6.21***
	54	38	6	5.93***
Forgotten > Remembered				
Cuneus, L	-4	-90	24	4.86*
Cuneus, R	16	-64	34	8.46***
Lingual gyrus, L	-16	-62	-4	5.74**
Middle frontal gyrus, R	38	34	34	5.04*
	28	52	22	4.87*

Table 1 (continued)

Region	MNI Coordinates			Peak
	x	y	z	T-value
<b>Main effect of stress</b>				
<i>Stress &gt; Control</i>				
Superior occipital gyrus, L	-8	-94	8	5.09*
Superior occipital gyrus, R	16	-92	20	4.99*
Lingual gyrus, R	8	-72	-2	5.86***
Fusiform gyrus, L	-36	-66	-16	3.88 <sup>+</sup>
Fusiform gyrus, R	28	-70	-6	5.28*
	28	-50	-2	4.88*
Inferior temporal gyrus, R	46	-48	-18	4.06 <sup>++</sup>
<b>Stress by SME interaction (negative)</b>				
Hippocampus, R	28	-26	-8	4.29 <sup>++</sup>
<i>Forgotten &gt; Remembered during stress</i>				
Hippocampus, R	28	-26	-8	5.01*
<b>Stress by SME conjunction</b>				
<i>Remembered &gt; Forgotten &amp; Stress &gt; Control</i>				
Inferior temporal gyrus, R	48	-52	-6	3.20 <sup>‡</sup>
<i>Stress &gt; Control &amp; Forgotten &gt; Remembered</i>				
Lingual gyrus, L	-8	-76	-6	4.21 <sup>++</sup>
	-20	-62	-4	3.68 <sup>+</sup>

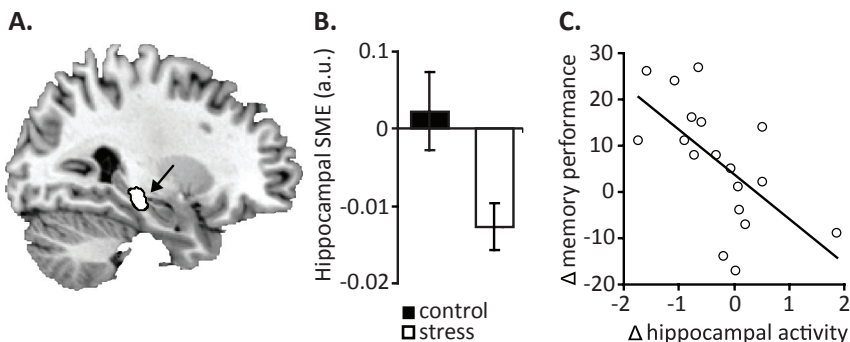
The peak x, y, z coordinates are given in MNI152 standard space coordinates. L and R denote left and right. SME: subsequent memory effect. \*:  $p < 0.05$  whole brain corrected, \*\*:  $p < 0.01$  whole brain corrected, \*\*\*:  $p < 0.001$  whole brain corrected, <sup>+</sup>:  $p < 0.05$  small volume corrected, <sup>++</sup>:  $p < 0.01$  small volume corrected, <sup>‡</sup>:  $p < 0.05$  small volume corrected ( $r = 10$  mm) centered on the maximum of the main contrast

## Brain activation maps: conjunction and interaction effects of stress and memory

To examine the main question at issue, how stress affects memory formation, we first identified those brain regions where activity was modulated by both stress and memory formation independently (i.e. overlapping effects), leaving the actual underlying memory processes unaffected. Both factors were associated with differential activity in the primary visual cortex and inferior temporal gyrus. To ensure actual spatial overlap, conjunction analyses (using the

minimum statistic compared to the conjunction null (MS/CN)) over the two orthogonal contrasts (Nichols et al. 2005) were performed. Activity in the primary visual cortex was significantly increased after stress induction and was negatively associated with subsequent remembrance (Fig. 6C), indicating that stress-induced activation of this region was related to less effective memory formation. In contrast, in the inferior temporal gyrus, a combined positive stress induction and subsequent memory effect was found (Fig. 6C). Enhanced activation after stress induction in this region was apparently associated with better memory formation.

Second, we investigated whether stress interacted with memory processes and thus influenced the subsequent memory effect itself. Stress induction modulated the subsequent memory effect focally in the right hippocampus (Table 1, Fig. 7A-B). Most interestingly, the observed interaction was carried by a negative subsequent memory effect in the stress induction condition: hippocampal responses to pictures were lower during encoding of subsequently remembered as compared to forgotten items. To determine whether this effect was related to the observed increases in memory performance, and thus could explain observed variance in stress effects on memory performance across participants, mean activity of the anatomically defined hippocampus (bilateral) was extracted (Tzourio-Mazoyer et al. 2002) and the differences in activity between the stress and control conditions were entered into regression analyses as a predictor for the difference in memory performance. The decrease in hippocampal response to pictures predicted the stress-induced improvement in memory performance ( $r = -0.615$ ;  $p = 0.007$ ), providing complementary evidence that reduced hippocampal activity is related to an increase in memory performance under stress (Fig. 7C).



**Figure 7.** Stress modulated the subsequent memory effect (SME) in the right hippocampus. (A) Statistical parametric maps, here thresholded at  $p < 0.001$  (uncorrected) for visualization purposes, revealed a negative stress induction by SME interaction ( $X = 28$ ). (B) Signal differences between subsequently remembered and forgotten trials separately depicted for the stress induction and control condition, based on averaged parameter estimates of the total volume of the anatomically defined hippocampus, revealed a negative SME during stress. (C) The observed stress-induced decrease in hippocampal responses predicted the stress-related improvement in memory performance across subjects. Error bars represent S.E.M. of the between subjects variance.

Table 2. Regions revealing main effects or interactions of picture valence

Region	MNI Coordinates			Peak
	x	y	z	T-value
<b>Main effect of picture valence</b>				
<i>Negative &gt; Neutral</i>				
Cerebellum, L	-18	-76	-38	8.23***
	-4	-54	-40	4.86*
Cerebellum, R	16	-72	-40	5.22*
Middle temporal gyrus, L	-52	-66	10	5.52***
Middle temporal gyrus, R	54	-64	6	9.48***
Posterior cingulate cortex	4	-52	30	9.54***
Superior parietal lobule, R	32	-48	58	5.95***
Supramarginal gyrus, L	-64	-26	36	7.95***
Supramarginal gyrus, R	64	-28	32	9.03***
Upper brain stem	2	-30	-2	8.43***
	-12	-22	-10	5.11*
Insula, L	-28	12	-20	6.69***
Insula, R	38	-2	-10	5.01*
Precentral gyrus, R	44	2	42	6.49***
Medial temporal pole, R	44	20	-36	6.85***
Inferior frontal gyrus, L	-50	34	0	6.11***
Inferior frontal gyrus, R	50	30	0	10.73***
Amygdala, L	-22	-6	-14	7.28***
Amygdala, R	24	-4	-16	9.47***
Superior medial gyrus	6	36	52	4.92*
	6	52	24	9.92***
Rectal gyrus	2	40	-18	4.99*
Mid orbital gyrus	2	54	-14	6.37***
<i>Neutral &gt; Negative</i>				
Precuneus, L	-6	-68	52	5.32**
Precuneus, R	10	-66	54	6.52***
	14	-42	8	5.23*
Calcarine, R	24	-62	20	7.52***
Cerebellum, L	-44	-62	-34	5.01*
	-40	-58	-40	4.94*
Inferior parietal lobule, L	-46	-52	46	6.65***
Inferior parietal lobule, R	48	-48	48	8.10***

Table 2 (continued)

Region	MNI Coordinates			Peak
	x	y	z	T-value
<i>Neutral &gt; Negative</i>				
Lingual gyrus, L	-30	-48	-4	10.29***
Lingual gyrus, R	30	-46	-4	9.02***
Inferior temporal gyrus, R	62	-40	-16	5.39**
Superior temporal gyrus, L	-40	-38	16	4.87*
	-38	-36	14	4.95*
Superior temporal gyrus, R	58	-10	4	7.58***
Paracentral lobule, L	-2	-32	62	4.98*
Paracentral lobule, R	6	-34	60	5.36**
Postcentral gyrus, L	-20	-30	62	6.73***
Postcentral gyrus, R	24	-30	62	6.76***
Rolandic operculum, L	-44	-30	16	5.07*
	-36	-16	20	5.58**
Middle frontal gyrus, L	-28	14	60	6.03***
	-34	48	16	6.05***
	-28	34	42	5.19*
Middle frontal gyrus, R	30	12	58	5.83**
	40	34	28	9.63***
Inferior frontal gyrus, L	-40	26	30	5.88***
Middle cingulate cortex, R	8	32	30	5.49**
Middle orbital gyrus, R	12	40	-6	6.00***
	30	48	-12	6.18***
<b>Valence x SME interaction (positive)</b>				
Hippocampus, R	16	-20	-12	4.25 <sup>++</sup>
Fusiform gyrus, L	-22	-36	-18	3.54 <sup>+</sup>

The peak *x*, *y*, *z* coordinates are given in MNI152 standard space coordinates. *L* and *R* denote left and right. *SME*: subsequent memory effect. \*:  $p < 0.05$  whole brain corrected, \*\*:  $p < 0.01$  whole brain corrected, \*\*\*:  $p < 0.001$  whole brain corrected, <sup>+</sup>:  $p < 0.05$  small volume corrected, <sup>++</sup>:  $p < 0.01$  small volume corrected.

## DISCUSSION

Here we show that acute stress profoundly affected the neural correlates of memory formation, and it did so in a region-specific manner. Reduced hippocampal responses were associated with better memory formation under stress, both within and across subjects. Furthermore, in early visual areas, stress led to an increase of activity, which was accompanied by a negative subsequent memory effect, while stress-enhanced activation in inferior temporal regions was accompanied by a positive subsequent memory effect.

The stress induction increased both psychological stress, as indicated by elevated self-reported negative affect, and physiological stress: both activity of the HPA axis and sympathetic tonus was increased. Moreover, decreased pupil dilation responses were found, which is widely regarded as a relatively direct index of LC activity (Koss 1986), with stimulus-locked pupil dilation reflecting a phasic LC response. During states of stress, the LC shifts towards a tonically hyperactive state, which is thought to result in a hypervigilant processing state and a concomitant decrease in stimulus-coupled phasic LC activity (Aston-Jones and Cohen 2005). Our finding of a decreased pupil dilation response during stress, together with the slightly elevated reaction times for picture rating, support this interpretation of a stress-induced hypervigilant state of unfocussed processing. The stress-enhanced activity in the primary visual cortex might also support the notion of such a state change. Previous studies have shown that both attentional and emotional states modulate visual processing (Wang et al. 2006; Vuilleumier and Driver 2007), and that hypervigilance is accompanied by potentiation of sensory input (Munk et al. 1996). The widespread neocortical projections of the LC might recruit additional neural resources in order to process an excess of sensory information. The negative conjunction of stress and subsequent memory effects in this region might indicate that the stress-induced activation, however, is supraoptimal for memory formation, and likely contains large amounts of task-irrelevant information. Since this effect in itself is not related to better memory, other additional factors are necessary to explain stress-induced memory enhancement.

One possible explanation for this memory improvement may lay in stress-enhanced filtering of excess sensory information in the ventral visual stream (Kastner et al. 1998; Kastner and Pinsk 2004). Visual-selective attention modulates the inferior temporal cortex (Moran and Desimone 1985), and lesions in these regions lead to attentional deficits (De Weerd et al. 1999). Under conditions of low attentional selection, cortical representations of simultaneous visual stimuli interact in a mutually suppressive fashion. Attentional selection of a single stimulus results in diminishment of the suppressive influence of nearby stimuli, thus providing a neural basis for filtering out irrelevant information (Kastner et al. 1998). Moreover, it has been proposed that tonic LC states are mirrored by increased activation of a ventral frontoparietal attention network, enhancing selective processing of salient stimuli (Corbetta et al. 2008). In line with this, we observed bilateral subsequent memory effects in these inferior temporal regions, but also

stress-induced activity increases. The latter can be taken to reflect reduction of ambient noise by focusing on task-relevant information. Conjunction effects of stress and subsequent memory, without interaction, indicate that activity in this region is modulated relatively independently by stress and memory formation. Thus, elevated stress may increase the likelihood of successful memory formation.

Consequently, adequate noise reduction may have led to less information relayed to the hippocampus. In line with this idea, the hippocampus showed less activity for later remembered than for later forgotten items under stress. Moreover, the overall decrease in hippocampal responses predicted the stress-related improvement in memory performance across subjects. Possibly, during stress, hippocampal input during subsequently forgotten items might be characterized by a large proportion of irrelevant information, thwarting clean separation between task-related and -unrelated information as required for the subsequent memory test. Thus, our findings suggest that stress-related memory improvements are related to a combination of increased noise reduction accompanied by, or leading to, a decreased hippocampal response.

In addition to these alterations in sensory and mnemonic operations, stress may promote a neural state optimized for memory formation. LC activation elevates hippocampal NE-levels leading to tonically increased activity (Berridge and Foote 1991). Therefore, the level of hippocampal activity might have been generally higher during the stress as compared to the control condition, but fMRI cannot detect such slowly modulated changes in baseline activity. Furthermore, corticosteroids and NE lower the threshold for synaptic modification (Groc et al. 2008). Therefore, sensitization of hippocampal plasticity – requiring less neural input for trace formation – possibly in combination with increased baseline activity – may provide a complementary mechanism through which acute stress can enhance memory formation. However, both this tonically increased activity and sensitized plasticity would result in smaller phasic responses, but cannot readily explain the observed reversal of the subsequent memory effect.

An alternative explanation for the stress enhanced memory is that it is carried by stress effects on memory consolidation. Our memory test was deliberately delayed precluding effects on memory retrieval (de Quervain et al. 1998; Roozendaal et al. 2006c), thus creating a time window during which consolidation may have been affected. Consolidation effects have been demonstrated in studies in which stress (hormone) manipulations were restricted to the post-learning period (Oitzl et al. 2001; Andreano and Cahill 2006; Roozendaal et al. 2006c). Therefore, effects on memory consolidation are likely to have contributed to the behavioral effect observed. It appears unlikely, however, that consolidation effects were the only contributing factor, since effects of acute stress on memory encoding were evident, and individual differences in stress-induced memory enhancement were predicted by hippocampal responses during encoding.

Remarkably, our stress induction resulted in a general improvement of memory which was not specific to negative pictures. In contrast, several studies have reported interactions between picture

valence and stress or cortisol (Buchanan and Lovallo 2001; Cahill et al. 2003; Abercrombie et al. 2006; Payne et al. 2006, 2007; Roozendaal et al. 2006c). This potential discrepancy may be explained by the dependence of glucocorticoid effects on simultaneous NE activation (Roozendaal et al. 2006c). Previous stress-induction studies have not always tested memory encoding during NE activation, since stressor and task were temporally separated. By integrating the memory task within the stress procedure – both in time and content – continuous NE activity was assured, likely enabling glucocorticoids to affect memory for negative and neutral items.

Some limitations of the current study should be considered. First, our findings are based on a specific memory test and may therefore not generalize. However, a picture cued recall test appears quite optimal for probing emotional memory formation (e.g. Buchanan and Lovallo 2001; Dolcos et al. 2004; Payne et al. 2006, 2007); it shows robustly the typical emotional bias effect, and provides a cleaner measure of episodic memory retrieval than for instance a recognition memory test, which can be confounded by familiarity judgments. Further, participants need to remember both the pictures' gist (in order to remember the corresponding picture) and details (which determined whether the picture would be scored as recalled). Therefore, our procedure provides a useful compound measure. Nevertheless, tests specific for memory of gist as opposed to details appear important for future research (Adolphs et al. 2005).

Second, we investigated men only and thus, we acknowledge that the obtained results cannot be readily generalized to women. The reason for excluding women was that they exhibit smaller and more variable stress responses (Kajantie and Phillips 2006), depending on menstrual cycle phase and use of contraceptives (Kirschbaum et al. 1999; Bouma et al. 2009). In this study, however, the stress response was not of primary interest in itself, but merely served as independent variable, which is why we opted to recruit the population with the most robust and stable stress response. Although important, sex and cycle specific effects were beyond the scope of this initial study.

Third, it would have been interesting to assess also movie-related memories, but practical reasons restrained us from doing so. The clips used, do not contain many distinct details that could be probed in a subsequent memory test and movies do not allow straightforward designs with subsequent memory effects. It is also impossible to align all physical and semantic features of the stress and the control movies. Thus, stress effects would have always been confounded by irrelevant factors. Instead, we show that memory formation for pictures that are identical across participants is affected by the state the participant is in.

In conclusion, the present study demonstrates that acute stress profoundly affects the neural substrates of memory formation, and it does so in a region-specific manner. Our findings suggest that acute stress is accompanied by a shift into a hypervigilant mode of sensory processing in combination with increased allocation of neural resources to noise reduction. This reduction of task-irrelevant ambient noise, in combination with a stress hormone induced optimal state for neural plasticity, may explain why stressful events attain a privileged position in memory. This interpretation provides a heuristic framework for further investigation into the mechanisms

underlying trauma etiology.

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**Dynamically changing effects of  
corticosteroids on human  
hippocampal and prefrontal  
processing**

**2.2**

**Marloes J.A.G. Henckens, Zhenwei Pu, Erno J. Hermans,  
Guido A. van Wingen, Marian Joëls, and Guillén Fernández**

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# 2.2

## ABSTRACT

Stress has a powerful impact on memory. Corticosteroids, released in response to stress, are thought to mediate, at least in part, these effects by affecting neuronal plasticity in brain regions involved in memory formation, including the hippocampus and prefrontal cortex. Animal studies have delineated aspects of the underlying physiological mechanisms, revealing rapid, non-genomic effects facilitating synaptic plasticity, followed several hours later by a gene-mediated suppression of this plasticity. Here, we tested the hypothesis that corticosteroids would also rapidly up- and slowly down-regulate brain regions critical for episodic memory formation in humans. To target rapid and slow effects of corticosteroids on neural processing associated with memory formation, we investigated 18 young, healthy men who received 20 mg hydrocortisone either 30 or 180 minutes prior to a memory encoding task in a double-blind, placebo-controlled, counter-balanced, crossover design. We used functional MRI to measure neural responses during these memory encoding sessions, which were separated by a month. Results revealed that corticosteroids' slow effects reduced both prefrontal and hippocampal responses, while no significant rapid actions of corticosteroids were observed. Thereby, this study provides initial evidence for dynamically changing corticosteroid effects on brain regions involved in memory formation in humans.

## INTRODUCTION

Aversive, stressful life-experiences are extremely well remembered (Joëls et al. 2006; Sandi and Pinelo-Nava 2007). Corticosteroids, released in response to stress, are thought to be critically involved in this memory enhancement by affecting neural plasticity (Joëls and de Kloet 1989; McEwen 1994). Recent animal studies on cellular excitability and long-term-potential (LTP), the alleged neurobiological substrate of memory formation (Martin and Morris 2002), suggest that corticosteroids alter neural plasticity in a time-dependent manner. On the one hand, corticosteroids were shown to rapidly enhance hippocampal excitability and LTP via a low-affinity mineralocorticoid receptor (MR) thought to reside in the plasma membrane (Karst et al. 2005). These rapid actions of corticosteroids work in concert with (and amplify) the effects of catecholamines (Roosendaal et al. 2006c) and are suggested to optimize rapid adaptive behavior by relocating neural resources away from higher-order cognitive processing regions in the prefrontal cortex (PFC) to the medial temporal lobe (MTL) (Diamond 2007). On the other hand, the initiation of a corticosteroid-induced genomic cascade by the binding of intracellular mineralocorticoid and glucocorticoid receptors (GRs) is known to suppress hippocampal LTP several hours later (Pavlidis et al. 1995; Wiegert et al. 2005); this delayed action is considered to promote consolidation of relevant information (de Kloet et al. 2008), possibly by impairing retroactive interference. Although these neurobiological mechanisms are quite well established in rodents, at present, it is unclear if and how they translate to the human brain.

Therefore, we tested the hypothesis that corticosteroids rapidly up-regulate and slowly down-regulate brain regions critical for episodic memory formation at the human system-level. We focused on two brain regions known to be affected by corticosteroids (de Kloet 1991), and critically involved in memory processing (Fernández and Tendolkar 2001); the hippocampus and prefrontal cortex. Rather than giving participants corticosteroids at one time point and follow them along the process of memory formation (which would involve rapidly succeeding fMRI sessions, inevitably inducing strong order effects), participants received either placebo, 20 mg hydrocortisone 30 min prior to the study phase to target the rapid actions of corticosteroids, or 20 mg hydrocortisone 180 min prior to the study phase to target the slow actions of corticosteroids. We used a double-blind, placebo-controlled, counter-balanced crossover design and invited participants for three study-test cycles each separated by approximately one month, receiving each time a different pharmacological manipulation. In every cycle, participants were instructed to memorize different sets of both emotionally negative and neutral pictures while brain activity was measured using fMRI. The memory for these pictures was tested 24 hours later. Moreover, to exclude potential physiological or psychological side-effects of hydrocortisone administration, heart rate and mood state were assessed throughout the experiment.

## MATERIALS & METHODS

### Participants

Eighteen young (ages 18–29, median 23), right-handed, healthy male volunteers participated in the study after signing written informed consent. Women were excluded from participation since previous research has indicated that they respond differently to hydrocortisone than men, both in behavior (Andreano and Cahill 2006; Bohnke et al. 2010) and brain activation (Merz et al. 2010; Stark et al. 2006). We presently focused on men, allowing easier comparison with the results from an earlier study in which subjects were exposed to stress (Henckens et al. 2009), a situation that is known to induce a more stable neuroendocrine response in men than in women (Bouma et al. 2009; Kajantie and Phillips 2006; Kirschbaum et al. 1999; Ossewaarde et al. 2010). Furthermore, individuals who met any of the following criteria were excluded from participation during screening: history of head injury, autonomic failure, history of or current psychiatric, neurological, or endocrine disorders, current periodontitis, acute inflammatory disease, acute peptic or duodenal ulcers, regular use of corticosteroids, treatment with psychotropic medications, narcotics, beta-blockers, steroids, or any other medication that affects central nervous system or endocrine systems, medical illness within the three weeks prior to testing, self-reported mental or substance use disorder, daily tobacco or alcohol use, regular night shift work, current stressful episode or major life event, and previous exposure to slides used in the study (i.e., International Affective Picture System; Lang et al. 1999). The study was executed in accordance with the declaration of Helsinki and approved by the local ethics committee (CMO region Arnhem-Nijmegen, Netherlands).

### Procedure

**Screening.** After granting informed consent, all participants were invited for an introductory interview, during which they were asked to complete an initial screenings questionnaire, the Beck Depression Inventory (Beck 2002), and NEO-FFI Personality Inventory (Costa and McCrae 1992). Further, a T<sub>1</sub>-weighted anatomical scan was made, familiarizing participants with the MRI environment before the study sessions began (see Fig. 8 for a schematic overview of the complete procedure).

**Prior to arrival.** To minimize differences in baseline cortisol levels, we instructed participants not to use any recreational drugs for three days and to refrain from drinking alcohol, exercising, and smoking for 24 h prior to each session. Furthermore, participants were requested not to brush their teeth, floss, or eat and drink anything but water for one hour prior to all sessions enabling adequate saliva sampling for cortisol assessment. They were asked to take a light lunch and do so no later than one hour before arrival; their lunch could not contain any citrus products, coffee, tea, milk, and sweets (Maheu et al. 2005). Throughout each session, they had no further food intake and had only water to drink.

**Arrival.** To reduce the impact of diurnal variation in cortisol levels, the experiment started in the afternoon, when hormone levels are relatively stable. After arrival at 12:00 h ( $\pm 45$  min) on the first day, participants rested 30 min prior to taking the first saliva sample, followed by another sample 15 min later. The average value of these two samples served as baseline cortisol level. To increase familiarity with the procedure and minimize task repetition effects, participants were explicitly informed about all details of the memory experiment. A financial reward was promised proportional to the participant's performance in the recall test to encourage motivation. During the entire period ( $\sim 3.75$  h) prior to the encoding task, the participants had to wait in a quiet, isolated room where they were free to conduct any activities except for anything potentially arousing (e.g. video games).

<b>Day 1: Intake</b>		<b>3x:</b> different drug manipulations & different picture sets	<b>Day n:</b>	
t = 0	Screening Questionnaire		t = 0	Arrival; explanation procedure
t = 30	Personality Questionnaires		t = 30	Saliva sample 1 Mood Questionnaire 1
t = 60	Structural scan		t = 45	Saliva sample 2 Drug 1
			t = 75	Saliva sample 3
			t = 105	Saliva sample 4
			t = 135	Saliva sample 5
			t = 195	Saliva sample 6 Drug 2
			t = 225	Entering MRI scanner Saliva sample 7 Mood Questionnaire 2 Start Picture Encoding
			t = 285	End Picture Encoding Saliva sample 8 Mood Questionnaire 3
			<b>Day n+1:</b>	
			t = 0	Arrival; explanation procedure
			t = 15	Free Recall Memory Test
			t = 75	Cued Recall Memory Test
			t = 135	End of session

**Figure 8.** Time line of the experiment. Participants were first invited for an intake interview, after which they returned for three sessions consisting of two subsequent days and separated by approximately a month. *t* = time in minutes

**Drug administration.** Implementing a double-blind, placebo-controlled, counter-balanced crossover design, each participant underwent three experimental sessions, with an approximate inter-session interval of one month (mean interval  $\pm$  S.E.M.;  $40 \pm 4$  days). The whole procedure for individual sessions remained identical except that the drug administration schemes differed from session to session. All drug capsules, containing either 20 mg CORT (Hydrocortison CF 20 mg tablets, Centrafarm Services B.V. Etten-Leur, The Netherlands) or placebo (cellulose) were administered orally. The administration dose of 20 mg was based on previous studies using a similar dose (Buchanan and Lovallo 2001; van Stegeren et al. 2010) showing that this dose elevated cortisol levels to those observed during exposure to severe stress (Morgan et al. 2000). In order to ensure a double-blind paradigm and to monitor the time-dependent effect of cortisol, participants received two capsules at distinct time points; at 180 min prior to the start of picture encoding ( $t = 45$ ) and at 30 min prior to the start of picture encoding ( $t = 195$ ). At these time points they received either: 1) 1st capsule containing CORT, 2nd placebo – to reveal the slow effect of cortisol; 2) 1st placebo, 2nd CORT – to disclose the rapid cortisol effect; and 3) 1st placebo, 2nd placebo – the control. Timing of administration at 30 min prior to encoding was based on previous studies in humans showing rather immediate ( $<15$  min) increases in salivary cortisol levels following hydrocortisone intake (van Stegeren et al. 2010) and a high correlation between salivary cortisol levels and serum levels of free (i.e. active) cortisol (Kirschbaum and Hellhammer 1994). Cortisol is known to pass the blood-brain barrier quite well (Karssen et al. 2001), and rodent studies have shown a strong correlation between plasma and brain corticosteroid levels (Droste et al. 2008), but with a small time delay ( $\sim 20$  min) between plasma peak corticosteroid levels and those in the brain after exposure to stress. Based on these studies, brain cortisol levels are expected to rise approximately 30 minutes after hydrocortisone administration. Given that the rapid, non-genomic corticosteroid effects on the brain are known to occur almost immediately upon brain exposure to elevated corticosteroid levels (Karst et al. 2005), we therefore optimally targeted rapid effects by administering hydrocortisone 30 min prior to scanning. The slow, genomic effects of corticosteroids were not expected to start earlier than approximately 90 min after corticosteroid administration, and last for hours (Joëls and de Kloet 1992; Joëls and de Kloet 1994; Joëls et al. 2003). The timing for targeting these effects, i.e. 3 hours post-administration, was based on previous work showing suppressed LTP (Pavlidis et al. 1995; Wiegert et al. 2005), and strongest corticosteroid effects on hippocampal gene expression at this time-delay (Morsink et al. 2006).

**Scanning.** Participants lay supine in the scanner and viewed the screen through a mirror positioned on the head coil. They were asked to lie as still as possible, keep their eyes open, and look directly and continuously at the center of the screen in front of them. Participants were instructed to view each picture for the entire time that it was displayed. Pictures belonged to two categories, either with a neutral or negatively arousing content. Participants were asked to memorize each picture and to rate its aversiveness. Ratings were given with right-hand button presses, with the index finger for negative and the middle finger for neutral pictures. Pictures were shown in a pseudorandom order (no more than two pictures of the same category consecutively), and all first

slides were neutral to avoid ceiling effects in recall that might result from the combined effect of arousal and primacy on memory.

**Stimulus materials.** Participants viewed a distinct stimulus set during each picture encoding session (Henckens et al. 2009); resulting in the requirement of three different stimulus sets. Each of these sets consisted of 80 negative and 80 neutral pictures, supplemented with 41 null events (fixation). Pictures were selected from both a standard set of affective pictures (International Affective Picture System (IAPS) (Lang et al. 1999)) and an additional set of newly rated pictures. New pictures were previously (Henckens et al. 2009) downloaded from the internet and selected on the authors' assessment of emotionality and similarity to IAPS pictures. New pictures were rated on a scale from 1 to 9 on both arousal and valence using the Self-Assessment Manikin (SAM) scales (Bradley and Lang 1994) by an additional 20 male volunteers. To assure reliable rating that did not significantly differ from IAPS ratings, and to serve as a reference frame, positive and negative IAPS pictures were added. Negative slides were chosen for their moderate to high arousal quality (mean  $\pm$  S.E.M.;  $5.5 \pm 0.7$ ), and negative valence (mean  $\pm$  S.E.M.;  $3.1 \pm 0.7$ ), rated on a 1-9 point rating scale as determined by the SAM (Bradley and Lang 1994). Neutral slides were selected for their relatively low arousal (mean  $\pm$  S.E.M.;  $2.5 \pm 0.7$ ) and neutral valence (mean  $\pm$  S.E.M.;  $5.3 \pm 0.3$ ). Used picture sets contained about 50 percent newly rated neutral and 15 percent newly rated negative pictures and were matched on chromatic features and complexity, while overlap in contents within one set was minimized. Stimulus sets did not differ in mean arousal and valence ratings. All slides were presented for 6 s with a 4-8 s inter-trial interval (fixation cross), resulting in a total scanning time of ~40 min for each session.

**Subsequent memory test.** To exclude any corticosteroid effects on memory retrieval, participants came back on the day after each encoding session (at 14:15 h ( $\pm$  45 min)) to perform a free and a cued recall test, both lasting 60 minutes. In both tests, participants were required to write to the utmost detail all the characteristics of the pictures they could remember, so that an outsider would be able to identify the pictures as distinctively recognizable with the information provided (Dolcos et al. 2004). A short introduction was written to help the participants in listing characteristics. The cued recall test differed from the free recall in that the participant received one- or two-word written cues (of similar arousal to that of the picture) that may facilitate his recall. This cue could e.g. be the negative one "wounded hand", to which participants could mention the details "left hand, few fingers missing, tendons sticking out, held above a metal bowl, etc". The cue could also describe a neutral picture, e.g. "bike", to which participants could write down "pink bike, put against a brick wall, basket on steering wheel, etc". These written descriptions provided by the participants were evaluated by a researcher blind to the drug condition the participant was in, and only pictures with a description that allowed both identification and discrimination were classified as remembered. Since some pictures that were mentioned during free recall were not described in the cued recall test (due to motivational issues or specifics of the cues), but were obviously remembered, all pictures mentioned in either the free or cued recall test were considered remembered for further analyses. Pictures with no recollection of characteristics were

considered forgotten.

## Physiological and behavioral measures

**Saliva collection and analysis.** Cortisol levels were measured from saliva at eight time points: baseline measurements at the beginning of the experiment (twice) ( $t = 30, 45$  min), and six samples ( $t = 75, 105, 135, 195, 225, 285$  min) to assess cortisol changes throughout the experiment. Saliva was collected using a commercially available collection device (Salivette®, Sarstedt, Germany). For each sample, the participant first placed the cotton swab provided in each Salivette tube in his mouth and chewed gently on it for 1 min to produce saliva. The swab was then placed back in the salivette tube, and the samples were stored in a freezer at  $-25^{\circ}\text{C}$  until assayed. Laboratory analyses were performed at the Department of Biopsychology, TU Dresden, Germany. After thawing, salivettes were centrifuged at 3,000 rpm for 5 min, which resulted in a clear supernatant of low viscosity. Salivary free cortisol concentrations were subsequently measured using a commercially available chemiluminescence-immuno-assay (CLIA) with high sensitivity of 0.16 ng/mL (IBL, Hamburg, Germany).

**Heart rate.** Cardiac rhythm of the participants was measured during scanning using a pulse oximeter placed on their left index finger. Participants were instructed to keep their hands as still as possible during the measurement. Heart rate frequency was calculated using in-house software. Data of one subject were discarded from analyses, due to excessive artifacts in the recorded signal. **Mood State.** Mood state was assessed using the Positive and Negative Affect Schedule questionnaire (Watson et al. 1988) at three time points: at the beginning of the experiment ( $t = 30$  min), just prior to encoding ( $t = 225$  min), and immediately after encoding ( $t = 285$  min).

## Physiological and behavioral statistical analysis

Behavioral and physiological data were analyzed in SPSS 15.0 (SPSS, Inc., Chicago, IL, USA) using repeated measures ANOVAs with drug manipulation (slow CORT vs. rapid CORT vs. placebo), subsequent memory (remembered vs. forgotten), and picture arousal (aversive vs. neutral) as within subject factors, and paired samples t-test statistics. Although the implemented session order (of the placebo, rapid CORT, and slow CORT sessions) was counterbalanced over participants, we also tested whether this factor still potentially modulated the effects of drug administration. Therefore, we tested whether session order had any influence on the drug effects observed by including it as a covariate in the analyses of the drug effects. This did not change the observed pattern of results, which made us to exclude this factor in all further analyses. Alpha was set at 0.05 throughout.

## MRI acquisition

Participants were scanned in a Siemens (Erlangen, Germany) MAGNETOM Avanto 1.5 Tesla MRI scanner equipped with an 8-channel head coil. During each of the three scanning sessions,

a series of blood oxygenation level dependent (BOLD)  $T_2^*$ -weighted gradient echo EPI images were acquired with the following parameters: TR = 2340 ms, TE = 35 ms, FA = 90°, 32 axial slices approximately aligned with AC-PC plane, slice matrix size = 64 x 64, slice thickness = 3.5 mm, slice gap = 0.35 mm, FOV = 212 x 212 mm<sup>2</sup>. High resolution anatomical images were acquired using a  $T_1$ -weighted 3D Magnetization-Prepared RAPid Gradient Echo (MP-RAGE) sequence with the following parameters: TR = 2250 ms, TE = 2.95 ms, FA = 15°, orientation: sagittal, FOV = 256 x 256 mm<sup>2</sup>, voxel size = 1.0 mm isotropic.

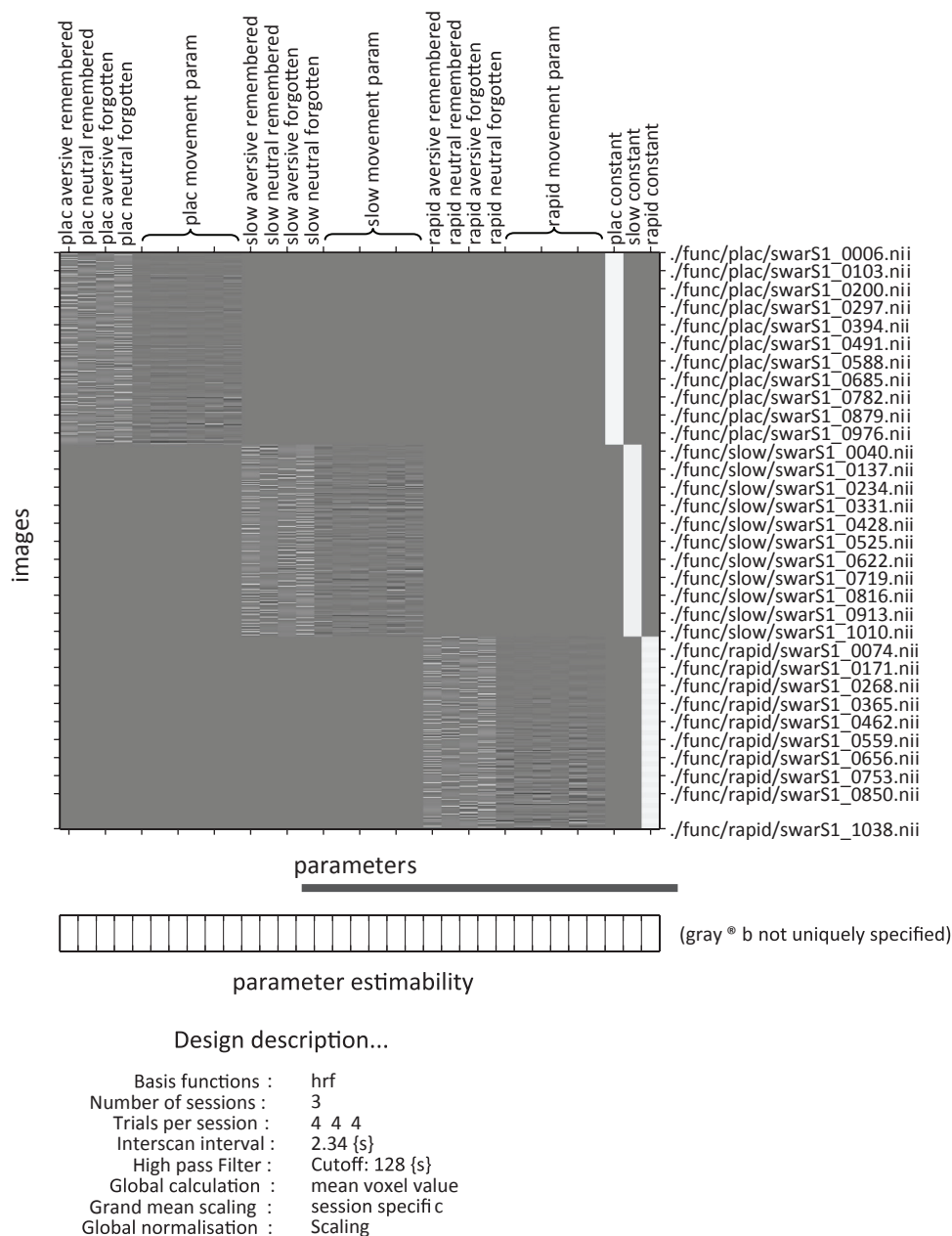
## fMRI data analysis

Data were analyzed using Statistical Parametric Mapping software (SPM5; UCL, London) and in-house software. The first five EPI-volumes were discarded to allow for  $T_1$ -equilibration. Prior to fMRI analysis, the images were motion corrected using rigid body transformations and least sum of squares minimization. Subsequently, they were temporally adjusted to account for differences in sampling times across different slices. All functional images were then co-registered with the high-resolution  $T_1$ -weighted structural image using normalized mutual information maximization. The anatomical image was subsequently used to normalize all scans into MNI152 (Montreal Neurological Institute) space. All functional images were resampled with a voxel size of 2 mm isotropic. Finally, all images were smoothed with an isotropic 8-mm full-width-at-half-maximum Gaussian kernel in order to accommodate residual functional/anatomical variance between subjects.

Subsequently, data were analyzed using a general linear model, in which individual events were modeled based on drug condition (slow CORT vs. rapid CORT vs. placebo), subsequent memory (remembered vs. forgotten), and picture arousal (aversive vs. neutral). Regressors were temporally convolved with the canonical hemodynamic response function of SPM5. The six covariates corresponding to the movement parameters obtained from the realignment procedure for every session were also included in the model. To reduce unspecific differences between scan sessions, global normalization using proportional scaling was applied (see Fig. 9 for the first level model applied). The single subject parameter estimates of each session and condition obtained from the first level analysis were included in subsequent random effects analyses. For the second level analysis a factorial ANOVA was used, with drug manipulation, subsequent memory, and picture arousal as within subject factors.

Statistical tests were family-wise error (FWE) rate corrected ( $p(\text{fwe}) < 0.05$ ) for multiple comparisons at the cluster-level using a height threshold of  $p < 0.001$ . F-contrast cluster-level statistics in SPM were performed by implementing the random field theory (RFT) version of cluster size inference (under stationarity) extended to F-tests (Ashburner and Friston 2000; Hayasaka et al. 2004). Correction for multiple comparisons was done across the entire brain, or for the search volume for regions of interest using a small volume correction. Given strong neurophysiological evidence for the locus of CORT receptors (de Kloet 1991), and their known involvement in memory formation (Fernández and Tendolkar 2001), the hippocampus and PFC were *a priori* considered regions of interest. The search volumes for these ROIs were anatomically

## Statistical analysis: Design



**Figure 9.** Exemplary design matrix for the first model used in fMRI data analysis. All individual events were modeled based on drug condition (slow CORT vs. rapid CORT vs. placebo), subsequent memory (remembered vs. forgotten), and item aversiveness (aversive vs. neutral). Besides these regressors the six covariates corresponding to the movement for every session were included in the model, as well as a constant.

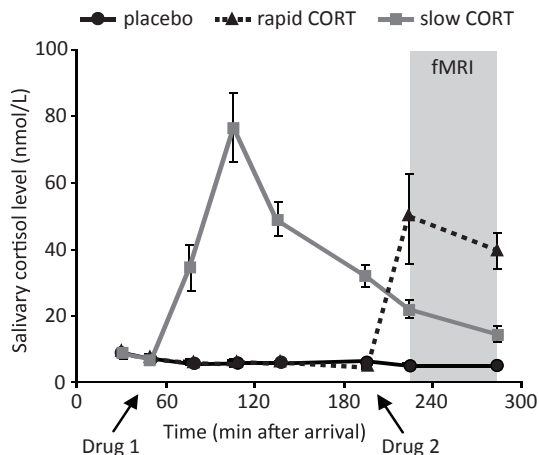
defined using the WFU PickAtlas Tool (version 2.4) toolbox implemented in SPM5 (Maldjian et al. 2003). The specific masks used were those for the hippocampus (bilaterally) and the frontal lobe.

To test for distributed drug effects on hippocampal activity specifically, mean activity of the anatomically defined hippocampus was extracted and analyzed in SPSS. Visualizations of activations were created using MRIcroN (<http://www.sph.sc.edu/comd/rorden/mricron/>) by superimposing statistical parametric maps thresholded at  $p < 0.005$  uncorrected and an extended cluster-size of 500 voxels (to filter out effects that did not reach our statistical threshold corrected for multiple testing), onto a canonical  $T_1$ -weighted image in standard MNI152 space.

## RESULTS

### Cortisol level

Twenty mg of hydrocortisone (CORT) was effective in elevating salivary cortisol levels to levels observed during severe stress (Morgan et al. 2000). Both drug administration conditions increased cortisol levels (peak level rapid CORT vs. placebo:  $T(17) = 4.45$ ,  $p < 0.001$ ), peak level slow CORT vs. placebo:  $T(17) = 8.10$ ,  $p < 0.001$ ), with levels either peaking during or at 120 min prior to the study phase, respectively (Fig. 10). As intended, in the rapid CORT condition cortisol levels during scanning were higher than those in both the placebo ( $F(1,17) = 21.73$ ,  $p < 0.001$ ) and the slow CORT condition ( $F(1,17) = 11.88$ ,  $p = 0.003$ ). However, in the slow CORT condition cortisol



**Figure 10.** Salivary cortisol levels. Participants received two capsules (drug1 & drug2) containing either 20 mg hydrocortisone (CORT) or placebo at different time-points prior to picture encoding during fMRI scanning. CORT intake significantly elevated salivary cortisol levels to levels observed during severe stress in both CORT administration conditions. Rapid CORT: 20 mg CORT administered 30 min prior to encoding, Slow CORT: 20 mg CORT administered 180 min prior to encoding, Placebo: mere placebo administered. Error bars represent S.E.M.

levels were still slightly, but significantly, elevated compared to placebo during memory encoding ( $F(1,17) = 32.38, p < 0.001$ ). To correct for any potential effect of this remainder of circulating cortisol in the slow CORT condition, the absolute difference in cortisol levels as compared to placebo was included as a covariate in all comparisons between these drug conditions.

## Physiological and psychological measures

Hydrocortisone did not have any subjective, noticeable effects. Post-experiment debriefing revealed that participants were not able to identify the substance received. As expected, hydrocortisone administration did not affect autonomic measures of heart rate (main effect of drug:  $F(2,15) = 2.39, p = 0.125$ ) and heart rate variability ( $F(2,15) = 1.72, p = 0.213$ ) (Table 3). Further, hydrocortisone administration did not affect psychological state as assessed by the Positive and Negative Affect Schedule questionnaire (Watson et al. 1988). A consistent reduction in positive affect over time was observed in all drug conditions ( $F(2,16) = 18.18, p < 0.001$ ), independently of drug administration (main effect of drug:  $F(2,16) = 1.56, p = 0.241$ , drug  $\times$  time interaction:  $F(4,14) = 1.54, p = 0.244$ ). Negative affect did not change throughout the experiment (main effect of time:  $F(2,16) = 2.53, p = 0.111$ ), nor was it affected by drug administration (main effect of drug  $F(2,16) = 2.68, p = 0.099$ , drug  $\times$  time interaction:  $F(4,14) < 1$ ) (Table 3). Hence, differences in brain activity due to drug administration cannot readily be explained by autonomic or psychological side effects of the drug.

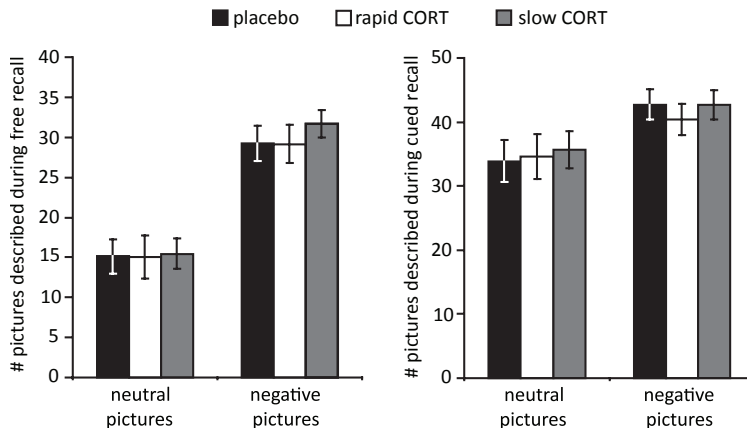
**Table 3. Psychological and physiological measures**

	Placebo	Rapid CORT	Slow CORT
<b>Affective State (PANAS)</b>			
Positive affect: baseline	30.78 (1.54)	32.39 (1.28)	32.11 (1.65)
Positive affect: prior to encoding	29.28 (1.40)	30.67 (1.01)	31.50 (1.32)
Positive affect: after encoding	26.00 (1.30)	25.22 (1.24)	28.00 (1.49)
Negative affect: baseline	16.06 (1.97)	13.06 (1.23)	14.50 (1.51)
Negative affect: prior to encoding	16.17 (1.78)	14.78 (1.51)	15.17 (1.53)
Negative affect: after encoding	16.11 (1.83)	15.22 (1.65)	15.22 (1.54)
<b>Heart rate (BPM)</b>	62.00 (1.77)	59.58 (1.41)	61.96 (1.24)
<b>Heart rate variability (<math>\text{ms}^2</math>)</b>	134.71 (20.08)	123.39 (16.85)	109.70 (11.58)
<b>Picture rating (# congruent)</b>			
Neutral pictures	77.67 (0.44)	77.17 (0.42)	78.06 (0.41)
Negative pictures	73.39 (0.98)	75.28 (0.64)	74.89 (1.02)
<b>Memory performance</b>			
Free recall: # neutral pictures	15.11 (2.11)	15.06 (2.66)	15.44 (1.88)
Free recall: # negative pictures	29.22 (2.19)	29.17 (2.36)	31.67 (1.75)
Cued recall: # neutral pictures	33.89 (3.27)	34.61 (3.51)	35.67 (2.86)
Cued recall: # negative pictures	42.72 (2.37)	40.39 (3.27)	42.67 (2.30)

Mean values (S.E.M.). PANAS; Positive and Negative Affect Schedule questionnaire (Watson et al. 1988).

## Memory performance

As intended, about 50% of the pictures were recalled the subsequent day (mean  $\pm$  S.E.M.;  $47.56 \pm 1.78$  aversive pictures,  $36.72 \pm 3.08$  neutral pictures, see Table 3, and Fig. 11). Hydrocortisone administration did not induce any significant effects on memory performance ( $F(2,16) < 1$ ). As expected, we did observe a strong effect of picture arousal, with participants recalling more aversive than neutral items ( $F(1,17) = 32.11$ ,  $p < 0.001$ , Table 3), but also this effect was not modulated by CORT administration ( $F(2,16) < 1$ ).



**Figure 11.** Number of pictures recalled in the free and cued recall tests. CORT; hydrocortisone

## Brain activation

First, regions supporting successful memory formation were identified. Confirming earlier findings (Brewer et al. 1998; Henckens et al. 2009; Wagner et al. 1998), regions displaying larger neural activity during encoding of subsequently remembered than forgotten pictures included the hippocampus, bilateral inferior temporal gyrus, inferior, middle, and superior frontal gyrus, inferior parietal gyrus, and the mid/superior occipital lobe (Table 4). Second, brain imaging results revealed strong main effects of picture arousal (aversive > neutral) in the amygdala, hippocampus, insula, cerebellum, brain stem, inferior frontal cortex, and regions associated with visual processing (including the middle temporal gyri) (Henckens et al. 2009; Phan et al. 2002) (Table 4).

Next, we examined the main question at issue, how CORT affects brain regions involved in memory formation over time. We first tested whether there were any differences in brain activity between all three drug conditions (i.e., the main effect of drug) by performing an ANOVA with three levels of the factor drug. This analysis revealed a large cluster within the middle frontal gyrus (comprising Brodmann areas (BA) 9, 45, 46 and 48) affected by CORT administration (local maximum at  $[x = 32, y = 28, z = 26]$ ,  $F(2,204) = 13.16$ ,  $p(\text{fwe}) < 0.001$ ). Thus, CORT

Table 4. Regions revealing main effects or interactions of picture valence and subsequent memory

Region	MNI Coordinates				Peak T-value	Cluster- size	P-value
	Brodmann area	x	y	z			
Main effect of subsequent memory							
Remembered > Forgotten							
Middle occipital gyrus, L & R	7,19	32	-72	38	5.93	746	p < 0.001
Inferior temporal gyrus, L	19,37	-52	-58	-10	7.78	988	p < 0.001
Inferior temporal gyrus, R	37	54	-52	-14	5.00	430	p < 0.001
Inferior parietal lobule, L	2,40	-48	-40	50	6.28	1479	p < 0.001
Hippocampus / Amygdala, L	34,35	-14	-8	-12	5.95	623	p < 0.001
Superior temporal pole / Amygdala, R	34,36,38	30	8	-22	4.64	187	p = 0.041
Middle cingulate gyrus	24	2	-2	36	4.70	240	p = 0.015
Inferior & Middle frontal gyrus, L	6,44,48	-50	10	26	6.81	1698	p < 0.001
Inferior frontal gyrus, R	44	50	10	24	5.36	263	p = 0.010
Supplemental motor area, L	6	-6	10	62	5.34	191	p = 0.038
Superior frontal gyrus, L	9	-20	32	48	5.20	299	p = 0.005
Inferior frontal & Orbitofrontal gyrus, L	45	-46	40	14	5.28	789	p < 0.001
Forgotten > Remembered							
Precuneus	7,23,26	4	-64	36	6.63	2279	p < 0.001
Middle temporal gyrus, R	20-22,37	56	-48	16	5.63	672	p < 0.001
Middle frontal gyrus, R	10,11,46	36	48	14	4.28	467	p < 0.001
Main effect of picture valence							
Aversive > Neutral							
Cerebellum, L	/	-14	-74	-44	9.15	949	p < 0.001
Middle temporal gyrus, L	19-21,37,39	-50	-64	8	15.97	5821	p < 0.001
Middle temporal gyrus, R (incl. hippocampus, amygdala, and insula)	19-22,28,34,37,41,42,48	54	-66	0	16.79	15089	p < 0.001
Precuneus / Posterior cingulate cortex	23,26	4	-54	30	9.44	1310	p < 0.001
Inferior frontal gyrus, L & R	45	-48	32	4	5.16	303	p = 0.005
Superior medial frontal gyrus	8-10,32	6	52	32	8.97	1821	p < 0.001
Rectal gyrus	11	4	56	-16	7.54	420	p < 0.001
Neutral > Aversive							
Cerebellum, L	/	-40	-62	-44	5.37	236	p = 0.016
Calcarine, L & R	17-19,23,27,29,30,37	-16	-58	14	9.40	2069	p < 0.001
Inferior parietal lobule, L	7,39,40	-48	-54	48	6.76	637	p < 0.001
Inferior parietal lobule, R	7,39,40	50	-48	48	8.36	1017	p < 0.001
Inferior temporal gyrus, L & R	20-22,37,48	62	-40	-14	8.46	2595	p < 0.001
Fusiform, L & R	17,19,23,2730,37	28	-44	-10	9.37	1787	p < 0.001

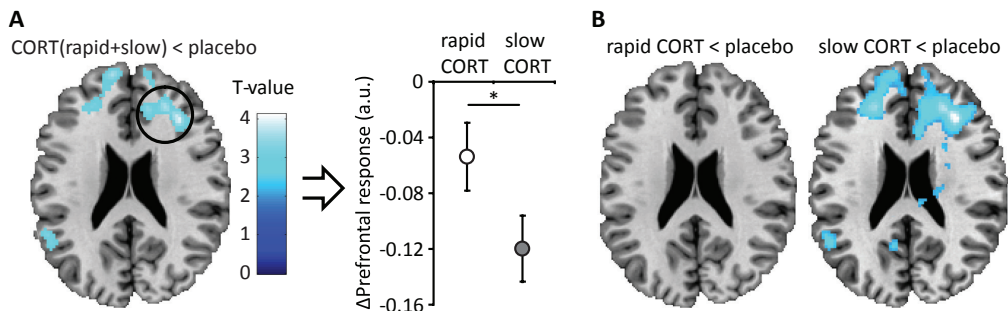
Table 4 (continued)

Region	MNI Coordinates						
	Brodmann area	x	y	z	Peak T-value	Cluster-size	P-value
Main effect of picture valence							
Neutral > Aversive							
Superior temporal gyrus, L & R	20-22,43,48	-58	-4	4	7.25	2798	p < 0.001
Middle frontal gyrus, L & R	8-11,45,46	42	36	28	8.29	7901	p < 0.001
Valence by SME interaction (positive)							
Orbitofrontal cortex, L	38	-34	20	-18	4.39	210	p = 0.026
Medial superior frontal gyrus	10,11,32	0	66	20	5.28	1141	p < 0.001

The peak x, y, z coordinates are given in MNI152 standard space coordinates. L and R denote left and right. SME: subsequent memory effect. All effects were analyzed using whole brain family wise error (FWE) correction for multiple comparisons at the cluster-level ( $p(\text{fwe}) < 0.05$ ), after using a height threshold of  $p < 0.001$ .

administration clearly modulated prefrontal cortex activity. Next, we wanted to perform follow-up tests to investigate whether this observed effect for corticosteroids was time-dependent. However, we were not allowed to extract the data from this activation cluster, since the selection of voxels would have been biased towards differences between the three drug conditions (it would induce circularity arising from a non-independent selection of voxels (Kriegeskorte et al. 2009)). Therefore, we conducted a new contrast for corticosteroid modulation that was orthogonal (i.e. independent) to the timing effect by contrasting placebo to both drug conditions combined (CORT(rapid+slow) vs. placebo). This analysis revealed again a cluster in the middle frontal gyrus that exhibited a negative CORT effect for this contrast (Table 5, Fig. 12A; CORT(rapid+slow) < placebo). The parameter estimates for both CORT conditions were subsequently extracted and their direct comparison showed that the slow CORT condition was characterized by a stronger reduction in prefrontal cortex activity than the rapid CORT condition ( $F(1,17) = 4.46$ ,  $p = 0.050$ , Fig. 12A). The rapid CORT condition on the other hand, did not show a significant difference in activity in this region from placebo (Fig. 12B).

To correct for any potential effects of the remaining small but significant CORT increase during encoding in the slow CORT condition, a new general linear model was created using the normalized difference in hormone concentration between slow CORT and placebo conditions as a covariate. This correction did not change the pattern of results (Table 6 and Fig. 13), indicating that the observed effects cannot easily be explained by the acute effects of the remaining small elevation in CORT levels, but are rather caused by the slow actions of CORT.

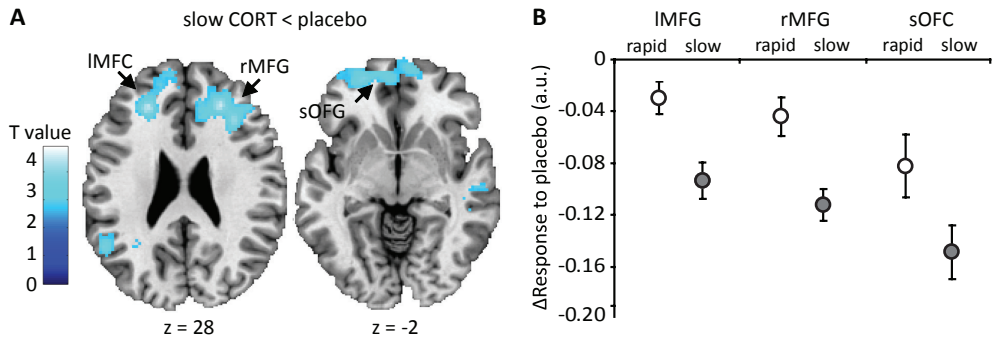


**Figure 12.** Effects of hydrocortisone (CORT) administration on brain activity during picture encoding. **(A)** Negative main effect of CORT administration regardless of timing ( $z = 26$ ); activity in prefrontal cortex was decreased due to CORT administration. Comparison of parameter estimates from this activation cluster (local maximum at  $[34, 26, 26]$ ) to placebo revealed that PFC activity was significantly down-regulated in the slow CORT condition. **(B)** Simple effect contrasts of brain regions that were more active during picture processing under placebo conditions than under CORT ( $z = 26$ ). The slow effects of corticosteroids clearly down-regulated prefrontal cortex activity, whereas the rapid effects of corticosteroids did not. Baseline represents activity under placebo conditions. \*:  $p = 0.050$ . See Table 5 for formal statistical tests. Error bars represent S.E.M.

**Table 5.** Peak voxel and corresponding F/T value of significantly activated clusters in the main effects of hydrocortisone (CORT)

	Brodmann area	MNI Coordinates			Peak T-value	Cluster size	P-value
		x	y	z			
Main effect of drug							
F-contrast:							
Placebo vs. Rapid CORT vs. Slow CORT							
Middle frontal gyrus, R	45,46,48	32	28	26	13.16	456	p < 0.001*
Placebo > Rapid & Slow CORT							
Angular gyrus, L	39	-46	-62	40	3.96	211	p = 0.026*
Middle frontal gyrus, R	9,45,46,48	34	26	26	3.93	133	p = 0.050+
Placebo > Slow CORT							
Mid occipital gyrus/Angular gyrus, L	39	-38	-66	36	4.34	489	p < 0.001*
Middle cingulate gyrus, R	23	8	-30	40	4.32	622	p < 0.001*
Middle frontal gyrus, R	9,32,45,48	34	26	26	5.09	2571	p < 0.001*
Middle frontal gyrus, L		-24	34	28	4.63		

The peak x, y, z coordinates are given in MNI152 standard space coordinates. MNI, Montreal Neurological Institute; R, right; L, left. All effects were analyzed using family wise error (FWE) correction for multiple comparisons at the cluster-level ( $p_{fwe} < 0.05$ ), after using a height threshold of  $p < 0.001$ . \*: FWE-corrected for whole brain volume, +: FWE-corrected for region of interest



**Figure 13.** Brain regions displaying decreased activity compared to placebo due to the slow effects of hydrocortisone (CORT). To correct for the residual elevation in salivary cortisol levels still present during scanning, the absolute difference was entered as a covariate in the general linear model. (A) This did not change the results: activity in middle prefrontal gyrus (MFG) and orbitofrontal cortex (OFC) was strongly reduced in the slow CORT condition. (B) parameter estimates of the observed activation clusters in Fig. 13A revealed significant downregulation due to the slow effects of corticosteroids.

**Table 6.** Regions revealing significant slow CORT effects, after correcting for the difference in cortisol level between placebo and slow CORT during scanning

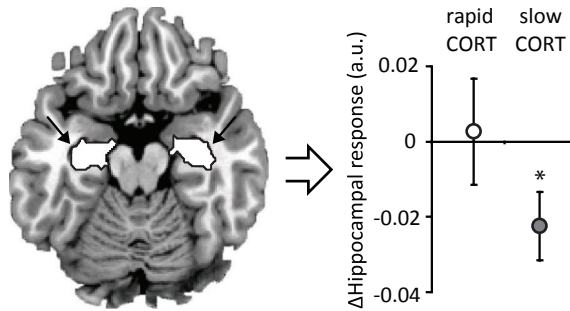
Region	MNI Coordinates				Peak T-value	Cluster size	P-value
	Brodmann area	x	y	z			
Placebo > Slow CORT							
Middle frontal gyrus, R	9,46,48	24	36	30	4.48	717***	$p < 0.001^*$
Middle frontal gyrus, L	9,46,48	-24	34	28	4.42	165 <sup>+</sup>	$p = 0.025^+$
Sup orbitofrontal lobule, L	11	-24	52	-2	3.82	155 <sup>+</sup>	$p = 0.030^+$

The peak x, y, z coordinates are given in MNI152 standard space coordinates. L and R denote left and right. All effects were analyzed using family wise error (FWE) correction for multiple comparisons at the cluster-level ( $p(\text{fwe}) < 0.05$ ), after using a height threshold of  $p < 0.001$ . \*: FWE-corrected for whole brain volume, <sup>+</sup>: FWE-corrected for region of interest

Second, we tested whether CORT had any effects on the hippocampus specifically. The initial analysis in SPM did not reveal a general main effect of CORT in this region, but since voxel-wise analyses are most efficient in detecting focal effects, any effect may remain below the detection threshold if it is widely distributed across the entire hippocampus. Therefore, we averaged data from the anatomically defined hippocampi and tested for time-specific CORT effects. As hypothesized, this analysis revealed reduced hippocampal responses compared to placebo due to the slow hydrocortisone effects ( $F(1,17) = 6.21$ ,  $p = 0.023$ , Fig. 14). The rapid actions of corticosteroids did not seem to affect hippocampal activity, as activity observed in the rapid CORT

condition was not significantly different from placebo ( $F(1,17) < 1$ ). However, the difference in activity between both drug conditions (rapid vs. slow CORT) failed to reach significance ( $F(1,17) = 3.12$ ,  $p = 0.095$ ). Therefore, the effect of rapid corticosteroid actions on hippocampal activation remains to be resolved.

To investigate whether CORT also affected neural processes underlying memory formation we tested for interaction effects between drug and subsequent memory. No such interaction effects were found. Also, the observed arousal effects did not interact with drug administration.



**Figure 14.** Effects of hydrocortisone (CORT) administration on hippocampal activity. The slow effects of corticosteroids reduced activity in the hippocampus bilaterally (anatomically defined), whereas corticosteroids' rapid effects did not have such an effect. Baseline represents activity under placebo conditions. \*:  $p < 0.05$ . Error bars represent S.E.M.

## DISCUSSION

In this study, we targeted time-specific effects of corticosteroids on human memory formation by administering 20 mg hydrocortisone orally at two different time points prior to a memory encoding task executed during fMRI scanning. In line with previous animal studies, we found that corticosteroids affect neural processing in brain regions involved in memory formation in a dynamically changing manner. Specifically, corticosteroids' slow effects inhibited hippocampal and prefrontal processing, whereas corticosteroids' rapid actions did not show such an effect.

Previous work in animals has indicated that corticosteroids exert both rapid, non-genomic, and slow, genomic effects (Karst et al. 2005; Pavlides et al. 1995; Wiegert et al. 2005). Here, we aimed to dissociate these two effects experimentally by administering 20 mg of hydrocortisone at either 30 or 180 minutes prior to the memory task. The timing of the rapid corticosteroid condition was based on previous studies revealing 1) elevated salivary cortisol levels in humans within 15 min after hydrocortisone intake (van Stegeren et al. 2010), 2) highly significant covariation between salivary and free plasma cortisol levels following administration (Tunn et al. 1992), 3) a time-delay between rodent peak plasma and brain levels of approximately 20 min (Droste et al. 2008), and 4) most prominent rapid, effects with corticosteroids administered directly to hippocampal slices (Karst et al. 2005). The slow effects of corticosteroids are not expected to start

earlier than approximately 90 min after corticosteroid administration, and last for hours (Joëls and de Kloet 1992; Joëls and de Kloet 1994; Joëls et al. 2003). We based the timing for targeting these effects on previous work showing suppressed LTP (Pavlidis et al. 1995; Wiegert et al. 2005), and strongest corticosteroid effects on hippocampal gene expression at this time-delay (Morsink et al. 2006). Thus, administration of hydrocortisone at either 30 or 180 minutes prior to scanning allowed us to disentangle most optimally the rapid and slow corticosteroid effects, respectively. Previous animal work on the genomic effects of corticosteroids showed that corticosteroid exposure suppresses hippocampal firing and LTP (Pavlidis et al. 1995; Wiegert et al. 2005), presumably by modulating expression of over 200 genes (Datson et al. 2001) involved in many different cellular processes. Here we show that, in line with this animal work, the slow corticosteroid effects result in inhibition of human hippocampal processing. Most imaging studies on corticosteroid effects have found similar MTL down-regulation by corticosteroid administration (de Quervain et al. 2003; Oei et al. 2007; van Stegeren et al. 2010), but lack time-specificity of these corticosteroid effects. However, one very recent study (Lovallo et al. 2010) reports on rapidly decreased hippocampal and amygdala activity due to the immediate (thus presumably non-genomic) effects of corticosteroids, using i.v. administration of hydrocortisone immediately followed by fMRI-scanning. The apparent discrepancy with our own findings (i.e., no effects at 30-75 min post hydrocortisone intake, but a decrease at 180-195 min post intake) could possibly be explained by differences in experimental setup; whereas Lovallo et al. investigated the effects of cortisol on resting BOLD signal in the brain (i.e., assessing a tonic state), we asked participants to memorize 160 complex pictures, and measured the brain responses to these stimuli (i.e., assessing phasic responses). Tonic and phasic brain responses may be altered differentially by corticosteroids, as is seen for other stress hormones (Valentino and Van Bockstaele 2008; Vijayraghavan et al. 2007), and might depend on the participants behavioral state (Makara and Haller 2001; Roozendaal 2002). Moreover, our results add to these findings in showing that corticosteroids' influence on hippocampal activity remains discernable even when they are out of circulation.

Although previous animal studies indicated that corticosteroids' rapid actions enhance hippocampal excitability (Karst et al. 2005) and LTP (Wiegert et al. 2006), we did not observe any rapid corticosteroid effects on hippocampal processing. One possible explanation for this null finding is that corticosteroids' rapid effects manifest themselves by interacting with concurrent noradrenergic activation (Roozendaal et al. 2006c). Although corticosteroids' rapid effects were capable of increasing LTP after mild tetanization in the CA1 region of the hippocampus (Wiegert et al. 2006), concurrent noradrenergic stimulation was necessary to establish this effect in hippocampus' dentate gyrus (Pu et al. 2007). For their augmenting effects on memory consolidation, corticosteroids also critically depend on noradrenergic activation (Roozendaal et al. 2006c). We tried to induce this activation by showing highly aversive pictures, but this effect might have been too subtle compared to a truly stressful event. Nevertheless, we show that corticosteroids' rapid, putatively non-genomic actions by themselves are not sufficient to amplify human hippocampal processing.

Besides affecting hippocampal processing, the slow effects of corticosteroids clearly down-regulated activity of the prefrontal cortex (PFC) in a time-specific manner. Time-specific effects on rodent prefrontal cortex function have been reported before for stress (Jackson and Moghaddam 2006); with acute stress producing immediate inhibition of PFC functioning, followed by subsequent recovery. Our findings in humans suggest that corticosteroids' rapid effects on their own are not able to induce such inhibition. Instead, other stress-related neuromodulators, such as norepinephrine and dopamine (Arnsten 2009), might cause this stress-induced impairment of PFC function, and their effects might potentially be amplified by corticosteroids' rapid actions (van Stegeren et al. 2010). Corticosteroids slow, putatively genomic effects did down-regulate the PFC. This novel finding is in line with previous studies on chronic stress, in which continuous (genomic) corticosteroid actions can be inferred, inducing both structural abnormalities (Liston et al. 2006) and functional disruption in the prefrontal cortex (Liston et al. 2006, 2009).

The PFC has traditionally been associated with cognitive control processes, but its role in memory and interaction with the MTL is just as crucial (Fernández and Tendolkar 2001). The PFC and MTL contribute in different ways to the process of memory encoding, and their interaction is vital for successful memory in order to provide discrete and elaborated representations that fit long-term storage (Fernández and Tendolkar 2001). Specifically, the region affected by corticosteroids in this study comprises parts of BA9, 45, 46 and 48 (Table 5). Whereas the exact function of BA48 - the retrosubicular area, located on the medial surface of the temporal lobe - remains unclear, all other regions have been implicated in memory processing. BA9 and 46 roughly correspond with the dorsolateral prefrontal cortex (DLPFC), which has traditionally been associated with its role in sustaining attention and working memory processing (WM). More recently, DLPFC has also been shown to promote long term memory formation through its role in WM-organization (Blumenfeld and Ranganath 2006), memory maintenance (Leung et al. 2002), and associative memory processing (Murray and Ranganath 2007). BA45 on the other hand has been typically associated with verbal processing, and has been implicated especially in intentional encoding paradigms (Braver et al. 2001) in which verbal elaboration has been shown to be an effective encoding strategy, predicting individual differences in memory performance (Kirchhoff and Buckner 2006). Also in this study we find greater activity in the inferior-middle frontal gyrus (BA9, 45, and 48) during the processing of items that are subsequently remembered compared to those later forgotten, implicating this region in memory formation (Table 4). Therefore, the observed down-regulation of both the hippocampus and the PFC indicates reduced processing due to the slow effects of corticosteroids.

Although such suppression of memory related areas by the slow effects of corticosteroids does not seem to be beneficial at first sight, since it could be related to impaired memory for events following a stressful experience, one could speculate that it might actually aid memory for the stressful experience by reducing retroactive interference into the initial memory trace. Retroactive interference is assumed to be a major cause of forgetting. Forgetting can be induced

by any subsequent task (Dewar et al. 2007), and has been shown to be reduced by preventing new learning (Sangha et al. 2005). Therefore, the suppression of memory related areas might actually protect against the forgetting of the stressful event by reducing retroactive interference.

Some limitations should be considered. First of all, we cannot claim that the peak cortisol levels in the rapid and slow CORT condition are the same. Figure 10 shows the salivary cortisol curves with cortisol levels peaking either during (rapid CORT condition) or 120 min prior to the scanning session. However, peak salivary cortisol levels in the rapid CORT condition seem lower than those induced in the slow CORT condition, although the dose of hydrocortisone administration was exactly the same. Possibly, cortisol-binding globulin (CBG) levels were higher in the rapid than in the slow CORT condition. Approximately 95% of total cortisol is bound to carrier proteins, of which 80–90% to CBG (Lewis et al. 2005). The measured levels in saliva represent the remaining cortisol that is unbound and free to diffuse across cell membranes and bind to intracellular glucocorticoid and mineralocorticoid receptors, and thus highly dependent on the level of carrier proteins present. Reports on the circadian variations in CBG level are somewhat conflicting (Droste et al. 2009; Hsu and Kuhn 1988; Lewis et al. 2006). Given that CBG binding affinity is temperature dependent (Henley and Lightman 2011), one would actually expect lower binding in the later afternoon (in the rapid CORT condition), i.e. the opposite of what we observed. Alternatively, free cortisol levels might have been influenced by circadian variations in  $11\beta$ -steroid dehydrogenase 1 efficacy (Veniant et al. 2009), which could indeed lead to lower peak levels. However, the most likely explanation for the difference in peak levels is that we might have missed the peak in salivary cortisol levels in the rapid CORT condition that is supposedly occurring 1 hour post-administration (as seen for the slow CORT condition). Practical reasons restrained us from taking a saliva sample at that exact same time point, which is half way the encoding session, when subjects are in the scanner; chewing on the cotton swap might induce head movement and require new realignment for the second half of the session (and thereby require more time) and disturb the encoding process. This is supported by the fact that when the saliva samples taken in both drug conditions were time-locked to the time of drug intake, they seemed to be comparable. IV-injection of hydrocortisone combined with regular blood sampling might have resolved this issue and also have increased the time-specificity of corticosteroid exposure to the brain. However, injections in general are known to induce stress in participants; a factor we would like to circumvent since we were specifically interested in the effects of corticosteroids. Secondly, although we clearly found time-dependent effects of corticosteroid application on neural responses in brain regions associated with memory encoding, we did not find a modulation of the subsequent memory effect in these regions (i.e., the difference in brain activation during the processing of subsequently remembered and forgotten items), nor a main effect of corticosteroids on memory performance. Significant effects on memory performance have been reported previously (Abercrombie et al. 2003; Buchanan and Lovullo 2001; Kuhlmann and Wolf 2006; Maheu et al. 2004; van Stegeren et al. 2010) and were also targeted in this study. One could speculate about the reason why we did not observe any of these effects. Most likely, specific

properties of the study design have contributed.

Firstly, the intentional learning instruction might have led to an elaborate processing strategy for all items overriding or reducing some basal differential neuromodulatory effects that could have affected the difference between later remembered and later forgotten items (Kensinger et al. 2005; Talmi et al. 2008). This might explain the absence of a corticosteroid (main) effect on memory performance that has been observed previously in incidental encoding paradigms (Abercrombie et al. 2003; Buchanan and Lovallo 2001; Kuhlmann and Wolf 2006; Maheu et al. 2004; van Stegeren et al. 2010). Moreover, despite the counter-balancing, the repeated testing could result in session order effects interacting with those of corticosteroids, as was seen in a recent study (Wirth et al. 2011). However, a crossover design with repeated testing requires an intentional instruction as the participants would expect a memory test after the initial session. The only alternative design to circumvent this intentional encoding instruction would have been the use of a between subjects design, but this has other disadvantages, such as decreased power by introducing between-subject variance. Moreover, incidental encoding would most likely have resulted in decreased memory performance because intentional encoding ensures deeper encoding by e.g. conscious semantic encoding strategies (Braver et al. 2001; Kirchhoff and Buckner 2006), increased motivation or elevated attention to the exact details of the encoded information. For fMRI analysis proper performance was required since a sufficient number of remembered neutral and aversive pictures were required. Using a recognition memory paradigm could have been an alternative approach, but recall measures provide a cleaner measure of episodic memory retrieval than recognition memory, which can be confounded by familiarity judgments, and seem to be more sensitive to corticosteroid-modulation (Buchanan and Lovallo 2001).

A second explanation might be a lack of power of our neuroimaging study in comparison to behavioral studies, which have tested larger groups of subjects (Abercrombie et al. 2003; Buchanan and Lovallo 2001; Kuhlmann and Wolf 2006; Maheu et al. 2004). Since brain activity is a more sensitive measure than behavioral output, which is the consequence of many parallel neural operations, regional differences in brain activity are more easily detected with smaller samples. However, these samples offer little power to observe behavioral effects. A third explanation for the absence of a behavioral effect might be corticosteroids' dependence on noradrenergic activation, which naturally joins corticosteroid release during exposure to stress. Since corticosteroids' rapid effects on hippocampal activity might depend on noradrenergic activation (Pu et al. 2007), the same might be true to corticosteroids' facilitating effects on memory formation under conditions of stress (Abercrombie et al. 2006). Moreover, previous animal work has shown that corticosteroids critically depend on noradrenergic activation for their augmenting effects on memory consolidation as well (Roozendaal et al. 2006c). Therefore, corticosteroids' delayed genomic effects might also depend on noradrenergic activation in preserving (by reducing retroactive interference) what was earlier encoded under stressful conditions. The fact that also corticosteroid's slow effects are modulated by noradrenergic activation is supported by a recent study that shows that gene binding of the GR is targeted to preexisting foci of accessible chromatin (John et al. 2011). Because of this dependence of GR-binding on preexisting chromatin

architecture, stress or arousal induced alterations in chromatin structure might modulate these effects. Previous research in rodents has indicated that stressful challenges (e.g. forced swimming (Bilang-Bleuel et al. 2005), novelty (Chandramohan et al. 2007), and fear conditioning (Chwang et al. 2006; Gupta et al. 2010) evoke such post-translational changes in dentate gyrus neurons. The rapid effects of corticosteroids were also shown to play a role in establishing the observed epigenetic modifications (histone modifications and DNA (de-)methylation) and conformational changes in the chromatin. These effects were mediated by GRs interacting with the NMDA-receptor activated ERK MAPK pathway in a rapid, non-genomic fashion (Trollope et al. 2012). This suggests that corticosteroids' slow genomic effects might be modulated by earlier rapidly induced changes by corticosteroid signaling and concurrent noradrenergic activation. Thus, both the rapid and slow effects of corticosteroids by themselves may not be sufficient to result in clear mnemonic effects. Although we tried to induce sufficient noradrenergic activation by showing highly aversive pictures to the participants, this effect might have been too subtle to generate the necessary interactions with corticosteroids.

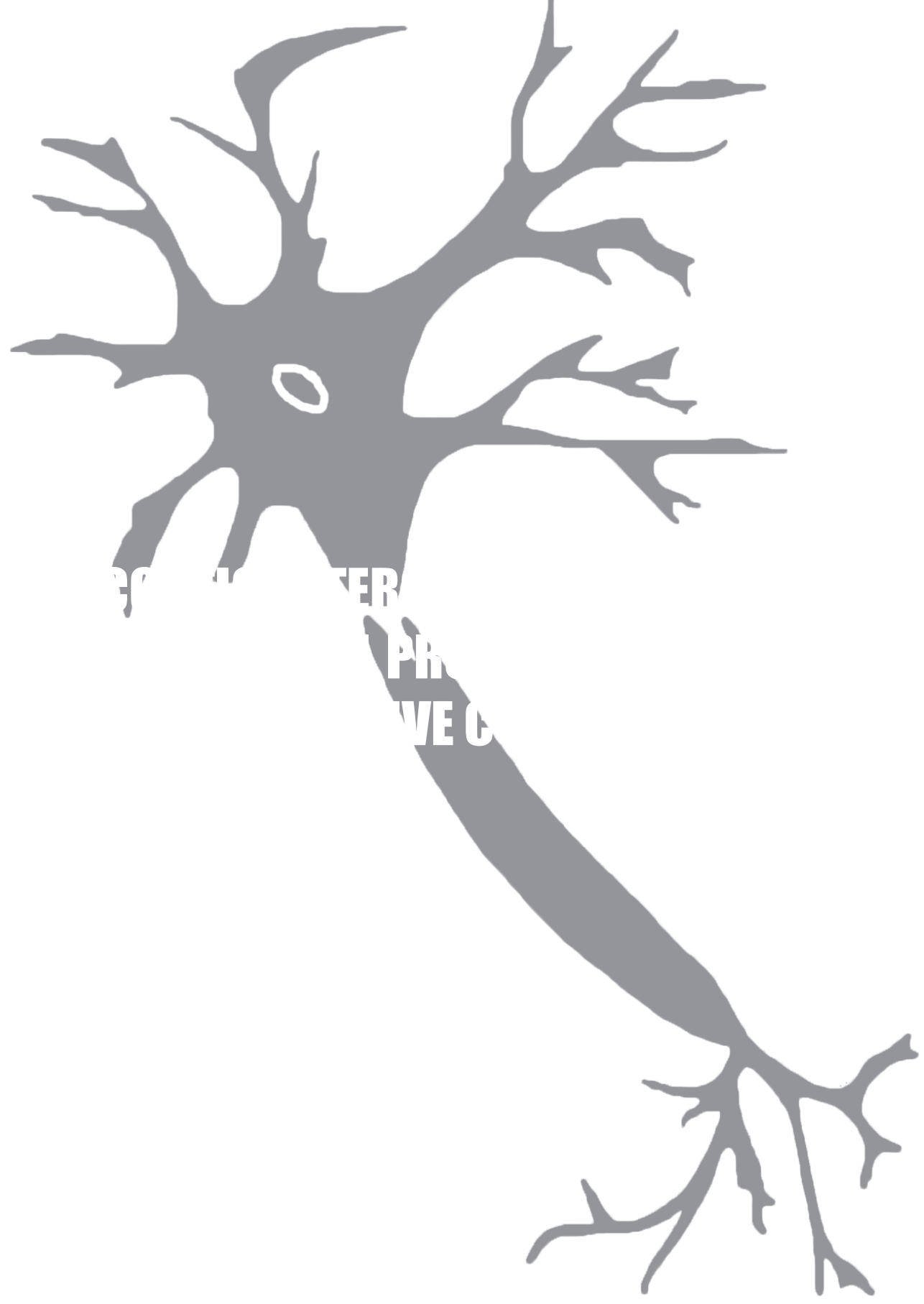
A final limitation to this study is that it investigated men only, thus the obtained results cannot be readily generalized to women. Hydrocortisone administration has been shown to result in differential effects between women and men, both in behavior (Andreano and Cahill 2006; Bohnke et al. 2010) and brain activation (Merz et al. 2010; Stark et al. 2006). Moreover, the hippocampus of women displays a more distinct affinity for corticosteroids than that of men (Madeira and Lieberman 1995), which might contribute to different effects on exposure to corticosteroids during memory formation. Although important, sex-differences were beyond the scope of this initial study, which is why we opted to recruit male subjects only, allowing easier comparison with an earlier study in stressed individuals (Henckens et al. 2009).

In conclusion, this study is first in showing that corticosteroids affect neural processing in brain regions involved in human memory formation in a time-dependent manner. Specifically, corticosteroid's slow, putatively genomic effects reduced activity in hippocampus and prefrontal cortex, whereas no changes were observed due to corticosteroid's rapid actions. Down-regulation of these memory related brain regions might minimize subsequent interference into the initial memory trace by post-stress experiences, and therefore aid consolidation of the stressful event most optimally. Thus, we provide an initial mechanistic account of how corticosteroids affect memory in humans.

## Acknowledgements

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ERGOTANERGIN  
PREVENTS  
SEROTONIN-INDUCED  
Vasoconstriction



**Time-dependent effects of  
corticosteroids on human  
amygdala processing**

**3.1**

**Marloes J.A.G. Henckens, Guido A. van Wingen,  
Marian Joëls, and Guillén Fernández**

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# 3.1

## ABSTRACT

Acute stress is associated with a sensitized amygdala. Corticosteroids, released in response to stress, are suggested to restore homeostasis by normalizing/desensitizing brain processing in the aftermath of stress. Here, we investigated the effects of corticosteroids on amygdala processing using fMRI. Since corticosteroids exert rapid non-genomic and slow genomic effects, we administered hydrocortisone either 75 min (rapid effects) or 285 min (slow effects) prior to scanning in a randomized, double-blind, placebo-controlled design. Seventy-two healthy males were scanned while viewing faces morphing from a neutral facial expression into fearful or happy expressions. Imaging results revealed that hydrocortisone desensitizes amygdala responsivity rapidly, while it selectively normalizes responses to negative stimuli slowly. Psycho-physiological interaction analyses suggested that this slow normalization is related to an altered coupling of the amygdala with the medial prefrontal cortex. These results reveal a temporarily fine-tuned mechanism critical for avoiding amygdala overshoot during stress and enabling adequate recovery thereafter.

## INTRODUCTION

It is of vital importance to an organism to respond adequately to potential threats during the exposure to a stressful experience, but also to subsequently recover when the threat has subsided. The immediate central release of norepinephrine (NE) during the initial phase of the stress response is known to induce a surge of vigilance, which optimizes the detection and assessment of these threats by prioritizing sensory processing (de Kloet et al. 2005) and activating the key modulator of vigilance and emotional processing in the brain; the amygdala (Phelps and LeDoux 2005; van Marle et al. 2009). Whereas this amygdala-mediated hypervigilant state of processing is highly beneficial during an initial fight-or-flight response, it may become maladaptive and culminate in mental diseases such as depression or post-traumatic stress disorder (PTSD) if this sensitization is not properly controlled (McEwen 2004; de Kloet et al. 2005).

Corticosteroids, released at a slightly slower time-scale in response to stress, have been suggested to be crucial factors in this regulation of the stress response (de Kloet et al. 2005). They restore homeostasis by diverting energy supply to challenged tissues and control the excitability of neuronal networks (de Kloet et al. 1999). Therefore, it can be hypothesized that they regulate amygdala activation and thus normalize vigilance in the aftermath of stress. Initial evidence for such a regulatory role of corticosteroids was derived from animal studies showing that corticosteroids induce anxiolytic effects in rodents (File et al. 1979; Andreatini and Leite 1994). Corticosteroid administration resulted in more explorative and socially interactive behaviour in rats, which was the exact opposite effect of acute stress. Recent studies have extended these findings to humans (Soravia et al. 2006; Het and Wolf 2007; Putman et al. 2007). Remarkably, these anxiolytic effects occur relatively instantly. This goes against the general assumption that the normalizing effects occur gradually by a process involving gene-transcription (de Kloet et al. 2005), but suggests that rapid non-genomic effects are involved as well.

To elucidate the role of corticosteroids in vigilance regulation, we targeted both the rapid non-genomic, and the slow genomic effects of corticosteroids on amygdala function. To assess the dynamic corticosteroid effects over time, we used a randomized, double-blind, placebo-controlled design, in which healthy male participants received either 10 mg hydrocortisone at 75 min (targeting the rapid effects) or 285 min (targeting the slow effects), or placebo prior to a task probing amygdala reactivity (van Marle et al. 2009) during functional magnetic resonance imaging (fMRI). Timing of hydrocortisone administration was based on animal work, targeting the non-genomic and genomic effects of corticosteroids. The task consisted of passive viewing of photographed faces morphing from a neutral expression into a fearful or happy facial one (Fig. 15), allowing us to test whether the normalization is specific for certain emotional input. Additionally, we used functional connectivity analyses to test whether corticosteroids affect amygdala coupling to brain regions involved in its control.

## MATERIALS & METHODS

### Participants

Seventy-two young (age range 18-29, median 21), right-handed, healthy male volunteers gave informed consent to participate in the study. In order to ensure stable effects of hydrocortisone over all participants, women were excluded from participation. Women are known to display different HPA-axis activity than men, exhibiting smaller and more variable cortisol responses to stress (Kajantie and Phillips 2006), depending on menstrual cycle phase and use of hormonal contraceptives (Kirschbaum et al. 1999; Bouma et al. 2009). Furthermore, individuals who met any of the following criteria were excluded from participation: history of head injury, autonomic failure, history of or current psychiatric, neurological, or endocrine disorders, current periodontitis, acute inflammatory disease, acute peptic or duodenal ulcers, regular use of corticosteroids, treatment with psychotropic medications, narcotics, beta-blockers, steroids, or any other medication that affects central nervous system or endocrine systems, medical illness within the three weeks prior to testing, self reported mental or substance use disorder, daily tobacco or alcohol use, regular night shift work, or current stressful episode or major life event. Moreover, volunteers with high scores on depression (score above 8 on the Beck Depression Inventory; Beck et al. 2002) were excluded from participation. Four participants were excluded from analyses because they displayed either abnormal basal salivary cortisol levels ( $> 3$  standard deviations above mean; 1 participant), or showed no elevation in salivary cortisol level in response to CORT intake, which means we ended up with 23 men in the placebo group, 23 in the slow CORT group, and 22 in the rapid CORT group. The study was approved by the local ethics committee (CMO region Arnhem-Nijmegen, Netherlands) and in accordance with the declaration of Helsinki.

### Study design

Participants were scanned in a randomized, double-blind, placebo-controlled, parallel group design. To target the time-differential effects of hydrocortisone (CORT), participants were divided over three groups, receiving 10 mg CORT either 75 min (rapid CORT effects), 285 min (slow CORT effects), or placebo prior to viewing an emotional processing task in the MRI-scanner. Physiological (cortisol level) and psychological (mood and attention) indices were measured to confirm cortisol level manipulation without additional side effects.

### Procedure

**Prior to arrival.** Prior to inclusion all eligible participants received an extensive information brochure, listing all in- and exclusion criteria and explaining the setup of the experiment. If criteria were met (according to the participant's own insights), an appointment was made. To minimize differences in baseline cortisol levels we instructed participants not to use any recreational drugs for three days and to refrain from drinking alcohol, exercising, and smoking for 24 h prior to the appointment. Furthermore, participants were requested not to brush their teeth, floss, or eat and

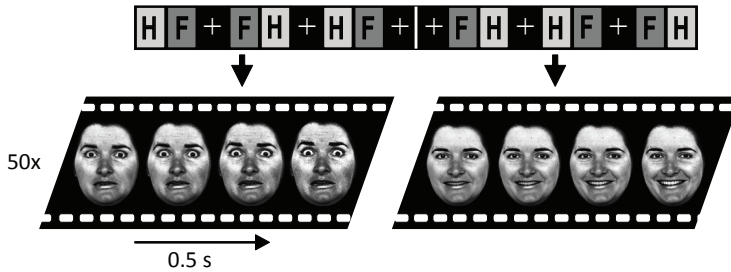
drink anything but water for one hour prior to the session, enabling adequate saliva sampling for cortisol assessment. They were asked to take a light lunch and do so no later than one hour before arrival; their lunch could not contain any citrus products, coffee, tea, milk or sweets (Maheu et al. 2005). Throughout the entire study period, participants were only given water to drink, except for a scheduled lunch at 135 min after arrival. To reduce the impact of diurnal variation in cortisol levels, all testing was performed in the afternoon, between 12:00 h ( $\pm 30$  min) and 18:00 h ( $\pm 30$  min), when hormone levels are relatively stable.

**Arrival.** Upon arrival, participants received an information brochure about the procedure, they gave written informed consent, and completed an intake questionnaire to ensure that in- and exclusion criteria were met. 30 min after arrival a first saliva sample was taken, followed by another one 15 min later, in order to measure a reliable baseline level. Participants were then asked to complete the NEO-FFI Personality Inventory (Costa and McCrae 1992), the Spielberger Trait Anxiety Inventory (trait anxiety) (van der Ploeg 1980, 1981) and a first Profile of Mood States (POMS) questionnaire (Reddon et al. 1985; Wald and Mellenbergh 1990; de Groot 1992). Immediately after the second saliva sample (at  $t = 45$  min) participants received the first capsule, containing either 10 mg CORT or placebo. During the entire period ( $\sim 3.5$  h) prior to scanning, the participants had to wait in a quiet room where they were free to conduct any activities except for anything potentially arousing (e.g. video games). At 255 min after arrival participants were asked to complete a second POMS questionnaire and received the second capsule. Both drug capsules, containing either 10 mg CORT or placebo (cellulose), were administered orally. This dose is known to elevate salivary cortisol levels to high-stress levels (Kirschbaum et al. 1996; Groschl et al. 2002; Tops et al. 2003). Depending on the group to which participants were (randomly) assigned they received either; the 1st capsule containing placebo, the 2nd containing placebo (placebo condition); the 1st capsule placebo, the 2nd CORT (rapid CORT condition); or the 1st capsule CORT, the 2nd placebo (slow CORT condition).

**Scanning.** At about 4.5 h after arrival participants were taken to the scanner room and the procedures were explained. Participants lay supine in the scanner and viewed the screen through a mirror positioned on the head coil. They were asked to lie as still as possible, keep their eyes open, and look directly and continuously at the center of the screen in front of them.

**Dynamic Facial Expression Task.** The Dynamic Facial Expression Task (Fig. 15) started 75 min after administration of the 2nd capsule (at  $t = 330$  min, Fig. 16). In brief, participants were asked to passively view blocks of faces morphing dynamically into either a fearful or happy facial expression. The perceptual processing of emotional faces has been shown to robustly engage the amygdala (Vuilleumier and Pourtois 2007) and even more so with a dynamic rather than static presentation (Sato et al. 2004). Stimuli consisted of short 133-ms animation clips for each of 10 different faces (taken from a standardized set (Ekman and Friesen 1976) and equalized in luminance and contrast), showing a morphing sequence consisting of four frames (55%, 70%, 85%, and 100% emotional expression). Within a block, each of these morphing sequences was immediately followed by the morphing sequence of a different face, resulting in the presentation

of distinct faces every 0.5 second. An experimental session lasted 8 min and consisted of six blocks of each emotion (25 s, 50 morphing sequences each) and six blocks of fixation cross (25 s, baseline for analysis). Blocks were presented in a mirrored design avoiding covariation with linear drift, and adjacent blocks of the same emotion were avoided. Participants were asked to make a right index finger response on a button box whenever the fixation cross appeared, as a control for attention.



**Figure 15.** Experimental task. The Dynamic Facial Expression Task consisted of blocks of emotional faces dynamically morphing into overtly fearful (F) or happy (H) expressions.

## Physiological and psychological measures

**Saliva collection and analysis.** Cortisol levels were measured from saliva at ten time points: baseline measurements at the beginning of the experiment ( $t = 30, 45$  min), and eight samples ( $t = 75, 105, 135, 255, 275, 315, 345,$  and  $375$  min) to assess cortisol changes throughout the experiment. Saliva was collected using a commercially available collection device (Salivette®, Sarstedt, Germany). For each sample, the participant first placed the cotton swab provided in each Salivette tube in his mouth and chewed gently on it for 1 min to produce saliva. The swab was then placed back in the salivette tube, and the samples were stored in a freezer at  $-25^{\circ}\text{C}$  until assayed. Laboratory analyses were performed at the Department of Biopsychology, TU Dresden, Germany. After thawing, salivettes were centrifuged at 3,000 rpm for 5 min, which resulted in a clear supernatant of low viscosity. Salivary free cortisol concentrations were subsequently measured using a commercially available chemiluminescence-immuno-assay (CLIA) with high sensitivity of 0.16 ng/mL (IBL, Hamburg, Germany).

**Mood state.** Mood state was assessed using the Profile of Mood States (POMS) questionnaire (Reddon et al. 1985; Wald and Mellenbergh 1990; de Groot 1992) at three time points: at the beginning of the experiment ( $t = 30$  min), just prior to the intake of the 2nd capsule ( $t = 255$  min), and at the end of the experiment ( $t = 375$  min).

**Attention.** Average reaction times to appearance of the fixation cross were calculated to assess the participant's attentiveness.

## Physiological and psychological statistical analysis

Behavioral and physiological data were analyzed in SPSS 15.0 (SPSS, Inc. Chicago, IL, USA)

using repeated measured ANOVAs with emotion type (fearful vs. happy) as within subject factor and drug condition (placebo vs. rapid CORT vs. slow CORT) as between subject factor. The level of neuroticism (as assessed by the NEO-FFI Personality Inventory (Costa and McCrae, 1992)) was included as covariate. Due to the high levels of skewness and kurtosis of the POMS questionnaire (Reddon et al. 1985; Wald and Mellenbergh 1990; de Groot 1992), mood data were analyzed using non-parametric tests. Changes over time in mood state were assessed by Friedman tests, and Kruskal-Wallis tests were used to assess potential drug effects on mood. Alpha was set at 0.05 throughout.

## **MRI acquisition**

Participants were scanned by a Siemens (Erlangen, Germany) MAGNETOM Avanto 1.5 Tesla MRI scanner equipped with an 8-channel head coil. A series of blood oxygenation level dependent (BOLD)  $T_2^*$ -weighted gradient echo EPI images was acquired with the following parameters: TR = 2340 ms, TE = 35 ms, FA = 90°, 32 axial slices approximately aligned with AC-PC plane, slice matrix size = 64 x 64, slice thickness = 3.5 mm, slice gap = 0.35 mm, FOV = 212 x 212 mm<sup>2</sup>. Owing to its relatively short TE, this sequence yields optimal contrast-to-noise ratio in the medial temporal lobes (Stocker et al. 2006). High resolution anatomical images were acquired for individuals by a  $T_1$ -weighted 3D Magnetization-Prepared RAPid Gradient Echo (MP-RAGE) sequence, which employed the following parameters: TR = 2250 ms, TE = 2.95 ms, FA = 15°, orientation: sagittal, FOV = 256 x 256 mm<sup>2</sup>, voxel size = 1.0 mm isotropic.

## **fMRI data analysis**

Data were analyzed using Statistical Parametric Mapping software (SPM5; UCL, London). The first five EPI-volumes were discarded to allow for  $T_1$ -equilibration. Prior to analysis, the images were motion corrected using rigid body transformations and least sum of squares minimization. Subsequently, they were temporally adjusted to account for differences in sampling times across different slices. All functional images were then co-registered with the high-resolution  $T_1$ -weighted structural image using normalized mutual information maximization. The anatomical image was subsequently used to normalize all scans into MNI152 (Montreal Neurological Institute) space. All functional images were resampled to a voxel size of 2 mm isotropic. Finally, all images were smoothed with an isotropic 8-mm full-width-at-half-maximum Gaussian kernel in order to accommodate residual functional/anatomical variance between subjects.

Data were analyzed using a general linear model, in which blocks were modeled based on emotion type. Regressors were temporally convolved with the canonical hemodynamic response function of SPM5. The six covariates corresponding to the movement parameters obtained from the realignment procedure were also included in the model. To reduce unspecific differences between scan sessions, and to correct for any unspecific, global effects of drug intake on hemodynamic response instead of neuronal activation (Desjardins et al. 2001; Peeters and Van der Linden 2002), global normalization using proportional scaling was applied. Although this method might

induce certain artifacts when local effects are strong enough to contribute substantially to global signal changes (Junghofer et al. 2005), all critical comparisons in this study (those between drug conditions) remain valid since this potential problem is similarly present in all drug conditions. The single subject parameter estimates from each session and condition obtained from the first level analysis were included in subsequent random effects analyses. For the second level analysis a factorial ANOVA was used, with emotion type (fearful vs. happy) as within subject factor, drug condition (placebo vs. rapid CORT vs. slow CORT) as between subject factor, and level of neuroticism (as assessed by the NEO-FFI Personality Inventory (Costa and McCrae, 1992), known to influence amygdala activity (Haas et al. 2007) as covariate.

Given the abundance of glucocorticoid and mineralocorticoid receptors in both the amygdala (de Kloet 1991) and mPFC, and their involvement in emotional processing (Joëls et al. 2004; Ochsner and Gross 2005), these regions were considered regions of interest. Data concerning these *a priori* regions of interest were corrected for reduced search volumes through anatomical masks as defined by the WFU PickAtlas Tool (version 2.4). A threshold of  $p < 0.05$  whole brain corrected was applied to all other regions. Visualizations of activations were created in SPM5 by superimposing statistical parametric maps thresholded at  $p < 0.001$  uncorrected (unless specified otherwise), onto a canonical  $T_1$ -weighted image in standard MNI152 space.

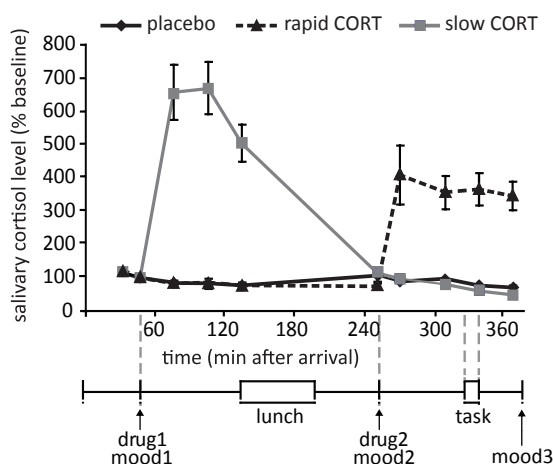
### **Functional connectivity analysis: Psycho–physiological interaction (PPI)**

Psycho–physiological interaction (PPI) analyses were used to assess how activity in a brain region of interest covaried with a source region in response to the experimental condition (Friston et al. 1997). We examined functional connectivity from the drug x emotion type interaction cluster in the left amygdala as a source region in order to investigate whether this interaction was related to altered connectivity due to CORT administration. In order to test this, we extracted the deconvolved time series from this cluster (thresholded at  $p < 0.001$  uncorrected). The PPI was calculated as the element by element product of this interaction cluster (the first eigenvariate from all voxels' time series) and a vector coding for the effect of task (the contrast 'faces > fixation') was entered. This product was subsequently re-convolved with the hemodynamic response function (HRF) and the resulting interaction term was entered as a regressor in a first level model together with the time series of the amygdalar interaction cluster and the vector coding for the task effect. The model was estimated and contrasts generated to test the effects of positive and negative PPIs. This analysis identified regions that display stronger functional connectivity with the amygdala during face processing. Next, the contrast images for the PPI effects were entered in a second level analysis for which we used a factorial ANOVA with drug condition (placebo vs. rapid CORT vs. slow CORT) as between subject factor, and neuroticism as covariate. Similar to the conventional fMRI analyses, regions of interest were corrected for reduced search regions through anatomical masks as defined by the WFU PickAtlas Tool (version 2.4). A threshold of  $p < 0.05$  whole brain corrected was applied to all other regions.

## RESULTS

### Endocrine and psychological measures

As expected, oral administration of 10 mg hydrocortisone increased salivary cortisol levels to those observed during severe stress (Morgan et al. 2000) (Fig. 16, see Table 7 for absolute values), which was evidenced by a significant time  $\times$  group interaction ( $F(18,114) = 28.43$ ,  $p < 0.001$ ). Increased levels were observed from 30 min post-administration onwards in both hydrocortisone administration conditions, and the levels remained elevated for at least 90 min. This resulted in elevated cortisol levels during fMRI scanning in the rapid hydrocortisone condition, whereas the levels in the slow condition had already returned to baseline.



**Figure 16.** Experimental design and salivary cortisol curves. Participants received two capsules (drug1 & drug2) containing either 10 mg hydrocortisone (CORT) or placebo at different time-points prior to the emotional processing task. Hydrocortisone intake significantly elevated salivary cortisol levels in both hydrocortisone administration conditions to levels observed during moderate-severe stress. mood: Profile of Mood States questionnaire (Reddon et al. 1985; Wald and Mellenbergh 1990; de Groot 1992). Error bars represent S.E.M.

Post-experiment debriefing revealed that participants were not able to identify the substance received. Furthermore, drug administration did not affect mood as assessed three times during the experiment using the Profile of Mood States (POMS) questionnaire (Table 7) (Reddon et al. 1985; Wald and Mellenbergh 1990; de Groot 1992). Although significant reductions in levels of depression scores (Friedman's ANOVA;  $\chi^2(2) = 10.53$ ,  $p = 0.005$ ), anger scores ( $\chi^2(2) = 9.09$ ,  $p = 0.011$ ), vigor scores ( $\chi^2(2) = 78.79$ ,  $p < 0.001$ ), and tension scores ( $\chi^2(2) = 21.88$ ,  $p < 0.001$ ) were observed over the course of the experiment, and levels of fatigue ( $\chi^2(2) = 51.18$ ,  $p < 0.001$ ) increased, none of these factors was affected by drug administration. Groups did not differ on any aspect of mood state at baseline, nor at any other time point during the experiment (all  $p > 0.05$ ). Changes in mood over time were also not affected by drug administration (all  $p > 0.05$ ). The drug administration did not affect participants' attentiveness, since average reaction

times to the appearance of the fixation cross were not different across groups ( $F(2) = 1.54$ , n.s.). Thus, differences in brain activity found after drug administration cannot readily be explained by changes in mood or attention.

**Table 7. Physiological and psychological measures**

		Placebo	Rapid CORT	Slow CORT
<b>Salivary cortisol level</b>				
	Sample 1 (t = 30 min)	9.58 (0.77)	11.44 (1.29)	9.67 (1.07)
	Sample 2 (t = 45 min)	8.49 (0.74)	9.62 (1.22)	8.37 (0.89)
	Sample 3 (t = 75 min)	6.29 (0.43)	7.42 (0.98)	46.01 (4.28)***
	Sample 4 (t = 105 min)	6.07 (0.60)	7.06 (0.85)	45.65 (2.72)***
	Sample 5 (t = 135 min)	5.50 (0.59)	6.01 (0.66)	35.06 (2.19)***
	Sample 6 (t = 255 min)	7.87 (0.75)	5.79 (0.42)	8.14 (0.62)
	Sample 7 (t = 275 min)	7.06 (0.64)	30.92 (6.03)***	6.97 (0.46)
	Sample 8 (t = 315 min)	7.48 (0.84)	25.80 (2.19)***	5.74 (0.42)
	Sample 9 (t = 345 min)	6.47 (0.69)	25.83 (2.01)***	4.34 (0.31)
	Sample 10 (t = 375 min)	5.60 (0.65)	24.58 (1.77)***	3.37 (0.22)
<b>Mood state</b>				
Depression score	1 (t = 30 min)	0.26 (0.13)	0.82 (0.37)	0.65 (0.32)
	2 (t = 255 min)	0.09 (0.06)	0.64 (0.35)	0.13 (0.07)
	3 (t = 375 min)	0.04 (0.04)	0.45 (0.24)	0.13 (0.10)
Anger score	1 (t = 30 min)	0.61 (0.23)	1.27 (0.40)	1.00 (0.43)
	2 (t = 255 min)	0.30 (0.19)	0.45 (0.23)	0.48 (0.20)
	3 (t = 375 min)	0.22 (0.18)	0.55 (0.24)	0.87 (0.32)
Fatigue score	1 (t = 30 min)	1.17 (0.30)	1.68 (0.50)	2.70 (0.61)
	2 (t = 255 min)	1.35 (0.44)	1.64 (0.51)	2.43 (0.56)
	3 (t = 375 min)	3.52 (0.67)	4.77 (0.62)	4.22 (0.71)
Vigor score	1 (t = 30 min)	12.65(0.79)	10.73 (0.80)	11.70 (0.90)
	2 (t = 255 min)	10.43 (0.68)	9.00 (0.81)	10.26 (0.96)
	3 (t = 375 min)	7.57 (0.88)	5.23 (0.79)	7.13 (0.91)
Tension score	1 (t = 30 min)	1.00 (0.27)	1.50 (0.29)	1.30 (0.46)
	2 (t = 255 min)	0.35 (0.13)	1.05 (0.34)	0.96 (0.30)
	3 (t = 375 min)	0.26 (0.16)	0.55 (0.19)	0.17 (0.10)

Mean values (S.E.M.). CORT: hydrocortisone, \*\*\*:  $p < 0.001$  compared to other drug conditions

## Brain activation

We first identified brain regions activated by viewing emotional faces in general. As expected, the face processing task activated the amygdala bilaterally. Furthermore, increased activity

was observed in a widespread visual processing network, including the primary visual cortex, extrastriate cortex, and occipitotemporal regions like the fusiform gyrus, as well as the inferior frontal gyrus, the angular and precentral gyrus (Table 8). Second, we looked into the effect of emotion type on brain activation. The left amygdala was the only brain region that displayed stronger responses towards fearful than happy faces, whereas the opposing contrast (happy > fearful) did not yield any significant differences in brain activity (Table 8).

**Table 8. Peak voxel and corresponding T values of significantly activated clusters in main effect**

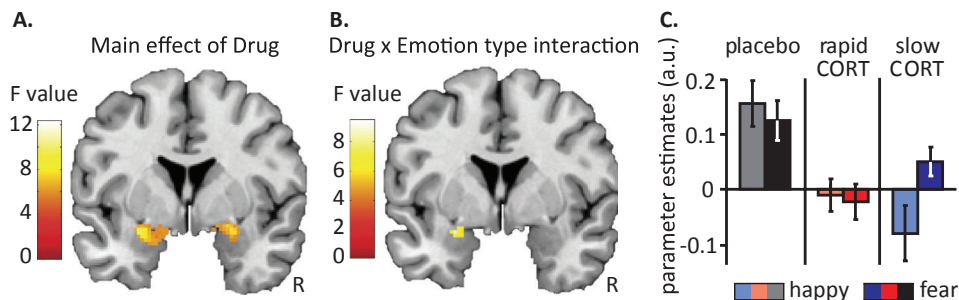
Region	MNI Coordinates			Peak
	x	y	z	T-value
<b>Main effect of task</b>				
<i>Task &gt; Fixation</i>				
Widespread visual processing network	14	-96	16	27.97***
Angular gyrus, R	30	-58	54	7.00***
Amygdala, L	-20	-6	-14	4.76+++
	22	-4	-14	4.89+++
Hippocampus, R	20	-6	-14	5.18*
Precentral gyrus, L	-46	-2	52	5.24*
Precentral gyrus, R	52	2	46	8.65***
Inferior Frontal Gyrus Tri, R	58	34	10	5.75***
<b>Main effect of emotion</b>				
<i>Fearful &gt; Happy</i>				
Medial prefrontal cortex	-4	56	42	4.12 <sup>+</sup>

MNI, Montreal Neurological Institute; BA, Brodmann Area; R, right; L, left; \*:  $p < 0.05$  whole brain corrected; \*\*\*:  $p < 0.001$  whole brain corrected; <sup>+</sup>:  $p < 0.05$  small volume corrected for region of interest; <sup>++</sup>:  $p < 0.01$  small volume corrected for region of interest; <sup>+++</sup>:  $p < 0.001$  small volume corrected for region of interest

To examine how corticosteroids affect emotional processing over time, we first identified those brain regions whose activity was modulated by any of the drug conditions. The main effect of drug revealed that hydrocortisone affected amygdala responsivity bilaterally ([ $x = -28$ ,  $y = -4$ ,  $z = -12$ ]  $F(2,129) = 9.64$ ,  $p(\text{corrected}) = 0.009$ ; [ $x = 26$ ,  $y = -4$ ,  $z = -12$ ]  $F(2,129) = 7.43$ ,  $p(\text{corrected}) = 0.048$ ). Further testing using directed t-tests showed that both hydrocortisone administration conditions significantly reduced responses in the amygdala, but did not significantly differ from each other. Thus, hydrocortisone administration in general reduced amygdala responsivity regardless of timing (Table 9, Fig. 17A).

Next, we assessed whether this corticosteroid modulation of brain activity was emotion specific, and tested for an interaction between drug condition and emotion type. This analysis revealed a significant interaction in the left amygdala ([ $x = -26$ ,  $y = -4$ ,  $z = -12$ ]  $F(2,129) = 8.17$ ,  $p(\text{corrected}) = 0.028$ , Fig. 17B). Further testing showed that this interaction was caused by an emotion-specific

response of the amygdala in the slow hydrocortisone condition only. Whereas corticosteroids rapidly reduced amygdala responsivity towards all emotional input, the slow corticosteroid effects enabled responses to emotionally negative information, while responses to positive stimuli remained reduced, resulting in emotion-specificity of the amygdala response (Table 9, Fig. 17C).



**Figure 17.** Hydrocortisone affected amygdala responsivity in a time- and emotion-specific manner. **(A)** Main effect of hydrocortisone administration on activity in the amygdala ( $y = -4$ ). Hydrocortisone administration reduced amygdala responsivity to faces in general, regardless of the timing of administration. **(B)** Drug x Emotion type interaction in the amygdala ( $y = -4$ ). The effects of hydrocortisone administration depended on the emotion type. **(C)** Extracted parameter estimates from the anatomically defined bilateral amygdala revealed that the Drug x Emotion type interaction was driven by a larger emotion effect (fearful > happy) in the slow hydrocortisone condition. Error bars represent S.E.M. For visualization purposes both statistical parametric maps are thresholded at  $p < 0.005$  uncorrected. See Table 9 for statistical tests.

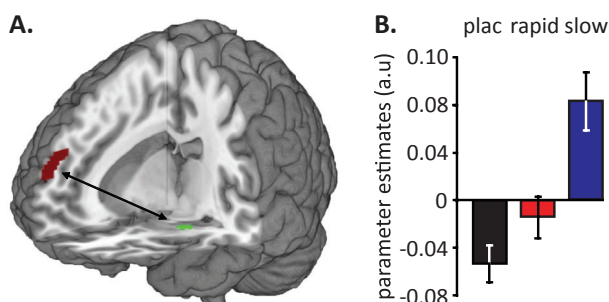
**Table 9.** Results for main effects of drug and drug x emotion interaction

Region	MNI Coordinates			Peak
	x	y	z	T-value
<b>Main effects of drug</b>				
<i>Slow CORT &lt; Placebo</i>				
Amygdala, L	-22	-8	-12	3.85 <sup>++</sup>
Amygdala, R	26	-4	-12	3.82 <sup>++</sup>
<i>Rapid CORT &lt; Placebo</i>				
Amygdala, L	-28	-4	-12	3.91 <sup>++</sup>
<b>Drug x emotion interaction</b>				
<i>Val(slow CORT) &gt; Val(placebo)</i>				
Amygdala, L	-26	-4	-12	3.69 <sup>++</sup>
Amygdala, R	24	-2	-12	3.24 <sup>+</sup>
<i>Val(slow CORT) &gt; Val(rapid CORT)</i>				
Amygdala, L	-26	-4	-12	3.31 <sup>+</sup>

Peak voxel and corresponding T values of significantly activated clusters. MNI, Montreal Neurological Institute; CORT, hydrocortisone; R, right; L, left; Val, emotional valence contrast: fearful > happy; <sup>+</sup>:  $p < 0.05$  small volume corrected for region of interest; <sup>++</sup>:  $p < 0.01$  small volume corrected for region of interest

## Brain connectivity

To investigate whether these corticosteroid effects on amygdala responsivity were related to altered amygdala coupling with brain regions involved in its regulation, we performed additional psycho-physiological interaction (PPI) analyses, seeding the drug by emotion type interaction in the amygdala. These analyses revealed that the slow effects of corticosteroids increased the coupling between the amygdala and the medial prefrontal cortex compared to the placebo condition (mPFC; [ $x = 10, y = 60, z = 24$ ]  $T(64) = 4.38$ ,  $p(\text{corrected}) = 0.032$ , Table 10, Fig. 18A). This effect was specific for the slow hydrocortisone condition, since it also differed from the rapid hydrocortisone condition ([ $x = 12, y = 38, z = 48$ ]  $T(64) = 4.26$ ,  $p(\text{corrected}) = 0.045$ , Table 10, Fig. 18B), the latter not being significantly different from placebo. Thus, the slow effects of corticosteroids induced both the emotion-specificity in amygdala responses and altered its connectivity to the mPFC.



**Figure 18.** The slow effects of corticosteroids strengthened connectivity between the amygdala and mPFC. (A) Psycho-physiological interaction (PPI) analyses showed that the slow effects of corticosteroids strengthened connectivity between the amygdala and a cluster within the mPFC. (B) Analysis of the parameter estimates of the observed mPFC cluster showed that this altered connectivity was specific to the slow hydrocortisone condition, since it significantly differed from that observed in the rapid hydrocortisone condition ( $T(43) = 3.22$ ,  $p = 0.002$ ). Error bars represent S.E.M. For visualization purposes the statistical parametric map is thresholded at  $p < 0.001$  uncorrected. See Table 10 for other statistical tests.

**Table 10.** Results psycho-physiological interaction analysis seeding the amygdala. Peak voxel and corresponding T values of significantly activated clusters in main effects of drug

Region	MNI Coordinates			Peak
	x	y	z	T-value
Main effects of drug				
Slow CORT > Placebo				
Medial prefrontal cortex, R	10	60	24	4.38 <sup>+</sup>
Slow CORT > Rapid CORT				
Medial prefrontal cortex, R	12	38	48	4.26 <sup>+</sup>

MNI, Montreal Neurological Institute; CORT, hydrocortisone; R, right; L, left; <sup>+</sup>:  $p < 0.05$  small volume corrected for region of interest

To test whether these two effects were actually associated rather than independent, we extracted the parameter estimates of both the emotion effect in amygdala responsivity and the amount of amygdala-mPFC coupling, and tested whether these measures were correlated across participants. Even though the PPI analysis was corrected for amygdala activity fluctuations within each participant, this analysis showed that these measures were positively correlated across participants ( $r = 0.223$ ). Although this correlation just failed to reach significance ( $p = 0.067$ ), this suggests that stronger amygdala-mPFC coupling was related to stronger emotion specific amygdala responses.

## DISCUSSION

In this study we targeted the time-specific effects of corticosteroids on human amygdala functioning by administering hydrocortisone at two different time points prior to an emotional processing task during fMRI scanning. We found that corticosteroids down-regulate amygdala responsivity to emotional stimuli in a time- and emotion-specific manner; whereas corticosteroids rapidly suppress amygdala responsivity towards all emotional stimuli, they only suppress responses to positive stimuli later on, while responses to negative emotional stimuli are normal again. This emotion-specific recovery of amygdala activity appears related to altered amygdala connectivity to the medial prefrontal cortex.

Previous work in animals has indicated that corticosteroids exert both rapid non-genomic and slow genomic effects that are functionally distinct (Joëls et al. 2006). At high concentrations, corticosteroids are shown to rapidly enhance hippocampal plasticity by binding to a mineralocorticoid receptor (MR) thought to reside in the plasma membrane, leading to an increase in glutamate release (Karst et al. 2005). At the same time, a corticosteroid-induced genomic cascade is initiated by the binding of primarily intracellular glucocorticoid receptors (GRs) that upon binding translocate to the nucleus where they function as transcription factors to modulate the expression of over 200 genes (Datson et al. 2001). These slow genomic effects of corticosteroids have been shown to inhibit hippocampal plasticity (Pavlidis et al. 1995; Wiegert et al. 2005). Here, we dissociated these two effects experimentally by administering 10 mg of hydrocortisone at either 75 or 285 minutes prior to the emotional processing task. The timing of the rapid corticosteroid condition was based on 1) previous studies in rodents revealing a delay between elevations in corticosteroid level in plasma versus brain (Droste et al. 2008) and 2) the observation in humans that salivary cortisol levels peak at one hour after intake (Abercrombie et al. 2003). Once in the brain, these non-genomic corticosteroid effects are rapid in onset and quickly reversible (Karst et al. 2005). The genomic effects of corticosteroids on the other hand generally do not start earlier than at least 3 hrs after exposure to high corticosteroid levels in vivo (Joëls et al. 2003; Morsink et al. 2006) and these effects last for hours (Joëls and de Kloet 1992, 1994; Joëls et al. 2003). Thus, administration of hydrocortisone at 75 minutes prior to scanning probably caused sufficiently high levels of the hormone in the brain to evoke rapid non-genomic

effects whereas this delay was much too short to allow development of gene-mediated events. Conversely, when hydrocortisone was applied at 285 min prior to testing, hormone levels were so low during the behavioral task that non-genomic actions are not likely to happen, yet allowed enough time for the gene-mediated actions to occur.

Here, we show that corticosteroids rapidly desensitize human amygdala responses to emotional stimuli. Corticosteroids may therefore be a crucial factor in terminating a critical feed-forward loop in the amygdala: Acute stress sensitizes the amygdala, and the amygdala boosts vigilance/anxiety and drives in turn the stress-response. This positive feed-forward loop constitutes a powerful mechanism leading to progressively augmented amygdala sensitization with repeated stress exposure. The fact that the HPA-axis is dysregulated in stress-related mental disorders such as depression and post-traumatic stress disorder (PTSD), but also that corticosteroids seem to be effective in preventing (Schelling et al. 2006) and treating (Aerni et al. 2004; de Quervain 2008) PTSD, may speak for their crucial role in interrupting this positive feed-forward loop.

The anxiolytic effects of corticosteroids observed in previous studies are in line with the proposed corticosteroid-induced desensitization and thus suppression of vigilance/anxiety. Behavioral studies in humans have shown that corticosteroids reduce the anxiety-driven selective attention to threat (Putman et al. 2007; van Peer et al. 2009), attenuate fear responses (Soravia et al. 2006), and protect mood during exposure to stressful situations (Het and Wolf, 2007). Here, we provide a mechanistic account for these observations, by showing that the rapid, non-genomic corticosteroid effects unspecifically desensitize the amygdala. This claim is supported by a previous study showing a tonic suppression of the acoustic startle reflex in humans, thought to be modulated by the amygdala, which was independent of emotional modulation (Buchanan and Lovullo 2001). Two possible, but not mutually exclusive, molecular mechanisms could underlie this corticosteroid-associated reduction in amygdala activation. First of all, corticosteroids might modulate amygdala activity in a direct manner by binding to its mineralocorticoid (MRs) and glucocorticoid receptors (GRs) (Sapolsky et al. 1983; Reul and de Kloet 1985). Corticosteroids have been shown to act in such direct manner in the hippocampus, where they rapidly increase neuronal excitability in a non-genomic fashion by binding to a low-affinity membrane MR (Karst et al. 2005; Olijslagers et al. 2008), and slowly impair hippocampal function by binding intracellular MRs and GRs. Corticosteroids could affect amygdala function in a similar manner, but supporting evidence for this idea is so far scarce (Karst et al. 2002; Duvarci and Pare 2007; Pu et al. 2009). Alternatively, the corticosteroid effects might be mediated by a reduction in brain levels of corticotrophin-releasing hormone (CRH). CRH is a coordinator of the central stress-response, and known to induce anxious behavior by activating the human amygdala both directly (Liang and Lee 1988) and indirectly by increasing locus ceruleus norepinephrine signaling (Valentino et al. 1983; Valentino and Foote 1988). Since CRH levels are known to be inhibited by the negative feedback actions of corticosteroids on the hypothalamus (Keller-Wood and Dallman 1984; Herman et al. 1996; Tasker 2006; Aguilera et al. 2007), corticosteroid induced reductions in circulating CRH levels

could also explain our findings. Thus, corticosteroids rapidly inhibit amygdala activity either by direct modulation or by reducing circulating CRH levels, and thereby protect the amygdala during stress from potential overshoot by the sensitizing actions of NE and CRH.

One might also argue that an altered mood state during scanning could underlie the observed changes in amygdala response. We cannot exclude this possibility since we did not assess mood just prior to or during scanning. However, mood state was assessed three times during the study and appeared not to be affected by either a history of elevation in cortisol levels (mood measure 2 and 3 for the slow CORT group (Fig. 16)), or an acutely elevated level (mood measure 3 rapid CORT group (Fig. 16)). Therefore, we consider it unlikely that mood during scanning was different between groups.

The slow effects of corticosteroids on the other hand normalized responses to negative input, while responses to positive input remained suppressed. Moreover, the induction of this emotion-specificity in the amygdala seemed to be related to increased coupling with the medial prefrontal cortex (mPFC). The mPFC is known to play a significant role in emotion regulation (Ochsner and Gross 2005), and suppresses the amygdala during the regulation of emotional responses to negative stimuli (Beauregard et al. 2001; Kompus et al. 2009). Further, the mPFC is known to play a critical role in the control over the hypothalamic-pituitary-adrenal (HPA) axis. The mPFC expresses high levels of glucocorticoid receptors (Diorio et al. 1993; Sanchez et al. 2000) and is a prominent target for the negative feedback control over the HPA-axis (Sullivan and Gratton 2002; Radley et al. 2009). Activation of the mPFC has been shown to reduce stress-induced salivary cortisol increases, but also amygdala activity and dispositional mood state (Kern et al. 2008). Here, we show that the connectivity between the amygdala and mPFC is strengthened by the slow, putatively genomic, actions of corticosteroids. Moreover, this strengthening seemed to enhance the preferential processing of negative over positive emotional stimuli. This suggests that the slow actions of corticosteroids ensure recovery of the rapid effects of corticosteroids on amygdala responses to negative input specifically by changing regulatory actions of the mPFC. This could entail a highly adaptive mechanism for survival, since it is most important to be capable to respond adequately to dangerous stimuli first.

Here we reveal an adaptive mechanism of time-dependent amygdala modulation by corticosteroids; corticosteroids' rapid non-genomic effects suppress overall amygdala activity in an unspecific manner, whereas corticosteroids' slow genomic actions up-regulate (i.e. normalize) responses to negative input specifically, by altered prefrontal control. In response to stress, corticosteroids thereby rapidly guard amygdala activation from potential overshoot by the sensitizing actions of NE and CRH, and normalize amygdala response later on, prioritizing negative emotional processing. Thus, corticosteroids control amygdala responsivity and vigilance/anxiety, and appear therefore as a crucial factor when the stress response has to be terminated adequately in the aftermath of traumatic experiences.

## **ACKNOWLEDGEMENTS**

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**Time-dependent effects of cortisol  
on selective attention  
and emotional interference:  
a functional MRI study**

**3.2**

**Marloes J.A.G. Henckens, Guido A. van Wingen,  
Marian Joëls, and Guillén Fernández**

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# 3.2

## ABSTRACT

Acute stress is known to induce a state of hypervigilance, allowing optimal detection of threats. Although one may benefit from sensitive sensory processing, it comes at the cost of unselective attention and increased distraction by irrelevant information. Corticosteroids, released in response to stress, have been shown to profoundly influence brain function in a time-dependent manner, causing rapid non-genomic and slow genomic effects. Here, we investigated how these time-dependent effects influence the neural mechanisms underlying selective attention and the inhibition of emotional distracters in humans. Implementing a randomized, double-blind, placebo-controlled design, 65 young healthy men received 10 mg hydrocortisone either 60 min (rapid effects) or 270 min (slow effects), or placebo prior to an emotional distraction task, consisting of color-naming of either neutral or aversive words. Overall, participants responded slower to aversive compared to neutral words, indicating emotional interference with selective attention. Importantly, the rapid effects of corticosteroids increased emotional interference, which was associated with reduced amygdala inhibition to aversive words. Moreover, they induced enhanced amygdala connectivity with frontoparietal brain regions, which may reflect increased influence of the amygdala on an executive network. The slow effects of corticosteroids acted on the neural correlates of sustained attention. They decreased overall activity in the cuneus, possibly indicating reduced bottom-up attentional processing, and disrupted amygdala connectivity to the insula, potentially reducing emotional interference. Altogether, these data suggest a time-specific corticosteroid modulation of attentive processing. Whereas high circulating corticosteroid levels acutely increase emotional interference, possibly facilitating the detection of threats, a history of elevation might promote sustained attention and thereby contribute to stress-recovery of cognitive function.

## INTRODUCTION

Stress has profound influence on the brain's attentional resources. When exposed to an acutely stressful situation, the brain shifts into a mode of hypervigilant processing in which the detection and assessment of potential threats is optimized by prioritized sensory processing (de Kloet et al. 2005; van Marle et al. 2009), and the amygdala, key modulator of vigilance and emotional processing in the brain (Phelps and LeDoux, 2005), is activated (van Marle et al. 2009). This surge in vigilance in immediate response to stress is thought to be mediated by the central release of norepinephrine (NE) by tonic activation of the locus coeruleus (LC) (Aston-Jones and Cohen 2005; Valentino and Van Bockstaele 2008; Cousijn et al. 2010). This state of hypervigilance is highly adaptive and enhances chances of survival during stressful situations, but it comes at the cost of specificity (van Marle et al. 2009), impaired selective attention (Tanji and Hoshi 2008; Henderson et al. 2012) and increased susceptibility to distraction (Skosnik et al. 2000; Braunstein-Bercovitz et al. 2001; Aston-Jones and Cohen 2005), resulting from impaired prefrontal cortex (PFC) processing underlying executive functioning (Arnsten 2009; Qin et al. 2009) and exhaustion of attentional resources (Sato et al. 2012). It might cumulate in stress-related disorders such as depression and post-traumatic stress disorder (PTSD), which are characterized by an attentional bias towards negative emotional information (Williams et al. 1996). Therefore, normalization of attentional processing some time after the stressful event is very important for well-being. Notably, these disorders are characterized by aberrant corticosteroid signaling (Yehuda et al. 2001).

Corticosteroids, released in response to stress as the end-product of the hypothalamic-pituitary-adrenal (HPA) axis, are well-known modulators of human cognition. The hormones exert their actions upon binding of the mineralocorticoid (MR) and glucocorticoid receptor (GR), which are abundantly expressed in the brain (Sapolsky et al. 1983; Reul and de Kloet 1985; de Kloet 1991). Recent research in rodents has indicated that corticosteroid-binding can induce both rapid non-genomic and slow genomic effects by acting on receptors that are respectively located in the plasma membrane and in the nucleus (Di et al. 2003; Karst et al. 2005; Wiegert et al. 2005). These distinct temporal pathways are thought to serve different functions (Joëls et al. 2006; Joëls et al. 2011). The rapid actions of corticosteroids on the one hand, have been suggested to work in concert with (and amplify) the effects of catecholamines (Rooszendaal et al. 2006c; Joëls and Baram, 2009) to optimize rapid adaptive behavior by relocating neural resources away from higher-order cognitive processing regions in the prefrontal cortex to the limbic structures (Diamond, 2007). Therefore, they might boost the effects of catecholamines on attentional processing, increasing emotional interference. The slow corticosteroid-induced genomic cascade is on the other hand thought to be responsible for the regulation of the stress response and the restoration of homeostasis in the aftermath of stress (de Kloet et al. 2005; Henckens et al. 2010, 2011). Thereby, the slow corticosteroid effects might contribute to the normalization of attentional processing in the aftermath of stress. However, these time-dependent effects of corticosteroids on

the neural substrates of selective attention have never been tested.

Here, we set out to investigate the time-dependent effects of corticosteroids on the neural correlates of selective attentional processing. In a randomized, double-blind, placebo-controlled design, 65 young healthy men received 10 mg hydrocortisone either 60 min (to target the rapid corticosteroid effects) or 270 min (slow corticosteroid effects), or placebo prior to functional MRI scanning. Selective attention was assessed by means of an emotional distraction task, in which participants were asked to identify the font color of neutral and highly aversive words as fast and accurate as they could (Mathews and MacLeod 1985; McKenna 1986). Proper selective attention is critical for task-execution, since it requires participants to focus on just one source of information for processing (i.e. font color) while ignoring competing information, including word meaning (e.g. emotion). It is well-known that under such competitive conditions, the presence of emotionally salient information disrupts the ability to attend selectively to the task-relevant information (Arnsten and Goldman-Rakic 1998; Dolcos and McCarthy 2006; Dolcos et al. 2011). Typically, this results in slower reaction times and lower accuracy for color naming of emotional words relative to neutral words, which serves as a measure of emotional interference. By measuring the corticosteroid effect on emotional interference induced by the emotional, attention-grabbing distracters (Bishop 2008; Wingenfeld et al. 2009), this task enabled us to assess corticosteroid effects on selective attention. Moreover, this task enabled us to assess corticosteroid effects on sustained attention, i.e. one's ability to maintain a consistent response during continuous (i.e. repetitive) task performance. In other words, it measures the ability to keep the selective attention maintained over time (McDowd 2007). Since sustained attention is required to complete any cognitively planned activity, here task execution, it could be assessed by analyzing overall task performance, regardless of the emotional valence of the words.

## **MATERIALS & METHODS**

### **Participants**

Seventy-two young (age range 18-29, median 21), right-handed, Dutch speaking, healthy male volunteers gave written informed consent to participate in the study. Women were excluded from participation, since previous research has indicated that women respond differently to hydrocortisone than men, both in behavior (Andreano and Cahill 2006; Bohnke et al. 2010) and brain activation (Stark et al. 2006; Merz et al. 2010). Moreover, their response to hydrocortisone is modulated by oral contraceptive use and varies over the menstrual cycle (Merz et al. 2011). Therefore, in order to reduce variance we here recruited the group with the most stable response to hydrocortisone. Furthermore, individuals who met any of the following criteria were excluded from participation: history of head injury, autonomic failure, history of or current psychiatric, neurological, or endocrine disorders, current periodontitis, acute inflammatory disease, acute peptic or duodenal ulcers, regular use of corticosteroids, treatment with psychotropic medications, narcotics, beta-blockers, steroids, or any other medication that affects central nervous system or

endocrine systems, medical illness within the three weeks prior to testing, self reported mental or substance use disorder, daily tobacco or alcohol use (or experienced inconvenience in refraining from these activities for three days), exercising at the professional level, regular night shift work, or current stressful episode or major life event. Four participants were excluded from analyses because of unreliable cortisol manipulation (abnormal basal cortisol levels (1 x placebo) or no elevation in salivary cortisol level in response to CORT intake (2 x rapid CORT, 1 x slow CORT)), and another three participants because of insufficient task performance (based on outlier analyses ( $> 3$  SD below average performance; 2 x placebo, 1 x slow CORT). Thus, the results comprise data of 21 men in the placebo group, and 22 men in the rapid CORT and 22 men in the slow CORT group. The study was approved by the local ethics committee (CMO region Arnhem-Nijmegen, Netherlands) and executed in accordance with the declaration of Helsinki.

## Study design

**Prior to arrival.** To minimize differences in baseline cortisol levels we instructed participants not to use any recreational drugs for three days and to refrain from drinking alcohol, exercising, and smoking for 24 h prior to the appointment. Furthermore, participants were requested not to brush their teeth, floss, or eat and drink anything but water for one hour prior to the session enabling adequate saliva sampling for cortisol assessment. They were asked to take a light lunch and do so no later than one hour before arrival; their lunch could not contain any citrus products, coffee, tea, milk or sweets (Maheu et al. 2005). Throughout the entire study period, participants were only given water to drink, except for a scheduled lunch at  $t = -180$  min.

**Arrival.** To reduce the impact of diurnal variation in cortisol levels, all testing was performed in the afternoon, between 12 P.M. ( $\pm 30$  min) and 6:00 P.M. ( $\pm 30$  min), when hormone levels are relatively stable. Upon arrival participants received an information brochure about the procedure, they gave informed consent, and completed an intake questionnaire to ensure that in- and exclusion criteria were met. 30 min after arrival, a first saliva sample was taken, followed by another one 15 min later, in order to measure a reliable baseline level. Participants were asked to complete a first Profile of Mood States (POMS) questionnaire (Reddon et al. 1985; Wald and Mellenbergh 1990; de Groot 1992), after which they briefly trained the emotional distraction task to ensure proper performance during scanning. Immediately after the second saliva sample (at  $t = -270$  min) participants received the first capsule. During the entire period ( $\sim 4$  h) prior to scanning, participants waited in a quiet room where they were free to conduct any activities except for anything potentially arousing (e.g. video games). At 60 min prior to the emotional distraction task participants were asked to complete another POMS questionnaire, and received the second capsule. Both drug capsules, containing either 10 mg CORT or placebo (cellulose), were administered orally. This dose is known to elevate salivary cortisol levels to moderate to high stress levels (Kirschbaum et al. 1996; Morgan et al. 2000; Tops et al. 2003), and has been shown to be successful in the induction of corticosteroid effects on declarative memory (Kirschbaum et al. 1996; Tops et al. 2003). Depending on the group to which the participant was

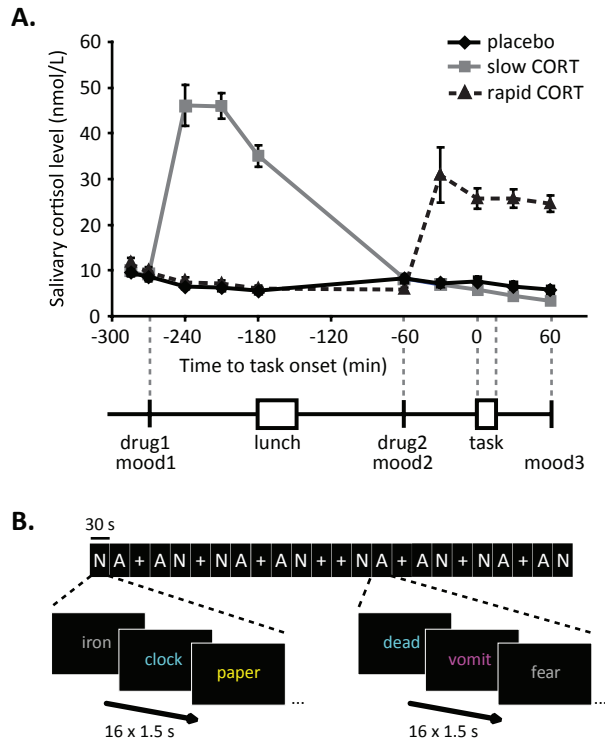
(randomly) assigned he received either; the 1st capsule containing placebo, the 2nd containing placebo (placebo group); the 1st capsule CORT, the 2nd placebo (slow CORT group); or the 1st capsule placebo, the 2nd CORT (rapid CORT group). The experiment described here was part of a larger study into the time-dependent effects of corticosteroids on emotional and cognitive brain function. Results on the other tasks have been reported elsewhere (Henckens et al. 2010, 2011, 2012b).

**Emotional interference task.** The emotional interference task started 60 min after administration of the second capsule (at  $t = 0$  min) (Fig. 19A). In brief, series of colored words were presented to the participants, and they were asked to press one of four buttons as fast as possible for the color in which the word was displayed. Words were presented either in blue, magenta, yellow or grey, which was counterbalanced across subjects, and colors were matched in luminosity. Colors were chosen for their distinctiveness, while any associations with go- or stop-signals (i.e. green and red) were excluded to prevent their confounding effects on reaction times, inducing increased variability between colors. Participants used both their index- and middle fingers to respond, ensuring proper fast responding.

Words belonged to one of two categories, neutral or aversive, and were selected for the emotional valence and arousal ratings of their translation in English in the Affective Norms for English Words (ANEW) database (Bradley and Lang 1999). Aversive words were selected for their high arousal and low valence, as rated on a 1-9 scale using the Self-Assessment Manikin (SAM) scales (Bradley and Lang, 1994), while neutral words were selected for their low arousal and neutral valence ratings. Subsequently, words were translated in Dutch and categories were matched on average word length (mean  $\pm$  S.E.M.;  $6.63 \pm 1.62$  (neutral),  $6.84 \pm 1.85$  (aversive)) and word form frequency ( $1006.56 \pm 103.77$  (neutral),  $920.60 \pm 88.97$  (aversive)), and lemma frequency ( $1528.63 \pm 154.16$  (neutral),  $1307.12 \pm 135.11$  (aversive)) based on the Dutch lexical database CELEX (Baayen et al. 1995). In total, 128 words of each category were selected. To confirm proper valence and arousal levels of these Dutch words, all participants were asked to rate the words one day after the experiment, using the Self-Assessment Manikin (SAM) scales (Bradley and Lang 1994). These ratings confirmed word categorization. The sets of aversive and neutral words differed on arousal (mean  $\pm$  S.E.M.;  $3.79 \pm 0.07$  (aversive),  $1.70 \pm 0.04$  (neutral),  $T(254) = 24.29$ ,  $p < 0.001$ ) and valence ( $3.13 \pm 0.06$  (aversive),  $5.31 \pm 0.04$  neutral),  $T(254) = -32.37$ ,  $p < 0.001$ ).

The total task lasted 12 min and consisted of eight blocks of each category (containing 16 words presented for 1.5 s, 0.15 s ISI, 3.6 s inter-block fixation), supplemented with eight fixation blocks. Words were presented in a pseudo-random color (immediate color repetition was not allowed) Blocks were presented in a mirrored design avoiding covariation with linear drift, and adjacent blocks of the same emotion were avoided (Fig. 19B) To ensure proper understanding and sufficient performance, participants had twice a short two-block practice of nonsense words (random letters); once earlier that day outside the MRI scanner (at  $t = -270$  min), and once inside the scanner immediately prior to the actual task ( $t = 0$  min) Since participants were instructed to respond as fast and as accurately as possible, task performance was assessed both in terms of

reaction times and error rates (Swick et al. 2002; Wagner et al. 2006; Weiss et al. 2007; Kertzman et al. 2010). Sustained attentional performance was defined by overall performance on the task combining both neutral and aversive trials, whereas selective attention (i.e. emotional interference) was assessed by contrasting performance between these trials (aversive vs. neutral). The session ended with a high resolution anatomical scan.



**Figure 19.** Salivary cortisol data and experimental design. (A) Participants received two capsules (drug1 and drug2) containing either 10 mg of hydrocortisone (CORT) or placebo at different time points before the emotional distraction task. Hydrocortisone intake significantly elevated salivary cortisol levels in both hydrocortisone administration groups to levels observed during moderate-to-severe stress (Morgan et al. 2000) (B) The emotional distraction task consisted of 30s-blocks of neutral (N) or aversive (A) words or fixation (+) Participants were requested to button press as fast as possible for the color in which the presented words were displayed. Mood: POMS questionnaire (Reddon et al. 1985; Wald and Mellenbergh, 1990; de Groot, 1992) Error bars represent SEM. N.B. In reality Dutch words were used, the words in Fig. 19B only serve an illustrative purpose.

## Physiological and psychological measures

**Saliva collection and analysis.** Cortisol levels were measured from saliva at ten time points: two baseline measurements at the beginning of the experimental day ( $t = -285, -270$  min), and eight samples thereafter ( $t = -240, -210, -180, -60, -30, 0, 30$ , and  $60$  min) to assess cortisol changes throughout the experiment. Saliva was collected using a commercially available collection device (Salivette®, Sarstedt, Germany) For each sample, the participant first placed the cotton swab

provided in each Salivette tube in his mouth and chewed gently on it for 1 min to produce saliva. The swab was then placed back in the Salivette tube, and the samples were stored in a freezer at  $-25^{\circ}\text{C}$  until assayed. Laboratory analyses were performed at the Department of Biopsychology, TU Dresden, Germany. After thawing, Salivettes were centrifuged at 3,000 rpm for 5 min, which resulted in a clear supernatant of low viscosity. Salivary free cortisol concentrations were subsequently measured using a commercially available chemiluminescence-immuno-assay (CLIA) with high sensitivity of 0.16 ng/mL (IBL, Hamburg, Germany).

**Mood state.** To determine whether hydrocortisone administration led to psychological side-effects, mood state was assessed using the Profile of Mood States (POMS) questionnaire (Reddon et al. 1985; Wald and Mellenbergh 1990; de Groot 1992) at three time points: at the beginning of the experiment ( $t = -285$  min), just prior to the intake of the second capsule ( $t = -60$  min), and at the end of the experiment ( $t = 60$  min).

## Physiological and psychological statistical analysis

Behavioral and physiological data were analyzed in SPSS 15.0 (SPSS, Inc. Chicago, IL, USA) using repeated measured ANOVAs with drug condition (placebo vs. rapid CORT vs. slow CORT) as between subject factor. Due to the high levels of skewness and kurtosis of the POMS questionnaire (Reddon et al. 1985; Wald and Mellenbergh 1990; de Groot 1992), mood data were analyzed using non-parametric tests. Changes over time in mood state were assessed by Friedman tests, and Mann-Whitney U tests were used to assess potential drug effects on mood. Alpha was set at 0.05 throughout.

## MRI acquisition

At approximately 4.5 h after arrival, participants were taken to the scanner room and the procedures were explained. Participants lay supine in the scanner and viewed the screen through a mirror positioned on the head coil. They were asked to lie as still as possible, keep their eyes open, and look directly and continuously at the center of the screen in front of them.

Participants were scanned by a Siemens (Erlangen, Germany) MAGNETOM Avanto 1.5 Tesla MRI scanner equipped with an 8-channel head coil. A series of blood oxygenation level dependent (BOLD)  $T_2^*$ -weighted gradient echo EPI images was acquired with the following parameters: TR = 2340 ms, TE = 35 ms, FA =  $90^{\circ}$ , 32 axial slices approximately aligned with AC-PC plane, slice matrix size =  $64 \times 64$ , slice thickness = 3.5 mm, slice gap = 0.35 mm, FOV =  $212 \times 212$  mm<sup>2</sup>. Owing to its relatively short TE, this sequence yields optimal contrast-to-noise ratio in the medial temporal lobes. High resolution anatomical images were acquired for individuals by a  $T_1$ -weighted 3D Magnetization-Prepared Rapid Gradient Echo (MP-RAGE) sequence, which employed the following parameters: TR = 2250 ms, TE = 2.95 ms, FA =  $15^{\circ}$ , orientation: sagittal, FOV =  $256 \times 256$  mm<sup>2</sup>, voxel size = 1.0 mm isotropic.

## fMRI data analysis

Data were analyzed using Statistical Parametric Mapping software (SPM5; UCL). The first five EPI volumes were discarded to allow for  $T_1$ -equilibration. Before analysis, the images were motion corrected using rigid body transformations and least sum of squares minimization. Subsequently, they were temporally adjusted to account for differences in sampling times across different slices. All functional images were then coregistered with the high-resolution  $T_1$ -weighted structural image using normalized mutual information maximization. The anatomical image was subsequently used to normalize all scans into Montreal Neurological Institute (MNI) 152 space. All functional images were resampled to a voxel size of 2 mm isotropic. Finally, all images were smoothed with an isotropic 8 mm full-width-at-half-maximum Gaussian kernel to accommodate residual functional/ anatomical variance between subjects. Data were analyzed using a general linear model, in which blocks were modeled based on emotion type. Regressors were temporally convolved with the canonical hemodynamic response function of SPM5. The six covariates corresponding to the movement parameters obtained from the realignment procedure were also included in the model. To reduce unspecific differences between scan sessions, and to correct for any unspecific, global effects of drug intake on hemodynamic response instead of neuronal activation (Desjardins et al. 2001; Peeters and Van der Linden 2002), global normalization using proportional scaling was applied. The single subject parameter estimates from each session and condition obtained from the first-level analysis were included in subsequent random-effects analyses. For the second-level analysis, a factorial ANOVA was used, with emotion (neutral vs. aversive) as the within-subject factor, and drug condition (placebo vs. rapid CORT vs. slow CORT) as the between-subject factor.

Statistical tests were family-wise error (FWE) rate corrected ( $p < 0.05$ ) for multiple comparisons at the voxel level for the main effects, and on the cluster-level using a height threshold of  $p < 0.01$  for the drug  $\times$  emotion interaction, depending on the robustness of the effects. Correction for multiple comparisons was done across the entire brain or for regions of interest (ROI) using a small volume correction. Given the abundance of glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) in the amygdala (de Kloet 1991) and its involvement in emotional processing (Phan et al. 2002; Ochsner and Gross 2005), this region was considered ROI. Data concerning the amygdala was corrected for a reduced search volume, defined as a sphere with 4 mm radius, centered on the locus of previously observed stress effects on amygdala responsivity (Ossewaarde et al. 2010).

## Functional connectivity analysis

For connectivity analyses, the time-course of amygdala activity was obtained by extracting the first eigenvariate of the anatomically defined bilateral amygdala (WFU PickAtlas Tool (version 2.4)). To obtain time-course correlation images irrespective of the experimental conditions, a new statistical model was constructed with the time-course of the amygdala as covariate of interest and the convolved regressors for the experimental conditions and realignment parameters as

covariates of no interest, as well as a constant. Time course correlation images were obtained for the amygdala and entered into subsequent random-effects analyses, using a factorial ANOVA with drug condition (placebo vs. rapid CORT vs. slow CORT) as the between-subject factor. Similar to the conventional fMRI analyses, statistical tests were family-wise error (FWE) rate corrected ( $p < 0.05$ ) for multiple comparisons at the voxel level for the main effects of amygdala coupling across drug conditions, and on the cluster-level using a height threshold of  $p < 0.01$  to assess cortisol effects. Visualizations of activations were created in SPM5 by superimposing statistical parametric maps thresholded at  $p < 0.01$  uncorrected (unless specified otherwise) onto a canonical  $T_1$ -weighted image in a standard MNI 152 space.

## RESULTS

### Physiological and psychological measures

As expected, oral administration of 10 mg hydrocortisone increased salivary cortisol levels to those observed during moderate-to-severe stress (Morgan et al. 2000) (Fig. 19A), which was evidenced by a significant main effect of group ( $F(2,62) = 41.63$ ,  $p < 0.001$ ) and a time  $\times$  group interaction ( $F(18,110) = 29.04$ ,  $p < 0.001$ ). Increased levels were observed from 30 min post-administration onwards in both hydrocortisone administration conditions, and the levels remained elevated for at least 90 min. As intended, treatment resulted in elevated cortisol levels during fMRI scanning in the rapid hydrocortisone condition, whereas the levels in the slow condition had already returned to baseline.

Post-experiment debriefing showed that participants were unable to identify the substance received. As expected, hydrocortisone administration did not affect mood as assessed three times during the experiment using the Profile of Mood States (POMS) questionnaire (Reddon et al. 1985; Wald and Mellenbergh 1990; de Groot 1992) (Table 11). Although significant reductions in levels of depression scores (Friedman's ANOVA;  $\chi^2(2) = 9.16$ ,  $p = 0.01$ ), anger scores ( $\chi^2(2) = 7.93$ ,  $p = 0.02$ ), vigor scores ( $\chi^2(2) = 73.17$ ,  $p < 0.001$ ), and tension scores ( $\chi^2(2) = 22.41$ ,  $p < 0.001$ ) were observed over the course of the experiment, and levels of fatigue ( $\chi^2(2) = 48.41$ ,  $p < 0.001$ ) increased, none of these factors were affected by drug administration. Groups did not differ on any aspect of mood state at baseline, nor at any other time point during the experiment (all  $p > 0.1$ ). Changes in mood over time were also not affected by drug administration (all  $p > 0.05$ ). Hence, differences in brain activity found between drug conditions cannot readily be explained by any psychological effects of drug administration.

### Emotional interference task

Overall task performance, assessing sustained attention by combining results on the neutral and aversive trials, was not significantly affected by hydrocortisone intake. No effects of group were found on reaction times ( $F(2,62) = 1.49$ ,  $p = 0.233$ ). Analysis of the error rates, however, seemed to indicate better performance due to the slow effects of corticosteroids. The slow corticosteroid

**Table 11. Behavioral performance on the emotional interference task**

	Placebo	Rapid CORT	Slow CORT
Reaction times neutral, in ms	674 (17)	702 (23)	650 (20)
Reaction times aversive, in ms	687 (17)	709 (23)	664 (20)
Emotional interference on reaction times, in $\Delta$ ms	12 (7)	7 (7)	14 (6)
Correct responses neutral, in %	95.03 (1.03)	95.29 (0.64)	96.63 (0.64)
Correct responses aversive, in %	95.24 (0.90)	93.96 (0.77)	96.80 (0.63)
Emotional interference on correct responses, in $\Delta\%$	0.21 (0.44)	-1.33 (0.50)*	0.17 (0.77)

*Mean values (S.E.M.). All groups were similarly affected in their reaction times by emotional interference, displaying slower responses to aversive compared to neutral words. However, the rapid corticosteroid (CORT) group specifically was impaired in its accuracy of responding due to emotional interference. The rapid CORT group made fewer correct responses to the aversive compared to the neutral words than placebo (\*:  $p < 0.05$ ), and this comparison reached a trend for the difference with corticosteroids' slow effects.*

group seemed to make fewer errors than the other groups, but significance just reached trend level (main effect of group:  $F(2,62) = 2.33$ ,  $p = 0.106$ , slow CORT vs. placebo:  $F(1,41) = 2.26$ ,  $p = 0.141$ , slow CORT vs. rapid CORT:  $F(1,42) = 6.27$ ,  $p = 0.016$ ). Processes of sustained attention might thus benefit from the slow effects of corticosteroids.

Next, we tested for the effects of emotion on task performance. As expected, emotion interfered with selective attention. Participants responded significantly slower to aversive words compared to neutral ones (Main effect of emotion (emotional interference):  $F(1,62) = 9.42$ ,  $p = 0.003$ ). Emotion did however not significantly affect error rates ( $F(1,62) < 1$ ) (Table 11).

Hydrocortisone intake had no significant influence on the emotional interference in terms of reaction times (Emotion x group interaction:  $F(2,62) < 1$ ), but did show a trend for correct response rate (Emotion x group interaction:  $F(2,62) = 2.20$ ,  $p = 0.12$ ). This trend appeared to be caused by the rapid corticosteroid (CORT) group, which was significantly affected ( $T(21) = -2.65$ ,  $p = 0.015$ ) in its accuracy of responding by emotional interference, whereas both other groups were not (both  $p$ 's  $> 0.6$ ). The rapid effects of corticosteroids induced fewer correct responses for the aversive relative to the neutral words than placebo ( $F(1,41) = 5.23$ ,  $p = 0.03$ ), and this comparison reached a trend for the difference with corticosteroids' slow effects (Emotion x group interaction (rapid CORT vs. placebo):  $F(1,42) = 2.65$ ,  $p = 0.11$ ). No such differences were observed between the slow effects of corticosteroids and placebo (Emotion x group interaction (rapid CORT vs. slow CORT):  $F(1,41) < 1$ ). Thus, the rapid effects of corticosteroids appeared to increase the susceptibility to emotional interference.

## Brain activation data

We first identified brain regions involved in task execution in comparison to rest (fixation). As expected, task execution recruited a large cluster of brain regions involved in visual processing, including the bilateral middle and inferior occipital lobe, calcarine, cuneus, cerebellum, lingual gyrus, and fusiform gyrus (Table 12). Moreover, brain regions involved in motor and executive

**Table 12. Peak voxels and corresponding T values of significantly activated clusters in main effects of task, emotion, and drug**

Region	MNI-coordinates			Peak
	x	y	z	T-value
Positive effect of task				
Extended cluster covering visual processing areas: inferior, middle, and superior occipital gyrus, calcarine, lingual gyrus, fusiform gyrus, cerebellum	16	-92	-4	28.90***
	-16	-92	-8	23.28***
Supplemental motor area, Middle cingulate cortex	-4	8	50	20.12***
Precentral cortex, R	32	-56	52	14.15***
Superior frontal cortex, R				
Inferior parietal cortex, R				
Angular cortex, R				
Inferior parietal cortex, L	-30	-52	48	19.06***
Angular cortex , L				
Precentral cortex, L	-28	-4	54	15.50***
Superior frontal cortex, L				
Middle frontal cortex, R	48	38	30	6.95***
Middle frontal cortex, L	-34	52	30	5.10*
Inferior frontal cortex, L	-40	28	24	5.93***
Insula, R	34	24	2	4.91*
Insula, L	-32	20	6	7.21***
Thalamus, R	12	-16	10	8.37***
Thalamus, L	-10	-18	10	10.65***
Putamen, L				
Putamen, R	26	4	-6	7.48***
Brain stem	-6	-28	-4	5.39**
Cerebellum, L	-20	-62	-50	5.66**
Negative effect of task				
Activation cluster covering the bilateral angular cortex, middle occipital cortex, cuneus, precuneus, posterior and middle cingulate cortex, middle temporal gyrus, lingual gyrus, parahippocampus gyrus, hippocampus, amygdala	-44	-76	32	17.86***
	46	-76	28	
Activation cluster covering the middle frontal cortex, superior frontal cortex, superior medial cortex, anterior cingulate cortex, rectus and middle orbitofrontal cortex	28	26	40	14.05***
	-24	30	44	
Inferior frontal cortex, L	-46	42	6	5.02*
	-58	32	2	4.96*
Middle orbitofrontal cortex, L	-48	50	0	4.91*
Insula, R	36	6	12	5.91***
Lingual gyrus, L	-14	-60	-4	5.57**
Cerebellum, R (Crus2)	44	-66	-40	6.84***

Table 12 (continued)

	MNI-coordinates			Peak T-value
	x	y	z	
Cerebellum, L (Crus2)	-42	-70	-40	5.10*
Cerebellum, R (9)	6	-50	-42	5.97***
<b>Positive effect of emotion</b>				
Inferior frontal cortex and inferior orbitofrontal cortex, L	-44	32	0	5.95***
Superior temporal pole, L	-58	6	-10	5.57**
Middle temporal pole, L	-52	14	-24	5.21*
<b>Main effect of drug</b>				
<i>Placebo &gt; slow CORT</i>				
Cuneus, L	-18	-72	36	5.51**

The peak x, y, z coordinates are given in MNI152 standard space coordinates. L and R denote left and right. Main effects of task are all  $p < 0.05$  FWE corrected at the voxel-level.

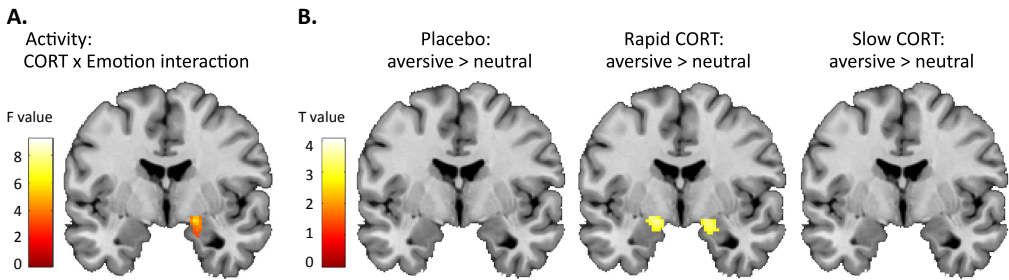
function were activated, including the angular, parietal and precentral cortex and the superior and middle frontal gyrus. Regions deactivated by task execution included regions of the default mode network; the medial prefrontal cortex (superior, middle and orbitofrontal cortex), the temporal lobe (covering the hippocampus and amygdala), cingulate gyrus (posterior, middle and anterior), precuneus and cuneus, and regions within the cerebellum (Table 12).

Subsequently, we tested for the effect of emotion during task execution. Regions that were more active during the processing of aversive compared to neutral words were mainly language-related areas in the left inferior frontal cortex (BA45), left inferior orbitofrontal cortex, superior temporal pole, and the middle temporal lobe (BA38). No regions were more active during the processing of neutral compared to aversive words (Table 12).

Next, we examined how corticosteroids affected sustained attentional processing. Looking into the main effect of drug (contrasting all three drug conditions) revealed a main effect in the cuneus ( $[-18, -72, 36]$ ,  $F(2,124) = 16.36$ ,  $p = 0.022$ ), which was driven by reduced activity due to the slow effects of corticosteroids (placebo > slow CORT:  $[-18, -72, 36]$ ,  $T(124) = 5.51$ ,  $p = 0.004$ ). Under basal (i.e. placebo) conditions this part of the cuneus was activated during task-execution, suggesting its involvement in visual processing (Hahn et al. 2006), but the slow effects of corticosteroids reduced its activation. In contrast, we did not find any main effect on brain processing in the rapid corticosteroid condition.

To test how corticosteroids influenced selective attention, or emotional interference, we next checked for a drug  $\times$  emotion interaction in the brain. Indeed, we found a trend towards such interaction in the amygdala specifically ( $[20, -4, -16]$ ,  $F(2,124) = 5.02$ ,  $p(\text{SVC}) = 0.077$ ) (Fig. 20A). This interaction appeared to be driven by an increased effect of emotional interference due to the rapid effects of corticosteroids (Emo(rapid CORT) > Emo(placebo):  $[20, -4, -16]$ ,  $T(124) = 3.15$ ,  $p(\text{SVC}) = 0.037$ ; Emo(rapid CORT) > Emo(slow CORT):  $[22, -4, -18]$ ,  $T(124) = 2.49$ ,  $p(\text{SVC}) = 0.071$ ). Whereas amygdala responsivity with placebo or under the influence of slow effects

of corticosteroids did not distinguish between neutral and aversive words, suggesting sufficient suppression of emotional interference, the rapid effects of corticosteroids induced significantly higher amygdala responses while color-naming aversive compared to neutral words ( $[22, -2, -16]$ ,  $T(124) = 3.96$ ,  $p(\text{SVC}) = 0.036$ ) (Fig. 20B). Thus, the increase in emotional interference observed in behavioral performance due to the rapid corticosteroid effects, was reflected in the brain as an enhanced emotion effect in the amygdala, indicating failed suppression of emotional processing.



**Figure 20.** Effects of corticosteroids on amygdala activity. (A) Hydrocortisone administration induced trend of a corticosteroid x emotion interaction in the amygdala ( $y = -4$ ) For visualization purposes the statistical parametric map is thresholded at  $p < 0.05$  uncorrected with a minimal cluster-size of 250 voxels. (B) This interaction appeared to be driven by a significant effect of emotion in the amygdala due to the rapid effects of corticosteroids, suggesting insufficient suppression of emotional interference in this group. The amygdala in the placebo and slow corticosteroid group did not distinguish between the processing of aversive vs. neutral words. For visualization purposes the statistical parametric maps are thresholded at  $p < 0.005$  uncorrected with a minimal cluster-size of 25 voxels.

## Brain connectivity data

Next, we assessed whether the corticosteroid-induced alterations in amygdala responses were related to any changes in functional connectivity of this region to the rest of the brain. First, brain regions were identified that were functionally coupled, i.e. displaying significantly correlated time courses of activity, to the amygdala across all drug conditions. Activity in the amygdala was positively associated to activity in a large cluster covering the bilateral amygdala itself, thalamus, pallidum, putamen, hippocampus, parahippocampal gyrus, fusiform, middle and superior temporal lobe, insula, and inferior, middle and superior orbitofrontal cortex. Other regions positively associated with amygdala activity included the brain stem (including the LC), regions within the anterior and middle ACC, superior frontal cortex, and regions within the cerebellum (Table 13). Conversely, amygdala activity was negatively associated with activity in frontal regions such as the medial superior frontal gyrus, superior, middle, and inferior frontal gyrus, and regions within the anterior and middle ACC, and with the insula, brain stem, and cerebellum. Overall, these patterns of functional connectivity are in line with previous studies (Roy et al. 2009; van Marle et al. 2010; Henckens et al. 2011b) and support models of emotion processing that suggest reciprocal ventral and dorsal systems (Phillips et al. 2003). However, one remarkable difference is the negative coupling of the amygdala to the insula observed in this study. This might suggest that the insula, during task execution, might be functioning as part of an executive network (Binder et

**Table 13. Peak voxels and corresponding T values of significantly activated clusters that show functional coupling with the bilateral amygdala**

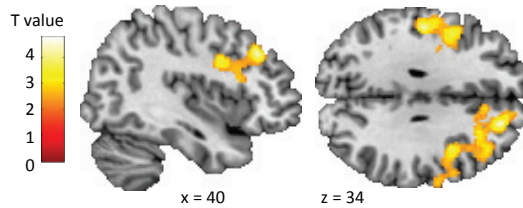
Region	MNI-coordinates			Peak
	x	y	z	T-value
Positive overall amygdala coupling				
Extended cluster covering the bilateral amygdala, brainstem (LC), thalamus, pallidum, putamen, hippocampus, parahippocampal gyrus, fusiform gyrus, middle and superior temporal lobe, inferior, middle and superior orbitofrontal cortex, anterior cingulate cortex and cerebellum	22	-2	-16	43.38***
Superior frontal cortex, R	20	70	8	5.45*
Middle cingulate cortex	0	0	46	5.50*
Caudate, L	-8	16	22	6.83***
Thalamus, R	16	-20	16	5.66*
Midbrain	14	-28	-26	7.99***
Cerebellum Crus2, L	-36	-80	-36	6.45***
Negative overall amygdala coupling				
Anterior and middle cingulate cortex, superior medial cortex, R	2	28	10	9.85***
Middle cingulate cortex, R	22	-16	32	9.08***
Middle cingulate cortex, L	-24	-4	36	9.80***
Inferior frontal gyrus, R	64	18	18	5.81**
Inferior frontal gyrus, L	-52	28	22	9.19***
	-42	14	32	5.40*
Inferior and middle frontal gyrus, L	46	40	26	6.57***
Middle frontal gyrus, L	-26	48	30	5.59*
Middle and superior frontal gyrus, L	-20	54	30	5.56*
Superior frontal gyrus, R	20	54	32	7.37***
	20	18	56	5.81**
Insula, R	-44	-2	6	8.70***
Insula, L	44	-4	4	8.86***
Thalamus, R	0	-18	6	9.42***
Middle temporal gyrus, R	58	-42	4	7.60***
Parahippocampal gyrus, R	16	-28	-16	8.20***
Inferior occipital and lingual gyrus, R	36	-86	-6	7.02***
Inferior occipital and lingual gyrus, middle temporal gyrus, L	-26	-90	-4	7.16***
Inferior parietal cortex, L	-50	-50	36	5.53*
Cerebellum, L	-18	-30	-18	5.85**
Cerebellum and brain stem, L	-16	-44	-28	13.39***
Brain stem	0	-8	-16	5.72*

The peak x, y, z coordinates are given in MNI152 standard space coordinates. L and R denote left and right. Overall amygdala coupling is  $p < 0.05$  FWE corrected at the voxel-level. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$  whole-brain corrected

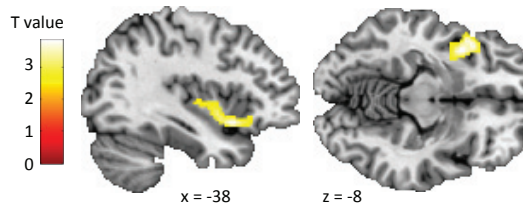
al. 2004; Nee et al. 2007) instead of the salience network (Seeley et al. 2007).

Second, when contrasting connectivity patterns between drug conditions, the rapid effects of corticosteroids influenced amygdala connectivity to regions involved in task execution including the middle frontal and precentral gyrus ([42, 26, 40],  $T(62) = 4.79$ ,  $p < 0.001$ ), and the postcentral gyrus ([−52, −20, 34],  $T(62) = 4.39$ ,  $p = 0.005$ ) (Fig. 21A). Whereas these structures displayed negative connectivity with the amygdala under basal (i.e. placebo) conditions, the rapid corticosteroid effects induced positive connectivity between the amygdala and this executive network. In addition, the slow effects of corticosteroids altered amygdala connectivity to the left insula ([−38, 10, −8],  $T(62) = 3.85$ ,  $p = 0.001$ ). The negative amygdala-insula coupling observed under placebo conditions was weakened by the slow effects of corticosteroids (Fig. 21B).

**A.** Amygdala connectivity: rapid CORT > placebo



**B.** Amygdala connectivity: slow CORT > placebo



**Figure 21.** Effects of corticosteroids on amygdala connectivity. **(A)** The rapid effects of corticosteroids increased the functional connectivity of the amygdala to regions involved in task execution (middle frontal gyrus, precentral gyrus, and postcentral gyrus), potentiating its influence on task execution. **(B)** The slow effects of corticosteroids disrupted the negative connectivity between the amygdala and insula, attenuating the effects the amygdala can exert on task execution. For visualization purposes the statistical parametric maps are thresholded at  $p < 0.01$  uncorrected with a minimal cluster-size of 250 voxels.

## DISCUSSION

In this study we investigated the time-dependent effects of corticosteroids on selective attention and emotional interference. The results suggest that the rapid effects of corticosteroids specifically increased emotional interference in terms of error rate, which was associated with reduced amygdala inhibition to aversive words. Moreover, they induced enhanced amygdala connectivity with frontoparietal brain regions, possibly reflecting increased influence of the amygdala on an executive control network. In contrast, the slow corticosteroid effects seemed to modulate the neural correlates of sustained attention by decreasing cuneus' activity, potentially

indicating reduced stimulus-driven (bottom-up) attentional processing. Furthermore, they altered the coupling of the amygdala to the insula, which might affect emotional interference. Thus, corticosteroids seemed to modulate different aspects of attentive processing in a time-specific manner.

Previous animal work has indicated that corticosteroids, next to their well-established slow genomic effects, also exert rapid non-genomic effects on brain function (Joëls et al. 2006). In the amygdala, the hormones have been shown to rapidly affect neuronal plasticity by binding to mineralocorticoid receptors (MR), leading to an increase in glutamate release (Karst et al. 2010). At the same time, the binding of primarily intracellular glucocorticoid receptors (GRs) initiates a corticosteroid-induced genomic cascade that modulates the expression of over 200 genes (Datson et al. 2001). Here, we aimed to dissociate these two effects experimentally by administering 10 mg of hydrocortisone at either 60 or 270 minutes prior to the emotional distraction task. The timing of the rapid corticosteroid condition was based (A) on a previous study in our lab revealing an elevation in human salivary cortisol levels from 30 min after hydrocortisone intake onwards (Henckens et al. 2012a), (B) previous rodent studies revealing a ~20 min delay between elevations in corticosteroid levels in plasma and brain (Droste et al. 2008), and (C) rapid effects of corticosteroids administered directly to amygdala slices in rodents from ~10 min post administration onwards (Karst et al. 2005). The genomic effects of corticosteroids on the other hand generally do not start earlier than at least 3 hrs after exposure to high corticosteroid levels in vivo (Joëls et al. 2003; Morsink et al. 2006) and these effects last for hours (Joëls and de Kloet 1992; Joëls et al. 2003). Thus, administration of hydrocortisone at 60 minutes prior to scanning probably caused sufficiently high levels of the hormone in the brain to evoke rapid non-genomic effects whereas this delay was too short to allow development of gene-mediated events. Conversely, when hydrocortisone was applied at 270 min prior to testing, hormone levels were back to baseline levels again during the behavioral task, making non-genomic actions not likely to happen, yet allowed enough time for the gene-mediated actions to occur. For these reasons, the rapid corticosteroid effects observed here most likely reflect corticosteroid's non-genomic effects, whereas the slow corticosteroid effect most likely involve a gene-mediated mechanism, although obviously this cannot be proven in the human brain.

Here we showed that the rapid corticosteroid effects increase emotional interference. Participants had difficulty ignoring emotional input; they made more mistakes for the aversive words and failed to down-regulate their amygdala response to this input. These findings are in line with the hypothesis that the rapid effects of corticosteroids act in concert with catecholamines in response to stress to optimize rapid adaptive behavior (Roosendaal et al. 2006c; Diamond 2007). Previous studies have already shown that during acute stress, the brain switches into a hypervigilant stimulus-driven reflex-like mode of processing, characterized by heightened overall attention, but also by increased susceptibility to (emotional) distraction (Skosnik et al. 2000; Braunstein-Bercovitz et al. 2001; Henderson et al. 2012) and impaired flexibility (Plessow et al. 2012). Performance

on relatively easy (e.g. perceptual) tasks seems to benefit by this state of increased arousal, but performance on more difficult tasks requiring executive control seems to deteriorate (Jasinska et al. 2012; Lee et al. 2012). Recent neuroimaging studies have indicated that this hypervigilant brain state is associated with enhanced sensory processing (Henckens et al. 2009), increased amygdala responsivity to emotional input (van Marle et al. 2009) and tightened amygdala connectivity to the salience network (van Marle et al. 2010). Moreover, prefrontal cortex function gets deteriorated (Qin et al. 2009). This state-change of brain processing has previously been attributed to the actions of catecholamines on brain function (Arnsten and Li 2005; Hermans et al. 2011). Our findings of increased emotional interference indicate that, next to the effects of catecholamines, the rapid effects of corticosteroids also contribute to this state of hypervigilance.

Earlier animal work already indicated that corticosteroids' rapid non-genomic effects, mediated by membrane-bound steroid receptors, boost amygdala activity (Kavushansky and Richter-Levin 2006; Karst et al. 2010), while impairing prefrontal cortex function (Barsegyan et al. 2010). Next to that, evidence for corticosteroid-modulation of noradrenergic function is abundant, both in animal (Roosendaal et al. 2006c; McReynolds et al. 2010; Zhou et al. 2012) and human research (van Stegeren et al. 2007; van Stegeren et al. 2010). Recent drug administration studies in humans for example showed that corticosteroid administration in combination with the administration of reboxetine (a noradrenaline-reuptake inhibitor) induced a negative response bias in the amygdala (Kukolja et al. 2008), and boosted emotion-induced retrograde amnesia (Hurlemann et al. 2007), in line with our findings of increased distraction by aversive input and increased susceptibility to the effects of emotion, respectively.

However, administration of hydrocortisone has produced quite conflicting results. One potential confounding factor is the type of brain function investigated in these studies. A widely accepted phenomenon from memory research for example is that corticosteroids influence processes of memory encoding and consolidation in an opposite manner than memory retrieval (Roosendaal 2002), although both processes heavily depend on hippocampal function. Nevertheless, corticosteroids boost memory encoding and associated hippocampal activation (van Stegeren et al. 2010), whereas they impair hippocampal activation during memory retrieval (de Quervain et al. 2003). Similarly, differential effects of corticosteroids have been observed depending on the function studied of the prefrontal cortex (Henckens et al. 2011, 2012a) and amygdala (Henckens et al. 2010; Lovallo et al. 2010; Tabbert et al. 2010; van Stegeren et al. 2010). Another crucial factor possibly explaining the discrepancies between studies is the dose in which hydrocortisone was administered (Lupien et al. 2007). Previous research has indicated that corticosteroids influence memory processing in an inverted U-shaped relationship (Lupien et al. 1997), with both low and high doses impairing memory consolidation, while moderate levels improve consolidation (Roosendaal 2000). Also the effects of corticosteroids on working memory (Lupien et al. 1999) and startle response (Buchanan et al. 2001) have been shown to be dose-dependent. This non-monotonic relationship between corticosteroids and their effects on cognitive function is hypothesized to be related to the differential activation of the MRs and GRs, which show distinct

affinity for the hormone (de Kloet 2003). The doses used in previous research range from 10-100 mg of hydrocortisone, and obviously produce different results. A recent review on the immediate effects of corticosteroids on selective attention concluded that corticosteroids actually facilitate stress-coping via the inhibition of autonomic processing of goal-irrelevant threatening information, when administered in a dose of  $>35$  mg (Putman and Roelofs 2011). The authors admit that lower doses might lead to different results. Here, we used a dose of 10 mg of hydrocortisone to mimic cortisol elevations in response to a moderate-to-severe stressor, and show that the rapid effects of corticosteroids increase emotional interference during executive function.

Moreover, the rapid effects of corticosteroids also affected amygdala connectivity. Connectivity to the middle frontal gyrus and precentral and postcentral gyrus was increased 60 min after hydrocortisone administration. Being part of an executive and motor network, these regions were recruited during task execution (Table 12). Whereas the pre- and postcentral gyrus are involved in more basic motor functions, the middle frontal gyrus is known for its role in response selection and suppression of automatic response tendencies (Forstmann et al. 2008), as well as in resolving interference (Nee et al. 2007). Under basal (i.e. placebo) conditions, all of these regions were negatively coupled to the amygdala, underlining their opposing roles in task execution. In contrast, the rapid effects of corticosteroids led to positive coupling between the amygdala and the executive network. Although one cannot infer any directionality from such correlative evidence, this might be suggestive for increased influence of the amygdala on brain regions crucially involved in task execution. This interpretation of the data would fit with the increase in emotional interference, but future research is needed to test this assumption.

Besides these rapid effects of corticosteroids on emotional interference we showed that the slow effects of corticosteroids modulated the neural correlates of sustained attention by reducing activity of the cuneus. This brain region is involved in basic visual processing, and has been shown to be engaged by stimulus-driven, bottom-up attentional processing (Hahn et al. 2006). Previous research has indicated that acute stress boosts visual processing (Henckens et al. 2009; van Marle et al. 2009), and more specifically, the rapid effects of corticosteroids have been shown to increase cuneus' regional cerebral activity during rest (Ganguli et al. 2002; Strelzyk et al. 2012). These data suggest that stress, or the rapid effects of corticosteroids, boost early visual processing and thereby shift the brain into a rather automated visually guided response-mode, which serves the fight-or-flight response. The slow effects of corticosteroids might in turn counteract these effects by reducing cuneus' activity, and shifting the brain back from a stimulus-driven response mode to a more controlled mode. This rationale fits with the general idea about the restorative role the slow corticosteroid effects serve in the aftermath of stress in order to return to homeostasis (de Kloet et al. 2005). The slow effects of corticosteroids have been shown to divert energy supply to challenged tissues and control the excitability of neuronal networks (de Kloet et al. 2008). Evidence from recent human neuroimaging studies also supports this hypothesis by showing that corticosteroids' slow effects are the exact opposite of those of acute stress. Whereas acute stress

impairs prefrontal cortex function (Qin et al. 2009) and boosts amygdala activity (van Marle et al. 2009), the slow effects of corticosteroids' enhanced prefrontal cortex function (Henckens et al. 2011) and suppressed amygdala responsivity to faces (Henckens et al. 2010). Here, we showed that the slow effects of corticosteroids reduced cuneus' activity, which might be another means to restore proper brain function in the aftermath of stress.

The slow effects of corticosteroids also reduced the negative connectivity between the amygdala and left anterior insula, seen under placebo conditions. The amygdala and anterior insula share widespread reciprocal connections (Mufson et al. 1981), and are known for their role in mediating autonomic arousal as part of the so-called salience network (Seeley et al. 2007). Connectivity in this network is known to be increased by acute stress (van Marle et al. 2010; Hermans et al. 2011) and serve the fight-or-flight response by promoting the information exchange between regions involved in autonomic-neuroendocrine control and vigilant attentional reorienting. However, next to the typical link to cortical control of autonomic function, the insula is consistently reported to be activated during experiments in which task conditions are challenging, and decisions have to be made (Binder et al. 2004). Therefore, it was recently suggested (Eckert et al. 2009) that the anterior insula engages brain regions selectively responsive to task demands and attention systems critical for coordinating task performance. In line with this hypothesis, a recent meta-analysis on neuroimaging studies into the resolution of interference pointed towards the involvement of the anterior insula in resolving interference (Nee et al. 2007). Although one cannot infer directionality from the correlative analysis performed, one could speculate that the negative connectivity between the amygdala and insula observed in our experiment reflects the interference of the amygdala with proper task performance. By reducing this connectivity, the slow effects of corticosteroids might attenuate the effect the amygdala can exert on task execution. Therefore, also the reduced amygdala-insula connectivity could entail a mechanism by which the slow effects of corticosteroids restore brain function in the aftermath of stress. However, this interpretation should be tested in future research.

Some limitations to the study should also be mentioned. First of all, this study involved a pharmacological manipulation to model the effects of corticosteroids, which does obviously not capture all aspects of the complex stress response. Real-life cortisol release in response to stress is accompanied by the release of many other neuromodulators, such as norepinephrine, corticotrophin-releasing hormone, dopamine, and serotonin (Joëls and Baram 2009), with which corticosteroids could potentially interact. Because we did not induce stress, the generalization from our results to stressful situations remains speculative. Nevertheless, mere administration of hydrocortisone reveals a cleaner mechanistic account for the corticosteroid effect, which was the aim of this study.

Secondly, we investigated men only, thus the obtained results cannot be readily generalized to women. Hydrocortisone administration has been shown to induce distinct effects in men and women, both in behavior (Andreano and Cahill 2006; Bohnke et al. 2010) and brain activation

(Stark et al. 2006; Merz et al. 2010). Although important, sex-differences were beyond the scope of this initial study, which is why we opted to recruit male subjects only, allowing easier comparison with an earlier study in stressed individuals (Henckens et al. 2009).

Furthermore, the increase in emotional interference by the rapid effects of corticosteroids was only observed in terms of error rate (i.e. lower correct response rate) and not in terms of reaction times (i.e. slower responding). On the other hand, the overall effect of emotion was only observed for reaction times. One could suggest that these findings reflect a shift in response strategy induced by the rapid effects of corticosteroids rather than an increase in emotional interference (Chen and Johnson 1991). This would mean that the rapid CORT group shifted from an accuracy-driven strategy, affecting reaction times while optimizing accuracy, towards a speed-driven strategy, affecting error rates but optimizing speed. However, besides the observed differences (i.e. increase in emotional interference) in error rate one would then also expect differences (i.e. reduced emotional interference effect) in reaction times. This does not seem to be the case. No differences between groups in overall reaction times ( $F(2,62) = 1.49$ ,  $p = 0.23$ ), nor emotional interference in reaction times ( $F(2,62) < 1$ ) were observed, indicating that the rapid CORT group is not different from the other groups in terms of reaction times. In terms of error rate, the rapid CORT group was significantly affected, indicating increased emotional interference in this group. Moreover, if it would be the case that the rapid CORT group shifted away from an accuracy-driven towards a speed-driven strategy, one would expect faster responding in this group, which is also not observed. All in all, it is difficult to speculate about the reason why we did find interference effects in one measure and not the other. However, small behavioral effects are not unprecedented in previous studies (Haas et al. 2006; Mincic 2010). Importantly, behavioral emotional interference effects are most consistently observed in psychopathological groups in response to words that are specific to their disorder (Daghighi 1995; Williams et al. 1996), and in normal subjects when the words are related to current concerns endorsed by them (Gilboa-Schechtman et al. 2000), reflecting their attentional bias. Overall, in normal subjects, behavioral interference by emotional distracters is either not detected at all (Williams et al. 1996), is depending on specific personality traits such as trait anxiety (Richards et al. 1992; Krug and Carter 2010) or extraversion (Haas et al. 2006) or habituates rapidly (McKenna 1986; Compton 2003). We used rather general aversive words, non-specific to the participants, which might explain why we only find overall effects in terms of reaction times and not error rate. Nevertheless, emotional interference can express itself in both reaction times and number of errors (Swick et al. 2002; Wagner et al. 2006; Weiss et al. 2007; Kertzman et al. 2010; Crocker et al. 2012).

Furthermore, the slow effects of corticosteroids manifested themselves only as altered brain activity, without translating to behavioral differences. The most likely explanation for the absence of a (clear) behavioral effect might be a lack of power of our neuroimaging study. Compared to behavioral studies, which tend to test large groups of subjects, our sample size is relatively small. Brain activity is supposed to be a more sensitive measure than behavioral output, which is the consequence of many parallel neural operations. Therefore, regional differences in brain activity are more easily detected with smaller samples, but these samples offer little power to

observe behavioral effects. However, a trend towards a better overall performance due to the slow effects of corticosteroids was observed in the behavioral data, since the slow corticosteroid group tended to make fewer errors than the other groups. These data therefore seem to support the enhanced sustained attention due to the slow effects of corticosteroids, but future studies using larger sample sizes are needed to confirm these effects.

Lastly, we interpreted the effects of emotional interference as a measure of selective attention, because this condition requires the attentional selection of relevant features while ignoring competing information. However, these findings cannot be readily generalized to other selective attention tasks. The emotional component might be critical in interfering with attentional processing, as corticosteroids have been shown to exert more prominent effects on the processing of emotional compared to neutral information (Roosendaal et al. 2006c). Therefore, future studies are necessary to determine whether the rapid effects of corticosteroids can be regarded as generally or emotion-specifically interfering with the neural processing of selective attention.

In conclusion, these results suggest that the rapid effects of corticosteroids increase emotional interference and selective attention. Although increased susceptibility to interference, and thus impaired selective attention, is often seen as a maladaptive response of attenuating higher-cognitive function, it is first and foremost a highly adaptive response in threatening situations. Wide-spread, unfocussed attention might contribute to the detection of potential threats in the environment (Aston-Jones and Cohen 2005), enhancing an organism's chances of survival. Moreover, it might have beneficial effects on memory processing (Henckens et al. 2009), since additional environmental cues can also be encoded during a salient event. Normalization some time after the stressful event is equally important. When not properly regulated, the increased processing of irrelevant emotional input due to combined corticosteroid and noradrenergic actions as well as the lack of normalization can be detrimental. Patients with stress-related disorders such as depression and post-traumatic stress disorder (PTSD) are known to be compromised in their capability to suppress emotional irrelevant information (Paunovi et al. 2002; Mitterschiffthaler et al. 2008), which is thought to reflect their attentional bias towards negative emotional information (Williams et al. 1996). Notably, these illnesses are characterized by aberrant corticosteroid signaling (Yehuda et al. 2001). Our results provide thus a mechanistic account for these problems with attention and emotional interference, by showing that the rapid effects of corticosteroids interfere with amygdala function, and the slow effects modulate the neural correlates of sustained attention.

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**Time-dependent corticosteroid  
modulation of prefrontal  
working memory processing**

**3.3**

**Marloes J.A.G. Henckens, Guido A. van Wingen,  
Marian Joëls, and Guillén Fernández**

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# 3.3

## ABSTRACT

Corticosteroids are potent modulators of higher cognitive function in humans. They are released in response to stress, and are thought to be involved in the modulation of cognitive function by inducing distinct rapid non-genomic, and slow genomic changes affecting neural plasticity throughout the brain. However, their exact effects on the neural correlates of higher-order cognitive function as performed by the prefrontal cortex at the human brain system level remain to be elucidated. Here, we targeted these time-dependent effects of corticosteroids on prefrontal cortex processing in humans using a working memory (WM) paradigm during functional MRI scanning. Implementing a randomized, double-blind, placebo-controlled design, 72 young, healthy men received 10 mg hydrocortisone either 30 min (rapid corticosteroid effects) or 240 min (slow corticosteroid effects), or placebo prior to a numerical n-back task with differential load (0- to 3-back). Corticosteroids' slow effects appeared to improve working memory performance and increased neuronal activity during WM-performance in the dorsolateral prefrontal cortex depending on WM-load, whereas no effects of corticosteroids rapid actions were observed. Thereby, corticosteroids' slow actions seem to facilitate adequate higher-order cognitive functioning, which may support recovery in the aftermath of stress exposure.

## INTRODUCTION

Corticosteroids are key modulators of human cognition. They are released in response to stress as the end product of the hypothalamic-adrenal pituitary (HPA) axis, and known to readily cross the blood-brain-barrier to affect brain processing (McEwen 1979). Corticosteroids ensure sufficient energy supply to challenged tissues and control the excitability of neuronal networks, and are thereby thought to support and regulate the stress response (de Kloet et al. 1999). The hormones exert their actions upon binding of the mineralocorticoid (Robertson et al.) and glucocorticoid receptor (GR), abundantly expressed in the brain (Sapolsky et al. 1983; Reul and de Kloet 1985; de Kloet, 1991). Recent animal research has indicated that receptor-binding causes both immediate non-genomic effects (Karst et al. 2005) and slow, genomic effects that manifest themselves several hours after stress exposure (Pavlidis et al. 1995; Wiegert et al. 2005). By these distinct mechanisms corticosteroids seem to influence neural plasticity in a time-dependent manner (Joëls et al. 2006).

So far, most research on modulation of cognition has focused on medial temporal lobe structures, where corticosteroids have been shown to affect neuronal excitability, synaptic plasticity, and processes of memory retrieval and consolidation (Roozendaal 2003; Joëls 2008). However, moderate to high levels of receptor expression in the prefrontal cortex (PFC) (de Kloet 1991), make this structure susceptible to corticosteroid-modulation as well. A current working hypothesis states that corticosteroids' rapid non-genomic effects work in concert with the effects of catecholamines during the early phase of the stress response (Joëls et al. 2006; Roozendaal et al. 2006c), and thereby optimize rapid adaptive behavior by reallocating neural resources away from higher-order cognitive processing regions in the PFC in order to promote vigilance, instinctive behavior and the encoding of the stressful experience into memory (Diamond 2007). Meanwhile, the corticosteroid-induced genomic cascade is initiated which is hypothesized to restore PFC function in the aftermath of stress (Diamond 2007). Although findings from both animal (Cerqueira et al. 2005; Cerqueira et al. 2007) and human literature (Dedovic et al. 2009a; van Stegeren et al. 2010) provide initial evidence for corticosteroid-modulation of PFC signaling, both the neural and functional consequences on higher-cognitive function and their time-dependency remain to be tested.

Here, we targeted both the rapid (putatively non-genomic) and slow (putatively genomic) effects of corticosteroids on PFC processing using a working memory (Bowman) paradigm during functional MRI in humans. WM refers to a system maintaining relevant information in a temporary buffer that is constantly updated to guide behavior (Baddeley, 2003). It is typically associated with the activation of a frontoparietal executive function network, including the dorsolateral prefrontal cortex (DLPFC) (Baddeley, 2003). Implementing a randomized, double-blind, placebo-controlled design 72 young, healthy men received 10 mg hydrocortisone – known to mimic corticosteroid levels observed during moderate to severe stress – either 30 (to target corticosteroid's rapid effects) or 240 min (to assess corticosteroid's slow effects) prior to a numerical n-back task. To investigate whether corticosteroid effects depend on task difficulty, we manipulated WM-load using a 0-, 1-, 2-, and 3-back condition.

## MATERIALS & METHODS

### Participants

Seventy-two young (age range 18-29, median 21), right-handed, healthy male volunteers gave written informed consent to participate in the study. In order to ensure stable effects of hydrocortisone over all participants, women were excluded from participation. Women are known to display different HPA-axis reactivity than men, exhibiting smaller and more variable cortisol responses to stress (Kajantie and Phillips 2006), depending on menstrual cycle phase and use of hormonal contraceptives (Kirschbaum et al. 1999; Bouma et al. 2009). Furthermore, individuals who met any of the following criteria were excluded from participation: history of head injury, autonomic failure, history of or current psychiatric, neurological, or endocrine disorders, current periodontitis, acute inflammatory disease, acute peptic or duodenal ulcers, regular use of corticosteroids, treatment with psychotropic medications, narcotics, beta-blockers, steroids, or any other medication that affects central nervous system or endocrine systems, medical illness within the three weeks prior to testing, self reported mental or substance use disorder, daily tobacco or alcohol use, regular night shift work, or current stressful episode or major life event. Moreover, volunteers with high scores on depression (score above 8 on the Beck Depression Inventory; Beck et al. 2002) were excluded from participation. Furthermore, four participants were excluded from analyses because they displayed either abnormal basal salivary cortisol levels ( $> 3$  standard deviations above mean; 1 participant), or showed no elevation in salivary cortisol level in response to CORT intake, ending up with 23 men in the placebo group, 23 in the slow CORT group, and 22 in the rapid CORT group. The study was executed in accordance with the declaration of Helsinki and approved by the local ethics committee (CMO region Arnhem-Nijmegen, Netherlands).

### Procedure

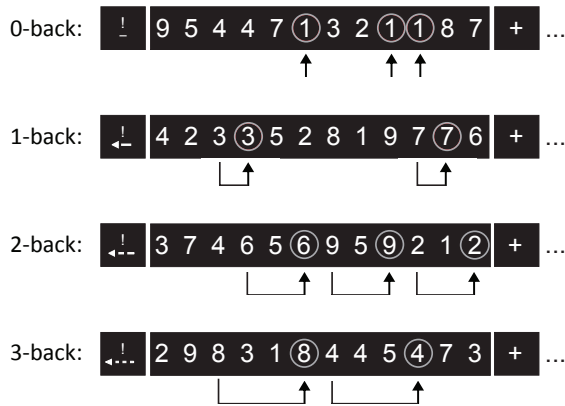
**Prior to arrival.** Prior to inclusion all eligible participants received an extensive information brochure, listing all in- and exclusion criteria and roughly explaining the setup of the experiment. If criteria were met (according to the participant's own insights), an appointment was made. To minimize differences in baseline cortisol levels we instructed participants not to use any recreational drugs for three days and to refrain from drinking alcohol, exercising, and smoking for 24 h prior to the appointment. Furthermore, participants were requested not to brush their teeth, floss, or eat and drink anything but water for one hour prior to the session enabling adequate saliva sampling for cortisol assessment. They were asked to take a light lunch and do so no later than one hour before arrival; their lunch could not contain any citrus products, coffee, tea, milk or sweets (Maheu et al. 2005). Throughout the entire study period, participants were only given water to drink, except for a scheduled lunch at 150 min prior to scanning.

**Experiment.** To reduce the impact of diurnal variation in cortisol levels, all testing was performed in the afternoon, between 12:00 h ( $\pm 30$  min) and 18:00 h ( $\pm 30$  min), when hormone levels are

relatively stable. Upon arrival participants received an information brochure about the procedure, they gave informed consent, and completed an intake questionnaire to ensure that in- and exclusion criteria were met. 30 min after arrival, a first saliva sample was taken, followed by another one 15 min later, in order to measure a reliable baseline level. Participants were asked to complete a first Profile of Mood States (POMS) questionnaire (Reddon et al. 1985; Wald and Mellenbergh 1990; de Groot 1992), after which they briefly trained the WM-task to ensure proper understanding during scanning. Immediately after the second saliva sample (at  $t = -240$  min) participants received the first capsule. During the entire period ( $\sim 3.5$  h) prior to scanning, participants waited in a quiet room where they were free to conduct any activities except for anything potentially arousing (e.g. video games). At 30 min prior to scanning participants were asked to complete another POMS questionnaire, and received the second capsule. Both drug capsules, containing either 10 mg CORT or placebo (cellulose), were administered orally. This dose is known to elevate salivary cortisol levels to moderate to high stress levels (Kirschbaum et al. 1996; Morgan et al. 2000; Tops et al. 2003), and has been shown to be successful in the induction of corticosteroid effects on declarative memory (Kirschbaum et al. 1996; Tops et al. 2003). Depending on the group to which the participant was (randomly) assigned he received either; the 1st capsule containing placebo, the 2nd containing placebo (placebo group); the 1st capsule CORT, the 2nd placebo (slow CORT group); or the 1st capsule placebo, the 2nd CORT (rapid CORT group).

**Scanning.** At about 4.5 hours after arrival, participants were taken to the scanner room and the procedures were explained. Participants lay supine in the scanner and viewed the screen through a mirror positioned on the head coil. They were asked to lie as still as possible, keep their eyes open, and look directly and continuously at the center of the screen in front of them

**N-Back task.** In the MRI scanner, participants were asked to conduct an n-back task. Using a blocked-design, participants completed eight cycles of alternating 0-, 1-, 2-, and 3-back conditions, interleaved by a short fixation period (2.4 s) (Fig. 22). Within each block, a pseudorandom digit sequence (no more than 2 repetitions) consisting of 12 single digits was shown to participants. Each digit was presented for 400 ms, followed by an inter-stimulus-interval of 1400 ms. Each block started with a 2 s cue presentation indicating the 0-, 1-, 2-, or 3-back condition, resulting in an inter-block-interval of 26 s. Blocks were presented in a mirrored design avoiding covariation with linear drift. During the 0-back condition, participants were asked to decide whether the current item on the screen was a “1” or not. During the 1-back condition, participants were asked to detect whether the current item had appeared one position back in the sequence. Similarly, in the 2- and 3-back condition participants were instructed to detect whether the current item had appeared two or three positions back, respectively. Each sequence contained either 2 or 3 targets and participants were asked to make a button press with their right index finger as fast as possible when detecting a target. To ensure proper understanding and sufficient performance, participants practiced each condition twice earlier that day outside the MRI scanner (at  $t = -240$  min), and twice inside immediately prior to the actual task ( $t = 0$  min).



**Figure 22.** N-back task containing a 0-, 1-, 2-, and 3-back condition. Participants were instructed to press a button when the currently viewed number was identical to the one they had seen n numbers before. For the 0-back condition they were asked to press whenever they saw a '1'. All participants completed 8 blocks of each condition, with every sequence consisting of 12 digits with either 2 or 3 targets.

## Physiological and psychological measures

**Salivary cortisol measure.** Cortisol levels were measured from saliva at ten time points (Fig. 23): baseline measurements at the beginning of the experiment (twice) ( $t = -255, -240$  min), and eight samples ( $t = -210, -180, -150, -30, 0, 30, 60, 90$  min) to assess cortisol changes throughout the experiment. Saliva was collected using a commercially available collection device (Salivette®, Sarstedt, Germany). For each sample, the participant first placed the cotton swab provided in each Salivette tube in his mouth and chewed gently on it for 1 min to produce saliva. The swab was then placed back in the salivette tube, and the samples were stored in a freezer at  $-25^{\circ}\text{C}$  until assayed. Laboratory analyses were performed at the Department of Biopsychology, TU Dresden, Germany. After thawing, salivettes were centrifuged at 3,000 rpm for 5 min, which resulted in a clear supernatant of low viscosity. Salivary free cortisol concentrations were subsequently measured using a commercially available chemiluminescence-immuno-assay (CLIA) with high sensitivity of 0.16 ng/mL (IBL, Hamburg, Germany).

**Heart rate.** Cardiac rhythm of the participants was measured during scanning using a pulse oximeter placed on their left index finger. Participants were instructed to keep their hands as still as possible during the measurement. Heart rate frequency was calculated using in-house software. **Mood state.** Mood state was assessed using the Profile of Mood States (POMS) questionnaire (Reddon et al. 1985; Wald and Mellenbergh 1990; de Groot 1992) at three time points: at the beginning of the experimental day ( $t = -255$  min), just prior to entering the fMRI scanner ( $t = -30$  min), and just prior to departure ( $t = 90$  min).

## Physiological and psychological statistical analysis

Behavioral and physiological data were analyzed in SPSS 15.0 (SPSS, Inc. Chicago, IL, USA)

using mixed model ANOVAs with WM-load (3- vs. 2- vs. 1- vs. 0-back) as within subject factor and CORT manipulation (placebo vs. slow CORT vs. rapid CORT) as between subject factor. Participant's age was included as covariate. Due to the high levels of skewness and kurtosis of the POMS questionnaire (Reddon et al. 1985; Wald and Mellenbergh 1990; de Groot 1992), mood data were analyzed using non-parametric tests (Friedman test). The two measures of working memory performance, accuracy and reaction times, were analyzed both separately and combined as one overall WM-performance measure using Stouffer's Z-score method (Stouffer et al. 1949). This method first applies a z-transformation to both independent variables and subsequently combines them (here by subtraction) into one overall z-score. Alpha was set at 0.05 throughout.

## **MRI acquisition**

Participants were scanned by a Siemens (Erlangen, Germany) MAGNETOM Avanto 1.5 Tesla MRI scanner equipped with an 8-channel head coil. A series of blood oxygenation level dependent (BOLD)  $T_2^*$ -weighted gradient echo EPI images was acquired with the following parameters: TR = 2340 ms, TE = 35 ms, FA = 90°, 32 axial slices approximately aligned with AC-PC plane, slice matrix size = 64 x 64, slice thickness = 3.5 mm, slice gap = 0.35 mm, FOV = 212 x 212 mm<sup>2</sup>. Owing to its relatively short TE, this sequence yields optimal contrast-to-noise ratio in the medial temporal lobes. High resolution anatomical images were acquired for individuals by a  $T_1$ -weighted 3D Magnetization-Prepared Rapid Gradient Echo (MP-RAGE) sequence, which employed the following parameters: TR = 2250 ms, TE = 2.95 ms, FA = 15°, orientation: sagittal, FOV = 256 x 256 mm<sup>2</sup>, voxel size = 1.0 mm isotropic.

## **fMRI data analysis**

Data were analyzed using Statistical Parametric Mapping software (SPM5; UCL, London). The first five EPI-volumes were discarded to allow for  $T_1$ -equilibration. Prior to fMRI analysis, the images were motion corrected using rigid body transformations and least sum of squares minimization. Subsequently, they were temporally adjusted to account for differences in sampling times across different slices. All functional images were then co-registered with the high-resolution  $T_1$ -weighted structural image using normalized mutual information maximization. The anatomical image was subsequently used to normalize all scans into MNI152 (Montreal Neurological Institute) space. All functional images were resampled with a voxel size of 2 mm isotropic. Finally, all images were smoothed with an isotropic 8-mm full-width-at-half-maximum Gaussian kernel in order to accommodate residual functional/anatomical variance between subjects. Following these preprocessing procedures, data were analyzed using a general linear model, in which individual events were modeled based on drug condition and working memory load (1-, 2- or 3-back contrasted versus 0-back (baseline)). Regressors were temporally convolved with the canonical hemodynamic response function of SPM5. The six covariates corresponding to the movement parameters obtained from the realignment procedure were also included in the model. To reduce unspecific differences between scan sessions, global normalization using proportional scaling

was applied. The single subject parameter estimates from each session and condition obtained from the first level analysis were included in subsequent random effects analyses. For the second level analysis a factorial ANOVA was used, with working memory load (1-, 2-, 3-back) as within subject factor, drug condition (placebo vs. slow CORT vs. rapid CORT) as between subject factor, and participant's age as covariate.

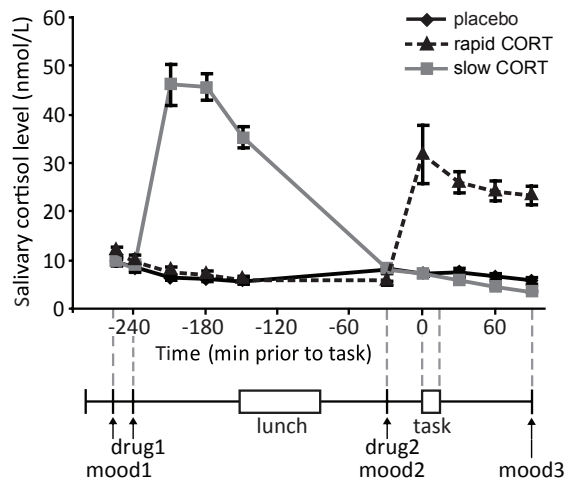
Given strong neurophysiological evidence for the locus of corticosteroid receptors (de Kloet 1991), and its involvement in working memory processing (Baddeley 2003) the DLPFC was a region of interest. Data concerning this region was corrected for reduced search volume through an anatomical mask as defined by the WFU PickAtlas Tool (version 2.4) (bilateral middle frontal gyrus). A threshold of  $p < 0.05$  FWE whole brain corrected was applied to all other regions.

Visualizations of activations were created using MRIcroN (<http://www.sph.sc.edu/comd/rorden/mricron/>) by superimposing statistical parametric maps thresholded at  $p < 0.001$  uncorrected (unless specified otherwise), onto a canonical  $T_1$ -weighted image in standard MNI152 space.

## RESULTS

### Physiological and psychological measures

As expected, oral administration of 10 mg hydrocortisone increased salivary cortisol levels to those observed during moderate to severe stress (Morgan et al. 2000) (Fig. 23, which was evidenced by a significant main effect of group ( $F(2,65) = 43.30$ ,  $p < 0.001$ ) and a time  $\times$  group interaction ( $F(18,116) = 26.17$ ,  $p < 0.001$ ). Increased levels were observed from 30 min post-



**Figure 23.** Experimental design and salivary cortisol curves. Participants received two capsules (drug1 & drug2) containing either 10 mg hydrocortisone (CORT) or placebo at different time-points prior to the numerical n-back task (0/1/2/3-back). Hydrocortisone intake significantly elevated salivary cortisol levels in both hydrocortisone administration conditions to levels observed during moderate to severe stress. mood: Profile of Mood States (POMS) questionnaire (Reddon et al. 1985; Wald and Mellenbergh 1990; de Groot 1992). Error bars represent S.E.M.

administration onwards in both hydrocortisone administration conditions, and the levels remained elevated for at least 90 min. As intended, treatment resulted in elevated cortisol levels during fMRI scanning in the rapid hydrocortisone condition, whereas the levels in the slow condition had already returned to baseline.

Post-experiment debriefing showed that participants were unable to identify the substance received. As expected, hydrocortisone administration did not affect autonomic measures of heart rate (main effect of drug:  $F(2,64) < 1$ ) and heart rate variability ( $F(2,64) < 1$ , n.s.) (Table 14). Further, drug administration did not affect mood as assessed three times during the experiment using the Profile of Mood States (POMS) questionnaire (Reddon et al. 1985; Wald and Mellenbergh 1990; de Groot 1992) (Table 14). Although significant reductions in levels of depression scores (Friedman's ANOVA;  $\chi^2(2) = 8.99$ ,  $p = 0.011$ ), anger scores ( $\chi^2(2) = 7.43$ ,  $p = 0.024$ ), vigor scores ( $\chi^2(2) = 79.05$ ,  $p < 0.001$ ), and tension scores ( $\chi^2(2) = 18.38$ ,  $p < 0.001$ ) were observed over the course of the experiment, and levels of fatigue ( $\chi^2(2) = 52.40$ ,  $p < 0.001$ ) increased, none of these factors was affected by drug administration. Hence, differences in brain activity found between drug conditions cannot readily be explained by any physiological or psychological side effects of drug administration.

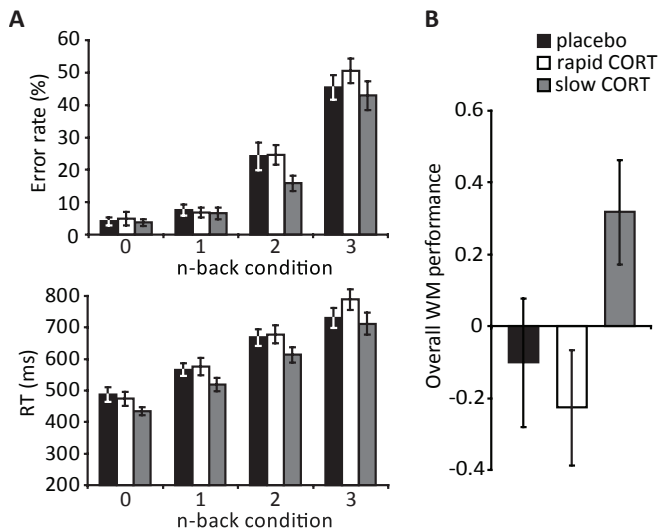
**Table 14. Physiological and psychological measures**

		Placebo	Rapid CORT	Slow CORT
<b>Mood State</b>				
Depression	1 (t = 30 min)	0.26 (0.13)	0.82 (0.37)	0.65 (0.32)
	2 (t = 255 min)	0.09 (0.06)	0.64 (0.35)	0.13 (0.07)
	3 (t = 375 min)	0.04 (0.04)	0.59 (0.24)	0.13 (0.10)
Anger	1 (t = 30 min)	0.61 (0.23)	1.18 (0.40)	1.00 (0.43)
	2 (t = 255 min)	0.30 (0.19)	0.45 (0.23)	0.48 (0.20)
	3 (t = 375 min)	0.22 (0.18)	0.73 (0.29)	0.87 (0.32)
Fatigue	1 (t = 30 min)	1.17 (0.30)	1.68 (0.50)	2.70 (0.61)
	2 (t = 255 min)	1.35 (0.44)	1.55 (0.52)	2.43 (0.56)
	3 (t = 375 min)	3.52 (0.67)	5.23 (0.69)	4.22 (0.71)
Vigor	1 (t = 30 min)	12.65(0.79)	10.50 (0.77)	11.70 (0.90)
	2 (t = 255 min)	10.43 (0.68)	8.73 (0.75)	10.26 (0.96)
	3 (t = 375 min)	7.57 (0.88)	4.86 (0.82)	7.13 (0.91)
Tension	1 (t = 30 min)	1.00 (0.27)	1.36 (0.29)	1.30 (0.46)
	2 (t = 255 min)	0.35 (0.13)	1.09 (0.35)	0.96 (0.30)
	3 (t = 375 min)	0.26 (0.16)	0.64 (0.20)	0.17 (0.10)
<b>Heart rate (BPM)</b>		65.60 (1.96)	67.04 (2.57)	68.30 (2.41)
<b>Heart rate variability (ms<sup>2</sup>)</b>		70.76 (4.95)	62.71 (4.99)	67.02 (6.60)

*Mean values (S.E.M.)*

## Working memory performance

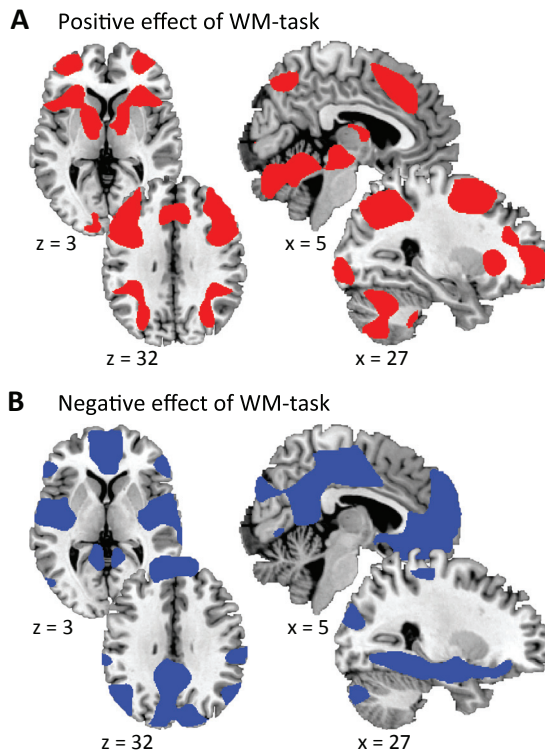
Separate ANOVAs for both performance measures of accuracy and reaction times (RTs) were conducted with WM-load as within-subject factor, and drug condition as between-subject factor. There were robust effects of WM-load on both accuracy ( $F(3,63) = 107.72$ ,  $p < 0.001$ ) and RTs ( $F(3,63) = 97.96$ ,  $p < 0.001$ ) (Fig. 24A). These analyses revealed no significant main effect of drug (accuracy:  $F(2,65) = 1.59$ ,  $p = 0.212$ ; RTs:  $F(2,65) = 1.98$ ,  $p = 0.146$ ) nor a WM-load  $\times$  drug interaction (accuracy and RTs:  $F$ 's  $< 1$ ) on both performance measures, although a tendency towards shorter RTs and improved accuracy could be observed for the slow CORT group (Fig. 24A). Since performance on the n-back task can be regarded as a combined measure of both accuracy and RTs of responding (both assessing voluntary attention (Prinzmetal et al. 2005) and efficacy of information processing (Pachella 1974)), the two measures were combined to create one overall WM-performance measure (Neubauer et al. 1992) (see Methods section). Analysis of this combined performance measure revealed that CORT administration indeed affected WM-performance ( $F(2,42) = 3.25$ ,  $p = 0.045$ ). This main effect of drug was driven by an improved performance of the slow CORT group compared to the rapid CORT group ( $T(42) = 6.58$ ,  $p = 0.014$ , Fig. 24B) and close to significant improvement compared to the placebo group ( $T(43) = 3.59$ ,  $p = 0.065$ ). The rapid CORT and placebo groups did not differ on WM-performance ( $T(42) = 0.233$ , n.s.). The observed effects seemed to be driven by drug effects at high WM-load (2- and 3-back conditions;  $F(2,64) = 3.34$ ,  $p = 0.042$ ), since there was no significant difference between drug conditions at low WM-load (0- and 1-back;  $F(2,64) = 1.68$ ,  $p = 0.195$ ). However, the drug  $\times$  load interaction failed to reach significance ( $F(2,64) < 1$ ).



**Figure 24.** Behavioral performance in the n-back task. (A) Mean error rates and reaction times (RT) of the 0-, 1-, 2-, and 3-back conditions for the three drug conditions did not reveal any effects of hydrocortisone (CORT). (B) The combination of error rates and RTs into one overall WM-performance measure revealed that the slow CORT group outperformed both other groups. Error bars represent S.E.M.

## Brain activation

We first identified brain regions activated by performing the numerical n-back task by contrasting 3-, 2-, 1-back with 0-back conditions (collapsing across groups). As expected, the WM-task activated an extended set of brain regions in the bilateral prefrontal cortex (including the DLPFC), bilateral inferior parietal cortex, inferior occipital lobe, cerebellum and other related regions (Table 15, Fig. 25A). The opposite contrast, regions deactivated by WM-processing, revealed the default mode network (DMN) including the posterior cingulate cortex, the ventral medial PFC extending into the orbitofrontal cortex and the medial temporal lobe (Table 15, Fig. 25B).



**Figure 25.** Brain activation related to WM-processing. (A) The n-back task activated a widespread set of brain regions in the bilateral prefrontal cortex (including the DLPFC), bilateral inferior parietal cortex, inferior occipital lobe, and cerebellum. (B) Regions deactivated by WM-processing were the default mode network (DMN) including the posterior cingulate cortex, the ventral medial PFC extending into the orbitofrontal cortex and the medial temporal lobe. Statistical parametric maps are family wise error (FWE) corrected. See Table 15 for the exact coordinates and values.

To examine how corticosteroids affect working memory processing over time, we first identified those brain regions whose activity was modulated by any of the drug conditions. This analysis showed that the only significant effect of hydrocortisone was observed in the left DLPFC ( $[x = -40, y = 42, z = 32]$   $F(2,194) = 11.52$ ,  $p(\text{corrected}) = 0.030$ , Fig. 26). We next extracted the data

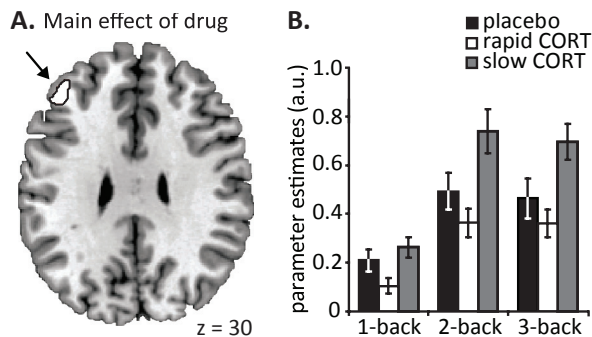
**Table 15. Peak voxel and corresponding F / T values of significantly activated clusters in main effects of working memory**

Region	Brodmann	MNI Coordinates			Peak
	area	x	y	z	T-value
Positive main effect of WM					
Extended activation cluster covering the precentral gyrus, superior & middle PFC, R	6,8,10,32, 44-46,48	-30	0	60	18.61***
Extended activation cluster covering the precentral gyrus, superior & middle PFC, L	6,8,10,32, 44-46,48	30	2	58	19.27***
Supplementary motor area, L	32	-4	16	46	21.57***
Inferior parietal cortex, L	40	-36	-46	44	22.15***
Inferior parietal cortex, R	40	44	-46	52	21.44***
Angular gyrus, R	40	38	-54	52	21.06***
Inferior temporal gyrus, R	37	56	-54	-12	9.21***
Cerebellum 9, L	L	-10	-58	-52	4.91*
Calcarine, R	17	14	-72	12	5.04*
Calcarine, L	17	-10	-98	0	6.48***
Inferior occipital lobe, R	18	30	-92	-6	8.49***
Inferior occipital lobe, L	18	-26	-94	-8	8.06***
Negative main effect of WM					
Ventral medial PFC, L	10	-4	60	18	17.62***
	10	-4	56	-4	18.95***
Rectus, L	11	-2	44	-16	19.01***
Inferior orbitofrontal gyrus, L	47	-32	34	-14	17.23***
Inferior temporal gyrus, L	21	-56	-4	-26	14.74***
Supramarginal / superior temporal gyrus, R	48	56	-26	24	13.87***
Fusiform gyrus / parahippocampal gyrus, L	37/20	-26	-42	-10	14.78***
incl. hippocampus	37	-30	-32	-12	10.78***
Fusiform gyrus / parahippocampal gyrus, R	37/20	30	-32	-16	12.72***
incl. hippocampus	20	28	-20	-16	10.92***
Middle temporal gyrus, L	21	-64	-44	-4	6.46***
Middle temporal gyrus, R	21	64	-2	-20	13.05***
Posterior cingulate cortex, L	23	-4	-46	30	23.21***
Precuneus, L	30	-6	-52	16	22.04***
Angular gyrus, L	39	-50	-66	32	17.72***
Cerebellum 9, R		4	-54	-44	5.06*
Cerebellum crus1/2, R		28	-82	-34	13.43***
Cerebellum crus1/2, L		-28	-82	-34	6.71***

MNI, Montreal Neurological Institute; BA, Brodmann Area; R, right; L, left. All effects are analyzed using voxel-level statistics. \*:  $p < 0.05$  whole brain corrected, \*\*:  $p < 0.01$  whole brain corrected, \*\*\*:  $p < 0.001$  whole brain corrected

from this cluster to analyze whether the effects of hydrocortisone were moderated by WM-load using orthogonal contrasts. First of all, the main effect of WM-load was significant ( $F(2,63) = 4.83$ ,  $p = 0.011$ ) and showed that the DLPFC displayed greater activity with increasing load. More importantly, the effect of WM-load on DLPFC activation was modulated by hydrocortisone administration (drug  $\times$  WM-load interaction;  $F(4,128) = 2.56$ ,  $p = 0.042$ ). Further analyses showed that this interaction was driven by more prominent drug effects at high compared to low WM-loads (drug  $\times$  WM-load (1- vs. 2-back);  $F(2,64) = 5.17$ ,  $p = 0.008$ , drug  $\times$  WM-load (1- vs. 3-back);  $F(2,64) = 4.57$ ,  $p = 0.014$ ), whereas the drug effect between the high WM-load conditions (2- and 3-back) did not differ significantly ( $F(2,64) < 1$ ).

To determine which of the drug conditions induced these DLPFC effects, we continued with pairwise follow-up tests between the three drug conditions. These analyses revealed that the observed drug  $\times$  WM-load interaction effect in the DLPFC was caused by the slow effects of corticosteroids under high WM-load. A history of corticosteroid elevation apparently induced increased high WM-load processing in the DLPFC compared to both placebo ( $F(1,43) = 6.31$ ,  $p = 0.016$ ) and the rapid corticosteroid conditions ( $F(1,42) = 12.82$ ,  $p = 0.001$ ). Current elevation in corticosteroid level had no such effect on DLPFC activation (rapid CORT vs. placebo:  $F(1,42) = 1.24$ , n.s.)



**Figure 26.** Main effect of drug on WM-related brain activity in the DLPFC. (A) Analysis of the main effect of drug revealed that corticosteroids specifically modulated activity in a prefrontal region. (B) Data extraction from this activation cluster revealed both a main effect of WM-load, and a drug  $\times$  WM-load interaction, caused by greater drug effects at higher WM-load. This interaction in the DLPFC was caused by the slow effects of corticosteroids increasing DLPFC-activity under high WM-load. Error bars represent S.E.M. For visualization purposes the statistical parametric map is thresholded at  $p < 0.001$  uncorrected.

## DISCUSSION

Here we targeted both the rapid presumably non-genomic, and slow presumably genomic effects of corticosteroids on prefrontal working memory processing. Results revealed time-differential effects for corticosteroids' actions, with their slow effects increasing WM-related activation of the DLPFC and thereby improving WM-performance, whereas corticosteroids' rapid effects did not induce any observable effect.

Previous work in animals has provided initial evidence that corticosteroids next to their well-established slow genomic effects also exert rapid non-genomic effects (Joëls et al. 2006). The hormones have been shown to rapidly affect neuronal plasticity by binding to membrane mineralocorticoid receptors (Robertson et al.), leading to a change in glutamate release (Karst et al. 2005). At the same time, a corticosteroid-induced genomic cascade is initiated by the binding of primarily intracellular glucocorticoid receptors (GRs) that upon binding translocate to the nucleus where they function as transcription factors to modulate the expression of over 200 genes (Datson et al. 2001). In the MTL, these rapid and slow actions of corticosteroids were shown to have fundamentally distinct consequences in that they either enhance or inhibit neuronal plasticity respectively (Pavlidis et al. 1995; Karst et al. 2005; Wiegert et al. 2005). Here, we aimed to dissociate these two effects on the human PFC experimentally by administering 10 mg of hydrocortisone at either 30 or 240 minutes prior to the WM-task. The timing of the rapid corticosteroid condition was based on a previous study in our lab revealing an elevation in human salivary cortisol levels from 30 min onwards (Henckens et al. 2010), and most prominent rapid, quickly reversible, effects with corticosteroids administered directly to hippocampal slices in rodents (Karst et al. 2005). The genomic effects of corticosteroids on the other hand generally do not start earlier than 3 hrs after exposure to high corticosteroid levels (Joëls et al. 2003; Morsink et al. 2006) and these effects last for hours (Joëls and de Kloet, 1992; Joëls et al. 2003). Thus, administration of hydrocortisone at 30 minutes prior to scanning probably caused sufficiently high levels of the hormone in the brain to evoke rapid non-genomic effects whereas this delay was too short to allow development of gene-mediated events. Conversely, when hydrocortisone was applied at 240 min prior to testing, hormone levels were so low (similar to baseline) during the behavioral task that non-genomic actions are not likely to happen, yet allowed enough time for the gene-mediated actions to occur.

Under conditions of acute stress working memory is generally impaired (Elzinga and Roelofs 2005; Oei et al. 2006; Schoofs et al. 2008), while neuronal firing and long-term potentiation in the PFC are known to be decreased (Maroun and Richter-Levin 2003; Birnbaum et al. 2004; Rocher et al. 2004). These effects are at least partly caused by the stress-related hormones norepinephrine and dopamine, which are known to impair prefrontal cortex function in higher doses (Arnsten 2009). They subserve the initial fight-or-flight response by prioritizing rapid instinctive behavior (as mediated by e.g. the amygdala) and emotional memory encoding (Henckens et al. 2009; van Marle et al. 2009) over complex higher-order cognitive functions as performed by the prefrontal cortex (Arnsten 2009; Qin et al. 2009). Since previous studies have shown that the rapid effects of corticosteroids act in concert with (and to amplify) the effects of catecholamines on long term memory (Joëls et al. 2006; Roozendaal et al. 2006c), we hypothesized impaired WM-performance in the rapid CORT condition. However, we did not observe any such rapid, non-genomic effects of corticosteroids on either WM-performance or DLPFC activation. Previous studies into corticosteroid-modulation of working memory performance show rather conflicting results on this topic. Studies have reported no effects on WM-performance (Monk and Nelson 2002;

Kumsta et al. 2010), corticosteroid-induced improvements in both humans (Oei et al. 2009) and animals (Yuen et al. 2009), as well as impairments (Wolf et al. 2001a) depending on concurrent sympathetic activation (Elzinga and Roelofs 2005) or WM-load (Lupien et al. 1999). The latter findings suggest that fast actions of corticosteroids have indeed additive effects to noradrenergic activation in WM impairment. Rodent work has shown that this concurrent noradrenergic activity of the amygdala is actually essential for corticosteroid-induced impaired WM to occur (Roozendaal et al. 2004a). In line with this, a recent human study into the effects of norepinephrine and corticosteroids on the neural correlates of memory formation, showed that specifically the administration of both hormones caused a strong deactivation in the prefrontal cortex, whereas no such effects were observed when only corticosteroids were administered (van Stegeren et al. 2010). Here we used different levels of difficulty (WM-load), which presumably triggered different levels of arousal, but did not observe any rapid modulatory effects of hydrocortisone on WM-performance or DLPFC processing. However, the levels of emotional arousal reached due to this manipulation most likely did not reach arousal levels observed under conditions of stress. Therefore, this issue of potentially interacting rapid corticosteroid and noradrenergic effects on PFC functioning remains open for future research. Regardless, our results show that corticosteroids by themselves do not modulate WM-performance or WM-related DLPFC activity in a rapid non-genomic manner.

Corticosteroids' slow, genomic effects on the other hand have often been seen as essential for adaptation and restoration of homeostasis following situations of acute stress (McEwen 2007). Here we provide the first demonstration that exactly these delayed effects of corticosteroids boost WM-processing. This effect was strongest at high WM-load when cognitive demand is highest. Our findings of enhanced WM by corticosteroids are supported by two recent rodent studies in which the administration of corticosterone in the prefrontal cortex was shown to enhance glutamatergic transmission in PFC pyramidal neurons by increasing surface levels of NMDA- and AMPA-receptor subunits (Yuen et al. 2009, 2011). Moreover, the first study showed that stress improved performance on a WM-task 4 hours later, but not immediately. Both this increase in glutamatergic transmission and improved behavioral performance were abolished by the administration of a selective GR antagonist, pointing towards the involvement of this receptor. Since the rapid stimulatory non-genomic effects of corticosteroids are thought to be mediated by corticosteroid-binding of membrane MRs (Karst et al. 2005), this observed corticosteroid-induced WM-improvement most likely involves a genomic mechanism. These findings in animals, together with the time-delay implemented for assessing the slow corticosteroid effects in this study, suggest that the observed improvement in WM-processing is mediated via a GR-dependent genomic mechanism. However, administration of a GR antagonist would be necessary to explicitly test this hypothesis in humans. Although extremely interesting and necessary for future understanding of corticosteroid effects, practical reasons currently prohibit realization of such experiment, since no selective GR antagonist is registered for human use yet. Mifepristone (RU-486) is the only compound commercially available (Pecci et al. 2009), but it is known to

cross the blood brain barrier only at very high concentrations (Heikinheimo and Kekkonen 1993) and, more importantly, to also act as a very potent progesterone receptor antagonist (Heikinheimo et al. 1987), which might cause many unwanted side effects. Future studies are therefore necessary to elucidate the exact underlying mechanism of the observed potentiation of WM-processing. Nevertheless, we here show that specifically corticosteroids' slow actions boost WM-processing in the DLPFC, which are likely mediated via a GR-dependent genomic mechanism.

Obviously, several limitations of this study should be mentioned. First of all, the behavioral effects observed in this study were not very strong. Although trends were seen in absolute measures of reaction time and error rate, these trends failed to reach significance. Only the combination of both measures revealed an indication for enhanced performance in the slow corticosteroid group. However, since both measures contribute to behavioral performance in their own distinct way (Pachella 1974; Prinzmetal et al. 2005), we think this combination is actually warranted. The combination of error rates and reaction times is often used to determine the speed-accuracy trade-off displayed by participants. This speed-accuracy trade-off refers to the fact that there is usually a trade-off between these two measures, with either short reaction times causing many errors, or longer reaction times reducing the number of errors (Wickelgren 1977). Here however, we observed both faster and more accurate responses by participants in the slow CORT group compared to the other groups, so instead of a shift in trade-off, we found additive effects both pointing towards improved performance. It cannot be excluded that the lack of a strong behavioral effect is partly caused by the relatively low number of subjects in our fMRI study; this number is obviously lower than for less laborious psychopharmacological studies. Behavioral output is dependent on a multitude of factors (e.g. intelligence or motivation), and the variation in WM-performance within each group is therefore quite substantial. For this reason, effects with rather small effect sizes, such as observed here, are not easily detected in behavior, certainly with the between-subject comparison that was used. Regardless, we found significant brain effects that were in line with the behavioral effect, providing corroborative evidence. A second explanation for the rather weak behavioral effect might be that the dose of hydrocortisone administered was too low to induce stronger effects. We used 10 mg hydrocortisone in this study, because this dose is known to increase salivary cortisol levels to physiological levels observed under conditions of moderate to severe stress (Kirschbaum et al. 1996; Morgan et al. 2000; Tops et al. 2003). Moreover, previous studies using a similar dose reported on the successful induction of corticosteroid effects on declarative memory (Kirschbaum et al. 1996; Tops et al. 2003), which has been shown to be less sensitive to corticosteroid-modulation than working memory (Lupien et al. 1999). However, several studies reporting on corticosteroid effects on human cognition have used higher doses of hydrocortisone (Buchanan and Lovallo 2001; Lupien et al. 2002; van Stegeren et al. 2010), and use of such dose might possibly have induced stronger behavioral effects.

Another limitation of this study is that we investigated men only, which limits the generalization of the obtained results to women. Women are known to display different HPA-axis reactivity than men and exhibit smaller and more variable responses to stress (Kajantie and Phillips 2006),

which appear to depend on the phase of the menstrual cycle and use of hormonal contraceptives (Kirschbaum et al. 1999). Although sex-differences are important to consider, this issue was beyond the scope of this initial study, which is why we opted to recruit the population with the most stable response to corticosteroids, and excluded women from participation.

Finally, this pharmacological study obviously is not an exact copy of naturally occurring circumstances. Real-life cortisol release in response to stress is accompanied by the release of many other neuromodulators, such as norepinephrine, CRH, dopamine, and serotonin (Joëls and Baram 2009). Mere administration of hydrocortisone lacks the interaction with these modulators, but does reveal a cleaner mechanistic account for the pure corticosteroid effect, which was the aim of this study.

Regardless these potential limitations, the present results reveal two major findings. First of all, this study provides clear evidence for the existence of time-dependent effects of corticosteroids on human brain processing. The importance of this timing-factor, although widely acknowledged in animal literature (de Kloet et al. 2008), has so far been neglected in human studies on corticosteroid effects. The majority of previous studies tested for corticosteroid effects about 1 h after hydrocortisone administration (de Quervain et al. 2003; Buss et al. 2004; Oei et al. 2007), most probably resulting in a mix of corticosteroids' rapid, non-genomic and slow, genomic effects. Our data suggests that future research on corticosteroids along with the understanding of their effects would greatly benefit from the incorporation of this crucial timing-factor in experimental designs. Secondly, corticosteroids' slow effects were shown to augment DLPFC processing and to facilitate WM-performance. Since previous research has indicated that working memory and prefrontal processing are impaired under conditions of acute stress by the rapid actions of catecholamines (Arnsten 2009), we speculate that these slow corticosteroid effects may counteract these changes and help the brain to recover in the aftermath of stress. Thereby, they may serve a highly adaptive function in normalizing brain processing when stress has subsided.

## **ACKNOWLEDGEMENTS**

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**Opposite effects of corticosterone  
on neuronal properties of the  
mouse lateral orbitofrontal cortex  
and CA1 hippocampal area**

**3.4**

**Anup G. Pillai, Marloes J.A.G. Henckens,  
Guillén Fernández, and Marian Joëls**

**(in preparation)**

# 3.4

## ABSTRACT

The rodent stress hormone corticosterone changes neuronal activity generally in a slow and persistent manner through transcriptional regulation. One of the most prominent targets of these slow actions is the amplitude of L-type calcium currents and downstream signaling pathways, e.g. the calcium-dependent potassium currents that regulate firing frequency accommodation and the lingering slow afterhyperpolarization (sAHP) at the end of a period of depolarization. However, these corticosteroid effects are liable to regional differentiation, with enhanced sAHP amplitude in the dorsal hippocampal CA1 region, but reduced amplitudes in neurons located in the ventral part of the CA1 region and the basolateral amygdala. We here addressed to what extent corticosterone slowly changes neuronal properties in the mouse orbitofrontal cortex (OFC). The OFC was selected because its dendritic complexity is – similar to that of the basolateral amygdala – enhanced after chronic stress, contrary to the reduction reported for pyramidal neurons in the dorsal hippocampus. We show that action potential properties of layer II/III pyramidal OFC neurons and passive membrane characteristics (resting membrane potential, tau) were unaltered 1-4 hrs after a 20-min pulse of corticosterone (100 nM) compared to vehicle treatment; a nearly significant corticosterone-induced reduction in the input resistance was observed. The amplitude of the sAHP – even when corrected for the input resistance – was significantly reduced after corticosterone treatment in the OFC, while the hormone enhanced sAHP amplitudes in dorsal CA1 hippocampal region. These contrasting effects at the single cell level might possibly contribute to the opposite effects on morphology earlier reported after chronic stress.

## INTRODUCTION

Exposure of an organism to stressful conditions causes the adrenal glands to release high amounts of corticosteroid hormones (corticosterone in most rodents). This hormone circulates in the body but also easily enters the brain where it binds to receptors, which are generally located intracellularly and act as transcriptional regulators (Revollo and Cidlowski 2009). Two types of receptors have been identified: the mineralocorticoid receptor (MR), which due to its high affinity for corticosterone is already substantially activated under rest, and the lower-affinity glucocorticoid receptor (GR), which is particularly occupied after stress (de Kloet et al. 2005).

One of the most prominent effects of stress and corticosterone via GRs is a slow and long-lasting enhancement in the amplitude of L-type calcium currents in the hippocampus (Kerr et al. 1992; Karst et al. 2000; Joëls et al. 2003; Chameau et al. 2007). Downstream of this calcium influx, activation of calcium-dependent potassium channels may occur and this pertains especially the current involved in the accommodation of firing frequency during periods of depolarization and the lingering afterhyperpolarization (sAHP) when the depolarization is terminated (e.g. Faber and Sah 2005). In line with this cascade, several studies have demonstrated that 1–4 hours after administration of a brief pulse of corticosterone to pyramidal neurons in the rodent dorsal CA1 hippocampal area, firing frequency accommodation and the amplitude of the sAHP are enhanced (Joëls et al. 1989; Kerr et al. 1989; Liebmann et al. 2008; Maggio and Segal 2009). This may have important consequences for the transfer of excitatory transmission in the area, potentially leading to attenuation of excitability in the aftermath of stress, which may normalize an earlier raise in excitability caused by rapidly acting stress hormones (Joëls et al. 2012).

Interestingly though, the corticosteroid effect on sAHP amplitude shows regional differentiation. In contrast to the enhanced sAHP amplitude several hours after corticosterone administration observed in dorsal CA1 neurons, pyramidal neurons in the most ventral part of the CA1 area show a reduction in the sAHP amplitude following corticosterone treatment (Maggio et al. 2009). A similar effect is seen for principal cells in the basolateral amygdala (BLA) (Duvarci and Paré 2007). Regional differentiation between BLA and hippocampal neurons has also been described with regard to morphological changes after chronic stress (Roozendaal et al. 2009). Whereas neurons in the CA3 – and to a lesser extent CA1 – hippocampal area display reduced dendritic complexity after chronic stress (McEwen and Magariños 1997), BLA neurons display dendritic hypertrophy (Vyas et al. 2002). The latter has also been observed for pyramidal neurons in the orbitofrontal cortex (OFC) (Liston et al. 2006). Despite these clear effects of chronic stress on OFC morphology, corticosteroid effects on physiological properties of OFC neurons – acute or chronic – have not been investigated at all. Given the similarity between the morphological changes in BLA and OFC neurons after chronic stress, we wondered whether OFC neurons respond to a single pulse of corticosterone in a comparable manner as neurons in the BLA, i.e. oppositely to CA1 neurons.

To test this hypothesis, we applied corticosterone (100 nM) for 20 min to OFC neurons in acutely prepared slices from young-adult male mice and compared the effect on passive (resting membrane potential, input resistance, membrane time constant ( $\tau$ )) and active (action potential, sAHP) membrane properties of pyramidal neurons in the OFC to corticosteroid effects on pyramidal neurons in the dorsal CA1 hippocampal area.

## MATERIALS & METHODS

### Animals

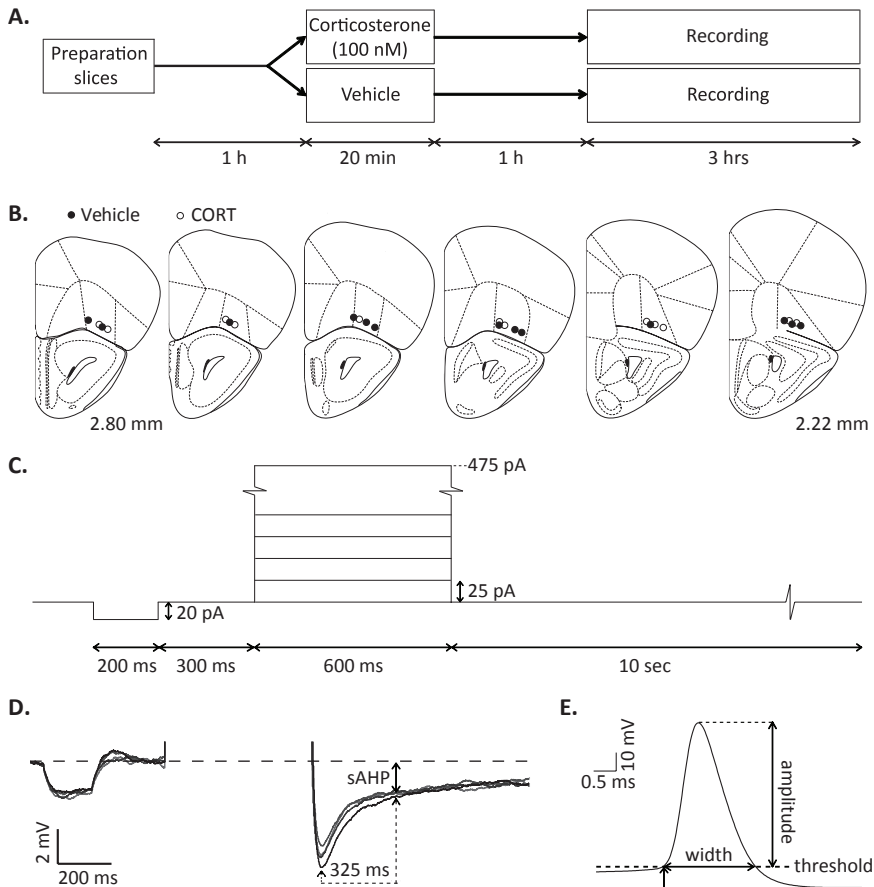
For the present study we used 19 (11 and 8 for CA1 and OFC respectively) male C57/Bl6 mice (~40 g; 4-10 weeks of age; Harlan CPB, Zeist, The Netherlands). All animals were acclimatized for a minimum of one week after transportation to the animal facility and were group housed (3-5) in a 12/12 h light/dark schedule (lights on at 07.00 A.M.) with ad libitum access to food and water. The local committee on animal bioethics of Utrecht University approved all experiments.

### Slice preparation and corticosterone treatment

Slices were obtained from naive animals and were always decapitated between 09.00-10.30 A.M., close to the circadian trough, without anesthesia. Immediately after decapitation, the brain was taken from the skull and chilled (at ~4 °C) in carbogenated (95:5% O<sub>2</sub>:CO<sub>2</sub>) artificial cerebrospinal fluid (aCSF) containing (in mmol/L): NaCl 126, KCl 3, NaHCO<sub>3</sub> 26, NaH<sub>2</sub>PO<sub>4</sub> 1.25, D-(+)-glucose 10, CaCl<sub>2</sub> 2 and MgSO<sub>4</sub> 1.3 (pH 7.4, ~290-300 mOsm). Coronal slices (300  $\mu$ m thick) containing either the hippocampus or the OFC were prepared with a vibroslicer (Leica VT 1000S; Leica Instruments, Nussloch, Germany). For the OFC slices the cerebellum was removed and for the hippocampal slices the frontal lobes and cerebellum were removed, and the caudal side of the brain was glued onto the slicing plateau. After preparation, slices were stored at room temperature in carbogenated aCSF. After at least 1 h, half of the slices from all experimental groups were subjected to vehicle treatment (0.009% ethanol), whereas the other half received corticosterone (100 nM in 0.009% ethanol), for 20 min at 32 °C (Fig. 27A). Previous studies have shown that this treatment is sufficient to observe changes in cellular properties that require homodimerization of the GR, 1-4 h later (Karst et al. 2000). After incubation in either corticosterone or vehicle, the slices were transferred back to the holding chamber containing carbogenated aCSF at room temperature.

### Electrophysiology setup

Patching and identification of neurons were carried out under a 40X objective (NA: 0.75) attached to a microscope (AX10 Examiner. A1, ZEISS, Germany) equipped with infrared differential contrast video microscopy in combination with a b/w high resolution CCD camera (TCCCD-624,



**Figure 27.** (A) *Experimental protocol.* Neurons were recorded 1–4 hrs after a 20 min administration period of 100 nM corticosterone or vehicle (aCSF containing 0.009% ethanol), to allow gene-mediated actions presumably via GRs to develop, and excluding putative rapid non-genomic actions. (B) *Schematic representation of the area from which neurons were recorded* (Paxinos and Franklin 1998). Circles indicate the position of the neurons from which was recorded in the present study (vehicle: closed circles ( $n = 13$ ), corticosterone: open circles ( $n = 12$ )). (C) *Recording protocol to determine action potential characteristics and slow afterhyperpolarization (sAHP).* Neurons were first subjected to a  $-20$  pA current to assess input resistance and membrane time constant ( $\tau$ ). Subsequently, a sequence of depolarizing current pulses, up to 475 pA, with increments of 25 pA were applied. (D) *Typical example traces from an OFC pyramidal neuron.* sAHP was measured by averaging (20 ms window) the voltage response after a delay (325 ms) from the mAHP peak. (E) *A typical action potential from an OFC pyramidal neuron.* The point of deflection in the signal was considered to represent the start of the action potential (vertical arrow). The corresponding voltage was designated as the action potential threshold. The height of the action potential was determined relative to this potential. The width of the action potential was determined as the time interval between the time at which the voltage response just rises above the threshold and the time it falls below threshold.

Monacor International) and video monitor (CDM-1702, Monacor International, Bremen, Germany). Patch electrodes ( $4\text{--}7\text{ M}\Omega$ ,  $\sim 2\text{ }\mu\text{m}$  tip diameter) were pulled from thick walled borosilicate glass capillaries (I.D./O.D. in mm: 1.5/0.86, Harward Apparatus, Kent, UK) on a P-97 Flaming/brown

micropipette puller (Sutter Instruments, Novato, USA). Recording of AHPs was made using an Axo Patch 200B amplifier (Axon Instruments, USA), filtered at 2 kHz and digitized at 50 kHz (Digidata 1322A, Axon Instruments, USA). All recording were carried out in carbogenated aCSF at 30 °C using a temperature controller (TC-324B, Warner Instrument Corp., USA) and perfused into the recording chamber using a peristaltic pump at 2-3 mL/min. Voltage- and current-clamp recording were acquired using pClamp 9.2 data acquisition software (Molecular devices, USA).

## Recording

The pipette solution contained (in mM): 135 K-methane-sulfonate, 10 HEPES, 4 MgATP, and 0.4 NaGTP, pH 7.3. The solution contained no calcium chelator EGTA, and thus minimally interfered with calcium-dependent properties of the cell, which made it optimal for recording firing frequency accommodation and the sAHP amplitude (Faber and Sah 2005). Neurons in the lateral part of the OFC (layer II/II) and dorsal CA1 hippocampal area were selected for recording if they displayed a pyramidal-shaped cell body. The approximate location of each recorded cell was drawn on paper (Fig. 27B), though not verified with intracellular staining. Only cells with a resting membrane potential more negative than -55 mV were included in this study. The fast (fAHP), medium (mAHP) and slow AHPs (sAHP) were measured from each neuron by injecting incremental steps (0-475 pA in steps of 25 pA; 600 ms duration) of suprathreshold currents from a baseline membrane potential (held at -70 mV), with 30 s inter-trial intervals. At the start of each AHP sweep (10 s total duration) we applied a brief hyperpolarizing pulse (200 ms, -20 pA) for measurement of passive membrane properties (input resistance and tau). Additionally, we also included a short zero-current step (200 ms, 0 pA) at the end of each sweep, to monitor resting membrane potential (Fig. 27C). We estimated series resistance, input resistance and tau using a non linear optimization technique (Nelder-Mead method) to find the minima of the sum of the squared errors (SSE) between the below function (Eq. 1) and the actual voltage response. Only the initial charging phase up to the peak of the voltage response to the hyperpolarizing step was used to find the SSE, which avoided interference with the voltage-sag due to activation of hyperpolarization-activated non-specific cationic currents (I<sub>h</sub>).

$$V_{(t)} = V_o + I_{inj} * R_p + I_{inj} * R_m * (1 - \exp(t/\tau_m)) \quad \text{----- Eq. 1}$$

where,  $V_{(t)}$ ,  $V_o$  are the instantaneous membrane voltage at time  $t$  and offset potential respectively,  $I_{inj}$  is the injected current,  $R_p$ ,  $R_m$  are the pipette and membrane input resistances and  $\tau_m$  the membrane time constant. We here report on an averaged value for each of these passive parameters, based on 19 sweeps, removing values that deviated  $>2 * SD$  from the overall mean. Active membrane properties were measured from the voltage responses (action potentials) during the first action potential of the supra-threshold current step. Spike threshold was assumed as the voltage at which the rate of voltage change ( $dv/dt$ ) crossed 20 V/s (Fig. 27E). Action potential width was calculated by subtracting the time at which the voltage response just rises above the threshold with the time it falls below threshold, both within the duration of the first action potential. Action potential amplitude was calculated as the difference between the absolute peak

of the spike and the threshold. Spike accommodation was computed for sweeps that had two or more spikes, but is not reported here.

The mAHP amplitude (not reported here) was considered as the averaged peak (6 ms window) of the negative voltage response that followed immediately after the end of the depolarizing current step. Latency to mAHP peak was measured as the time difference between the peak of the mAHP and the end of the depolarizing current step. Peak sAHP was measured by averaging (20 ms window) the voltage response after a delay (325 ms) from the mAHP peak (Fig. 27D). Spurious drifts in the values of the sAHP amplitude were manually checked and excluded in case of artifacts. All analyses were done offline using MATLAB (Mathworks MA, USA).

## Statistical analysis

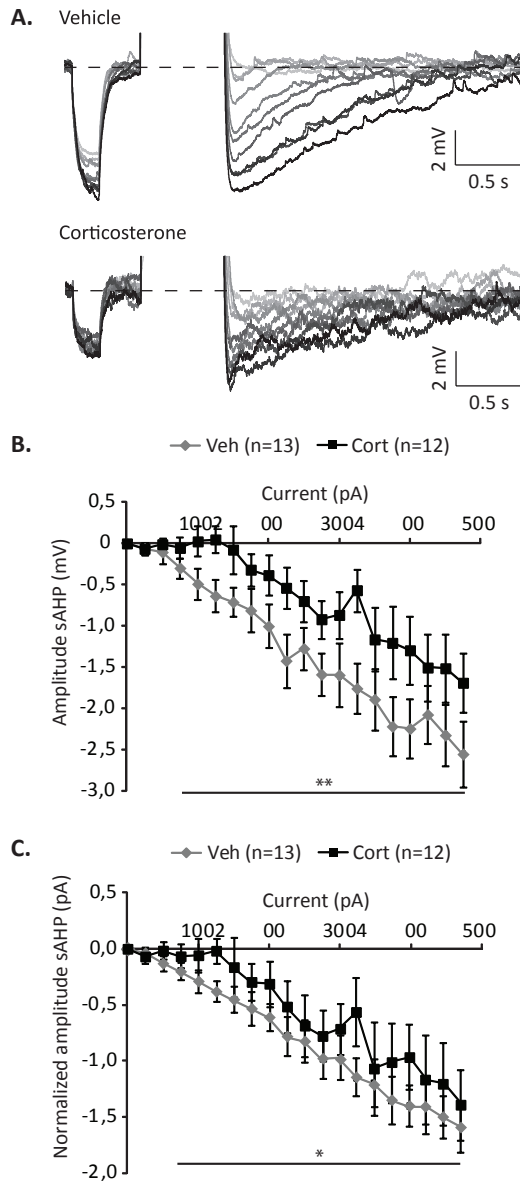
Results are expressed as means  $\pm$  S.E.M. and analyzed for statistical significance ( $p < 0.05$ ) using a Student's *t*-test. In the case of the sAHP amplitude evoked by a depolarizing pulse, data were analyzed with a generalized model for repeated measures.

# RESULTS

## Lateral orbitofrontal cortex

In total, 25 neurons identified under the microscope as pyramidal-like neurons in layers II/III of the lateral OFC were recorded (see Fig. 27B for approximate location). Passive membrane properties were not significantly affected by corticosterone treatment (Table 16). Despite the overall lack of significant corticosterone-dependent effects in the dataset, we nevertheless observed a tendency towards reduced input resistance in cells recorded 1-4 hrs after corticosterone treatment compared to those exposed to the vehicle; a trend that was just not significant ( $p = 0.057$ ). None of the characteristics of the action potential -threshold, height or width- were different between corticosterone and vehicle treated neurons in the OFC (Table 16).

As shown in Figure 28, the amplitude of the sAHP was reduced in neurons that had been exposed to corticosterone 1-4 hrs prior to recording, compared to the vehicle-treated control cells; this reached significance ( $p = 0.010$ ) for current steps ranging from 75 to 475 pA. The amplitude of the sAHP depends on the calcium influx during the preceding current step, which is related to the level of depolarization. The degree of depolarization seen with a particular current injection, in turn, is governed by the input resistance. Given the near-significant difference in input resistance between cells recorded after corticosterone compared to vehicle treatment, we normalized the amplitude of the sAHP for each of the sweeps to the corresponding input resistance. Even when normalized to the input resistance, the sAHP amplitude was significantly decreased in cells treated 1-4 hrs earlier with corticosterone compared to vehicle ( $p = 0.047$  for the range between 75 and 475 pA; Fig. 28).



**Figure 28.** Corticosterone decreases the amplitude of the sAHP in layer II/III OFC cells. **(A)** Typical traces of an OFC pyramidal neuron in response to current steps ranging from 0–475 pA, treated 1–4 hrs earlier with a brief pulse of vehicle (top) or with 100 nM corticosterone (bottom). **(B)** Averaged amplitude of the sAHP for the various current steps after vehicle or corticosterone treatment. Statistical analyses with a general linear model and repeated measures revealed a significant difference between the treatment groups for the current steps between 75 and 475 pA ( $p = 0.010$ ). **(C)** When sAHP amplitude for each current injection was normalized to the input resistance determined for the corresponding sweep (excluding traces where the input resistance deviated  $>2 \times \text{SD}$  from the mean of that cell), the values observed for the corticosterone-treated cells were still smaller than those observed for the vehicle-treated control group ( $p = 0.047$  for the range between 75 and 475 pA).

**Table 16.** Passive and active membrane properties 1-4 hrs after a brief pulse of 100 nM corticosterone or vehicle, recorded in pyramidal neurons in the lateral orbitofrontal cortex or hippocampus

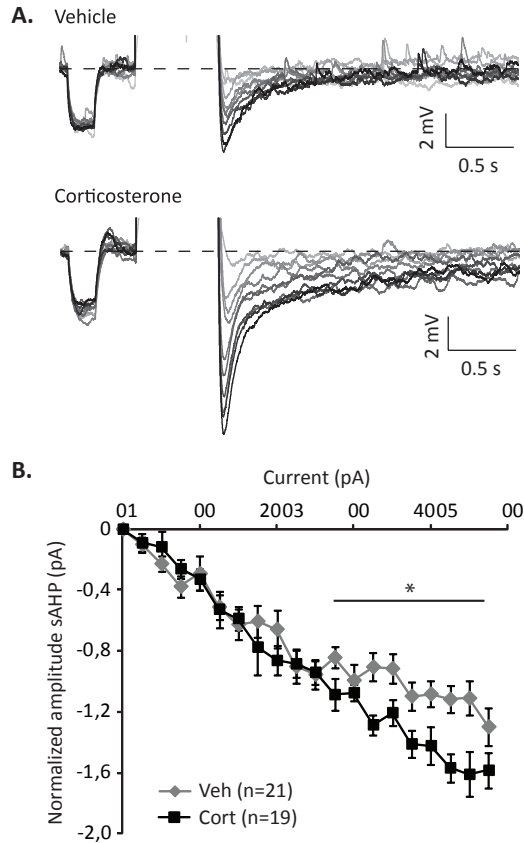
<b>Orbitofrontal cortex</b>			
	<b>Vehicle (n = 13)</b>	<b>Corticosterone (n = 12)</b>	<b>P value</b>
Resting membrane potential (mV)	-74.7 ± 2.1	-73.9 ± 3.0	0.815
Input resistance (MΩ)	168.4 ± 15.8	130 ± 12.7	0.072
Tau (ms)	25.3 ± 2.8	26 ± 1.9	0.819
Action potential threshold (mV)	-39.9 ± 0.9	-41.4 ± 0.8	0.224
Action potential height (mV)	89.9 ± 1.8	91.9 ± 1.6	0.399
Action potential width (ms)	0.67 ± 0.02	0.67 ± 0.03	0.893
<b>Hippocampus</b>			
	<b>Vehicle (n = 21)</b>	<b>Corticosterone (n = 19)</b>	<b>P value</b>
Resting membrane potential (mV)	-71.5 ± 0.9	-72.5 ± 1.4	0.579
Input resistance (MΩ)	226.7 ± 14.1	202.2 ± 18.3	0.296
Tau (ms)	30.9 ± 2.0	26.8 ± 1.5	0.101
Action potential threshold (mV)	-53.1 ± 1.0	-52.0 ± 1.3	0.499
Action potential height (mV)	108.9 ± 0.6	107.4 ± 0.9	0.176
Action potential width (ms)	0.70 ± 0.02	0.69 ± 0.01	0.708

*Mean ± S.E.M. None of the parameters showed significant differences.*

## Dorsal hippocampal CA1 area

In total we recorded 40 identified pyramidal neurons in the dorsal CA1 hippocampal area. Passive and active membrane properties were largely comparable to those described for CA1 pyramidal neurons in earlier studies (Joëls et al. 1989; Kerr et al. 1989; Liebmann et al. 2008; Maggio and Segal 2009). Input resistance, although on average reduced by 11% following corticosterone application (100 nM, 20 min duration; 1-4 hrs prior to recording), did not differ significantly between the two experimental groups, nor did resting membrane potential or the membrane tau (Table 16, bottom). As is evident from Table 16, action potential properties were also not different between the two groups (all  $p > 0.05$ ); thus, comparable values were observed with regard to the threshold for action potential generation, the average height of the action potential and the width of the action potential.

When we analyzed the sAHP amplitude in a comparable manner as done in the OFC, i.e. normalized for the input resistance, a significant increase in the corticosterone compared to vehicle treated group was apparent for the range between 275 and 450 pA ( $p = 0.041$ ; Fig. 29).



**Figure 29.** Corticosterone increases the amplitude of the sAHP in CA1 hippocampal neurons. **(A)** Typical sAHP of a CA1 pyramidal neuron response to current steps ranging from 0–475 pA, treated 1–4 hrs earlier with a brief pulse of vehicle (top) or with 100 nM corticosterone (bottom). **(B)** Averaged amplitude of the sAHP for the various current steps, normalized to the corresponding input resistance, after vehicle or corticosterone treatment. Statistical analyses with a general linear model and repeated measures revealed a significant difference between the treatment groups for current steps between 275 and 450 pA.

## DISCUSSION

The slow gene-mediated effects of corticosterone in the brain, presumably involving the activation of GRs, are well-documented (Joëls et al. 2012). Despite the moderately high expression levels of GR in cortical layers, including the OFC (Reul and de Kloet 1985; de Kloet 1991; Sinclair et al. 2012), corticosteroid actions in this region have been very much understudied. We here report that brief exposure to a relatively high concentration of corticosterone – sufficiently high to activate GRs (Karst et al. 2000) – reduces the amplitude of the sAHP after a period of depolarization, in absolute values or when normalized to the input resistance of the cell; an effect that is opposite in direction to that seen in dorsal CA1 hippocampal neurons. Other properties of OFC and

hippocampal pyramidal neurons, be it active or passive, were not significantly affected by the stress hormone, although the input resistance of OFC neurons tended to be reduced after steroid treatment. The latter requires follow-up studies in larger groups of cells. Overall, the present findings underline that pyramidal cells in the neocortex are affected in their function by slow gene-mediated corticosterone-dependent signaling pathways, as was earlier shown already for subcortical areas such as various subregions of the hippocampal formation (Joëls et al. 1989; Kerr et al., 1989; Kole et al. 2001; Liebmann et al. 2008; Maggio and Segal 2009) and the BLA (Duvarci and Paré 2007; Liebmann et al. 2008).

Recent studies demonstrated that glutamatergic transmission of layer V pyramidal neurons in the prelimbic cortex is sensitive to stress and GR activation (Yuen et al. 2009; Liu et al. 2010). In cultured prefrontal cortical cells, corticosterone, via SGK phosphorylation of GDI at Ser-213, is thought to increase the formation of GDI-Rab4 complexes, and thus to facilitate the functional cycle of Rab4 and Rab4-mediated recycling of AMPA receptors to the synaptic membrane (Liu et al. 2010; Yuen et al. 2011). This would enhance surface expression of AMPA receptors, explaining the enhanced AMPA (and NMDA) responses observed in prepubertal rats several hours after stress (Yuen et al. 2009), in addition to other changes in glutamatergic transmission in this same region (Caudal 2010; Usazzi et al. 2010). A highly comparable slow, GR-mediated increase in AMPA receptor surface expression and in the amplitude of spontaneous AMPA-receptor mediated postsynaptic currents has also been observed in hippocampal neurons of adult mice (Karst et al. 2005; Groc et al. 2008; Martin et al. 2009), pointing to a similarity in the response of layer V prelimbic and dorsal CA1 hippocampal neurons in this respect.

Apart from the effects of stress and corticosterone on layer V prelimbic neurons, the effect of corticosteroid hormones on electrical activity of other neurons in the frontal cortex was mostly unknown, except for a short report on GABAergic transmission (Hill et al. 2011). We here focused on the lateral OFC since neurons in this region show enhanced dendritic length after chronic stress exposure (Liston et al. 2006), a phenomenon that was similar to that observed for the BLA (Vyas et al. 2002). With respect to corticosteroid actions on the sAHP amplitude, BLA neurons respond very similarly to neurons in the ventral-most part of the CA1 hippocampal area (Duvarci and Paré 2007; Liebmann et al. 2008; Maggio and Segal 2009), but oppositely to what was seen in the dorsal CA1 and CA3 region. We therefore hypothesized that an acute, single exposure of OFC cells to corticosterone may result in effects that resemble those reported for BLA neurons and ventral hippocampal cells, rather than for cells in the dorsal hippocampus. This indeed appeared to be the case. The available data, although sparse, therefore suggest that exposure of the brain to a wave of corticosteroid hormones, such as happens after stress, leads to regionally differentiated electrical responses. There seem to be at least two systems that differently respond to corticosteroid exposure: on the one hand pyramidal neurons in the dorsal CA1 area and in layer V of the medial prefrontal cortex; and on the other hand principal neurons in the BLA, ventral-most part of the CA1 region and the lateral OFC.

At this moment we can only speculate why layer II/III OFC neurons respond in a different manner to corticosterone than CA1 dorsohippocampal neurons do. It seems unlikely that the involvement of a different receptor for corticosterone is responsible for the discrepancy. In CA1 neurons the effects of corticosterone on the sAHP are caused by activation of the GR (Joëls and de Kloet 1989; Kerr et al. 1989). OFC neurons express moderately high levels of GR but only low levels of MRs (Reul and de Kloet 1985; de Kloet 1991; Sinclair et al. 2012). Also, (nuclear) MRs are expected to be already extensively activated, even by the very low doses of corticosterone that circulate when slices are prepared from mice killed under rest and at the circadian nadir. Administration of a high dose of corticosterone will therefore almost exclusively activate GRs and not MRs. Downstream of GR activation however, regional differences may well explain why corticosteroid exposure of OFC cells results in an effect that differs from that seen in the dorsal CA1 area. For instance, granule neurons in the dentate gyrus show no effect of corticosterone on the amplitude of L-type calcium currents, despite their high GR expression levels (Van Gemert et al. 2009), due to a process between transcriptional and translational control of calcium channel subunits. Principal neurons in the BLA do show increased L-type calcium current amplitudes to GR activation (Karst et al., 2002), but this is not translated into a larger sAHP amplitude (Duvarci and Paré 2007; Liebmann et al. 2008). Preliminary evidence suggests that this may relate to the type of calcium channel subunits expressed by BLA versus CA1 hippocampal neurons (Liebmann et al. 2008). Local characteristics of translational regulators, ion channel subunits, or intracellular proteins may therefore cause large differences in the response of neurons to steroid hormones.

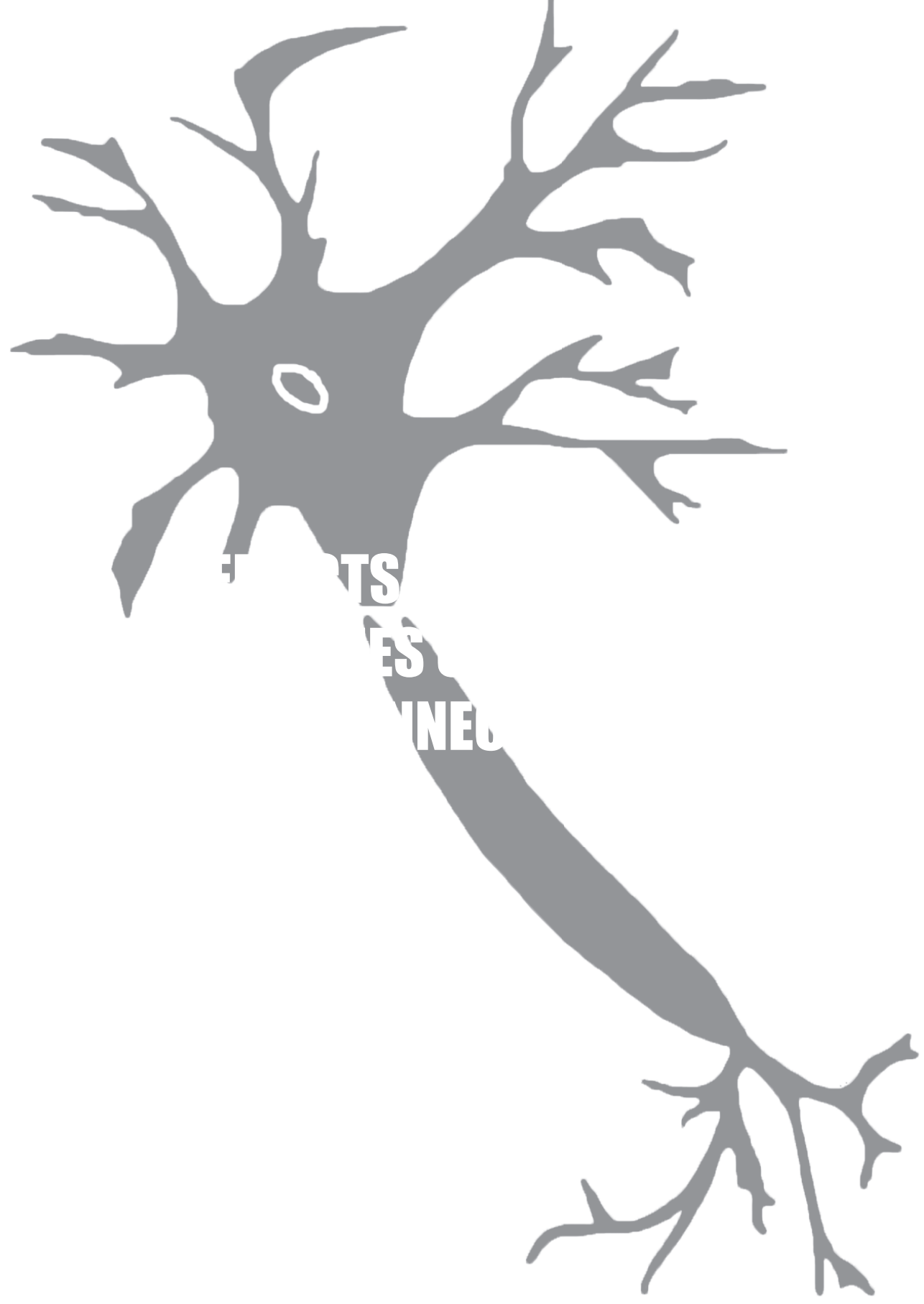
The fact that a brief pulse of corticosterone exerts long-lasting effects on OFC neuronal properties may have behavioral consequences. In rodents, the OFC has been implicated in the inhibition of many forms of behavior, including both impulsive and compulsive forms (Eagle and Baunez 2010). OFC lesions have been shown to induce impaired reversal learning (Schoenbaum et al. 2003; McAlonan and Brown 2003) and failure to adjust behavior; rats remained responding to a conditioned stimulus when the rewards predicted by the stimulus was devalued (Gallagher et al. 1999). The view that the OFC is involved in anticipating the value of actions and using this information to guide behavior is receiving growing empirical support (Cardinal et al. 2002). However, the OFC might be particularly specialized for simple (emotional) responses, such as fear and aggression, through its role in representing primary reinforcement or punishment (Rudebeck et al. 2008). Layer II/III pyramidal cells specifically, are both the origin and target of long-range corticocortical connections and are likely to play an important computational role in cognitive (e.g., attentional) functions mediated by a distributed network of structures (Dehaene et al. 1998). In humans, different roles for the anterior-posterior and lateral-medial divisions of the OFC in reinforcement learning and decision-making have been extensively shown and discussed (Elliott et al. 2000; Frank and Claus, 2006; Kable and Glimcher 2009; Kringelbach 2005; Mar et al. 2011; McClure et al. 2004; Noonan et al. 2010; O'Doherty et al. 2001; Rushworth et al. 2011; Sescousse et al. 2010; Windmann et al. 2006). The lateral OFC has been implicated in dealing

with unsteady or irregular patterns of rewarded actions, valuing punishments and adjusting choice behaviour as contingencies change, with a focus on distant or long-term rewards. Interestingly, activity of the OFC has been shown to be altered during stress exposure and to relate to the subsequent cortisol response to the stressor (Wang et al. 2005; Pruessner et al. 2008; Dedovic et al. 2009b). Abnormalities in OFC function and structure have also been related to stress-related mental disorders such as post-traumatic stress disorder (PTSD) (Liberzon et al., 2007; Carrion et al. 2008; Hakamata et al. 2007; Thomaes et al. 2010) and major depression (Koolschijn et al. 2009; Holsen et al. 2011) and to early life stress exposure (Hanson et al. 2010; Dannlowski et al. 2012). The observation that principal neurons in the OFC are clearly affected by corticosterone in a time-frame that is relevant for the aftermath of stress, together with the findings that these neurons are changed in morphology after a prolonged period of repetitive stress (Liston et al. 2006) and the observed relevance to psychopathology, urges more detailed investigation of both acute and longer-term stress exposure on OFC function.

## **ACKNOWLEDGEMENTS**

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# **Corticosteroid induced decoupling of the amygdala in men**

# **4.1**

**Marloes J.A.G. Henckens, Guido A. van Wingen,  
Marian Joëls, and Guillén Fernández**

**Cerebral Cortex 22(10): 2336-45 (2012)**

# 4.1

## ABSTRACT

The amygdala is a key regulator of vigilance and heightens attention towards threat. Its activity is boosted upon threat exposure and contributes to a neuroendocrine stress response via the hypothalamic-pituitary-adrenal (HPA) axis. Corticosteroids are known to control brain- as well as HPA-activity by providing negative feedback to the brain. However, it is unknown how corticosteroids affect the neural circuitry connected to the amygdala. Implementing a randomized, double-blind, placebo-controlled design, we here investigated the effects of 10 mg hydrocortisone on amygdala-centered functional connectivity patterns in men using resting state functional MRI. Results showed generally decreased functional connectivity of the amygdala by corticosteroids. Hydrocortisone reduced positive functional coupling of the amygdala to brain regions involved in the initiation and maintenance of the stress-response; the locus coeruleus, hypothalamus, and hippocampus. Furthermore, hydrocortisone reduced negative functional coupling of the amygdala to the middle frontal and temporal gyrus; brain regions known to be involved in executive control. A control analysis did not show significant corticosteroid modulation of visual cortex coupling, indicating that the amygdala decoupling was not reflecting a general reduction of network connectivity. These results suggest that corticosteroids may reduce amygdala's impact on brain processing in the aftermath of stress in men.

## INTRODUCTION

Corticosteroids are potent modulators of human cognitive function. The hormones are released in response to stress as the end product of the hypothalamic-pituitary-adrenal (HPA) axis, and known to readily cross the blood-brain-barrier to affect brain processing (McEwen 1979). Corticosteroids exert their actions upon binding of the mineralocorticoid (MR) and glucocorticoid receptor (GR), which are abundantly expressed in the brain (de Kloet 1991; Reul and de Kloet 1985; Sapolsky et al. 1983). By binding to these receptors, corticosteroids control the excitability of neuronal networks under rest, resulting in tonic inhibition of HPA axis activity (De Kloet and Reul 1987), but also during exposure to stress, contributing to behavioural adaptation (de Kloet et al. 1999). An additional prominent function of corticosteroids is to exert negative feedback on the HPA-axis after stress exposure, which makes the hormones crucial for the limitation and termination of the stress response (De Kloet and Reul 1987). This negative feedback is primarily established by direct inhibition of the core structures of the HPA-axis itself, the pituitary and the paraventricular nucleus (PVN) of the hypothalamus (Herman and Cullinan 1997), but the hippocampus has also been proposed to contribute to this negative feedback (de Kloet et al. 1993). Furthermore, corticosteroids are thought to provide both support and regulation of the sympatho-adrenomedullary (SAM) system, which subserves the initial ‘fight-or-flight’ response to threat. Activation of the SAM system occurs immediately upon threat exposure, and induces an elevation of central norepinephrine (NE) levels through increased tonic activity of the pontine locus coeruleus (LC) (Valentino and Van Bockstaele 2008). SAM-activation thereby induces a hypervigilant state of processing that optimizes the detection and assessment of threats by prioritizing sensory processing (Henckens et al. 2009; Shackman et al. 2011; van Marle et al. 2009), while suppressing higher-order executive function (Arnsten 2009; Diamond 2007; Qin et al. 2009). Although this response serves a clear adaptive purpose, sustained activation of vigilance-related brain circuits may become maladaptive and culminate in mental diseases such as depression (Siever and Davis 1985), and proper regulation is of critical importance to human mental health.

One of the main targets of the SAM-system and mediators of the initial surge in vigilance is the amygdala (de Kloet et al. 2005; Phelps and LeDoux 2005; van Marle et al. 2009). Its dense connectivity pattern places it at the center of the brain’s emotional processing network as a physical hub linking numerous distant regions, allowing emotions to influence brain processing from the first stages of perception (Vuilleumier and Driver 2007) to the regulation of social behavior (Adolphs 2010). It is reciprocally connected to a frontal executive system, which is on the one hand involved in the control of this emotional processing state (Phillips et al. 2003), but on the other hand subjected to influences of this emotional state. Previous imaging studies have shown that acute or prolonged stress increase amygdala reactivity (van Marle et al. 2009; van Wingen et al. 2011a), impair higher executive function (Arnsten 2009; Diamond 2007; Qin et al. 2009), and strengthen amygdala’s connectivity to the other regions of the vigilance-network, such

as the LC (Seeley et al. 2007; van Marle et al. 2010; van Wingen et al. 2011b, 2012). At the same time, animal and human studies have demonstrated that corticosteroids can reduce amygdala sensitivity (Henckens et al. 2010; Karst et al. 2010), suggesting that corticosteroids play a critical role in the restoration of homeostasis by normalizing/desensitizing brain processing following stress exposure (de Kloet et al. 2005). Corticosteroids might therefore also have an effect opposite to that of acute stress on amygdala connectivity, but this issue remains to be resolved.

Here, we investigated the effect of corticosteroids on amygdala-centered connectivity patterns in men during rest. We implemented a randomized, double-blind, placebo-controlled design, in which 48 male subjects received either placebo or 10 mg hydrocortisone prior to resting-state functional MRI. Functional connectivity was evaluated by exploring correlations in BOLD signal fluctuations over time between brain areas, enabling us to map patterns of connectivity under rest. Given the key role of the amygdala in the stress response, we used a seed-region approach correlating fluctuations in amygdala activity over time to the rest of the brain, and tested the hypothesis that corticosteroids affect amygdala connectivity, especially to regions involved in the initiation and regulation of the stress response, including the LC, hypothalamus (PVN), hippocampus, and the frontal regions exerting executive control. To check the specificity of these effects we included a control seed region analysis for the primary visual cortex, testing whether corticosteroids induced any general effects on network connectivity.

## **MATERIALS & METHODS**

### **Participants**

Forty-eight young (age range 19-28, median 21), right-handed, healthy male volunteers gave written informed consent to participate in the study. Women were excluded from participation for several reasons. First of all, functioning of the amygdala in women seems to differ from that in men; both amygdala responsivity (Cahill et al. 2004) and connectivity (Kilpatrick et al. 2006) are different between sexes. Furthermore, previous research has indicated that women respond differently to hydrocortisone than men, both in behavior (Andreano and Cahill 2006; Bohnke et al. 2010) and brain activation (Merz et al. 2010; Stark et al. 2006). We presently focused on men, allowing easier comparison with the results from an earlier study in which subjects were exposed to stress (Henckens et al. 2009), a situation that is known to induce more stable neuroendocrine response in men than in women (Bouma et al. 2009; Kajantie and Phillips 2006; Kirschbaum et al. 1999; Ossewaarde et al. 2010). Furthermore, individuals who met any of the following criteria were excluded from participation: history of head injury, autonomic failure, history of or current psychiatric, neurological, or endocrine disorders, current periodontitis, acute inflammatory disease, acute peptic or duodenal ulcers, regular use of corticosteroids, treatment with psychotropic medications, narcotics, beta-blockers, steroids, or any other medication that affects central nervous system or endocrine systems, medical illness within the three weeks

prior to testing, self reported mental or substance use disorder, daily tobacco or alcohol use (or experienced inconvenience in refraining from these activities for three days), exercising at the professional level, regular night shift work, or current stressful episode or major life event. Three participants were excluded from analyses because of unreliable cortisol manipulation (abnormal basal cortisol levels (1 x placebo) or no elevation in salivary cortisol level in response to CORT intake (2 x CORT)), and another two participants because of fMRI data drop-out (2 x CORT). Thus, the results are based on data of 23 men in the placebo group and 20 in the CORT group. The study was approved by the local ethics committee (CMO region Arnhem-Nijmegen, Netherlands) and executed in accordance with the declaration of Helsinki.

## Study design

**Prior to arrival.** Prior to inclusion all eligible participants received an extensive information brochure, listing all in- and exclusion criteria and roughly explaining the setup of the experiment. If criteria were met (according to the participant's own insights), an appointment was made. To minimize differences in baseline cortisol levels we instructed participants not to use any recreational drugs for three days and to refrain from drinking alcohol, exercising, and smoking for 24 h prior to the appointment. Furthermore, participants were requested not to brush their teeth, floss, or eat and drink anything but water for one hour prior to the session enabling adequate saliva sampling for cortisol assessment. They were asked to take a light lunch and do so no later than one hour before arrival; their lunch could not contain any citrus products, coffee, tea, milk or sweets (Maheu et al. 2005). Throughout the entire study period, participants were only given water to drink, except for a scheduled lunch at  $t = -120$  min.

**Arrival.** To minimize individual differences due to daily activities and to reduce the impact of diurnal variation in cortisol levels, all participants were invited to the laboratory in the early afternoon, between 12:00-13:00 h. Upon arrival, participants received an information brochure about the procedure, they gave written informed consent, and completed an intake questionnaire to ensure that in- and exclusion criteria were met. Furthermore, participants were asked to complete a first Profile of Mood States (POMS) questionnaire (de Groot 1992; Reddon et al. 1985; Wald and Mellenbergh 1990). During the entire waiting period (~3.5 h) prior to scanning, participants had to wait in a quiet room where they were free to conduct any activities except for anything potentially arousing. At specific time points, the experimenter entered to room to take a saliva sample. At 105 min prior to the resting-state scan (at  $t = 0$ ) participants were asked to complete a second POMS questionnaire and received the drug capsule. Drug administration occurred in a randomized, double-blind, placebo-controlled manner in which participants received either 10 mg CORT or placebo (cellulose), depending on the group to which the participant was (randomly) assigned. Capsules were administered orally. This dose of hydrocortisone is known to elevate salivary cortisol levels to moderate-to-high stress levels (Groschl et al. 2002; Henckens et al. 2010; Kirschbaum et al. 1996; Tops et al. 2003).

At about 4.5 h after arrival participants were taken to the scanner room. The resting-state run

started 105 min after administration of the capsule and lasted for 8 min. Participants were asked to lay as still as possible, close their eyes, and think of nothing in particular. They were instructed to relax, but not fall asleep, which was checked by verbal debriefing immediately afterwards. The session ended with a structural scan.

## Physiological and psychological measures

**Saliva collection and analysis.** Cortisol levels were measured from saliva at ten time points: two baseline measurements at the beginning of the experimental day ( $t = -225, -210$  min), and eight samples thereafter ( $t = -180, -150, -120, 0, 30, 60, 90, \text{ and } 120$  min) to assess cortisol changes throughout the experiment. Saliva was collected using a commercially available collection device (Salivette®, Sarstedt, Germany). For each sample, the participant first placed the cotton swab provided in each Salivette tube in his mouth and chewed gently on it for 1 min to produce saliva. The swab was then placed back in the salivette tube, and the samples were stored in a freezer at  $-25^{\circ}\text{C}$  until assayed. Laboratory analyses were performed at the Department of Biopsychology, TU Dresden, Germany. After thawing, salivettes were centrifuged at 3,000 rpm for 5 min, which resulted in a clear supernatant of low viscosity. Salivary free cortisol concentrations were subsequently measured using a commercially available chemiluminescence-immuno-assay (CLIA) with high sensitivity of 0.16 ng/mL (IBL, Hamburg, Germany).

**Heart rate.** Cardiac rhythm of the participants was measured during scanning using a pulse oximeter placed on their left index finger. Participants were instructed to keep their hands as still as possible during the measurement. After the completion of scanning, in-house software was used for offline artifact correction and the analysis of heart rate signal, calculating heart rate frequency (HRF) and heart rate variability (HRV). The HRF was calculated as  $60/\text{mean interbeat interval}$  and HRV as the root mean squares of successive differences (rMSSD) between successive interbeat intervals. This method assesses high-frequency variability in HR, which is thought to result from parasympathetic action mainly and is expected to show a decrease as a function of stress (Berntson et al. 1997; Goedhart et al. 2007).

**Mood state.** To exclude potential psychological side-effects of hydrocortisone administration, mood state was assessed using the Profile of Mood States (POMS) questionnaire (de Groot 1992; Reddon et al. 1985; Wald and Mellenbergh 1990) at three time points: at the beginning of the experiment ( $t = -225$  min), just prior to the intake of the capsule ( $t = 0$  min), and at the end of the experiment ( $t = 120$  min).

## Physiological and psychological statistical analysis

Behavioral and physiological data were analyzed in SPSS 15.0 (SPSS, Inc., Chicago, IL, USA) using repeated measured ANOVAs with drug condition (placebo vs. CORT) as between subject factor. Due to the high levels of skewness and kurtosis of the POMS questionnaire (de Groot 1992; Reddon et al. 1985; Wald and Mellenbergh 1990), mood data were analyzed using non-parametric

tests. Changes over time in mood state were assessed by Friedman tests, and Mann-Whitney U tests were used to assess potential drug effects on mood. Alpha was set at 0.05 throughout.

## **MRI acquisition**

Participants were scanned by a Siemens (Erlangen, Germany) MAGNETOM Avanto 1.5 Tesla MRI scanner equipped with an 8-channel head coil. A series of 265 blood oxygenation level dependent (BOLD)  $T_2^*$ -weighted gradient echo EPI images ( $\approx 8$  min) was acquired with the following parameters: TR = 1870 ms, TE = 35 ms, FA =  $80^\circ$ , 39 axial slices approximately aligned with AC-PC plane, slice matrix size =  $64 \times 64$ , slice thickness = 3 mm, slice gap = 0.35 mm, FOV =  $224 \times 224$  mm<sup>2</sup>. Owing to its relatively short TE, this sequence yields optimal contrast-to-noise ratio in the medial temporal lobes (Stocker et al. 2006).

## **fMRI data analysis**

Data were analyzed using Statistical Parametric Mapping software (SPM5; UCL, London). The first five EPI-volumes were discarded to allow for  $T_1$ -equilibration. Prior to analysis, the images were motion corrected using rigid body transformations and least sum of squares minimization. Subsequently, they were temporally adjusted to account for differences in sampling times across different slices. All functional images were then co-registered with the high-resolution  $T_1$ -weighted structural image using normalized mutual information maximization. The anatomical image was subsequently used to normalize all scans into MNI152 (Montreal Neurological Institute) space. All functional images were resampled to a voxel size of 2 mm isotropic. Finally, all images were smoothed with an isotropic 8-mm full-width-at-half-maximum Gaussian kernel in order to accommodate residual functional/anatomical variance between subjects.

Next, we extracted the amygdala time course using an anatomical mask that was created based on the locus of previously observed corticosteroid effects on amygdala responsivity (4 mm sphere around the peak coordinates [ $x = -28, y = -4, z = -12$ ] and [ $x = 26, y = -4, z = -12$ ]) (Henckens et al. 2010). Second, the first eigenvariate of the set of time courses from voxels comprising the amygdala was calculated for each subject. The resulting time series was used as a covariate of interest in a whole-brain, linear regression, statistical parametric analysis. Correlating this pattern of activity (the time series) to that observed in the rest of the brain, provides information on regions that are 'coupled' in activity and supposedly functionally connected. Besides this regressor the amygdala time-course, the realignment parameters, consisting of six parameter rigid body transformations (3 translations and 3 rotations) used for motion correction, were additionally included to model potential movement artifacts. Furthermore, global fluctuations, originating presumably from such systemic effects as respiration and cardiac-induced pulsations (Birn et al. 2006; Macey et al. 2004), were accounted for by extracting signal from individually defined white matter-, grey matter-, and cerebrospinal fluid (CSF)-masks and including these in the model. Masks were generated by segmenting the high-resolution structural images in SPM5 and down-sampling the obtained white matter and CSF masks to the same resolution as the functional data.

Contrast parameter images for the seed region covariate generated at the single subject level were then submitted to 2nd level random effects analysis.

To evaluate whether any corticosteroid effects on amygdala connectivity were related to general corticosteroid effects on brain connectivity, we performed the same analyses on a control seed region consisting of the primary visual cortex defined by a 4 mm sphere (similar to the amygdala) around the probabilistic cytoarchitectonic center of Brodmann area 17, the occipital cortex (hOC1) ( $[x = -10, y = -89, z = 8]$  and  $[x = 15, y = -85, z = 8]$ ) (Amunts et al. 2000).

Statistical parametric maps were created within SPM5 using a two sample t-test contrasting the CORT group versus the placebo group. Statistical tests were family-wise error (FWE) rate corrected ( $p < 0.05$ ) for multiple comparisons at the voxel level for main effects of amygdala coupling across drug conditions, and on the cluster-level using a height threshold of  $p < 0.005$  to assess cortisol effects. Correction for multiple comparisons was done across the entire brain, or for the search volume for regions of interest (ROIs) using a small volume correction. Given our *a priori* hypotheses on corticosteroid modulation, the LC, hypothalamus (PVN), hippocampus and frontal cortex were targeted as ROI's in our analysis of amygdala coupling. Specifically, we implemented a reduced spherical search volume (5 mm radius) around anatomically defined center coordinates for the LC (Astafiev et al. 2010) and hypothalamus, which was centered on the PVN (Baroncini et al. 2012). Data concerning the hippocampus and prefrontal cortex were corrected for reduced search regions through anatomical masks as defined by the WFU PickAtlas Tool (version 2.4) (Maldjian et al. 2003). In analyzing potential effects of corticosteroids on primary visual cortex coupling, four other ROI's were selected, based on their known dense connectivity pattern of these regions to the primary visual cortex, and their role in visual processing (Lowe et al. 1998; McIntosh et al. 1994). Data concerning the cuneus, calcarine, lingual gyrus, and fusiform gyrus were corrected for reduced search regions through anatomical masks as defined by the WFU PickAtlas Tool (version 2.4) (Maldjian et al. 2003). Furthermore, the targeted ROI's for amygdala coupling, i.e. the LC, hypothalamus (PVN), hippocampus and prefrontal cortex, were included into this analysis.

Visualizations of activations were created in SPM5 by superimposing statistical parametric maps thresholded at  $p < 0.001$  uncorrected (unless specified otherwise) onto a canonical  $T_1$ -weighted image in a standard MNI 152 space.

## RESULTS

### Physiological and psychological measures

As expected, oral administration of 10 mg hydrocortisone increased salivary cortisol levels to those observed during moderate to high levels of stress (Morgan et al. 2000; Schommer et al. 1999) (Table 17), which was evidenced by a significant main effect of group ( $F(1,41) = 55.34$ ,  $p < 0.001$ ) and a time x group interaction ( $F(9,33) = 16.46$ ,  $p < 0.001$ ). Groups did not differ on baseline cortisol levels ( $F(1,41) = 1.50$ , n.s). Increased levels were observed from 30 min post-

**Table 17. Cortisol manipulation**

Time	Salivary cortisol (nmol/L)	
	Placebo	Hydrocortisone
t = -225 min	9.58 (0.77)	11.80 (1.40)
t = -210 min	8.49 (0.74)	9.91 (1.32)
t = -180 min	6.29 (0.43)	7.74 (1.05)
t = -150 min	6.07 (0.60)	7.38 (0.90)
t = -120 min	5.50 (0.59)	6.14 (0.72)
t = 0 min	7.87 (0.75)	5.85 (0.45)
t = 30 min	7.06 (0.64)	32.64 (6.51)***
t = 60 min	7.48 (0.84)	25.71 (2.21)***
t = 90 min	6.47 (0.69)	25.62 (1.96)***
t = 120 min	5.60 (0.65)	24.51 (1.90)***

*The resting state scan was recorded at t = 105 min.*

*Mean values (S.E.M.) \*\*\*:  $p < 0.001$*

administration onwards (t = 30 min), and the levels remained similarly elevated for at least 90 min (t = 120 min). Thus, during resting state fMRI scanning (at t = 105 min) the CORT group displayed significantly higher cortisol levels than the control group.

Post-experiment debriefing showed that participants were unable to identify the substance received. As expected, hydrocortisone administration did not affect autonomic measures of heart rate frequency (main effect of drug:  $T(39) = -1.73$ , n.s.) and heart rate variability ( $T(39) = 1.24$ , n.s.) (Table 18). Further, drug administration did not affect mood as assessed three times during the experiment using the Profile of Mood States (POMS) questionnaire (de Groot 1992; Reddon et al. 1985; Wald and Mellenbergh 1990) (Table 18). Although significant reductions in levels of anger scores ( $\chi^2(2) = 11.22$ ,  $p = 0.004$ ), vigor scores ( $\chi^2(2) = 45.72$ ,  $p < 0.001$ ), tension scores ( $\chi^2(2) = 13.27$ ,  $p < 0.001$ ), and close to significant reductions in depression scores (Friedman's ANOVA;  $\chi^2(2) = 5.78$ ,  $p = 0.056$ ) were observed over the course of the experiment, and levels of fatigue increased ( $\chi^2(2) = 36.01$ ,  $p < 0.001$ ), none of these factors was affected by drug administration (all  $p$ 's  $> 0.05$ ). Groups did also not differ on baseline levels of these mood measures at intake (all  $p$ 's  $> 0.05$ ). Hence, differences in brain activity found between drug conditions cannot readily be explained by any physiological or psychological side effects of hydrocortisone, nor because of initial group differences in physiological or psychological traits.

## Amygdala coupling

To investigate the effect of corticosteroids on amygdala connectivity, we analyzed resting-state data using a seed region approach. First, brain regions were identified that were functionally coupled, i.e. displaying significantly correlated patterns of activity, to the amygdala across both experimental groups (taking the CORT and control groups together). Spontaneous activity in the amygdala positively predicted spontaneous activity in a large activation cluster covering

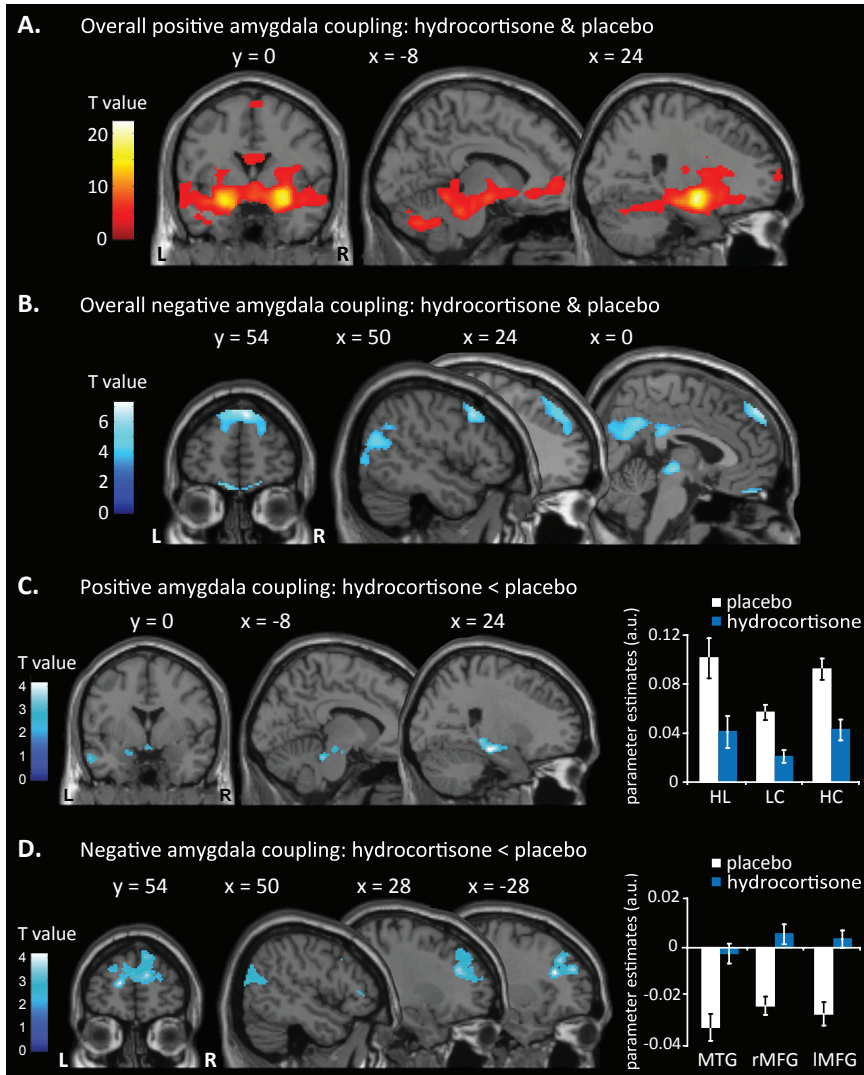
Table 18. Physiological and psychological measures

	Placebo	Hydrocortisone
<b>Mood state</b>		
Depression score 1 (t = -225 min)	0.26 (0.13)	0.85 (0.42)
2 (t = 0 min)	0.09 (0.06)	0.65 (0.39)
3 (t = 120 min)	0.04 (0.04)	0.50 (0.24)
Anger score 1 (t = -225 min)	0.61 (0.23)	1.40 (0.44)
2 (t = 0 min)	0.30 (0.19)	0.50 (0.26)
3 (t = 120 min)	0.22 (0.18)	0.60 (0.27)
Fatigue score 1 (t = -225 min)	1.17 (0.30)	1.75 (0.56)
2 (t = 0 min)	1.35 (0.44)	1.80 (0.56)
3 (t = 120 min)	3.52 (0.67)	4.80 (0.65)
Vigor score 1 (t = -225 min)	12.65(0.79)	10.85 (0.89)
2 (t = 0 min)	10.43 (0.68)	9.50 (0.82)
3 (t = 120 min)	7.57 (0.88)	5.45 (0.88)
Tension score 1 (t = -225 min)	1.00 (0.27)	1.50 (0.33)
2 (t = 0 min)	0.35 (0.13)	1.00 (0.39)
3 (t = 120 min)	0.26 (0.16)	0.60 (0.22)
<b>Heart rate frequency (BPM)</b>	54.91 (1.41)	58.45 (1.48)
<b>Heart rate variability (ms)</b>	96.16 (9.19)	80.30 (8.73)

Mean values (S.E.M.). The resting state scan was recorded at t = 105 min.

the bilateral amygdala itself, the brain stem (including the LC), hippocampus, hypothalamus, parahippocampal gyrus, temporal pole, pallidum, putamen, insula, and inferior frontal cortex. Other regions positively predicted by amygdala activity included the fusiform, ACC, middle orbitofrontal cortex, and regions within the cerebellum ( $p(\text{corr}) < 0.05$ , see Table 19, Fig. 30A). Conversely, amygdala activity was negatively associated with activity in frontal and posterior regions such as the middle frontal gyrus, medial superior frontal gyrus, superior frontal gyrus, middle temporal gyrus, cuneus, brain stem, and cerebellum (Table 19, Fig. 30B). Overall, these patterns of functional connectivity are in line with previous studies (Roy et al. 2009; van Marle et al. 2010) and support models of emotion processing that suggest reciprocal ventral and dorsal systems (Phillips et al. 2003).

Second, when contrasting the CORT and the control group, we found reduced correlated activity of the amygdala with two regions critically involved in the initiation of the stress response; the LC ( $[x = -8, y = -36, z = -22]$   $T(41) = 3.61$ ,  $p(\text{SVC}) = 0.047$ ) and the hypothalamus ( $[x = 0, y = 0, z = -14]$   $T(41) = 2.99$ ,  $p(\text{SVC}) = 0.044$ , Table 19, Fig. 30C). The peak coordinates of the cluster observed in the hypothalamus seemed to co-localize with the location of the PVN ( $[x = 2, y = 1, z = -12]$  Baroncini et al. 2012), which is the expected target for corticosteroid-mediated negative feedback (Herman and Cullinan 1997).



**Figure 30.** Functional connectivity pattern of the bilateral amygdala during resting state and its modulation by corticosteroids. **(A)** Amygdala's spontaneous activity was positively correlated to an extended network of emotional processing regions, encompassing the brain stem (including the LC), hypothalamus, medial temporal lobe (including the hippocampus), insula, inferior frontal cortex, and the ACC. **(B)** Regions in frontal and posterior brain areas exerting executive control, such as the middle and superior frontal gyrus, displayed negative correlations with activity patterns of the amygdala during rest. **(C)** Hydrocortisone intake attenuated the positive coupling between the amygdala and the hypothalamus (HL), locus coeruleus (LC), and hippocampus (HC) (all  $p(\text{SVC}) < 0.05$ ). **(D)** The negative coupling between the amygdala and regions within middle temporal gyrus (MTG) and the bilateral middle frontal gyrus (rMFG, right; lMFG, left) was also reduced under levels of high cortisol (all  $p(\text{corr}) < 0.05$ ). Error bars represent S.E.M. See Table 18 for exact statistical tests. For visualization purposes the statistical parametric maps of Fig. 30A and 30B are thresholded at  $p < 0.05$  FWE whole brain corrected at voxel level. Fig. 30C and 30D are thresholded at  $p < 0.01$  uncorrected with a minimal cluster-size of 25 voxels (small-volume corrected clusters; Fig. 30C) and 250 voxels (whole-brain corrected clusters; Fig. 30D) respectively.

**Table 19. Peak voxels and corresponding T values of significantly activated clusters that show functional coupling with the bilateral amygdala for both groups combined and for main effects of hydrocortisone**

Region	Cluster size	MNI Coordinates			Peak
		x	y	z	T-value
Positive coupling amygdala					
Extended cluster covering the bilateral amygdala, brain stem (incl. LC), hippocampus, hypothalamus, parahippocampal gyrus, temporal pole, pallidum, putamen, insula, and inferior frontal cortex	6323***	28	-6	-12	22.30
Putamen, L	11**	-26	10	8	6.22
	5**	36	6	14	6.16
Fusiform, L	1*	-34	-36	-14	5.82
Anterior cingulate cortex	13***	-6	50	0	6.20
	12***	-4	30	-6	5.98
Middle orbitofrontal gyrus	110***	4	48	2	7.52
Superior frontal cortex, R	1*	16	70	12	5.83
Cerebellum 6, L	7**	-32	-52	-26	6.18
Cerebellum 6, R	52***	32	-50	-26	6.50
Cerebellum 8, L	62***	-4	-68	-30	7.33
Cerebellum 9, L	3*	-6	-56	-32	6.04
Cerebellum 9, R	3*	10	-58	-34	5.98
Cerebellum Crus1, L	3**	-42	-50	-30	5.86
Cerebellum Crus1, R	2*	46	-50	-34	5.74
Negative coupling amygdala					
Cuneus	30***	0	-94	16	6.69
Medial superior frontal gyrus	148***	8	52	44	7.28
Middle frontal gyrus, R	74***	48	18	42	6.72
Superior orbitofrontal cortex, R	4**	12	46	-22	6.05
Cerebellum	13***	-12	-50	-24	6.91
	1*	20	-34	-46	6.00
Brain stem	24***	-2	-18	-4	6.60
Negative main effect of hydrocortisone					
Hypothalamus	5 <sup>#</sup>	0	0	-14	2.99
Hippocampus, R	335 <sup>+</sup>	24	-22	-14	4.15
Locus Coeruleus	4 <sup>#</sup>	-8	-36	-22	3.13
Positive main effect of hydrocortisone					
Middle frontal gyrus, L	574 <sup>+++</sup>	-28	30	24	4.42
Middle frontal gyrus, R	1142 <sup>+++</sup>	32	34	24	4.45
Middle temporal gyrus, R	320 <sup>+</sup>	58	-66	20	4.16

MNI, Montreal Neurological Institute; R, right; L, left. All effects are analyzed using cluster-level statistics. \*\*\*:  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*:  $p < 0.05$  (whole brain corrected, height threshold at  $p < 0.05$  FWE corrected at voxel level); +++:  $p < 0.001$ ; +:  $p < 0.05$  (whole brain corrected, height threshold at  $p < 0.005$  uncorrected at voxel level); #:  $p < 0.05$  (small-volume corrected for region of interest, height threshold at  $p < 0.005$  uncorrected at voxel level)

Moreover, significantly reduced correlated activity was observed between the amygdala and hippocampus ( $[x = 24, y = -22, z = -14]$   $T(41) = 4.15$ ,  $p(\text{corr}) = 0.025$ ). Extraction of the parameter estimates of these regions showed that their activity was positively correlated with activity of the amygdala in the placebo conditions and that hydrocortisone reduced this positive coupling (Fig. 30C).

Looking at the opposite contrast, i.e., CORT-increased correlations in activity patterns of the amygdala, revealed bilateral clusters in the middle frontal cortex ( $[x = 32, y = 34, z = 24]$   $T(41) = 4.45$ ,  $p(\text{corr}) < 0.001$ ,  $[x = -28, y = 30, z = 24]$ ,  $T(41) = 4.42$ ,  $p(\text{corr}) < 0.001$ ) and a region within the middle temporal gyrus ( $[x = 58, y = -66, z = 20]$ ,  $T(41) = 4.16$ ,  $p(\text{corr}) = 0.032$ , Table 19, Fig. 30D). Further data analyses showed that this increased correlation between activity in the amygdala and these frontal and temporal regions in fact reflected reduced negatively correlated activity between these regions; whereas activity in these regions was negatively related to amygdala activity in the placebo condition, this negative relationship was non-existent in the hydrocortisone condition (Fig. 30D). Thus, corticosteroids seem to ‘decouple’, or disconnect, the amygdala from the rest of the brain by reducing functional connectivity to regions that are positively as well as negatively correlated with its activity.

## Visual cortex coupling

To evaluate whether the observed corticosteroid-induced decoupling within the amygdala network was due to a general corticosteroid-induced network decoupling, we performed the same analyses on a control seed region in the primary visual cortex. The overall connectivity analysis showed a pattern of positive coupling in an extended visual processing network covering the occipital lobe, cuneus, calcarine, lingual gyrus, and fusiform gyrus, which is in line with previous studies (Lowe et al. 1998; McIntosh et al. 1994). Negative coupling of the primary visual cortex was observed for the cerebellum and a region within the brain stem (Table 20). Importantly, this connectivity pattern was not significantly affected by corticosteroid administration, neither within the visual processing network itself, nor to the regions observed for which altered amygdala coupling was observed (minimum  $p(\text{corr}) = 0.565$ ). These findings suggest that the observed corticosteroid-induced amygdala decoupling did not reflect a general reduction of network connectivity.

## DISCUSSION

The present study aimed at investigating how corticosteroids influence the functional amygdala network. The amygdala showed decreased positive coupling in response to corticosteroids to brain areas implicated in the initiation and regulation of the stress-response: the LC, hypothalamus, and hippocampus. Diminished negative amygdala-coupling due to corticosteroids was observed in executive control areas: the middle frontal gyrus and middle temporal gyrus. No strengthening of any connections was observed. Analysis of the connectivity patterns of a control seed region, the primary visual cortex, revealed no such corticosteroid modulation, indicating that these alterations

**Table 20. Peak voxels and corresponding T values of significantly activated clusters that show functional coupling with the primary visual cortex for both groups combined and for main effects of hydrocortisone**

Region	Cluster size	MNI Coordinates			Peak
		x	y	z	T-value
Positive coupling primary visual cortex					
Extended cluster covering the inferior, middle, and superior occipital lobe, cuneus, calcarine, lingual gyrus, and fusiform gyrus	13287***	20	-88	6	12.13
Supramarginal, R	5**	70	-16	24	6.15
Superior temporal gyrus, L	524***	-50	-32	24	8.77
Superior temporal gyrus, R	12**	68	-30	26	6.45
Middle temporal gyrus, R	1*	48	-52	14	5.71
Postcentral gyrus, L	268***	-58	-6	16	7.88
	4**	-66	-14	34	6.00
	6**	-44	-8	28	5.96
	1*	-46	-28	48	5.88
	1*	-64	-18	36	5.72
Postcentral gyrus, R	567***	52	-10	28	8.30
Middle frontal gyrus, L	23***	-26	46	28	6.53
	1*	-30	38	36	5.90
Middle frontal gyrus, R	98***	36	38	26	6.74
	1*	42	34	32	5.81
Inferior frontal gyrus, L	49***	-64	-20	20	7.01
Inferior frontal gyrus, R	19***	40	20	18	6.19
Anterior cingulate cortex, R	7**	12	30	32	5.88
Rolandic oper, L	49***	-38	-14	18	6.68
Rolandic oper, R	16***	42	-16	18	6.30
	17***	46	-30	22	5.97
Thalamus, L	3*	-18	-28	2	6.04
	1*	-6	-22	0	5.88
Brain stem, L	1*	-6	-24	-4	5.72
Negative coupling primary visual cortex					
Cerebellum Crus1, R	171***	10	-86	-24	7.73
	7**	50	-70	-22	6.55
Brain stem, R	6**	10	0	-16	6.12
Caudate nucleus	1*	-6	2	22	5.72
Negative main effect of hydrocortisone	No significant clusters				
Positive main effect of hydrocortisone	No significant clusters				

MNI, Montreal Neurological Institute; R, right; L, left. All effects are analyzed using cluster-level statistics. \*\*\*:  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*:  $p < 0.05$  (whole brain corrected, height threshold at  $p < 0.05$  FWE corrected at the voxel level)

did not reflect a general reduction of network connectivity. Thus, in men, corticosteroids appear to ‘decouple’ rather specifically the amygdala from the rest of the brain.

The amygdala is the key modulator of vigilance and emotional processing in the brain (Phelps and LeDoux 2005). Functional connectivity studies have indicated that it is part of a ventral emotional processing system, comprising the insula, ventral striatum, and ventral regions of the ACC and prefrontal cortex. This network of regions is known to be involved in the identification of the emotional significance of a stimulus and the production of an affective state (Phillips et al. 2003; Roy et al. 2009). It is reciprocally connected to a dorsal control system that includes the hippocampus and dorsal regions of the anterior cingulate and prefrontal cortex, which is responsible for the regulation of this affective state (Phillips et al. 2003). Our findings on overall amygdala connectivity patterns, displaying positively and negatively correlated activity with ventral and dorsal regions respectively, are in line with these networks and previous literature on amygdala connectivity during rest (Roy et al. 2009; Stein et al. 2007).

Corticosteroids reduced positive coupling between the amygdala and the hypothalamus and hippocampus, which may have consequences for cognitive functioning and control of the HPA-axis under rest and possibly also after stress. For instance, both the hypothalamus and hippocampus exert a tonic inhibitory influence over HPA axis activity under rest via MR activation, while negative feedback after stress may take place via GR activation (De Kloet and Reul 1987). By contrast, activation of amygdala’s GRs is thought to stimulate the HPA axis (Herman et al. 2003). If cortisol would act in a similar way in the aftermath of stress as presently observed in non-stressed subjects, it might promote normalization of the HPA axis by decoupling of the amygdala from these regions. The peak coordinates of the changes observed in the hypothalamus seemed to co-localize with the location of the PVN (Baroncini et al. 2012), which is the expected target for corticosteroid-mediated negative feedback (Herman and Cullinan 1997). However, fMRI lacks the spatial resolution to pinpoint signal activation or co-activation to anatomically minute structures such as distinct hypothalamic nuclei, thereby limiting our conclusions to altered connectivity of the amygdala to the entire hypothalamus.

Interestingly, hydrocortisone also reduced the positive coupling of the amygdala to the LC, the forebrain’s main source of norepinephrine and activator of the SAM-system (Sara 2009). During the initiation of the stress response, the amygdala relies heavily on its reciprocal connections to both the LC and hypothalamus (Silverman et al. 1981; Valentino and Van Bockstaele 2008; Van Bockstaele et al. 2001). Functional coupling between these stress regions increases during acute stress (van Marle et al. 2010), LC firing increases (Valentino and Van Bockstaele 2008), and hypothalamic CRH release is elevated (Feldman et al. 1995; Gray 1991), resulting in elevated levels of arousal (Aston-Jones et al. 1991; Joëls and Baram 2009; Valentino and Van Bockstaele 2008). Thereby, the brain is shifted into a hypervigilant state of processing in which limbic pathways prevail over prefrontal cortical pathways in the control of affect (Arnsten 2009; Diamond 2007).

Corticosteroids reduce the coupling between the amygdala and LC, and thereby could prevent subsequent activation of the SAM-system. We furthermore speculate that corticosteroids may act similarly in the aftermath of stress exposure, which could curtail prior activation of the LC and thereby normalize the hypervigilant state.

Besides reducing positive functional coupling of the amygdala, corticosteroids also reduced amygdala's negative coupling to the middle frontal gyrus and middle temporal gyrus. Activity in these regions was negatively correlated with activity in the amygdala under control conditions, but this coupling was diminished under conditions of high cortisol. The observed clusters are part of the so-called executive control network (Seeley et al. 2007), which enables an organism to sustain attention, and supports working memory (Curtis and D'Esposito 2003) and response selection (Lau et al. 2006). Activity in this network ensures response flexibility, by directing attention to pertinent stimuli as behavioral choices are weighed against shifting conditions, background homeostatic demands, and context (Seeley et al. 2007). Animal research has already shown that the induction of long-term potentiation of the amygdala-prefrontal cortex pathway by stimulation of the amygdala was impaired in the aftermath of stress (Maroun and Richter-Levin 2003). This, together with our findings, suggests that corticosteroids reduce amygdala's influence on executive function. Such reduction might aid cognitive control processes in the aftermath of stress and contribute to the return to homeostasis.

Some limitations to this design should be mentioned. First of all, recent research has pointed out that corticosteroids are capable in inducing distinct rapid and slow effects by activating non-genomic and genomic cascades respectively (Joëls et al. 2006). Whereas the rapid effects can occur within minutes after brain exposure to corticosteroids (Karst et al. 2005), the typical slow genomic effects take several hours to develop and can last for days (Pavlidis et al. 1995; Wiegert et al. 2005). Here, we assessed the effects of corticosteroids ~105 min after hydrocortisone intake. This design ensured elevated corticosteroid levels during fMRI scanning, but maximal rapid effects of corticosteroids might have occurred earlier. Moreover, the rather long delay permitted genomic effects to occur as well, which makes that the corticosteroid effects as reported here are most likely the result of a mixture of both non-genomic and genomic effects on amygdala's functional connectivity patterns, and future studies will be necessary to disentangle both effects. Secondly, results are not based on a randomly selected, population-based sample, and are therefore by definition not representative for the entire population. We opted to recruit participants with the most stable response to corticosteroids, making that they had to meet rather strict in- and exclusion criteria in order to be enrolled in this study. Most important, we only included men as participants. Women were excluded because amygdala functioning appears to differ between sexes; both amygdala's responsivity (Cahill et al. 2004) and connectivity (Kilpatrick et al. 2006) have been shown to be different in men and women. Furthermore, women are known to respond differently to hydrocortisone than men, both in behavior (Andreano and Cahill 2006; Bohnke et al. 2010) and brain activation (Merz et al. 2010; Stark et al. 2006). Therefore, we restricted this

study to men only. Obviously, because of these in- and exclusion criteria, the results cannot be readily generalized. Future studies will be needed to test whether corticosteroids exert similar effects in women.

Another factor to investigate in future studies is the effect of corticosteroids on the psychological state the participants are in. Mood state is known to modulate amygdala's functional connectivity patterns (Harrison et al. 2008), and could thereby be related to the observed effects in amygdala connectivity. We assessed this state using the Profile Of Mood States (POMS) questionnaire (de Groot 1992; Reddon et al. 1985; Wald and Mellenbergh 1990) and did not observe a significant difference between groups. However, this lack of significance could be due to too low statistical power to detect any changes in behavioral output. Therefore, future studies implementing larger sample sizes should determine whether hydrocortisone administration induces any effect on mood, potentially related to our findings of altered amygdala connectivity.

Furthermore, the pharmacological model for the effects of corticosteroids used in this study obviously does not capture all aspects of the complex stress response. Real-life cortisol release in response to stress is accompanied by the release of many other neuromodulators, such as norepinephrine, CRH, dopamine, and serotonin (Joëls and Baram 2009), with which corticosteroids could potentially interact. Because we did not induce stress, the generalization from our results to stressful situations remains speculative. Nevertheless, mere administration of hydrocortisone reveals a cleaner mechanistic account for the corticosteroid effect, which was the aim of this study.

Moreover, we did not check for all environmental factors known to modulate amygdala function and HPA axis dynamics. Although participants with any history of or current psychiatric illness, any past or current use of antidepressants or anxiolytics, or participants currently in a stressful period or undergoing a major life event were excluded from participation, we did not check for stress during early life (e.g. childhood trauma). Early life adversity is known to constitute a major risk factor for psychiatric disorders and has been associated with structural and functional brain alterations (Cohen et al. 2006a; Frodl et al. 2010; Kessler et al. 1997), as well as alterations in HPA axis functioning (Bremner 2003; Gillespie et al. 2009). Moreover, also recent trauma can trigger the onset of psychiatric disorders (e.g. post-traumatic stress disorder) and cause long lasting changes in amygdala functioning (van Wingen et al. 2012). We obtained this data from the majority of subjects ( $n = 23$ ; 12 x placebo, 11 x CORT) retrospectively, using an adapted version of the List of Threatening Life Events developed by Brugha and colleagues (Brugha et al. 1985). This inventory encompasses life events, which are likely to occur relatively frequently and score relatively high on long-term threat. Groups did not differ in the occurrence of overall nor severe life events as assessed by this questionnaire, neither during childhood nor later during life (all  $p$ 's  $> 0.05$ ). Therefore, the obtained results cannot be readily explained by differences in early life stress.

Lastly, one should note that although findings on the hypothalamus and locus coeruleus were corrected for all comparisons done over the specific region of interest, i.e. family wise error (FWE) rate corrected, these findings would not remain significant after correction for testing of

multiple ROIs. Future studies should aim to replicate these findings.

In conclusion, corticosteroids inhibit amygdala connectivity to several regions in the male brain. Amygdala's positive connectivity patterns to the stress-related structures including the LC, hypothalamus, and hippocampus were reduced, as well as its negative connectivity patterns to executive control regions including the middle frontal gyri and middle temporal gyrus. These effects of cortisol on amygdala connectivity in men appear to be opposite to the effects of rapidly acting stress hormones. Acute stress has been shown to strengthen amygdala's connectivity to other regions of the salience-network, to boost emotional processing (van Marle et al. 2009), and impair executive control (Arnsten 2009; Qin et al. 2009), resulting in a state of hypervigilance. If corticosteroids would act in a similar way after stress exposure as observed in the current study, the hormones might play a critical role in the restoration of homeostasis following stress exposure by desensitizing and normalizing brain processing (de Kloet et al. 2005). In line with this hypothesis it was recently shown that corticosteroids suppress amygdala responsivity (Henckens et al. 2010), and boost executive control function (Henckens et al. 2011c). Here, we provide additional evidence by showing that corticosteroids reduce amygdala's influence on brain processing by weakening its connectivity patterns. 'Disconnecting' the amygdala in the aftermath of stress might contribute to a curtailed neuroendocrine stress response and minimized stress influence on executive control function, which suggests an essential role of corticosteroids in normalizing brain function in the aftermath of stress. Such normalization may be compromised in individuals liable to PTSD which are thought to have only brief exposure to cortisol after stress due to a stronger negative feedback mechanism (Yehuda et al. 1993). Moreover, our results suggest that the low ambient cortisol levels as observed in PTSD (Rohleder et al. 2004) might contribute to the increased amygdala connectivity detected in these patients (Gilboa et al. 2004; Lanius et al. 2010; Osuch et al. 2008). Conversely, continuously elevated cortisol levels such as observed in major depression (Parker et al. 2003) might result in a more chronic state of amygdala 'decoupling', as was indeed described for depressed individuals (Moses-Kolko et al. 2010; Veer et al. 2010). In sum, these observations underline the critical role of corticosteroids in the regulation of amygdala's influence on the brain.

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**Chronic stress-induced effects  
on macroscopic structure and  
functional connectivity patterns  
in the rodent brain**

**4.2**

**Marloes J.A.G. Henckens, Kajo van der Marel,  
Annette van der Toorn, Anup G. Pillai, Guillén Fernández,  
Rick M. Dijkhuizen, and Marian Joëls**

**(submitted)**

# 4.2

## ABSTRACT

Stress exerts a profound influence on brain functioning. Whereas the stress response first and foremost constitutes a highly adaptive mechanism that enables an organism to respond optimally to potential threats in the environment, dysregulation of this response or prolonged stress exposure can cumulate in stress-related psychopathology, such as depression or post-traumatic stress disorder. Recent developments in the field have extended our knowledge on the functional and structural abnormalities observed in these mental illnesses, and sketch a view of dysfunctional brain networks with aberrant patterns of activity and connectivity. Meanwhile, animal research has expanded our understanding of the supposed cellular underpinnings of these effects by investigating the influence of chronic stress exposure on regional morphology, excitability, and plasticity in the rodent brain. Here, we set out to link the two fields, by investigating how the regional alterations in cellular structure and function translate to inter-regional changes in structural integrity and functional connectivity patterns observed in the rodent brain, using MRI. Implementing a controlled design, male rats were exposed to 10 days of chronic immobilization stress, which resulted in a significantly lower body weight of the animals, increased adrenal weight, and the expected dendritic hypertrophy in the amygdala, and hypotrophy in the hippocampus and medial prefrontal cortex. Resting-state functional MRI revealed that functional connectivity strength was significantly increased in the somatosensory cortex, visual cortex, and default mode network in response to stress. Moreover, chronic stress exposure was associated with an increased volume and mean diffusivity of the lateral ventricles, as measured by *post-mortem* high-resolution structural MRI and diffusion kurtosis imaging. Thus, this study shows that chronic stress exposure in rodents induces macroscopic structural changes and alterations in functional network connectivity strength similar to those observed in stress-related psychopathology.

## INTRODUCTION

Stress has a major impact on brain functioning. Whereas the stress response first and foremost constitutes a highly adaptive mechanism that enables an organism to respond optimally to potential threats in the environment, dysregulation of this response or prolonged stress exposure can cumulate in stress-related psychopathology, such as depression or post-traumatic stress disorder (PTSD) (de Kloet et al. 2005). Implementing a top-down approach, cognitive neuroimaging studies have investigated the functional and structural neural abnormalities observed in these mental illnesses, in order to improve our understanding of stress-related psychopathology. Studies have been focusing on the main structures involved in the regulation of the stress response and emotional processing: the amygdala, hippocampus, and prefrontal cortex. Patient studies have revealed volumetric reductions in hippocampal, prefrontal cortex, and anterior cingulate volume (Drevets et al. 1998; Bremner 1999; Shin et al. 2006; Lorenzetti et al. 2009), together with their functional impairment as observed in memory performance (McEwen 1997; Sapolsky 2000), attention (Mialet et al. 1996), and emotion regulation (Phillips et al. 2003; Taylor and Liberzon 2007). The amygdala seems to be hyperresponsive in both depression and PTSD, especially in response to negative emotional stimuli (Drevets 1999; Shin et al. 2006; Leppänen 2006). However, recent advances in the field have elicited a shift away from such region-of-interest-based approaches towards network based approaches, in which the brain is regarded as a set of functional networks, each representing a unique brain function. Importantly, these analyses revealed that stress-related psychopathology is also characterized by alterations in structural integrity and functional connectivity patterns throughout the brain (Gilboa et al. 2004; Zeng et al. 2012; Whitfield-Gabrieli and Ford 2012; Admon et al. 2012; Patel et al. 2012). Alterations in the strength of the default mode network (DMN), a set of regions typically active during rest (Raichle et al. 2001), but also the affective/vigilance network and visual cortical areas have been observed, and differences in their connectivity patterns were even shown to separate the healthy from the diseased brain (Zeng et al. 2012).

Animal research, using a bottom-up approach, has investigated the effects of prolonged (i.e. chronic) stress exposure on neuronal function and structure, in order to elucidate the potential neural underpinnings of stress-related illnesses. Chronic stress affects both brain function and structure in a region-specific manner. Higher-order cognitive function, performed by the hippocampus and medial prefrontal cortex, typically deteriorates as a consequence of chronic stress; chronic restraint, unpredictable, or psychosocial stress impairs memory performance (McEwen 2001), reduces hippocampal LTP (Pavlidis et al. 2002), increases hippocampal excitability (for review see Joëls et al. 2012), and impairs attentional set-shifting (Liston et al. 2006) and working memory performance (Cerqueira et al. 2007) in rats. Structurally, this deterioration of function is associated with a reduced hippocampal volume (Lee et al. 2009), and dendritic hypotrophy in hippocampal (CA3) and medial prefrontal (prelimbic and cingulate cortex) cells. Pyramidal neurons in these regions showed reduced dendritic complexity (i.e., a decrease in the total length and reduced

branching of the apical dendritic tree; Woolley et al. 1990; Watanabe et al. 1992; Magariños and McEwen 1995a; Cook and Wellman 2004; Radley et al. 2004; Liston et al. 2006), and a reduction in spine number (Fuchs et al. 2006; Li et al. 2011) as a consequence of chronic stress. Conversely, the amygdala displays dendritic hypertrophy (increased dendritic length and larger amount of branch points; Vyas et al. 2002) and increased spine density (Mitra et al. 2005). Behaviorally, these structural changes were shown to translate into an increased anxiety phenotype (Vyas et al. 2002). Remarkably, similar to the amygdala, chronic stress boosts the function of the most ventral part of the hippocampus (in contrast to its dorsal part), as was reflected in an increase in LTP in response to chronic stress (Maggio and Segal 2011).

Despite the detailed knowledge about regional effects of chronic stress, it is currently entirely unknown whether these effects in neuronal morphology, excitability, and synaptic plasticity translate to altered connectivity as observed in the diseased human brain. Therefore, we here set out to investigate the effects of chronic stress exposure on structural integrity and the functional connectivity patterns observed in the rodent brain. Implementing a controlled design, we exposed male rats to 10 days of chronic immobilization stress, and tested its effects on functional connectivity networks as identified by independent component analysis (ICA) of resting-state functional MRI (rs-fMRI). Subsequently, we performed *post-mortem* high-resolution structural MRI and diffusion kurtosis imaging (DKI) to assess structural changes resulting from stress exposure. Furthermore, a subset of rats was used for Golgi-staining to confirm the presence of the expected chronic stress-induced morphological changes in neuronal structure in the hippocampus (CA3), amygdala (BLA), and mPFC (PrL).

## MATERIALS & METHODS

### Animals

Thirty-six male Wistar rats (RccHan<sup>TM</sup>, Harlan) were housed in groups of three animals per cage with *ad libitum* access to food and water, except for the periods specified otherwise. Animals were kept in a temperature-controlled room (22–24 °C), with a light/dark cycle of 12 hrs (lights on at 7:00 A.M.). At the beginning of the experiments, animals were approximately 3 months old and weighed 325–400 grams. The animal experimental protocol was approved by the Utrecht University Ethical Committee on Animal Experiments, and the experiments were carried out in accordance with the guidelines of the European Communities Council Directive.

### Stress manipulation

The rats were randomly assigned to one of two experimental groups, entering either a chronic immobilization stress (CIS) or control protocol for 10 consecutive days. CIS consisted of complete immobilization (2 hrs/d, 10 A.M.-noon) in rodent immobilization bags without access to food

or water (Vyas et al. 2002). Control animals were deprived from food and water for the same period of time (2 hr/d, 10 A.M.-noon), but were otherwise left undisturbed in their home cage. All animals within one cage were assigned to the same protocol. To monitor the overall effects of the stress paradigm, animals were weighed daily, and the adrenal glands were removed and weighed after completion of the experiment.

## Functional MRI

**Protocol.** One day after the end of the chronic stress/control procedure (day 11), the majority of the animals entered a MRI protocol ( $n = 20$ ). The animals were anesthetized with 4% isoflurane for endotracheal intubation, followed by mechanic ventilation (TOPO; Kent Scientific) with 1.5% isoflurane in a mixture of air with 30% O<sub>2</sub> (55 beats/min). Rats were placed in a MR-compatible stereotactic holder and immobilized with earplugs and a tooth holder. During MRI, blood oxygen saturation and heart rate were continuously monitored by a pulse oximeter (8600V; Nonin Medical) with the probe positioned on a hind paw. In addition, expired CO<sub>2</sub> was continuously monitored with a capnograph (Multinex 4200; Datascope Corporation), and body temperature was maintained at  $37.0 \pm 0.5$  °C using a feedback-controlled heating pad. All *in vivo* MRI scanning took place in the morning (8:30-12:30 A.M.), when corticosteroid levels are relatively low. After scanning, animals were euthanized with 1 mL pentobarbital i.p. and transcardially perfused with cold phosphate-buffered saline (PBS) followed by a fixative containing 4% paraformaldehyde (PFA) in PBS. After overnight post-fixation at 4 °C, all extracranial tissue was removed and brains were left in the skulls. The samples were left intact and were subsequently stored at 4 °C in PBS with sodium azide for subsequent high-resolution *post-mortem* MRI.

**Resting-state functional MRI.** Resting-state functional MRI (rs-fMRI) was acquired on a 4.7 T horizontal bore MR system (Agilent) with use of a custom-built 90 mm-diameter Helmholtz volume coil (for signal excitation) and an inductively coupled 25 mm-diameter surface coil (for signal reception). Two series of blood oxygenation level-dependent (BOLD) images were acquired, under 1.0% and 1.5% isoflurane, respectively. T<sub>2</sub>\*-weighted BOLD images were obtained with a ventilation-triggered single-shot 3D gradient-echo EPI sequence (repetition time (TR) / echo time (TE) = 32 / 19 ms; FOV =  $32 \times 24 \times 12$  mm<sup>3</sup>; acquisition matrix =  $64 \times 48 \times 32$ ; voxel resolution =  $0.5 \times 0.5 \times 0.5$  mm<sup>3</sup>; 12° pulse angle; approximately 1 s temporal resolution; 600 BOLD images; total scan time = 10 min). Exactly ten minutes prior to the first rs-fMRI acquisition, end-tidal isoflurane anesthesia concentration was reduced to and maintained at 1.0%. At this level of isoflurane anesthesia, coherence of low-frequency BOLD signal fluctuations between functionally connected regions has been shown to be preserved (Wang et al. 2011). Following the first rs-fMRI run, the level of anesthesia was increased to 1.5% of isoflurane, and a structural scan was obtained with a 3D gradient-echo sequence (TR / TE = 6 / 2.25 ms; 40° flip angle; 4 averages;  $256 \times 128 \times 128$  matrix; field of view =  $60 \times 40 \times 40$  mm<sup>3</sup>).

**Post-processing.** After bias-field inhomogeneity correction (Sled et al. 1998) and masking out non-brain structures (Smith 2002), within-subject functional and anatomical images were non-

rigidly aligned using ANTS (Avants et al. 2011), and subsequently registered to an anatomical reference image that was matched to a 3D reconstruction of a stereotaxic rat brain atlas (Paxinos and Watson 2005).

**Resting-state fMRI analysis.** Resting-state fMRI was obtained to assess functional connectivity in the brain. BOLD time series were corrected for subject motion using FLIRT (Jenkinson et al. 2002), and the middle 7.5 min were interpolated on a 2 seconds temporal grid, spatially smoothed (Gaussian kernel, full width at half maximum = 1.0 mm), and corrected for linear drift. Non-neuronal signal contributions were removed by linear regression, as outlined in Weissenbacher et al. 2009, with (1) the global mean time-varying signal; (2) six motion correction parameter estimates; (3) a linear trend. Low-frequency fluctuations of the BOLD signal were obtained by band-pass filtering between 0.01 and 0.1 Hz. Group-level independent component analysis (ICA) was performed using temporal concatenated spatial ICA as implemented in MELODIC (FSL software package, <http://fsl.fmrib.ox.ac.uk/fsl/melodic>). Subject-level spatial maps and associated time courses were obtained with the dual-regression approach (Filippini et al. 2009) for subsequent voxel-wise statistical testing (two-sample t-tests). To verify that the results did not depend on the *a priori* defined number of components (i.e., 20), the analysis was replicated with 10, 30, and 40 components. The reported ICA-components were robustly identified across the different runs, and similar effects of chronic stress exposure were found within each of these networks. For each reported ICA component, its within-subject network strength was calculated as the average Fisher-transformed partial correlation coefficient of its subject-level time course with the signal in all voxels identified by the group-level spatial map (thresholded at  $p > 0.5$ ), thus correcting for the correlation with the time courses of the remaining components. Statistical testing of network strength differences between control and chronic stress groups was performed using a single linear mixed-model analysis (R software, lme4 package), with component, group, and their interaction as fixed effects, and taking subject as a random effect. P-values were computed by Markov Chain Monte Carlo (MCMC)-simulation (R software; languageR package).

## Structural MRI

**Protocol.** After approximately 6 weeks, *post-mortem* high-resolution structural MRI was obtained on a 9.4 T horizontal bore MR system equipped with a  $600 \text{ mT} \cdot \text{m}^{-1}$  gradient coil (Agilent), and a custom-built 20 mm-diameter solenoid coil for signal excitation and reception. Intact skulls were placed in a custom-made holder and immersed in non-magnetic oil (Fomblin, Solvay Solexis). First, diffusion kurtosis imaging was performed using a 3D diffusion-weighted 4-shot spin-echo EPI sequence (TR / TE = 500 / 39.2 ms;  $96 \times 80 \times 136$  matrix; field of view =  $19 \times 16 \times 27 \text{ mm}^3$ ;  $b = 1289, 2508, 3772, \text{ and } 5023 \text{ s/mm}^2$ ,  $\delta = 8.5 \text{ ms}$ ,  $\Delta = 18.4 \text{ ms}$ ; 30 diffusion-weighted images in non-collinear directions per b-value, and 8 images without diffusion-weighting ( $b = 0$ )). Subsequently, 3D gradient-echo images with a resolution of  $75 \times 75 \times 75 \mu\text{m}^3$  were acquired (TR / TE = 16 / 10 ms;  $7^\circ$  flip angle; at least 14 averages;  $360 \times 214 \times 214$  matrix; field of view =  $27 \times 16 \times 16 \text{ mm}^3$ ).

**Template construction.** A template image for morphometry analysis was iteratively refined by applying (1) non-rigid registration of subject images to the current template using ANTS (Avants et al. 2011), (2) averaging of the affine transforms (Woods 2003) and deformation fields, (3) resampling subject images to the new template space, and (4) averaging the resampled images to construct the updated template.

**Diffusion kurtosis imaging (DKI) analysis.** Conventionally, Diffusion Tensor Imaging (DTI) informs on the magnitude and orientation-dependency of apparent diffusivity of tissue water, which is quantified by the mean diffusivity (MD) and fractional anisotropy (FA), respectively. In the brain, movement of water molecules is relatively unconstrained in the ventricular system, whereas myelin sheaths (Barkovich 2000), axonal structure, and cell membranes (Prayer et al. 2001) provide significant restrictions to diffusion magnitude and direction. However, because of its near-isotropic tissue water properties, diffusion MD and FA provide little contrast in gray matter. Here, DKI additionally captures kurtosis, i.e., the extent to which the water diffusion displacement profile is non-Gaussian (Wu and Cheung 2010), which may yield increased sensitivity for tissue microstructure composition. We applied DKI to obtain quantitative maps of MD and FA, as well as mean kurtosis (MK), parallel kurtosis ( $K_{\text{par}}$ ), and perpendicular kurtosis ( $K_{\text{perp}}$ ) (Tabesh et al. 2011). DKI parameters were analyzed both voxel-based and by using a region-of-interest (ROI) approach. The ROIs included the fornix, fimbria, stria terminalis, and the medial and subgenual corpus callosum. These white matter structures are known to play a major role in conducting hippocampal, amygdala, and prefrontal cortex signaling, and their integrity has been shown to be affected by stress in previous studies (Jackowski et al. 2008; Teicher et al. 2012; Oliveira et al. 2012). The ROIs were outlined on the FA template (Fig. 34A) and average diffusion parameters were obtained.

**Deformation-based morphometry (DBM) analysis.** To test whether chronic stress exposure affects tissue volume locally, a DBM analysis was performed using the deformation fields that were calculated from the non-rigid registrations of the high-resolution anatomical images to the template. At the voxel level, the determinant of the Jacobian matrix of the final deformation field describes the volume scaling factor relative to the template. These local volume differences were subsequently tested for effects of chronic stress exposure. Moreover, based on previous reports on hippocampal atrophy and volume reductions following chronic stress in rodents (Woolley et al. 1990; Watanabe et al. 1992; Magariños and McEwen 1995a; Lee et al. 2009) and hippocampal volume reductions observed in psychopathology (Bremner 1999; Sapolsky 2000; Koolschijn et al. 2009; Savitz and Drevets 2009), the hippocampus was included as an ROI. Therefore, the hippocampus was manually delineated on the average  $T_2^*$ -weighted 3D volume and volumes were calculated. A representative  $T_2^*$ -weighted 3D volume is shown in Fig. 34D. Since most pronounced and well-established alterations have been reported on the CA3 hippocampal region (Woolley et al. 1990; Watanabe et al. 1992; Magariños and McEwen 1995a; Magariños et al. 2011; Wang et al. 2010), this region was manually segmented as well. Moreover, we delineated the dorsal and the most ventral part of the hippocampus separately, based on regional functional

segregation within the hippocampus (Fanselow and Dong 2010), and differential stress effects on these regions (Maggio and Segal 2010). The dorsal hippocampus comprised the part of the hippocampus from Bregma y-coordinates -2.3: -4.28 (Paxinos and Watson 1998), whereas the ventral hippocampus was defined as the region from Bregma y-coordinates -7.34: -8.82.

**Hippocampal shape analysis.** A point-based morphometry model (Styner et al. 2004) was used to quantitatively assess variations in hippocampal shape, as previously described (Otte et al. 2012). Briefly, the left and right hippocampus were manually outlined on the template image and separately projected onto individual subject images. Delineations were converted to surface meshes. An area-preserving, distortion-minimizing spherical parametrization was computed to obtain 1002 coordinates per surface. Procrustes analysis (Gower and Dijksterhuis 2004) was applied to spatially align hippocampal shapes, providing point correspondence for all mesh vertices and thus enabling direct statistical analysis per vertex between groups by means of a multivariate analysis of variance (MANOVA), with the coordinates as the dependent variables and group as the independent variable. Reported p-values were false discovery rate (FDR)-adjusted to account for multiple comparisons. A representative 3D-reconstruction of the smoothed variant of the originally delineated hippocampus used for morphometric analysis is shown in Fig. 34E.

## Morphological cell analysis

**Protocol.** The remainder of the animals ( $n = 16$ ) were sacrificed in the late morning on day 11 and used for Golgi staining ( $n = 16$ ). After decapitation, the brain was removed quickly, and coronally cut at approximately Bregma 0 mm to process the mPFC and the hippocampus-amygdala separately. The blocks of tissue were processed for rapid Golgi staining technique as described earlier (Castano et al. 1995; Gibb and Kolb 1998; Shankaranarayana Rao et al. 2001). mPFC tissue was impregnated for 12 days, while impregnation of the hippocampus and amygdala was restricted to 8 days. After completion of the staining protocol, one hemisphere of each brain was used for preparing transverse sections from the dorsal hippocampus, and the other hemisphere was used for obtaining coronal sections from the amygdala. The mPFC was also coronally cut. For all regions, 150- $\mu\text{m}$ -thick sections were obtained using a vibratome, and sections were collected serially, dehydrated in absolute alcohol, cleared in xylene, and coverslipped. Slides were coded before quantitative analysis, and the code was broken only after the analysis was completed. To be selected for analysis, Golgi-impregnated neurons had to satisfy the following criteria: (1) presence of untruncated primary or apical dendrites, (2) consistent and dark impregnation along the entire extent of all of the dendrites, and (3) relative isolation from neighboring impregnated neurons to avoid interference with analysis. For morphological quantification of hippocampal and mPFC neurons, 5-10 pyramidal neurons from each animal in each group were analyzed from the dorsal CA3 region and prelimbic cortex (PrL) layer II/III, respectively. For the analysis of amygdalar morphology, 5-9 cells were selected from the basolateral complex of the amygdala (BLA) (between Bregma -2.0 mm and -3.2 mm). Based on morphological criteria described in the literature (McDonald 1982; McDonald 1992), only pyramidal and stellate neurons were

selected for analysis. Images were obtained (63x for CA3, and 40x for BLA and mPFC) from the selected neurons using Zen 2011 (Carl Zeiss) in combination with an automated stage and focus control connected to the microscope. Image stacks of 1  $\mu\text{m}$  thickness were automatically acquired and combined. Next, neurons were traced using NeuroLucida software (MicroBrightField, Inc. Colchester, Vt, USA), to obtain a 3D representation of each cell. Numerical analysis and graphical processing were performed with NeuroExplorer (MicroBrightFields). Sholl plots (Sholl 1953) were constructed by plotting the dendritic length as a function of radial distance from the soma center, which was automatically set to zero. The length of the dendrites within each subsequent radial bin at 30  $\mu\text{m}$  increments was summed. Besides the Sholl analysis, results were expressed in terms of total (apical) dendritic length, total number of branch points, and total number of branch tips.

**Statistical analysis.** For the statistical analysis of the morphological data, cells displaying characteristics that deviated  $>3$  standard deviations from the mean were considered outliers, and were removed from subsequent analysis. In total, 63 control and 60 stress BLA neurons were included into the analysis of BLA morphology. For the hippocampal CA3 region, we analyzed 57 control and 57 stress cells. For the mPFC, a total of 50 neurons of control and 48 neurons of stressed animals were included into analysis. Because earlier studies reported that chronic stress mostly affects the apical and not basal dendritic tree (Magariños et al. 1996; Vyas et al. 2002; Radley et al. 2004; Cook and Wellman 2004), we confined our analyses to the former. Statistical testing was performed using a mixed factors (within and between) ANOVA, and values were represented as the mean  $\pm$  S.E.M. The Sholl analysis was tested using a repeated measures ANOVA, followed by a mixed factors ANOVA to test the group differences in dendritic length at a specific distance from the soma. Differences were considered statistically significant at  $p < 0.05$ .

## RESULTS

### Effects of chronic immobilization stress on body and adrenal weight

Chronic stress abolished the weight gain that was observed in control animals. At the start of the experiment (day 1), the control and stress groups did not differ on average weight ( $p > 0.2$ ). However, whereas the control animals significantly gained weight over the course of the experiment ( $F(10,8) = 51.0$ ,  $p < 0.001$ ), the stressed animals did not ( $F(10,8) = 1.6$ ,  $p > 0.2$ ). This resulted in a time  $\times$  group interaction in weight gain ( $F(10,25) = 25.2$ ,  $p < 0.001$ ), and a significantly lower body weight of the stress animals at the end of the experiment (day 11) ( $T = 5.7$ ,  $p < 0.001$ , Table 21). Adrenal weight was increased by chronic stress. At the end of the experiment stressed animals showed significantly larger adrenal glands relative to their body weight than control animals ( $T = 3.6$ ,  $p = 0.002$ , Table 21). Thus, changes in both body weight and weight of the adrenal glands confirmed successful stress manipulation.

**Table 21. Effects of chronic immobilization stress on body and adrenal weight, and dendritic morphology**

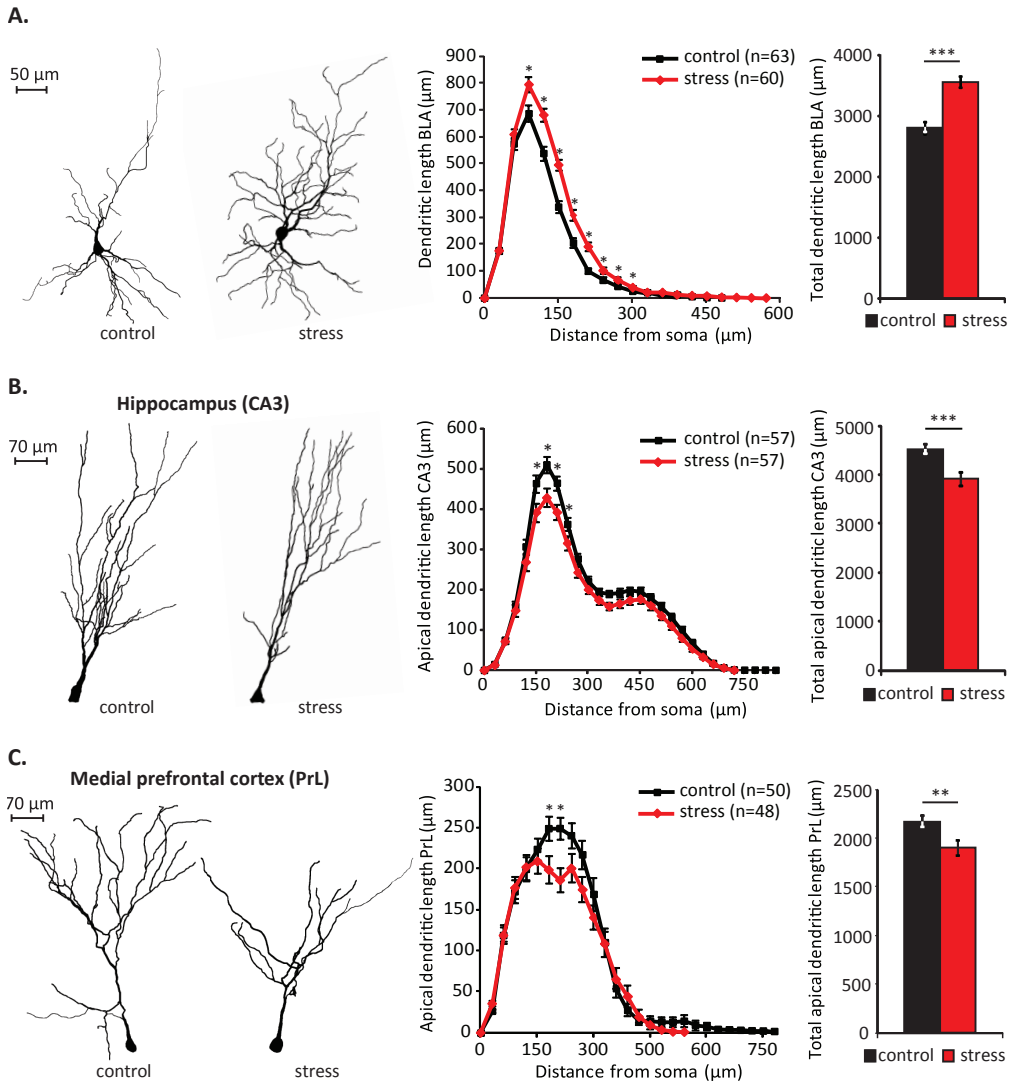
	Control	Stress
<b>Body weight at day 1 (g)</b>	368.4 ± 3.7	360.7 ± 5.0
<b>Body weight at day 11 (g)</b>	395.3 ± 3.8	360.9 ± 4.7***
<b>Relative adrenal weight</b>	17.9 ± 0.8	19.8 ± 0.7*
<b>Amygdala (BLA)</b>		
Number of branch points	22.8 ± 0.8	26.6 ± 0.7***
Number of branch tips	29.0 ± 0.9	33.3 ± 0.8***
Total dendritic length (μm)	2807.9 ± 103.9	3560.8 ± 85.1***
<b>Hippocampus (CA3)</b>		
Number of branch points	29.5 ± 0.9	25.0 ± 1.2***
Number of branch tips	31.2 ± 0.9	26.5 ± 1.2***
Total apical dendritic length (μm)	4521.8 ± 117.7	3913.9 ± 134.2**
<b>Medial prefrontal cortex (PrL)</b>		
Number of branch points	18.0 ± 0.7	15.4 ± 0.6**
Number of branch tips	19.1 ± 0.7	16.5 ± 0.6**
Total apical dendritic length (μm)	2160.9 ± 76.6	1895.7 ± 74.4**

Mean values ± S.E.M. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$

## Effects of chronic immobilization stress on dendritic morphology

Chronic stress induced a significant increase in the dendritic length ( $F(1,110) = 25.06$ ,  $p < 0.001$ ), number of branch points ( $F(1,83) = 16.36$ ,  $p < 0.001$ ) and number of branch tips ( $F(1,73) = 16.56$ ,  $p < 0.001$ ) in principal neurons of the basolateral nucleus of the amygdala (BLA) (Table 21). No significant effect was found for the total number of dendrites emerging from the soma ( $F(1,96) = 1.29$ , n.s.), nor on the outline ( $F(1,110) < 1$ ) or surface of the soma ( $F(1,115) = 1.21$ , n.s.), suggesting that the neurons selected in both groups were not different. To investigate the effects of chronic stress in greater detail, a segmental Sholl analysis was performed to track the changes in dendritic length as a function of radial distance from the cell soma (Fig. 31A). Sholl analysis revealed a main effect of stress ( $F(1,121) = 27.6$ ,  $p < 0.001$ ) and radial distance from the soma ( $F(19,2299) = 782.2$ ,  $p < 0.001$ ) on apical dendritic length, as well as a stress x radial distance interaction ( $F(19,2299) = 9.3$ ,  $p < 0.001$ ), indicating a radial distance-dependent effect of stress on dendritic length. Further analysis showed that chronic stress induced the most pronounced increase in dendritic length within a distance of 60–300 μm from the soma.

In contrast to the BLA, hippocampal CA3 pyramidal neurons were reduced in apical dendritic length ( $F(1,99) = 12.35$ ,  $p < 0.001$ ), and the number of branch points ( $F(1,101) = 13.03$ ,  $p < 0.001$ ) and branch tips ( $F(1,96) = 13.89$ ,  $p < 0.001$ ) in the apical dendritic tree in response to chronic stress exposure (Table 21). Sholl analysis revealed a main effect of stress ( $F(1,112) = 10.8$ ,  $p = 0.001$ ) and radial distance from the soma ( $F(28,3136) = 370.1$ ,  $p < 0.001$ ) on apical dendritic



**Figure 31.** Effects of chronic immobilization stress on dendritic morphology in the amygdala, hippocampus, and mPFC. (A) Chronic stress induced dendritic hypertrophy in basolateral amygdala (BLA) principal neurons, increasing dendritic branching and total apical dendritic length. (B) Hypotrophy was observed for CA3 hippocampal pyramidal neurons as a result of chronic stress; total apical dendritic length and branching were found to be reduced. (C) Layer II/III pyramidal neurons in the prelimbic cortex (PrL) also showed dendritic hypotrophy in response to chronic stress exposure. Apical dendritic length was reduced in these cells, as well as the dendritic branching. Error bars represent S.E.M. \*:  $p < 0.05$

length, as well as a stress  $\times$  radial distance interaction ( $F(28,3136) = 2.4$ ,  $p < 0.001$ ). *Post hoc* contrasts indicated that chronic stress induced the most profound reduction in apical dendritic length within a distance of 120–240  $\mu\text{m}$  from the soma (Fig. 31B).

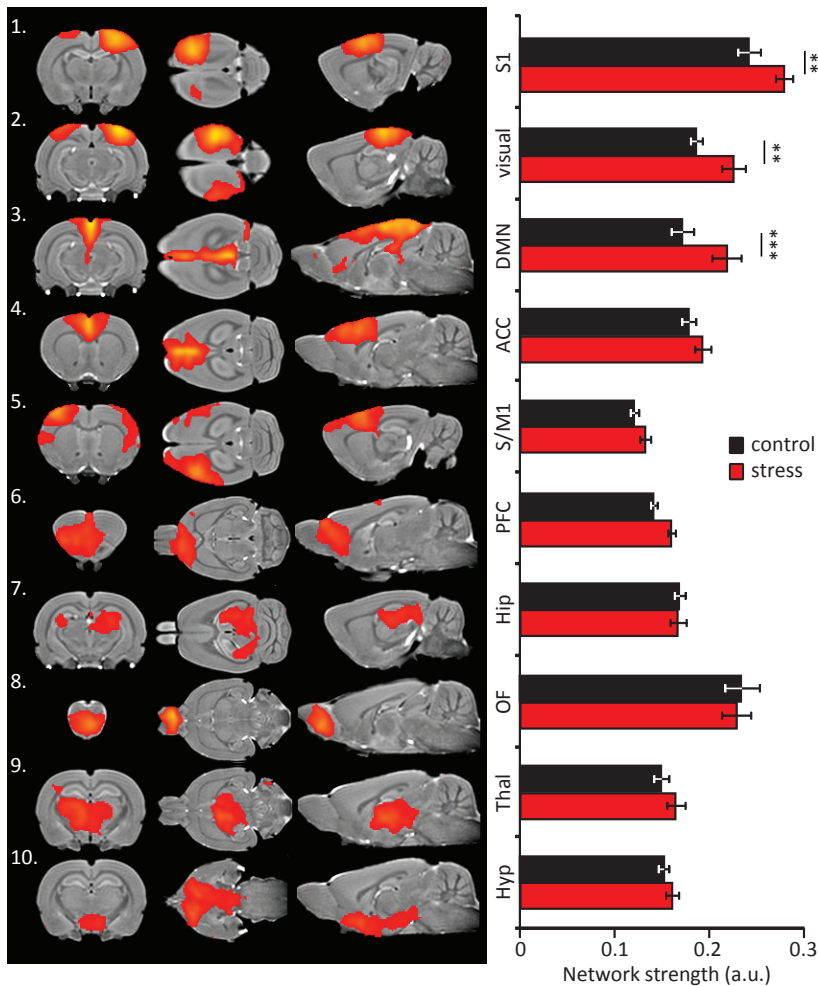
Furthermore, chronic stress induced a decrease in apical dendritic length ( $F(1,67) = 10.99$ ,  $p = 0.001$ ), and number of branch points ( $F(1,81) = 9.21$ ,  $p = 0.003$ ) and branch tips ( $F(1,83) = 8.92$ ,  $p = 0.004$ ) of the apical dendrite in layer II/III PrL pyramidal neurons (Table 21). Sholl analysis revealed a main effect of stress ( $F(1,96) = 4.6$ ,  $p = 0.035$ ) and radial distance from the soma ( $F(18,1728) = 123.1$ ,  $p < 0.001$ ) on apical dendritic length, as well as a stress x radial distance interaction ( $F(18,1728) = 1.9$ ,  $p = 0.010$ ). The most pronounced effect of chronic stress was found at a distance of 150-210  $\mu\text{m}$  from the soma (Fig. 31C).

All together, these data are in line with previous studies reporting on dendritic hypertrophy in the BLA and hypotrophy in the CA3 hippocampal area and the mPFC as a result of chronic stress (Magariños et al. 1995a; Vyas et al. 2002; Cook and Wellman 2004; Radley et al. 2004; Liston et al. 2006). Therefore, these region-specific alterations in dendritic morphology support the notion of successful stress induction.

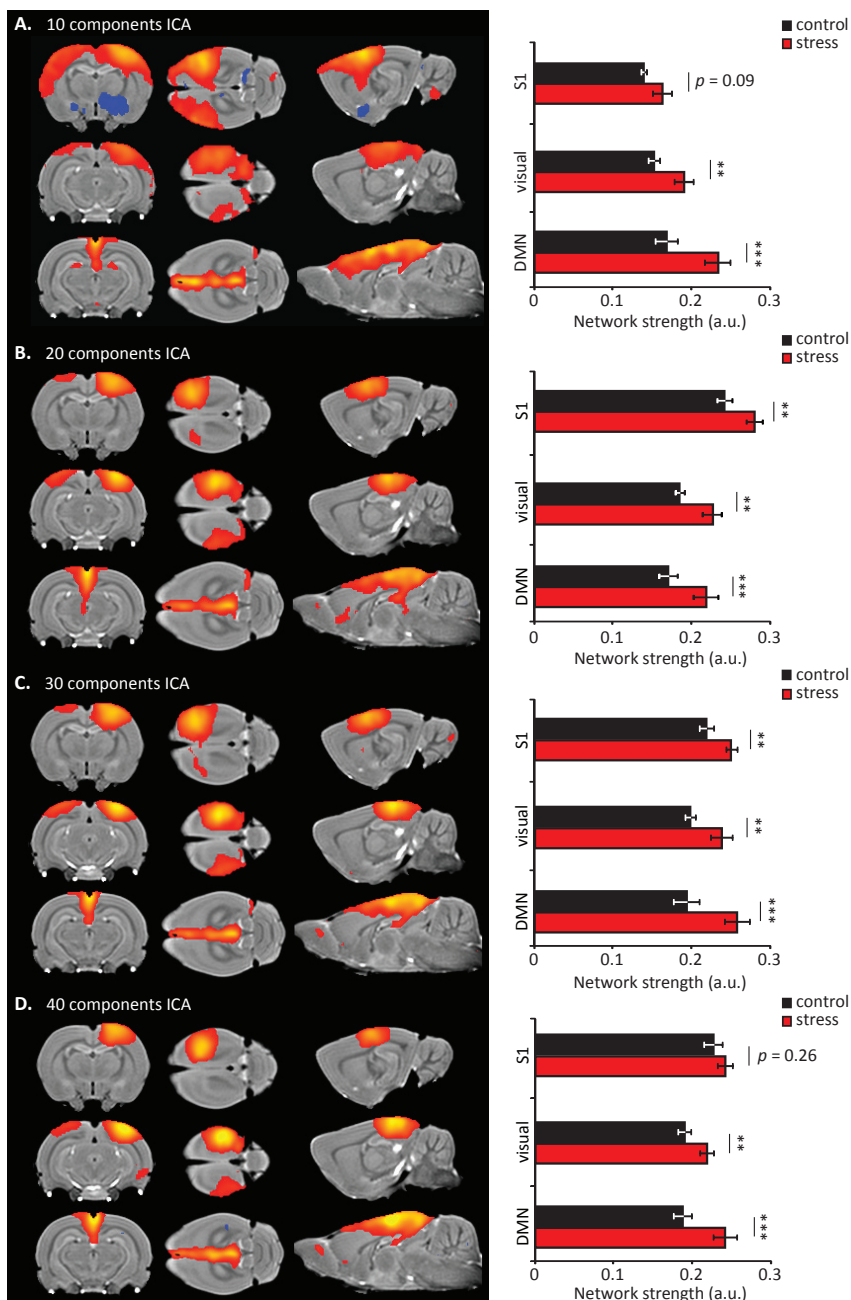
## Effects of chronic stress on functional connectivity networks

To determine whether chronic stress affected functional connectivity networks within the rodent brain, we implemented an ICA-analysis to identify functional connectivity maps from rs-fMRI data. Without *a priori* defined templates or constrained modeling, clearly identifiable networks were apparent from visual inspection alone. Comparing the components with known neuroanatomical regions, 10 of the 20 components were identified as anatomically and functionally meaningful circuits. These mean components are shown in Fig. 32 and include the bilateral primary somatosensory cortex, motor cortex, visual cortex (V1 and V2), anterior cingulate cortex, prefrontal cortex, hippocampus, thalamus, hypothalamus and olfactory bulb. The networks are in line with those previously reported in both awake and anaesthetized rats (Hutchison et al. 2010; Liang et al. 2011; Jonckers et al. 2011). Moreover, a network was found that corresponds to the default mode network (DMN), as described very recently in rats (Lu et al. 2012), and comprised regions within the orbitofrontal cortex, medial prefrontal cortex, cingulate cortex, retrosplenial cortex, hippocampus, granular and dysgranular cortex, parietal cortex, and medial visual cortex (Fig. 32-3). To test whether stress exposure affected these connectivity networks, we performed a dual-regression analysis to obtain subject-specific representations of these networks (Fillipini et al. 2009), and compared both groups using voxel-wise permutation tests. This analysis did not reveal any voxel-wise different connectivity networks between groups. However, since voxel-wise analyses are most efficient in detecting focal effects, effects may remain below the detection threshold if they are widely distributed across the entire network. Therefore, we next tested whether the *overall* connectivity within the observed networks was affected by chronic stress exposure, by comparing network strengths (see Materials & Methods). This analysis did reveal significantly altered connectivity within three distinct bilateral networks; the somatosensory cortex, visual cortex, and DMN, whereas the other components were not affected (Fig. 32). Connectivity within the somatosensory cortex ( $T = 2.87$ ,  $p = 0.004$ ), visual cortex ( $T = 3.07$ ,  $p = 0.002$ ), and DMN ( $T = 3.59$ ,  $p < 0.001$ ) was increased due to chronic stress exposure. Importantly, these findings on

increased connectivity within the visual cortex and DMN were replicated when implementing an ICA approach with 10, 30, or 40 components (Fig. 33, all  $p$ 's < 0.01), while the somatosensory cortex failed to reach significance using the 10- ( $T = 1.72$ ,  $p = 0.09$ ) and 40-component ( $T = 1.13$ ,  $p = 0.26$ ) variant.



**Figure 32.** Group independent component analysis (ICA) of functionally relevant resting-state networks in the rat brain, revealed 10 meaningful circuits, including the primary somatosensory cortex (1), visual cortex (2), default mode network (3), anterior cingulate cortex (4), motor cortex (5), prefrontal cortex (6), hippocampus (7), olfactory bulb (8), thalamus (9), and hypothalamus (10). The strength of these individual networks was determined by the average partial correlation value within the network. Chronic stress exposure appeared to increase the connectivity strength within the visual network ( $p = 0.002$ ), the primary somatosensory network ( $p = 0.004$ ) and the DMN ( $p < 0.001$ ), without affecting the other components. Error bars represent S.E.M. \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$



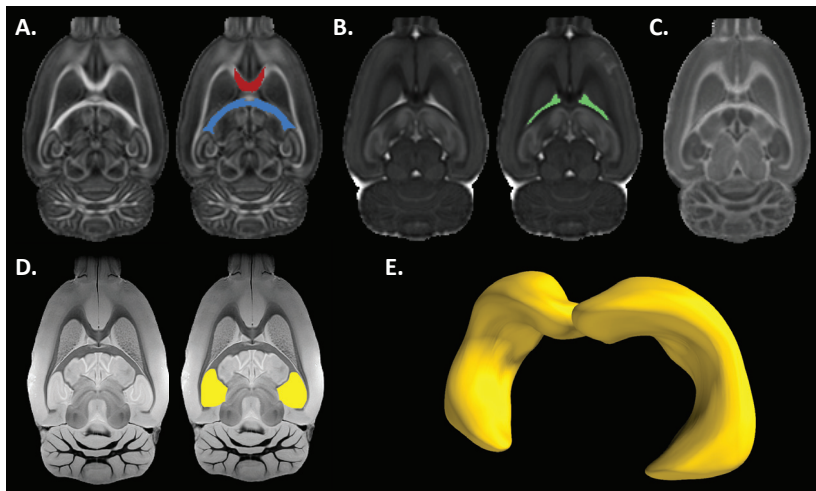
**Figure 33.** Group independent component analysis (ICA) using (A) 10, (C) 30, and (D) 40 components largely replicated the findings on the increased functional connectivity in the somatosensory cortex, visual cortex, and DMN due to chronic stress. All analyses indicated significantly stress-induced increased connectivity within the visual cortex and DMN (all  $p$ 's < 0.01), while the somatosensory cortex failed to reach significance using the 10- ( $p = 0.09$ ) and 40-component ( $p = 0.26$ ) variant. Thus, results did not seem to depend on the a priori defined number of components (20) (B). Error bars represent S.E.M. \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$

## Effects of chronic stress on structural integrity

To test the effects of chronic immobilization stress on the brain's structural integrity, we tested its effects on several microstructural white matter properties measures based on DKI (Jensen and Helpert 2003; Jensen et al. 2005; Lu et al. 2006).

A whole brain voxel-wise comparison of these measures (mean diffusivity (MD), fractional anisotropy (FA), and parallel, perpendicular, and mean kurtosis (MK)) revealed a significant effect of chronic stress on MD. Voxel-wise two-sample t-tests revealed significantly increased MD values in the lateral ventricles (LV) in response to chronic stress (Fig. 35A). In order to ensure that this increase in MD was localized in the LV, we manually outlined the LV on the MD template (Fig. 34B) and calculated its average MD. MD was significantly increased in the LV of the chronically stressed compared to control rats (mean  $\pm$  S.E.M., control:  $4.42 \pm 0.22 \times 10^{-4} \text{ mm}^2\text{s}^{-1}$ , stress:  $5.15 \pm 0.25 \times 10^{-4} \text{ mm}^2\text{s}^{-1}$ ,  $T = 2.17$ ,  $p = 0.04$ , Fig. 35B). All other DKI-measures were not affected by stress.

Next, we tested for changes in white matter characteristics in our regions-of-interest: the medial corpus callosum, subgenual corpus callosum, stria terminalis, fimbria, and the fornix. None of these structures showed any difference in either MD (all  $p$ 's  $> 0.1$ ), FA (all  $p$ 's  $> 0.25$ ), or kurtosis measures (all  $p$ 's  $> 0.3$ ) between groups.



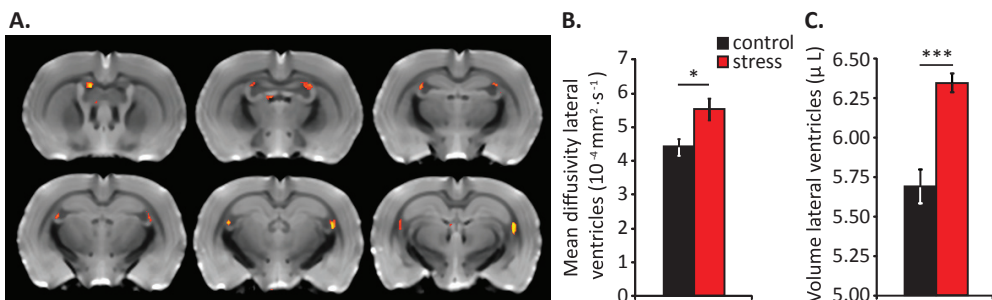
**Figure 34.** (A) Fractional Anisotropy (FA) template ( $200 \times 200 \times 200 \mu\text{m}^3$ ) scaled from 0-1 (isotropic-anisotropic), here with the subgenual corpus callosum (red) and fornix (blue) ROI overlaid. (B) Mean Diffusivity (MD) template ( $200 \times 200 \times 200 \mu\text{m}^3$ ) scaled from  $1-10 \times 10^{-4} \text{ mm}^2\text{s}^{-1}$ , with the lateral ventricles ROI (green) overlaid. (C) Mean Kurtosis (MK) template ( $200 \times 200 \times 200 \mu\text{m}^3$ ) scaled from 0-3. (D) High-resolution ( $75 \times 75 \times 75 \mu\text{m}^3$ ) post-mortem anatomical ( $T_2^*$ -weighted) image of the average rat brain. Individual scans were registered to this template to perform voxel-wise permutation tests on brain volume. Moreover, this average image was used as template to manually draw our region-of-interest for volumetric analysis; the hippocampus (yellow). (E) 3D-reconstruction from the manually delineated bilateral hippocampus used for the shape analysis.

## Volumetric changes induced by chronic stress

Next, we assessed whether chronic stress affected brain structure by regional volumetric changes. Whole brain deformation-based morphometry (DBM) analysis did however not reveal any significant differences between groups.

Subsequently, we examined whether the hippocampal volume changed as a result of the chronic immobilization stress by including it as a region-of-interest. Manual segmentation of the hippocampus did not reveal any differences in either the total hippocampal volume (mean  $\pm$  S.E.M., control:  $109.14 \pm 2.05 \text{ mm}^3$ , stress:  $107.90 \pm 1.83 \text{ mm}^3$ , n.s.), or the volume of the hippocampal CA3 region (control:  $19.35 \pm 6.32 \text{ mm}^3$ , stress:  $19.31 \pm 0.40 \text{ mm}^3$ , n.s.), known to be affected most profoundly by chronic stress (Magariños and McEwen 1995a; Magariños et al. 2011; Wang et al. 2010). Based on reports on region-specific effects of stress within the hippocampus (Maggio and Segal 2010), we tested the dorsal and ventral hippocampus separately. However, no difference in either of these regions was found as a result of chronic stress (ventral hippocampus, control:  $18.64 \pm 0.44 \text{ mm}^3$ , stress:  $18.52 \pm 0.42 \text{ mm}^3$ , n.s.; dorsal hippocampus, control:  $62.51 \pm 1.20 \text{ mm}^3$ , stress:  $61.39 \pm 1.26 \text{ mm}^3$ , n.s.), nor in their ratio ( $p > 0.5$ ). To check for any other regional changes in hippocampal volume we pursued a shape analysis in which the hippocampus of each animal was 3D-reconstructed and its shape compared between groups. This analysis also did not reveal any significant differences between groups.

Since the observed increase in mean diffusivity of the LV could potentially be caused by an enlargement of the ventricles, as is observed in stress-related disorders such as depression (Zipursky et al. 1997; Kumar et al. 1997; Strakowski et al. 2002), we next tested the effects of chronic stress exposure on LV volume. LV were manually segmented on the average  $T_2^*$ -weighted 3D volume (see Fig. 34D), and the average volumes were calculated. This analysis revealed that chronic stress significantly enlarged the LV volume (mean  $\pm$  S.E.M., control:  $5.69 \pm 0.11 \text{ mm}^3$ , stress:  $6.34 \pm 0.06 \text{ mm}^3$ ,  $T = 5.44$ ,  $p < 0.001$ , Fig. 35C), likely explaining the increased diffusivity of the area.



**Figure 35.** Chronic immobilization stress increased mean diffusivity and volume of the lateral ventricles (LV). (A) Statistical parametric map depicting the increase in mean diffusivity in the chronic stress compared to the control group, thresholded at  $p < 0.05$  corrected. (B) Extraction of the mean diffusivity values from the manually segmented LV showed a significantly increased mean diffusivity of the LV in the chronically stressed animals. (C) Manual delineation of the LV from the high-resolution anatomical scan (Fig. 33D) revealed that chronic stress increased LV volume. Error bars represent S.E.M. \*:  $p < 0.05$ , \*\*\*:  $p < 0.001$

## DISCUSSION

Here we investigated the effects of chronic stress (10 days of immobilization stress) on the structural integrity and functional connectivity patterns observed in the rodent brain. The stress procedure resulted in a significantly lower body weight in the stressed animals, as well as an increased weight of the adrenal glands; confirming successful stress induction. Moreover, stress induced hypertrophy in the basolateral amygdala, and hypotrophy in the hippocampal CA3 region and the prelimbic cortex, confirming previous reports (Woolley et al. 1990; Watanabe et al. 1992; Magariños and McEwen 1995a; Vyas et al. 2002, 2004; Cook and Wellman 2004; Radley et al. 2004; Liston et al. 2006). No differences were however found in hippocampal volume or shape, but higher mean diffusivity was observed in the lateral ventricles (LV), which was related to an increase in their volume. Functional connectivity was shown to be significantly increased in the somatosensory cortex, visual cortex, and default mode network (DMN) in response to stress. Thus, this study shows that chronic stress exposure in rodents induces macroscopic structural changes and alterations in functional network connectivity strength similar to those observed in stress-related psychopathology.

In this study we used restraint stress as chronic stress paradigm, since this paradigm was shown to reliably induce a structural and behavioral phenotype; chronically restrained rats are highly anxious (Vyas et al. 2002; Suvrathan et al. 2010), and impaired in their memory performance (McEwen 2001), attentional-set shifting (Liston et al. 2006) and working memory capacity (Cerqueira et al. 2007). In the brain, these behavioral alterations are associated with dendritic hypertrophy of amygdalar neurons (Vyas et al. 2002, 2003) and dendritic atrophy in the hippocampus and mPFC (Woolley et al. 1990; Watanabe et al. 1992; Magariños and McEwen 1995a; Cook and Wellman 2004; Radley et al. 2004; Liston et al. 2006). Chronic restraint stress was shown to be more effective in inducing these morphological changes than chronic unpredictable stress, although this might depend on the severity of the stressors included (Vyas et al. 2002). Furthermore, the use of an intense stressor for a rather short period of time (10 days 2hrs/day restraint stress in immobilization bags), reduces potential habituation of the stress response (Magariños and McEwen 1995a), and minimizes the effects of daily handling stress in the control group. Thus, we implemented a well-established stress paradigm known to induce robust stress effects in the brain. Also in our hands, the chronic restraint stress paradigm was effective in inducing stress; stressed animals showed a strong reduction in body weight gain, together with adrenal hypertrophy. Moreover, chronic stress induced the expected morphological changes; the amygdala displayed dendritic hypertrophy as reflected in an increased dendritic length and branching, whereas the hippocampus and mPFC showed dendritic atrophy of pyramidal neurons, as indicated by a reduction in their apical dendritic length and reduced branching of their apical dendritic tree. To our knowledge, this is the first study showing these stress-induced morphological alterations in all three brain regions at once.

Despite these morphological differences at the cellular level, we did not observe any stress-induced alterations in hippocampal volume or morphometry. Previous research in patients with

stress-related mental disorders such as depression and PTSD, has shown volume reductions in the hippocampus (Bremner 1999; Sapolsky 2000; Koolschijn et al. 2009; Savitz and Drevets 2009), although the effect size is moderate (Koolschijn et al. 2009) and might be overestimated by a positive publication bias. Rodent studies investigating the impact of chronic stress have also reported on hippocampal volume reductions, but show rather modest effects, which require prolonged and severe stress exposure before only small reductions become detectable (Lucassen et al. 2006). Therefore, our neuroimaging method might not be sensitive enough to pick up these subtle changes. A recent MRI study by Lee and colleagues (2010) however did show detectable reductions in hippocampal volume following chronic stress. In contrast to our study, they implemented a longitudinal design in which rats were scanned both before and after a period of stress, which enabled them to show that chronic restraint stress produced approximately 3% reduction in hippocampal volume. We however relied on a between-subjects comparison, with considerable variation within groups (standard deviation of 5-10%), which could partly explain why we failed to find a significant effect.

The mechanisms underlying the hippocampal volume reductions observed in stress-related psychopathology are currently unresolved, and previous studies have proposed dendritic retraction (Sousa and Almeida 2002), suppressed neurogenesis (Henn and Vollmayr 2004) and neuronal death (Lucassen et al. 2001; Pham et al. 2003), all due to elevated levels of glucocorticoids, as causative factors for hippocampal shrinkage. However, histopathological studies examining hippocampi of depressed individuals have so far failed to confirm either a massive neuronal loss or a suppression of dentate neurogenesis, an event that is notably very rare in adult or elderly humans. Alternatively, reductions in glial numbers, reduced gliogenesis, and alterations in glial morphology caused by chronic stress have been related to hippocampal volume reductions (Czeh and Lucassen 2007). Glia play critical roles in regulating synaptic glutamate concentrations and energy homeostasis in the central nervous system, as well as in releasing trophic factors that participate in the development and maintenance of synaptic networks formed by neuronal and glial processes (Ongur et al. 1998; Rajkowska et al. 1999; Rajkowska 2000; Coyle and Schwarcz 2000; Haydon 2001; Ullian et al. 2001). Preliminary evidence for reduced glial numbers and reduced glia density has been found in psychopathology (Ongur et al. 1998; Rajkowska et al. 1999; Cotter et al. 2001, 2002; Bowley et al. 2002; Miguel-Hidalgo and Rajkowska 2002), but future studies are necessary to firmly establish their involvement. Interestingly, one has also speculated on a shift in fluid balance between the ventricles and brain tissue. Besides reporting on reductions in hippocampal volume, numerous clinical studies have also reported on enlarged ventricles in patients with stress-related mental disorders (Zipursky et al. 1997; Kumar et al. 1997; Strakowski et al. 2002; Salokangas et al. 2002; Cardoner et al. 2003), or on reductions in water content or balance after treatment with high levels of corticosteroids (Manji et al. 2003). The idea that altered water content is apparent in the hippocampi of depressed patients is further supported by the observation that when frozen, their tissue shrinks differently compared with controls (Stockmeier et al. 2004). Interestingly, we found increased mean diffusivity of the LV

in this study, together with a relatively large ( $> 10\%$ ) increase in their volume. Enlarged LV have been reported before in depression (Zipursky et al. 1997; Kumar et al. 1997; Strakowski et al. 2002), and the enlargement of CSF spaces has been shown to predict poor treatment response of the disease, accounting for 35% of the remission time variance (Cardoner et al. 2003). Here we show that chronic stress induces enlargement of the lateral ventricles, a factor that may contribute to the development of psychopathology.

Next to inducing structural changes, chronic stress also affected the functional connectivity networks in the rodent brain. To our knowledge, so far, no studies have ever investigated the influence of chronic stress on functional connectivity at the network scale. Previous rodent studies have often inferred effects on synaptic connectivity by analyzing changes in neuronal morphology and spine profiles or synaptic vesicle density (Vyas et al. 2006; Magariños et al. 2006). Furthermore, chronic stress effects on functional connectivity have been studied by measuring neuronal plasticity as assessed by long-term-potential (LTP). These studies have provided important insights in the effects of (chronic) stress on hippocampus (Pavlidis et al. 1993; Holderbach et al. 2007), prefrontal cortex (Goldwater et al. 2009; Quan et al. 2011) and amygdalar (Kavushansky and Richter-Levin 2006; Conrad et al. 2011; Sarabdjitsingh et al. 2012) neuronal plasticity. Moreover, to a limited extent they allowed the study of stress effects on the functional connectivity between these regions (Maroun and Richter-Levin 2003; Cerqueira et al. 2007; Richter-Levin and Maroun 2010). However, all these studies implemented a hypothesis-driven approach and were only capable of assessing the connectivity within 2 regions at a time. Here, we show that similar to the human brain, also brain activity and connectivity in the rodent brain is organized in functionally distinct networks with discrete localization patterns. We were able to dissociate 10 functional networks covering the bilateral primary somatosensory cortex, motor cortex, visual cortex (V1 and V2), anterior cingulate cortex, prefrontal cortex, hippocampus, thalamus, hypothalamus, and olfactory bulb, which are in line with those previously reported in both awake and anaesthetized rats (Hutchison et al. 2010; Liang et al. 2011; Jonckers et al. 2011). Furthermore, we showed that chronic stress increases connectivity within the DMN of the rodent brain. The DMN has been suggested to support a variety of self-referential functions, such as understanding other's mental state, recollection and imagination (Buckner et al. 2008), conceptual processing (Binder et al. 1999), and even in the sustenance of conscious awareness (Horovitz et al. 2009). Although these higher-order cognitive functions have been considered to be unique to humans, a similar DMN has been reported in non-human primates (Vincent et al. 2007; Rilling et al. 2007), and very recently also in rodents (Lu et al. 2012). Importantly, both the activity and the connectivity within the DMN were recently reported to be increased in depression (Grimm et al. 2009; Sheline et al. 2009, 2010; Greicius et al. 2007, reviewed by Whitfield-Gabrieli and Ford 2012) and the patterns of physiological activity within the DMN in depression have been hypothesized to relate to self-absorption or obsessive ruminations accompanying the major depressive syndrome (Raichle et al. 2001; Gusnard et al. 2001; Drevets et al. 2002; Grimm et al. 2009). As regions within the DMN (the medial network and limbic

system) exert forebrain modulation over visceral responses mediated via the hypothalamus and brainstem, dysfunction within these circuits also may contribute to the disturbances in autonomic and neuroendocrine function that have been associated with depression. These hypotheses are compatible with treatments for depression, involving pharmacological, neurosurgical, and deep brain stimulation methods, that appear to suppress pathological activity within components of the extended DMN such as the posterior cingulate and subgenual anterior cingulate cortex and the ventromedial frontal cortex (Drevets et al. 2002; Mayberg et al. 2005).

Next to affecting the strength of the DMN, chronic stress also increased the functional connectivity within the visual and somatosensory cortex. In line with these findings, previous studies have shown hypertrophy in the somatosensory cortex after repeated stress exposure (Bock et al. 2005), and impaired inhibition of sensory processing (Stevens et al. 2001) and decreased sensory thresholds in response to corticosteroid treatment (Myers et al. 2007). Moreover, boosted visual processing has been reported during acute stress exposure in humans (van Marle et al. 2009; Henckens et al. 2009), and has been suggested to reflect a hypervigilant state of processing, in which sensory-based processing is prioritized over higher-order executive function (Hermans et al. 2011). The observed increased network strength of the early sensory networks might therefore reflect a prominent role of hypervigilant, sensory-based processing in the chronically stressed brain. Interestingly, aberrant visual processing has also been reported in major depressives (Deseilles et al. 2009; Veer et al. 2010). Depressed patients showed abnormal filtering of irrelevant information in the visual cortex, together with an altered functional connectivity between frontoparietal networks and visual cortices (Deseilles et al. 2009). Moreover, a recent study by Zeng and colleagues (2012) indicated that the majority of the most discriminating connections between depressed patients and healthy controls were located within or across the DMN, affective network, and visual cortical areas. Here, we show that chronic stress profoundly affects the connectivity within two of these three networks, supporting its involvement in the development of psychopathology and providing a handle to study these abnormalities in rodents.

Some limitations to this study should be mentioned. Firstly, the present study was performed under anesthesia. Isoflurane is a vasodilator (Farber et al. 1997) that can alter cerebrovascular activity and has been shown to have dose-dependent effects on task-elicited BOLD responses in the rat cortex (Masamoto et al. 2009). It is known to suppress overall functional connectivity in a dose-dependent manner, although the coherent spontaneous BOLD fluctuations persist (Williams et al. 2010). Here, we used a dose of isoflurane (1%) that approached the minimum required for maintaining immobility. Although it was demonstrated that low-frequency BOLD fluctuations are largely preserved under light to mild isoflurane anesthesia (Wang et al. 2011), the correlation of spontaneous BOLD fluctuations during resting-state fMRI acquisition and therefore the strengths of the connectivity networks may have been lower than under awake conditions. Nevertheless, we found connectivity networks that were similar to those observed in the awake rat brain (Liang et al. 2011), and network specific modulation by chronic stress that cannot be explained by any

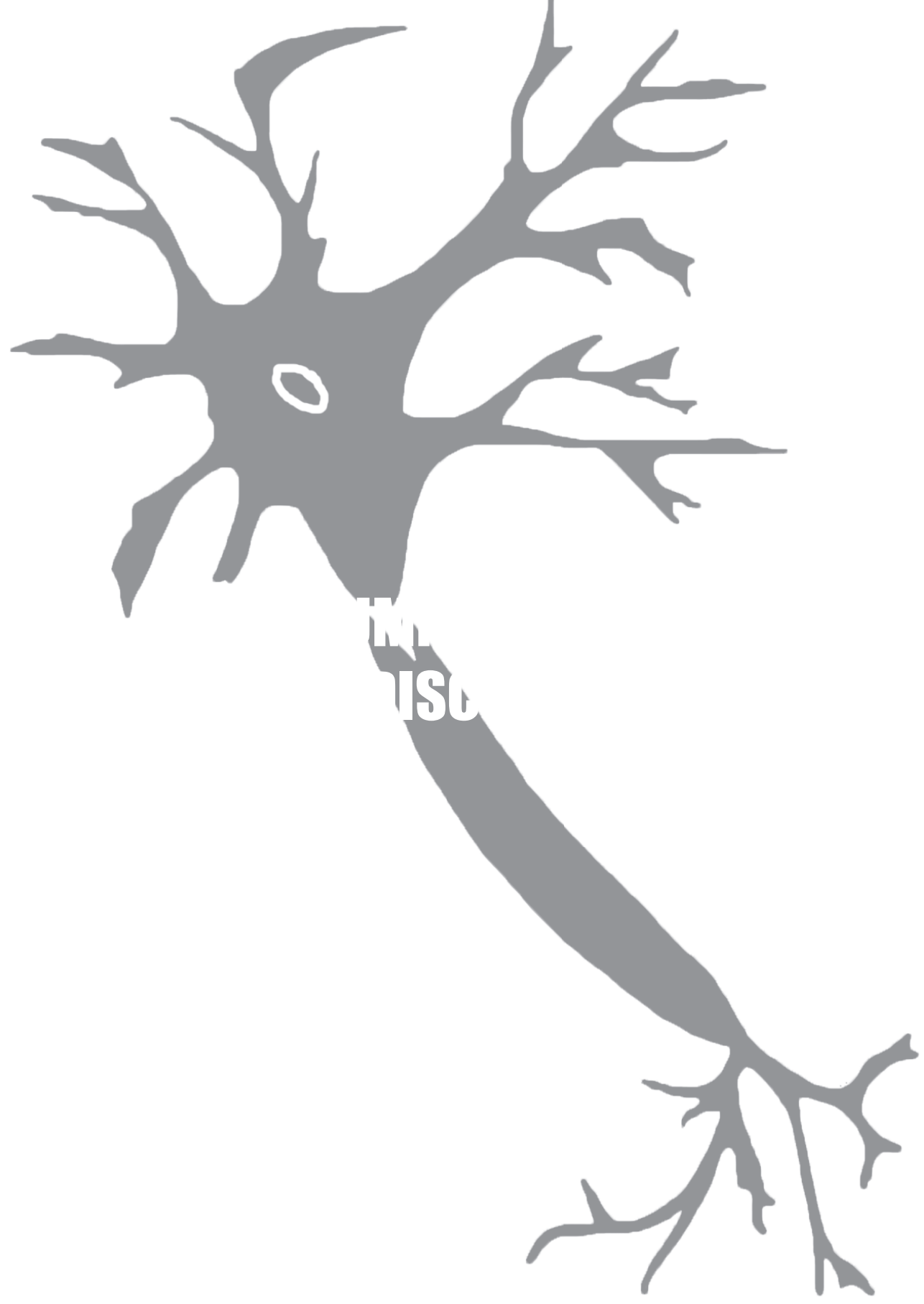
general effects of anesthesia. Secondly, although we did find volumetric alterations in the size of the lateral ventricles, we were unable to replicate earlier reports on reduced hippocampal volume due to chronic stress exposure. Considering the relative small effect size and the large variation between animals within each group, a lack of power in the current design might explain this null-finding. Repeated testing of the animals would have increased detection power, but would also have required the acquisition of the anatomical scans *in vivo*. This would have significantly limited the scanning time and thereby the resolution of the obtained images. Thirdly, technical limitations restricted our ability to assess the effects of chronic stress on amygdala connectivity. First of all, the relative distance from the surface (i.e. detection) coil to the most ventral regions of the rodent brain was rather long, decreasing signal intensity from these regions. Moreover, due to strong magnetic field inhomogeneity that exists in the interface between the ear canal and brain tissue, our imaging coverage of the caudal ventrolateral structures, in particular the amygdala, was relatively poor. These factors might explain why we did not find any indications for the existence of an affective salience network in the rodent brain, as is observed in humans (Seeley et al. 2007). Moreover, these factors could also have affected the signal from the temporal areas, including the perirhinal and entorhinal cortices, which may have compromised the detection of some of the temporal areas that could potentially be involved in the rodent DMN. Lastly, although the effects of chronic stress exposure on the DMN and visual cortex network were highly consistent over ICA analyses with a different number of components, the chronic stress induced increase in somatosensory cortex network strength could not be reliably reproduced. Therefore, future studies are necessary to replicate these findings.

In conclusion, we here present the first study investigating the effects of chronic stress exposure on the macroscopic structure and the functional connectivity patterns in the rodent brain. We showed that stress increases mean diffusivity and the size of the LV, a phenomenon also observed in stress-related mental disorders such as depression. Moreover, chronic stress increased functional connectivity in early sensory regions (the visual cortex and primary somatosensory cortex) and the DMN, potentially indicating a more prominent role of sensory based and self-referential processing in the chronically stressed brain. These animal studies, performed under highly controlled conditions, could therefore provide a valuable approach to better understand the mechanisms by which chronic stress disrupts human brain function, ultimately leading to psychopathology.

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The work presented in this thesis provides initial evidence for the time- and region-specific effects of stress and corticosteroids in both the human and rodent brain. The regional differentiation of responses was most clearly addressed in the electrophysiological study (**Part 3.4**), reporting opposite effects of acute corticosteroid exposure on cellular properties of neurons in the dorsal CA1 hippocampal area and layer II/III of the orbitofrontal cortex (OFC). In the human brain, stress and corticosteroids were shown to affect both activity and connectivity in a time- and region-specific manner.

## **Temporal effects of stress hormones on memory formation**

We started out with investigating the effects of acute stress exposure on the neural correlates of human memory formation (**Part 2.1**). Stress was induced using short, highly aversive movie clips that were embedding an intentional memory encoding task for both neutral and aversive pictures, creating one coherent stressful experience. Stress induction was successful, since heart rate, salivary cortisol level, and reported negative affective feelings increased. Moreover, (phasic) pupil dilation responses were decreased, most likely reflecting a tonically hyperactive state of the locus coeruleus (LC) (Aston-Jones and Cohen 2005). Memory performance was improved by stress, regardless of the emotionality of the studied material. In the brain, stress induced an increase in early visual processing and inferior temporal regions, which were displaying a negative and a positive subsequent memory effect, respectively. Furthermore, reduced hippocampal responses were associated with better memory formation under stress, both within and across subjects. These findings of decreased pupil dilation responses during stress, the slightly elevated reaction times for picture rating, and increase in early visual processing activity, support the idea of a stress-induced hypervigilant state of unfocussed processing. Previous studies have shown that both attentional and emotional states modulate visual processing (Wang et al. 2006; Vuilleumier and Driver 2007), and that hypervigilance is accompanied by a potentiation of sensory input (Munk et al. 1996). The widespread neocortical projections of the LC might recruit additional neural resources in order to process an excess of sensory information. However, the negative subsequent memory effect observed in these early visual regions suggests that their activation is in fact not beneficial for memory formation. Possibly, an increase in the amount of visual input processed causes a large ratio of irrelevant information, which should be filtered out for events to be properly encoded into memory. Our data suggest that the stress-induced increase in the inferior temporal regions might exactly be doing that. Activity in the inferior temporal cortex has been related to visual-selective attention (Moran and Desimone 1985; De Weerd et al. 1999) and filtering out irrelevant information (Kastner et al. 1998). Moreover, it has been proposed that tonic LC states are mirrored by increased activation of a ventral frontoparietal attention network, enhancing the selective processing of salient stimuli (Corbetta et al. 2008). In line with this, we observed bilateral subsequent memory effects in these inferior temporal regions, but also stress-induced activity increases, which might reflect a reduction of ambient noise by focusing on task-

relevant information. Consequently, adequate noise reduction may have led to less information relayed to the hippocampus. In line with this idea, the hippocampus showed less activity for later remembered than for later forgotten items under stress. Moreover, the overall decrease in hippocampal responses predicted the stress-related improvement in memory performance across subjects.

In addition to these alterations in sensory and mnemonic operations, stress may promote a neural state optimized for memory formation in the hippocampus. LC activation elevates hippocampal NE-levels leading to tonically increased activity (Berridge and Foote 1991). Thus, hippocampal activity might have been generally higher during the stress as compared to the control condition, but fMRI cannot detect such slowly modulated changes in baseline activity. Furthermore, corticosteroids and noradrenalin rapidly lower the threshold for synaptic modification (Groc et al. 2008). Therefore, sensitization of hippocampal plasticity – requiring less neural input for trace formation – possibly in combination with increased baseline activity – may provide a complementary mechanism through which acute stress can enhance memory formation. However, both this tonically increased activity and sensitized plasticity would result in smaller phasic responses, and cannot readily explain the observed reversal of the subsequent memory effect. Next to these effects, stress and corticosteroids might also have acted during the memory consolidation process to improve memory performance (Oitzl et al. 2001; Andreano and Cahill 2006; Roozendaal et al. 2006c). However, it is unlikely that such consolidation effects were the only contributing factor, since effects of acute stress on encoding-related brain activity were evident, and individual differences in stress-induced memory enhancement were predicted by hippocampal responses during encoding.

Thus, this first study shows how acute stress enhances memory encoding by profoundly affecting the neural substrates of memory formation in a region-specific manner. Our findings indicate that acute stress is accompanied by a shift into a hypervigilant mode of sensory processing in combination with increased allocation of neural resources to noise reduction. This reduction of task-irrelevant ambient noise, in combination with a stress hormone induced optimal state for neural plasticity, may explain why stressful events attain a privileged position in memory. This interpretation provides a heuristic framework for further investigation into the mechanisms underlying trauma etiology.

To test the involvement of corticosteroids in this stress-induced modulation of memory processing, we next set out to investigate the time-dependent effects of corticosteroids on memory formation using the exact same intentional encoding paradigm (**Part 2.2**). To target the rapid non-genomic and slow genomic effects, 20 mg of hydrocortisone was administered at respectively 30 min and 180 min prior to the start of memory encoding. Timing of administration for targeting the rapid corticosteroid effects was based on previous studies revealing 1) elevated cortisol levels in humans within 15-30 min after hydrocortisone intake (van Stegeren et al. 2010), 2) a time-delay between rodent peak plasma corticosteroid levels and peak brain levels of approximately 20 min (Droste et al. 2008), and 3) most prominent rapid effects with corticosteroids administered directly

to hippocampal slices (Karst et al. 2005). The slow effects of corticosteroids on the other hand were not expected to start earlier than approximately 90 min after corticosteroid administration, and known to last for hours (Joëls and de Kloet 1992, 1994; Joëls et al. 2003). Timing for targeting these effects was based on previous work showing suppressed LTP (Pavlidis et al. 1995; Wiegert et al. 2005), and strongest corticosteroid effects on hippocampal gene expression 3 hrs after exposure (Morsink et al. 2006). Thus, administration of hydrocortisone at either 30 or 180 minutes prior to scanning allowed us to disentangle most optimally the rapid and slow corticosteroid effects, respectively.

Administration of 20 mg of hydrocortisone increased salivary cortisol levels to those observed under situation of severe stress (Morgan et al. 2000). Furthermore, imaging results revealed that corticosteroids' slow effects reduced both prefrontal and hippocampal responses, while no significant rapid actions of corticosteroids were observed. Previous animal work on the genomic effects of corticosteroids showed that corticosteroid exposure suppresses hippocampal firing and LTP (Pavlidis et al. 1995; Wiegert et al. 2005), presumably by modulating the expression of over 200 genes involved in many different cellular processes (Datson et al. 2001). Here we found, in line with this animal work, that the slow corticosteroid effects suppressed human hippocampal processing. Other neuroimaging studies have also reported on MTL down-regulation by corticosteroid administration (de Quervain et al. 2003; Oei et al. 2007; van Stegeren et al. 2010), but overall lack time-specificity of these corticosteroid effects. Moreover, our data indicated that the slow effects of corticosteroids down-regulated activity of the prefrontal cortex (PFC) as well. The PFC has traditionally been associated with cognitive control processes, but its role in memory and interaction with the MTL is just as crucial (Fernández and Tendolkar 2001). Specifically, the regions affected by corticosteroids comprise parts of the dorsomedial (dmPFC) and dorsolateral prefrontal cortex (dlPFC), shown to promote long term memory formation through its role in working memory (WM) (Leung et al. 2002; Blumenfeld and Ranganath 2006), and associative memory processing (Murray and Ranganath 2007), and parts of the inferior frontal cortex, which is associated with verbal processing, and especially implicated in intentional encoding paradigms (Braver et al. 2001; Kirchhoff and Buckner 2006). Thus, the slow effects of corticosteroids suppressed processing in memory related areas, which might not seem beneficial at first sight. However, we speculate that this suppression in real-life conditions might actually aid memory for the stressful experience that initially induced the release of corticosteroids, by reducing retroactive interference into its memory trace. Retroactive interference is assumed to be a major cause of forgetting. Forgetting can be induced by any subsequent task (Dewar et al. 2007), and has been shown to be reduced by preventing new learning (Sangha et al. 2005). Therefore, the suppression of memory related areas might actually protect against the forgetting of the stressful event by reducing retroactive interference, and thus boost memory for the stressful event.

The rapid effects of corticosteroids did not induce any clear effects. Animal studies have shown rapidly enhanced hippocampal excitability (Karst et al. 2005) and LTP (Wiegert et al. 2006) by corticosteroids, but we did not observe any rapid corticosteroid effects on hippocampal processing. Possibly, these effects critically depend on concurrent noradrenergic activation, as is seen in some

animal studies (Pu et al. 2007; Roozendaal et al. 2006c). We tried to induce this activation by showing highly aversive pictures, but this effect might have been too subtle compared to a truly stressful event. Nevertheless, we showed that corticosteroids' rapid, putatively non-genomic actions by themselves are not sufficient to amplify human hippocampal processing.

There is one important limitation that should be mentioned in discussing these findings. In contrast to what was seen for stress (Part 2.1), we did not find a modulation of the subsequent memory effect (i.e., the difference in brain activation during the processing of subsequently remembered and forgotten items) due to the effects of corticosteroids, nor an effect on memory performance. One could only speculate about the reasons why we did not observe any of these effects. Possibly, specific properties of the study design have contributed, such as the intentional nature of the memory task, which might have overruled other basal differential neuromodulatory effects that could have affected the difference between later remembered and later forgotten items (Kensinger et al. 2005; Talmi et al. 2008), or the repeated testing of participants in this design, potentially causing session order effects (Wirth et al. 2011). Alternatively, both corticosteroids' rapid and slow effects might depend on noradrenergic activation (as described in Part 1), which naturally joins corticosteroid release during exposure to stress. Nevertheless, this study provides initial evidence in how corticosteroids affect neural processing in brain regions specifically involved in human memory formation.

In conclusion, this study is first in showing that corticosteroids affect neural processing in brain regions involved in human memory formation in a time-dependent manner. We showed that corticosteroid's slow, putatively genomic effects specifically reduced activity in the hippocampus and prefrontal cortex, whereas no changes were observed due to corticosteroid's rapid actions. Down-regulated activation of these memory related brain regions might minimize subsequent interference into the memory trace for the stressor by post-stress experiences, and therefore aid consolidation of the stressful event most optimally. Thus, this study provides an initial mechanistic account of how corticosteroids affect memory processing in humans.

## **Corticosteroid effects on emotional processing and cognitive control**

Next, we investigated the effects of corticosteroids on emotional processing and cognitive control function in the human brain. For this line of studies, the hydrocortisone administration protocol as described earlier (Part 2.2), was slightly adjusted. Hydrocortisone was applied in a lower dose and at an earlier time point, i.e., 240 min prior to the onset of fMRI scanning in the slow corticosteroid condition, to ensure cortisol levels to be back at baseline during testing in the slow corticosteroid group. Thus, for this series of studies, the rapid and slow effects of corticosteroids were targeted by administering 10 mg of hydrocortisone at 30 min or 240 min prior to the onset of scanning, respectively. This dose still elevated cortisol levels to those observed during moderate-to-severe stress (Morgan et al. 2000) and indeed allowed cortisol levels to have returned to baseline before

the start of fMRI scanning in the slow corticosteroid condition.

The first task participants were asked to complete in the MRI scanner was a WM-task (**Part 3.3**). The slow effects of corticosteroids appeared to boost working memory performance (measured as a combined measure of reaction times and accuracy of responding) and to increase WM-related activity in the dlPFC. This improvement in WM by the slow effects of corticosteroids is in line with animal studies where WM-performance was enhanced 4 hrs after initial corticosteroid exposure (Yuen et al. 2009), which was most likely related to initially enhanced glutamatergic transmission (increased mEPSCs amplitude) by increased surface levels of NMDA- and AMPA-receptor subunits (Yuen et al. 2011). These findings indicate that the slow effects of corticosteroids induce the exact opposite effect of acute stress, which is typically detrimental for PFC function (Qin et al. 2009; Arnsten 2009).

The rapid actions of corticosteroids did neither induce a clear behavioral nor brain effect. Since previous studies have shown that the rapid effects of corticosteroids act in concert with (and to amplify) the effects of catecholamines on long term memory (Joëls et al. 2006; Roozendaal et al. 2006c), we hypothesized impaired WM-performance in the rapid CORT condition. However, we did not observe any rapid, non-genomic effects of corticosteroids on either WM-performance or dlPFC activation. The explanation for the absence of a rapid corticosteroid effect might lie in the essential role for concurrent noradrenergic activity of the amygdala in order for corticosteroid-induced impaired WM to occur, as is seen for rodents (Roozendaal et al. 2004a). In line with this, a recent human study into the effects of norepinephrine and corticosteroids on the neural correlates of memory formation, showed that specifically the combined administration of both hormones caused a strong deactivation in the prefrontal cortex, whereas no such effects were observed when solely corticosteroids were administered (van Stegeren et al. 2010). Here we used different levels of difficulty (WM-load), which presumably triggered different levels of arousal, but did not observe any rapid modulatory effects of hydrocortisone on WM-performance or dlPFC processing. However, the levels of emotional arousal reached due to this manipulation most likely did not reach arousal levels observed under conditions of stress. Therefore, the issue of potentially interacting rapid corticosteroid and noradrenergic effects on PFC functioning remains open for future research. Regardless, our results show that corticosteroids by themselves do not modulate WM-performance or WM-related dlPFC activity in a rapid non-genomic manner.

In conclusion, this study provides clear evidence that corticosteroids modulate human PFC WM-processing in a highly time-dependent manner. The rapid effects of corticosteroids by themselves did not modulate WM-performance or WM-related dlPFC activity. By contrast, corticosteroids' slow effects were shown to augment dlPFC processing and to facilitate WM-performance. Since previous research has indicated that working memory and prefrontal processing are impaired under conditions of acute stress by the rapid actions of catecholamines (Arnsten 2009), we speculate that these slow corticosteroid effects may counteract the more rapidly induced changes and help the brain to recover in the aftermath of stress. Thereby, they may serve a highly adaptive function in normalizing brain processing when stress has subsided.

The next task participants were asked to perform was an emotional distraction task (**Part 3.2**), consisting of color-naming of either neutral or aversive words. This task allowed us first of all to assess corticosteroid effects on the neural correlates of selective attention by measuring emotional interference. Selective attention is critical for task-execution since it requires participants to focus on just one source of information for processing (i.e., font color) while ignoring competing information (i.e., word meaning). It is well-known that under such competitive conditions, the presence of emotionally salient information disrupts the ability to attend selectively to the task-relevant information (Arnsten and Goldman-Rakic 1998; Dolcos and McCarthy 2006; Dolcos et al. 2011), which results in slower reaction times and lower accuracy of responding (i.e., emotional interference). By measuring the corticosteroid effect on emotional interference induced by the emotional, attention-grabbing distracters (Bishop 2008; Wingenfeld et al. 2009), this task enabled us to assess corticosteroid effects on selective attention. Moreover, this task allowed us to assess corticosteroid effects on sustained attention by analyzing overall task performance, regardless of emotional valence of the material. Sustained attention measures the ability to keep the selective attention maintained over time (McDowd 2007) and is therefore required to complete any cognitively planned activity, here task execution.

Participants responded slower to aversive compared to neutral words, confirming emotional interference with selective attention in this task. Importantly, the rapid effects of corticosteroids (here assessed at 60 min post hydrocortisone intake) were shown to increase emotional interference, which was associated with reduced amygdala inhibition to aversive words. Moreover, the rapid effects of corticosteroids enhanced amygdala connectivity with frontoparietal brain regions; the middle frontal gyrus, and the pre- and postcentral gyrus. These regions are typically involved in basic motor functioning, but also in response selection and suppression of automatic response tendencies (Forstmann et al. 2008), as well as in resolving interference (Nee et al. 2007). As such, these regions were recruited during task execution, and displayed a negatively coupling to the amygdala, underlining their opposing roles in optimal task execution. The rapid effects of corticosteroids however induced a positive coupling between the amygdala and the executive network, which might reflect an increased influence of the amygdala on these brain regions. Altogether, these data indicate that the rapid effects of corticosteroids increase emotional interference and might thus act in concert with catecholamines to optimize rapid adaptive behavior in response to stress (Roozendaal et al. 2006c; Diamond et al. 2007). Acute stress had already been shown to increase susceptibility to (emotional) distraction (Skosnik et al. 2000; Braunstein-Bercovitz et al. 2001; Henderson et al. 2012) and to switch brain processing into a hypervigilant state in which sensory processing is enhanced (Part 2.1), as is amygdala responsivity (van Marle et al. 2009) and connectivity (van Marle et al. 2010), in order to facilitate the detection of potential threats. This state-change of brain processing was previously attributed to the actions of catecholamines on brain function (Arnsten and Li 2005; Hermans et al. 2011), but our findings of increased emotional interference by the rapid effects of corticosteroids indicate that they might play a role as well. Recent other studies seem to support this notion of additive or interactive noradrenergic and corticosteroid effects on amygdala functioning. Corticosteroid

administration in combination with the administration of reboxetine (a noradrenalin-reuptake inhibitor) was shown to induce a negative response bias in the amygdala (Kukolja et al. 2008), and to boost emotion-induced retrograde amnesia (Hurlemann et al. 2007). These findings are in line with our data on increased distraction by aversive input and increased susceptibility to the effects of emotion.

The slow effects of corticosteroids appeared to influence the neural correlates of sustained attention. They decreased overall activity in the cuneus, possibly indicating reduced stimulus-driven, bottom-up attentional processing (Hahn et al. 2006), and disrupted amygdala connectivity to the insula, potentially reducing emotional interference. The insula are well-known for their role in the mediation of autonomic arousal as part of the so-called salience network (Seeley et al. 2007), but have also been reported to exert a role in attention and the coordination of task performance during demanding tasks (Binder et al. 2004; Eckert et al. 2009) and resolving interference (Nee et al. 2007). This might explain the negative connectivity between the amygdala and insula observed in our experiment, which might reflect the interference of the amygdala with proper task performance. By reducing this connectivity, the slow effects of corticosteroids might attenuate the amygdala's influence on task execution, and thereby ensure proper performance. Moreover, the slow effects of corticosteroids induced a trend towards better overall performance in the behavioral data, as the slow corticosteroid group tended to make fewer errors than the other groups. Altogether, these data suggest an active role for the slow effects of corticosteroids in the stress-recovery of cognitive function.

In conclusion, these results suggest that the rapid effects of corticosteroids increase emotional interference in selective attention. The wide-spread, unfocussed attention might contribute to the detection of potential threats in the environment (Aston-Jones and Cohen 2005), enhancing an organism's chances of survival. Moreover, it might have beneficial effects on memory processing (Part 2.1), since additional environmental cues can also be encoded during a salient event. Furthermore, our results suggest a boost in the neural correlates of sustained attention by the slow effects of corticosteroids, which might thereby be involved in the restoration of cognitive function in the aftermath of stress. This rationale fits with the general idea about the restorative role the slow corticosteroid effects serve in order to ensure proper cognitive function once the stress has subsided (de Kloet et al. 2005), as was also seen for the working memory task (Part 3.3).

Subsequently, participants were subjected to an emotional processing task (**Part 3.1**). In this task participants were instructed to passively view faces that were morphing from a rather neutral to either an overtly fearful or happy facial expression. Imaging results revealed that the rapid effects of hydrocortisone (here assessed at 75 min post administration) desensitized amygdala responsivity, whereas the slow effects of corticosteroids selectively normalized responses to negative stimuli. Psycho-physiological interaction analyses suggested that this slow normalization was related to an altered coupling of the amygdala with the medial prefrontal cortex.

Although these findings seem at odds with those reported in Part 3.2 of this thesis (explanations for this apparent inconsistency will be discussed later), this reduced amygdala responsivity

due to the rapid effects of corticosteroids is in line with previous reports on anxiolytic effects of corticosteroid administration. These effects were first of all shown in rodents, in which corticosteroid administration resulted in more explorative and socially interactive behavior (File et al. 1979; Andreatini and Leite 1994). Recent studies extended these findings to humans, showing that corticosteroids attenuate fear responses (Soravia et al. 2006), and protect mood during exposure to stressful situations (Het and Wolf 2007, Het et al. 2012). Here, we provide a mechanistic account for these observations, by showing that the relatively rapid corticosteroid effects unspecifically desensitize the amygdala. This claim is supported by a study in humans showing a tonic suppression of the acoustic startle reflex, thought to be modulated by the amygdala, which was independent of emotional modulation (Buchanan et al. 2001).

The slow effects of corticosteroids seemed to normalize responses to negative input, while responses to positive input remained suppressed. Moreover, the induction of this emotion-specificity in the amygdala appeared to be related to increased amygdala coupling with the medial prefrontal cortex (mPFC), known to be involved in emotion regulation (Beauregard et al. 2001; Ochsner and Gross 2005; Kompus et al. 2009), and control over the HPA-axis (Sullivan and Gratton 2002; Kern et al. 2008; Radley et al. 2009). These data suggest an active role of the slow effects of corticosteroids to ensure recovery from the rapid effects of corticosteroids (on amygdala responses) to negative input specifically by changing regulatory actions of the mPFC. This could entail a highly adaptive mechanism for survival, since it is most important to be capable to respond adequately to dangerous stimuli first.

Thus, corticosteroids seem to rapidly guard amygdala activation during stress from potential overshoot by the sensitizing actions of noradrenalin and CRH, and to normalize amygdala response later on, prioritizing negative emotional processing. Thereby, they may be a crucial factor when the stress response has to be adequately terminated in the aftermath of traumatic experiences.

## Effects of stress hormones on brain connectivity

In Part 4 of this thesis we addressed how corticosteroids and stress exposure affect functional connectivity patterns in the brain. Both acute (van Marle et al. 2010) and prolonged (van Wingen et al. 2011a, 2011b) stress exposure had already been shown to profoundly affect, i.e., strengthen, amygdala functional connectivity in humans. Here, we investigated the effects of corticosteroids specifically on this functional amygdala network (**Part 4.1**). Time-dependency of corticosteroid effects could unfortunately not be assessed in this study since the resting state scan was obtained ~105 min post hydrocortisone administration. Maximal rapid effects of corticosteroids might have occurred earlier, whereas the rather long delay permitted genomic effects to occur as well. Thus, this design ensured elevated corticosteroid levels during MRI scanning, but the reported corticosteroid effects are most likely the result of a mixture of both non-genomic and genomic effects on amygdala's functional connectivity patterns, and future studies will be necessary to disentangle both effects. Corticosteroid administration was shown to generally decrease functional

connectivity of the amygdala. First of all, corticosteroids reduced *positive* functional coupling of the amygdala to brain regions involved in the initiation and maintenance of the stress-response; the LC, hypothalamus, and hippocampus. Activation of the amygdala is typically thought to stimulate the SAM-system (Silverman et al. 1981; Valentino and Van Bockstaele 2008; Van Bockstaele et al. 2001) and HPA-axis (Herman et al. 2003; Feldman et al. 1995; Gray 1991) by acting on these regions, and acute stress has been shown to further increase their connectivity (van Marle et al. 2010). By reducing amygdala connectivity to these stress-related brain regions, corticosteroids could potentially prevent subsequent activation of the systems, curtail the stress-response, and promote the return to homeostasis.

Besides reducing *positive* functional coupling of the amygdala, corticosteroids also reduced the *negative* functional coupling of the amygdala to the middle frontal and temporal gyrus; brain regions known to be involved in executive control. The observed clusters are part of the so-called executive control network (Seeley et al. 2007), which enables an organism to sustain attention, and supports working memory (Curtis and D'Esposito 2003) and response selection (Lau et al. 2006). Animal research has already shown that the induction of long-term potentiation of the amygdala-prefrontal cortex pathway by stimulation of the amygdala was impaired in the aftermath of stress (Maroun and Richter-Levin 2003). This, together with our findings, suggests that corticosteroids reduce the amygdala's influence on executive function. Such reduction might aid cognitive control processes in the aftermath of stress and also contribute to the return to homeostasis.

In conclusion, corticosteroids inhibit amygdala connectivity to several regions, reducing its influence on brain processing. Amygdala's positive connectivity patterns to the stress-related structures were reduced, as well as its negative connectivity patterns to executive control regions. These effects of corticosteroids on amygdala connectivity appear to be opposite to the effects of rapidly acting stress hormones, such as the catecholamines (and possibly the rapid effects of corticosteroids), suggesting they might play a critical role in the restoration of homeostasis following stress exposure by 'disconnecting' the amygdala from the rest of the brain.

Acute exposure to stress and/or corticosteroids does not necessarily affect the brain in the same way as more prolonged periods of corticosteroid over-exposure. The latter is a clinically relevant situation, since exposure to chronic stress has been linked to the development of psychopathology (Mazure 1995; Sapolsky 1996; McEwen 1998). Moreover, patients suffering from a depression are characterized by (continuous) excessive corticosteroid signaling (Murphy 1991; Parker et al. 2003; Wolkowitz et al. 2009), which makes it of obvious interest for further study. We chose to investigate the effects of chronic corticosteroid over-exposure on brain connectivity under highly controlled experimental conditions, making use of an established animal model for chronic stress (Vyas et al. 2002). This model was earlier reported to cause dendritic hypertrophy in the hippocampal CA3 region and mPFC pyramidal neurons, and dendritic hypertrophy in the basolateral amygdala (BLA) (Vyas et al. 2002). We used this model to induce chronic stress in male rats, and tested its effects on the structural integrity and functional connectivity patterns in the rodent brain, using diffusion kurtosis imaging (DKI), high-resolution structural scanning, and

resting state fMRI (**Part 4.2**).

Chronic stress exposure resulted in a significantly lower body weight of the animals, increased adrenal weight, and the expected dendritic hypertrophy in the amygdala, and hypotrophy in the hippocampus and medial prefrontal cortex. Resting-state functional MRI revealed that functional connectivity strength was significantly increased in the somatosensory cortex, visual cortex, and default mode network (DMN) in response to stress. Interestingly, increased activity in visual processing areas in humans has been reported during acute stress exposure (Part 2.1, van Marle et al. 2009), and has been suggested to reflect a hypervigilant state of processing, in which bottom-up stimulus-driven processing is prioritized over higher-order executive function (Hermans et al. 2011). The observed increased network strength of the visual and somatosensory cortex might similarly reflect a more prominent role of sensory based processing in the chronically stressed brain. Interestingly, abnormal visual processing has been reported before in patients suffering from major depression (Deseilles et al. 2009; Veer et al. 2010). Depressed patients showed abnormal filtering of irrelevant information in the visual cortex, together with an altered functional connectivity between frontoparietal networks and visual cortices (Deseilles et al. 2009). Also the activity and the connectivity within the DMN have been shown to be increased in this depression (Grimm et al. 2009; Sheline et al. 2009, 2010; Greicius et al. 2007, reviewed by Whitfield-Gabrieli and Ford 2012), relating our findings of stress-induced increased DMN connectivity to this illness as well. The patterns of physiological activity within the DMN in depression have been hypothesized to relate to the self-absorption or obsessive ruminations accompanying the major depressive syndrome (Raichle et al. 2001; Gusnard et al. 2001; Drevets et al. 2002; Grimm et al. 2009). Moreover, as regions within the DMN (the medial network and limbic system) exert forebrain modulation over visceral responses mediated via the hypothalamus and brainstem, dysfunction within these circuits also may contribute to the disturbances in autonomic and neuroendocrine function that have been associated with depression. These hypotheses are compatible with treatments for depression, involving pharmacological, neurosurgical, and deep brain stimulation methods, that appear to suppress pathological activity within components of the extended DMN such as the posterior cingulate and subgenual anterior cingulate cortex and the ventromedial frontal cortex (Drevets et al. 2002; Mayberg et al. 2005). Moreover, a recent study by Zeng and colleagues (2012) indicated that the majority of the most discriminating connections between depressed patients and healthy controls were located within or across the DMN, affective network, and visual cortical areas. Here, we show that chronic stress profoundly affects the connectivity within two of these three networks, supporting its involvement in the development of psychopathology and providing a handle to study these abnormalities in rodents.

Next to affecting functional connectivity, chronic stress exposure induced an increased volume and mean diffusivity of the lateral ventricles, as measured with *post-mortem* high-resolution structural MRI and DKI. Numerous clinical studies have also reported on enlarged ventricles in patients with stress-related mental disorders (Zipursky et al. 1997; Kumar et al. 1997; Strakowski et al. 2002; Salokangas et al. 2002; Cardoner et al. 2003). Moreover, the enlargement of CSF spaces has been shown to predict poor treatment response of depression, accounting for 35% of the remission

time variance (Cardoner et al. 2003). Here we show that chronic stress induces enlargement of the lateral ventricles, a factor that may contribute to the development of psychopathology.

Despite the morphological differences at the cellular level, we did not find any stress-induced alterations in hippocampal volume or morphometry. Previous research in patients with stress-related mental disorders such as depression and PTSD, has shown volume reductions in the hippocampus (Bremner 1999; Sapolsky 2000; Koolschijn et al. 2009; Savitz and Drevets 2009), although the effect size is moderate (Koolschijn et al. 2009) and might be overestimated by a positive publication bias. Rodent studies investigating the impact of chronic stress have also reported on hippocampal volume reductions, but show rather modest effects as well, which require prolonged and severe stress exposure before only small reductions become detectable (Lucassen et al. 2006). Therefore, our neuroimaging method might not have been sensitive enough to pick up these subtle changes.

All in all, this study shows that chronic stress exposure in rodents induces macroscopic structural changes and alterations in functional network connectivity strength similar to those observed in stress-related psychopathology. Thereby, such chronic stress paradigms provide a handle to study these abnormalities in rodents under highly controlled experimental conditions.

## **An integrative perspective: time-dependent corticosteroid effects on the brain**

In this thesis we showed that stress and corticosteroids clearly influence brain function (activity and connectivity) in a time-dependent manner. In general, a quite consistent view on the slow, putatively genomic effects of corticosteroids emerged. The slow corticosteroid actions seemed to induce effects that were the exact opposite as what is generally seen for acute stress. Corticosteroids slowly improved prefrontal cortex executive control function, as reflected by improved working memory processing (Part 3.3) and enhanced sustained attention (Part 3.2). Neurally, these effects were observed as increases in dlPFC WM-related activity (Part 3.3), and a reduction in cuneus activity and connectivity between the amygdala and insula (Part 3.2), minimizing stimulus-driven emotional distraction. Moreover, the slow effects of corticosteroids induced an emotion-specific response mode in the amygdala (Part 3.1) and an increased connectivity between the amygdala and mPFC during emotional processing (Part 3.1). By exerting these effects the slow actions of corticosteroids might facilitate adequate higher-order cognitive functioning, which might contribute to stress recovery and return to homeostasis in the aftermath of stress. Moreover, they might prevent an overshoot in emotional processing during extensive stress. The findings on suppressed hippocampal and prefrontal cortex activity during memory encoding (Part 2.2) are slightly more difficult to interpret in this framework of enhanced executive function due to the slow effects of corticosteroids. Although some of the differences in experimental setup between these studies (e.g., the different dose of hydrocortisone administered, and the different time-interval of fMRI scanning relative to hydrocortisone intake) could be held responsible for these

apparent inconsistencies in suppressed hippocampal and PFC activity and the hypothesized boost of executive function, this suppression might contribute to another critical function of the brain in the sequels of stress. It may optimize the consolidation of the memory trace for the stressful event into memory, enabling proper responding upon future exposure, by reducing retroactive interference by new encoding. Moreover, the suppression of hippocampal and PFC activity might serve a neuroprotective function, since the initial activation upon stress exposure might cause neurotoxicity and eventual damage to these brain structures (de Kloet et al. 2008). Furthermore, it is noteworthy that the locations of the slow corticosteroid-modulation of PFC-activity observed during WM-processing (increase in activity, Part 3.3) and memory encoding (decrease in activity, Part 2.2) do not overlap. Therefore, it could be very well possible that the slow effects of corticosteroids contribute to the restoration of homeostasis by altering PFC function in a (sub) region-specific manner.

The findings on the rapid effects of corticosteroids are less clear-cut than those observed for their slow effects. In Part 3.1, we showed that corticosteroids rapidly suppressed emotional processing in the amygdala, potentially to prevent an overshoot in activity during stress. However, these data seem at odds with the data presented in Part 3.2, which shows rapid effects of corticosteroids that were similar to those of acute stress, boosting amygdala responsivity and disrupting its connectivity to frontoparietal regions. One crucial difference between the two studies is the type of brain function investigated, and the role of the amygdala in each of these tasks. Whereas the first study examined the effects of corticosteroids on amygdala function during basic emotional processing without any additional cognitive task (Part 3.1), the other study assessed amygdala functioning during the active suppression of emotional processing by higher-order cognitive control (i.e., the emotional distraction task, Part 3.2). Therefore, the causal role of the observed effects in the amygdala might be different and potentially caused by altered control. Such task-dependent modulation of brain activity by corticosteroids has already been shown for other tasks. A widely accepted phenomenon from memory research for example is that corticosteroids influence the processes of memory encoding and consolidation in an opposite manner compared to memory retrieval (Roozendaal 2002), although they all heavily depend on hippocampal activation. Nevertheless, corticosteroids boost memory encoding and associated hippocampal activity (van Stegeren et al. 2010), whereas they impair hippocampal activation during memory retrieval (de Quervain et al. 2003; Oei et al. 2007). As mentioned before, differential effects of corticosteroids are also observed depending on the function studied of the prefrontal cortex, displaying corticosteroid-induced decreased activity during long-term memory encoding (Part 2.2) and corticosteroid-induced increased activity during WM-processing (Part 3.3). Previous studies on corticosteroid effects on amygdala function suggest similar task-dependent effects; corticosteroids increased amygdala activity during memory encoding (van Stegeren et al. 2010), whereas they reduced its responding during fear conditioning (Merz et al. 2010) and extinction learning (Tabbert et al. 2010). One possible explanation for these differential rapid corticosteroid effects on amygdala functioning could be the extent to which the amygdala is activated in these

tasks. Possibly, corticosteroids rapidly increase vigilance for rather infrequently occurring, or less intense threatening stimuli, whereas they prevent amygdala overshoot during continuous exposure to threatening (emotional) input. For example, in the previously mentioned studies, the amygdala might be more active during fear conditioning (Merz et al. 2010) and extinction (Tabbert et al. 2010) compared to the encoding of complex pictures (van Stegeren et al. 2010), inducing rapid corticosteroid-induced suppression and activation, respectively. Similarly, in our tasks, the amygdala would be generally less activated by the occasional processing of aversive words, than by the continuous processing of morphing emotional faces. This hypothesized anxiolytic role for the rapid effects of corticosteroids during continuously high amygdala activation is in line with the anxiolytic effects of corticosteroids during stress exposure (Het and Wolf 2007, Het et al. 2012), or reduction of phobic fear response (Soravia et al. 2006). Furthermore, it might be related to the metaplasticity in response to corticosteroid exposure observed in the amygdala. Previous research has indicated that initial corticosteroid exposure (i.e., when amygdala activity was low) increased amygdala's spontaneous activity, whereas subsequent exposure to corticosteroids (i.e., when amygdala activity was still elevated) reduced amygdala's spontaneous activity, potentially preventing overshoot (Karst et al. 2010). Thus, corticosteroids' rapid actions seem to depend on the current state the amygdala is in; they boost amygdala activity under conditions of low basal activation, whereas they reduce amygdala activity under conditions of high basal activity. However, this line of reasoning remains rather speculative and should be tested in future studies.

A second explanation between the apparent discrepancy between the rapid corticosteroid effects on amygdala function, might be their critical dependence on (a sufficient level of) autonomic arousal and concurrent activation of noradrenalin or potentially other neurotransmitter systems in the amygdala. The rapid effects of corticosteroids have been shown to interact with noradrenergic signaling in the amygdala (the BLA in specific) (see Box 2 in Part 1 of this thesis). This interaction was shown to be critical for corticosteroid-modulation of memory consolidation processes, but the same may hold true for amygdala processing in general. Since demanding cognitive tasks are known to induce a higher level of arousal than passive viewing tasks, there is reason to assume that noradrenergic activation was in fact higher during the emotional distraction (Part 3.2) compared to the emotional processing task (Part 3.1). Also in our own line of studies we found that participants' heart rate (HR), reflecting autonomic arousal, was significantly higher during the cognitively demanding WM-task (Part 3.3) compared to resting state (Part 4.1) (mean  $\pm$  S.E.M., HR =  $66.5 \pm 3.2$  bpm during working memory, HR =  $56.7 \pm 0.9$  bpm during resting state,  $p < 0.001$ ). Assuming a similar noradrenergic activation during the emotional distraction task, might mean that the rapid effects of corticosteroids boost amygdala processing in interaction with noradrenergic activation, as is seen in other studies (e.g., van Stegeren et al. 2007, Hurlermann et al. 2007). The rapid effects of corticosteroids in isolation might on the other hand suppress amygdala activity (Lovallo et al. 2010), as is observed for the emotional processing task. Unfortunately, we did not assess heart rate during the amygdala tasks, nor any other measures of autonomic arousal, which leaves this issue open for future research.

A final explanation might lie in the critical time frame during which the rapid corticosteroid effects are assessed. Here, we targeted the rapid effects of corticosteroids on amygdala functioning at 60 min (emotional interference, Part 3.2) and 75 min (emotional processing, Part 3.1) post administration, respectively. Previous studies have shown elevated salivary cortisol levels from 15 min post administration onwards (van Stegeren et al. 2010), salivary cortisol levels that resemble plasma levels (Tunn et al. 1992), a delay of approximately 20 min between rises in plasma and brain corticosteroid levels (Droste et al. 2008), and *in vitro* amygdala effects from ~10 min post corticosteroid brain exposure onwards (Karst et al. 2005). This makes that one could expect the first effects of corticosteroids in the human amygdala to start at 45 min post administration. On the other hand, the first genomic effects of corticosteroids on the amygdala *in vitro* have been observed 60 min after brain exposure (Karst et al. unpublished data), which would mean that the earliest genomic corticosteroid effects on amygdala functioning arise at ~95 min post administration. Thus, assessing corticosteroid effects on amygdala functioning at 60 or 75 min post administration was too early to measure any genomic effects, but about 15 or 30 min after the start of the first rapid effects. Interestingly, in the hippocampus also intermediate effects of corticosteroids have been observed at about 20-30 min after brain exposure to corticosteroids (Joëls et al. 2012). Overall, these effects seemed to inhibit hippocampal function, similar to the genomic effects of corticosteroids; glutamate release (Zhang et al. 2005) and spontaneous firing rate (Pfaff et al. 1971) were reduced, LTP suppressed (Joëls and de Kloet 1993), and LTD enhanced (Zhang et al. 2005) at this time delay. Although this ambiguous time-domain seems to be rather slow for non-genomic corticosteroid actions, it is too rapid for any genomic actions to have occurred. Possibly, such intermediate corticosteroid effects also occur in the amygdala. A recent human study assessing the effects of an IV-injection of hydrocortisone on baseline brain activity (Lovallo et al. 2010) might point towards the existence of corticosteroid effects at such an intermediate time frame. It reported on an increase in amygdala and hippocampal activity 5-10 min post CORT-injection (possibly reflecting the rapid corticosteroid actions), but a strong decrease at 30 min. Based on these findings we might speculate that we assessed the truly rapid effects of corticosteroids only in the first amygdala task, the emotional interference task (Part 3.2), in which we found an increase in the processing of emotional distracters in the amygdala and in behavioral performance. The corticosteroid effects in the second amygdala task, the emotional processing task (Part 3.1), might in turn reflect the intermediate effects of corticosteroids observed at ~30 min post brain exposure to the hormone. Here, we observed a suppression of emotional processing in the amygdala, which is in line with the reduced activity observed in the study by Lovallo and colleagues (2010). However, at this stage this interpretation is rather speculative, and future studies should determine whether corticosteroid indeed affect (i.e., suppress) amygdala processing at intermediate time delays.

In conclusion, the data presented in this thesis suggest an active role for the slow effects of corticosteroids in the stress-recovery of cognitive function. They seem to shift the brain away from the hypervigilant stimulus-driven, reflex-mode of functioning as induced by acute stress, by

restoring cognitive control and boosting executive function. The rapid effects of corticosteroids were only shown to affect amygdala processing in the human brain. Corticosteroids rapidly increased vigilance for rather infrequently occurring threatening stimuli, whereas they suppressed amygdala responsivity during continuous exposure to emotional input. These data could suggest that corticosteroids' rapid actions affect amygdala processing depending on the concurrent brain state, as is seen in animals (Karst et al. 2010); boosting responses during conditions of low basal activation, while suppressing additional activation when basal activity is high. Alternatively, this differential modulation of amygdala responsivity by corticosteroids might be the result of distinct timing of the effects (first potentiation, quickly followed by suppression) or differential interaction with other stress hormones such as noradrenalin. Future studies should therefore further investigate the possible factors involved.

## **An integrative perspective: region-specific effects of corticosteroids on the brain**

Next to affecting brain functioning in a time-dependent manner, the data presented in this thesis show that stress and corticosteroids influence the brain in a region-specific manner. The previous section already dissociated between their effects on emotional processing as occurring in the amygdala on the one hand, and cognitive processing occurring in the PFC on the other. Here, we want to add the proof obtained from the electrophysiology data on the OFC to these findings and discuss the observed alterations in regional connectivity patterns as a consequence of stress or corticosteroid exposure. In Part 3.4 of this thesis, we investigated the effects of corticosteroids on OFC neuronal function. Human studies already pointed towards a modulation of OFC-function by stress hormone exposure; activity of the OFC was shown to be altered during stress exposure and to relate to the cortisol response to stress (Wang et al. 2005; Pruessner et al. 2008; Dedovic et al. 2009b). Moreover, abnormalities in OFC function and structure have been associated with stress-related mental disorders such as post-traumatic stress disorder (PTSD) (Liberzon et al. 2007; Carrion et al. 2008; Hakamata et al. 2007; Thomaes et al. 2010) and major depression (Koolschijn et al. 2009; Holsen et al. 2011) and to early life stress exposure (Hanson et al. 2010; Dannlowski et al. 2012). However, to the best of our knowledge, no previous study has ever examined the mechanistic underpinnings of these effects in the rodent OFC. Here, we showed that the slow, putatively genomic, effects of corticosteroids increase the amplitude of the sAHP after depolarization of OFC neurons. Thereby, the slow effects of corticosteroids seem to contribute to an increase in excitability of this region. The effects observed in the OFC were opposite to what was seen in dorsal CA1 hippocampal neurons, where an enhanced sAHP was observed after corticosteroid treatment. Remarkably, the OFC effects were similar to those reported for the most ventral part of the CA1 area (Maggio et al. 2009), and the basolateral amygdala (BLA) (Duvarci and Paré 2007). Similar regional differentiation between BLA and hippocampal neurons has been described with regard to morphological changes after chronic stress (Roosendaal et al. 2009, and

Part 4.2 of this thesis); whereas neurons in the CA3 – and to a lesser extent CA1 – hippocampal area display reduced dendritic complexity after chronic stress (McEwen and Magarinos 1997), BLA neurons show dendritic hypertrophy (Vyas et al. 2002). Also OFC neurons display hypertrophy in response to chronic stress (Liston et al. 2006), whereas hypotrophy is observed for the mPFC (PrL and ACg) (Cook and Wellman 2004, Radley et al. 2004, Liston et al. 2006). These data seem to suggest that there is a functional distinction between the effects of stress hormone exposure on the ventral emotional processing regions (BLA and OFC) on the one hand, and the dorsal cognitive control regions (hippocampus, mPFC) on the other. These effects are also reflected in behavior, since chronic stress is known to increase anxiety (Vyas et al. 2002), but to impair memory performance (McEwen 2001), attentional set-shifting (Liston et al. 2006), and working memory processing (Cerqueira et al. 2007). A similar effect has been described for acute stress exposure in the human brain; increased activity in visual processing regions and the amygdala was observed (van Marle et al. 2009), whereas PFC activity was suppressed (Qin et al. 2009) following acute stress exposure. At this stage, we can only speculate about the underlying mechanism of such differential neuronal response to stress and corticosterone between regions. It is highly unlikely that these differences are established by the activation of different types of receptors, but downstream effects of receptor activation might well differ between regions. Preliminary evidence suggests that the observed differences between CA1 and BLA neurons in response to corticosteroids may relate to the type of calcium channel subunits expressed by these neurons (Liebmann et al. 2008). Local characteristics of translational regulators, ion channel subunits or intracellular proteins may therefore cause large differences in the response of neurons to steroid hormones, and future studies are needed to determine these factors. Nevertheless, we are first in showing that the slow, putatively genomic, effects of corticosteroids affect OFC neurons in a different way than they affect the CA1.

These regional differences in stress and corticosteroid effects between ventral emotional processing areas and dorsal cognitive control areas are also observed in terms of connectivity. In this thesis (Part 4.1), we investigated the effects of acutely elevated corticosteroid levels on amygdala centered connectivity patterns in the human brain. It had already been shown that acute (van Marle et al. 2009; Hermans et al. 2011) or more prolonged (van Wingen et al. 2011a, 2011b) exposure to stress not only increase activity of the amygdala, but also strengthen its connectivity to regions within the salience network (Seeley et al. 2007), comprising the orbital frontoinsulae cortices, subcortical and limbic structures, and the anterior cingulate cortex. Moreover, it had been shown that this altered connectivity might even last long after the stressor is gone (van Wingen et al. 2012). Here, we showed that corticosteroids reduced functional connectivity of the amygdala (at ~100 min post administration). First of all, corticosteroids reduced positive functional coupling of the amygdala to brain regions involved in the initiation and maintenance of the stress-response (LC, hypothalamus, and hippocampus). By reducing amygdala connectivity to these stress-related brain regions, corticosteroids could prevent subsequent activation of the systems, curtail the stress-response, and promote the return to homeostasis. Secondly, corticosteroids reduced the negative

functional coupling of the amygdala to brain regions known to be involved in executive control (the middle frontal and temporal gyrus). Thus, by these actions corticosteroids seem to impair the connection between the salience and executive-control network, and within the salience network itself (Seeley et al. 2007), potentially reducing amygdala's influence on executive function. Such reduction might aid cognitive control processes in the aftermath of stress. By these mechanisms, corticosteroids thus seem to reduce the prominent role of ventral emotional processing induced by acute stress exposure, and contribute to the return to homeostasis.

Next to investigating the effects of an acute elevation of corticosteroid levels on functional connectivity as observed in the human brain, we also investigated the effects of prolonged exposure to elevated corticosteroid levels. This could be done under very controlled conditions by making use of an animal model of chronic stress (Part 4.2). Besides inducing the characteristic hypertrophy of dendrites in the BLA and dendritic hypotrophy in the CA3 and mPFC, chronic stress increased connectivity within the visual cortex, somatosensory cortex, and the DMN. This increased connectivity between visual processing and somatosensory regions is in line with a more prominent role of bottom-up stimulus-driven processing in response to stress, as described in humans in response to acute stress exposure (Part 2.1, van Marle et al. 2009, Hermans et al. 2011). Moreover, these findings relate to what is observed in stress-related psychopathology such as depression (Lanius et al. 2010; Daniels et al. 2010) and PTSD (Zeng et al. 2012; Whitfield-Gabrieli and Ford 2012). Connectivity within the visual cortical areas, the salience, and DMN has been shown to be the main predictor of major depression (Zeng et al. 2012; Whitfield-Gabrieli and Ford 2012), and connectivity within the DMN the main predictor of PTSD symptom severity (Lanius et al. 2010). Thus, these mental illnesses are not only characterized by highly regional abnormalities in brain activity, but also connectivity.

All in all, these studies show that stress and corticosteroids affect brain activity and connectivity in a highly region-specific manner. Whereas stimulus-driven, bottom-up emotional processing as occurring in the visual processing and salience network is boosted upon acute stress exposure, and the connectivity between these regions is strengthened, higher-order cognitive function deteriorates under conditions of acute stress. A similar effect is seen upon prolonged stress exposure, which boosts the function of ventral emotional processing areas (inducing hypertrophy in the BLA, increasing connectivity in the visual cortex), while suppressing regions involved in higher-order cognition (reflected in hypotrophy in the CA3 and mPFC). Conversely, acute elevation of corticosteroid levels appeared to acutely reduce the influence of the salience network on brain processing, by 'disconnecting' the amygdala from the rest of the brain.

## Limitations

Several limitations to these studies should be mentioned. First of all, in the majority of the studies the effects of corticosteroids on brain functioning were assessed by administering cortisol/corticosterone exogenously. Such a pharmacological model for the effects of corticosteroids does obviously not capture all aspects of the complex stress response and does not necessarily mimic the role of corticosteroids in mediating this stress response. Real-life cortisol release to stress is accompanied by the release of many other neuromodulators, such as noradrenalin, CRH, dopamine, and serotonin (Joëls and Baram 2009), with which corticosteroids could potentially interact. Since we did not induce stress in the majority of studies, the generalization from our results to stressful situations remains speculative. Nevertheless, mere administration of hydrocortisone reveals a cleaner mechanistic account for the corticosteroid effect, which was the aim of these studies.

Secondly, results are not based on a randomly selected, population-based sample, and are therefore by definition not representative for the entire population. In the human studies, we opted to recruit participants with the most stable response to stress and corticosteroids, making that they had to meet rather strict in- and exclusion criteria in order to be enrolled in the study. Most importantly, we only included men as participants; women were excluded for several reasons. First of all, women are known to exhibit smaller and more variable stress responses (Kajantie and Phillips 2006), depending on menstrual cycle phase and use of contraceptives (Kirschbaum et al. 1999; Bouma et al. 2009; Ossewaarde et al. 2011). Also the effects of stress exposure on brain functioning seem to be depending on menstrual cycle phase (Ossewaarde et al. 2010). Furthermore, women are known to respond differently to hydrocortisone than men, both in behavior (Andreano and Cahill 2006; Bohnke et al. 2010) and brain activation (Merz et al. 2010; Stark et al. 2006). For all these reasons, we restricted the human studies to men only. Reports on gender by stress interactions in rodent literature are also abundant (Louvart et al. 2006; Uriarte et al. 2009; Kikusui et al. 2009; Noschang et al. 2009; Iwasaki-Sekino et al. 2009), which made us restrict our animal studies to male mice and rats as well. Furthermore, both human and animal studies tested solely young adult subjects. Age was kept consistent, since it is known to be an important factor in determining the effects of corticosteroids on brain functioning (Wolf et al. 2001a; Heffelfinger and Newcomer 2001; Kukolja et al. 2008). Obviously, because of these in- and exclusion criteria, the results cannot be readily generalized, and future studies will be needed to test whether corticosteroids exert similar effects in other groups.

Thirdly, most of the reported behavioral effects are small effects (Part 2.2, 3.2, and 3.3). Although explanations for this lack of a strong behavioral effects can often be found in specifics of the study designs optimized for fMRI, it is probably also partially caused by the relatively low number of subjects in our fMRI studies; this number is obviously lower than for purely behavioral studies. Behavioral output is dependent on a multitude of factors (e.g., intelligence and motivation), and the variation in task performance amongst individuals within a condition is therefore quite

substantial. This is certainly the case for the between-subject comparisons that were used (Part 3.2 and 3.3), but also for within-subject comparisons when e.g. session order might influence results (Part 2.2) (Wirth et al. 2011). For this reason, effects with rather small effect sizes, such as observed here for stress and corticosteroid administration, are not easily detected in behavior with small sample sizes. The absence of clear behavioral effects is not unprecedented in neuroimaging studies (Monk and Nelson 2002; Oei et al. 2007; Qin et al. 2009; Kumsta et al. 2010; Kukulja et al. 2011). Regardless, we found robust brain effects that were in line with the behavioral effects, providing corroborative evidence. An alternative explanation for the rather small behavioral effects might be that the dose of hydrocortisone administered was too low to induce stronger effects. We used 10 or 20 mg hydrocortisone in these studies, because these doses are known to increase salivary cortisol levels to physiological levels observed under conditions of moderate or severe stress, respectively (Kirschbaum et al. 1996; Morgan et al. 2000; Tops et al. 2003). Moreover, previous studies using similar doses reported on the successful induction of corticosteroid effects on declarative memory (Kirschbaum et al. 1996; Buchanan and Lovallo 2001; Abercrombie et al. 2003; Tops et al. 2003; van Stegeren et al. 2010), and working memory had been shown to be even more sensitive to corticosteroid-modulation (Lupien et al. 1999). However, several studies reporting on corticosteroid effects on human cognition have used higher doses of hydrocortisone (Lupien et al. 2002; Kuhlmann and Wolf 2006; Putman et al. 2007a, 2007b, 2010; Tollenaar et al. 2009), and use of such dose might possibly have induced stronger behavioral effects. However, we chose to manipulate cortisol levels within the normal physiological range, which made the data ecologically more valid. Moreover, our design of targeting the slow, genomic effects of corticosteroids restricted the dose of hydrocortisone we could administer, since cortisol levels should be back to baseline a few hours post administration.

Furthermore, peak cortisol levels in the rapid and slow corticosteroid conditions in the studies described in Part 2.2 and 3.1-3.3, were not the same. Peak salivary cortisol levels in the rapid corticosteroid conditions were lower than those observed for the slow corticosteroid conditions, although the dose of hydrocortisone administration was exactly the same. Most likely, this difference is caused by a time of day effect since the hydrocortisone tablets were ingested in the early or late afternoon, to target the slow and rapid corticosteroid effects, respectively. Possibly, cortisol-binding globulin (CBG) levels were higher in the late afternoon, inducing lower levels of free cortisol (measured in saliva) in the rapid corticosteroid condition as measured in saliva. Reports on the circadian variations in CBG level are somewhat conflicting (Droste et al. 2009; Hsu and Kuhn 1988; Lewis et al. 2006). Given that CBG binding affinity is temperature dependent (Henley and Lightman 2011), one would actually expect lower binding in the later afternoon (in the rapid CORT condition), i.e., the opposite to what we observed. Alternatively, free cortisol levels might have been influenced by circadian variations in  $11\beta$ -steroid dehydrogenase 1 efficacy (Veniant et al. 2009), which could indeed lead to lower peak levels. Nevertheless, inclusion of the difference in peak corticosteroid level as a regressor into the analyses did not change the observed results. Therefore, differences between corticosteroid administration conditions are highly likely

the result of differential timing effects instead of dose effects.

Another limitation is the inability of fMRI to assess baseline differences in brain activity between conditions (stress/control, hydrocortisone/placebo). In traditional task-related fMRI designs (such as implemented in Part 2.1, 2.2, and 3.1-3.3) brain activity is analyzed by contrasting carefully modeled active with control conditions to target a contrast of interest. However, one is unable to detect slowly modulated changes in tonic baseline activity, which may occur alongside the phasic responses to discrete stimuli. Such differences in baseline cerebral blood flow might first of all occur by rather unspecific neurovascular effects caused by the manipulation. Stress is known to increase heart rate and blood pressure, and to induce vasoconstriction, and hydrocortisone has been shown to decrease venous oxygenation (Brown et al. 2012); factors known to affect the blood-oxygen-level-dependent (BOLD) fMRI response (Cohen et al. 2002; Behzadi and Liu 2005; Lu et al. 2008; Liu and Liau 2010). However, since such vascular, non-specific effects would be expected to change the BOLD response to a similar degree in all brain regions and regardless of the cognitive task, they are unlikely to explain the effects reported in our studies (which are region-specific and stimulus-dependent). In order to correct for such effects, global normalization using proportional scaling was applied in the studies described in Part 2.1, 2.2, and 3.1-3.3 (Desjardins et al. 2001; Peeters and Van der Linden 2002). Although this method might induce certain artifacts when local effects are strong enough to contribute substantially to global signal changes (Junghöfer et al. 2005), all critical comparisons in these studies (those between drug conditions) remain valid since this potential problem is similarly present in all drug conditions. In the connectivity study described in Part 4.1, global fluctuations were accounted for by extracting signal from individually defined white matter-, grey matter-, and cerebrospinal fluid (CSF)-masks and including these in the model. Moreover, in this study a control region was included to exclude any general effects on network connectivity. However, stress and corticosteroid manipulation might also cause region-specific changes in brain activity that have a neural basis. Stress has been shown to change tonic activity in both the amygdala and the hippocampus (Tillfors et al. 2001; Peres et al. 2007; Cousijn et al. 2010), potentially by elevating noradrenalin levels leading to tonically increased activity (Berridge and Foote 1991), and also in stress-related mental disorders perfusion of these regions seems to be altered (Drevets 1999; Liberzon et al. 2007). No evidence for such regional increase in perfusion has been reported as a consequence of hydrocortisone administration, but it has been found for other pharmacological compounds (Chen et al. 2011). Future studies should measure the effects of stress and corticosteroids on perfusion patterns of the brain, in order to assess their effects on regional baseline brain activity.

A further limitation is that we cannot infer any directionality from the correlative evidence for the connectivity analyses presented in Part 3.1, 3.2, and 4.1 of this thesis. As shown in all three studies, corticosteroids clearly affected amygdala connectivity, but we could only speculate about the directionality of these effects. We interpreted our findings either from a bottom-up (i.e., the amygdala influencing other regions) or top-down (i.e., other regions influencing the amygdala)

approach based on the supporting behavioral findings (e.g., increased emotional interference as described in Part 3.2), effects observed in activity patterns (Part 3.1 and 3.2), or previous findings from animal studies (Part 3.1 and 4.1). Future assessment of the directionality of the observed connectivity effects of corticosteroids using e.g., dynamic causal modeling (DCM; Friston et al. 2003), would be a very interesting next step into the elucidation of this corticosteroid-modulation of functional connectivity.

A final limitation is that these studies did not allow us to assess the mechanistic underpinnings of the observed corticosteroids effects. First of all, we do not know whether the rapidly induced changes by hydrocortisone administration are a direct effect of corticosteroid binding to their receptors, as shown in animal studies for the modulation of hippocampus and amygdala functioning (Karst et al. 2002, 2005, 2010; Duvarci and Paré 2007; Olijslagers et al. 2008; Pu et al. 2009), or an indirect effect mediated by a reduction in brain levels of corticotrophin-releasing hormone (CRH). CRH levels are known to be inhibited by the negative feedback actions of corticosteroids on the hypothalamus (Keller-Wood and Dallman 1984; Herman et al. 1996; Tasker 2006; Aguilera et al. 2007), though primarily under activated conditions which may not apply to our experimental setting. Since CRH is known to induce anxious behavior by activating the human amygdala both directly (Liang and Lee 1988) and indirectly by increasing locus coeruleus noradrenalin signaling (Valentino et al. 1983; Valentino and Foote 1988), corticosteroid-induced reductions in circulating CRH levels could also explain the suppressed amygdala responsivity as observed in Part 3.1. Secondly, we cannot claim that the observed slow effects of corticosteroids are in fact entailing a genomic mechanism. The evidence from animal studies showing that these slow effects of corticosteroids are depending on gene-transcription (i.e., depending on protein synthesis and GR-binding to the DNA (Kerr et al. 1992; Karst et al. 2000)) is strong. However, it is impossible to indisputably prove that the slow effects of corticosteroids induced in our studies involve a gene-mediated mechanism, as we cannot expose living human beings to the same manipulation as brain slices. Nevertheless, based on the evidence from animal studies and the time frame in which we assessed the effects, it is highly likely that the slow corticosteroid effects observed involved indeed a genomic pathway. Furthermore, it is very likely that the slow effects of corticosteroids entail a GR-dependent genomic mechanism, but we did not prove that. In order to do so, we could have tried administering a GR antagonist, but practical reasons currently prohibit realization of such experiment, since no selective GR antagonist is registered for human use yet. Mifepristone (RU-486) is the only compound commercially available (Pecci et al. 2009), but it is known to cross the blood brain barrier only at very high concentrations (Heikinheimo and Kekkonen 1993) and, more importantly, to also act as a very potent progesterone receptor antagonist (Heikinheimo et al. 1987), which might cause many unwanted side effects. Future studies are therefore necessary to elucidate the exact underlying mechanism of the observed slow corticosteroid effects.

## Conclusions

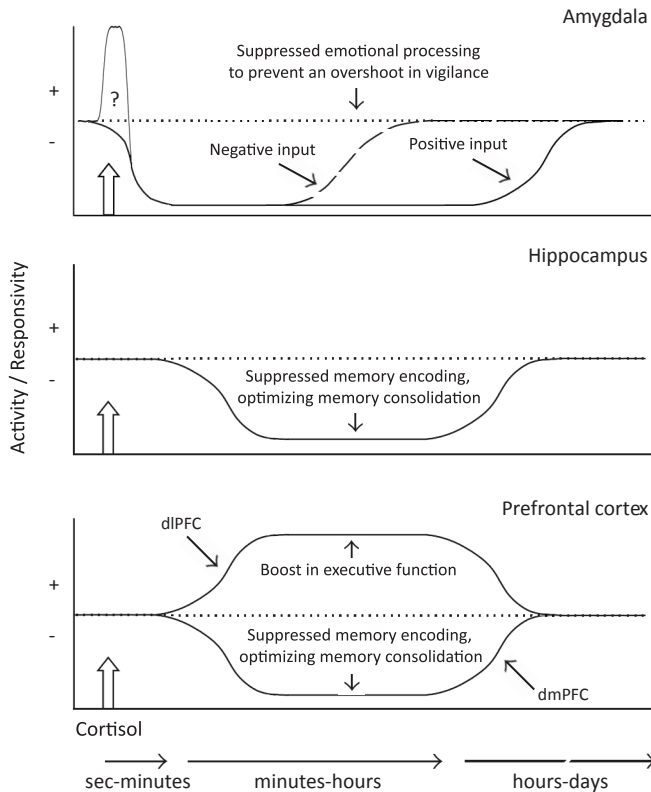
In conclusion, the data presented in this thesis suggests that acute stress and corticosteroids modulate brain activity and connectivity in a highly time- and region-specific manner. Although region-specific effects of corticosteroids have been reported before in the human brain (e.g., van Stegeren et al. 2010), the importance of the timing-factor has so far been neglected in human studies, while it is widely acknowledged in animal literature (Joëls et al. 2006). Our data suggests that future research on corticosteroids along with the understanding of their effects would greatly benefit from the incorporation of this crucial timing-factor in experimental designs.

In general, acute stress was shown to induce a hypervigilant state of processing in the brain, which was characterized by potentiated, but rather unspecific visual processing. This increase in sensory processing, in combination with an increased allocation of neural resources to noise reduction and potentially an optimal state for neural plasticity, enhanced stressful memory encoding. Corticosteroids might further contribute to the optimal storage of this memory trace and optimize consolidation processes by suppressing activity in memory-related brain regions and thereby reducing retro-active interference. Furthermore, corticosteroids were shown to time-dependently and region-specifically affect brain processing involved in emotion regulation and executive control to optimally cope with stress exposure. The rapid effects of corticosteroids seemed to affect amygdala processing in a brain-state dependent manner; whereas they boosted vigilance for rather infrequently occurring, or less intense threatening stimuli, they prevented amygdala overshoot during continuous exposure to threatening (emotional) input. However, this differential modulation of amygdala responsivity by corticosteroids might also be the result of distinct timing of the effects or differential interaction with other stress hormones such as noradrenalin. Future studies should therefore further investigate the rapid effects of corticosteroids on amygdala function.

The slow effects of corticosteroids seemed to play an active role in the stress-recovery of cognitive function. Data suggested that they shift the brain away from the hypervigilant stimulus-driven, reflex-mode of functioning as induced by acute stress, by restoring cognitive control and boosting executive function. Moreover, later corticosteroid effects were shown to ‘disconnect’ the amygdala from the rest of the brain, possibly to reduce its influence on brain functioning in the aftermath of stress exposure. Thus, corticosteroids in general seemed to induce effects that were the opposite of those of acute stress. This made us adapt the temporal dynamics model of Diamond et al. (2007) and compose a new model for temporal corticosteroid modulation of human brain function (Fig. 36), which obviously should be validated by future research.

The effects of prolonged exposure to corticosteroids, as occurring during chronic stress, were however different from acute corticosteroid modulation. They boosted the function of ventral emotional processing areas (inducing hypertrophy in the BLA, and increasing connectivity in the visual and somatosensory cortex), while it suppressed regions involved in higher-order cognition (reflected in hypotrophy in the CA3 and mPFC) and increased self-referential processing (increased connectivity of the DMN). Thereby, the effects of prolonged corticosteroid exposure

do not serve a similar protective function and may in fact be involved in the development of stress-related psychopathology.



**Figure 36.** Temporal dynamics model of how corticosteroids affected activity/responsivity of the amygdala, hippocampus, and prefrontal cortex. Whereas corticosteroids did not affect hippocampal and PFC processing in a rapid manner, their slow putatively genomic effects suppressed both hippocampal and dorsomedial PFC (dmPFC) processing during memory encoding. Thereby, they might contribute to the optimization of memory consolidation by reducing retro-active interference into the initial (stressful) memory trace. Moreover, corticosteroids were shown to slowly upregulate dorsolateral PFC (dlPFC) executive functioning, potentially contributing to the restoration of higher-order cognitive function in the aftermath of stress exposure. The rapid effects of corticosteroids were less straight-forward. Corticosteroids boosted amygdala responsivity to rather infrequently occurring, mildly threatening stimuli in an initial study, while it reduced amygdala responses during continuous exposure to (threatening) emotional input. These data could either indicate that corticosteroid effects on the amygdala are brain-state dependent; boosting responses during conditions of low basal activation, while suppressing additional activation (and thus preventing overshoot) when basal activity is high. Alternatively, this differential modulation of amygdala responsivity by corticosteroids might be the result of distinct timing of the effects (first potentiation, quickly followed by suppression, as indicated in the figure) or differential interaction with other stress hormones such as noradrenalin. Future studies should therefore further investigate the rapid effects of corticosteroids on amygdala function. The slow effects of corticosteroids seemed to play an active role in the normalization of amygdala responses to negative stimuli specifically. This could aid survival, since it is most important to be capable to respond adequately to dangerous stimuli first.

## Future perspectives

### Remaining open questions

As mentioned before, we base these claims on corticosteroid modulation of brain function on findings from studies investigating the effects of exogenous administration of corticosteroids. This raises the question whether endogenously released corticosteroids in response to stress exert similar effects on brain function. First evidence for a similar role of endogenous corticosteroids comes from a recent study by Het et al. (2012), in which they reviewed 5 of their previous studies (comprising 232 participants in total) to relate the salivary cortisol response to the TSST to the participants' increase in negative affective feelings in response to the stressor. The two measures appeared to be inversely correlated; the higher the cortisol response, the less emotionally affected participants were by the stress procedure. Remarkably, Het and colleagues observed a positive correlation between the increase in negative affect and levels of salivary alpha-amylase, a marker of noradrenergic activity. A similar negative correlation between cortisol response and negative affect was seen in social phobics upon exposure to the TSST (Soravia et al. 2006). These data suggest that endogenously released corticosteroids upon stress exposure serve a role in protecting mood and aid coping with the stressor. Thereby, they seem to counteract the effects of increased noradrenergic activation upon stress exposure, which corresponds to our (interpretation of the) findings of exogenously administered corticosteroids. Future studies are however necessary to test whether endogenous corticosteroids indeed counteract the acute stress response and increase in vigilance and contribute to the restoration of normal of brain function afterwards. The most straight-forward ways of testing this hypothesis is (A) by treatment with corticosteroids prior to acute stress exposure, and (B) blocking corticosteroid release (by the administration of metyrapone) during the stress response. Assessment of the effects of acute stress on psychological and physiological state, as well as brain activity in both these experiments will be informative to address (A) whether corticosteroids serve a protective function and suppress the effects of acute stress on brain functioning, and (B) whether corticosteroids are essential for the regulation and termination of the stress response.

Furthermore, our data do not fully resolve the rapid corticosteroids effects on brain functioning. In two studies (targeting memory formation (Part 2.2) and WM-processing (Part 3.3)) we did not find any evidence of modulation by the rapid corticosteroid effects. In two other tasks (investigating emotional processing (Part 3.1) and emotional interference (Part 3.2)) we found contradictory effects of the rapid actions of corticosteroids on amygdala functioning. These findings could first of all suggest that the rapid effects of corticosteroids depend on their interactions with other neuromodulators affected by stress exposure, such as noradrenalin, CRH, dopamine, and serotonin. Of special interest is the interaction between the rapid effects corticosteroids and noradrenalin, since both boosting and counteracting interaction effects are suggested by the amygdala findings. Secondly, they might suggest the possibility of a brain state-dependent (meta-plastic) modulation of amygdala function by the rapid effects of corticosteroids; whereas they increased vigilance for rather infrequently occurring, or less intense threatening stimuli, they suppressed amygdala

responsivity during continuous exposure to (threatening) emotional input. Thereby, they seem to potentiate amygdala responding when its basal activity is low, whereas they prevent an overshoot in activation during high basal activation levels. Such meta-plasticity of the amygdala in response to corticosteroids has been reported already in animals (Karst et al. 2010), and future studies combining amygdala-dependent functional tasks with arterial spin labeling to measure brain perfusion could possibly address this issue in humans.

Alternatively, the differential modulation of amygdala responsivity by corticosteroids might be the result of distinct timing of the assessment of the rapid effects, and entail a rapid potentiation of responding, followed at an intermediate time frame by a suppression. Effects at such an intermediate time frame (at about 20-30 min after brain exposure) have been observed for the hippocampus (Joëls et al. 2012), and future studies should determine whether such intermediate corticosteroid effects also occur in the amygdala.

## **Clinical implications for stress-related psychopathology**

The data presented in this thesis suggests a critical time-dependent role for corticosteroids in the regulation of the stress response, emotion, and cognition. Corticosteroids rapidly suppress amygdala activity (upon strong stimulation) and later on ‘disconnect’ the amygdala from other brain regions. Thereby, they may play a crucial role in terminating the critical feed-forward loop in the amygdala (i.e., the sensitization of the amygdala by acute stress, which boosts vigilance/anxiety and drives in turn the stress-response) and in limiting the effect the amygdala can exert on brain functioning. If uncontrolled, this positive feed-forward loop constitutes a powerful mechanism leading to progressively augmented amygdala sensitization with repeated stress exposure, and an increased influence of the amygdala on brain functioning. Our data suggest that the rapid effects of corticosteroids might be involved in curtailing this response. The fact that the HPA-axis is dysregulated in stress-related mental disorders such as depression and post-traumatic stress disorder (PTSD), but also the effectiveness of corticosteroids in preventing (Schelling et al. 2006) and treating (Aerni et al. 2004; de Quervain 2008) PTSD, may speak for their crucial role in interrupting the positive feed-forward loop. Furthermore, the slow effects of corticosteroids seem to boost higher-order cognitive function as performed by the PFC. Thereby, they might contribute to the restoration of proper cognitive function in the aftermath of acute stress exposure. As such corticosteroids could potentially be effective in the treatment and possibly prevention of stress-related mental disorders.

**Preventing PTSD by corticosteroid treatment.** PTSD patients are thought to be characterized by an overall stronger negative feedback mechanism of the HPA-axis, leading to only brief exposure to cortisol after stress (Yehuda et al. 1993). Moreover, previous studies have indicated that lower cortisol levels in the acute aftermath of trauma were predictors for subsequent PTSD symptoms (McFarlane et al. 1997; Delahanty et al. 2000; McFarlane 2000; Witteveen et al. 2010). These findings, together with our own data, suggest that corticosteroids are essential regulators of the

stress-response and critical factors for the restoration of proper brain functioning in the aftermath of stress. Ensuring properly high corticosteroid levels following trauma might therefore help to prevent PTSD-symptoms to develop. Previous animal studies have also related blunted HPA responses to stress to the development of PTSD (Cohen et al. 2006), and have shown that early post-stress treatment with high-dose corticosterone reduced the prevalence of PTSD-like behavioral responses (Cohen et al. 2008). Also human studies administering hydrocortisone following septic shock have shown a reduced incidence of PTSD due to this treatment (Schelling et al. 1999, 2001, 2003). Also preliminary data from Zohar et al. (2011), investigating the effects of administration of high levels of hydrocortisone at less than 6 hours after (poly)trauma exposure, showed that hydrocortisone was effective in reducing both acute stress disorder (20 % in the CORT vs. 66.7 % in the placebo group) as well as rates of PTSD (12.5 % vs. 37.5 % at 1 month (n.s.), and 0 % vs. 37.5 % at 3-month follow-up). Thus, post-trauma administration of hydrocortisone reduced the risk on the development of PTSD and attenuated core stress symptoms.

Other studies have investigated whether the administration of corticosteroids prior to trauma-exposure also reduces the incidence for the development of PTSD, since initial evidence for this phenomenon was found in animals (Cohen et al. 2006). Indeed, also (high-dose) hydrocortisone treatment prior to cardiac surgery was shown to be effective in reducing the stress symptoms during surgery (Weis et al. 2006), reducing later PTSD symptoms and traumatic memories (Schelling et al. 2004), and in improving overall quality of life (Weis et al. 2006).

Thus, initial studies indicate that corticosteroid administration might be an effective tool in preventing the development of PTSD. The exact mechanism by which corticosteroids do so, is currently unknown. Our data indicate that corticosteroids might accomplish these effects by manipulating prefrontal cortex and amygdala functioning in a highly time-dependent manner, and thereby might contribute to the restoration of proper brain functioning in the aftermath of stress.

**Treating PTSD with corticosteroids.** Besides the stronger negative feedback mechanism of the HPA-axis observed in PTSD, PTSD patients also seem to be characterized by lower basal cortisol levels, although some controversy exists (Yehuda 2001; Rohleder et al. 2004; Wessa et al. 2006). Our findings indicate that insufficient cortisol levels might contribute to the increased amygdala connectivity detected in these patients (Gilboa et al. 2004; Lanius et al. 2010; Osuch et al. 2008), as well as their increased amygdala activity (Hull 2002; Hayes et al. 2012), which has been shown to correlate to their anxiety scores and PTSD symptomatology (El Khoury-Malhame et al. 2011; Dickie et al. 2011). Our studies would indicate that the administration of exogenous cortisol might restore proper cortisol function in these patients, and thereby contribute to the proper regulation of brain processing. One very preliminary study, comprising 3 patients, has suggested that chronic hydrocortisone treatment indeed relieves PTSD symptoms in patients; 1 month of hydrocortisone treatment was shown to reduce PTSD symptomatology, traumatic memories, and reexperiencing symptoms (Aerni et al. 2004). Corticosteroid treatment in phobia also seems to be effective in reducing fear; an effect that remains until 2 days after corticosteroid exposure (Soravia et al. 2006). A prominent current theory is that these beneficial effects of corticosteroids are caused

by the inhibition of memory retrieval. Aversive memories are thought to play an important role in the pathogenesis and symptomatology of PTSD (and phobia); the persistent retrieval and reconsolidation of traumatic memories is thought to keep these memories vivid and thereby the disorder alive. Corticosteroids are known to impair memory retrieval (de Quervain et al. 1998, 2000, 2003; Tops et al. 2003; Kuhlmann et al. 2005a, 2005b; Kuhlmann and Wolf 2006; Buchanan and Tranel 2006, 2008; Tollenaar et al. 2009), and to suppress the activation of the medial temporal lobe (de Quervain et al. 2003; Oei et al. 2007, Weerda et al. 2010). Patients might benefit from this inhibition of their aversive memories. Moreover, studies have indicated that corticosteroids enhance the consolidation of extinction memory (Barrett and Gonzalez-Lima 2004; Cai et al. 2006). By inhibiting memory retrieval and promoting extinction, corticosteroids could therefore partly interrupt the vicious cycle of spontaneous retrieving, re-experiencing, and reconsolidating traumatic memories in PTSD and, thereby, promote forgetting. In this theory, particular emphasis is put on trauma-related memory, and the modulation of hippocampal function (i.e., suppression) by corticosteroids. Although this rationale is theoretically well-based, more studies are needed to further evaluate the beneficial effects of corticosteroids on PTSD symptomatology and their underlying mechanisms of action.

Our data indicate that the effects of corticosteroids on amygdala processing might play a prominent role in ameliorating PTSD symptoms as well. First of all, the corticosteroid-induced reduction in amygdala connectivity as observed in Part 4.1 of this thesis might be involved. Corticosteroid effects on memory retrieval have been shown to be amygdala-dependent (Roosendaal et al. 2003, 2004b), and reducing the connectivity between the amygdala and hippocampus might thus impair memory retrieval. Furthermore, the suppression of amygdala responsivity by corticosteroids might also contribute to the effects. Previous studies already indicated that corticosteroids can induce anxiolytic effects (File et al. 1979; Andreatini and Leite 1994; Het and Wolf 2007) and suppress arousal in both healthy individuals (Buchanan et al. 2001) and PTSD patients (Miller et al. 2011). By suppressing amygdala responsivity and reducing anxiety, the negative arousal induced by the memory (or exposure) might be attenuated, making it less emotionally salient. Removing the salience from the memory will make it more neutral in nature, and more easily forgotten. These data thus suggest that exposure techniques in cognitive-behavioral therapy might benefit from additional corticosteroid treatment in order to facilitate the extinction of aversive memories. More studies are needed to further evaluate the therapeutic efficacy of corticosteroids in the treatment of anxiety disorders and to determine their exact neural underpinnings.

**Blocking corticosteroid effects to treat depression.** In contrast to PTSD patients, patients suffering from major depression are thought to be characterized by continuously elevated cortisol levels (Murphy 1991; Parker et al. 2003; Wolkowitz et al. 2009), potentially caused by impaired GR-mediated negative feedback on the HPA-axis (Burke et al. 2005). Our data (Part 4.1) suggest that this increased cortisol signaling might induce a state of amygdala ‘decoupling’ as has been described for depressed individuals (Savitz et al. 2009; Moses-Kolko et al. 2010; Veer et al. 2010; Etkin and Schatzberg 2011; Lui et al. 2011; Luking et al. 2011), and has been shown to predict

symptom severity (Matthews et al. 2008; Dannlowski et al. 2009). Moreover, our data (Part 3.2) indicate that it could mediate their attentional bias towards negative emotional information (Williams et al. 1996). Depressed patients are known to be compromised in their capability to suppress emotional irrelevant information (Mitterschiffthaler et al. 2008), and we here show a role for the rapid effects of corticosteroids in mediating this effect. However, with continuously elevated corticosteroid signaling one would also expect increased slow genomic effects of corticosteroids, which are not observed in the brain. Prefrontal cortex function is in general deteriorated in depressed patients and cognitive control over emotions impaired (Phillips et al. 2003; Drevets et al. 2008; Mitterschiffthaler et al. 2008). It is therefore difficult to pinpoint the exact involvement of excessive corticosteroid signaling in establishing these effects in depression, and the mechanism by which they influence symptomatology.

This does however not exclude the possibility that depressed patients might benefit from interference with the aberrant corticosteroid signaling. One could think of treating depression by direct inhibition of corticosteroid synthesis or by reducing corticosteroid levels indirectly by improving the negative feedback. Current antidepressants have already been shown to modulate GR-function, mainly by increasing feedback-regulation of the HPA axis (for review see Anacker et al. 2011). Treatment with glucocorticoid synthesis inhibitors (e.g., ketoconazole) has also been proven to rapidly ameliorate depression in treatment-resistant depression (Reus and Wolkowitz 2001). Moreover, studies administering GR- or MR-agonists such as hydrocortisone, dexamethasone, and prednisolone have shown antidepressive effects in clinical populations (Dinan et al. 1997; Bouwer et al. 2000; DeBattista et al. 2000), potentially by restoring negative feedback on the HPA-axis. However, in preclinical studies these agonists have been shown to induce depressive effects and decrease neurogenesis (David et al. 2009).

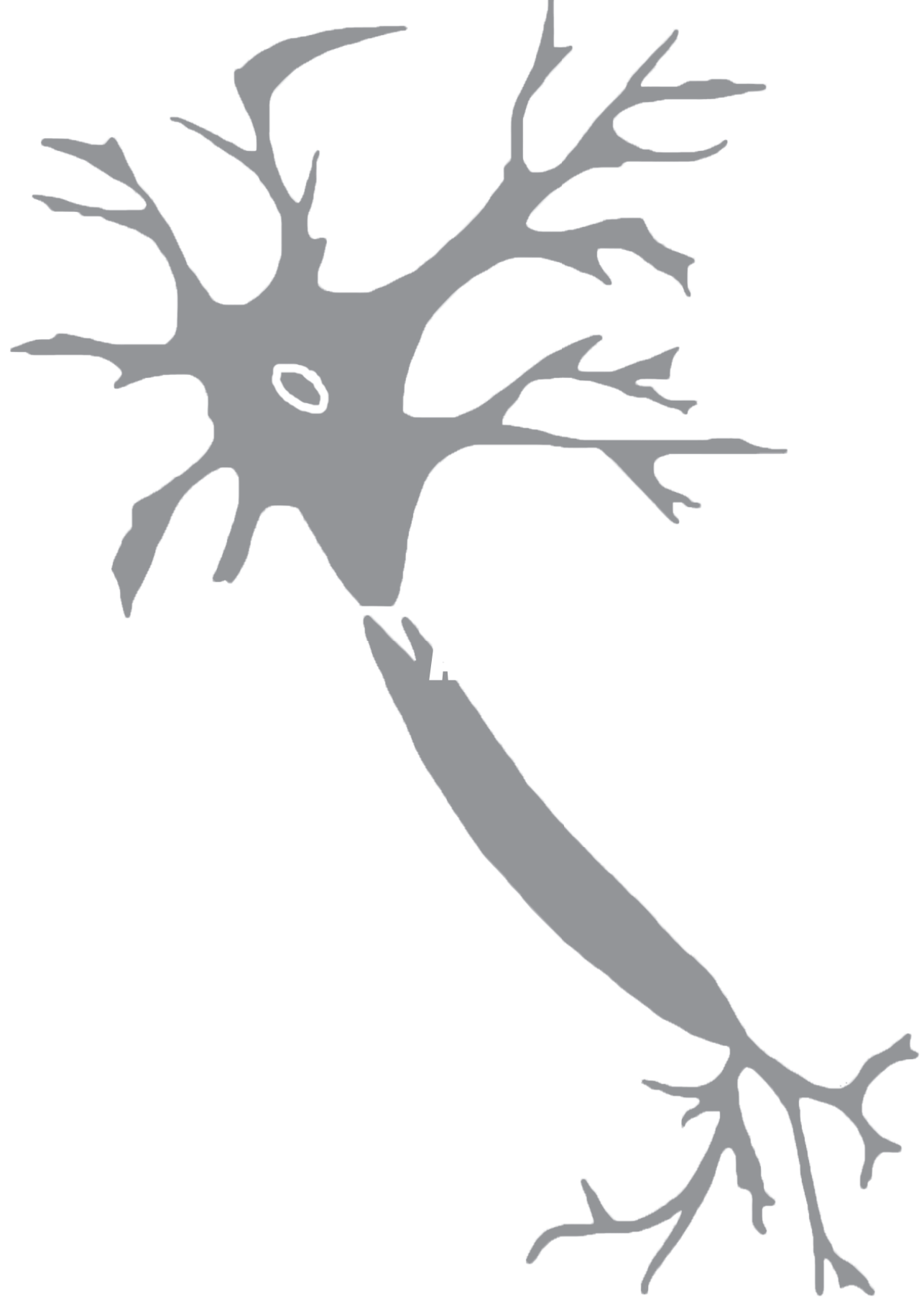
Another potential way of blocking the effects of aberrant corticosteroid signaling is by blocking GR-function by the administration of mifepristone. Prolonged administration of this drug has been shown to improve neurocognitive impairments, depression ratings, and psychotic symptoms (Murphy et al. 1993; Belanoff et al. 2001, 2002; Young et al. 2004); effects that remained stable for >7 weeks after the termination of treatment (Simpson et al. 2005). Data on larger trials of mifepristone in psychotic depression (DeBattista and Belanoff 2006; DeBattista et al. 2006; Flores et al. 2006), suggested that the effect on particularly the psychotic symptoms may be rapid and persistent. A recent animal study by Wulsin et al. (2010) showed that mifepristone reduces anxiety during an acute stressor, and increases neuronal activity (i.e., c-Fos expression) in the mPFC and ventral subiculum, whereas it reduces activity in the hippocampus and central amygdala. Thereby it was suggested that it may ameliorate stress dysfunction associated with depressive illness (Wulsin et al. 2010). Mifepristone has also been shown to reverse the reduction in adult hippocampal neurogenesis as observed in chronically stressed or glucocorticoid treated rats (Mayer et al. 2006; Oomen et al. 2007). However, one can imagine that blocking GR-function has its down side as well. Mifepristone administration in humans is known to elevate cortisol levels, possibly by blocking GR-mediated negative feedback (Flores et al. 2006). Moreover, chronic injection of mifepristone into the dentate gyrus has been shown to induce, rather than

ameliorate, depressive symptoms in animals (Papolos et al. 1993).

Thus, findings on treating depression by manipulating corticosteroid signaling, either by increasing negative feedback or blocking corticosteroid effects in the brain, are still confusing. Moreover, there is currently no selective GR antagonist registered for human use yet. Mifepristone is a very potent progesterone receptor antagonist as well (Heikinheimo et al. 1987) and known to cross the blood brain barrier only at very high concentrations (Heikinheimo and Kekkonen 1993). Testing the effects of GR-blockage in depression might benefit from the development of such a selective antagonist. Preliminary reports already suggested that a specific GR-antagonist (ORG 34517; Høyberg et al. 2002) is more effective in reducing symptoms and HPA-dysfunction, and much effort is put into the development of such compounds (Li et al. 2010; Brown et al. 2011). Future studies will determine the potential of such GR-antagonist in treating depression.









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## Nederlandse samenvatting

Stress, iedereen heeft het wel eens meegemaakt. Tijdens een presentatie, een examen of een optreden; je voelt je hart bonzen, je spieren spannen zich aan, je gaat sneller ademen, begint te zweten, en trilt misschien zelfs een beetje. Op zo'n moment ben je extra alert, maar ook sneller afgeleid, en je denkt alleen aan het hier en nu; al het andere lijkt even onbelangrijk. Je kunt je vaak moeilijk dingen herinneren, en krijgt misschien zelfs een black-out, maar tegelijkertijd wordt de stressvolle gebeurtenis die je op dat moment meemaakt in je geheugen gegrift.

Al deze reacties, in je lichaam maar ook in je brein, zijn het resultaat van de effecten van hormonen die tijdens een stressreactie worden aangemaakt. Dit is een gezonde reactie van het lichaam; er wordt namelijk extra energie vrijgemaakt die je in staat stelt om te gaan met de situatie. Stress is echter ongezond wanneer deze te lang aanhoudt of te hevig is, en kan dan ernstige gevolgen hebben, zoals een burn-out, overspannenheid, of de ontwikkeling van een trauma van de stressvolle gebeurtenis. Daar komt bij dat een verkeerde regulatie van stresshormonen gerelateerd is aan verschillende psychische aandoeningen, zoals depressie en posttraumatische stress stoornis (PTSS). Hoewel het voor de behandeling van deze ziekten erg belangrijk te weten is hoe stress ons brein precies beïnvloedt, is er nog maar weinig over bekend.

Het doel van het onderzoek dat in dit proefschrift staat beschreven is ons begrip van de effecten van stresshormonen in het brein te vergroten en daarmee bij te dragen aan onze inzichten in stress-gerelateerde psychische aandoeningen. Om de effecten van stresshormonen op het menselijk brein te onderzoeken hebben we gebruik gemaakt van beeldvormend hersenonderzoek (functionele MRI) in combinatie met experimentele stressinductie, of farmacologische manipulatie van het stresshormoon cortisol. Dierstudies naar de effecten van cortisol op het functioneren van hersencellen (neuronen) hadden al eerder aangetoond dat deze effecten afhankelijk zijn van welk hersengebied men precies bestudeert en op welk tijdstip men dat doet. Cortisol heeft namelijk zowel snelle als langzame effecten die veroorzaakt worden door verschillende mechanismen, en een verschillende rol lijken te spelen bij stress. Dierstudies hebben gesuggereerd dat de snelle effecten van cortisol bijdragen aan de acute stressreactie, en de werking van andere stresshormonen, zoals noradrenaline, versterken. De langzame effecten van cortisol lijken daarentegen bij te dragen aan de normalisatie van het brein wanneer de stressvolle gebeurtenis voorbij is. Of cortisol vergelijkbare effecten in het menselijk brein heeft, was tot nu toe echter onbekend. Daarom hebben we de effecten van stress en cortisol op het functioneren van verschillende hersengebieden over de tijd onderzocht. Verder hebben we ook bestudeerd hoe het nu kan dat stresshormonen een hersengebiedafhankelijk effect hebben. Daartoe hebben we gebruik gemaakt van elektrofysiologie, een techniek waarmee men de elektrische eigenschappen van hersencellen en hun communicatie kan meten aan de hand van elektrische signalen.

## De effecten van stresshormonen op het geheugen

In deel 2 van dit proefschrift hebben we allereerst onderzocht hoe het kan dat we stressvolle gebeurtenissen zo goed kunnen herinneren. Soms herinneren we ons nare gebeurtenissen zelf zo sterk dat we ze niet meer kunnen vergeten, al zouden we dat graag willen. Om te onderzoeken wat er nu precies gebeurt met de geheugenprocessen in ons brein onder stress, hebben we allereerst een methode ontwikkeld waarmee we proefpersonen in de MRI scanner gestresst konden maken (**deel 2.1**). We hebben ze daartoe erg gewelddadige korte filmfragmenten laten zien, die ervoor zorgden dat onze proefpersonen inderdaad psychisch en lichamelijk gestresst raakten; ze rapporteerden een toename in negatieve gevoelens, hun hartslag ging omhoog, en ze lieten ook een stijging zien in hun niveau van het stresshormoon cortisol dat we konden meten in hun speeksel. We lieten deze fragmenten zien voorafgaand, tijdens en na afloop van een geheugentaak in de MRI scanner, waarin proefpersonen de opdracht kregen de foto's die ze te zien kregen zo goed mogelijk in hun geheugen op te slaan. Deze foto's waren neutraal of negatief, en de proefpersonen moesten met een druk op de knop aangeven tot welke categorie de foto's behoorden. De volgende dag kwamen de proefpersonen terug voor een geheugentest, waarin ze zo veel mogelijk foto's die ze gezien hadden moesten proberen te omschrijven. Om te onderzoeken wat nu het verschil is tussen geheugenvorming in het brein onder stressvolle en neutrale omstandigheden, vergeleken we deze geheugensessie met een neutrale geheugensessie, waarin de proefpersonen precies dezelfde geheugentaak te doen kregen, maar de nare filmfragmenten vervangen waren door neutrale beelden. Wat bleek? Het geheugen van de proefpersonen was inderdaad beter voor de foto's die ze bestudeerd hadden onder de stressvolle omstandigheden, vergeleken met de neutrale omstandigheden. Het maakte daarbij niet uit of het neutrale of negatieve foto's waren, al werden de laatste over het algemeen beter onthouden. In het brein waren ook effecten te zien van stress, die mogelijk konden verklaren waarom ons geheugen onder stress beter werkt. We zagen een toename in hersenactiviteit in het gebied dat visuele informatie verwerkt, de visuele cortex, door stress. Ons brein krijgt dus meer (visuele) informatie binnen tijdens stress, maar die toename bleek niet goed te zijn voor ons geheugen, waarschijnlijk omdat er ook heel veel overbodige informatie binnenkomt. Verder verhoogde stress ook de activiteit in gebieden die de visuele informatie vervolgens filteren tot informatie die bruikbaar is voor ons brein (zodat we niet 'overgestimuleerd' worden); de fusiform gyrus. Deze activiteit bleek wel goed te zijn voor het geheugen. Daarnaast bleken foto's onthouden te worden wanneer de hippocampus, een erg belangrijk gebied voor geheugenvorming, *minder* activiteit vertoonde. De data suggereerde dus dat stress een toename in visuele verwerking veroorzaakt, maar dat dit alleen voor een beter geheugen zorgt wanneer ook de 'filter-gebieden' toenemen in activiteit. Wanneer dat gebeurt, krijgt de hippocampus minder, maar specifiekere, informatie binnen, die deze vervolgens op een goede manier kan verwerken tot een herinnering. Met deze studie hebben we dus laten zien wat er gebeurt met de processen van geheugenvorming in het brein tijdens stress, en aangetoond hoe het kan dat stressvolle gebeurtenissen zo goed worden onthouden.

Het onderliggende mechanisme waarmee stress deze effecten veroorzaakt was hiermee echter nog niet duidelijk. Om de rol van het stresshormoon cortisol hierin te onderzoeken, vervolgden we deze studie met een farmacologisch onderzoek, waarin proefpersonen een capsule met cortisol innamen (**deel 2.2**). Omdat dieronderzoek had aangetoond dat cortisol verschillende snelle en langzame effecten heeft, dienden we cortisol toe op twee verschillende momenten. Proefpersonen kregen cortisol óf 3 uur voor een geheugentaak (om de langzame effecten van cortisol te meten) óf 30 min van te voren (voor de snelle effecten), óf ze kregen een neppil (placebo) om eventuele effecten van hun verwachtingen uit te sluiten. De proefpersonen wisten dus niet welke pil ze kregen. De geheugentaak in de MRI scanner was precies dezelfde als die in het stressonderzoek, en proefpersonen kregen wederom neutrale en negatieve foto's te zien die ze moesten proberen te onthouden en waar hun geheugen de volgende dag voor werd getest. We vonden in deze studie geen gedragseffect van cortisoltoediening; de prestatie in de geheugentest veranderde niet door cortisolinname. In het brein vonden we echter wel sterke aanwijzingen dat cortisol de activiteit in geheugengebieden beïnvloedt. We vonden geen snelle effecten van cortisol in het brein, maar de trage effecten verlaagden de activiteit in de hippocampus en gebieden in de prefrontale cortex (PFC), een andere structuur die erg belangrijk is voor geheugenvorming. Soortgelijke onderdrukking van geheugenprocessen in de hippocampus was al eerder aangetoond in dierstudies voor de trage effecten van cortisol. Hier lieten we dus zien dat ze eenzelfde effect hebben in het menselijk brein. De onderdrukking van activiteit in deze gebieden na stress zou ervoor kunnen zorgen dat er minder verstoring optreedt in de herinnering voor de stressvolle gebeurtenis, waardoor deze extra goed onthouden wordt. Op deze manier draagt cortisol dus mogelijk ook bij aan een beter geheugen voor stressvolle gebeurtenissen.

## **De effecten van cortisol op het verwerken en controleren van emoties**

Vervolgens wilden we de rol van cortisol testen op andere hersenfuncties waarvan eerder onderzoek al had aangetoond dat ze worden beïnvloedt door stress. In deel 3 van dit proefschrift beschrijven we de tijdsafhankelijke effecten van cortisol op emotionele verwerking, aandacht, en hogere cognitieve functies zoals het werkgeheugen in het brein. Vergelijkbaar aan de vorige studie, kregen proefpersonen weer óf enkele uren van te voren (4 uur in dit geval) cortisol toegediend om diens trage effecten te meten, óf 30 min van te voren om diens snelle effecten te bepalen, óf placebo. De effecten van cortisol op emotionele verwerking werden getest aan de hand van een gezichtentaak in de MRI scanner (**deel 3.1**). In deze taak kregen de proefpersonen foto's van gezichten te zien die heel snel van een vrij neutrale uitdrukking in een expliciet blijde of angstige uitdrukking veranderden. Af en toe kregen de proefpersonen ook een kruisje te zien op het scherm, en hun enige opdracht was om naar de gezichten te kijken en op de knop te drukken wanneer het kruisje in beeld kwam. Deze gezichtentaak werd gebruikt omdat we weten dat deze een belangrijk emotiegebied in het brein, de amygdala, sterk activeert. Bovendien stelde deze taak ons in staat om te kijken naar emotiespecifieke effecten van cortisol, door de reactie van het brein op de angstige en blijde gezichten met elkaar te vergelijken. De hersendata liet zien dat

de amygdala van proefpersonen die een placebo hadden gekregen erg sterk reageerde op zowel de blij als angstige gezichten. De snelle effecten van cortisol onderdrukten deze reactie echter volledig. In de proefpersonen die 30 min voorafgaand het scannen cortisol hadden gekregen, reageerde de amygdala totaal niet meer op de gezichten, maar vertoonde vergelijkbare activiteit als bij het zien van de kruisjes. In de proefpersonen die cortisol 4 uur voor het scannen hadden gekregen vertoonde de amygdala een vergelijkbaar onderdrukte reactie op de blij gezichten, maar reageerde normaal op de angstige gezichten. Deze emotiespecifieke reactie bleek gerelateerd te zijn aan een veranderde communicatie tussen de amygdala en een gebied in de prefrontale cortex, dat erom bekend staat dat het de activiteit van de amygdala reguleert. De resultaten van dit onderzoek toonden dus aan dat de snelle effecten van cortisol de algehele reactie van de amygdala onderdrukken, en dat de langzame effecten deze reactie normaliseren voor negatieve informatie, terwijl het verwerken van positieve informatie nog steeds onderdrukt blijft. Onze bevindingen voor cortisol komen overeen met vorig onderzoek dat aangetoond had dat zowel ratten als mensen minder angstig zijn wanneer ze net cortisol hebben gekregen. Opmerkelijk genoeg veroorzaakt acute stress een toename in amygdala activiteit en een toename in angstige gevoelens. Deze data suggereert dus dat de snelle effecten van cortisol een tegenovergesteld effect hebben op de amygdala dan acute stress. Het is daarom goed mogelijk dat cortisol een rol speelt in de regulatie van de amygdala en het brein mogelijk beschermt tegen een te hoge activiteit. Als de stress voorbij is, is het echter weer belangrijk dat de amygdala normaal reageert. De langzame effecten van cortisol lijken een rol te spelen in deze normalisatie, maar stellen daarbij prioriteit aan een normale reactie op negatieve t.o.v. positieve stimuli.

Vervolgens onderzochten we in hetzelfde experiment ook de effecten van cortisol op aandacht (**deel 3.2**). Om de aandacht van onze proefpersonen te testen in de MRI scanner, gebruikten we een emotionele interferentie taak. Deze taak hield in dat de proefpersonen woorden in een bepaalde kleur te zien kregen, en zo snel mogelijk op een knop moesten drukken om aan te geven welke kleur dat was. De woorden waren neutraal (zoals papier, ijzer, of klok) of negatief (zoals dood, braaksel, of angst). Het is bekend dat mensen de betekenis van deze woorden automatisch verwerken wanneer ze gepresenteerd worden, ook al proberen ze de informatie te negeren. Men wordt in deze taak daardoor afgeleid door de woorden, en met name de emotionele, waardoor men langzamer op de knop drukt voor de juiste kleur, of meer fouten maakt voor deze woorden. Deze taak konden we dus gebruiken om te testen hoe goed proefpersonen in staat zijn emotionele informatie te onderdrukken (door de emotionele en de neutrale trials met elkaar te vergelijken), en hoe goed ze überhaupt in staat zijn hun aandacht bij de taak te houden (door hun algehele prestatie te vergelijken). Zoals we hadden verwacht, reageerden onze proefpersonen langzamer op emotionele dan neutrale woorden, maar de proefpersonen die net van de te voren cortisol hadden gekregen maakten ook nog meer fouten voor de emotionele woorden, terwijl de andere groepen dat niet deden. De snelle effecten van cortisol zorgden er dus voor dat de proefpersonen meer afgeleid werden door emotie. Dat zagen we ook terug in het brein. De snelle effecten van cortisol zorgden voor een hogere activiteit in de amygdala tijdens het zien van de emotionele

dan de neutrale woorden. Normaal gesproken (in de placebo conditie) was men goed in staat deze activiteit te onderdrukken en dus de emotie te negeren, maar de snelle effecten van cortisol zorgden er dus voor dat de proefpersonen vatbaarder werden voor afleiding door emotionele stimuli. Daarnaast nam ook de communicatie tussen de amygdala en gebieden in de prefrontale en pariëtale cortex toe, waardoor de amygdala mogelijk meer invloed had op het uitvoeren van de taak. Deze effecten zouden mogelijk tijdens de blootstelling van stress handig kunnen zijn voor het oppikken van gevaarlijke signalen in de omgeving. De proefpersonen die enkele uren van te voren cortisol hadden gekregen leken de taak daarentegen over het algemeen beter te doen; ze maakten minder fouten, al was dat effect statistisch gezien net niet betrouwbaar. In het brein zagen we dat de trage cortisol effecten de activiteit van de cuneus onderdrukten, een gebied dat betrokken is bij visuele verwerking, wat zou kunnen duiden op een minder visueel ingestelde toestand van het brein. Daarnaast verzwakte de communicatie tussen de amygdala en de insula in het brein, waardoor de amygdala mogelijk minder invloed kon uitoefenen op de uitvoering van de taak. Deze studie liet dus zien dat cortisol ook aandachtsprocessen in een tijdsafhankelijke manier beïnvloedt.

De proefpersonen kregen ook nog een werkgeheugen taak te doen in de MRI scanner (**deel 3.3**). Met deze taak wilden we de effecten van cortisol op de prefrontale cortex bepalen. Deze hersenstructuur is betrokken bij hogere cognitieve functies, zoals het nemen van beslissingen, plannen, sociaal gedrag en de controle van emoties. Van deze taak is het bekend dat hij voornamelijk de dorsolaterale PFC (dlPFC) activeert. Proefpersonen kregen cijferreeksen te zien die ze moesten proberen te houden. Wanneer het cijfer dat ze op een bepaald moment zagen hetzelfde was als dat ze afwisselend 0, 1, 2 of 3 plekken terug hadden gezien moesten ze op een knop drukken. De snelle effecten van cortisol leken geen invloed te hebben op het uitoefenen van de taak; we zagen geen verschil in de prestatie of in de hersenactiviteit tussen deze groep en de groep die placebo had gekregen. De langzame effecten van cortisol daarentegen zorgden ervoor dat de proefpersonen beter presteerden; ze reageerden sneller en maakten minder fouten. In het brein zagen we dat dit samenging met een hogere activiteit van de dlPFC. De trage effecten van cortisol verbeterden dus de functie van de dlPFC.

Met deze studies hebben we het eerste bewijs geleverd voor tijdsafhankelijke effecten van cortisol in het menselijk brein. De langzame effecten van cortisol bleken de functie van de prefrontale cortex te verbeteren; ze verhoogden de activiteit van de dlPFC, verbeterden het werkgeheugen en ze versterkten de communicatie tussen de amygdala en PFC. Daarnaast leken ze de algehele aandacht te verbeteren in de aandachtstaak door visuele gebieden te onderdrukken, en de invloed van de amygdala te beperken. Daarmee lijken de langzame effecten van cortisol precies het tegenovergestelde effect te hebben van acute stress, en is het dus waarschijnlijk dat ze bijdragen aan de normalisatie van hersenfunctie als de stressvolle gebeurtenis voorbij is. De snelle effecten van cortisol waren minder eenduidig. We vonden geen snelle effecten op de hippocampus en PFC, wat erop zou kunnen duiden dat de snelle effecten van cortisol afhankelijk zijn van hun interactie

met andere stresshormonen om deze gebieden te kunnen beïnvloeden. In de amygdala vonden we ogenschijnlijk tegenstrijdige resultaten. In de emotietaak onderdrukten de snelle effecten van cortisol de reactie van de amygdala, terwijl deze juist vergroot werd in de aandachtstaak. Dit zou mogelijk kunnen komen door de verschillende context tijdens de verschillende taken; cortisol zou de reactie van de amygdala op minder emotionele stimuli die maar af en toe voorkomen kunnen vergroten, terwijl het een overactivatie van de amygdala tijdens continue blootstelling aan sterk emotionele stimuli voorkomt. Zo'n contextafhankelijk effect van cortisol in de amygdala is al eerder aangetoond in dierstudies. Toekomstig onderzoek zal echter moeten uitwijzen of dit ook het geval is bij mensen.

Cortisol heeft dus verschillende effecten op de hippocampus, amygdala en PFC. Maar hoe kan dat? Welk mechanisme ligt daaraan ten grondslag? Om dit te onderzoeken maakten we gebruik van een diermodel (muis), waarin we de hippocampus en de orbitofrontale cortex (OFC) onderzochten (**deel 3.4**). We focusten op deze twee gebieden omdat bekend was dat hun neuronen anders reageren op chronische stress; de cellen in de hippocampus krimpen, terwijl die in de OFC groeien als gevolg van stress. Om te onderzoeken welk effect de langzame acties van cortisol hebben op de elektrische eigenschappen van de cellen, onderzochten we hersenplakjes van deze gebieden nadat ze 1-4 uur eerder blootgesteld waren aan een hoge concentratie cortisol of 'placebo' (ethanol) voor 20 min. We maten deze eigenschappen door gebruik te maken van een speciale methode binnen de elektrofysiologie, genaamd 'patch-clamp'. Bij deze techniek gebruikt men een heel dunne pipet met daarin een elektrode, die men tegen de hersencel aanzet en een stukje van de rand (het membraan) stuk maakt, waardoor men metingen kan verrichten aan de elektrische stroompjes die door de hersencel heen lopen. We vonden geen effecten van cortisol op de elektrische eigenschappen van neuronen wanneer deze zich in een relatieve rusttoestand bevonden; de rustpotentiaal, elektrische weerstand, en de eigenschappen van de signalen (actiepotentialen) die de neuronen doorstuurden werden niet beïnvloed door cortisol. We vonden echter wel dat na een periode van veel activiteit van een neuron (onder depolarisatie), de langzame na-hyperpolarisatie van de cellen in de OFC verkleind werd door cortisol. Normaal gesproken beschermt deze na-hyperpolarisatie hersencellen tegen een te hoge activiteit door de drempel voor het afgeven van een nieuw elektrisch signaal te verhogen. Cortisol bleek het afgeven van veel signalen in de OFC dus te vergemakkelijken; een vergelijkbaar effect aan wat al eerder werd gezien voor de amygdala. In de hippocampus veroorzaakte cortisol precies het tegenovergestelde effect; daar vergrootte cortisol de na-hyperpolarisatie juist, waardoor het moeilijker voor de hippocampus werd om nieuwe signalen door te sturen. Met deze studie hebben we dus verder bijgedragen aan het begrip van de hersengebiedafhankelijke effecten van cortisol, door aan te tonen dat het hormoon de langzame na-hyperpolarisatie van de hersencellen in de OFC en de hippocampus op een andere manier beïnvloedt.

## De effecten van cortisol en stress op de communicatie in de hersenen

Naast de effecten van cortisol op hersenactiviteit te onderzoeken, hebben we ook gekeken of het hormoon de communicatie tussen verschillende hersengebieden beïnvloedt; de hersenconnectiviteit (**deel 4.1**). We hebben deze connectiviteit gemeten tijdens rust. Proefpersonen hoefden niets te doen, alleen wakker te blijven in de MRI scanner. Deze zogenaamde ‘resting state’ (rusttoestand) scan stelde ons in staat de spontane fluctuaties in de activiteit van verschillende gebieden in het brein met elkaar te correleren. Het idee is dat wanneer twee gebieden een vergelijkbaar patroon van activiteit vertonen, deze met elkaar communiceren. We waren vooral geïnteresseerd in hoe cortisol de communicatie van de amygdala met andere gebieden in het brein beïnvloedt. Het was namelijk al bekend dat deze toeneemt tijdens acute en langdurige stress. We onderzochten de communicatie van de amygdala in proefpersonen na inname van placebo, en na inname van cortisol. Hoge concentraties cortisol bleken de connectiviteit van de amygdala af te zwakken. De positieve communicatie die het gebied normaliter vertoont met hersengebieden betrokken bij de stressreactie, de hippocampus, locus coeruleus, en hypothalamus, namen allemaal af in sterkte. Daarnaast verzwakte cortisol ook de negatieve communicatie van de amygdala met gebieden in de prefrontale cortex en de pariëtale cortex, gebieden betrokken bij hoger cognitieve functies. Cortisol leek er dus voor te zorgen dat de amygdala minder goed verbonden was met de rest van het brein. Waarschijnlijk is de amygdala daardoor ook minder goed in staat andere hersengebieden te beïnvloeden; een verschijnsel dat mogelijk bijdraagt aan het herstel van het brein na een stressvolle gebeurtenis.

Daarnaast waren we ook geïnteresseerd in de effecten van langdurige blootstelling aan hoge niveaus van cortisol, zoals het geval is bij chronische stress. De blootstelling aan chronische stress is namelijk gerelateerd aan de ontwikkeling van psychopathologie. Om de effecten van chronische stress op een goed gecontroleerde manier te kunnen onderzoeken, maakten we hierbij gebruik van een diermodel (**deel 4.2**). Ratten werden 10 dagen lang gestresst en vervolgens getest in de MRI scanner om de effecten van deze stress op de functionele connectiviteit (de communicatie) en de structurele integriteit van hun brein te bepalen door ze te vergelijken met een controle groep. Daarnaast werd een subgroep van de ratten gebruikt om te controleren of de verwachte effecten van stress op de structuur van hun neuronen ook opgetreden waren. Het stressprotocol zorgde ervoor dat de ratten minder in gewicht toenamen dan de controle groep, maar ook dat de complexiteit van de structuur van de neuronen in de hippocampus en PFC afnam, terwijl deze in de amygdala toenam. Dit was al eerder gerapporteerd voor chronische stress, en bevestigde dus dat de ratten daadwerkelijk gestresst waren. In het brein zagen we dat de laterale ventrikels groter waren geworden door stress. Dit zijn holtes in het brein die gevuld zijn met hersenvocht (cerebrospinale vloeistof), en ook de beweging van dit hersenvocht in de ventrikels nam toe door stress. Vergrote ventrikels zijn ook gevonden in patiënten met psychische stoornissen, zoals schizofrenie of depressie. Daarnaast zagen we dat door chronische stress de functionele connectiviteit in de visuele cortex, de somatosensorische cortex en het zogenaamde “default mode” netwerk toenam. Dit “default mode” netwerk is een netwerk aan hersengebieden

die het meest actief zijn wanneer het brein in een rusttoestand verkeert. Er wordt gedacht dat het betrokken is bij hersenfuncties als dagdromen, het bewustzijn, het zich kunnen inbeelden van dingen en het conceptueel denkvermogen. Het is interessant te weten dat een toename in dit netwerk ook bij depressieve patiënten gevonden is. Ook een veranderde connectiviteit in de visuele cortex was al eerder gerapporteerd in depressie. De toename in connectiviteit in de visuele cortex die wij observeerden als resultaat van chronische stress, samen met die in de somatosensorische cortex, zou erop kunnen duiden dat het brein meer vanuit een sensorische modus werkt als gevolg van chronische stress. Deze studie toont dus aan dat chronische stress leidt tot vergrote laterale ventrikels en een toegenomen functionele hersenconnectiviteit in de visuele cortex, somatosensorische cortex en het “default mode” netwerk; symptomen die ook gevonden worden in stress-gerelateerde ziekten zoals depressie. Daarmee relateert deze studie chronische stress aan verschijnselen die ook gevonden worden in de psychopathologie en biedt deze experimentele opzet een goede mogelijkheid tot het gecontroleerd bestuderen van diens symptomen in diermodellen.

## Conclusies

De studies die in dit proefschrift beschreven zijn, tonen aan dat stress en het stresshormoon cortisol het menselijk en dierlijk brein in een regiospecifieke (i.e., hersengebiedafhankelijke) en tijdsafhankelijke manier beïnvloeden. Daarnaast tonen ze aan dat ze niet alleen de hersenactiviteit aantasten, maar ook de functionele verbindingen tussen gebieden.

Eerder onderzoek heeft aangetoond dat acute stress het brein in een staat van paraatheid brengt; de hersenactiviteit in gebieden die visuele informatie verwerken neemt toe, en ook de amygdala reageert gevoeliger. De activiteit in de PFC vermindert daarentegen, waardoor hogere cognitieve functies verslechteren. Ons onderzoek wijst uit dat een goede filtering van deze toegenomen visuele informatie, en daarmee verlaagde activiteit van de hippocampus, ervoor zorgt dat het brein de stressvolle gebeurtenis beter opslaat in het geheugen. De trage effecten van cortisol onderdrukken vervolgens de activiteit van de geheugengebieden, zodat er zo min mogelijk verstoring in de stressvolle herinnering optreedt en deze optimaal opgeslagen wordt.

Verder hebben we hier laten zien dat de langzame effecten van cortisol de tegenovergestelde effecten veroorzaken van acute stress; ze verminderen activiteit in visuele gebieden, zorgen voor toegenomen controle over de amygdala, en verhogen de activiteit in de PFC tijdens een werkgeheugentaak. Deze tegenwerking van de effecten van acute stress door de langzame acties van cortisol lijkt daarmee bij te dragen aan de normalisatie van de hersenen wanneer de stressvolle gebeurtenis voorbij is.

De rol van de snelle effecten van cortisol tijdens de stressreactie is nog niet helemaal duidelijk. In isolatie bleken ze niet in staat de hippocampus en PFC te beïnvloeden, maar mogelijk kunnen ze dat wel in samenwerking met andere stresshormonen, zoals noradrenaline. In de amygdala zorgden de snelle effecten van cortisol er enerzijds voor dat deze gevoeliger was voor matig emotionele stimuli die maar af en toe waargenomen werden, terwijl ze een overactivatie van de

amygdala tijdens continue blootstelling aan sterk emotionele stimuli voorkwamen. Dit suggereert dus een contextafhankelijke regulatie van de amygdala door de snelle effecten van cortisol, maar toekomstig onderzoek zal dat verder moeten vaststellen.

Al met al lijkt cortisol dus een cruciale speler in de controle van de stressreactie in het brein en de normalisatie van hersenfunctie achteraf. Herstel van een normale cortisol-huishouding in patiënten met stressgerelateerde stoornissen zoals depressie en of PTSS, zou daarmee kunnen bijdragen aan hun behandeling.



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Erno, jij hebt me opgevangen toen ik als studentje stage kwam lopen in de ‘Memory group’. Ik miste een achtergrond in de neuroscience of psychologie en het was eigenlijk helemaal niet je taak me te begeleiden, maar dat deed je toch. Ik vond je meteen al bizar slim, en jij bent er dan ook medeverantwoordelijk voor dat ik psychologen nu toch als volwaardige wetenschappers ben gaan beschouwen (hier maak ik vast geen vrienden mee), hoewel je af en toe meer een natuurkundige lijkt. Guido en jij waren er tevens voor verantwoordelijk dat ik lange tijd betwijfelde of er wel een carrière in de wetenschap voor me weggelegd was; ik was immers geen Erno of Guido. Ik hoop dat we in de toekomst nog samen aan mooie stressprojecten mogen werken, al ga ik dit keer liever niet naar de videotheek voor de gruwelijkste films die er maar bestaan.

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Hein, hoewel ik in het begin als provinciaaltje even moest wennen aan jou als rasechte Amsterdammer, zijn mijn reservaties over de jaren geheel verdwenen. Hoewel je je immer kritische houding nooit hebt verloren, heb je wel geleerd deze iets beter te doseren (en heb ik geleerd deze met een korreltje zout te nemen). Ik weet nog goed hoe ik achter je weg kon krimpen op congressen als je geheel in Hein-stijl mensen aanviel op hun posters. Later kreeg je door dat het misschien toch wat vriendelijker was ze af en toe ook te complimenteren, en dan deed je het afzeiken van hun werk daarna wel gewoon tegen mij. Je scherpe commentaren waren overigens altijd raak! Ik heb ervan genoten deel te nemen aan de discussies tussen jou en Shaozheng, en hoewel iedereen dacht dat jij de dominante van de twee was, weet ik hoe het werkelijk zat. Ook je goedbedoelde psychiater-commentaren (“dat Israël daar hebben we het nog over”) weet ik inmiddels op waarde te schatten. Hein, het is een eer dat je mijn paranimf wil zijn.

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op orde te houden ook bewonderenswaardig.

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## Publication list

- Hermans E.J., **Henckens M.J.A.G.**, Roelofs K., Fernández G. (2012) Fear bradycardia and activation of the human periaqueductal grey. *Neuroimage* 66C: 278-87
- Henckens M.J.A.G.**, van Wingen G.A., Joëls M., Fernández G. (2012) Time-dependent corticosteroid modulation of emotional interference. *Frontiers in Integrative Neuroscience* 6(66): 1-14
- Henckens M.J.A.G.**, van Wingen G.A., Joëls M., Fernández G. (2012) Corticosteroid induced decoupling of the human amygdala. *Cerebral Cortex* 22(10): 2336-45
- Henckens M.J.A.G.**, Pu Z., Hermans E.J., Joëls M., Fernández G. (2012) Dynamically changing effects of corticosteroids on human hippocampal and prefrontal processing. *Human Brain Mapping* 33(12): 2885-97
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- Korosi A., Veening J.G., Kozicz T., **Henckens M.J.A.G.**, Dederen J., Groenink L., Gugten van der J., Olivier B., Roubos E.W. (2006) Distribution and expression of CRF receptor 1 and 2 mRNAs in the CRF over-expressing mouse brain. *Brain Research* 1072:46-54

## Publications in preparation

- Pillai A.G., **Henckens M.J.A.G.**, Fernández G., Joëls M. (*in preparation*) Opposite effects of corticosterone on neuronal properties of the mouse lateral orbitofrontal cortex and CA1 hippocampal area.
- Henckens M.J.A.G.**, van der Marel K., van der Toorn A., Pillai A.G., Fernández G., Dijkhuizen R.M., Joëls M. (*submitted*) Stress-induced alterations in functional connectivity networks of the rodent brain.





## Curriculum vitae

Marloes Henckens was born on May 19th 1984 in Beegden. In 2002, she graduated from the Gymnasium at Sg. St Ursula in Horn and started her studies in Natural Sciences at the Radboud University Nijmegen. During her masters she performed a research internship in the lab of Prof. Guillén Fernández at the Donders Institute for Brain, Cognition and Behaviour, Centre for Cognitive Neuroimaging, investigating how acute stress affects the neural correlates of memory formation using functional MRI. After completing this research, she graduated *cum laude* in Natural Sciences in 2008. She left the lab for three months of voluntary work in Ghana, but returned to continue the work in the lab of Prof. Guillén Fernández as a research assistant, and applied for a Toptalent grant from the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) to conduct her own PhD research. She received this grant and started working on a project into the time-dependent effects of corticosteroids on brain function. To do so, she combined human functional neuroimaging work in the lab of Prof. Guillén Fernández, with electrophysiology and neuroimaging in rodents in the lab of Prof. Marian Joëls at the Rudolf Magnus Institute of Neuroscience at the University Medical Center in Utrecht. After completing her thesis she conducted another study into the inter-individual differences in genetic makeup for the glucocorticoid receptor and its effects on the stress response and influence on human brain function. As of January 2013 she will be working as a postdoctoral fellow in the group of Prof. Alon Chen at the Weizmann Institute for Science, in Rehovot, Israel, where she will study the effects of corticotrophin-releasing factor on the functioning of amygdalar subnuclei using viral vectors and optogenetics. For this work she received a Niels Stensen Fellowship and a Dean of Faculty Postdoctoral Fellowship from the Feinberg Graduate School of the Weizmann Institute of Science.







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