The good and bad of stress

Wichmann, Romy

THE GOOD AND BAD OF STRESS

implications for memory and adaptive processes

Romy Wichmann

THE GOOD AND BAD OF STRESS

implications for memory and adaptive processes

Romy Wichmann

ISBN: 978-94-6182-162-1

Cover Design by Beate Hanitzsch (www.lemonhead.de) & Romy Wichmann

Layout and Printing by Offpage, Amsterdam, www.offpage.nl

The research projects described in this thesis were performed at the department of Neuroscience, Section Anatomy (University of Groningen, The Netherlands) within the framework of the Groningen Graduate School of Behavioral and Cognitive Neurosciences (BCN), and were financially supported by a Hazewinkel Behringer Scholarship from the University of Groningen.

Printing of this thesis was financially supported by:
University of Groningen
University Medical Center Groningen
Graduate School of Behavioral and Cognitive Neuroscience

STELLINGEN

Behorende bij het proefschrift

The Good and Bad of Stress
Implications for memory and adaptive processes

1. The pure presence of a neuroendocrine response is not sufficient anymore to label a stimulus as stressful.
2. Glucocorticoid hormones do not care whether an experience is positive or negative.
3. We all have our time machines. Some take us back, they´re called memories. Some take us forward, they´re called dreams. (Jeremy Irons)
4. Changes in the brain as response to stress should not immediately be interpreted as maladaptive and disease-promoting.
5. Including female subjects in research is essential to explain neurobiological mechanisms underlying stress-related diseases in women.
6. If the brain was so simple we could understand it, we would be so simple that we couldn´t.
7. Success is the ability to go from one failure to another with no loss of enthusiasm. (Winston Churchill)
8. To achieve great things, two things are needed: a plan and not quite enough time. (Leonard Bernstein)
9. Sometimes it´s important to work for that pot of gold. But sometimes it´s essential to take time off and to make sure that your most important decision in the day simply consists of choosing which color to slide down on the rainbow. (Douglas Pagels)
10. Horseback riding is life, the rest is just details.

Romy Wichmann, 29 Oktober 2012

CMB.

rijksuniversiteit groningen

THE GOOD AND BAD OF STRESS

implications for memory and adaptive processes

Proefschrift
ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
maandag 29 oktober 2012
om 12.45 uur

door

Romy Wichmann
geboren op 1 september 1980
te Schwerin, Duitsland

Promotores:	Prof. dr. B. Roozendaal Prof. dr. G.J. ter Horst
Beoordelingscommissie:	Prof. dr. J.M. Koolhaas Prof. dr. P.G.M. Luiten Prof. dr. K. Roelofs

Für meine Eltern,
als Dank für ihre
endlose Ermutigung,
Unterstützung und Liebe.

TABLE OF CONTENTS

Chapter 1	General introduction	8
Chapter 2	Rodent stereotaxic surgery and animal welfare outcome improvements for behavioral neuroscience	40
Chapter 3	Involvement of the insular cortex in regulating glucocorticoid effects on memory consolidation of inhibitory avoidance training	48
Chapter 4	Glucocorticoids interact with the noradrenergic arousal system in the nucleus accumbens shell to enhance memory consolidation of both appetitive and aversive taste learning	68
Chapter 5	Glucocorticoid administration into the basolateral amygdala enhances memory consolidation of an appetitive taste learning experience	88
Chapter 6	Sex differences in the effects of acute and chronic stress and recovery after long-term stress on stress-related brain regions in rats	98
Chapter 7	Sex-specific alterations in camp response element-binding protein in the nucleus accumbens shell after acute and chronic stress	120
Chapter 8	Summary & general discussion	130
Appendix		148
	Nederlandse Samenvatting	150
	Deutsche Zusammenfassung	153
	Acknowledgement	157

GENERAL INTRODUCTION

1

STRESS

'Stress is an ignorant state. It believes everything is an emergency'
Nathalie Goldberg

Stress is a common occurrence in our modern society that affects all of us at some point in life. Hans Selye defined the concept of stress, as the '*non-specific bodily response to any demand which leads to a variety of temporary or permanent changes*' [1]. Stress is thus a normal and necessary physiological defense reaction, which allows us to respond appropriately to environmental or physiological demands. Therefore, contrary to general belief, stress is not necessarily a negative event. In small amounts, it can help us to perform under pressure, motivate us and increase our cognitive abilities.

With the response to stress, our body is equipped with an alarm system that helps us to adjust to new demands as quickly as possible. It is composed of altered behavioral, endocrine and autonomic function and the secretion of multiple hormones and neurotransmitters. When working properly, it helps us to stay focused, energetic, and alert [2,3] and highlights things that are important in the environment and require closer attention.

THE STRESS RESPONSE

A situation perceived as (potentially) stressful immediately leads to an activation of the sympatho-adrenomedullary (SAM) system. It triggers the secretion of catecholamines (adrenaline and noradrenaline) from the adrenal medulla and the release of noradrenaline from sympathetic nerve terminals [4]. These hormones alert the body for emergency action and lead to physiological alterations, all known to us as the feeling of being stressed: the heart pounds faster, blood pressure rises, breath quickens, the hand palms get sweaty and the senses become sharper. The blood flow to the brain and muscles increases dramatically and muscle tension intensifies. These physical changes prepare the body to react quickly and effectively to any threat and to increase chances of survival. This is also referred to as the 'fight or flight' reaction [5].

A second, slower reaction takes place via the activation of the neuroendocrine system and more specifically the hypothalamus-pituitary-adrenal (HPA) axis [6]. Stress stimulates the central control station of the stress system, the hypothalamus, which results in the secretion of various hormones, like corticotropin-releasing hormone (CRH) and vasopressin (AVP). They in turn stimulate the release of adrenocorticotropic hormone (ACTH) from the pituitary gland into the peripheral blood stream. ACTH reaches and stimulates its target organ, the adrenal glands. The cortex of the adrenal glands is responsible for the synthesis and release of glucocorticoids (GCs), the final key messenger in this cascade (Figure 1).

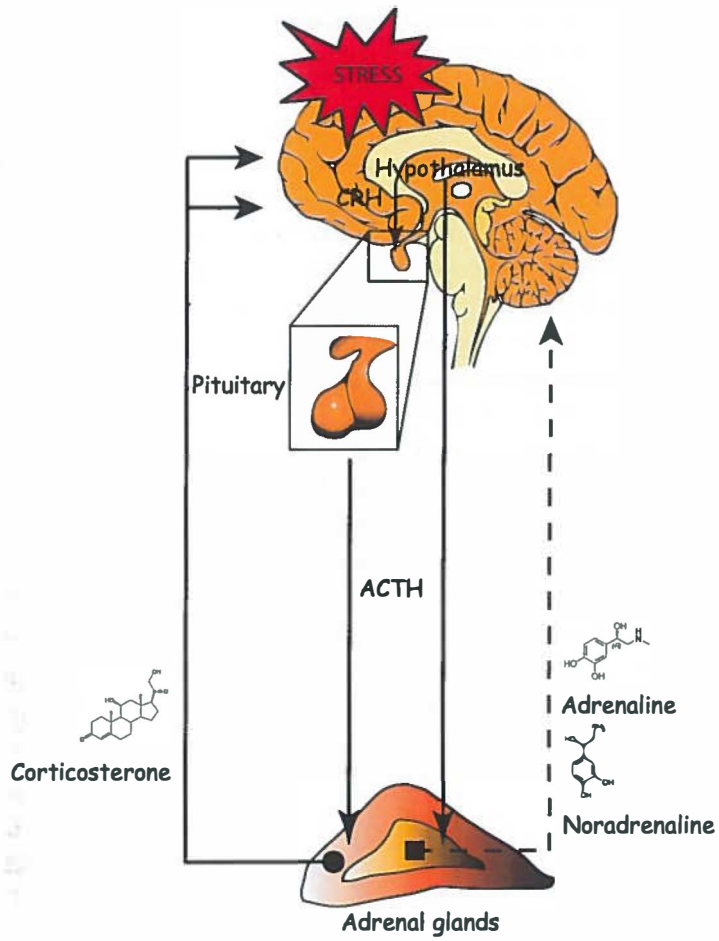


Figure 1. Simplified schematic representation of the hypothalamo-pituitary (HPA) axis and the sympatho-adenomedullary (SAM) system.

GLUCOCORTICOIDS

GCs – cortisol in humans and corticosterone in rodents – are a class of steroid hormones, synthesized from cholesterol and released into the general circulation. They regulate and support a variety of important cardiovascular, metabolic, immunological and homeostatic functions and ultimately travel back to the hypothalamus, inhibit further release of CRH and turn off the HPA-axis response (negative-feedback loop). Next to their multitudinous effects in the periphery, GCs easily cross the blood-brain barrier and also induce plenty of effects in different brain regions. All brain cells are in principle exposed to corticosteroid hormones. Nevertheless, the sites where GCs are effective are restricted by the distribution of corticosteroid receptors [7]. There are two known intracellular receptors, glucocorticoid receptors (GRs) and mineralocorticoid

receptors (MRs). Both bind the same hormone, yet with a ten-fold different affinity [8]. The higher affinity MR is predominantly occupied at lower, basal levels of glucocorticoids. The GR has a much lower affinity for GCs and is therefore only heavily occupied when there are high levels of GCs, as seen during stress [9]. Upon binding the hormone, the receptors undergo a conformational change and form dimers. These translocate into the nucleus, where they subsequently activate or repress appropriate hormone-responsive genes [10-12]. Consequently, GC effects on neuronal function usually require at least an hour to develop and can last hours to days [7]. However, GCs can also exert more rapid effects that are not compatible with genomic regulation [13]. Studies suggest that these effects are instead mediated by interactions of the hormone with specific membrane-associated receptors to cause changes within the cell that are similar to those initiated by neurotransmitters [14-18].

The activation of the stress response is commonly connected to challenges of an aversive nature. As a result, the release and presence of (nor)adrenaline and GCs is often regarded as an indicator of exposure to aversive stimuli. However, it should be recognized that the stimulus that triggers the stress response does not necessarily has to have a noxious or adverse nature. Multiple appetitive and rewarding stimuli such as sexual behavior, food, drugs of abuse or victory elicit the activation of the HPA axis with a similar magnitude as highly aversive situations [19-21]. Thus, any situation that puts high demands on an organism – ranging from aversive stressors to highly rewarding events – activates the stress response and results in a state of physiological or emotional arousal [22,23].

PART I
LEARNING AND MEMORY

'That which touches the heart is engraved in the memory'
Voltaire

One of the most extraordinary traits we possess is our ability to store information by experience and to retrieve much of this information at will. The process of encoding, storing and retrieving this information is generally defined as memory. It is a complex biological process involving multiple brain systems and many molecular and cellular mechanisms that process and consolidate information in the brain.

Following any learning event, the fresh memory trace is not instantaneously hardwired. New memories are initially labile and sensitive to disruption before undergoing a series of processes that slowly strengthen and transform them into a more stable and persistent form over time 24. The hypothesis that new memories consolidate slowly over time was proposed already over a century ago by Müller and Pilzecker [25,26]. Their 'perseveration-consolidation' hypothesis provides the opportunity for endogenous neurobiological processes to modulate and adapt the strength of subsequent memories [26,27]. We know from our own experience that not all information is equally well transferred into long-term memory. Our brain is particularly good at storing information with strong emotional content. We remember life´s significant moments especially well, such as memories of where we were and what we were doing when we met the men (or women) of our dreams; when we had our graduation ceremony or got an awesome birthday present. Unfortunately, strong memories develop just as well when there is bad news, such as the death of a loved one or the terrorist attacks of September 11. Most people remember vividly what they were doing when they heard of these events. Hence, events that emotionally touch (arouse) us appear to activate neurobiological processes and systems and increase the likelihood of these events to be converted into permanent memories. Memories for nonarousing events on the other hand are more vulnerable for disruption and often are quickly forgotten [28-32]. The association between the emotionally arousing characteristics of events and facilitated memory lead to the hypothesis that the degree to which memories are lasting may be influenced by hormonal systems activated by these experiences [33-35]. The link to stress hormones was quickly made and it was proposed that stress hormones released during and immediately after emotionally arousing experiences are the endogenous modulators of the neurobiological processes underlying memory consolidation [26,36,37]. Extensive evidence from many different studies in animals as well as humans now supports this theory [22,28,37-41].

STRESS HORMONE EFFECTS ON MEMORY CONSOLIDATION

CATECHOLAMINES

Adrenaline (also known as epinephrine) is perhaps the hormone best studied and understood as an enhancer of memory formation processes. Numerous studies

repeatedly demonstrated the role of the adrenergic system in consolidation of memory for emotionally significant experiences [28,33,42,49]. Since adrenaline does not readily cross the blood-brain barrier [50] the question arose how it affects the central brain function to modulate memory consolidation [33]. Today it is believed that it activates ß-adrenergic receptors on the ascending vagus nerve terminating in the nucleus of the solitary tract (NTS), a brainstem structure with a high population of noradrenergic cell groups [51-57]. Noradrenergic projections originating from the NTS in turn innervate forebrain structures involved in learning and memory either directly [58-60] or indirectly via the locus coeruleus (LC), another brainstem structure with widespread noradrenergic projections to forebrain regions [35]. The NTS thus appears to be the interface between the peripheral adrenergic activation and the neural noradrenergic brain mechanisms regulating memory consolidation [56,61]. This further implicates a crucial role for noradrenaline (norepinephrine) in the central effects leading to enhanced memory consolidation. Support for this assumption comes from a plethora of experiments showing that injections of noradrenaline or a ß-adrenoceptor agonist into various brain regions at times when memories are encoded or shortly afterwards can enhance memory performance [45,46,62-67]. Conversely, posttraining intra-cerebral injections of adrenergic receptor antagonists block the memory-enhancing effects of emotionally arousing experiences [48] or systemically administered adrenaline [45,67].

GLUCOCORTICOIDS

Emotionally arousing experiences additionally activate the HPA-axis, resulting in elevated plasma levels of GCs, as already described in the previous section. Compelling evidence indicates that elevated GC levels are also involved in modulating consolidation processes resulting in better long-term memory [37,39,41,68-72]. Blockade of the corticosterone response to stress or arousal impairs memory consolidation and prevents arousal-induced memory enhancement in both animals and humans [38,73]. On the other hand, administration of moderate doses of GCs or a specific GR agonist facilitates memory consolidation, in a dose- and time-dependent manner in different kinds of learning tasks, including inhibitory avoidance, contextual and cued fear conditioning, spatial discrimination tasks, conditioned taste aversion, and object recognition [34,74-80]. In humans beneficial effects of GCs or stress exposure on (emotional) memory consolidation were likewise found in several studies [72,81,82]. Extensive evidence indicates that the low-affinity GR, and not the high-affinity MR, is involved in mediating GC effects on memory consolidation [38,69,83-85].

Different findings propose that GCs facilitate memory consolidation of emotionally arousing stimuli or of experiences occurring during states of emotional arousal, but do not affect memory consolidation of neutral information [29,72,77,81,86,87]. These selective effects of arousing material suggest that GCs interact with other components of emotional arousal in influencing memory consolidation.

ADRENERGIC-GLUCOCORTICOID INTERACTION

Indeed, the major hormonal stress systems interact to influence memory consolidation of emotional events and information [88-92]. Arousal-induced noradrenergic activation seems to be indispensable for mediating the enhancing effects of glucocorticoids on memory consolidation in a variety of emotional learning experiences. Administration of a ß-adrenoceptor antagonist immediately after training blocks corticosterone-induced memory enhancement [87,93-95]. Likewise, GCs alter the sensitivity of adrenaline in influencing memory consolidation [96,97]. Disruption of GC synthesis prevents memory enhancement induced by posttraining adrenaline injections or exposure to psychological stress [98,99].

GCs appear to exert their influence via increasing the activation of postsynaptic ß-adrenoceptors. These receptors are directly coupled to adenylate cyclase (AC) to stimulate 3'-5'-cyclic adenosine monophosphate (cAMP) formation. Intracellular cAMP subsequently initiates a cascade of molecular events, including the activation of the cAMP-dependent protein kinase (PKA), which ultimately results in the phosphorylation of the transcription factor cAMP response element binding (CREB) protein [100,101]. Manipulation of CREB levels was shown to influence long-term memory formation for aversive conditioning [102-105]. Recent evidence indicates that the influence of GCs on the ß-adrenergic receptor cascade is mediated by the release of endocannabinoids and endocannabinoid effects on GABAergic interneurons [14,106,107]. GCs initiate the release of endocannabinoids in the synaptic cleft by binding a membrane-bound GR. Endocannabinoids then bind their receptor type 1 (CB1) on GABAergic terminals to inhibit the release of GABA. This inhibition disinhibits noradrenaline release and thus increases the activation of ß-adrenergic receptors and the succeeding cascade (see Figure 2).

THE BRAIN NETWORK INVOLVED IN EMOTIONAL MEMORY

Several brain regions, especially in the limbic system, are influenced by stress and stress hormones and are responsible for the influence of emotions and emotional arousal on the formation of memories. Some of these members relevant for this thesis and their role in emotional memory consolidation will be explained in more detail below.

The **amygdala**, a small almond shaped region in the anteromedial temporal lobe, is the area most strongly implicated in regulating emotional arousal and stress effects on memory [53]. Among the different nuclei of the amygdala, the basolateral complex (BLA) seems to be selectively and crucially involved in mediating these neuromodulatory influences, whereas the adjacent central nucleus does not appear to play a significant role in modulating memory consolidation [26,76,85,107,108].

It is well established that emotional experiences that induce the release of stress hormones also activate the amygdala. Patients with a lesion in this region show a diminished long-term memory enhancement for emotionally arousing aversive stimulation [30,109-111]. Additional research in humans has revealed a link between the amount of amygdala activation at encoding and the likelihood of remembering

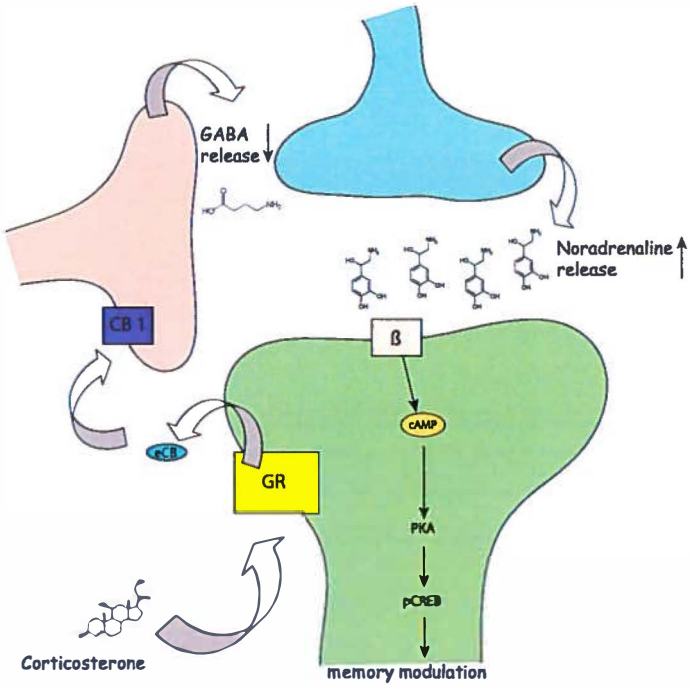


Figure 2. Current model of glucocorticoid-adrenergic interaction during emotional memory consolidation. Corticosterone is released during training in emotionally arousing tasks and binds to a membrane-bound glucocorticoid receptor (GR). This activates endocannabinoid synthesis. Endocannabinoids are then released into the synapse where they bind to CB1 receptors probably on GABAergic terminals, and thereby inhibit the release of GABA. This suppression of GABA release subsequently disinhibits noradrenaline release, and this results in an activation of the postsynaptic ß-adrenoceptor and the downstream cAMP/PKA/pCREB intracellular signaling pathway. (adapted from Atsak et al 2012)

emotional information [30,31,47,110,112-114]. Noradrenaline release in the amygdala seems to play an important, possibly critical, role in mediating the emotional arousal effects on memory consolidation [45,67,115-121]. Emotionally arousing learning experiences as well as drugs and hormones that enhance memory consolidation induce the release of noradrenaline within the amygdala and the amount released highly correlates with later retention performance [122-124]. Lesions of the amygdala, on the other hand, block the enhancing effects of emotional arousal or systemic administration of adrenaline [53,98]. There is extensive evidence that also the effects of GCs on memory consolidation are mediated through influences involving the amygdala [76,79,98,101,104,125,126] and this process requires noradrenergic co-activation within the amygdala [90,93,127].

Although the evidence summarized above indicates that the amygdala is a critical brain site for integrating adrenergic and GC influences on memory consolidation, the amygdala has a clear modulatory role and is not the locus of long-term memory storage [22,128]. Rather, the amygdala enhances emotional memory encoding via

modulation of responses in many efferent brain regions, including various cortical regions, the hippocampus, and the nucleus accumbens [53,129-136].

The **prefrontal cortex (PFC)** is the anterior part of the frontal lobes and is involved in higher cognitive functions like decision making and reasoning. It is highly interconnected with other cortical regions as well as subcortical areas and organized in a topographical manner [137]. The emotional and stress responses are mainly modulated by the medial PFC (mPFC) and its dorsal region (i.e., prelimbic cortex) in particular. Yet, all the PFC regions interconnect to orchestrate the brain´s activity for regulation of behavior, thought and emotion.

The PFC has a specific role in working memory [137,138], the ability to temporary store and manage information necessary to execute complex cognitive tasks [139]. However an involvement in memory consolidation has also been shown [18,140-142]. The PFC is also a target of stress processes. Stress hormone actions within the PFC influence structural and functional plasticity, and modulate working memory as well as memory consolidation processes. Stress, and increased noradrenaline and GC levels, inhibit PFC neuronal activity and induce working memory impairment via an activation of the intracellular cAMP/PKA signaling cascade [18,137,143-146]. On the other hand, the same stress-hormone induced disruption of PFC function during working memory processes enhances long-term memory consolidation [18,146-150]. This is confirmed by a functional imaging study in humans, showing that combined administration of GC and noradrenaline agonists during the encoding of emotionally arousing material induces a strong deactivation of the PFC and that the level of deactivation positively correlates with enhanced performance on a delayed retention task [92].

The **hippocampus (HC)** is part of the cerebral cortex. It consists of three major subdivisions: the dentate gyrus, the Ammon's horn (fields CA1, CA2 and CA3) and the subiculum. Its role in learning and memory is known since decades. Early evidence comes from studies in patients who had both hippocampi removed which resulted in a profound impairment of their ability to retain new information [151,152]. These early studies as well as more recent reports show that this region is predominantly involved in explicit/declarative and spatial memory process [153-157]. Similar to amygdala activation, the level of hippocampal activation predicts later memory strength [158].

The HC is also a critical target in the regulation of the stress response and is at the same time affected by it [159-161]. Noradrenaline release in the HC enhances cellular excitability, synaptic transmission, and plasticity [162] and modulates the encoding efficacy [37]. The HC contains a very high density of GRs as well as MRs [9,163,164]. Both animal models and human studies reveal that stress and stress levels of GCs induce structural and functional changes in the hippocampal circuitry [9,165]. Glucocorticoids influence the neuronal excitability and the induction of LTP in the HC [68,166-170]. This is consistent with behavioral evidence suggesting that GCs regulate memory storage [166-168]. Experiments using posttraining infusions of corticosterone or specific agonists or antagonists of GRs into the HC affect memory consolidation of different learning tasks [38,94,105,171,172].

As seen in the amygdala, elevated corticosteroid levels require the presence of elevated noradrenaline levels to promote processing of emotional stimuli [173]. Thus, also in the HC corticosteroids increase the effects of noradrenergic stimulation on encoding of emotional information [158].

The **insular cortex (IC)** is a part of the cerebral cortex within the lateral sulcus between the temporal and the frontal lobe. It is divided into two parts, the large anterior and smaller posterior insula. It plays a role in various functions linked to emotion and homeostasis. Human neuroimaging studies point to a key role of the IC in emotionally influenced learning and memory [174-178]. Increased activity of the anterior IC has been reported during the encoding and recall of a broad spectrum of emotionally aversive learning tasks [179-184] but see [92,185]. However, most animal studies investigating the role of the IC in emotional arousal effects on learning and memory have been limited to the involvement in the formation and maintenance of taste memory [79,186-190], although several findings also reported the involvement of the IC in other learning tasks, including spatial water maze learning, inhibitory avoidance

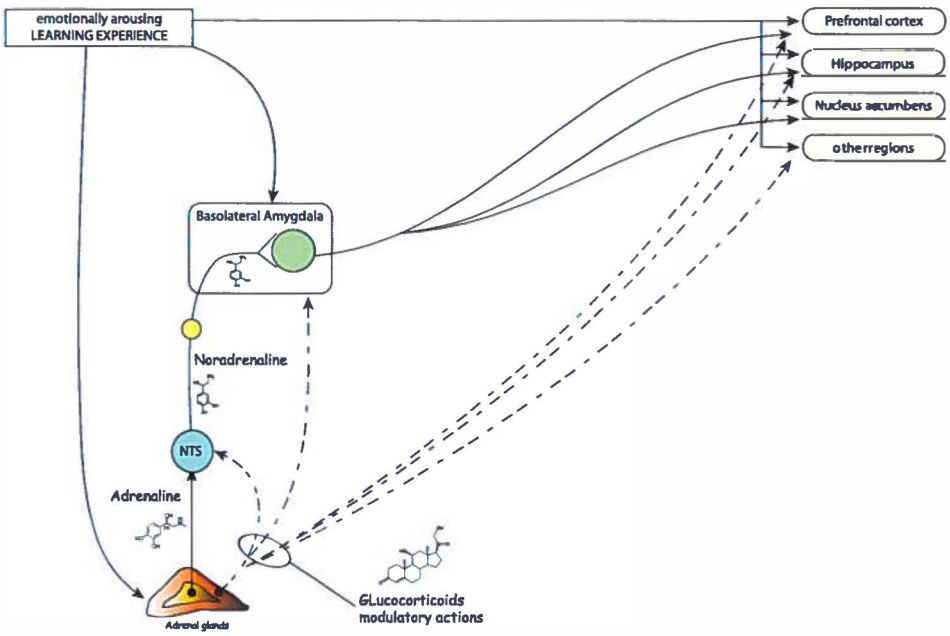


Figure 3. Schematic representation of emotional arousal-induced modulation of memory consolidation. Learning experiences initiate time-dependent storage processes in several brain regions. In addition, emotional arousal (e.g. stress) associated with these learning experiences stimulates the release of the stress hormones, catecholamines and glucocorticoids, from the adrenal glands. Catecholamines, not able to cross the blood-brain barrier, activate noradrenergic cells in the nucleus of the solitary tract (NTS). These in turn stimulate, among other regions, the basolateral amygdala (BLA). Glucocorticoids directly influence memory processes in several brain regions. They also interact with noradrenaline in the BLA. This modulates the memory storage processes by influencing neuroplasticity in other brain areas. (adapted from McGaugh et al 2000)

and object recognition [105,191-197]. Despite the obvious involvement of the IC in emotional memory consolidation, studies investigating the influence of stress hormone treatments on memory function in this region are few and far between [79,105].

The **nucleus accumbens** (NAc) is a basal forebrain structure located at the top of the brainstem. It can be divided into two structures; the nucleus accumbens core (NAcC) and shell (NAcS). The NAc is commonly referred to as '*the pleasure center of the brain*' due to its important role in reward, pleasure and motivation [24,198-200]. Nonetheless, a role in learning and memory is also well established [24,201]. Extensive evidence, including lesion studies and pharmacological manipulation of different neurotransmitter systems in animals [202-205] as well as functional imaging studies in humans [206-209], showed an involvement of the NAc in the encoding and consolidation of emotional experiences.

Different studies have shown that the NAc is activated during stressful situations [210,211]. However, the involvement of the NAc regarding stress hormone-induced facilitation of memory consolidation of emotionally arousing experiences is only scarcely and mostly indirectly studied. It was recently shown that noradrenaline release in the NAc, in response to emotionally arousing negative experiences, is necessary for the consolidation of these events [60,212]. Studies investigating the influence of GCs on NAc activity have mainly focused on the effects of increased GC levels on the release of the neurotransmitter dopamine in this region and subsequent behavioral outcomes [211,213-215]. Other studies show that lesions of the NAc block the memory-enhancing effects of systemically administered dexamethasone [131] or intracranial administered GR agonist into the BLA or HC [130]. Although it is known that the NAc expresses GRs [163] only one recent study has so far investigated the direct actions of glucocorticoids within the NAc on memory formation [216].

In summary, the limbic brain system, influenced by the stress hormones, exerts diverse emotional, cognitive and mnemonic functions, involved in the regulation of emotional arousal or stress induced memory consolidation.

PART II
STRESS-INDUCED PSYCHOPATHOLOGY

'A sick thought can devour the body's flesh more than fever or consumption'
Guy the Maupassant

The body's stress response is perfect in the short-term, but damaging if it goes on for weeks and years. Many of the modern stressful events are psychological in origin and they are chronic, lasting days, weeks, months, or even years. They do not involve an actual threat and cannot be resolved by fighting or running away. However, the brain cannot distinguish between a real or potential threat. Thus, these stressors still trigger the SAM system and the HPA-axis, which makes them as genuine as possible. This constant activation of a system designed to be activated occasionally can make it go into overdrive and cause stress-related health problems. It is now evident that intensely emotional events or chronic exposure to stressful experiences can result in the development of cardiovascular diseases, diverse endocrine and metabolic disorders and psychopathologies, including post-traumatic stress disorder (PTSD), burn out and depression [217,218].

Chronic stress can also be used as a valuable tool to investigate the neurobiological mechanisms underlying psychopathologies in animals [219]. The chronic mild stress model is the most valid and widely used animal model of depression [220]. It aims to model a chronic depression-like state that develops gradually over time in response to a series of mild and unpredictable stressors during a prolonged period of time [218,220-222]. This has been reported to result in long-lasting changes of behavioral, neurochemical and neuroendocrinological variables resembling a depressed state that can be reversed by chronic, but not acute, treatment with a variety of antidepressants [218,220].

DEPRESSION

Depression is a chronic, recurring and potentially life-threatening illness that affects up to 20% of the population across the globe [217,223]. Its dramatic increase in prevalence over the last years caused it to be one of the leading reasons of medical disability, morbidity and mortality worldwide [217,224]. Since the 1960's a clinical diagnosis of depression is based on symptomatic criteria set forth in the Diagnostic and Statistical Manual of Mental Disorders [217,225]. Depression is often viewed as a heterogeneous syndrome comprised of numerous diseases with distinct causes and pathophysiologies [217]. It has a highly variable set of symptoms and an inconsistent response to treatment [226]. The core symptoms include long-lasting mood disturbances (characterized by profound sadness and irritability) and anhedonia, in association with feelings of guilt and worthlessness, disturbances of psychomotor activity, sleep, appetite and weight and recurrent thoughts of death and suicide [217,227]. Anhedonia, the loss of motivation, reward and pleasure in activities previously enjoyed, is one of the most common symptoms in depression.

Research attempts to elucidate the mechanisms responsible for the development of depression has yielded valuable insight, but still the etiology and pathophysiology

remain poorly understood. Roughly 40-50 % of the risk of depression seems to be genetic [228,229], however, no specific genetic abnormality has been identified so far [217,229]. Most likely, depression is caused by a combination of genetic, biological, environmental, and psychological factors [229].

The current picture of the pathophysiology of depression is largely incomplete and thus a variety of hypotheses have been postulated in search for the molecular causes of depression. Three representative examples are briefly reviewed in the following section.

THE MONOAMINE HYPOTHESIS

For half a century, the hottest topic of neurobiological research and classical pharmacotherapy regimens trying to explain depression has been the monoamine hypothesis [230,231]. This hypothesis proposes that an imbalance or deficiency of brain monoamine neurotransmitters, such as serotonin, noradrenaline and dopamine, are responsible for the development of depressive symptoms [217,231]. This theory is based and supported by the fact that most used antidepressant drugs acutely boost monoamine transmitter function in different brain regions [231-234] and patients with depression show alterations in the production of these neurotransmitters [235,236]. However, current antidepressant pharmacotherapies based on this principle alleviate symptoms only in an average of 50% of patients [217,237,238]. Furthermore, even though the increase in monoamine levels is very rapidly detectable, antidepressant drugs often take 2-6 weeks before full clinical efficiency is observed [231,239]. Thus, the immediate biochemical effects of antidepressants cannot fully explain this delay in the treatment response. Therefore, although the monoaminergic transmitters are undoubtedly involved in the mechanism and treatment of depression, enhanced neurotransmission is only part of the story and not sufficient to explain the clinical actions of these drugs entirely [217]. Rather, it suggests that some gradually developing adaptive downstream changes would be ultimately responsible for clinical symptom improvement and resulted in the formulation of the neuroplasticity hypothesis [217,232,240-242]. This hypothesis is explained in more detail further below in the text.

THE HPA-AXIS HYPOTHESIS

There is considerable evidence that abnormal, excessive activation of the HPA-axis is implicated in depression as well [217,243-245]. Stress, especially when chronic, has long been associated with a substantial increase in risk for the onset of depression and can trigger depressive episodes [232,246-248]. Depression, particularly when severe, is characterized by an over activity of the HPA-axis, associated with hypersecretion of cortisol; pituitary and adrenal gland enlargement and an increased production of CRF [217,227,236]. Increased HPA-axis activity is evident in about 50% of the patients and chronic antidepressant treatment can often reverse this phenomenon [231,249-251].

Additional support comes from animal studies, showing that stress-induced behavioral and neuroendocrine disturbances resemble some of the symptoms seen

in depressed patients. CRH, injected into the brain of experimental animals, mimics some effects of depression in humans, such as diminished activity, loss of appetite, and increased signs of anxiety. Additionally, chronic exposure to corticosterone induces persistent and long-lasting behaviors resembling anhedonia in combination with other molecular correlates of depression and these effects are reversible by adrenalectomy or chronic antidepressant treatment [224,252,253].

Despite the compelling evidence outlined above, it is still unknown whether HPA axis abnormalities are a primary cause of depression or, instead, secondary to depressed mood [254]. Thus, a major liability of the HPA axis hypothesis is the difficulty of defining the relationship between stress and depression.

THE NEUROPLASTICITY HYPOTHESIS AND CREB

Intracellular signaling pathways linked to neuroplasticity and cellular survival are undoubtedly involved in events that regulate complex psychological and cognitive processes, as well as diverse vegetative functions [232] and disturbed regulation of these

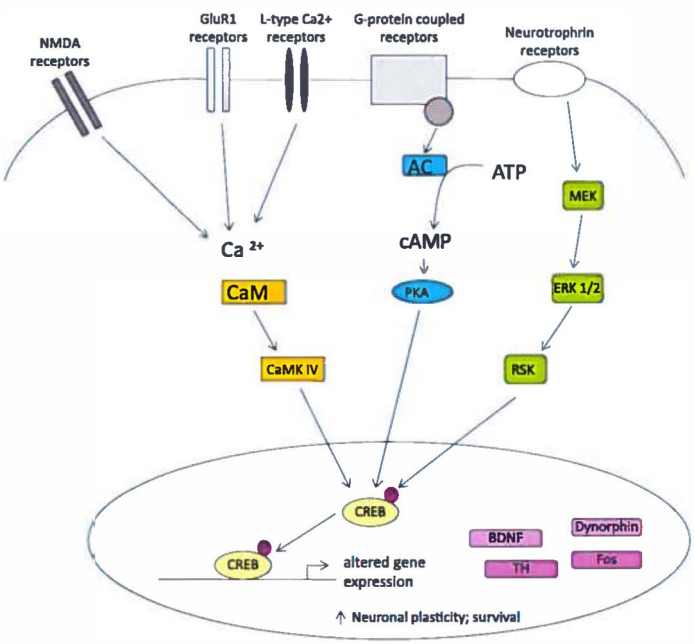


Figure 4. Simplified depiction of the different cellular events in regulation of CREB. Neurotransmitters and peptides act at membrane receptors to trigger the different intracellular signal transduction cascades. All these pathways culminate in the phosphorylation (P) of CREB. This activates CREB-mediated gene transcription and results in changes in synaptic function and neural plasticity. (adapted from Carlezon et al 2005)

pathways is suggested as a key event in the development of depression [255]. Preclinical and clinical studies have further shown that factors involved in signaling pathways regulating cell survival and cell death are also affected by antidepressants which may explain some of the delayed, long-term beneficial effects observed in patients receiving these drugs [232]. Therefore, as mentioned above, more recent research into the pathophysiology and treatment of depression focuses toward molecular mechanisms underlying long-lasting downstream changes in the brain after chronic antidepressant treatment [231,232,242].

Monoamine neurotransmitters regulate the 3'-5'-cyclic adenosine monophosphate (cAMP) signal transduction cascade. Activation of the G protein-coupled receptor (GPCR) subunit Gs leads to the activation of adenylyl cyclase, which, in turn, catalyzes the conversion of ATP into cAMP. All further effects of cAMP are then mediated by the activation of cAMP-dependent protein kinase (PKA). Once PKA is activated, it further regulates cellular function by phosphorylation of specific target proteins. One of these proteins is the already mentioned transcription factor CREB. CREB is a key regulator of neuroplasticity in the brain [238,256] and seems to be implicated in both stress- and antidepressant-induced effects on transcriptional regulation [255]. Postmortem studies in brains of depressed patients found reduced levels of CREB whereas CREB levels were increased in subjects taking antidepressants at time of death [257]. Exposure of animals to chronic stress decreases CREB levels in different cortical and limbic regions [258,259] and an upregulation of CREB expression and function is observed with chronic administration of virtually all classes of antidepressants [217,260,261]. However, this effect is not seen in all brain regions. In the NAc, for example, elevated CREB levels produce depressive-like effects whereas reduction in CREB activity has antidepressant-like effects [217,256,262,263]. CREB has numerous phosphorylation sites that differentially regulate its activity. In addition to PKA, CREB is phosphorylated by several other kinases, including the mitogen-activated protein (MAP) kinase signaling pathway (MEK/ERK), the calcium-calmodulin kinase II and IV (CaMK II/IV) as well as phospholipase C (PLC) [263-265]. Upon activation, CREB forms dimers and binds to the cAMP-response element (CRE) in promotor regions of certain genes, and by regulation of these genes influences synaptic plasticity [266-270]. One such gene influenced by CREB is brain-derived neurotrophic factor (BDNF). BDNF belongs to the family of neurotrophins, factors essential for the survival and function of neurons [265]. The importance of BDNF in stress and depression has been extensively documented [260,265,271]. It was shown that prolonged exposure to different stressors results in a down-regulation of BDNF expression [272-274]. Studies have also clearly indicated that BDNF is increased by long-term antidepressant treatment [273-276] and also can exert antidepressant effects itself [277,278]. However, reminiscent to CREB, the spatial expression of BDNF seems to be critical for its antidepressant activity [279].

Thus, in summary, CREB and BDNF appear to be key mediators in stress responses and a target of antidepressant induced neural plasticity [265].

SEX DIFFERENCES IN DEPRESSION

When discussing depression, one cannot bypass the intriguing sex difference accompanying this disorder. The prevalence for depression is twice as high in women than in men [219,247,280-282]. Additionally, the cause of depression and even the symptom pattern exhibited by men and women can be quite different, especially when it comes to atypical symptoms of depression [283]. Recent evidence also points towards sex-specific antidepressant responses [219,284]. Many factors have been implicated in this gender discrepancy, including neuroanatomical differences, hormonal influences and environmental factors [282].

As mentioned before, chronic stress or major stressful life events are seen as an underlying cause of depression [226,282,285]. There is accumulating evidence that men and women activate different brain regions and neuronal circuits in response to stressful experiences and apply different coping strategies [219,282,286-288]. Research found that men often react to stress with the typical 'fight or flight' response mentioned in the beginning of this chapter, whereas women are more likely to manage their stress with a 'tend and befriend' response. Females respond to stressful conditions by protecting and nurturing their young (tend) and by seeking social contact and support from others – especially other females (befriend) [289]. Women also produce more stress hormones than men do, and the female sex hormone progesterone prevents the stress hormone system from turning itself off. Even when women and men are confronted with similar stressors, women may be more vulnerable than men to developing depression and related anxiety disorders such as PTSD [290,291]. The underlying mechanism of these sex differences is still insufficiently studied and remains virtually unknown [247,281].

Chronic mild stress models were used in preclinical studies to understand not just the neurobiology of depression but also the underlying cause of sex differences within this disorder. A variety of parameters, including behavioral responses, hormonal levels as well as pattern of neuronal activity, and gene expression showed differences between both sexes [288,292,293]. Male and female rats, as humans, show differences in magnitude of the stress response [294-297] and females are generally more behaviorally active to stressors [247]. Male rats generally show increased plasma corticosterone levels as well as increased adrenal weights [258,298-301] and reduced exploration in an open field [299]. Female rats subjected to the same paradigm show similar increases of plasma corticosterone levels and adrenal weights however show increased exploration and distance moved [299-301]. However, the opposite was also found [288]. Concerning neuronal activity pattern, chronically stressed male rats show increased activity in various limbic brain regions, whereas females rather respond with decreased limbic activity [282,298,300,302].

Despite a growing interest in sex differences in depression, most preclinical studies are still done in males and comparative studies on males and females are still limited. Therefore, the neurobiological underpinnings that may contribute to this difference remain elusive. This represents a major research interest and further investigation into the role of sex in the development of depression will reveal new insights on the underlying neurobiology of this debilitating disorder.

OUTLINE OF THE THESIS

As probably noticed, this thesis is divided into two parts. The major aim of Part 1 is to conduct fundamental research in the field of emotional learning and memory consolidation, whereas Part 2 concentrates on the deleterious effects of chronic stress with a special emphasis on gender differences.

Part I starts off in **Chapter 2** with a detailed introduction of the main surgical technique used throughout this part. Due to a few modifications we were able to refine the long standing procedure of stereotaxic surgery to significantly increase animal welfare and survival.

In **Chapter 3** this method was then used to investigate the role of glucocorticoids within the insular cortex in an aversively motivated learning paradigm. Although glucocorticoids are known to enhance memory consolidation of emotionally arousing experiences, little is known about the influence of the insular cortex or other cortical regions in regulating learning and memory. Therefore, we examined the influence of the IC in the regulation of glucocorticoid effects on memory consolidation of an emotionally arousing training experience. Adult Sprague-Dawley rats were subjected to inhibitory avoidance training followed by bilateral infusion of the specific GR agonist RU 28362 into the IC immediately after training in the one-trial as well as modified, two-phase inhibitory avoidance task. Retention performance was assessed 48-h later. In different groups of rats, the training experience was followed by immediate systemic injection of corticosterone or vehicle and 30 min later brain collection for determination of training induced phosphorylation of ERK1/2.

It was already mentioned that the stress response is also activated by appetitive and rewarding stimuli [19-21]. However, so far the vast majority of studies, in animals as well as humans, investigating the neurobiological mechanism underlying stress hormone-induced facilitation of emotional memory consolidation made use of learning tasks with a strong aversive or fear-motivated component. Although, there is some evidence indicating a possible involvement of glucocorticoids in the consolidation of emotionally arousing appetitive experiences [72,81,171,303,304], little is known about the brain circuit and mechanisms involved in the influence of glucocorticoids on the consolidation of memory of positively motivated learning experiences.

In **Chapter 4 and 5**, we therefore aimed to investigate the influence of glucocorticoids on appetitive memory consolidation in two brain regions known to be involved in emotional learning and memory. To this aim, rats were administered RU 28362, the specific GR agonist already used in Chapter 3, into the BLA (**Chapter 4**) or NAc (**Chapter 5**) immediately after training on an appetitive taste learning paradigm and retention of this positively motivated task was assessed 24 h later. The mechanism of glucocorticoid action on aversive memory consolidation is extensively studied and is believed to involve an interaction with the arousal-induced noradrenergic system (see above). However, this interaction has never been investigated in the context

of appetitive rewarding memory consolidation. For that reason, in **Chapter 5**, we additionally investigated whether the mechanism used by glucocorticoids to enhance memory consolidation of appetitive learning experiences is comparable to that seen in aversively motivated tasks. To address this question we examined a potential interaction with the noradrenergic system by administration of the ß-adrenoceptor blocker propranolol concurrently with RU 28362.

Part II

In contrast to the mostly adaptive and positive effects of acute stress and glucocorticoids seen in Part 1, chronic stress can have deleterious effects on the brain and is a risk factor in the development of depression. Even though the sex difference in depression is widely recognized, the underlying biological mechanisms have not been fully investigated. In **Chapter 6 and 7** we describe some of the effects induced by acute and chronic stress in different stress-related brain regions in male and female rats. We focus especially on the expression of CREB and its active, phosphorylated form, pCREB. CREB is believed to be one of the major targets of downstream antidepressant action. **Chapter 6** examined the effects of acute and chronic footshock stress on CREB, pCREB and BDNF levels in male and female hippocampus and prefrontal cortex regions. In **Chapter 7** we then report the consequences of acute and chronic stress on CREB and pCREB expression levels in male and female rats in the NAc shell and core.

Finally, in **Chapter 8**, the work addressed in this thesis is summarized and discussed.

REFERENCES

1. Selye, H. *A syndrome produced by diverse noxious agents.* (1936).
2. Van de Kar, L. & Blair, M. Forebrain pathways mediating stress-induced hormone secretion. *Frontiers in Neuroendocrinology* (1999).
3. McEwen, B. S. The neurobiology of stress: from serendipity to clinical relevance. *Brain Research* **886**, 172–189 (2000).
4. Johnson, E. O., Kamilaris, T. C., Chrousos, G. P. & Gold, P. W. Mechanisms of stress: a dynamic overview of hormonal and behavioral homeostasis. *Neuroscience and Biobehavioral Reviews* **16**, 115–130 (1992).
5. Cannon, W. B. *The wisdom of the body.* (W W Norton & Co: 1932).
6. Tsigos, C. & Chrousos, G. P. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *J Psychosom Res* **53**, 865–871 (2002).
7. Joëls, M. & Baram, T. Z. The neuro-symphony of stress. *Nature Reviews Neuroscience* (2009). doi:10.1038/nrn2632
8. Reul, J. M. & de Kloet, E. R. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. *Endocrinology* **117**, 2505–2511 (1985).
9. de Kloet, E. R., Vreugdenhil, E., Oitzl, M. S. & Joels, M. Brain corticosteroid receptor balance in health and disease. *Endocr. Rev.* **19**, 269–301 (1998).
10. Pratt, W. B. Glucocorticoid receptor structure and the initial events in signal transduction. *Prog. Clin. Biol. Res.* **322**, 119–132 (1990).
11. Beato, M., Herrlich, P. & Schütz, G. Steroid hormone receptors: many actors in search of a plot. *Cell* **83**, 851–857 (1995).
12. Oitzl, M. S., Reichardt, H. M., Joels, M. & de Kloet, E. R. Point mutation in the mouse glucocorticoid receptor preventing DNA binding impairs spatial memory. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 12790–12795 (2001).
13. de Kloet, E. R., Karst, H. & Joëls, M. Corticosteroid hormones in the central stress response: Quick-and-slow. *Frontiers in Neuroendocrinology* **29**, 268–272 (2008).
14. Atsak, P., Roozendaal, B. & Campolongo, P. Role of the endocannabinoid system in regulating glucocorticoid effects on memory for emotional experiences. *Neuroscience* **204**, 104–116 (2012).
15. Tasker, J. G., Di, S. & Malcher-Lopes, R. Rapid Glucocorticoid Signaling via Membrane-Associated Receptors. *Endocrinology* **147**, 5549–5556 (2006).
16. Tasker, J. G. & Herman, J. P. Mechanisms of rapid glucocorticoid feedback inhibition of the hypothalamic-pituitary-adrenal axis. *Stress* **14**, 398–406 (2011).
17. Joels, M. & de Kloet, E. R. Mineralocorticoid and glucocorticoid receptors in the brain. Implications for ion permeability and transmitter systems. *Progress in Neurobiology* **43**, 1–36 (1994).
18. Barsegyan, A., Mackenzie, S. M., Kurose, B. D., McGaugh, J. L. & Roozendaal, B. Glucocorticoids in the prefrontal cortex enhance memory consolidation and impair working memory by a common neural mechanism. *Proceedings of the National Academy of Sciences* **107**, 16655–16660 (2010).
19. Koolhaas, J. M. *et al.* Stress revisited: A critical evaluation of the stress concept. *Neuroscience and Biobehavioral Reviews* **35**, 1291–1301 (2011).
20. Piazza, P. V. & Le Moal, M. Glucocorticoids as a biological substrate of reward: physiological and pathophysiological implications. *Brain Research Reviews* **25**, 359–372 (1997).
21. Buwalda, B., Scholte, J., de Boer, S. F., Coppens, C. M. & Koolhaas, J. M. The acute glucocorticoid stress response does not differentiate between rewarding and aversive social stimuli in rats. *Hormones and Behavior* **61**, 218–226 (2012).
22. Cahill, L. & McGaugh, J. L. Mechanisms of emotional arousal and lasting declarative memory. *Trends in Neurosciences* **21**, 294–299 (1998).
23. Thoits, P. A. Stress, coping, and social support processes: Where are we? What next? *Journal of health and social behavior* 53–79 (1995).
24. Setlow, B. The nucleus accumbens and learning and memory. *J. Neurosci. Res.* **49**, 515–521 (1997).
25. Mueller, G. & Pilzecker, A. Experimentelle Beiträge zur Lehre vom Gedächtnis. *Zeitschrift fuer Psychologie* (1900).
26. McGaugh, J. L. Memory--a Century of Consolidation. *Science* **287**, 248–251 (2000).
27. McGaugh, J. L. Hormonal influences on memory. *Annu. Rev. Psychol.* **34**, 297–323 (1983).
28. McGaugh, J. L. Make mild moments memorable: add a little arousal. *Trends in Cognitive Sciences* **10**, 345–347 (2006).
29. Bradley, M. M., Greenwald, M. K., Petry, M. C. & Lang, P. J. Remembering pictures: pleasure and arousal in memory. *Journal of Experimental Psychology: Learning, Memory, and Cognition* **18**, 379–390 (1992).

30. Hamann, S. Cognitive and neural mechanisms of emotional memory. *Trends in Cognitive Sciences* **5**, 394–400 (2001).

31. Cahill, L. & McGaugh, J. L. A novel demonstration of enhanced memory associated with emotional arousal. *Conscious Cogn* **4**,410–421 (1995).

32. LaBar, K. S. & Cabeza, R. Cognitive neuroscience of emotional memory. *Nature Reviews Neuroscience* **7**, 54–64 (2006).

33. Gold, P. E. & van Buskirk, R. Posttraining brain norepinephrine concentrations: correlation with retention performance of avoidance training and with peripheral epinephrine modulation of memory processing. *Behav Biol* **23**, 509–520 (1978).

34. Flood, J. F. *et al.* Memory facilitating and anti-amnesic effects of corticosteroids. *Pharmacology, Biochemistry and Behavior* **8**, 81–87 (1978).

35. McGaugh, J. L. & Roozendaal, B. Drug enhancement of memory consolidation: historical perspective and neurobiological implications. *Psychopharmacology* **202**, 3–14 (2009).

36. McGaugh, J. L. Time-dependent processes in memory storage. *Science* **153**, 1351–1358 (1966).

37. McGaugh, J. L. & Roozendaal, B. Role of adrenal stress hormones in forming lasting memories in the brain. *Current Opinion in Neurobiology* **12**, 205–210 (2002).

38. Oitzl, M. S. & de Kloet, E. R. Selective corticosteroid antagonists modulate specific aspects of spatial orientation learning. *Behavioral Neuroscience* **106**, 62–71 (1992).

39. Roozendaal, B. Glucocorticoids and the regulation of memory consolidation. *Psychoneuroendocrinology* **25**, 213–238 (2000).

40. Roozendaal, B., McEwen, B. S. & Chattarji, S. Stress, memory and the amygdala. *Nature Reviews Neuroscience* **10**, 423–433 (2009).

41. Roozendaal, B. & McGaugh, J. L. Memory modulation. *Behavioral Neuroscience* **125**, 797–824 (2011).

42. Gold, P. E. & Van Buskirk, R. B. Facilitation of time-dependent memory processes with posttrial epinephrine injections. *Behav Biol* **13**, 145–153 (1975).

43. Introini-Collison, I. B. & McGaugh, J. L. Epinephrine modulates long-term retention of an aversively motivated discrimination. *Behavioral and Neural Biology* **45**, 358–365 (1986).

44. Izquierdo, I. & Dias, R. D. Influence on memory of posttraining or pre-test injections of ACTH, vasopressin, epinephrine, and [beta]-endorphin, and their interaction with naloxone. *Psychoneuroendocrinology* **10**, 165–172 (1985).

45. Liang, K. C., Juler, R. G. & McGaugh, J. L. Modulating effects of posttraining epinephrine on memory: involvement of the amygdala noradrenergic system. *Brain Research* **368**, 125–133 (1986).

46. Cahill, L. & Alkire, M. T. Epinephrine enhancement of human memory consolidation: interaction with arousal at encoding. *Neurobiology of Learning and Memory* **79**, 194–198 (2003).

47. Cahill, L. Enhanced Human Memory Consolidation With Post-Learning Stress: Interaction With the Degree of Arousal at Encoding. *Learning & Memory* **10**, 270–274 (2003).

48. Cahill, L., Prins, B., Weber, M. & McGaugh, J. L. Beta-adrenergic activation and memory for emotional events. *nature* **371**, 702–704 (1994).

49. van Stegeren, A. H., Everaerd, W., Cahill, L., McGaugh, J. L. & Gooren, L. J. Memory for emotional events: differential effects of centrally versus peripherally acting beta-blocking agents. *Psychopharmacology* **138**, 305–310 (1998).

50. Weil-Malherbe, H., Axelrod, J. & Tomchick, R. Blood-brain barrier for adrenaline. *Science* **129**, 1226–1227 (1959).

51. Kalia, M. & Sullivan, J. M. Brainstem projections of sensory and motor components of the vagus nerve in the rat. *J. Comp. Neurol.* **211**, 248–265 (1982).

52. Introini-Collison, I., Saghafi, D., Novack, G. D. & McGaugh, J. L. Memory-enhancing effects of post-training dipivefrin and epinephrine: involvement of peripheral and central adrenergic receptors. *Brain Research* **572**, 81–86 (1992).

53. McGaugh, J. L., Cahill, L. & Roozendaal, B. Involvement of the amygdala in memory storage: interaction with other brain systems. *Proceedings of the National Academy of Sciences* **93**, 13508–13514 (1996).

54. Schreurs, J., Seelig, T. & Schulman, H. Beta 2-adrenergic receptors on peripheral nerves. *J Neurochem* **46**, 294–296 (1986).

55. Lawrence, A. J. Neurotransmitter mechanisms of rat vagal afferent neurons. *Clin. Exp. Pharmacol. Physiol.* **22**, 869–873 (1995).

56. Williams, C. L. & McGaugh, J. L. Reversible lesions of the nucleus of the solitary tract attenuate the memory-modulating effects of posttraining epinephrine. *Behavioral Neuroscience* **107**, 955–962 (1993).

57. Williams, C. L., Men, D. & Clayton, E. C. The effects of noradrenergic activation of the nucleus tractus solitarius on memory and in potentiating norepinephrine release in the amygdala. *Behavioral Neuroscience* **114**, 1131–1144 (2000).
58. Ricardo, J. A. & Koh, E. T. Anatomical evidence of direct projections from the nucleus of the solitary tract to the hypothalamus, amygdala, and other forebrain structures in the rat. *Brain Research* **153**, 1–26 (1978).
59. Mehendale, S., Xie, J.-T., Aung, H. H., Guan, X.-F. & Yuan, C.-S. Nucleus accumbens receives gastric vagal inputs. *Acta Pharmacol. Sin.* **25**, 271–275 (2004).
60. Kerfoot, E. C., Chattillion, E. A. & Williams, C. L. Functional interactions between the nucleus tractus solitarius (NTS) and nucleus accumbens shell in modulating memory for arousing experiences. *Neurobiology of Learning and Memory* **89**, 47–60 (2008).
61. Williams, C. L. & Clayton, E. C. *Memory consolidation: Essays in honor of James L. McGaugh.* 141–163 (American Psychological Association: Washington, DC, US, 2001). doi:10.1037/10413-008
62. van Stegeren, A. H. The role of the noradrenergic system in emotional memory. *Acta Psychologica* **127**, 532–541 (2008).
63. McGaugh, J. L. *et al.* Neuromodulatory systems and memory storage: role of the amygdala. *Behavioural Brain Research* **58**, 81–90 (1993).
64. Introini-Collison, I. B., Miyazaki, B. & McGaugh, J. L. Involvement of the amygdala in the memory-enhancing effects of clenbuterol. *Psychopharmacology* **104**, 541–544 (1991).
65. Introini-Collison, I. B., Ford, L. & McGaugh, J. L. Memory impairment induced by intraamygdala beta-endorphin is mediated by noradrenergic influences. *Neurobiology of Learning and Memory* **63**, 200–205 (1995).
66. Liang, K. C., McGaugh, J. L. & Yao, H. Y. Involvement of amygdala pathways in the influence of post-training intra-amygdala norepinephrine and peripheral epinephrine on memory storage. *Brain Research* **508**, 225–233 (1990).
67. Liang, K. C., Chen, L. L. & Huang, T. E. The role of amygdala norepinephrine in memory formation: involvement in the memory enhancing effect of peripheral epinephrine. *Chin J Physiol* **38**, 81–91 (1995).
68. de Kloet, E. R., Oitzl, M. S. & Joels, M. Stress and cognition: are corticosteroids good or bad guys? *Trends in Neurosciences* **22**, 422–426 (1999).
69. Lupien, S. J. & McEwen, B. S. The acute effects of corticosteroids on cognition: integration of animal and human model studies. *Brain Res. Brain Res. Rev.* **24**, 1–27 (1997).
70. Sandi, C. & Pinelo-Nava, M. T. Stress and Memory: Behavioral Effects and Neurobiological Mechanisms. *Neural Plast.* **2007**, 1–20 (2007).
71. de Quervain, D. J. F., Aerni, A., Schelling, G. & Roozendaal, B. Glucocorticoids and the regulation of memory in health and disease. *Frontiers in Neuroendocrinology* **30**, 358–370 (2009).
72. Buchanan, T. W. & Lovallo, W. R. Enhanced memory for emotional material following stress-level cortisol treatment in humans. *Psychoneuroendocrinology* **26**, 307–317 (2001).
73. Maheu, F. S., Joober, R., Beaulieu, S. & Lupien, S. J. Differential Effects of Adrenergic and Corticosteroid Hormonal Systems on Human Short- and Long-Term Declarative Memory for Emotionally Arousing Material. *Behavioral Neuroscience* **118**, 420–428 (2004).
74. Cordero, M. I. & Sandi, C. A role for brain glucocorticoid receptors in contextual fear conditioning: dependence upon training intensity. *Brain Research* **786**, 11–17 (1998).
75. Roozendaal, B., Williams, C. L. & McGaugh, J. L. Glucocorticoid receptor activation in the rat nucleus of the solitary tract facilitates memory consolidation: involvement of the basolateral amygdala. *European Journal of Neuroscience* **11**, 1317–1323 (1999).
76. Roozendaal, B. & McGaugh, J. L. Glucocorticoid receptor agonist and antagonist administration into the basolateral but not central amygdala modulates memory storage. *Neurobiology of Learning and Memory* **67**, 176–179 (1997).
77. Okuda, S., Roozendaal, B. & McGaugh, J. Glucocorticoid effects on object recognition memory require training-associated emotional arousal. *Proceedings of the National Academy of Sciences* **101**, 853–858 (2004).
78. Sandi, C. & Rose, S. P. Corticosterone enhances long-term retention in one-day-old chicks trained in a weak passive avoidance learning paradigm. *Brain Research* **647**, 106–112 (1994).
79. Miranda, M. I., Quirarte, G. L., Rodriguez-Garcia, G., McGaugh, J. L. & Roozendaal, B. Glucocorticoids enhance taste aversion memory via actions in the insular cortex and basolateral amygdala. *Learning & Memory* **15**, 468–476 (2008).
80. Zorawski, M. & Killcross, S. Glucocorticoid Receptor Agonist Enhances Pavlovian Appetitive Conditioning but Disrupts Outcome-Specific Associations. *Behavioral Neuroscience* **117**, 1453–1457 (2003).

81. Kuhlmann, S. & Wolf, O. T. Arousal and cortisol interact in modulating memory consolidation in healthy young men. *Behavioral Neuroscience* **120**, 217–223 (2006).

82. Beckner, V. E., Tucker, D. M., Delville, Y. & Mohr, D. C. Stress facilitates consolidation of verbal memory for a film but does not affect retrieval. *Behavioral Neuroscience* **120**, 518–527 (2006).

83. Oitzl, M. S., Fluttert, M. & de Kloet, E. R. Acute blockade of hippocampal glucocorticoid receptors facilitates spatial learning in rats. *Brain Research* 797, 159–162 (1998).

84. Conrad, C. D., Lupien, S. J. & McEwen, B. S. Support for a bimodal role for type II adrenal steroid receptors in spatial memory. *Neurobiology of Learning and Memory* **72**, 39–46 (1999).

85. Roozendaal, B., Portillo-Marquez, G. & McGaugh, J. L. Basolateral amygdala lesions block glucocorticoid-induced modulation of memory for spatial learning. *Behavioral Neuroscience* **110**, 1074–1083 (1996).

86. Abercrombie, H., Speck, N. & Monticelli, R. Endogenous cortisol elevations are related to memory facilitation only in individuals who are emotionally aroused. *Psychoneuroendocrinology* **31**, 187–196 (2006).

87. Roozendaal, B., Okuda, S., Van der Zee, E. A. & McGaugh, J. L. Glucocorticoid enhancement of memory requires arousal-induced noradrenergic activation in the basolateral amygdala. *Proceedings of the National Academy of Sciences* **103**, 6741–6746 (2006).

88. Joëls, M., Fernández, G. & Roozendaal, B. Stress and emotional memory: a matter of timing. *Trends in Cognitive Sciences* **15**, 280–288 (2011).

89. Pu, Z., Krugers, H. J. & Joëls, M. Beta-adrenergic facilitation of synaptic plasticity in the rat basolateral amygdala in vitro is gradually reversed by corticosterone. *Learn. Mem.* **16**, 155–160 (2009).

90. Roozendaal, B. *et al.* Basolateral amygdala noradrenergic activity mediates corticosterone-induced enhancement of auditory fear conditioning. *Neurobiology of Learning and Memory* **86**, 249–255 (2006).

91. Roozendaal, B., Barsegyan, A. & Lee, S. Adrenal stress hormones, amygdala activation, and memory for emotionally arousing experiences. *Progress in Brain Research* **167**, 79–97 (2008).

92. van Stegeren, A. H., Roozendaal, B., Kindt, M., Wolf, O. T. & Joëls, M. Interacting noradrenergic and corticosteroid systems shift human brain activation patterns during encoding. *Neurobiology of Learning and Memory* **93**, 56–65 (2010).

93. Quirarte, G. L., Roozendaal, B. & McGaugh, J. L. Glucocorticoid enhancement of memory storage involves noradrenergic activation in the basolateral amygdala. *Proceedings of the National Academy of Sciences* **94**, 14048–14053 (1997).

94. Roozendaal, B., Nguyen, B. T., Power, A. E. & McGaugh, J. L. Basolateral amygdala noradrenergic influence enables enhancement of memory consolidation induced by hippocampal glucocorticoid receptor activation. *Proceedings of the National Academy of Sciences* **96**, 11642–11647 (1999).

95. Roozendaal, B., Okuda, S., de Quervain, D. & McGaugh, J. Glucocorticoids interact with emotion-induced noradrenergic activation in influencing different memory functions. *Neuroscience* **138**, 901–910 (2006).

96. Borrell, J., De Kloet, E. & Versteeg, D. Inhibitory avoidance deficit following short-term adrenalectomy in the rat: the role of adrenal catecholamines. *Behavioral and neural ...* (1983).

97. Borrell, J., de Kloet, E. R. & Bohus, B. Corticosterone decreases the efficacy of adrenaline to affect passive avoidance retention of adrenalectomized rats. *Life Sciences* **34**, 99–104 (1984).

98. Roozendaal, B., Carmi, O. & McGaugh, J. L. Adrenocortical suppression blocks the memory-enhancing effects of amphetamine and epinephrine. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1429–1433 (1996).

99. Liu, L., Tsuji, M., Takeda, H., Takada, K. & Matsumiya, T. Adrenocortical suppression blocks the enhancement of memory storage produced by exposure to psychological stress in rats. *Brain Research* **821**, 134–140 (1999).

100. Carew, T. J. Molecular enhancement of memory formation. *Neuron* **16**, 5–8 (1996).

101. McGaugh, J. L. The amygdala modulates the consolidation of memories of emotionally arousing experiences. *Annual Review of Neuroscience* **27**, 1–28 (2004).

102. Lamprecht, R., Hazvi, S. & Dudai, Y. cAMP response element-binding protein in the amygdala is required for long- but not short-term conditioned taste aversion memory. *J. Neurosci.* **17**, 8443–8450 (1997).

103. Josselyn, S. A. *et al.* Long-term memory is facilitated by cAMP response element-binding protein overexpression in the amygdala. *Journal of Neuroscience* **21**, 2404–2412 (2001).

104. Roozendaal, B., Quirarte, G. L. & McGaugh, J. L. Glucocorticoids interact with the basolateral amygdala beta-adrenoceptor–cAMP/cAMP/

PKA system in influencing memory consolidation. *European Journal of Neuroscience* **15**, 553–560 (2002).

105. Roozendaal, B. *et al.* Membrane-Associated Glucocorticoid Activity Is Necessary for Modulation of Long-Term Memory via Chromatin Modification. *Journal of Neuroscience* **30**, 5037–5046 (2010).

106. Hill, M. N. & McEwen, B. S. Endocannabinoids: The silent partner of glucocorticoids in the synapse. *Proceedings of the National Academy of Sciences* **106**, 4579–4580 (2009).

107. Campolongo, P. *et al.* Endocannabinoids in the rat basolateral amygdala enhance memory consolidation and enable glucocorticoid modulation of memory. *Proceedings of the National Academy of Sciences* **106**, 4888–4893 (2009).

108. Tomaz, C., Dickinson-Anson, H. & McGaugh, J. L. Basolateral amygdala lesions block diazepam-induced anterograde amnesia in an inhibitory avoidance task. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3615–3619 (1992).

109. Cahill, L., Babinsky, R., Markowitsch, H. J. & McGaugh, J. L. The amygdala and emotional memory. *nature* **377**, 295–296 (1995).

110. Adolphs, R., Cahill, L., Schul, R. & Babinsky, R. Impaired declarative memory for emotional material following bilateral amygdala damage in humans. *Learning & Memory* **4**, 291–300 (1997).

111. Strange, B. A., Hurlemann, R. & Dolan, R. J. An emotion-induced retrograde amnesia in humans is amygdala- and beta-adrenergic-dependent. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 13626–13631 (2003).

112. Cahill, L. *et al.* Amygdala activity at encoding correlated with long-term, free recall of emotional information. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8016–8021 (1996).

113. Canli, T., Zhao, Z., Brewer, J., Gabrieli, J. D. & Cahill, L. Event-related activation in the human amygdala associates with later memory for individual emotional experience. *Journal of Neuroscience* **20**, RC99 (2000).

114. Hamann, S. B., Ely, T. D., Grafton, S. T. & Kilts, C. D. Amygdala activity related to enhanced memory for pleasant and aversive stimuli. *Nature Neuroscience* **2**, 289–293 (1999).

115. Ferry, B., Roozendaal, B. & McGaugh, J. L. Basolateral amygdala noradrenergic influences on memory storage are mediated by an interaction between beta- and alpha1-adrenoceptors. *Journal of Neuroscience* **19**, 5119–5123 (1999).

116. Hatfield, T., Spanis, C. & McGaugh, J. L. Response of amygdalar norepinephrine to footshock and GABAergic drugs using in vivo microdialysis and HPLC. *Brain Research* **835**, 340–345 (1999).

117. Introini-Collison, I. B., Dalmaz, C. & McGaugh, J. L. Amygdala beta-noradrenergic influences on memory storage involve cholinergic activation. *Neurobiology of Learning and Memory* **65**, 57–64 (1996).

118. Izquierdo, I. *et al.* Neurotransmitter receptors involved in post-training memory processing by the amygdala, medial septum, and hippocampus of the rat. *Behavioral and Neural Biology* **58**, 16–26 (1992).

119. LaLumiere, R. T. Memory enhancement induced by post-training intrabasolateral amygdala infusions of -adrenergic or muscarinic agonists requires activation of dopamine receptors: Involvement of right, but not left, basolateral amygdala. *Learning & Memory* **12**, 527–532 (2005).

120. LaLumiere, R. T., Buen, T.-V. & McGaugh, J. L. Post-training intra-basolateral amygdala infusions of norepinephrine enhance consolidation of memory for contextual fear conditioning. *Journal of Neuroscience* **23**, 6754–6758 (2003).

121. Roozendaal, B., Castello, N. A., Vedana, G., Barsegyan, A. & McGaugh, J. L. Noradrenergic activation of the basolateral amygdala modulates consolidation of object recognition memory☆. *Neurobiology of Learning and Memory* **90**, 576–579 (2008).

122. Galvez, R., Mesches, M. H. & McGaugh, J. L. Norepinephrine release in the amygdala in response to footshock stimulation. *Neurobiology of Learning and Memory* **66**, 253–257 (1996).

123. McIntyre, C. K., Hatfield, T. & McGaugh, J. L. Amygdala norepinephrine levels after training predict inhibitory avoidance retention performance in rats. *European Journal of Neuroscience* **16**, 1223–1226 (2002).

124. Williams, C. L., Men, D., Clayton, E. C. & Gold, P. E. Norepinephrine release in the amygdala after systemic injection of epinephrine or escapable footshock: contribution of the nucleus of the solitary tract. *Behavioral Neuroscience* **112**, 1414–1422 (1998).

125. Roozendaal, B. & McGaugh, J. L. Amygdaloid nuclei lesions differentially affect glucocorticoid-induced memory enhancement in an inhibitory avoidance task. *Neurobiology of Learning and Memory* **65**, 1–8 (1996).

126. Donley, M. P., Schulkin, J. & Rosen, J. B. Glucocorticoid receptor antagonism in the basolateral amygdala and ventral hippocampus interferes with long-term memory of contextual

fear. *Behavioural Brain Research* **164**, 197–205 (2005).

127. van Stegeren, A. H. *et al.* Endogenous cortisol level interacts with noradrenergic activation in the human amygdala. *Neurobiology of Learning and Memory* 87, 57–66 (2007).

128. Packard, M. G., Cahill, L. & McGaugh, J. L. Amygdala modulation of hippocampal-dependent and caudate nucleus-dependent memory processes. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8477–8481 (1994).

129. Pitkänen, A., Pikkarainen, M., Nurminen, N. & Ylinen, A. Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat. A review. *Annals of the New York Academy of Sciences* **911**, 369–391 (2000).

130. Roozendaal, B., de Quervain, D. J., Ferry, B., Setlow, B. & McGaugh, J. L. Basolateral amygdala-nucleus accumbens interactions in mediating glucocorticoid enhancement of memory consolidation. *Journal of Neuroscience* **21**, 2518–2525 (2001).

131. Setlow, B., Roozendaal, B. & McGaugh, J. L. Involvement of a basolateral amygdala complex–nucleus accumbens pathway in glucocorticoid-induced modulation of memory consolidation. *European Journal of Neuroscience* **12**, 367–375 (2000).

132. Ikegaya, Y., Nakanishi, K., Saito, H. & Abe, K. Amygdala beta-noradrenergic influence on hippocampal long-term potentiation in vivo. *NeuroReport* **8**, 3143–3146 (1997).

133. Ikegaya, Y., Saito, H. & Abe, K. Dentate gyrus field potentials evoked by stimulation of the basolateral amygdaloid nucleus in anesthetized rats. *Brain Research* **718**, 53–60 (1996).

134. McIntyre, C. K. *et al.* Memory-influencing intra-basolateral amygdala drug infusions modulate expression of Arc protein in the hippocampus. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 10718–10723 (2005).

135. McReynolds, J. R. *et al.* Memory-enhancing corticosterone treatment increases amygdala norepinephrine and Arc protein expression in hippocampal synaptic fractions. *Neurobiology of Learning and Memory* **93**, 312–321 (2010).

136. Holloway-Erickson, C. M., McReynolds, J. R. & McIntyre, C. K. Memory-enhancing intra-basolateral amygdala infusions of clenbuterol increase Arc and CaMKIIα protein expression in the rostral anterior cingulate cortex. *Frontiers in Behavioral Neuroscience* **6**, (2012).

137. Arnsten, A. F. T. Stress signalling pathways that impair prefrontal cortex structure and function. *Nature Reviews Neuroscience* 1–13 (2009).doi:10.1038/nrn2648

138. McEwen, B. S. Glucocorticoids, depression, and mood disorders: structural remodeling in the brain. *Metabolism* **54**, 20–23 (2005).

139. Baddeley, A. Working memory. *Science* **255**, 556–559 (1992).

140. Liang, K. & Chen, H. Posttraining Infusion of Norepinephrine and Corticotropin Releasing Factor into the Bed Nucleus of the Stria Terminalis Enhanced Retention in an Inhibitory .... *Chinese Journal of Physiology* (2001).

141. Runyan, J. D., Moore, A. N. & Dash, P. K. A role for prefrontal cortex in memory storage for trace fear conditioning. *Journal of Neuroscience* **24**, 1288–1295 (2004).

142. Izquierdo, L. A. *et al.* A link between role of two prefrontal areas in immediate memory and in long-term memory consolidation. *Neurobiology of Learning and Memory* **88**, 160–166 (2007).

143. Fuster, J. M. The prefrontal cortex and its relation to behavior. *Progress in Brain Research* **87**, 201–211 (1991).

144. Li, B. M., Mao, Z. M., Wang, M. & Mei, Z. T. Alpha-2 adrenergic modulation of prefrontal cortical neuronal activity related to spatial working memory in monkeys. *Neuropsychopharmacology* **21**, 601–610 (1999).

145. Elzinga, B. M. & Roelofs, K. Cortisol-induced impairments of working memory require acute sympathetic activation. *Behavioral Neuroscience* **119**, 98–103 (2005).

146. Roozendaal, B., McReynolds, J. & McGaugh, J. The Basolateral Amygdala Interacts with the Medial Prefrontal Cortex in Regulating Glucocorticoid Effects on Working Memory Impairment. *Journal of Neuroscience* **24**, 1385–1392 (2004).

147. Mingote, S. Noradrenaline and dopamine efflux in the prefrontal cortex in relation to appetitive classical conditioning. *Journal of Neuroscience* **24**, 2475–2480 (2004).

148. Ventura, R., Latagliata, E. C., Morrone, C., La Mela, I. & Puglisi-Allegra, S. Prefrontal Norepinephrine Determines Attribution of "High" Motivational Salience. *PLoS ONE* **3**, e3044 (2008).

149. Roozendaal, B. *et al.* Glucocorticoid Effects on Memory Consolidation Depend on Functional Interactions between the Medial Prefrontal Cortex and Basolateral Amygdala. *Journal of Neuroscience* **29**, 14299–14308 (2009).

150. Reyes-López, J., Nuñez-Jaramillo, L., Morán-Guel, E. & Miranda, M. I. Differential effects of β-adrenergic receptor blockade in the medial

prefrontal cortex during aversive and incidental taste memory formation. *Neuroscience* **169**, 195–202 (2010).

151. Milner, D. The memory defect in bilateral hippocampal lesions. *Psychiatr Res Rep Am Psychiatr Assoc* **11**, 43–58 (1959).

152. Milner, B. & Penfield, W. The effect of hippocampal lesions on recent memory. *Trans Am Neurol Assoc* 42–48 (1955).

153. Izquierdo, I. *et al.* The connection between the hippocampal and the striatal memory systems of the brain: a review of recent findings. *Neurotoxicity Research* **10**, 113–121 (2006).

154. Squire, L. R., Stark, C. E. L. & Clark, R. E. The medial temporal lobe. *Annual Review of Neuroscience* 27, 279–306 (2004).

155. Eichenbaum, H. The hippocampus and declarative memory: cognitive mechanisms and neural codes. *Behavioural Brain Research* **127**, 199–207 (2001).

156. Eichenbaum, H. The hippocampus and mechanisms of declarative memory. *Behavioural Brain Research* **103**, 123–133 (1999).

157. Squire, L. R. Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. *Psychological Review* **99**, 195–231 (1992).

158. Kukolja, J., Klingmuller, D., Maier, W., Fink, G. R. & Hurlemann, R. Noradrenergic-glucocorticoid modulation of emotional memory encoding in the human hippocampus. *Psychological Medicine* **41**, 2167–2176 (2011).

159. McEwen, B. S. Stress, adaptation, and disease. Allostasis and allostatic load. *Annals of the New York Academy of Sciences* **840**, 33–44 (1998).

160. Sapolsky, R. M., Zola-Morgan, S. & Squire, L. R. Inhibition of glucocorticoid secretion by the hippocampal formation in the primate. *J. Neurosci.* **11**, 3695–3704 (1991).

161. Mora, F., Segovia, G., Del Arco, A., de Blas, M. & Garrido, P. Stress, neurotransmitters, corticosterone and body-brain integration. *Brain Research* (2012).doi:10.1016/j.brainres.2011.12.049

162. Harley, C. Noradrenergic and locus coeruleus modulation of the perforant path-evoked potential in rat dentate gyrus supports a role for the locus coeruleus in attentional and memorial processes. *Progress in Brain Research* **88**, 307–321 (1991).

163. Morimoto, M., Morita, N., Ozawa, H., Yokoyama, K. & Kawata, M. Distribution of glucocorticoid receptor immunoreactivity and mRNA in the rat brain: an immunohistochemical and in situ hybridization study. *Neuroscience Research* **26**, 235–269 (1996).

164. Patel, P. D. *et al.* Glucocorticoid and mineralocorticoid receptor mRNA expression in squirrel monkey brain. *Journal of Psychiatric Research* **34**, 383–392 (2000).

165. Sapolsky, R. M., Uno, H., Rebert, C. S. & Finch, C. E. Hippocampal damage associated with prolonged glucocorticoid exposure in primates. *J. Neurosci.* **10**, 2897–2902 (1990).

166. Diamond, D. M., Bennett, M. C., Fleshner, M. & Rose, G. M. Inverted-U relationship between the level of peripheral corticosterone and the magnitude of hippocampal primed burst potentiation. *Hippocampus* 2, 421–430 (1992).

167. Joels, M. & de Kloet, E. R. Control of neuronal excitability by corticosteroid hormones. *Trends in Neurosciences* **15**, 25–30 (1992).

168. Pavlides, C., Watanabe, Y. & McEwen, B. S. Effects of glucocorticoids on hippocampal long-term potentiation. *Hippocampus* 3, 183–192 (1993).

169. Joëls, M. & Krugers, H. J. LTP after stress: up or down? *Neural Plast.* **2007**, 93202 (2007).

170. Maroun, M. & Richter-Levin, G. Exposure to acute stress blocks the induction of long-term potentiation of the amygdala-prefrontal cortex pathway in vivo. *Journal of Neuroscience* 23, 4406–4409 (2003).

171. Micheau, J., Destrade, C. & Soumireu-Mourat, B. Time-dependent effects of posttraining intrahippocampal injections of corticosterone on retention of appetitive learning tasks in mice. *European Journal of Pharmacology* **106**, 39–46 (1985).

172. Roozendaal, B. & McGaugh, J. L. Basolateral amygdala lesions block the memory-enhancing effect of glucocorticoid administration in the dorsal hippocampus of rats. *Eur. J. Neurosci.* 9, 76–83 (1997).

173. Strange, B. A. & Dolan, R. J. Beta-adrenergic modulation of emotional memory-evoked human amygdala and hippocampal responses. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 11454–11458 (2004).

174. Craig, A. D. B. How do you feel--now? The anterior insula and human awareness. *Nature Reviews Neuroscience* **10**, 59–70 (2009).

175. Menon, V. & Uddin, L. Q. Saliency, switching, attention and control: a network model of insula function. *Brain Struct Funct* **214**, 655–667 (2010).

176. Shin, L. M. & Liberzon, I. The neurocircuitry of fear, stress, and anxiety disorders. *Neuropsychopharmacology* **35**, 169–191 (2010).

177. Hartley, C. A., Fischl, B. & Phelps, E. A. Brain structure correlates of individual differences in

the acquisition and inhibition of conditioned fear. *Cereb. Cortex* **21**, 1954–1962 (2011).

178. Ille, R. *et al.* Emotion recognition and experience in Huntington disease: a voxel-based morphometry study. *J Psychiatr y Neurosci* **36**, 383–390 (2011).

179. Büchel, C., Morris, J., Dolan, R. J. & Friston, K. J. Brain systems mediating aversive conditioning: an event-related fMRI study. *Neuron* **20**, 947–957 (1998).

180. Alvarez, R. P., Biggs, A., Chen, G., Pine, D. S. & Grillon, C. Contextual fear conditioning in humans: cortical-hippocampal and amygdala contributions. *Journal of Neuroscience* **28**, 6211–6219 (2008).

181. Alvarez, R. P., Chen, G., Bodurka, J., Kaplan, R. & Grillon, C. Phasic and sustained fear in humans elicits distinct patterns of brain activity. *NeuroImage* **55**, 389–400 (2011).

182. Marschner, A., Kalisch, R., Vervliet, B., Vansteenwegen, D. & Büchel, C. Dissociable roles for the hippocampus and the amygdala in human cued versus context fear conditioning. *Journal of Neuroscience* **28**, 9030–9036 (2008).

183. King, A. P. *et al.* Medial prefrontal cortex and right insula activity predict plasma ACTH response to trauma recall. *NeuroImage* **47**, 872–880 (2009).

184. Rasch, B. *et al.* A genetic variation of the noradrenergic system is related to differential amygdala activation during encoding of emotional memories. *Proceedings of the National Academy of Sciences* **106**, 19191–19196 (2009).

185. Pruessner, J. C. *et al.* Deactivation of the Limbic System During Acute Psychosocial Stress: Evidence from Positron Emission Tomography and Functional Magnetic Resonance Imaging Studies. *Biological Psychiatry* **63**, 234–240 (2008).

186. Bermudez-Rattoni, F. Molecular mechanisms of taste-recognition memory. *Nature Reviews Neuroscience* **5**, 209–217 (2004).

187. Shema, R., Sacktor, T. C. & Dudai, Y. Rapid Erasure of Long-Term Memory Associations in the Cortex by an Inhibitor of PKM. *Science* **317**, 951–953 (2007).

188. Nuñez-Jaramillo, L., rez-Lugo, L. R., Herrera-Morales, W. & Miranda, M. A. I. Taste memory formation: Latest advances and challenges. *Behavioural Brain Research* 1–17 (2009). doi:10.1016/j.bbr.2009.10.040

189. Stehberg, J., Moraga-Amaro, R. & Simon, F. The role of the insular cortex in taste function. *Neurobiology of Learning and Memory* 1–6 (2011).doi:10.1016/j.nlm.2011.03.005

190. Miranda, M. I. & McGaugh, J. L. Enhancement of inhibitory avoidance and conditioned taste aversion memory with insular cortex infusions of 8-Br-cAMP: involvement of the basolateral amygdala. *Learning & Memory* **11**, 312–317 (2004).

191. Bermudez-Rattoni, F., Introini-Collison, I. B. & McGaugh, J. L. Reversible inactivation of the insular cortex by tetrodotoxin produces retrograde and anterograde amnesia for inhibitory avoidance and spatial learning. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5379–5382 (1991).

192. Bermudez-Rattoni, F., Introini-Collison, I., Coleman-Mesches, K. & McGaugh, J. L. Insular cortex and amygdala lesions induced after aversive training impair retention: effects of degree of training. *Neurobiology of Learning and Memory* **67**, 57–63 (1997).

193. Bermudez-Rattoni, F., Okuda, S., Roozendaal, B. & McGaugh, J. L. Insular cortex is involved in consolidation of object recognition memory. *Learning & Memory* **12**, 447–449 (2005).

194. Bermudez-Rattoni, F. & McGaugh, J. L. Insular cortex and amygdala lesions differentially affect acquisition on inhibitory avoidance and conditioned taste aversion. *Brain Research* **549**, 165–170 (1991).

195. Nerad, L., Ramírez-Amaya, V., Ormsby, C. E. & Bermudez-Rattoni, F. Differential effects of anterior and posterior insular cortex lesions on the acquisition of conditioned taste aversion and spatial learning. *Neurobiology of Learning and Memory* **66**, 44–50 (1996).

196. Gutiérrez, H., Hernández-Echeagaray, E., Ramírez-Amaya, V. & Bermudez-Rattoni, F. Blockade of N-methyl-D-aspartate receptors in the insular cortex disrupts taste aversion and spatial memory formation. *NSC* **89**, 751–758 (1999).

197. Miranda, M. I. & Bermudez-Rattoni, F. Cholinergic activity in the insular cortex is necessary for acquisition and consolidation of contextual memory. *Neurobiology of Learning and Memory* **87**, 343–351 (2007).

198. Robbins, T. W. & Everitt, B. J. Neurobehavioural mechanisms of reward and motivation. *Current Opinion in Neurobiology* **6**, 228–236 (1996).

199. Salamone, J. D. The behavioral neurochemistry of motivation: methodological and conceptual issues in studies of the dynamic activity of nucleus accumbens dopamine. *Journal of Neuroscience Methods* **64**, 137–149 (1996).

200. Wise, R. A. Dopamine, learning and motivation. *Nature Reviews Neuroscience* **5**, 483–494 (2004).

201. Ramírez-Lugo, L., Nuñez-Jaramillo, L. & Bermudez-Rattoni, F. Taste Memory Formation:

Role of Nucleus Accumbens. *Chemical Senses* 32, 93–97 (2006).

202. Seamans, J. K. & Phillips, A. G. Selective memory impairments produced by transient lidocaine-induced lesions of the nucleus accumbens in rats. *Behavioral Neuroscience* **108**, 456–468 (1994).

203. Lorenzini, C. A., Baldi, E., Bucherelli, C. & Tassoni, G. Time-Dependent Deficits of Rat's Memory Consolidation Induced by Tetrodotoxin Injections into the Caudate-Putamen, Nucleus Accumbens, and Globus Pallidus. *Neurobiology of Learning and Memory* **63**, 87–93 (1995).

204. Manago, F., Castellano, C., Oliverio, A., Mele, A. & De Leonibus, E. Role of dopamine receptors subtypes, D1-like and D2-like, within the nucleus accumbens subregions, core and shell, on memory consolidation in the one-trial inhibitory avoidance task. *Learning & Memory* **16**, 46–52 (2008).

205. Pedroza-Llinás, R., Ramírez-Lugo, L., Guzmán-Ramos, K., Zavala-Vega, S. & Bermudez-Rattoni, F. Safe taste memory consolidation is disrupted by a protein synthesis inhibitor in the nucleus accumbens shell. *Neurobiology of Learning and Memory* 1–8 (2009).doi:10.1016/j.nlm.2009.02.011

206. Goldenberg, G., Schuri, U., Grömminger, O. & Arnold, U. Basal forebrain amnesia: does the nucleus accumbens contribute to human memory? *J. Neurol. Neurosurg. Psychiatr.* **67**, 163–168 (1999).

207. Epstein, J. *et al.* Lack of ventral striatal response to positive stimuli in depressed versus normal subjects. *Am J Psychiatry* **163**, 1784–1790 (2006).

208. Sabatinelli, D., Bradley, M. M., Lang, P. J., Costa, V. D. & Versace, F. Pleasure Rather Than Salience Activates Human Nucleus Accumbens and Medial Prefrontal Cortex. *Journal of Neurophysiology* **98**, 1374–1379 (2007).

209. Phan, K. L. *et al.* Neural correlates of individual ratings of emotional salience: a trial-related fMRI study. *NeuroImage* **21**, 768–780 (2004).

210. Sorg, B. A. & Kalivas, P. W. Effects of cocaine and footshock stress on extracellular dopamine levels in the ventral striatum. *Brain Research* **559**, 29–36 (1991).

211. Rougé-Pont, F., Deroche, V., Le Moal, M. & Piazza, P. V. Individual differences in stress-induced dopamine release in the nucleus accumbens are influenced by corticosterone. *Eur. J. Neurosci.* **10**, 3903–3907 (1998).

212. Kerfoot, E. C. & Williams, C. L. Interactions between brainstem noradrenergic neurons and the nucleus accumbens shell in modulating memory for emotionally arousing events. *Learning & Memory* **18**, 405–413 (2011).

213. Piazza, P. V. *et al.* Glucocorticoids have state-dependent stimulant effects on the mesencephalic dopaminergic transmission. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8716–8720 (1996).

214. Marinelli, M., Aouizerate, B., Barrot, M., Le Moal, M. & Piazza, P. V. Dopamine-dependent responses to morphine depend on glucocorticoid receptors. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7742–7747 (1998).

215. Barrot, M. *et al.* The dopaminergic hyper-responsiveness of the shell of the nucleus accumbens is hormone-dependent. *European Journal of Neuroscience* (2000).

216. Dong, Z. *et al.* Morphine conditioned place preference depends on glucocorticoid receptors in both hippocampus and nucleus accumbens. *Hippocampus* **16**, 809–813 (2006).

217. Nestler, E. J. *et al.* Neurobiology of depression. *Neuron* **34**, 13–25 (2002).

218. Pollak, D. D., Rey, C. E. & Monje, F. J. Rodent models in depression research: Classical strategies and new directions. *Ann Med* **42**, 252–264 (2010).

219. Dalla, C., Pitychoutis, P. M., Kokras, N. & Papadopoulou-Daifoti, Z. Sex differences in animal models of depression and antidepressant response. *Basic Clin. Pharmacol. Toxicol.* **106**, 226–233 (2010).

220. Yan, H.-C., Cao, X., Das, M., Zhu, X.-H. & Gao, T.-M. Behavioral animal models of depression. *Neurosci. Bull.* **26**, 327–337 (2010).

221. Willner, P., Towell, A., Sampson, D., Sophokleous, S. & Muscat, R. Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. *Psychopharmacology* **93**, 358–364 (1987).

222. Willner, P. The mesolimbic dopamine system as a target for rapid antidepressant action. *Int Clin Psychopharmacol* **12 Suppl 3**, S7–14 (1997).

223. Gillespie, N. A., Whitfield, J. B., Williams, B., Heath, A. C. & Martin, N. G. The relationship between stressful life events, the serotonin transporter (5-HTTLPR) genotype and major depression. *Psychological Medicine* **35**, 101–111 (2005).

224. Krishnan, V. & Nestler, E. J. Linking Molecules to Mood: New Insight Into the Biology of Depression. *American Journal of Psychiatry* **167**, 1305–1320 (2010).

225. DSM-IV, P. A. T. F. O. *Diagnostic and statistical manual of mental disorders: DSM-IV-TR.* (2000).

226. Belmaker, R. H. & Agam, G. Major depressive disorder. *N. Engl. J. Med.* **358**, 55–68 (2008).

227. Drevets, W. C., Price, J. L. & Furey, M. L. Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression. *Brain Struct Funct* **213**, 93–118 (2008).

228. Sanders, A. & Detera-Wadleigh, S. *Molecular genetics of mood disorders.* (... of Mental Illness. New York: Oxford: 1999).

229. Fava, M. & Kendler, K. S. Major depressive disorder. *Neuron* **28**, 335–341 (2000).

230. Schildkraut, J. J. The catecholamine hypothesis of affective disorders: a review of supporting evidence. *Am J Psychiatry* **122**, 509–522 (1965).

231. Lee, S., Jeong, J., Kwak, Y. & Park, S. K. Depression research: where are we now? *Mol Brain* **3**, 8 (2010).

232. Manji, H. K., Drevets, W. C. & Charney, D. S. The cellular neurobiology of depression. *Nat. Med.* **7**, 541–547 (2001).

233. Morilak, D. A. & Frazer, A. Antidepressants and brain monoaminergic systems: a dimensional approach to understanding their behavioural effects in depression and anxiety disorders. *Int. J. Neuropsychopharmacol.* **7**, 193–218 (2004).

234. Berton, O. & Nestler, E. J. New approaches to antidepressant drug discovery: beyond monoamines. *Nature Reviews Neuroscience* **7**, 137–151 (2006).

235. Lambert, G., Johansson, M., Agren, H. & Friberg, P. Reduced brain norepinephrine and dopamine release in treatment-refractory depressive illness: evidence in support of the catecholamine hypothesis of mood disorders. *Arch. Gen. Psychiatry* **57**, 787–793 (2000).

236. Swaab, D. F., Bao, A.-M. & Lucassen, P. J. The stress system in the human brain in depression and neurodegeneration. *Ageing Res. Rev.* **4**, 141–194 (2005).

237. Nelson, J. C. A review of the efficacy of serotonergic and noradrenergic reuptake inhibitors for treatment of major depression. *BPS* **46**, 1301–1308 (1999).

238. Blendy, J. A. The Role of CREB in Depression and Antidepressant Treatment. *Biological Psychiatry* **59**, 1144–1150 (2006).

239. Hyman, S. E. & Nestler, E. J. Initiation and adaptation: a paradigm for understanding psychotropic drug action. *Am J Psychiatry* **153**, 151–162 (1996).

240. Krishnan, V. & Nestler, E. J. The molecular neurobiology of depression. *Nature* **455**, 894–902 (2008).

241. Pittenger, C. & Duman, R. S. Stress, Depression, and Neuroplasticity: A Convergence of Mechanisms. *Neuropsychopharmacology* **33**, 88–109 (2007).

242. Mill, J. & Petronis, A. Molecular studies of major depressive disorder: the epigenetic perspective. *Molecular Psychiatry* **12**, 799–814 (2007).

243. Sachar, E. J. & Baron, M. The biology of affective disorders. *Annual Review of Neuroscience* **2**, 505–517 (1979).

244. Arborelius, L., Owens, M. J., Plotsky, P. M. & Nemeroff, C. B. The role of corticotropin-releasing factor in depression and anxiety disorders. *J. Endocrinol.* **160**, 1–12 (1999).

245. Holsboer, F. Stress, hypercortisolism and corticosteroid receptors in depression: implications for therapy. *Journal of Affective Disorders* **62**, 77–91 (2001).

246. Kessler, R. C. The effects of stressful life events on depression. *Annu. Rev. Psychol.* **48**, 191–214 (1997).

247. Altemus, M. Sex differences in depression and anxiety disorders: Potential biological determinants. *Hormones and Behavior* **50**, 534–538 (2006).

248. Paykel, E. S. Stress and affective disorders in humans. *Semin Clin Neuropsychiatry* **6**, 4–11 (2001).

249. Holsboer, F. The corticosteroid receptor hypothesis of depression. *Neuropsychopharmacology* **23**, 477–501 (2000).

250. Burke, H. M., Davis, M. C., Otte, C. & Mohr, D. C. Depression and cortisol responses to psychological stress: a meta-analysis. *Psychoneuroendocrinology* **30**, 846–856 (2005).

251. Parker, K. J., Schatzberg, A. F. & Lyons, D. M. Neuroendocrine aspects of hypercortisolism in major depression. *Hormones and Behavior* **43**, 60–66 (2003).

252. McEwen, B. S. Stress and hippocampal plasticity. *Annual Review of Neuroscience* **22**, 105–122 (1999).

253. Sapolsky, R. M. Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. *Arch. Gen. Psychiatry* **57**, 925–935 (2000).

254. Pariante, C. M. & Lightman, S. L. The HPA axis in major depression: classical theories and new developments. *Trends in Neurosciences* **31**, 464–468 (2008).

255. Duman, R. S., Heninger, G. R. & Nestler, E. J. A molecular and cellular theory of depression. *Arch. Gen. Psychiatry* **54**, 597–606 (1997).

256. Barrot, M. *et al.* CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 11435–11440 (2002).

257. Dowlatshahi, D., MacQueen, G. M., Wang, J. F. & Young, L. T. Increased temporal cortex CREB concentrations and antidepressant treatment in major depression. *Lancet* **352**, 1754–1755 (1998).

258. Trentani, A., Kuipers, S. D., Horst, Ter, G. J. & Boer, Den, J. A. Selective chronic stress-induced in vivo ERK1/2 hyperphosphorylation in medial prefrontocortical dendrites: implications for stress-related cortical pathology? *European Journal of Neuroscience* **15**, 1681–1691 (2002).

259. Lin, Y. *et al.* Effects of Long-Term Stress and Recovery on the Prefrontal Cortex and Dentate Gyrus in Male and Female Rats. *Cereb. Cortex* **18**, 2762–2774 (2008).

260. Nibuya, M., Nestler, E. J. & Duman, R. S. Chronic antidepressant administration increases the expression of cAMP response element binding protein (CREB) in rat hippocampus. *J. Neurosci.* **16**, 2365–2372 (1996).

261. Thome, J. *et al.* cAMP response element-mediated gene transcription is upregulated by chronic antidepressant treatment. *Journal of Neuroscience* **20**, 4030–4036 (2000).

262. Pliakas, A. M. *et al.* Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. *Journal of Neuroscience* **21**, 7397–7403 (2001).

263. Carlezon, W., Jr, Duman, R. & Nestler, E. The many faces of CREB. *Trends in Neurosciences* **28**, 436–445 (2005).

264. Gonzalez, G. A. *et al.* A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. *nature* **337**, 749–752 (1989).

265. D'Sa, C. & Duman, R. S. Antidepressants and neuroplasticity. *Bipolar Disord* **4**, 183–194 (2002).

266. Shaywitz, A. J. & Greenberg, M. E. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu. Rev. Biochem.* **68**, 821–861 (1999).

267. Mayr, B. & Montminy, M. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat. Rev. Mol. Cell Biol.* **2**, 599–609 (2001).

268. Conti, A. C., Cryan, J. F., Dalvi, A., Lucki, I. & Blendy, J. A. cAMP response element-binding protein is essential for the upregulation of brain-derived neurotrophic factor transcription, but not the behavioral or endocrine responses to antidepressant drugs. *Journal of Neuroscience* **22**, 3262–3268 (2002).

269. Finkbeiner, S. CREB couples neurotrophin signals to survival messages. *Neuron* **25**, 11–14 (2000).

270. Tao, X., Finkbeiner, S., Arnold, D. B., Shaywitz, A. J. & Greenberg, M. E. Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. *Neuron* **20**, 709–726 (1998).

271. Smith, M. A., Makino, S., Kvetnansky, R. & Post, R. M. Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J. Neurosci.* **15**, 1768–1777 (1995).

272. Grønli, J. *et al.* Chronic mild stress inhibits BDNF protein expression and CREB activation in the dentate gyrus but not in the hippocampus proper. *Pharmacology, Biochemistry and Behavior* **85**, 842–849 (2006).

273. Alfonso, J. *et al.* Regulation of hippocampal gene expression is conserved in two species subjected to different stressors and antidepressant treatments. *BPS* **59**, 244–251 (2006).

274. Duman, R. S. & Monteggia, L. M. A Neurotrophic Model for Stress-Related Mood Disorders. *Biological Psychiatry* **59**, 1116–1127 (2006).

275. Nibuya, M., Morinobu, S. & Duman, R. S. Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. *J. Neurosci.* **15**, 7539–7547 (1995).

276. Dias, B. G., Banerjee, S. B., Duman, R. S. & Vaidya, V. A. Differential regulation of brain derived neurotrophic factor transcripts by antidepressant treatments in the adult rat brain. *Neuropharmacology* **45**, 553–563 (2003).

277. Shirayama, Y., Chen, A. C.-H., Nakagawa, S., Russell, D. S. & Duman, R. S. Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. *Journal of Neuroscience* **22**, 3251–3261 (2002).

278. Siuciak, J. A., Boylan, C., Fritsche, M., Altar, C. A. & Lindsay, R. M. BDNF increases monoaminergic activity in rat brain following intracerebroventricular or intraparenchymal administration. *Brain Research* **710**, 11–20 (1996).

279. Eisch, A. J. *et al.* Brain-derived neurotrophic factor in the ventral midbrain-nucleus accumbens pathway: a role in depression. *BPS* **54**, 994–1005 (2003).

280. Kessler, R. C. *et al.* Sex and depression in the National Comorbidity Survey. II: Cohort effects. *Journal of Affective Disorders* **30**, 15–26 (1994).

281. Baler, R. D. & Volkow, N. D. Drug addiction: the neurobiology of disrupted self-control. *Trends in Molecular Medicine* **12**, 559–566 (2006).

282. Horst, Ter, G. J., Wichmann, R., Gerrits, M., Westenbroek, C. & Lin, Y. Sex differences in stress responses: Focus on ovarian hormones. *Physiology & Behavior* **97**, 239–249 (2009).

283. Parker, G. & Brotchie, H. Gender differences in depression. *Int Rev Psychiatry* **22**, 429–436 (2010).

284. Sloan, D. M. E. & Kornstein, S. G. Gender differences in depression and response to antidepressant treatment. *Psychiatr. Clin. North Am.* **26**, 581–594 (2003).

285. Gold, P. W. & Chrousos, G. P. Organization of the stress system and its dysregulation in melancholic and atypical depression: high vs low CRH/NE states. *Molecular Psychiatry* **7**, 254–275 (2002).

286. Kajantie, E. & Phillips, D. I. W. The effects of sex and hormonal status on the physiological response to acute psychosocial stress. *Psychoneuroendocrinology* **31**, 151–178 (2006).

287. Whittle, S., Yücel, M., Yap, M. B. H. & Allen, N. B. Sex differences in the neural correlates of emotion: Evidence from neuroimaging. *Biological Psychology* **87**, 319–333 (2011).

288. Dalla, C. *et al.* Chronic mild stress impact: Are females more vulnerable? *Neuroscience* **135**, 703–714 (2005).

289. Taylor, S. E. *et al.* Biobehavioral responses to stress in females: Tend-and-befriend, not fight-or-flight. *Psychological Review* **107**, 411–429 (2000).

290. Breslau, N., Davis, G. C., Andreski, P., Peterson, E. L. & Schultz, L. R. Sex differences in posttraumatic stress disorder. *Arch. Gen. Psychiatry* **54**, 1044–1048 (1997).

291. Nolen-Hoeksema, S. Gender Differences in Depression. *Current Directions in Psychol Sci* **10**, 173–176 (2001).

292. Drossopoulou, G. Sex differences in behavioral, neurochemical and neuroendocrine effects induced by the forced swim test in rats. *Neuroscience* **126**, 849–857 (2004).

293. Palanza, P. Animal models of anxiety and depression: how are females different? *Neuroscience and Biobehavioral Reviews* **25**, 219–233 (2001).

294. Weinstock, M., Razin, M., Schorer-Apelbaum, D., Men, D. & McCarty, R. Gender differences in sympathoadrenal activity in rats at rest and in response to footshock stress. *Int. J. Dev. Neurosci.* **16**, 289–295 (1998).

295. Tinnikov, A. A. Responses of serum corticosterone and corticosteroid-binding globulin to acute and prolonged stress in the rat. *Endocrine* **11**, 145–150 (1999).

296. Rivier, C. Gender, sex steroids, corticotropin-releasing factor, nitric oxide, and the HPA response to stress. *Pharmacology, Biochemistry and Behavior* **64**, 739–751 (1999).

297. McCormick, C. M., Linkroum, W., Sallinen, B. J. & Miller, N. W. Peripheral and central sex steroids have differential effects on the HPA axis of male and female rats. *Stress* **5**, 235–247 (2002).

298. Trentani, A. *et al.* Immunohistochemical changes induced by repeated footshock stress: revelations of gender-based differences. *Neurobiology of Disease* **14**, 602–618 (2003).

299. Westenbroek, C. *et al.* Gender-specific effects of social housing in rats after chronic mild stress exposure. *Progress in Neuro-Psychopharmacology & Biological Psychiatry* **27**, 21–30 (2003).

300. Westenbroek, C., Boer, Den, J. A. & Horst, Ter, G. J. Gender-specific effects of social housing on chronic stress-induced limbic FOS expression. *Neuroscience* **121**, 189–199 (2003).

301. Westenbroek, C. *et al.* Pair-housing of male and female rats during chronic stress exposure results in gender-specific behavioral responses. *Hormones and Behavior* **47**, 620–628 (2005).

302. Kuipers, S. D. *et al.* Unique patterns of FOS, phospho-CREB and BrdU immunoreactivity in the female rat brain following chronic stress and citalopram treatment. *Neuropharmacology* **50**, 428–440 (2006).

303. Micheau, J., Destrade, C. & Soumireu-Mourat, B. Intraventricular corticosterone injection facilitates memory of an appetitive discriminative task in mice. *Behavioral and Neural Biology* **31**, 100–104 (1981).

304. Zorawski, M. & Killcross, S. Posttraining glucocorticoid receptor agonist enhances memory in appetitive and aversive Pavlovian discrete-cue conditioning paradigms. *Neurobiology of Learning and Memory* **78**, 458–464 (2002).

2

RODENT STEREOTAXIC SURGERY AND ANIMAL WELFARE OUTCOME IMPROVEMENTS FOR BEHAVIORAL NEUROSCIENCE

Raquel V. Fornari[1]
Romy Wichmann[1]
Piray Atsak[1]
Erika Atucha[1]
Areg Barsegyan[1]
Hassiba Beldjoud[1]
Fany Messanvi[1]
Catrien M.A. Thuring[2]
Benno Roozendaal[1]

[1] Department of Neuroscience, Section Anatomy, University Medical Center Groningen, University of Groningen
[2] Animal Welfare Office, University of Groningen

Journal of Visualized Experiments, January 2012, Issue 59, e3528

ABSTRACT

Stereotaxic surgery for the implantation of cannulae into specific brain regions has for many decades been a very successful experimental technique to investigate the effects of locally manipulated neurotransmitter and signaling pathways in awake, behaving animals. Moreover, the stereotaxic implantation of electrodes for electrophysiological stimulation and recording studies has been instrumental to our current understanding of neuroplasticity and brain networks in behaving animals. Ever-increasing knowledge about optimizing surgical techniques in rodents [1-4], public awareness concerning animal welfare issues and stringent legislation (e.g., the 2010 European Union Directive on the use of laboratory animals [5]) prompted us to refine these surgical procedures, particularly with respect to implementing new procedures for oxygen supplementation and the continuous monitoring of blood oxygenation and heart rate levels during the surgery as well as introducing a standardized protocol for post-surgical care. Our observations indicate that these modifications resulted in an increased survival rate and an improvement in the general condition of the animals after surgery (e.g. less weight loss and a more active animal). This video presentation will show the general procedures involved in this type of stereotaxic surgery with special attention to our several modifications. We will illustrate these surgical procedures in rats, but it is also possible to perform this type of surgery in mice or other small laboratory animals by using special adaptors for the stereotaxic apparatus [6].

PRE-SURGICAL PROCEDURES

Note: Antiseptic techniques should be employed during the whole procedure. All the instruments and materials (cotton-tipped swabs, gauze, etc.) that will be used during the surgery should be sterilized by autoclaving. A surgical mask, hair bonnet and sterile gloves should be worn. The working area and the stereotaxic apparatus should be cleaned thoroughly, and disinfected with a 70% ethanol solution.

1. Set up the stereotaxic apparatus and all materials needed. Pre-warm the heating pad.
2. Place the cannula in its support and check if it is straight.
3. Turn on the gas system - mixture of ambient air and oxygen (30-35% of total flow should be oxygen).
4. Weigh the rat and administer the anesthetic. We are using a mixture of ketamine (37.5 mg/kg) and dexmedetomidine (0.25 mg/kg) injected subcutaneously. For different anesthesia protocols, see Flecknell [4] and Hellebrekers et al. [7].
5. After the rat lost consciousness, shave the head area going from the ears to just in between the eyes with an electric razor.
6. Place the rat on the heating pad, with its nose in front of the air tubing. Use an oximeter to ensure that the rat has an adequate blood oxygenation level (should not drop <90%). Please follow the manufacturer's instructions for proper use of equipment.
7. Apply eye cream (Duratears Z, Alcon) on both corneas to avoid dehydration.
8. Check the rat's reflexes (tail reflex or toe-pinch reflex, as demonstrated in Walantus et al. 8) to ensure that it is adequately anesthetized. If the rat continues to show strong reflexes, supplementation of anesthesia might be needed.
9. If no toe-pinch reflex is shown, place the rat in the stereotaxic apparatus, adjust the ear bars so that it shows equal reading on both sides, and place again the air tubing in front of the animal by fixing it with the nose bar. Check again if the rat shows a blood oxygenation level of 90% or higher. If not, adjust either the tubing, bringing it closer to the nose, or increase the flow of oxygen. Monitor the blood oxygenation level and heart rate throughout the surgery.
10. Continuously monitor the rat's temperature with a rectal thermometer (preferentially connected to a heating pad) and record the values at the beginning and end of the surgery. Adjust the heating pad or use a blanket to maintain a body temperature of 37.5 to 38.5°C.

SURGERY

1. Inject the analgesic. We are using a single peri-operative administration of carprofen (4.0-5.0 mg/kg, subcutaneously). For different analgesic protocols, see Hellebrekers et al. [7].
2. Clean the shaved area of the skin from the center to the haired perimeter three times with a disinfectant (e.g., chlorhexidine 0.5%) and locally inject a mixture

of lidocaine (20 mg/ml) and adrenaline (5 mg/ml) for local anesthesia and vasoconstriction (to prevent excessive bleeding).

3. Make an anterior-posterior incision of about 2.5 cm on the midline of the scalp, going from between the eyes until the back of the ears. Use 4-6 bulldog clamps to pinch off the skin and to keep the incision open. Remove any conjunctive tissue with a spatula and/or cotton swabs and clean the area to expose the skull surface.
4. Check if the head is level: First, find Lambda and place the guide cannula exactly over this location, touching the skull. Record the dorso-ventral coordinate. Next, place the guide cannula exactly over Bregma, touching the skull, and record its dorso-ventral coordinate. These two coordinates should be identical. If the difference is >0.3 mm, adjust the nose bar to correct it.
5. Make two small holes for fixing the skull screws using a sterilized hand drill (one approximately 5 mm anterior to the cannula location in one of the hemispheres and the other 5 mm posterior to the cannula location in the contralateral hemisphere). Place two sterile screws into these holes until they are tightly anchored, without being inserted completely into the skull.
6. With the guide cannula placed exactly at Bregma, record the anterior-posterior and lateral coordinates. The correct location of guide cannula placement for each brain region can be calculated by adding or subtracting from Bregma, with the aid of a stereotaxic atlas [9-11].
7. Position the guide cannula in its correct location, slightly touching the skull. Record the dorso-ventral coordinate. For bilateral cannula placement, find next the cannula location in the other hemisphere, and again record the dorso-ventral coordinate. Both coordinates should be identical (or differ <0.3 mm).
8. Mark the cannula locations on the skull with a sterile pencil and, with the hand driller, make the burr holes, checking the size and the correct location with the aid of the guide cannula. Once the holes are made, use a sterile needle to gently punch the meninges to allow for unobstructed insertion of the cannula.
9. Place the cannula into the first hole and lower it carefully until it reaches the final ventral coordinate. Prepare the dental cement and generously apply it around the cannula and one or both screws in order to fix the cannula. Wait until the cement has dried completely. Afterwards, carefully remove the cannula support by turning the dorso-ventral bar upwards.
10. Place the second cannula into the support and go to the cannula location in the other hemisphere. Place the cannula into the hole and repeat the previous step. Cover the screws and a large surface of the cannulas with the cement, and before the cement is dry, remove any surplus from the skin.
11. Inject warm (~37°C) sterile saline (~10 ml/kg, s.c.) to ensure rehydration.
12. After the cement has completely dried, remove the cannula support and place a sterile pin into each cannula to prevent obstruction.
13. Clean the wound area with sterile saline and suture the front and the back of the wound.

14. Remove the animal from the stereotaxic apparatus, replacing the gas tubing in front of its nose. Continue to monitor the oxygen saturation level and body temperature.
15. If an injectable anesthetic with dexmedetomidine is used, inject its antagonist atipamezole (0.25 mg/kg, s.c.) and wait until the animal wakes up (approximately 5 minutes).
16. Place the rat into a recovery cage. To avoid hypothermia, place the cage in an incubator at 28°C or on a heating pad in a place where you can observe the animal for at least one hour, before returning it to the vivarium room.

POST-SURGICAL CARE

1. During the first 4 days after surgery, monitor the rat's recovery by keeping daily records of weight and other observations concerning the condition of the animal in laboratory logbooks or "animal welfare diaries".
2. Animals that show overt signs of sickness, infection of the wound, loss of body weight or other signs of reduced well-being must undergo special care: e.g. an extra dose of analgesics to minimize post-operative pain, a mixture of powder food and water in addition to standard chow to stimulate the rat's appetite, and/or a subcutaneous injection of saline to support rehydration.
3. If the rat does not show any improvement after these interventions, or the loss of body weight is >15% (compared to pre-surgery weight), sacrifice the animal with an overdose of anesthetic (humane end-point).
4. Rats usually need to recover for at least 7 days before commencement of behavioral experiments.

REPRESENTATIVE RESULTS

To determine whether the various modifications to our surgical procedure, particularly with respect to oxygen supplementation and the continuous monitoring of blood oxygenation levels, heart rate and body temperature, enhanced the animal's survival and improved its general condition after surgery, we compared the non-survival rate of 20 cohorts of animals (consisting of 20 rats each) that underwent surgery after we implemented these modifications with the non-survival rate of 24 cohorts (20 rats each) operated with the standard protocol. As is shown in Figure 1a, the non-survival rate was significantly reduced in the sample of cohorts that was operated with the modified protocol ($P < 0.05$; Mann-Whitney U test, two tailed). Moreover, as is shown in Figure 1b, post-surgical weight loss of rats operated with the modified protocol was also significantly reduced as compared to that of rats operated with the standard procedure (post-operative day 1: $P < 0.05$; post-operative day 2: $P < 0.01$; post-operative day 3: $P = 0.17$; Student t-tests).

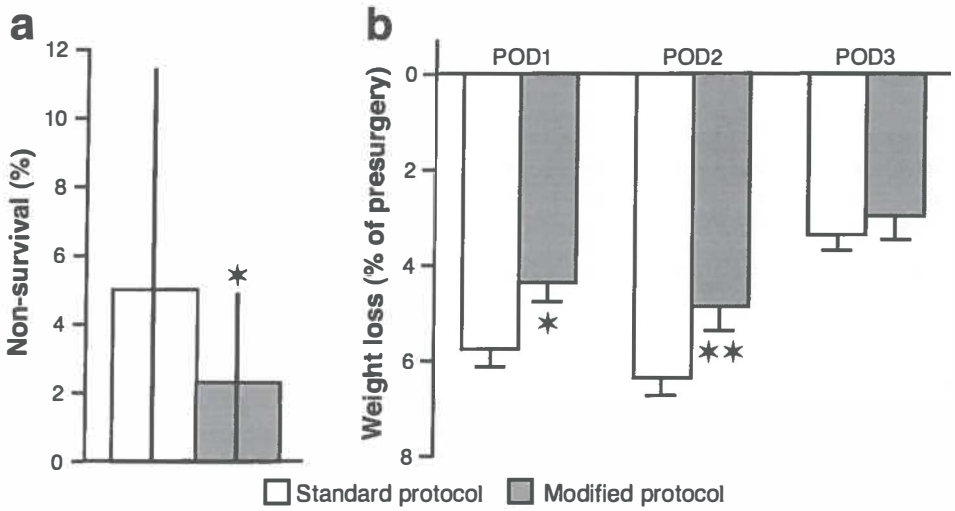


Figure 1. Effect of surgical modifications on non-survival rate and post-surgical weight loss. (A.) Non-survival rate of rats operated with the modified protocol as compared to that of rats operated with the standard protocol. The non-survival rate (median ± interquartile ranges) was calculated as the percentage of rats, per cohort of 20 rats, that did not survive surgery. *p < 0.05, Mann-Whitney U test two tailed (n = 20 cohorts for the modified protocol and 24 cohorts for the standard protocol). (B.) Weight loss (mean ± SEM as percentage of pre-surgery weight) during the first (POD1), second (POD2) and third (POD3) post-operative day. *P < 0.05, **P < 0.01, Student t-test (n = 60 per group).

DISCUSSION

The main purpose of this video presentation is to familiarize behavioral neuroscientists with the basic principles of stereotaxic surgery. Researchers who are already performing stereotaxic surgery might also benefit from this video and consider some of the procedural refinements for use in their own laboratory. An ever-increasing knowledge about optimizing surgical techniques [1,3], the development of new anesthetics and analgesics for use in human and veterinary medicine [4], public awareness concerning animal welfare issues and stringent legislation (e.g., the 2010 European Union Directive on the use of laboratory animals [5]) prompted us to implement new procedures for oxygen supplementation and the continuous monitoring of blood oxygenation and heart rate levels during the surgery. We observed, as shown for a representative sample of animals, an overall increased survival rate and a significantly reduced post-surgical weight loss. Such a reduced post-surgical weight loss might reflect a smaller burden of the surgical procedure on the animal and, consequently, result in a more active animal in the immediate aftermath of surgery. Whether it also has beneficial effects on its long-term health is not clear. However, a remarkable observation was that removing the oxygen supply temporarily led to a marked and reliable decrease in blood oxygen saturation levels, which could drop even below 50% (See the video presentation for a demonstration

hereof). It is conceivable that prolonged inadequate blood levels of oxygen, possibly as occurring in animals not provided with any oxygen supplementation during surgery, might result in hypoxia with long-term behavioral consequences and also negatively impact the outcome and/or quality of behavioral experimentation. We do not know whether such a depression of oxygen blood supply during surgery is specific to the anesthetic protocol used in our laboratory (i.e., a mixture of ketamine and dexmedetomidine) or whether it is a more general phenomenon associated with injectable anesthetics. The use of inhalation anesthesia, with a mixture of ambient air and oxygen, might be an alternative method to overcome the depression of blood oxygen levels.

REFERENCES

1. Russell, W. & Burch, R. *The Principles of Humane Experimental Technique.* (Methuen: London, 1959).
2. Richardson, C. A. & Flecknell, P. A. Anaesthesia and post-operative analgesia following experimental surgery in laboratory rodents: are we making progress? *Altern Lab Anim* **33**, 119–127 (2005).
3. Stokes, E. L., Flecknell, P. A. & Richardson, C. A. Reported analgesic and anaesthetic administration to rodents undergoing experimental surgical procedures. *Lab. Anim.* **43**, 149–154 (2009).
4. Flecknell, P. *Laboratory Animal Anaesthesia - A Practical Introduction for Research Workers and Technicians.* (Academic Press: 2009).
5. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Official Journal of the European Union* 1–47 (2010).
6. Geiger, B. M., Frank, L. E., Caldera-Siu, A. D. & Pothos, E. N. Survivable Stereotaxic Surgery in Rodents. *Journal of Visualized Experiments* (2008).doi:10.3791/880
7. Hellebrekers, L., Booij, L. & Flecknell, P. Anaesthesia, analgesia and euthanasia. *Principles of Laboratory Animal Science* (2001).
8. Walantus, W., Elias, L. & Kriegstein, A. In utero intraventricular injection and electroporation of E16 rat embryos. *Journal of Visualized Experiments* 236 (2007).doi:10.3791/236
9. Paxinos, G. & Watson, C. *The Rat Brain in Stereotaxic coordinates.* (Academic Press: 1986).
10. Paxinos, G. & Watson, C. *The Rat Brain in Stereotaxic Coordinates.* (Amsterdam: Elsevier: 2007).
11. Swanson, L. *Brain maps: the structure of the adult rat brain.* (Elsevier Science BV: 1992).

3

INVOLVEMENT OF THE INSULAR CORTEX IN REGULATING GLUCOCORTICOID EFFECTS ON MEMORY CONSOLIDATION OF INHIBITORY AVOIDANCE TRAINING

Raquel V. Fornari [1,2]

Romy Wichmann [1]

Erika Atucha [1]

Tifany Deprez [1]

Ellie Eggens Meijer [1]

Benno Roozendaal [1]

[1] Department of Neuroscience, Section Anatomy, University Medical Center Groningen, University of Groningen
[2] Núcleo de Cognição e Sistemas Complexos, Centro de Matemática, Computação e Cognição, Universidade Federal do ABC, Santo André, SP, Brasil

Frontiers in Behavioral Neuroscience, March 2012, Volume 6, Article 10

ABSTRACT

Glucocorticoids are known to enhance the consolidation of memory of emotionally arousing experiences by acting upon a network of interconnected brain regions. Although animal studies typically do not consider the insular cortex (IC) to be part of this network, the present findings indicate that the IC is importantly involved in regulating glucocorticoid effects on memory consolidation of emotionally arousing inhibitory avoidance training. The specific glucocorticoid receptor agonist RU 28362 (3 or 10 ng in 0.5 μl) infused bilaterally into the IC of male Sprague-Dawley rats immediately after one-trial inhibitory avoidance training dose-dependently enhanced 48-h retention performance. Moreover, training on the inhibitory avoidance task increased neuronal activity of the IC, as assessed by an increased number of cells expressing immunoreactivity for phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2). However, systemic administration of a memory-enhancing dose of corticosterone (1 mg/kg) after inhibitory avoidance training rapidly reduced the number of pERK1/2-positive cells in the IC, suggesting that glucocorticoid administration reduces overall neuronal activity of the IC. To investigate which components of the inhibitory avoidance training experience were influenced by the intra-IC glucocorticoid administration, in the last experiment rats were trained on a modified inhibitory avoidance task in which context exposure and footshock training occur on two sequential days. RU 28362 administration into the IC enhanced later retention when infused immediately after either the context or footshock training. Thus, these findings indicate that the IC mediates glucocorticoid effects on the consolidation of memory of different components of inhibitory avoidance training and suggest that the IC might be an important element of the rodent brain network involved in emotional regulation of learning and memory.

INTRODUCTION

Glucocorticoid hormones – stress hormones released from the adrenal cortex – are known to strengthen the consolidation of memory of emotionally arousing experiences [1-7].Most animal studies investigating glucocorticoid-induced enhancement of memory consolidation examined their effects on a network of interacting brain regions involved in emotional regulation of memory, including the basolateral amygdala (BLA), prefrontal cortex and hippocampus [6,8-13]. Although human neuroimaging studies generally support an involvement of these brain regions, as well as their functional interaction, in emotionally influenced learning and memory [14-17], they also consistently point to a key role for the insular cortex (IC) [17-21]. Extensive evidence indicates that the IC, which receives autonomic, visceral and somatosensory inputs [22,23], might be part of a 'salience network' involved in the detection of novel and salient information [19,24,25] that is collectively upregulated in response to an acute stressful event and after highly stressful experiences [26-28]. Accordingly, increased anterior insula activity has been reported during the subjective awareness of both positive and negative emotions [18,19] as well as during the encoding and recall of a broad spectrum of emotionally salient learning tasks.

Contrasting the human literature, the IC never received a prominent position in animal research investigating stress hormone or emotional arousal effects on learning and memory. Although Bermudez-Rattoni and colleagues have reported several findings supporting the view that the IC might be involved herein [33-39], most animal studies have been limited to investigating its involvement in the formation and maintenance of taste memory [40-44]. The present study investigated whether the IC is implicated in regulating glucocorticoid effects on the consolidation of memory of emotionally arousing inhibitory avoidance training. In the first experiment, we investigated whether the specific glucocorticoid receptor (GR) agonist RU 28362 administered into the IC immediately after one-trial inhibitory avoidance training enhances long-term retention of the training experience. Next, we examined whether a systemic injection of corticosterone, the major endogenous glucocorticoid in rodents, given immediately after inhibitory avoidance training recruits the IC and induces changes in cellular activity within this brain region. Cellular activity was assessed by determining phosphorylation levels of extracellular signal-regulated kinase 1/2 (pERK1/2), a signaling cascade implicated in neuronal activity and synaptic plasticity [45,46]. To gain a better understanding of the precise role of the IC in regulating glucocorticoid effects on inhibitory avoidance memory, in the last experiment we employed a modified inhibitory avoidance procedure [13,47,48] that allows investigating which components of the inhibitory avoidance experience (i.e., memory of the context or the footshock) were influenced by the glucocorticoid infusion.

MATERIALS AND METHODS

Subjects

Male adult Sprague-Dawley rats (280-320 g at time of surgery) from Charles River Breeding Laboratories (Kisslegg, Germany) were kept individually in a temperature-controlled (22°C) colony room and maintained on a standard 12-h light: 12-h dark cycle (07:00-19:00 h lights on) with *ad libitum* access to food and water. Training and testing were performed during the light phase of the cycle between 10:00 and 15:00 h. All procedures were in compliance with the European Community's Council Directive (86/609/EEC) and approved by the Institutional Animal Care and Use Committee of the University of Groningen, The Netherlands.

Surgery for cannula implantation

Animals, adapted to the vivarium for at least 1 week, were anesthetized with a subcutaneous injection of ketamine (37.5 mg/kg of body weight; Alfasan) and dexmedetomidine (0.25 mg/kg; Orion), and received the non-steroidal analgesic carprofen (4 mg/kg, s.c.; Pfizer). Oxygen (35%) mixed with ambient air was administered during surgery such that blood oxygenation levels would not drop below 90% (Fornari et al 2012)[49]. The skull was positioned in a stereotaxic frame (Kopf Instruments), and two stainless-steel guide cannulae (15 mm; 23 gauge; Small Parts, Inc) were implanted bilaterally with the cannula tips 2.0 mm above the anterior IC. The coordinates were based on the atlas of Paxinos and Watson (2007): anteroposterior: +1.0 mm from Bregma; mediolateral: ±5.5 mm from midline; dorsoventral: -4.8 mm from Bregma; incisor bar: −3.3 mm from interaural. The cannulae were affixed to the skull with two anchoring screws and dental cement. Stylets (15-mm long 00-insect dissection pins), inserted into each cannula to maintain patency, were removed only for the infusion of drugs. After surgery, the rats received a subcutaneous injection of 3 ml of saline to facilitate clearance of drugs and prevent dehydration, and were subsequently administered atipamezole hydrochloride (Antisedan, 0.25 mg/kg, s.c.; Orion) to reverse anesthesia. The rats were allowed to recover for a minimum of 7 days before initiation of training and were handled 3 times for 1 min each during this recovery period to accustom them to the infusion procedure.

Inhibitory avoidance apparatus and procedures

For all experiments, rats were trained and tested in an inhibitory avoidance apparatus, consisting of a trough-shaped alley (91 cm long, 15 cm deep, 20 cm wide at the top, and 6.4 cm wide at the bottom) divided into two compartments, separated by a sliding door that opened by retracting into the floor . The starting compartment (30 cm) was made of opaque white plastic and was well lit; the shock compartment (60 cm) was made of dark, electrifiable metal plates and was not illuminated. Training and testing were conducted in a sound- and light-attenuated room.

For one-trial inhibitory avoidance training, the rats were placed in the starting compartment of the apparatus, facing away from the door, and were allowed to enter the dark (shock) compartment. After the rat stepped completely into the dark compartment, the sliding door was closed and a single inescapable footshock (0.35 mA; 1 s) was delivered. The rats were removed from the shock compartment 15 s later and, after drug treatment, returned to their home cages. For the modified, two-phase inhibitory avoidance procedure [13,47], on the first day (context training), the rat was placed into the starting compartment, facing away from the door, and allowed to freely explore the inhibitory avoidance apparatus for 3 min. On day 2 (shock training), each rat was placed directly into the dark compartment, facing away from the starting compartment, with the retractable door closed. The rat then received an inescapable footshock (0.75 mA; 1 s) and immediately afterward was removed from the training apparatus. For both one-trial and two-phase inhibitory avoidance, retention was tested 48 h after training by placing the rat into the starting compartment of the inhibitory avoidance apparatus and measuring the latency to re-enter the former shock compartment with all four paws (maximum latency of 600 s). Longer latencies were interpreted as indicating better retention. Shock was not administered on the retention test trial.

Local drug infusion into the insular cortex

The specific GR agonist RU 28362 (11β,17β-dihydroxy-6,21-dimethyl-17α-pregna-4,6-trien-20yn-3-one, 3 or 10 ng; a generous gift of Aventis, Frankfurt, Germany) was first dissolved in 100% ethanol and subsequently diluted in 0.9% saline to reach a final ethanol concentration of 0.5%. Receptor binding studies have shown that this compound has selective and high affinity for GRs [52]. Bilateral infusions of RU 28362 or an equivalent volume of vehicle (0.5% ethanol in saline) into the IC were given immediately after one-trial inhibitory avoidance training or after either the context or footshock components of the modified, two-phase inhibitory avoidance task by using 30-gauge injection needles connected to a 10-μl Hamilton microsyringe with polyethylene (PE-20) tubing. The injection needle protruded 2.0 mm beyond the tip of the cannula and a 0.5-μl injection volume was infused over a period of 50 s by an automated syringe pump (Stoelting Co). The injection needles were retained within the cannulae for an additional 20 s after drug infusion to maximize diffusion and to prevent backflow of drug into the cannulae. The infusion volume was based on previous findings indicating that infusion of this volume into the IC [53,54], but not the cortex dorsal to the IC [38], modulates memory consolidation. To control for time- and site specificity, additional groups of rats received delayed infusions of RU 28362 or vehicle into the IC 3 h after the training trial or immediate posttraining infusions into the somatosensory cortex, located approximately 1 mm dorsal to the IC. The use of posttraining drug administration provides direct support for the view that the treatment affects memory consolidation processes and that retention performance is, thus, not confounded by

possible effects on attentional, motivational, or sensory-perceptual mechanisms at the time of training or test [55].

Systemic corticosterone treatment

Corticosterone (1 mg/kg, Sigma-Aldrich) or vehicle, in a volume of 2 ml/kg body weight, was given subcutaneously immediately after the training trial. Corticosterone was dissolved in 5% ethanol in saline. This dose of corticosterone is known to enhance memory consolidation of different types of training [53,56,57].

Cannula placement verification

Rats were deeply anesthetized with an overdose of sodium pentobarbital (~100 mg/kg, i.p.) and perfused transcardially with a 0.9% saline solution followed by 4% formaldehyde. Following decapitation, the brains were removed and immersed in fresh 4% formaldehyde. At least 24 h before sectioning, the brains were submerged in a 25% sucrose (wt/vol) solution in water for cryoprotection. Coronal sections of 50 µm were cut on a cryostat, mounted on gelatin-coated slides, and stained with cresyl violet. The sections were examined under a light microscope and determination of the location of injection needle tips in the IC was made according to the atlas plates of Paxinos & Watson (2007)[50], by an observer blind to drug treatment condition. Rats with injection needle placements outside the IC or with extensive tissue damage at the injection needle tips were excluded from analysis.

Immunohistochemistry

Thirty minutes after training and systemic corticosterone treatment, rats were perfused transcardially with ice-cold 0.01 M phosphate-buffered saline (PBS), pH 7.4, followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed, postfixed overnight at 4°C, and then transferred to a 25% sucrose solution in 0.1 M PB for 3-6 days at 4°C. Frozen coronal sections at the level of the anterior IC (0.2 - 2.5 mm anterior to Bregma) were cut at a thickness of 20 µm on a cryostat and collected in Tris-buffered saline (TBS) with 0.1% sodium azide and phosphatase inhibitors (20 mM sodium fluoride and 2 mM sodium orthovanadate).

Every eighth section was used for quantification. Free-floating sections were rinsed in 0.3% Triton X-100 in TBS with phosphatase inhibitors. To block non-specific binding, sections were incubated with TBS containing phosphatase inhibitors, 0.3 % Triton X-100 and 5% normal donkey serum (nds, Jackson ImmunoResearch) for 1 h. Subsequently, sections were incubated with a cocktail of primary antibodies for pERK1/2 (rabbit anti-phospho-p44/42 MAP kinase, 1:1000; Cell Signaling # 9101) and NeuN (mouse anti-NeuN, 1:100; Millipore Mab 377) or pERK1/2 and CaMK II (mouse anti-calcium/calmodulin-dependent protein kinase II, 1:400; Millipore #05-532) in TBS containing phosphatase inhibitors, 0.3 % Triton X-100 and 1% nds for 48 h at 4°C. Sections were then rinsed several times with TBS and incubated with a cocktail

of specific fluorochrome-conjugated antibodies (Alexa Fluor 488 donkey anti-rabbit 1:500 and Alexa Fluor 594 donkey anti-mouse 1:1000 or Alexa Fluor 633 goat anti-rabbit 1:500 and Alexa 488 donkey anti-mouse 1:500; Invitrogen) for 2 h in the dark. Sections were rinsed again in TBS, incubated with Hoechst dye (0.1 µg/ml, Invitrogen) for 30 min and mounted on gelatin-coated slides, using non-fading mounting medium for coverslipping and stored in the dark for further analysis.

Images of each section were acquired with TissueFaxs®, Zeiss AxioObserver Z1 Microscope System (Tissue-Gnostics GmbH, Vienna, Austria). The number of pERK1/2 immunopositive nuclei was quantified with ImageJ 1.43m software (Girish and Vijayalakshmi, 2004;). Cell counts were determined at two levels within the anterior IC (2.5-1.7 mm and 1.6-0.7 mm anterior to Bregma), according to the standard atlas plates of Paxinos & Watson (2007), and expressed as number of pERK1/2-positive nuclei per mm2. Quantitative analysis of cell counts was performed blind to treatment condition. Double labeling was examined with a Leica SP2 AOBS confocal microscope.

Statistics

Data are expressed as mean ± SEM. Inhibitory avoidance training and retention test latencies were analyzed with one-way ANOVAs. Further analysis used Fisher's *post-hoc* tests to determine the source of the detected significances. To determine whether learning had occurred, paired *t*-tests were used to compare the training and retention latencies. Quantitative measures of pERK1/2 immunoreactivity in the IC were analyzed with two-way-ANOVAs, using Training (training versus no training) and Corticosterone treatment (vehicle versus corticosterone) as independent factors. The analyses were followed by Fisher's *post-hoc* tests, when appropriate. Individual comparisons with home-cage control groups were performed with Student *t*-tests for independent samples. For all comparisons, a probability level of < 0.05 was accepted as statistical significance. The number of rats per group is indicated in the figure legends.

RESULTS

GR agonist administration into the insular cortex enhances memory consolidation of inhibitory avoidance training

This experiment examined whether the GR agonist RU 28362 infused into the IC enhances the consolidation of memory of inhibitory avoidance training. For that, bilateral infusions of RU 28362 (3 or 10 ng in 0.5 µl) or vehicle were administered into the IC immediately after one-trial inhibitory avoidance training and retention of the training was tested 48 h later. Control groups received delayed infusions of the GR agonist into the IC 3 h after the training trial to determine whether the GR agonist enhances retention by influencing time-dependent processes underlying memory consolidation.

Average step-through latencies for all groups during training, before footshock or drug treatment, were 9.9 ± 0.8 s (mean ± SEM). One-way ANOVA for training

latencies revealed no significant differences between groups [$F(2,31) = 0.17$, $p = 0.84$, data not shown]. Forty-eight hour retention latencies of rats administered vehicle into the IC immediately after training were significantly longer than their latencies during the training trial (paired t-test: $p < 0.05$), indicating that the rats retained memory of the inhibitory avoidance experience. As is shown in Fig. 1A, rats treated with the GR agonist immediately after training had significantly longer retention test latencies as compared with rats that received vehicle [$F(2,31) = 3.59$, $p < 0.05$]. Fisher's *post hoc* tests revealed that the lower dose of RU 28362 (3 ng) enhanced retention ($p < 0.05$, compared with vehicle), whereas retention latencies of animals given the higher dose (10 ng) approached, but failed to reach, significance ($p = 0.07$). As is shown in Fig. 1B, the GR agonist administered into the IC 3 h after the training did not significantly alter retention latencies [$F(2,16) = 0.26$, $p = 0.78$], indicating a time-limited involvement of the IC in mediating glucocorticoid effects on the consolidation of inhibitory avoidance memory.

Fig. 1C and 1D show cannula placement within the IC. All injection needle tips of rats included in the analysis were localized within the granular and dysgranular subdivisions of the IC. To control for site specificity, other groups of rats received immediate posttraining infusions of vehicle or the GR agonist (3 or 10 ng in 0.5 µl) into the somatosensory cortex, approximately 1 mm above the IC (see, Fig. 1C). One-way ANOVA for 48-h retention latencies revealed no significant GR agonist effect ($F(2,12) = 0.29$, $p = 0.75$, Fig. 1E), indicating that the memory-modulatory effects of RU 28362 are localized within the IC.

Systemic corticosterone administration immediately after one-trial inhibitory avoidance training reduces pERK1/2-immunoreactivity in the insular cortex

The findings described above indicate that direct pharmacological activation of GRs in the IC after inhibitory avoidance training enhances the consolidation of memory of this experience in a dose-, time- and site-specific manner. However, these findings do not indicate whether circulating glucocorticoids normally act upon the IC in regulating memory consolidation. Therefore, the next experiment examined whether a systemic injection of corticosterone after inhibitory avoidance training changes neuronal activity of the IC. Rats received a subcutaneous injection of corticosterone (1 mg/kg) or vehicle immediately after one-trial inhibitory avoidance training (or without training) and were sacrificed 30 min later. Brains were processed to investigate training- and drug-induced changes in the number of pERK1/2-positive cells at two locations within the anterior IC. ERK1/2, a member of the mitogen-activated protein (MAP) kinase family, is considered to be phosphorylated by elevated neuronal activity and synaptic plasticity. Home-cage control groups did not receive any training or drug treatment. Other rats received the same posttraining injection of corticosterone or vehicle and their retention was tested 48 h later

As is shown in Fig. 2A, posttraining injection of this dose of corticosterone significantly enhanced 48-h retention latencies ($p < 0.05$). Fig. 2B shows the pattern

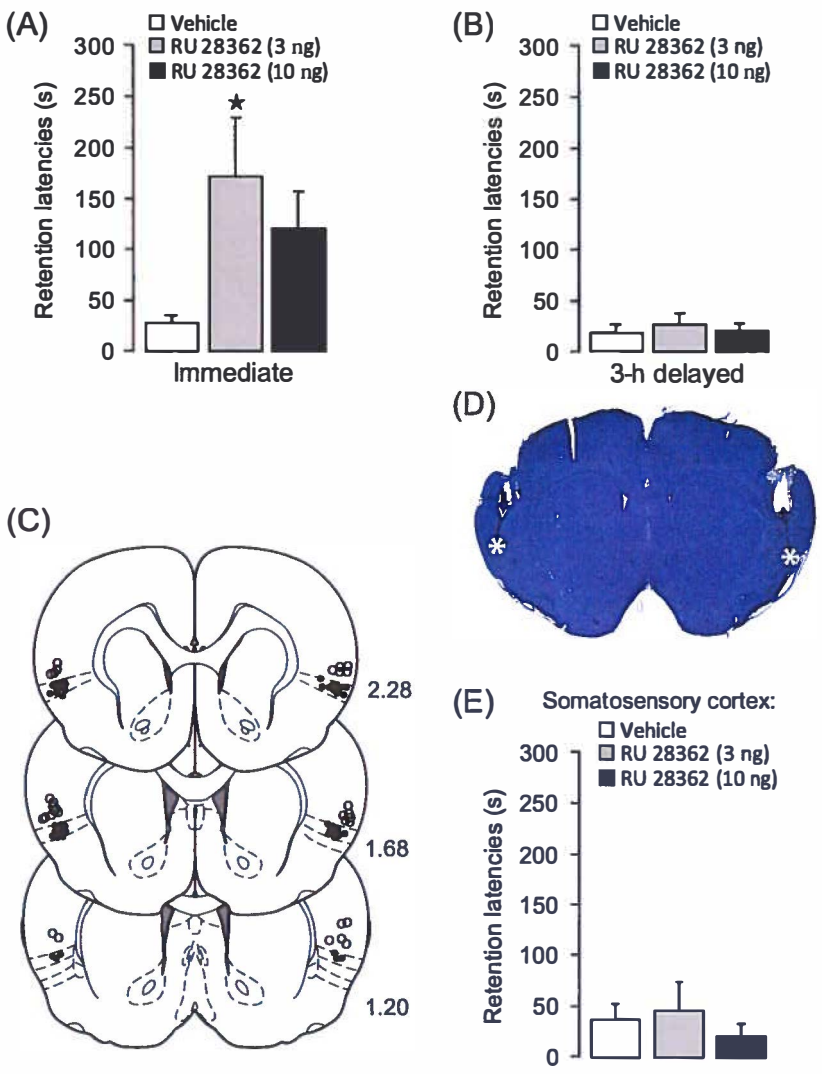


Figure 1. Glucocorticoid receptor agonist administration into the insular cortex enhances memory consolidation of inhibitory avoidance training. A, Step-through latencies (mean ± SEM) in seconds on the 48 h inhibitory avoidance retention test of rats given bilateral infusions of the glucocorticoid receptor agonist RU 28362 (3 or 10 ng in 0.5 µl) into the IC immediately after training. * $p < 0.05$ as compared with the vehicle-treated group (n = 11-12 per group). B, Step-through latencies (mean ± SEM) in seconds on the 48 h inhibitory avoidance retention test of rats given the glucocorticoid receptor agonist RU 28362 (3 or 10 ng in 0.5 µl) into the IC 3 h after training (n = 6-7 per group). C, Location of injection needle tips within the IC of all rats included in the immediate infusions groups (black circles) and 15 rats with infusion needle tips in the somatosensory cortex as a control for site specificity (open circles). Adapted from Paxinos & Watson (2007). D, Representative photomicrograph illustrating placement of cannulae and needle tips within the insular cortex. E, Step-through latencies (mean ± SEM) in seconds on the 48 h inhibitory avoidance retention test of rats given bilateral infusions of the glucocorticoid receptor agonist RU 28362 (3 or 10 ng in 0.5 µl) into the somatosensory cortex, approximately 1 mm above the IC, immediately after training (n = 4-6 per group).

of pERK1/2 expression in the IC. Immunoreactivity for pERK1/2 was found in somata as well as fibers (Fig. 2C). Qualitative double-labeling indicated that the vast majority of pERK1/2-positive cells in the IC also showed immunoreactivity for the neuronal marker NeuN (Fig. 2C) or CaMKII (Fig. 2D), a marker for glutamatergic pyramidal cells . These observations strongly suggest that pERK1/2 immunoreactivity within somata was mainly expressed in glutamate-rich pyramidal cells. Because most pERK1/2 immunoreactivity was concentrated in the superficial layers (II-III) of the agranular and dysgranular IC, cell counts were restricted to these regions, at two levels (2.5-1.7 mm and 1.6-0.7 mm anterior to Bregma), according to the standard atlas plates of Paxinos & Watson (2007). Fig. 2E shows the number of pERK1/2-positive cells at these two levels of the IC of rats treated with corticosterone or vehicle immediately after inhibitory avoidance training or of rats that were not trained. Across all groups, the number of pERK1/2-positive cells at the more rostral level appeared to be higher than that at the more caudal level. At the rostral level, two-way ANOVA indicated a significant training effect [$F(1,14) = 11.63, p < 0.01$] but no corticosterone effect [$F(1,14) = 1.95, p = 0.18$] or interaction effect between these two parameters [$F(1,14) = 0.53, p = 0.48$]. Fisher's *post-hoc* analyses indicated that rats that were trained on the inhibitory avoidance task and subsequently treated with vehicle had more pERK1/2-positive cells than non-trained vehicle-treated rats ($p < 0.05$). Corticosterone administration after inhibitory avoidance training did not significantly alter the number of pERK1/2-positive cells as compared to vehicle-treated rats. When compared to home cage controls, student *t*-test analyses indicated that rats treated with either vehicle ($p < 0.05$) or corticosterone ($p < 0.05$) immediately after training had more pERK1/2-positive cells, whereas the number of pERK1/2-positive cells of non-trained rats administered vehicle or corticosterone did not differ from those of home cage controls.

On the other hand, two-way ANOVA for the number of pERK1/2-positive cells at the more caudal level revealed significant effects of training [$F(1,14) = 21.66, p < 0.01$], corticosterone treatment [$F(1,14) = 10.55, p < 0.01$] and the interaction between them [$F(1,14) = 6.64, p < 0.05$, Fig. 2E]. Fisher's *post-hoc* analyses indicated that trained rats that were subsequently injected with vehicle had significantly more pERK1/2-positive cells than rats that were administered corticosterone after training ($p < 0.01$) or non-trained rats treated with either vehicle or corticosterone ($p < 0.01$). When compared to home cage controls, the group that received vehicle immediately after training was the only one that showed increased cell counts ($p < 0.0001$). These findings indicate that training on the inhibitory avoidance task increases the number of pERK1/2-positive cells within this area of the IC, and that posttraining administration of a memory-enhancing dose of corticosterone resulted in a significant decrease in the number of pERK1/2-positive cells (as compared to vehicle-treated trained rats), which did not differ significantly from home cage controls or non-trained rats.

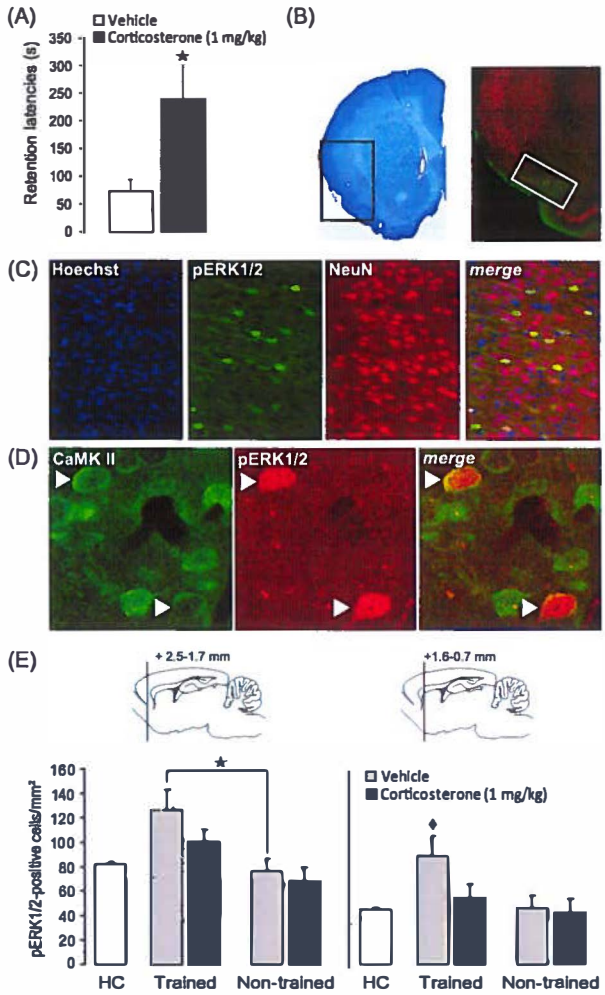


Figure 2. Systemic injection of a memory-enhancing dose of corticosterone after one-trial inhibitory avoidance training reduces pERK1/2-immunoreactivity in the insular cortex. A, Step-through latencies (mean ± SEM) in seconds on a 48 h inhibitory avoidance retention test of rats given a subcutaneous injection of corticosterone (1 mg/kg) or vehicle immediately after training. * $p < 0.05$ (n = 8-9 per group). B, Example of a Nissl-stained coronal section depicting the region that was acquired for the analyses of immunostaining and the distribution pattern of pERK1/2 expression in the insular cortex. The white square indicates the region analyzed for cell counts. C, Triple localization of Hoechst (blue), pERK1/2 (green) and the neuronal marker NeuN (red) in the insular cortex. Merging the three channels demonstrates that all pERK1/2-positive cells exhibit immunoreactivity for NeuN. D, Dual localization of pERK1/2 (red) and CamKII (green) immunoreactivity in the insular cortex. Merging of the red and green channels demonstrates that pERK1/2-positive cells also showed immunoreactivity for CamKII. Arrows point to cells with combined pERK1/2 and CamKII immunoreactivity. E, Number of pERK1/2-positive cells (mean ± SEM) at two levels within the insular cortex as assessed 30 min after inhibitory avoidance training and systemic injection of corticosterone (n = 4) or vehicle (n = 4). Non-trained rats received an injection of corticosterone (n = 5) or vehicle (n = 5) but were not trained on the inhibitory avoidance task. Home cage (HC) control rats (n = 6) did not receive any training or systemic injection. * $p < 0.05$ as compared with non-trained vehicle-treated rats. ♦ $p < 0.01$ as compared with all other groups.

GR agonist infusions into the insular cortex enhance memory for both the context and footshock components of inhibitory avoidance training

The findings described above indicate that the IC is an important target structure for glucocorticoids in regulating memory consolidation of inhibitory avoidance training. However, with one-trial inhibitory avoidance it is not possible to investigate the relative involvement of a brain region in memory consolidation of the contextual information independently from that of the footshock. To address this issue, a modified two-phase inhibitory avoidance trainingprocedure had been developed in which context training and footshock training occur on two sequential days . In the next experiment, we used this modified inhibitory avoidance training procedure to investigate whether GR agonist administration into the IC enhances memory consolidation of contextual information, the footshock experience, or of both components of training.

Fig. 3 shows 48-h retention latencies of rats administered the GR agonist (3 or 10 ng in 0.5 µl) into the IC after either the context or footshock component of inhibitory avoidance training. One-way ANOVAs for retention latencies revealed significant group effects of rats given intra-IC infusions of RU 28362 immediately after either context [$F(2,37) = 3.50$, $p < 0.05$] or footshock training [$F(2,37) = 4.04$, $p < 0.05$]. Retention latencies of rats given intra-IC infusions of the 3 and 10 ng doses of RU 28362 immediately after context training (both, $p < 0.05$), or infusions of the 3 ng dose of RU 28362 after footshock training ($p < 0.01$) were significantly longer than those of their respective vehicle controls. Importantly, as is shown in Table 1, both

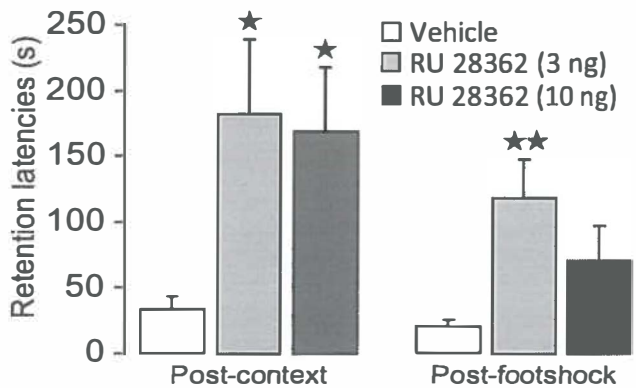


Figure 3. Glucocorticoid receptor agonist infusions into the insular cortex enhance memory consolidation of both the context and footshock components of inhibitory avoidance training. Step-through latencies (mean ± SEM) in seconds on the 48 h inhibitory avoidance retention test of rats given bilateral infusions of the glucocorticoid receptor agonist RU 28362 (3 or 10 ng in 0.5 µl) into the insular cortex immediately after either context or shock training on the two-phase modified inhibitory avoidance task (n = 12-14 per group). * $p < 0.05$, ** $p < 0.01$, as compared with the corresponding vehicle-treated group.

Table 1. Effect of RU 28362 after context or footshock exposure alone.

	Vehicle	RU 28362 (3 ng)	RU 28362 (10 ng)	p
Context exposure alone	4.2 ± 0.9 (5)	3.1 ± 0.3 (7)	3.2 ± 0.9 (6)	0.51
Footshock exposure alone	8.7 ± 4.2 (7)	19.0 ± 5.2 (6)	13.0 ± 8.3 (5)	0.44

Data are presented as mean ± SEM. Number of animals per group is shown in parentheses.

doses of RU 28362 administered into the IC after either context or shock training to animals that did not receive the other component of training did not enhance 48-h retention latencies [context: $F(2,15) = 0.70$, $p = 0.51$; footshock: $F(2,15) = 0.88$, $p = 0.44$], indicating that the expression of the enhanced memory depends on the learning about both context and footshock.

DISCUSSION

The aim of this study was to investigate whether the IC is involved in regulating glucocorticoid effects on memory consolidation of inhibitory avoidance training. Our findings indicate that the specific GR agonist RU 28362 infused bilaterally into the IC after inhibitory avoidance training induced dose- and time-dependent enhancement of the consolidation of memory of this training experience. Moreover, training on the inhibitory avoidance task increased neuronal activity of the IC, as assessed with immunoreactivity for pERK1/2. Unexpectedly, systemic injection of a memory-enhancing dose of corticosterone after inhibitory avoidance training rapidly reduced pERK1/2 expression in the IC. Lastly, we show that posttraining activation of GRs in the IC enhanced the consolidation of memory of both the footshock and contextual components of inhibitory avoidance training. These findings provide evidence that the IC is an important target structure for glucocorticoids in regulating the consolidation of different components of inhibitory avoidance memory.

It is well established that glucocorticoid hormones, via GR activation, enhance memory consolidation of emotionally arousing training experiences . However, most studies investigating glucocorticoid effects on the formation and stabilization of memory, and its molecular underpinnings, examined glucocorticoid actions on a network of interconnected brain regions such as the BLA and hippocampus . Surprisingly little is known concerning glucocorticoid effects on the IC or other cortical regions in regulating learning and memory. Our finding that RU 28362 infused into the IC immediately after inhibitory avoidance training enhanced later retention is in line with other recent evidence indicating that corticosterone or GR agonist administration into the IC also enhances the consolidation of memory of conditioned taste aversion and object recognition training [54]. More generally, findings from human and, to a lesser extent, animal studies indicate that the IC is involved in memory formation of emotionally arousing experiences. Human neuroimaging studies reported activation of

the IC during the encoding of aversive conditioning paradigms [14,16,29,32]. In rats, lesions of the IC, made before training, disrupted memory of emotionally arousing water-maze spatial [34, 35] and inhibitory avoidance training . Although such neuroimaging studies in humans and permanent lesion studies in animals obviously cannot determine whether the memory effects were attributable to specific influences on memory consolidation, our findings using immediate posttraining or delayed drug delivery provide compelling evidence for the view that the IC is involved in time-dependent processes underlying the consolidation of memory.

To investigate whether inhibitory avoidance training normally recruits the IC, we examined the pattern of pERK1/2 expression in this cortical region 30 min after inhibitory avoidance training. ERK is a rapidly activated protein that has been implicated in neuronal activity as well as neuroplasticity and memory consolidation [45,62,63]. Exposure to the inhibitory avoidance training procedure increased the number of pERK1/2-positive cells within the IC of rats treated with vehicle. Qualitative immuno-staining indicated that most pERK1/2-positive cells were glutamate-rich pyramidal cells. These findings are consistent with the evidence that ERK1/2 is activated in neurons in response to excitatory glutamatergic activity [64,65], and suggest that the elevated pERK1/2 immunoreactivity reflects an increased neuronal activity within this region [66]. Such findings are in agreement with the above-mentioned human neuroimaging studies indicating enhanced IC activity during affective processing and the encoding of emotionally arousing information [14,16,27,29-32]. The pattern of pERK1/2 expression in the present study was similar to that of Kobayashi et al. (2010)[67] reporting that novel sucrose stimulation increased the number of pERK1/2-positive cells in the IC, when compared to sucrose-experienced rats. As in our study, they found that pyramidal neurons, but not GABAergic interneurons, in the upper layers of the IC were most susceptible to ERK1/2 phosphorylation. However, the difference between naïve and sucrose-experienced rats was only found in the gustatory region -dysgranular and granular subdivisions- of the IC [67], whereas in the present study most pERK1/2 immunoreactivity was concentrated in the agranular and dysgranular regions, and not in the gustatory region. Other studies also indicated increased pERK1/2 levels in the IC in response to an unfamiliar taste stimulus [68,69], and that such activation is necessary for taste learning [68]. These findings suggest that ERK1/2 activation in the IC may contribute to the detection of novelty and/or memory formation [41,67,68].

A more puzzling observation is that a memory-enhancing dose of corticosterone administered immediately after inhibitory avoidance training resulted in a rapid decrease in the number of pERK1/2-positive cells within the IC. Corticosterone administration to non-trained rats did not induce such a reduction. The selective influence of corticosterone on trained, and not naïve, rats is consistent with prior evidence indicating that RU 28362 infused into the IC interacts with training-induced noradrenergic activation in enhancing memory consolidation [54]. However, the direction of the corticosterone effect on pERK1/2 immunoreactivity appears to

contrast prior evidence indicating that glucocorticoid effects on memory consolidation involving other brain regions require an upregulation of pERK1/2-MAP kinase activity. Previously, we reported that a memory-enhancing dose of RU 28362 infused into either the BLA or medial prefrontal cortex after inhibitory avoidance training rapidly elevated pERK1/2 levels in the other brain site (medial prefrontal cortex or BLA, respectively) [13]. Moreover, stress and corticosterone are known to interact with training-associated glutamatergic activity within the hippocampus to increase the expression and enzymatic activity of the MAP kinase pathway [59,70]. A blockade of pERK1/2 signaling with a MEK inhibitor infused into any of these brain regions prevented glucocorticoid effects on memory consolidation. Thus, although we only examined pERK1/2 immunoreactivity in somata, and not in fibers, these findings suggest that glucocorticoids, at least at a 30-min time interval, might exert an opposite influence on the pERK1/2-MAP-kinase pathway, and possibly neuronal activity, in the IC as compared to the BLA or hippocampus. Emerging evidence supports the view that glucocorticoids can activate or suppress synaptic plasticity and neuronal activity in a highly time-dependent and brain region-specific manner [71].

Our finding that corticosterone-induced enhancement of inhibitory avoidance memory is associated with a reduced pERK1/2 expression, and possibly neuronal activity, of the IC is in agreement with that of a recent functional magnetic resonance imaging study in humans showing that the combined oral administration of cortisol and the noradrenergic stimulant yohimbine shortly before the encoding of emotionally arousing pictures led to a strong deactivation of the IC, along with the hippocampus and orbitofrontal cortex [72]. Moreover, the magnitude of this deactivation correlated with enhanced recall of the material assessed 1 week later. Highly comparable, another study reported that human participants who responded with a large increase in cortisol when confronted with a psychosocial stressor, as opposed to low-responders, also showed deactivation of a network of limbic regions that includes the right anterior insula [73]. The crucial question is whether and how such overall deactivation of the IC might contribute to the enhanced consolidation of memory processing. Pruessner et al. (2008)[73] interpreted the deactivation of limbic regions in their study as a stress-mediated regulatory influence on hypothalamic-pituitary-adrenal (HPA) axis activity. However, no findings are available indicating whether or not the IC per se is involved in HPA-axis regulation. Alternatively, based on the involvement of frontal areas, including the anterior IC, in emotional regulation, attention and focusing [74,75], Van Stegeren et al. (2010)[72] suggested that a reduced BOLD signal in these areas could reflect either a loss of top-down inhibition, and therefore activation (disinhibition) of other brain regions, or an increased signal-to-noise ratio, resulting in a shift of attentional processing from a peripherally to a centrally focused view. In line with this hypothesis, others have proposed that the anterior IC is part of a "salience network" which detects salient stimuli (events) and triggers appropriate control signals to regulate behavior and homeostatic state [19,24,25]. It is possible, therefore, that a reduced neuronal activity

of the IC after exogenous glucocorticoid administration induces a "reallocation of resources", thereby increasing the detection of the most relevant stimuli and enhancing the consolidation of memory of these experiences [72,76].

Such a potential role of the IC, as part of the salience network, in novelty and salience detection fits well with our finding that local activation of GRs with RU 28362 enhanced the memory of exposure to both the contextual and aversive components of inhibitory avoidance training. Similarly, findings of human neuroimaging studies indicated activation of the IC in fear conditioning studies during the presentation of either specific conditioned stimuli or context [14,16,29,77]. A broad involvement of the IC in regulating the consolidation of memory of emotionally salient experiences fits also well with other findings of animal studies indicating that posttraining pharmacological manipulation of IC activity modulates memory consolidation of training on many different kinds of learning tasks, including spatial water maze [37], conditioned taste aversion [11,53,61,78] and object recognition [38,54]. The extensive network of connections between the IC and other cortical regions, including the prefrontal, cingulate, perirhinal and entorhinal cortices [23,79 81], might account for such a general modulatory influence on memory. There are also dense reciprocal connections between the IC and BLA [80,82,83]. Extensive evidence indicates that the BLA influences memory consolidation of emotionally arousing training experiences by regulating neuroplasticity and information storage processes in other brain regions, including the hippocampus and dorsal striatum [6,84]. Therefore, it is possible that the IC and BLA share a functional commonality and cooperate in regulating memory consolidation. Indeed, some findings provide evidence for a necessary interaction between the IC and BLA in strengthening the consolidation of memory of emotionally arousing training. Posttraining infusion of a cAMP analog into the IC is known to enhance memory consolidation of both conditioned taste aversion and inhibitory avoidance training. However, concurrent blockade of noradrenergic transmission in the BLA with the α-adrenoceptor antagonist propranolol prevents this memory enhancement [61]. Findings of human studies indicating an increased connectivity between the amygdala and IC during the encoding of emotionally arousing material [31] support a functional interaction between the BLA and IC in regulating memory consolidation.

In summary, the present findings provide evidence for the view that the IC is importantly involved in regulating glucocorticoid effects on memory consolidation of emotionally arousing inhibitory avoidance training. These findings indicate that the IC deserves a prominent position in animal research investigating the neural basis of emotional regulation of learning and memory.

REFERENCES

1. de Kloet, E. R., Oitzl, M. S. & Joels, M. Stress and cognition: are corticosteroids good or bad guys? *Trends in Neurosciences* **22**, 422–426 (1999).
2. Roozendaal, B. Glucocorticoids and the regulation of memory consolidation. *Psychoneuroendocrinology* **25**, 213–238 (2000).
3. Joëls, M., Pu, Z., Wiegert, O., Oitzl, M. S. & Krugers, H. J. Learning under stress: how does it work? *Trends in Cognitive Sciences* **10**, 152–158 (2006).
4. Sandi, C. & Pinelo-Nava, M. T. Stress and Memory: Behavioral Effects and Neurobiological Mechanisms. *Neural Plast.* **2007**, 1–20 (2007).
5. de Quervain, D. J. F., Aerni, A., Schelling, G. & Roozendaal, B. Glucocorticoids and the regulation of memory in health and disease. *Frontiers in Neuroendocrinology* **30**, 358–370 (2009).
6. Roozendaal, B. & McGaugh, J. L. Memory modulation. *Behavioral Neuroscience* **125**, 797–824 (2011).
7. Schwabe, L., Joëls, M., Roozendaal, B., Wolf, O. T. & Oitzl, M. S. Stress effects on memory: An update and integration. *Neuroscience and Biobehavioral Reviews* 1–10 (2011).doi:10.1016/j.neubiorev.2011.07.002
8. Micheau, J., Destrade, C. & Soumireu-Mourat, B. Intraventricular corticosterone injection facilitates memory of an appetitive discriminative task in mice. *Behavioral and Neural Biology* **31**, 100–104 (1981).
9. Roozendaal, B. & McGaugh, J. L. Basolateral amygdala lesions block the memory-enhancing effect of glucocorticoid administration in the dorsal hippocampus of rats. *Eur. J. Neurosci.* **9**, 76–83 (1997).
10. Roozendaal, B. & McGaugh, J. L. Glucocorticoid receptor agonist and antagonist administration into the basolateral but not central amygdala modulates memory storage. *Neurobiology of Learning and Memory* **67**, 176–179 (1997).
11. Miranda, M. I., Rodríguez-García, G., Reyes-López, J. V., Ferry, B. & Ferreira, G. Differential effects of beta-adrenergic receptor blockade in basolateral amygdala or insular cortex on incidental and associative taste learning. *Neurobiology of Learning and Memory* **90**, 54–61 (2008).
12. Roozendaal, B., Barsegyan, A. & Lee, S. Adrenal stress hormones, amygdala activation, and memory for emotionally arousing experiences. *Progress in Brain Research* **167**, 79–97 (2008).
13. Roozendaal, B. *et al.* Glucocorticoid Effects on Memory Consolidation Depend on Functional Interactions between the Medial Prefrontal Cortex and Basolateral Amygdala. *Journal of Neuroscience* **29**, 14299–14308 (2009).
14. Büchel, C., Morris, J., Dolan, R. J. & Friston, K. J. Brain systems mediating aversive conditioning: an event-related fMRI study. *Neuron* **20**, 947–957 (1998).
15. Richardson, M. P., Strange, B. A. & Dolan, R. J. Encoding of emotional memories depends on amygdala and hippocampus and their interactions. *Nature Neuroscience* **7**, 278–285 (2004).
16. Marschner, A., Kalisch, R., Vervliet, B., Vansteenwegen, D. & Büchel, C. Dissociable roles for the hippocampus and the amygdala in human cued versus context fear conditioning. *Journal of Neuroscience* **28**, 9030–9036 (2008).
17. Shin, L. M. & Liberzon, I. The neurocircuitry of fear, stress, and anxiety disorders. *Neuropsychopharmacology* **35**, 169–191 (2010).
18. Craig, A. D. B. How do you feel--now? The anterior insula and human awareness. *Nature Reviews Neuroscience* **10**, 59–70 (2009).
19. Menon, V. & Uddin, L. Q. Saliency, switching, attention and control: a network model of insula function. *Brain Struct Funct* **214**, 655–667 (2010).
20. Hartley, C. A., Fischl, B. & Phelps, E. A. Brain structure correlates of individual differences in the acquisition and inhibition of conditioned fear. *Cereb. Cortex* **21**, 1954–1962 (2011).
21. Ille, R. *et al.* Emotion recognition and experience in Huntington disease: a voxel-based morphometry study. *J Psychiatry Neurosci* **36**, 383–390 (2011).
22. Saper, C. B. Convergence of autonomic and limbic connections in the insular cortex of the rat. *J. Comp. Neurol.* **210**, 163–173 (1982).
23. Augustine, J. R. Circuitry and functional aspects of the insular lobe in primates including humans. *Brain Res. Brain Res. Rev.* **22**, 229–244 (1996).
24. Downar, J., Crawley, A. P., Mikulis, D. J. & Davis, K. D. A cortical network sensitive to stimulus salience in a neutral behavioral context across multiple sensory modalities. *Journal of Neurophysiology* **87**, 615–620 (2002).
25. Seeley, W. W. *et al.* Dissociable intrinsic connectivity networks for salience processing and executive control. *Journal of Neuroscience* **27**, 2349–2356 (2007).

26. Van Marle, H., Hermans, E., Qin, S. & Fernandez, G. Enhanced resting-state connectivity of amygdala in the immediate aftermath of acute psychological stress. *NeuroImage* (2010).
27. Hermans, E. J. *et al.* Stress-related noradrenergic activity prompts large-scale neural network reconfiguration. *Science* **334**, 1151–1153 (2011).
28. van Wingen, G., Geuze, E. & Vermetten, E. Perceived threat predicts the neural sequelae of combat stress. *Molecular* ... (2011).
29. Alvarez, R. P., Biggs, A., Chen, G., Pine, D. S. & Grillon, C. Contextual fear conditioning in humans: cortical-hippocampal and amygdala contributions. *Journal of Neuroscience* **28**, 6211–6219 (2008).
30. King, A. P. *et al.* Medial prefrontal cortex and right insula activity predict plasma ACTH response to trauma recall. *NeuroImage* **47**, 872–880 (2009).
31. Rasch, B. *et al.* A genetic variation of the noradrenergic system is related to differential amygdala activation during encoding of emotional memories. *Proceedings of the National Academy of Sciences* **106**, 19191–19196 (2009).
32. Alvarez, R. P., Chen, G., Bodurka, J., Kaplan, R. & Grillon, C. Phasic and sustained fear in humans elicits distinct patterns of brain activity. *NeuroImage* **55**, 389–400 (2011).
33. Bermudez-Rattoni, F., Introini-Collison, I., Coleman-Mesches, K. & McGaugh, J. L. Insular cortex and amygdala lesions induced after aversive training impair retention: effects of degree of training. *Neurobiology of Learning and Memory* **67**, 57–63 (1997).
34. Bermudez-Rattoni, F. & McGaugh, J. L. Insular cortex and amygdala lesions differentially affect acquisition on inhibitory avoidance and conditioned taste aversion. *Brain Research* **549**, 165–170 (1991).
35. Nerad, L., Ramirez-Amaya, V., Ormsby, C. E. & Bermudez-Rattoni, F. Differential effects of anterior and posterior insular cortex lesions on the acquisition of conditioned taste aversion and spatial learning. *Neurobiology of Learning and Memory* **66**, 44–50 (1996).
36. Bermudez-Rattoni, F., Introini-Collison, I. B. & McGaugh, J. L. Reversible inactivation of the insular cortex by tetrodotoxin produces retrograde and anterograde amnesia for inhibitory avoidance and spatial learning. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5379–5382 (1991).
37. Gutiérrez, H., Hernández-Echeagaray, E., Ramírez-Amaya, V. & Bermudez-Rattoni, F. Blockade of N-methyl-D-aspartate receptors in the insular cortex disrupts taste aversion and spatial memory formation. *NSC* **89**, 751–758 (1999).
38. Bermudez-Rattoni, F., Okuda, S., Roozendaal, B. & McGaugh, J. L. Insular cortex is involved in consolidation of object recognition memory. *Learning & Memory* **12**, 447–449 (2005).
39. Miranda, M. I. & Bermudez-Rattoni, F. Cholinergic activity in the insular cortex is necessary for acquisition and consolidation of contextual memory. *Neurobiology of Learning and Memory* **87**, 343–351 (2007).
40. Berman, D. E. & Dudai, Y. Memory extinction, learning anew, and learning the new: dissociations in the molecular machinery of learning in cortex. *Science* **291**, 2417–2419 (2001).
41. Bermudez-Rattoni, F. Molecular mechanisms of taste-recognition memory. *Nature Reviews Neuroscience* **5**, 209–217 (2004).
42. Shema, R., Sacktor, T. C. & Dudai, Y. Rapid Erasure of Long-Term Memory Associations in the Cortex by an Inhibitor of PKM. *Science* **317**, 951–953 (2007).
43. Nuñez-Jaramillo, L., rez-Lugo, L. R., Herrera-Morales, W. & Miranda, M. A. I. Taste memory formation: Latest advances and challenges. *Behavioural Brain Research* 1–17 (2009). doi:10.1016/j.bbr.2009.10.040
44. Stehberg, J., Moraga-Amaro, R. & Simon, F. The role of the insular cortex in taste function. *Neurobiology of Learning and Memory* 1–6 (2011).doi:10.1016/j.nlm.2011.03.005
45. Thomas, G. M. & Huganir, R. L. MAPK cascade signalling and synaptic plasticity. *Nature Reviews Neuroscience* **5**, 173–183 (2004).
46. Peng, S., Zhang, Y., Zhang, J., Wang, H. & Ren, B. ERK in learning and memory: A review of recent research. *Int J Mol Sci* **11**, 222–232 (2010).
47. Malin, E. L. & McGaugh, J. L. Differential involvement of the hippocampus, anterior cingulate cortex, and basolateral amygdala in memory for context and footshock. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 1959–1963 (2006).
48. Medina, A. C. *et al.* Glucocorticoid administration into the dorsal stratium facilitates memory consolidation of inhibitory avoidance training but not of the context or footshock components. *Learning & Memory* **14**, 673–677 (2007).
49. Fornari, R. V. *et al.* Rodent stereotaxic surgery and animal welfare outcome improvements for behavioral neuroscience. *J Vis Exp* (2012). doi:10.3791/3528

50. Paxinos, G. & Watson, C. *The Rat Brain in Stereotaxic Coordinates*. (Amsterdam: Elsevier: 2007).

51. McGaugh, J. L., Introini-Collison, I. B. & Nagahara, A. H. Memory-enhancing effects of posttraining naloxone: involvement of beta-noradrenergic influences in the amygdaloid complex. *Brain Research* **446**, 37–49 (1988).

52. Teutsch, G. *et al.* 17 alpha-alkynyl-11 beta, 17-dihydroxyandrostane derivatives : a new class of potent glucocorticoids. *Steroids* **38**, 651–665 (1981).

53. Miranda, M. I., Quirarte, G. L., Rodriguez-Garcia, G., McGaugh, J. L. & Roozendaal, B. Glucocorticoids enhance taste aversion memory via actions in the insular cortex and basolateral amygdala. *Learning & Memory* **15**, 468–476 (2008).

54. Roozendaal, B. *et al.* Membrane-Associated Glucocorticoid Activity Is Necessary for Modulation of Long-Term Memory via Chromatin Modification. *Journal of Neuroscience* **30**, 5037–5046 (2010).

55. McGaugh, J. L. Time-dependent processes in memory storage. *Science* **153**, 1351–1358 (1966).

56. Hui, G. K. *et al.* Memory enhancement of classical fear conditioning by post-training injections of corticosterone in rats. *Neurobiology of Learning and Memory* **81**, 67–74 (2004).

57. Okuda, S., Roozendaal, B. & McGaugh, J. Glucocorticoid effects on object recognition memory require training-associated emotional arousal. *Proceedings of the National Academy of Sciences* **101**, 853–858 (2004).

58. Roozendaal, B., McEwen, B. S. & Chattarji, S. Stress, memory and the amygdala. *Nature Reviews Neuroscience* **10**, 423–433 (2009).

59. Revest, J.-M. *et al.* The MAPK pathway and Egr-1 mediate stress-related behavioral effects of glucocorticoids. *Nature Neuroscience* **8**, 664–672 (2005).

60. Groeneweg, F. L., Karst, H., de Kloet, E. R. & Joels, M. Rapid non-genomic effects of corticosteroids and their role in the central stress response. *Journal of Endocrinology* **209**, 153–167 (2011).

61. Miranda, M. I. & McGaugh, J. L. Enhancement of inhibitory avoidance and conditioned taste aversion memory with insular cortex infusions of 8-Br-cAMP: involvement of the basolateral amygdala. *Learning & Memory* **11**, 312–317 (2004).

62. Atkins, C. M., Selcher, J. C., Petraitis, J. J., Trzaskos, J. M. & Sweatt, J. D. The MAPK cascade is required for mammalian associative learning. *Nature Neuroscience* **1**, 602–609 (1998).

63. Schafe, G. E. *et al.* Activation of ERK/MAP kinase in the amygdala is required for memory consolidation of pavlovian fear conditioning. *Journal of Neuroscience* **20**, 8177–8187 (2000).

64. Fiore, R. S., Murphy, T. H., Sanghera, J. S., Pelech, S. L. & Baraban, J. M. Activation of p42 mitogen-activated protein kinase by glutamate receptor stimulation in rat primary cortical cultures. *J Neurochem* **61**, 1626–1633 (1993).

65. Xia, Z., Dudek, H., Miranti, C. K. & Greenberg, M. E. Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. *J. Neurosci.* **16**, 5425–5436 (1996).

66. Fujita, S., Adachi, K., Koshikawa, N. & Kobayashi, M. Spatiotemporal dynamics of excitation in rat insular cortex: intrinsic corticocortical circuit regulates caudal-rostro excitatory propagation from the insular to frontal cortex. *Neuroscience* **165**, 278–292 (2010).

67. Kobayashi, M. *et al.* Functional mapping of gustatory neurons in the insular cortex revealed by pERK-immunohistochemistry and in vivo optical imaging. *Synapse* **64**, 323–334 (2010).

68. Berman, D. E., Hazvi, S., Rosenblum, K., Seger, R. & Dudai, Y. Specific and differential activation of mitogen-activated protein kinase cascades by unfamiliar taste in the insular cortex of the behaving rat. *J. Neurosci.* **18**, 10037–10044 (1998).

69. Berman, D. E., Hazvi, S., Neduva, V. & Dudai, Y. The role of identified neurotransmitter systems in the response of insular cortex to unfamiliar taste: activation of ERK1-2 and formation of a memory trace. *J. Neurosci.* **20**, 7017–7023 (2000).

70. Gutièrrez-Mecinas, M. *et al.* Long-lasting behavioral responses to stress involve a direct interaction of glucocorticoid receptors with ERK1/2-MSK1-Elk-1 signaling. *Proceedings of the National Academy of Sciences* **108**, 13806–13811 (2011).

71. Joëls, M., Fernández, G. & Roozendaal, B. Stress and emotional memory: a matter of timing. *Trends in Cognitive Sciences* **15**, 280–288 (2011).

72. van Stegeren, A. H., Roozendaal, B., Kindt, M., Wolf, O. T. & Joëls, M. Interacting noradrenergic and corticosteroid systems shift human brain activation patterns during encoding. *Neurobiology of Learning and Memory* **93**, 56–65 (2010).

73. Pruessner, J. C. *et al.* Deactivation of the Limbic System During Acute Psychosocial Stress:

Evidence from Positron Emission Tomography and Functional Magnetic Resonance Imaging Studies. *Biological Psychiatry* **63**, 234–240 (2008).

74. Dolcos, F., LaBar, K. S. & Cabeza, R. Dissociable effects of arousal and valence on prefrontal activity indexing emotional evaluation and subsequent memory: an event-related fMRI study. *NeuroImage* **23**, 64–74 (2004).
75. Dolcos, F., Miller, B., Kragel, P., Jha, A. & McCarthy, G. Regional brain differences in the effect of distraction during the delay interval of a working memory task. *Brain Research* **1152**, 171–181 (2007).
76. Cahill, L. & van Stegeren, A. Sex-related impairment of memory for emotional events with beta-adrenergic blockade. *Neurobiology of Learning and Memory* **79**, 81–88 (2003).
77. Büchel, C., Dolan, R. J., Armony, J. L. & Friston, K. J. Amygdala-hippocampal involvement in human aversive trace conditioning revealed through event-related functional magnetic resonance imaging. *Journal of Neuroscience* **19**, 10869–10876 (1999).
78. Shema, R., Hazvi, S., Sacktor, T. C. & Dudai, Y. Boundary conditions for the maintenance of memory by PKMzeta in neocortex. *Learn. Mem.* **16**, 122–128 (2009).
79. Mufson, E. J., Mesulam, M. M. & Pandya, D. N. Insular interconnections with the amygdala in the rhesus monkey. *NSC* **6**, 1231–1248 (1981).
80. Shi, C. J. & Cassell, M. D. Cortical, thalamic, and amygdaloid connections of the anterior and posterior insular cortices. *J. Comp. Neurol.* **399**, 440–468 (1998).
81. Höistad, M. & Barbas, H. Sequence of information processing for emotions through pathways linking temporal and insular cortices with the amygdala. *NeuroImage* **40**, 1016–1033 (2008).
82. McDonald, A. J. & Jackson, T. R. Amygdaloid connections with posterior insular and temporal cortical areas in the rat. *J. Comp. Neurol.* **262**, 59–77 (1987).
83. Shi, C. J. & Cassell, M. D. Cascade projections from somatosensory cortex to the rat basolateral amygdala via the parietal insular cortex. *J. Comp. Neurol.* **399**, 469–491 (1998).
84. McGaugh, J. L. Memory consolidation and the amygdala: a systems perspective. *Trends in Neurosciences* **25**, 456 (2002).

4

GLUCOCORTICOIDS INTERACT WITH THE NORADRENERGIC AROUSAL SYSTEM IN THE NUCLEUS ACCUMBENS SHELL TO ENHANCE MEMORY CONSOLIDATION OF BOTH APPETITIVE AND AVERSIVE TASTE LEARNING

Romy Wichmann[1]

Raquel V. Fornari[12]

Benno Roozendaal[1]

[1] Department of Neuroscience, Section Anatomy, University Medical Center Groningen, University of Groningen
[2] Núcleo de Cognição e Sistemas Complexos, Centro de Matemática, Computação e Cognição, Universidade Federal do ABC, Santo André, SP, Brasil

Neurobiology of Learning and Memory 98 (2012) 197-205

ABSTRACT

It is well established that glucocorticoid hormones strengthen the consolidation of long-term memory of emotionally arousing experiences but have little effect on memory of low-arousing experiences. Although both positive and negative emotionally arousing events tend to be well remembered, studies investigating the neural mechanism underlying glucocorticoid-induced memory enhancement focused primarily on negatively motivated training experiences. In the present study we show an involvement of glucocorticoids within the nucleus accumbens (NAc) in enhancing memory consolidation of both an appetitive and aversive form of taste learning. The specific glucocorticoid receptor (GR) agonist RU 28362 (1 or 3 ng) administered bilaterally into the NAc shell, but not core, of male Sprague-Dawley rats immediately after an appetitive saccharin drinking experience dose-dependently enhanced 24-h retention of the safe taste, resulting in a facilitated attenuation of neophobia. Similarly, GR agonist infusions given into the NAc shell immediately after pairing of the saccharin taste with a malaise-inducing agent enhanced memory of this negative experience, resulting in an intensified conditioned aversion. Importantly, a suppression of noradrenergic activity within the NAc shell with the ß-adrenoceptor antagonist propranolol blocked the facilitating effect of a concurrently administered GR agonist on memory consolidation in both the appetitive and aversive learning task. Thus, these findings indicate that GR activation interacts with the noradrenergic arousal system within the NAc to enhance memory consolidation of emotionally arousing training experiences regardless of valence.

INTRODUCTION

Emotional memory enhancement is a well-recognized phenomenon that helps us to remember important life events. Both positive and negative emotionally arousing experiences are more likely to be recalled with greater detail and vividness than events that lack emotional significance [1,2]. However, studies investigating the neural mechanisms underlying arousal-induced memory enhancement have focused almost exclusively on negatively motivated experiences. Such studies indicate that glucocorticoid hormones (corticosterone in rodents, cortisol in humans), released from the adrenal cortex during arousing episodes, are crucially involved in facilitating the consolidation of long-term memory of these experiences [3-6]. Corticosterone or specific glucocorticoid receptor (GR) agonists are known to act upon different loci within the emotional memory network, including the basolateral amygdala, hippocampus and various cortical regions, to enhance memory consolidation of training on a wide variety of aversively motivated learning tasks [7-12]. Further studies report that arousal-induced noradrenergic activity is required for enabling the effects of glucocorticoids on memory consolidation [13-15], a mechanism that might explain why glucocorticoids selectively affect memory formation of experiences that are emotionally arousing [3,4,14]. However, and importantly, despite extensive evidence indicating that the release of endogenous glucocorticoids is initiated not only during aversive or noxious stimulation but that corticosterone levels also mount in response to appetitive and rewarding stimuli such as food, drugs of abuse or sexual activity [16-18], little is known concerning the influence of glucocorticoids on the consolidation of memory of positively motivated learning experiences [19-21].

Substantial evidence indicates that the nucleus accumbens (NAc), which receives major input from the basolateral amygdala, hippocampus and prefrontal cortex [22,23], is importantly involved in the processing of positively motivated or rewarding information [24-26]; however, it is also uniquely positioned to subserve a role in the formation and/or retrieval of emotionally influenced memory [27,28]. Indeed, lesion and pharmacological manipulation studies indicate an involvement of the NAc in memory for both appetitive [29-31] and aversive training experiences [32-34], including appetitive [35,36] and aversive taste learning [35,37-39]. Taste learning, and its corresponding gastro-intestinal consequences, is a commonly used and evolutionary significant experimental model for the study of memory formation [40,41]. If the ingestion of a novel appetitive drinking solution is not followed by any negative consequences, the animal learns that the taste is safe, a process termed attenuation of neophobia (AN) occurs and the animal will increase its consumption upon subsequent taste presentations [36,40]. On the other hand, if the ingestion is followed by malaise, a conditioned taste aversion (CTA) develops and the animal will reject the taste on the next encounter [40,42].

The present study investigated the effects of glucocorticoid administration into the NAc on memory consolidation of both appetitive and aversive taste learning. In the first experiment, we investigated whether the specific GR agonist RU 28362 administered

into the shell or core subregions of the NAc immediately after ingestion of a novel saccharin drinking solution enhances the consolidation of memory of the appetitive taste learning experience. In the second experiment, we investigated whether GR agonist administration into the NAc induces comparable memory consolidation enhancement of an aversive taste learning experience. In the last experiment we investigated whether noradrenergic activity within the NAc is essential for enabling the facilitating effects of glucocorticoids on memory of both appetitive and aversive learning experiences.

MATERIAL & METHODS

Subjects

Adult male Sprague-Dawley rats (300-350 g at time of surgery) from Charles River Breeding Laboratories (Kisslegg, Germany) were housed individually in a temperature-controlled (22°C) colony room and maintained on a standard 12-h:12-h light:dark cycle (07:00-19:00 h lights on) with *ad libitum* access to food and water, except when noted in the behavioral procedures. They were kept in these conditions for at least 7 d before surgery for cannula implantation. Training and testing were performed during the light phase of the cycle between 10:00 and 13:00 h, at the rat nadir of the diurnal rhythm of corticosterone. All experimental procedures were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Institutional Animal Care and Use Committee of the University of Groningen, The Netherlands.

Surgery for cannula implantation

For a detailed description of surgical procedures, see Fornari et al (2012) [43]. Briefly, rats were anesthetized with a subcutaneous injection of ketamine (37.5 mg/kg body weight; Alfasan), dexmedetomidine (0.25 mg/kg; Orion) and atropine sulfate (0.4 mg kg; Pharmachemie BV), and received the non-steroidal analgesic carprofen (4 mg/kg, sc; Pfizer). The skull was positioned in a stereotaxic frame (Kopf Instruments) and two stainless-steel guide cannulae (15 mm long; 23 gauge, Small Parts Inc.) were implanted bilaterally with the cannula tips 2.0 mm above the NAc shell or core. The coordinates were based on the atlas of Paxinos and Watson (2007) [44]; for NAc shell: anteroposterior [AP], 1.6 mm anterior to Bregma; mediolateral [ML], ±1.0 mm from midline; dorsoventral [DV], 5.7 mm below skull surface; for NAc core: AP, 1.6 mm anterior to Bregma; ML, ±1.7 mm from midline; DV, 5.0 mm below skull surface; incisor bar -3.3 mm from interaural. The cannulae were affixed to the skull with two surgical screws and dental acrylic cement. Stylets (15-mm long 00-insect pins) were inserted into each guide cannula to maintain patency. After surgery, the rats received a subcutaneous injection of 3 ml of saline to facilitate clearance of drugs and prevent dehydration, and were subsequently administered atipamezole hydrochloride (Antisedan, 0.25 mg/kg, sc; Orion) to reverse anesthesia. The rats were allowed to recover for a minimum of 7 d

before initiation of training and were handled three times, for 1 min each, during this recovery period to accustom them to the infusion procedure.

Appetitive taste learning

Rats were initially deprived of water for 24 h and then habituated to drink water for 20 min/d for 5 d. After a stable water consumption baseline was reached, the animals were divided into several treatment groups, counterbalanced according to their mean baseline water consumption. On the acquisition trial, rats were allowed to freely drink a novel 0.5% saccharin (saccharin sodium salt hydrate, Sigma-Aldrich) solution for 20 min in their home cages. A neophobic response was calculated as the ratio of saccharin intake relative to water baseline consumption. Due to the high relative novelty of this saccharin concentration, a large neophobic response is observed [45]. Immediately or 3 h after the saccharin presentation, the rats received microinfusions of drug or vehicle. On the 24-h retention test, rats had access to the same (familiar) saccharin solution for 20 min and the volume drunk was recorded. An AN index was calculated as the saccharin consumption on the retention test relative to the acquisition trial. A larger AN index was interpreted as indicating better retention of the appetitive taste learning experience. Rats had additional access to water for 10 min at the end of each day to assure that all animals consumed their daily fluid requirements.

Aversive taste learning

As on the appetitive taste learning task, rats were initially deprived of water for 24 h and subsequently habituated to drink water for 20 min/d for 5 d. After a stable water consumption baseline was reached, the animals were divided into several treatment groups, counterbalanced according to their mean baseline water consumption, and acquisition of aversive taste learning was performed [10,46]. For conditioning, the rats were allowed to freely drink a novel 0.1% sodium saccharin solution for 20 min in their home cages, and the volume drunk was recorded. This concentration of saccharin is highly preferred but, in contrast to higher saccharin concentrations, only induces a slight neophobic reaction [47]. Thirty minutes after completion of saccharin intake, the animals were injected intraperitoneally with 0.15 M lithium chloride (LiCl), a dose that produces only mild gastric malaise. Immediately, 3 or 6 h after the LiCl injection, rats received microinfusions of drug or vehicle. For the next three days, rats could drink water for 20 min/d. Four days after conditioning, the 0.1% saccharin solution was presented again for 20 min to test for retention of the acquired taste aversion (i.e., single bottle test). A CTA index was calculated as the saccharin intake on the 96-h retention test relative to that ingested on the acquisition trial. A smaller CTA index, i.e., a larger conditioned avoidance of the taste, was interpreted as indicating better retention of the aversive taste learning experience.

Drugs and infusion procedures

The specific GR agonist RU 28362 (1 or 3 ng in 0.3 µl; 11β,17β-dihydroxy-6,21-dimethyl-17α-pregna-4,6-trien-20yn-3-one, a generous gift of Aventis, Frankfurt, Germany) was first dissolved in 100% ethanol and subsequently diluted in saline to reach the appropriate concentration. The final concentration of ethanol was 0.5%. Receptor binding studies have shown that this compound has selective and high affinity for GRs [48]. For the last experiment, the ß-adrenoceptor antagonist DL-propranolol hydrochloride (0.3 µg in 0.3 µl; Sigma-Aldrich) was infused together with a memory-enhancing dose of the GR agonist (3 ng) or vehicle (0.5% ethanol in saline). Bilateral infusions of drug, or an equivalent volume of vehicle, were made by using 30-gauge injection needles connected to 10-µl Hamilton microsyringes by polyethylene (PE-20) tubing, driven by an automated microinfusion pump (Stoelting Inc.). The injection needles protruded 2.0 mm beyond the tip of the cannula, and an injection volume of 0.3 µl per hemisphere was delivered over 30 s. The injection needles were retained within the cannulae for an additional 30 s following drug infusion to maximize diffusion and prevent backflow of drug along the cannula track. Drug doses were chosen on the basis of previous findings [7,15]. The drug volume and infusion sites selected have been reported previously to induce selective activation/blockade of the shell and/or core subregions of the NAc [38,49].

Histology

Following completion of behavioral testing, rats were injected with an overdose of sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 0.9% saline, followed by 4% formaldehyde (w/v) in water. After decapitation, the brains were removed and stored in a fresh 4% formaldehyde solution at 4°C for 24 h. The brains were then immersed in 25% sucrose (w/v) for cryoprotection. Coronal sections of 50 µm were cut on a cryostat, mounted on gelatin-coated slides, and stained with cresyl violet. The sections were examined under a light microscope and determination of the location of injection needle tips within the NAc shell or core, respectively, was made according to the atlas plates of Paxinos and Watson (2007) [41], by an observer blind to drug treatment condition. Rats with injection needle placements outside the NAc shell or core or with extensive tissue damage at the injection needle tips were excluded from analysis.

Statistics

Data are presented as mean ± SEM. The AN and CTA indices were analyzed with one- or two-way ANOVAs. Further analyses used pairwise Fisher's LSD post-hoc tests, when appropriate. Additionally, one-sample *t*-tests were used to examine whether the AN and CTA index was significantly different from one, indicating that animals showed retention of the learning experience. For all comparisons, a probability level of < 0.05 was accepted as statistical significance. The number of animals per group is indicated in the figure legends.

RESULTS

Posttraining administration of a GR agonist into the nucleus accumbens shell, but not core, enhances consolidation of appetitive taste memory

This experiment investigated whether the GR agonist RU 28362 administered bilaterally into the NAc shell immediately after an appetitive saccharin drinking experience enhances the consolidation of memory of this safe taste learning experience, resulting in a facilitated AN response upon re-exposure to the taste stimulus 24 h later.

Baseline water consumption was stable over the 3 d of registration prior to conditioning. One-way ANOVA for mean baseline water intake revealed no significant differences between later drug treatment groups [$F(2,35) = 0.49$; $p = 0.62$; Table I]. On the acquisition day, novel saccharin ingestion also did not differ between groups [$F(2,35) = 1.99$; $p = 0.15$; Table I] and all rats showed a robust neophobic response, as assessed by a significantly lower intake of the novel saccharin solution compared to water intake on the previous day ($p < 0.0001$; Table I). As is shown in Fig. 1A, one-way ANOVA for AN index, i.e., saccharin consumption on the 24-h retention test relative to that on the acquisition trial, indicated a significant drug effect [$F(2,35) = 3.40$; $p < 0.05$]. One-sample *t*-test revealed that the AN index of rats treated with vehicle was significantly larger than 1 (2.56 ± 0.33; $p < 0.01$), indicating that control rats retained memory of the appetitive taste stimulus. Fisher's LSD post-hoc analysis revealed that the AN index of rats administered the higher dose of RU 28362 (3 ng) was significantly larger than that of vehicle control rats ($p < 0.05$) and thus reflects an enhanced retention of the appetitive taste learning experience. In contrast, the lower dose of RU 28362 (1 ng) did not induce significant retention enhancement ($p = 0.35$). To determine whether the GR agonist enhanced retention by influencing time-dependent processes underlying the consolidation phase of memory, other groups of rats received delayed infusions of the GR agonist after the saccharin drinking experience. As is shown in Fig. 1B, RU 28362 (1 or 3 ng) administered into the NAc shell 3 h after the appetitive

Table 1 Fluid intake prior to conditioning and on the acquisition day.

	Drug groups	H_2O baseline (ml)	Acquisition (ml)
Appetitive	vehicle	17.9 ± 0.8	4.3 ± 0.6
taste learning	RU 28362 (1 ng)	17.5 ± 0.6	3.9 ± 0.6
	RU 28362 (3 ng)	18.7 ± 0.9	3.0 ± 0.4
Aversive	vehicle	17.3 ± 0.7	12.5 ± 1.0
taste learning	RU 28362 (1 ng)	16.0 ± 0.7	13.0 ± 1.1
	RU 28362 (3 ng)	17.3 ± 0.5	11.8 ± 1.2

Data are presented as mean ± SEM of baseline water consumption on 3 consecutive days prior to conditioning and novel saccharin consumption on the acquisition day.

taste learning experience did not significantly alter the AN index [$F(2,40) = 0.02$; $p = 0.98$], indicating that the posttraining drug treatment did not directly affect behavior on the 24-h retention test.

To determine whether the GR agonist effect was localized within the NAc shell, other rats received infusions of RU 28362 (1 or 3 ng) or vehicle into the adjacent core subregion of the NAc immediately after saccharin drinking. One-way ANOVA for AN index did not reveal a significant drug effect [$F(2,31) = 1.22$; $p = 0.31$; Fig 1C], indicating a site-specific involvement of the NAc shell in mediating GR agonist effects on memory consolidation of the appetitive taste experience.

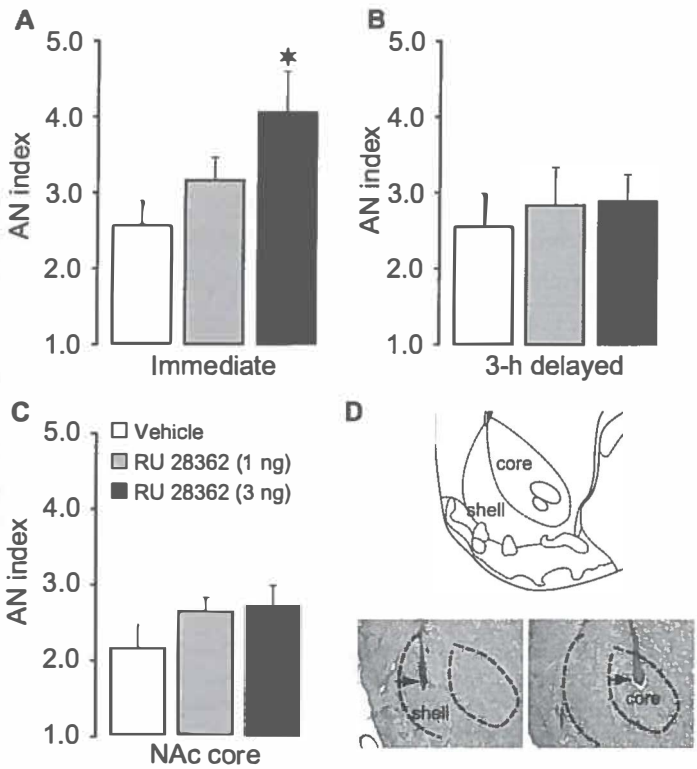


Figure 1. Effect of the glucocorticoid receptor agonist RU 28362 administered posttraining into the nucleus accumbens (NAc) on memory consolidation of appetitive taste learning. (A) RU 28362 (1 or 3 ng in 0.3 µl) administered into the NAc shell immediately after novel saccharin drinking enhanced 24-h retention performance, as indicated by an increased attenuation of neophobia (AN). The AN index (mean ± SEM) is expressed as saccharin drinking on the retention test relative to the acquisition trial (n = 11-13 per group). (B) In contrast, RU 28362 (1 or 3 ng in 0.3 µl) administered into the NAc core after saccharin drinking did not enhance the AN index (n = 10-13 per group). (C) Delayed infusions of RU 28362 (1 or 3 ng in 0.3 µl) administered into the NAc shell 3 h after saccharin drinking also did not enhance the AN index (n = 14-15 per group). *$p < 0.05$ compared with the vehicle group. (D) Representative diagram and photomicrographs illustrating placement of the cannulae and needle tips in the NAc shell and core, respectively. Arrows point to the infusion needle tips.

Fig. 1D shows representative photomicrographs of cannula placement within the NAc shell and core. All injection needle tips of rats included in the analysis were localized exclusively within the shell or core, respectively.

Posttraining administration of a GR agonist into the nucleus accumbens shell, but not core, enhances consolidation of aversive taste memory

To investigate whether posttraining activation of GRs within the NAc induces comparable enhancement of memory consolidation of an aversive taste learning experience, bilateral infusions of RU 28362 (1 or 3 ng) or vehicle were administered into the NAc shell or core immediately after aversive conditioning with an intraperitoneal injection of the malaise-inducing agent LiCl, 30 min after completion of saccharin intake.

Baseline water consumption was stable over the 3 d of registration prior to conditioning. One-way ANOVA for mean baseline water intake revealed no significant differences between later drug groups [$F(2,36) = 1.46$; $p = 0.25$; Table I]. There were also no group differences in novel saccharin ingestion during training [$F(2,36) = 0.29$; $p = 0.75$; Table I]. As is shown in Fig. 2A, one-way ANOVA for CTA index, i.e., saccharin consumption on the 96-h retention test relative to that on the acquisition trial, revealed a significant drug effect [$F(2,36) = 4.17$; $p < 0.05$]. One sample *t*-test indicated that the CTA index of rats treated with vehicle was significantly smaller than 1 (0.69 ± 0.06; $p < 0.001$), indicating that control rats exhibited a mild aversion to the saccharin taste on the retention test. Fisher's post-hoc comparison tests revealed that rats administered either dose of RU 28362 showed a significantly greater conditioned aversion to the saccharin taste (1 ng: $p < 0.05$; 3 ng: $p < 0.05$, compared to vehicle-treated rats). Control groups received RU 28362 immediately after pseudo-conditioning with an intraperitoneal injection of saline. In these rats, the GR agonist did not induce any saccharin aversion on a 96-h retention test [$F(2,30) = 0.08$; $p = 0.93$; Fig. 2B], indicating that the RU 28362 enhanced memory of the pairing of the saccharin taste with gastric malaise.

We further found that the GR agonist administration induced a time-dependent enhancement of the consolidation of aversive taste memory. As is shown in Fig. 2C, RU 28362 administered into the NAc shell 3 h after conditioning still enhanced 96-h retention [$F(2,31) = 3.95$; $p < 0.05$]. Post-hoc Fisher's LSD tests revealed that both doses of RU 28362 enhanced conditioned aversion on the 96-h retention test (1 ng: $p < 0.05$; 3 ng: $p < 0.05$, compared to vehicle). However, when RU 28362 was administered 6 h after conditioning, no increase in saccharin aversion on the 96-h retention test was found [$F(2,30) = 2.25$; $p = 0.12$; Fig. 2D]. Also comparable to the findings of the first experiment, RU 28362 (1 or 3 ng) administered into the adjacent NAc core immediately after aversive conditioning did not significantly alter the CTA index on the 96-h retention test [$F(2,45) = 0.82$; $p = 0.45$; Fig. 2E], indicating a site-specific involvement of the GR agonist within the NAc shell in enhancing the consolidation of aversive taste memory.

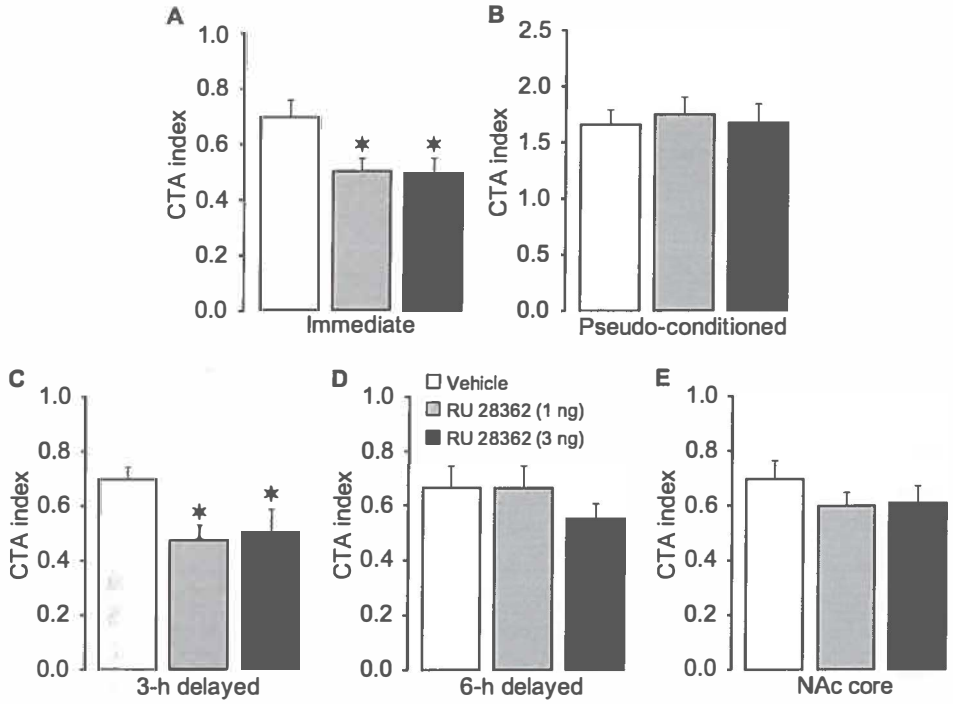


Figure 2. Effect of the glucocorticoid receptor agonist RU 28362 administered posttraining into the nucleus accumbens (NAc) on memory consolidation of aversive taste learning. (A) RU 28362 (1 or 3 ng in 0.3 µl) administered into the NAc shell immediately after pairing of a novel saccharin drinking solution with the malaise-inducing agent lithium chloride enhanced 96-h retention performance, as indicated by an increased conditioned taste aversion (CTA). The CTA index (mean ± SEM) is expressed as saccharin drinking on the retention test relative to the acquisition trial (n = 11-15 per group). (B) RU 28362 (1 or 3 ng in 0.3 µl) administered into the NAc shell immediately after pseudo-conditioning with an injection of saline did not induced taste aversion on a 96-h retention test (n = 9-13 per group). (C) Delayed infusions of RU 28362 (1 or 3 ng in 0.3 µl) administered into the NAc shell 3 h after aversive taste learning enhanced the CTA index on the 96-h retention test, whereas (D) RU 28362 administered 6 h after aversive conditioning did not alter the CTA index (n = 9-13 per group). (E) RU 28362 (1 or 3 ng in 0.3 µl) administered into the NAc core after aversive taste learning did not alter the CTA index (n = 14-18 per group). *$p < 0.05$ compared with the vehicle group.

Role of the noradrenergic system within the nucleus accumbens shell in enabling GR agonist effects on memory of appetitive and aversive taste learning

It is well established from studies in other brain regions that arousal-induced noradrenergic activity is a prerequisite for enabling glucocorticoid effects on memory consolidation of aversive training experiences [13,15,50-52]. As described above, such an essential interplay between these two neuromodulatory systems can possibly explain why glucocorticoids selectively enhance the consolidation of memory of emotionally arousing, and not emotionally neutral, training experiences [51,53]. However, it is presently unknown whether the enhancing effect of glucocorticoid administration on

the consolidation of memory of positively motivated learning experiences requires a similar interaction with the noradrenergic arousal system. To address this issue, we first investigated whether a suppression of noradrenergic activity within the NAc shell with local infusions of the ß-adrenoceptor antagonist propranolol (0.3 µg in 0.3 µl) would prevent the GR agonist-induced consolidation enhancement of appetitive taste memory. As is shown in Fig. 3A, two-way ANOVA for AN index on a 24-h retention test showed no GR agonist [$F(1,43) = 2.05$; $p = 0.16$] or propranolol effect [$F(1,43) = 0.89$; $p = 0.35$] but a significant interaction effect between GR agonist and propranolol [$F(1,43) = 4.46$; $p < 0.05$]. Post-hoc Fisher's LSD tests indicated that RU 28362 (3 ng) administered alone into the NAc shell significantly increased the AN index ($p < 0.05$, compared to vehicle-treated rats) and that concurrent administration of propranolol blocked this memory enhancement ($p < 0.05$). This low dose of propranolol administered alone into the NAc shell did not impair retention.

Next, we examined whether posttraining antagonism of ß-adrenoceptors within the NAc shell with propranolol (0.3 µg in 0.3 µl) induces a comparable blockade of the effect of RU 28362 (3 ng) in the aversive taste learning paradigm. As is shown in Fig. 3B, two-way ANOVA for CTA index on a 96-h retention test showed no GR agonist [$F(1,38) = 0.42$; $p = 0.52$] or propranolol effect [$F(1,38) = 1.39$; $p = 0.25$] but a significant interaction effect between GR agonist and propranolol [$F(1,38) = 4.63$;

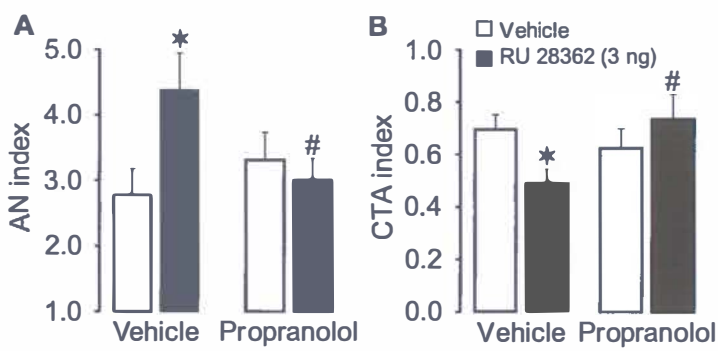


Figure 3. Effect of blockade of ß-adrenoceptor activity within the nucleus accumbens (NAc) shell on glucocorticoid-induced enhancement of memory consolidation of appetitive and aversive taste learning. (A) The glucocorticoid receptor agonist RU 28362 (3 ng in 0.3 µl) infused into the NAc shell immediately after appetitive taste learning enhanced 24-h retention of this experience, as indicated by an increased attenuation of neophobia (AN) index. Concurrent administration of the ß-adrenoceptor antagonist propranolol (0.3 µg) prevented this RU 28362 effect. The AN index (mean ± SEM) is expressed as saccharin drinking on the retention test relative to the acquisition trial ($n = 8$-14 per group). (B) RU 28362 (3 ng in 0.3 µl) infused into the NAc shell immediately after aversive taste learning enhanced 96-h retention of this experience, as indicated by an increased conditioned taste aversion (CTA). Concurrent administration of the ß-adrenoceptor antagonist propranolol (0.3 µg) prevented this RU 28362 effect. The CTA index (mean ± SEM) is expressed as saccharin drinking on the retention test relative to the acquisition trial. ($n = 11$-13 per group). *$p < 0.05$ as compared with the vehicle group; #$p < 0.05$ as compared with RU 28362 alone group.

$p < 0.05$]. Post-hoc Fisher's LSD tests indicated that RU 28362 infused alone into the NAc shell significantly increased conditioned saccharin aversion ($p < 0.05$, compared to vehicle-treated rats) and that concurrent administration of propranolol blocked this GR agonist effect ($p < 0.05$). Thus, these findings indicate that GR agonist administration into the NAc shell interacts with the noradrenergic arousal system in enhancing the consolidation of memory of both appetitive and aversive taste learning.

DISCUSSION

The present study investigated whether glucocorticoids are involved in modulating the consolidation of memory of emotionally arousing training experiences regardless of valence. In strong support of this view, we found that the specific GR agonist RU 28362 administered bilaterally into the NAc shell after ingestion of an appetitive saccharin drinking solution induced dose-, site- and time-specific enhancement of long-term retention of the safe taste experience. Comparably, GR agonist administration into the NAc shell after pairing of the saccharin taste with a malaise-inducing agent enhanced retention of the aversive taste learning experience. Furthermore, and importantly, as concurrent antagonism of ß-adrenoceptor activity within the NAc blocked the GR agonist-induced retention enhancement on both tasks, the findings indicate that noradrenergic signaling within the NAc is essential for enabling the enhancing effects of glucocorticoids on memory consolidation of both positively and negatively motivated learning experiences. The use of posttraining treatment ensures that animals were drug-free at the time of training and, thus, that drug effects are not attributable to possible influences on attentional, motivational or perceptual processes during memory acquisition [54,55]. Additionally, the finding that delayed drug infusions did not affect behavior during the retention test trial further strengthens the conclusion that drug effects are solely caused by time-dependent influences on neurobiological processes underlying the consolidation of long-term memory.

Our finding that the GR agonist RU 28362 infused into the NAc shell immediately after either appetitive or aversive taste learning enhanced retention of the training experience indicates that glucocorticoids induce a prioritized storage of emotionally arousing information into long-term memory regardless of the valence of the stimulus. The use of gustatory learning as experimental model to examine the effects of a GR agonist on memory consolidation of both positively and negatively valenced information has several advantages. Both tasks share multiple characteristics in their setup, which facilitate a comparison of effects [46,56]. In both tasks, animals learn to associate the comsumption of a novel saccharin solution with their respective outcome. In the aversive taste learning task the ingestion of saccharin is followed by gastric malaise, which induces an association of this experience with a negative consequence, and aversion develops. In the appetitive taste learning task, rats associate the saccharin taste with a positive reinforcement by learning that no negative consequences are associated

with the taste (learned safety) [57,58]. The ingestion of a novel saccharin solution induces an initial neophobic response [45], which easily disrupts the ingestion of these solutions [15,57,59]. Repeated exposure enhances familiarization and reduces the neophobic response. Therefore, the GR agonist effect to enhance this attenuation of neophobia response in the appetitive taste learning task could also be interpreted as an enhanced extinction of this innate fear response. Several prior studies indicated that glucocorticoids administered systemically after extinction learning on fear-associated tasks enhance the consolidation of extinction memory [60,61]. Despite these qualitative similarities, training on these two tasks likely induces different levels of endogenous emotional arousal and consequent circulating levels of corticosterone [10,62], which could explain our finding that the memory-enhancing doses of RU 28362 were not identical on both tasks.

Although it is well established that both positive and negative emotionally arousing events are remembered better than neutral ones [1,2,63,64], prior studies investigating the neurobiological mechanism underlying this memory facilitation have focused almost exclusively on aversive or fear-motivated learning. Findings from these studies indicate that glucocorticoids, via GR activation, act within a network of interconnected brain regions, including the basolateral amygdala, hippocampus and prefrontal cortex, to produce dose- and time-dependent enhancement of the consolidation of memory on various aversive learning tasks [7,10,11,65-70]. Such investigations have also provided valuable insight into the putative molecular machinery underlying the modulatory influence of glucocorticoids on neural plasticity within this memory network [71-75]. However, although it is now well established that appetitive and rewarding experiences also induce the release of glucocorticoid hormones from the adrenal cortex [16-18], only a few prior studies, using either animals or humans, have investigated a possible involvement of glucocorticoids in memory consolidation of positively motivated learning. In general agreement with the present findings, Zorawski and Killcross (2002) reported that systemic administration of dexamethasone, a synthetic glucocorticoid, to rats enhances memory consolidation of both an appetitive and aversive Pavlovian conditioning procedure [21]. Moreover, intraventricular or intrahippocampal administration of corticosterone was shown to enhance memory on an appetitive operant conditioning task in mice [19,20]. A small number of studies in human subjects indicated that stress exposure or the administration of cortisol, given either shortly before or after learning, enhances memory for arousing pleasant words and pictures to a similar extent as it does for arousing negatively valenced material [53,63]. Although none of these previous studies investigated the underlying brain mechanism, this prior evidence supports our present findings that glucocorticoids enhance the consolidation of memory of both appetitive and aversive training experiences and, thus, that the level of arousal associated with the learning experience tends to be a better prospect for memory strength than stimulus valence [1].

Our current finding that noradrenergic activity within the NAc is essential for enabling the effects of GR agonist administration on memory consolidation of both appetitive and aversive taste learning is congruent with extensive prior evidence indicating that

arousal-induced noradrenergic activation is indispensable for mediating the enhancing effects of glucocorticoids on memory consolidation of a variety of emotionally negative learning experiences [4,13,14,50,51]. However, this glucocorticoid dependence on noradrenergic activity had never been investigated within the NAc or in regard to glucocorticoid effects on memory of appetitive learning experiences. Previous studies in the basolateral amygdala (but also prefrontal and insular cortices) investigating the neural mechanism underlying this permissive interaction have shown an involvement of a rapid, nongenomically mediated action of glucocorticoids on the noradrenergic system [13-15,51,52,75-78]. Such a glucocorticoid-induced facilitation of noradrenergic transmission led to downstream CREB protein activation and changes in neuronal plasticity and memory [15,51,76,78]. As norepinephrine signaling is low during non-arousing conditions, this mechanism of interaction could thus explain why glucocorticoids might selectively affect memory formation of emotionally arousing experiences and have little effect on memory for neutral material [3,4,53,63,77]. As both highly rewarding and aversive emotionally arousing experiences are known to induce the release of norepinephrine into different brain regions, including the NAc [79-84], these findings suggest that a similar mechanism might underlie glucocorticoid effects on memory of appetitive taste learning. However, recent findings suggested that the interaction between glucocorticoids and norepinephrine, particularly within the amygdala, might lead to a facilitated processing of negative stimuli at the expense of positive stimuli [85-87] and that heightened amygdala activation is linked to an implicit negative cognitive bias [88]. This negative bias suggests that glucocorticoids in conjunction with noradrenergic acitivity give rise to a valence-specific facilitation of emotionally negative information. Our present findings indicating that a suppression of noradrenergic activity within the NAc shell prevented GR agonist-induced enhancement of memory on an appetitive as well as aversive taste learning task clearly do not support this view. Rather, they suggest that glucocorticoid-induced enhancement of memory consolidation for positively and negatively motivated emotionally arousing learning experiences might depend on a highly comparable recruitment of the noradrenergic system. Although we cannot exclude the possibility of brain region-specific differences between the NAc and basolateral amygdala, recent findings from our laboratory indicate that a GR agonist administered into the basolateral amygdala induces a near identical enhancement of long-term memory of appetitive taste learning (Wichmann & Roozendaal unpublished observations).

As the recollection of highly appetitive experiences has the same evolutionary significance as the remembrance of highly aversive stimuli, a privileged storage of highly affective stimuli or experiences regardless of valence is advantageous. At present, it is highly debated whether the role of arousal within the larger emotional memory network may differ depending on valence [89,90]. The NAc has a well-established role in processing rewards and reward-associated stimuli [24-26]. Emerging evidence from functional neuroimaging studies additionally associates the NAc with reward-related learning [91,92]. Some studies suggest that this brain region is exclusively involved in

reward-related processing [93,96]. However, our findings showing that local activation of GRs within the NAc shell enhances the memory of an appetitive as well as aversive taste learning experience fit better with a potential broad involvement of the NAc in the detection of biological significant experiences [97]. Substantial evidence indicates that the NAc encodes the emotional intensity (salience) and arousing properties of stimuli, independent of their valence [98-102]. As the core and shell subregion of the NAc differ substantially in their efferent connections [22,103], this raises the possibility of functionally independent neuronal networks [103,104]. Whereas the core projects mainly to the basal gangliacircuitry, the shell projects extensively to subcortical limbic structures [105]. Hence, especially the NAc shell is functionally and anatomically well positioned to integrate the components of salience. It is a crucial element of the mesocorticolimbic dopaminergic system [106], and receives highly processed information from the basolateral amygdala, hippocampus and prefrontal cortex [22,23]. Earlier findings indicate that an intact NAc is essential for mediating glucocorticoid-induced memory enhancement of emotionally arousing information [27]. Lesions of the NAc block memory enhancement induced by a GR agonist administered into either the basolateral amygdala or hippocampus [8]. Thus, the NAc may integrate influences derived from these brain regions and subsequently convey this information to cortical areas for permanent storage [107]. The present findings indicate that the NAc is not just processing information of new learning experiences coming from other brain regions but that the NAc itself is an important target for mediating the modulatory effects of glucocorticoids (and norepinephrine) on memory consolidation of emotionally arousing experiences.

In conclusion, the present findings indicating that GR agonist administration into the NAc shell enhances the consolidation of memory of both appetitive and aversive taste learning adds to the view that adrenal stress hormones play a critical role in consolidating lasting memories. Additionally, and importantly, the present findings provide evidence that the interaction of glucocorticoids with the noradrenergic system is not restricted to facilitating memory of aversive stimuli but that it might be equally involved in consolidating memories of appetitive emotionally arousing learning experiences.

REFERENCES

1. Bradley, M. M., Greenwald, M. K., Petry, M. C. & Lang, P. J. Remembering pictures: pleasure and arousal in memory. *Journal of Experimental Psychology: Learning, Memory, and Cognition* **18**, 379–390 (1992).
2. McGaugh, J. L. Make mild moments memorable: add a little arousal. *Trends in Cognitive Sciences* **10**, 345–347 (2006).
3. Okuda, S., Roozendaal, B. & McGaugh, J. Glucocorticoid effects on object recognition memory require training-associated emotional arousal. *Proceedings of the National Academy of Sciences* **101**, 853–858 (2004).
4. Abercrombie, H., Speck, N. & Monticelli, R. Endogenous cortisol elevations are related to memory facilitation only in individuals who are emotionally aroused. *Psychoneuroendocrinology* **31**, 187–196 (2006).
5. Schwabe, L., Joëls, M., Roozendaal, B., Wolf, O. T. & Oitzl, M. S. Stress effects on memory: An update and integration. *Neuroscience and Biobehavioral Reviews* 1–10 (2011).doi:10.1016/j.neubiorev.2011.07.002
6. Roozendaal, B. & McGaugh, J. L. Memory modulation. *Behavioral Neuroscience* **125**, 797–824 (2011).
7. Roozendaal, B. & McGaugh, J. L. Glucocorticoid receptor agonist and antagonist administration into the basolateral but not central amygdala modulates memory storage. *Neurobiology of Learning and Memory* **67**, 176–179 (1997).
8. Roozendaal, B., de Quervain, D. J., Ferry, B., Setlow, B. & McGaugh, J. L. Basolateral amygdala-nucleus accumbens interactions in mediating glucocorticoid enhancement of memory consolidation. *Journal of Neuroscience* **21**, 2518–2525 (2001).
9. Roozendaal, B., McEwen, B. S. & Chattarji, S. Stress, memory and the amygdala. *Nature Reviews Neuroscience* **10**, 423–433 (2009).
10. Miranda, M. I., Quirarte, G. L., Rodriguez-Garcia, G., McGaugh, J. L. & Roozendaal, B. Glucocorticoids enhance taste aversion memory via actions in the insular cortex and basolateral amygdala. *Learning & Memory* **15**, 468–476 (2008).
11. Quirarte, G. L. *et al.* Corticosterone infused into the dorsal striatum selectively enhances memory consolidation of cued water-maze training. *Learning & Memory* **16**, 586–589 (2009).
12. Fornari, R. V. *et al.* Involvement of the insular cortex in regulating glucocorticoid effects on memory consolidation of inhibitory avoidance training. *Frontiers in Behavioral Neuroscience* **6**, (2012).
13. Quirarte, G. L., Roozendaal, B. & McGaugh, J. L. Glucocorticoid enhancement of memory storage involves noradrenergic activation in the basolateral amygdala. *Proceedings of the National Academy of Sciences* **94**, 14048–14053 (1997).
14. Roozendaal, B., Okuda, S., de Quervain, D. & McGaugh, J. Glucocorticoids interact with emotion-induced noradrenergic activation in influencing different memory functions. *Neuroscience* **138**, 901–910 (2006).
15. Barsegyan, A., Mackenzie, S. M., Kurose, B. D., McGaugh, J. L. & Roozendaal, B. Glucocorticoids in the prefrontal cortex enhance memory consolidation and impair working memory by a common neural mechanism. *Proceedings of the National Academy of Sciences* **107**, 16655–16660 (2010).
16. Piazza, P. V. & Le Moal, M. Glucocorticoids as a biological substrate of reward: physiological and pathophysiological implications. *Brain Research Reviews* **25**, 359–372 (1997).
17. Koolhaas, J. M. *et al.* Stress revisited: A critical evaluation of the stress concept. *Neuroscience and Biobehavioral Reviews* **35**, 1291–1301 (2011).
18. Buwalda, B., Scholte, J., de Boer, S. F., Coppens, C. M. & Koolhaas, J. M. The acute glucocorticoid stress response does not differentiate between rewarding and aversive social stimuli in rats. *Hormones and Behavior* **61**, 218–226 (2012).
19. Micheau, J., Destrade, C. & Soumireu-Mourat, B. Intraventricular corticosterone injection facilitates memory of an appetitive discriminative task in mice. *Behavioral and Neural Biology* **31**, 100–104 (1981).
20. Micheau, J., Destrade, C. & Soumireu-Mourat, B. Time-dependent effects of posttraining intrahippocampal injections of corticosterone on retention of appetitive learning tasks in mice. *European Journal of Pharmacology* **106**, 39–46 (1985).
21. Zorawski, M. & Killcross, S. Posttraining glucocorticoid receptor agonist enhances memory in appetitive and aversive Pavlovian discrete-cue conditioning paradigms. *Neurobiology of Learning and Memory* **78**, 458–464 (2002).
22. Groenewegen, H. J., Wright, C. I., Beijer, A. V. & Voorn, P. Convergence and segregation of ventral striatal inputs and outputs. *Annals of the New York Academy of Sciences* **877**, 49–63 (1999).

23. Zahm, D. S. An integrative neuroanatomical perspective on some subcortical substrates of adaptive responding with emphasis on the nucleus accumbens. *Neuroscience and Biobehavioral Reviews* **24**, 85–105 (2000).
24. Robbins, T. W. & Everitt, B. J. Neurobehavioural mechanisms of reward and motivation. *Current Opinion in Neurobiology* **6**, 228–236 (1996).
25. Salamone, J. D. The behavioral neurochemistry of motivation: methodological and conceptual issues in studies of the dynamic activity of nucleus accumbens dopamine. *Journal of Neuroscience Methods* **64**, 137–149 (1996).
26. Wise, R. A. Dopamine, learning and motivation. *Nature Reviews Neuroscience* **5**, 483–494 (2004).
27. Setlow, B., Roozendaal, B. & McGaugh, J. L. Involvement of a basolateral amygdala complex–nucleus accumbens pathway in glucocorticoid-induced modulation of memory consolidation. *European Journal of Neuroscience* **12**, 367–375 (2000).
28. McGaugh, J. L. The amygdala modulates the consolidation of memories of emotionally arousing experiences. *Annual Review of Neuroscience* **27**, 1–28 (2004).
29. Parkinson, J. A. *et al.* Nucleus accumbens dopamine depletion impairs both acquisition and performance of appetitive Pavlovian approach behaviour: implications for mesoaccumbens dopamine function. *Behavioural Brain Research* **137**, 149–163 (2002).
30. Hernandez, P. J., Sadeghian, K. & Kelley, A. E. Early consolidation of instrumental learning requires protein synthesis in the nucleus accumbens. *Nature Neuroscience* **5**, 1327–1331 (2002).
31. Dalley, J. W. *et al.* Time-limited modulation of appetitive Pavlovian memory by D1 and NMDA receptors in the nucleus accumbens. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 6189–6194 (2005).
32. Seamans, J. K. & Phillips, A. G. Selective memory impairments produced by transient lidocaine-induced lesions of the nucleus accumbens in rats. *Behavioral Neuroscience* **108**, 456–468 (1994).
33. Ploeger, G. E., Spruijt, B. M. & Cools, A. R. Spatial localization in the Morris water maze in rats: acquisition is affected by intra-accumbens injections of the dopaminergic antagonist haloperidol. *Behavioral Neuroscience* **108**, 927–934 (1994).
34. Fadok, J. P., Darvas, M., Dickerson, T. M. K. & Palmiter, R. D. Long-term memory for pavlovian fear conditioning requires dopamine in the nucleus accumbens and basolateral amygdala. *PLoS ONE* **5**, e12751 (2010).
35. Ramírez-Lugo, L., Zavala-Vega, S. & Bermudez-Rattoni, F. NMDA and muscarinic receptors of the nucleus accumbens have differential effects on taste memory formation. *Learning & Memory* **13**, 45–51 (2006).
36. Pedroza-Llinás, R., Ramírez-Lugo, L., Guzmán-Ramos, K., Zavala-Vega, S. & Bermudez-Rattoni, F. Safe taste memory consolidation is disrupted by a protein synthesis inhibitor in the nucleus accumbens shell. *Neurobiology of Learning and Memory* 1–8 (2009).doi:10.1016/j.nlm.2009.02.011
37. Mark, G. P., Blander, D. S. & Hoebel, B. G. A conditioned stimulus decreases extracellular dopamine in the nucleus accumbens after the development of a learned taste aversion. *Brain Research* **551**, 308–310 (1991).
38. Fenu, S., Bassareo, V. & Di Chiara, G. A role for dopamine D1 receptors of the nucleus accumbens shell in conditioned taste aversion learning. *Journal of Neuroscience* **21**, 6897–6904 (2001).
39. Ferreira, G., Ferry, B., Meurisse, M. & Lévy, F. Forebrain structures specifically activated by conditioned taste aversion. *Behavioral Neuroscience* **120**, 952–962 (2006).
40. Bermudez-Rattoni, F. Molecular mechanisms of taste-recognition memory. *Nature Reviews Neuroscience* **5**, 209–217 (2004).
41. Shema, R. *et al.* Enhancement of Consolidated Long-Term Memory by Overexpression of Protein Kinase M in the Neocortex. *Science* **331**, 1207–1210 (2011).
42. Garcia, J., Kimeldorf, D. J. & Koelling, R. A. Conditioned aversion to saccharin resulting from exposure to gamma radiation. *Science* **122**, 157–158 (1955).
43. Fornari, R. V. *et al.* Rodent stereotaxic surgery and animal welfare outcome improvements for behavioral neuroscience. *J Vis Exp* (2012). doi:10.3791/3528
44. Paxinos, G. & Watson, C. *The Rat Brain in Stereotaxic Coordinates.* (Amsterdam: Elsevier: 2007).
45. Domjan, M. & Gillan, D. Role of novelty in the aversion for increasingly concentrated saccharin solutions. *Physiology & Behavior* **16**, 537–542 (1976).
46. Nuñez-Jaramillo, L., Rangel-Hernández, J. A., Burgueño-Zúñiga, B. & Miranda, M. I. Activation of nucleus accumbens NMDA receptors differentially affects appetitive or aversive taste learning and memory. *Frontiers in Behavioral Neuroscience* **6**, (2012).
47. Stehberg, J., Moraga-Amaro, R. & Simon, F. The role of the insular cortex in taste function.

Neurobiology of Learning and Memory 1–6 (2011).doi:10.1016/j.nlm.2011.03.005

48. Teutsch, G. *et al.* 17 alpha-alkynyl-11 beta, 17-dihydroxyandrostane derivatives : a new class of potent glucocorticoids. *Steroids* **38**, 651–665 (1981).
49. LaLumiere, R. T., Nawar, E. M. & McGaugh, J. L. Modulation of memory consolidation by the basolateral amygdala or nucleus accumbens shell requires concurrent dopamine receptor activation in both brain regions. *Learning & Memory* **12**, 296–301 (2005).
50. Roozendaal, B., Nguyen, B. T., Power, A. E. & McGaugh, J. L. Basolateral amygdala noradrenergic influence enables enhancement of memory consolidation induced by hippocampal glucocorticoid receptor activation. *Proceedings of the National Academy of Sciences* **96**, 11642–11647 (1999).
51. Roozendaal, B., Okuda, S., Van der Zee, E. A. & McGaugh, J. L. Glucocorticoid enhancement of memory requires arousal-induced noradrenergic activation in the basolateral amygdala. *Proceedings of the National Academy of Sciences* **103**, 6741–6746 (2006).
52. Roozendaal, B. *et al.* Basolateral amygdala noradrenergic activity mediates corticosterone-induced enhancement of auditory fear conditioning. *Neurobiology of Learning and Memory* **86**, 249–255 (2006).
53. Kuhlmann, S. & Wolf, O. T. Arousal and cortisol interact in modulating memory consolidation in healthy young men. *Behavioral Neuroscience* **120**, 217–223 (2006).
54. McGaugh, J. L. Time-dependent processes in memory storage. *Science* **153**, 1351–1358 (1966).
55. McGaugh, J. L. & Roozendaal, B. Drug enhancement of memory consolidation: historical perspective and neurobiological implications. *Psychopharmacology* **202**, 3–14 (2009).
56. Nuñez-Jaramillo, L., Delint-Ramirez, I. & Bermudez-Rattoni, F. PKC blockade differentially affects aversive but not appetitive gustatory memories. *Brain Research* **1148**, 177–182 (2007).
57. Siegel, S. Flavor preexposure and "learned safety". *J Comp Physiol Psychol* **87**, 1073–1082 (1974).
58. Rozin, P. & Kalat, J. Specific hungers and poison avoidance as adaptive specializations of learning. *Psychological Review* (1971).
59. Carroll, M., Dinc, H. & Levy, C. Demonstrations of neophobia and enhanced neophobia in the albino rat. *J Comp Physiol Psychol* (1975).
60. Cai, W. H., Blundell, J., Han, J., Greene, R. W. & Powell, C. M. Postreactivation Glucocorticoids Impair Recall of Established Fear Memory. *Journal of Neuroscience* **26**, 9560–9566 (2006).
61. Yang, Y.-L., Chao, P.-K. & Lu, K.-T. Systemic and Intra-Amygdala Administration of Glucocorticoid Agonist and Antagonist Modulate Extinction of Conditioned Fear. *Neuropsychopharmacology* **31**, 912–924 (2006).
62. Smotherman, W. P. Glucocorticoid and other hormonal substrates of conditioned taste aversion. *Annals of the New York Academy of Sciences* **443**, 126–144 (1985).
63. Buchanan, T. W. & Lovallo, W. R. Enhanced memory for emotional material following stress-level cortisol treatment in humans. *Psychoneuroendocrinology* **26**, 307–317 (2001).
64. Versace, F., Bradley, M. M. & Lang, P. J. Memory and event-related potentials for rapidly presented emotional pictures. *Exp Brain Res* **205**, 223–233 (2010).
65. Flood, J. F. *et al.* Memory facilitating and anti-amnesic effects of corticosteroids. *Pharmacology, Biochemistry and Behavior* **8**, 81–87 (1978).
66. Oitzl, M. S. & de Kloet, E. R. Selective corticosteroid antagonists modulate specific aspects of spatial orientation learning. *Behavioral Neuroscience* **106**, 62–71 (1992).
67. Cordero, M. I. & Sandi, C. A role for brain glucocorticoid receptors in contextual fear conditioning: dependence upon training intensity. *Brain Research* **786**, 11–17 (1998).
68. Hui, G. K. *et al.* Memory enhancement of classical fear conditioning by post-training injections of corticosterone in rats. *Neurobiology of Learning and Memory* **81**, 67–74 (2004).
69. Donley, M. P., Schulkin, J. & Rosen, J. B. Glucocorticoid receptor antagonism in the basolateral amygdala and ventral hippocampus interferes with long-term memory of contextual fear. *Behavioural Brain Research* **164**, 197–205 (2005).
70. Medina, A. C. *et al.* Glucocorticoid administration into the dorsal stratium facilitates memory consolidation of inhibitory avoidance training but not of the context or footshock components. *Learning & Memory* **14**, 673–677 (2007).
71. Sandi, C. The role and mechanisms of action of glucocorticoid involvement in memory storage. *Neural Plast.* **6**, 41–52 (1998).
72. Karst, H. *et al.* Glucocorticoids alter calcium conductances and calcium channel subunit expression in basolateral amygdala neurons. *Eur. J. Neurosci.* **16**, 1083–1089 (2002).

73. Revest, J.-M. *et al.* The MAPK pathway and Egr-1 mediate stress-related behavioral effects of glucocorticoids. *Nature Neuroscience* **8**, 664-672 (2005).

74. Duvarci, S. & Paré, D. Glucocorticoids enhance the excitability of principal basolateral amygdala neurons. *Journal of Neuroscience* **27**, 4482-4491 (2007).

75. McReynolds, J. R. *et al.* Memory-enhancing corticosterone treatment increases amygdala norepinephrine and Arc protein expression in hippocampal synaptic fractions. *Neurobiology of Learning and Memory* **93**, 312-321 (2010).

76. Roozendaal, B., Quirarte, G. L. & McGaugh, J. L. Glucocorticoids interact with the basolateral amygdala beta-adrenoceptor--cAMP/cAMP/PKA system in influencing memory consolidation. *European Journal of Neuroscience* **15**, 553-560 (2002).

77. van Stegeren, A. H. *et al.* Endogenous cortisol level interacts with noradrenergic activation in the human amygdala. *Neurobiology of Learning and Memory* **87**, 57-66 (2007).

78. Roozendaal, B. *et al.* Membrane-Associated Glucocorticoid Activity Is Necessary for Modulation of Long-Term Memory via Chromatin Modification. *Journal of Neuroscience* **30**, 5037-5046 (2010).

79. Galvez, R., Mesches, M. H. & McGaugh, J. L. Norepinephrine release in the amygdala in response to footshock stimulation. *Neurobiology of Learning and Memory* **66**, 253-257 (1996).

80. Feenstra, M. G., Teske, G., Botterblom, M. H. & De Bruin, J. P. Dopamine and noradrenaline release in the prefrontal cortex of rats during classical aversive and appetitive conditioning to a contextual stimulus: interference by novelty effects. *Neurosci. Lett.* **272**, 179-182 (1999).

81. McIntyre, C. K., Hatfield, T. & McGaugh, J. L. Amygdala norepinephrine levels after training predict inhibitory avoidance retention performance in rats. *European Journal of Neuroscience* **16**, 1223-1226 (2002).

82. Mingote, S. Noradrenaline and dopamine efflux in the prefrontal cortex in relation to appetitive classical conditioning. *Journal of Neuroscience* **24**, 2475-2480 (2004).

83. Ventura, R., Morrone, C. & Puglisi-Allegra, S. Prefrontal/accumbal catecholamine system determines motivational salience attribution to both reward-and aversion-related stimuli. *Proceedings of the National Academy of Sciences* **104**, 5181 (2007).

84. Kerfoot, E. C. & Williams, C. L. Interactions between brainstem noradrenergic neurons and the nucleus accumbens shell in modulating memory for emotionally arousing events. *Learning & Memory* **18**, 405-413 (2011).

85. Kukolja, J. *et al.* Modeling a Negative Response Bias in the Human Amygdala by Noradrenergic-Glucocorticoid Interactions. *Journal of Neuroscience* **28**, 12868-12876 (2008).

86. Enkel, T. *et al.* Ambiguous-cue interpretation is biased under stress- and depression-like states in rats. *Neuropsychopharmacology* **35**, 1008-1015 (2010).

87. Harmer, C. J., Hill, S. A., Taylor, M. J., Cowen, P. J. & Goodwin, G. M. Toward a neuropsychological theory of antidepressant drug action: increase in positive emotional bias after potentiation of norepinephrine activity. *American Journal of Psychiatry* **160**, 990-992 (2003).

88. Dannlowski, U. *et al.* Amygdala reactivity predicts automatic negative evaluations for facial emotions. *Psychiatry Research* **154**, 13-20 (2007).

89. Hamann, S. B., Ely, T. D., Hoffman, J. M. & Kilts, C. D. Ecstasy and agony: activation of the human amygdala in positive and negative emotion. *Psychol Sci* **13**, 135-141 (2002).

90. Mickley Steinmetz, K. R., Addis, D. R. & Kensinger, E. A. The effect of arousal on the emotional memory network depends on valence. *Neuroimage* **53**, 318-324 (2010).

91. Delgado, M. Reward-Related Responses in the Human Striatum. *Annals of the New York Academy of Sciences* **1104**, 70-88 (2007).

92. Costa, V. D., Lang, P. J., Sabatinelli, D., Versace, F. & Bradley, M. M. Emotional imagery: Assessing pleasure and arousal in the brain's reward circuitry. *Human Brain Mapping* **31**, 1446-1457 (2010).

93. Breiter, H. C., Aharon, I., Kahneman, D., Dale, A. & Shizgal, P. Functional imaging of neural responses to expectancy and experience of monetary gains and losses. *Neuron* **30**, 619-639 (2001).

94. Knutson, B., Adams, C. M., Fong, G. W. & Hommer, D. Anticipation of increasing monetary reward selectively recruits nucleus accumbens. *Journal of Neuroscience* **21**, RC159 (2001).

95. Knutson, B. & Cooper, J. C. Functional magnetic resonance imaging of reward prediction. *Current Opinion in Neurology* **18**, 411-417 (2005).

96. Sabatinelli, D., Bradley, M. M., Lang, P. J., Costa, V. D. & Versace, F. Pleasure Rather Than Salience Activates Human Nucleus Accumbens and Medial Prefrontal Cortex. *Journal of Neurophysiology* **98**, 1374-1379 (2007).

97. Setlow, B., Schoenbaum, G. & Gallagher, M. Neural encoding in ventral striatum during olfactory discrimination learning. *Neuron* **38**, 625–636 (2003).

98. Horvitz, J. C. Mesolimbocortical and nigrostriatal dopamine responses to salient non-reward events. *Neuroscience* **96**, 651–656 (2000).

99. Reynolds, S. M. & Berridge, K. C. Positive and negative motivation in nucleus accumbens shell: bivalent rostrocaudal gradients for GABA-elicited eating, taste "liking"/"disliking" reactions, place preference/avoidance, and fear. *Journal of Neuroscience* **22**, 7308–7320 (2002).

100. Zink, C. F., Pagnoni, G., Martin, M. E., Dhamala, M. & Berns, G. S. Human striatal response to salient nonrewarding stimuli. *Journal of Neuroscience* **23**, 8092–8097 (2003).

101. Phan, K. L. *et al*. Neural correlates of individual ratings of emotional salience: a trial-related fMRI study. *NeuroImage* **21**, 768–780 (2004).

102. Niznikiewicz, M. A. & Delgado, M. R. Two sides of the same coin: Learning via positive and negative reinforcers in the human striatum. *Developmental Cognitive Neuroscience* 1, 494–505 (2011).

103. Pennartz, C. M., Groenewegen, H. J. & Lopes da Silva, F. H. The nucleus accumbens as a complex of functionally distinct neuronal ensembles: an integration of behavioural, electrophysiological and anatomical data. *Progress in Neurobiology* **42**, 719–761 (1994).

104. Corbit, L. H., Muir, J. L. & Balleine, B. W. The role of the nucleus accumbens in instrumental conditioning: Evidence of a functional dissociation between accumbens core and shell. *Journal of Neuroscience* **21**, 3251–3260 (2001).

105. Zahm, D. S. & Brog, J. S. On the significance of subterritories in the "accumbens" part of the rat ventral striatum. *Neuroscience* **50**, 751–767 (1992).

106. Carlezon, W. A., Jr & Thomas, M. J. Biological substrates of reward and aversion: A nucleus accumbens activity hypothesis. *Neuropharmacology* 1–11 (2008).doi:10.1016/j.neuropharm.2008.06.075

107. Floresco, S. B., Blaha, C. D., Yang, C. R. & Phillips, A. G. Dopamine D1 and NMDA receptors mediate potentiation of basolateral amygdala-evoked firing of nucleus accumbens neurons. *Journal of Neuroscience* **21**, 6370–6376 (2001).

5

GLUCOCORTICOID ADMINISTRATION INTO THE BASOLATERAL AMYGDALA ENHANCES MEMORY CONSOLIDATION OF AN APPETITIVE TASTE LEARNING EXPERIENCE

Romy Wichmann [1]
Rodrigo Moraga-Amaro [1,2]
Jimmy Stehberg [1,2]
Benno Roozendaal [1]

[1]Department of Neuroscience, Section Anatomy, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

[2] Laboratorio de Neurobiologia, Departamento de Ciencias Biologicas, Facultad de Ciencias Biologicas & Facultad de Medicina, Universidad Andres Bello, Santiago, Chile

Manuscript in preparation

ABSTRACT

The basolateral complex of the amygdala (BLA) plays a central role in regulating the enhancing effects of glucocorticoid hormones on the consolidation of memory of aversively motivated learning. It is currently unknown, however, whether the BLA is also involved in mediating glucocorticoid effects on memory consolidation of appetitively motivated learning. Here, we report that the specific glucocorticoid receptor agonist RU 28362 administered bilaterally into the BLA immediately after ingestion of an appetitive saccharin drinking solution enhanced 24-h retention of the novel taste learning experience, as assessed by a facilitated attenuation of neophobia response. The findings suggest that glucocorticoids in the BLA induce strengthening of the consolidation of memory of emotionally arousing experiences regardless of stimulus valence.

It is well established that emotionally arousing experiences are typically remembered more vividly and with more detail than similar neutral experiences [1]. Animal and human studies investigating the neural mechanisms underlying this memory facilitation indicate that glucocorticoid hormones, released from the adrenal cortex during arousing episodes, are crucially involved in enhancing the consolidation of long-term memory of emotionally arousing experiences [2-5]. There is extensive evidence that glucocorticoid effects on memory consolidation are mediated, at least in part, via an activation of glucocorticoid receptors (GRs) within the basolateral complex of the amygdala (BLA) [5,6]. Prior studies indicate that corticosterone or a specific GR agonist administered into the BLA, but not the adjacent central amygdala, immediately after training on a wide variety of aversively motivated and/or fear-related tasks enhances the consolidation of memory of this training [7-11]. Conversely, selective lesions of the BLA or a blockade of glucocorticoid action within the BLA, via GR antagonists, prevent memory enhancement induced by systemically administered glucocorticoids [7,12,13]. Further studies reporting that arousal-induced noradrenergic activity within the BLA is required for enabling glucocorticoid effects on memory consolidation [14,15] might explain why glucocorticoids selectively affect memory formation of experiences that are emotionally arousing [3,15,16].

Despite extensive evidence that the release of endogenous glucocorticoids is not limited to negatively motivated experiences but is also initiated in response to appetitive and rewarding experiences [17-19], only a few studies, either in animals or humans, investigated the effects of glucocorticoids on the consolidation of memory of positively motivated learning experiences. Generally, these studies indicate that systemically administered glucocorticoids induce a highly comparable strengthening of the consolidation of appetitive memory in rodents [20-22] and of positively valenced material in human subjects [2,4]. However, a possible involvement of the BLA in mediating such glucocorticoid effects on memory consolidation of positively motivated learning has not, as yet, been investigated. To address this issue, the present experiment investigated whether the specific GR agonist RU 28362 administered into the BLA immediately after an appetitive taste learning experience enhances the consolidation of memory of this training. All experimental procedures were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Institutional Animal Care and Use Committee of the University of Groningen, The Netherlands.

Male adult Sprague-Dawley rats (300 - 350 g) from Charles River Breeding Laboratories (Kisslegg, Germany) were housed individually for at least 7 d before surgery for cannula implantation. For a detailed description of surgical procedures, see Fornari et al (2012)[23]. Briefly, rats were anesthetized with a subcutaneous injection of ketamine (37.5 mg/kg; Alfasan), dexmedetomidine (0.25 mg/kg; Orion) and atropine sulfate (0.4 mg/kg; Pharmachemie BV) and their head was positioned in a stereotaxic apparatus (Kopf Instruments). Two stainless-steel guide cannulae (15 mm long; 23 gauge; Small Parts Inc) were implanted bilaterally 2.0 mm above the BLA [coordinates: anteroposterior,

3.0 mm posterior to Bregma; mediolateral, ±5.0 mm from midline; dorsoventral, 6.4 mm below skull surface] according to the atlas of Paxinos and Watson (2007). Stylets (15-mm long 00-insect pins) were inserted into each cannula to maintain patency. The rats were allowed to recover for a minimum of 7 d before initiation of training.

For training on the appetitive taste learning task, rats were initially deprived of water for 24 h and then habituated to drink water for 20 min/d for 5 d. On the acquisition trial, rats were allowed to freely drink an appetitive 0.5% saccharin solution (saccharin sodium salt hydrate, Sigma-Aldrich; novel taste) for 20 min in their home cages and immediately afterwards were given bilateral microinfusions of either the specific GR agonist RU 28362 [11β,17β-dihydroxy-6,21-dimethyl-17α-pregna-4,6-trien-20yn-3-one; a generous gift of Aventis, Frankfurt, Germany] (1 or 3 ng in 0.2 μl) or vehicle into the BLA. Receptor binding studies have shown that this compound has selective and high affinity for GRs [24]. RU 28362 was first dissolved in 100% ethanol and subsequently diluted in saline. The final ethanol concentration was 0.5%. Drug doses were chosen on the basis of previous findings [8,25] and the drug volume and infusion sites selected are known to selectively influence the BLA division of the amygdala [8,26,27]. Thirty-gauge injection needles (17 mm) were connected to 10-μl Hamilton microsyringes with polyethylene tubing and inserted into the BLA, protruding 2 mm beyond the cannula tip. A 0.2-μl volume of vehicle or drug per hemisphere was infused over 25 s. The injection needles were retained in place for an additional 30 s following drug infusion and the rat was then returned to its home cage. On the 24-h retention test, rats had access to the same saccharin concentration for 20 min and the volume drunk was recorded. If the taste is remembered as being safe, the animal will increase its consumption during the retention test, a process termed attenuation of neophobia (AN) [28]. An AN index was calculated as the saccharin consumption during the retention test relative to the acquisition trial. A larger AN index was interpreted as indicating better retention.

Following completion of behavioral testing, rats were perfused transcardially with isotonic saline followed by 4% formaldehyde and the brains were removed and stored in a 4% formaldehyde solution. Coronal sections of 50 μm were cut on a cryostat, mounted on gelatin-coated glass slides and stained with cresyl violet. Rats with injection needle placements outside the BLA or with extensive tissue damage at the injection needle tips were excluded from statistical analysis. Fig. 1A and B show cannula placement within the BLA.

One-way ANOVA for mean baseline water intake revealed no significant differences between later drug treatment groups [$F(2,28) = 0.13$; $p = 0.88$]. Novel saccharin ingestion also did not differ between groups [$F(2,28) = 2.25$; $p = 0.13$] and all rats showed a robust neophobic response, as assessed by a significantly lower intake of the novel saccharin solution compared to water intake the previous day ($p < 0.0001$). As is shown in Fig. 2, one-way ANOVA for AN index, i.e., saccharin consumption on the retention test relative to the acquisition trial, indicated a significant drug effect [$F(2,28) = 4.68$; $p = 0.02$]. One-sample *t*-test revealed that the AN index of rats treated

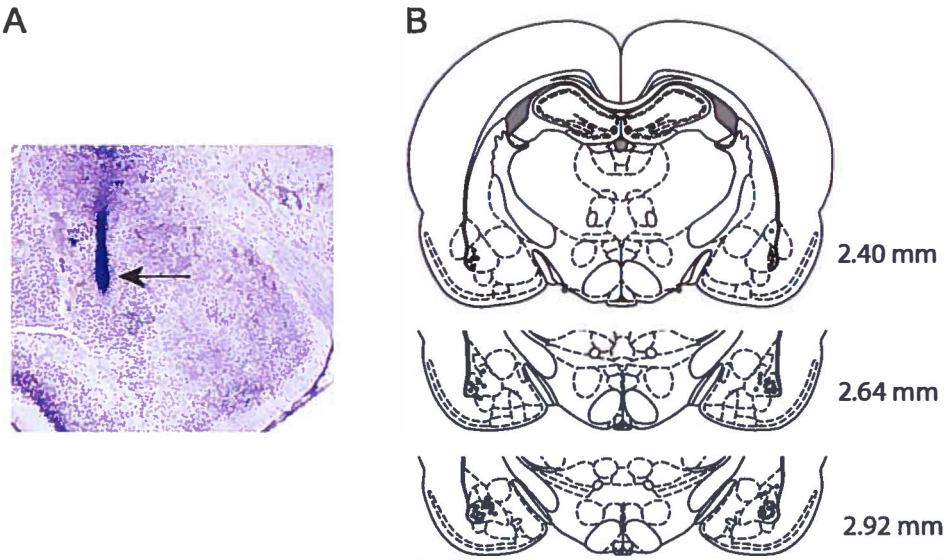


Figure 1. Representative photomicrograph (A) and diagrams (B) illustrating placement of cannulae and needle tips in the basolateral amygdala (BLA). (A) Arrow points to the infusion needle tip. (B) Dots represent the areas considered as correct infusion needle tip placements.

with vehicle was significantly larger than 1 (2.76 ± 0.24; $p < 0.0001$), indicating that control rats retained memory of the appetitive taste stimulus. Post-hoc analysis with Fisher's LSD test revealed that posttraining infusions of the GR agonist into the BLA dose-dependently enhanced retention of the appetitive taste learning experience. The AN index of rats given the lower dose of RU 28362 (1 ng) was significantly larger than that of vehicle control rats ($p < 0.01$). In contrast, the higher dose (3 ng) did not induce significant retention enhancement ($p = 0.94$).

The present findings indicate that the BLA is involved in regulating the facilitating effects of glucocorticoids on memory consolidation of appetitive training experiences. We found that the specific GR agonist RU 28362 administered bilaterally into the BLA immediately after ingestion of an appetitive saccharin drinking solution induced dose-dependent enhancement of long-term retention of this safe taste experience. To our knowledge, this is the first report relating glucocorticoid action within the BLA to memory enhancement of appetitive learning experiences. These findings appear thus highly comparable to an extensive literature investigating the effects of glucocorticoids within the BLA in modulating memory consolidation of aversively motivated training. Corticosterone or specific GR agonists administered into the BLA immediately, but not several hours, after training, are known to enhance the consolidation of memory of aversively motivated and/or fear-related training experiences [8-11,29], whereas selective lesions of the BLA or an infusion of a GR antagonist into the BLA prevent memory

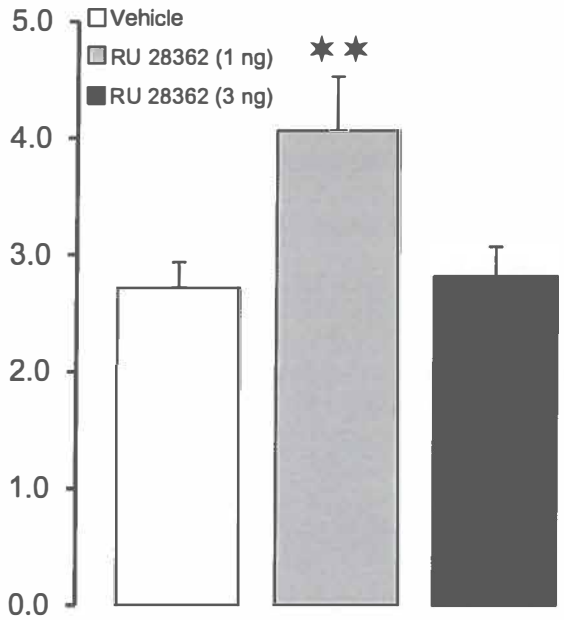


Figure 2. Effect of the glucocorticoid receptor agonist RU 28362 administered posttraining into the basolateral amygdala (BLA) on memory consolidation of appetitive taste learning. RU 28362 (1 or 3 ng in 0.2 µl) administered into the BLA immediately after novel saccharin drinking enhanced 24-h retention performance, as indicated by an increased attenuation of neophobia (AN). The AN index (mean ± SEM) is expressed as saccharin drinking on the retention test relative to the acquisition trial (n = 10-11 per group). **p < 0.01 compared with the vehicle group.

enhancement induced by systemically administered glucocorticoids [7,8,13,30]. Moreover, glucocorticoids have been reported to increase the activity of BLA pyramidal cells [31,32] and augment learning-associated neural plasticity within the BLA [33,34]. The present finding showing that local activation of GRs within the BLA immediately after appetitive taste learning enhances safe taste memory consolidation suggests that the BLA might have a similar role in regulating the memory-facilitatory effects of glucocorticoids on appetitively motivated emotionally arousing experiences. This contravenes recent findings suggesting that glucocorticoids, in conjunction with noradrenergic activity, give rise to a valence-specific facilitation of emotionally negative information, likely via heightened amygdala activation [35-37]. Our findings are consistent with the view suggesting a broad involvement of the amygdala in learning and memory of emotionally arousing experiences irrespective of the valence of the stimulus [1,38,39]. The amygdala's involvement in memory consolidation for emotionally unpleasant and fear-relevant stimuli is well established [40,41]. However, comparable effects have been obtained in experiments using appetitively motivated training experiences [42-44]. Further evidence implicating the amygdala also in the processing of positive stimuli comes from a growing number of human imaging and neuropsychological studies [38,45,46].

The BLA is a key structure in a memory-modulatory system that regulates stress and glucocorticoid effects on neural plasticity underlying the long-term storage of information within an integrated network of brain regions, involving among others the hippocampus [47], nucleus accumbens [29,48], insular cortex [49], entorhinal cortex [50] and medial prefrontal cortex [51]. Further support for the view that BLA activation induces memory storage processes in other brain regions comes from human imaging studies indicating enhanced functional connectivity between the amygdala and the above-mentioned target regions during successful encoding of emotional material [52-54]. Thus, exposure to emotionally arousing training experiences, by evoking glucocorticoid release from the adrenal cortex, might induce BLA activation in concert with effects on different target regions to create a brain state that promotes the long-term storage of these emotionally arousing events and thus preserves significant information [55]. Although it is now well established that arousal, induced by either positive or negative emotionally arousing stimuli, is essential for activation of the amygdala, there is still debate of whether the role of arousal in the larger emotional memory network may differ depending on valence [56,57]. It is hypothesized that arousal can have profoundly different effects on amygdala connectivity with other components of the emotion network, depending upon the valence of the information [51,57]. The effect of arousal on memory of positive items appears to be more focused on direct amygdala efferents whereas its influence on the encoding of negative items might be associated with more global effects, involving recruitment of additional brain regions outside the amygdala network 57. Thus, depending on the valence of the stimulus, the amygdala might interact with a different set of efferent brain regions in strengthening the consolidation of memories of appetitive and aversive experiences.

In summary, the present findings indicate that glucocorticoids within the BLA are importantly involved in modulating the consolidation of memory of appetitive learning experiences, comparable to their influence on aversively motivated learning. These findings support the notion that the amygdala enhances memory consolidation for events that are emotionally significant, regardless of whether the nature of the emotion is pleasant or aversive.

REFERENCES

1. McGaugh, J. L. The amygdala modulates the consolidation of memories of emotionally arousing experiences. *Annual Review of Neuroscience* **27**, 1–28 (2004).
2. Buchanan, T. W. & Lovallo, W. R. Enhanced memory for emotional material following stress-level cortisol treatment in humans. *Psychoneuroendocrinology* **26**, 307–317 (2001).
3. Okuda, S., Roozendaal, B. & McGaugh, J. Glucocorticoid effects on object recognition memory require training-associated emotional arousal. *Proceedings of the National Academy of Sciences* **101**, 853–858 (2004).
4. Kuhlmann, S. & Wolf, O. T. Arousal and cortisol interact in modulating memory consolidation in healthy young men. *Behavioral Neuroscience* **120**, 217–223 (2006).
5. Roozendaal, B. & McGaugh, J. L. Memory modulation. *Behavioral Neuroscience* **125**, 797–824 (2011).
6. Roozendaal, B., McEwen, B. S. & Chattarji, S. Stress, memory and the amygdala. *Nature Reviews Neuroscience* **10**, 423–433 (2009).
7. Roozendaal, B. & McGaugh, J. L. Amygdaloid nuclei lesions differentially affect glucocorticoid-induced memory enhancement in an inhibitory avoidance task. *Neurobiology of Learning and Memory* **65**, 1–8 (1996).
8. Roozendaal, B. & McGaugh, J. L. Glucocorticoid receptor agonist and antagonist administration into the basolateral but not central amygdala modulates memory storage. *Neurobiology of Learning and Memory* **67**, 176–179 (1997).
9. Roozendaal, B., Quirarte, G. L. & McGaugh, J. L. Glucocorticoids interact with the basolateral amygdala beta-adrenoceptor--cAMP/cAMP/PKA system in influencing memory consolidation. *European Journal of Neuroscience* **15**, 553–560 (2002).
10. Miranda, M. I., Quirarte, G. L., Rodriguez-Garcia, G., McGaugh, J. L. & Roozendaal, B. Glucocorticoids enhance taste aversion memory via actions in the insular cortex and basolateral amygdala. *Learning & Memory* **15**, 468–476 (2008).
11. Roozendaal, B. *et al.* Glucocorticoid Effects on Memory Consolidation Depend on Functional Interactions between the Medial Prefrontal Cortex and Basolateral Amygdala. *Journal of Neuroscience* **29**, 14299–14308 (2009).
12. Roozendaal, B. & McGaugh, J. L. Basolateral amygdala lesions block the memory-enhancing effect of glucocorticoid administration in the dorsal hippocampus of rats. *Eur. J. Neurosci.* **9**, 76–83 (1997).
13. Donley, M. P., Schulkin, J. & Rosen, J. B. Glucocorticoid receptor antagonism in the basolateral amygdala and ventral hippocampus interferes with long-term memory of contextual fear. *Behavioural Brain Research* **164**, 197–205 (2005).
14. Quirarte, G. L., Roozendaal, B. & McGaugh, J. L. Glucocorticoid enhancement of memory storage involves noradrenergic activation in the basolateral amygdala. *Proceedings of the National Academy of Sciences* **94**, 14048–14053 (1997).
15. Roozendaal, B., Okuda, S., Van der Zee, E. A. & McGaugh, J. L. Glucocorticoid enhancement of memory requires arousal-induced noradrenergic activation in the basolateral amygdala. *Proceedings of the National Academy of Sciences* **103**, 6741–6746 (2006).
16. Abercrombie, H., Speck, N. & Monticelli, R. Endogenous cortisol elevations are related to memory facilitation only in individuals who are emotionally aroused. *Psychoneuroendocrinology* **31**, 187–196 (2006).
17. Piazza, P. V. & Le Moal, M. Glucocorticoids as a biological substrate of reward: physiological and pathophysiological implications. *Brain Research Reviews* **25**, 359–372 (1997).
18. Koolhaas, J. M. *et al.* Stress revisited: A critical evaluation of the stress concept. *Neuroscience and Biobehavioral Reviews* **35**, 1291–1301 (2011).
19. Buwalda, B., Scholte, J., de Boer, S. F., Coppens, C. M. & Koolhaas, J. M. The acute glucocorticoid stress response does not differentiate between rewarding and aversive social stimuli in rats. *Hormones and Behavior* **61**, 218–226 (2012).
20. Micheau, J., Destrade, C. & Soumireu-Mourat, B. Intraventricular corticosterone injection facilitates memory of an appetitive discriminative task in mice. *Behavioral and Neural Biology* **31**, 100–104 (1981).
21. Micheau, J., Destrade, C. & Soumireu-Mourat, B. Time-dependent effects of posttraining intrahippocampal injections of corticosterone on retention of appetitive learning tasks in mice. *European Journal of Pharmacology* **106**, 39–46 (1985).
22. Zorawski, M. & Killcross, S. Glucocorticoid Receptor Agonist Enhances Pavlovian Appetitive Conditioning but Disrupts Outcome-Specific Associations. *Behavioral Neuroscience* **117**, 1453–1457 (2003).

23. Fornari, R. V. *et al.* Rodent stereotaxic surgery and animal welfare outcome improvements for behavioral neuroscience. *J Vis Exp* (2012). doi:10.3791/3528

24. Teutsch, G. *et al.* 17 alpha-alkynyl-11 beta, 17-dihydroxyandrostane derivatives : a new class of potent glucocorticoids. *Steroids* **38**, 651–665 (1981).

25. Barsegyan, A., Mackenzie, S. M., Kurose, B. D., McGaugh, J. L. & Roozendaal, B. Glucocorticoids in the prefrontal cortex enhance memory consolidation and impair working memory by a common neural mechanism. *Proceedings of the National Academy of Sciences* **107**, 16655–16660 (2010).

26. Roozendaal, B., Lengvilas, R., McGaugh, J. L., Civelli, O. & Reinscheid, R. K. Orphanin FQ/nociceptin interacts with the basolateral amygdala noradrenergic system in memory consolidation. *Learning & Memory* **14**, 29–35 (2007).

27. Campolongo, P. *et al.* Endocannabinoids in the rat basolateral amygdala enhance memory consolidation and enable glucocorticoid modulation of memory. *Proceedings of the National Academy of Sciences* **106**, 4888–4893 (2009).

28. Stehberg, J., Moraga-Amaro, R. & Simon, F. The role of the insular cortex in taste function. *Neurobiology of Learning and Memory* 1–6 (2011). doi:10.1016/j.nlm.2011.03.005

29. Roozendaal, B., de Quervain, D. J., Ferry, B., Setlow, B. & McGaugh, J. L. Basolateral amygdala-nucleus accumbens interactions in mediating glucocorticoid enhancement of memory consolidation. *Journal of Neuroscience* **21**, 2518–2525 (2001).

30. Roozendaal, B., Portillo-Marquez, G. & McGaugh, J. L. Basolateral amygdala lesions block glucocorticoid-induced modulation of memory for spatial learning. *Behavioral Neuroscience* **110**, 1074–1083 (1996).

31. Pelletier, J. G. Lasting increases in basolateral amygdala activity after emotional arousal: Implications for facilitated consolidation of emotional memories. *Learning & Memory* **12**, 96–102 (2005).

32. Duvarci, S. & Paré, D. Glucocorticoids enhance the excitability of principal basolateral amygdala neurons. *Journal of Neuroscience* **27**, 4482–4491 (2007).

33. Mitra, R. & Sapolsky, R. M. Acute corticosterone treatment is sufficient to induce anxiety and amygdaloid dendritic hypertrophy. *Proceedings of the National Academy of Sciences* **105**, 5573–5578 (2008).

34. Karst, H., Berger, S., Erdmann, G., Schütz, G. & Joëls, M. Metaplasticity of amygdalar responses to the stress hormone corticosterone. *Proceedings of the National Academy of Sciences* **107**, 14449–14454 (2010).

35. Dannlowski, U. *et al.* Amygdala reactivity predicts automatic negative evaluations for facial emotions. *Psychiatry Research* **154**, 13–20 (2007).

36. Kukolja, J. *et al.* Modeling a Negative Response Bias in the Human Amygdala by Noradrenergic-Glucocorticoid Interactions. *Journal of Neuroscience* **28**, 12868–12876 (2008).

37. Enkel, T. *et al.* Ambiguous-cue interpretation is biased under stress- and depression-like states in rats. *Neuropsychopharmacology* **35**, 1008–1015 (2010).

38. Hamann, S. B., Ely, T. D., Grafton, S. T. & Kilts, C. D. Amygdala activity related to enhanced memory for pleasant and aversive stimuli. *Nature Neuroscience* **2**, 289–293 (1999).

39. Stuber, G. D. *et al.* Excitatory transmission from the amygdala to nucleus accumbens facilitates reward seeking. *Nature* **475**, 377–380 (2011).

40. Phelps, E. A. & LeDoux, J. E. Contributions of the Amygdala to Emotion Processing: From Animal Models to Human Behavior. *Neuron* **48**, 175–187 (2005).

41. Gerdes, A. Brain activations to emotional pictures are differentially associated with valence and arousal ratings. *Front. Hum. Neurosci.* **4**, (2010).

42. Hsu, E. H., Schroeder, J. P. & Packard, M. G. The amygdala mediates memory consolidation for an amphetamine conditioned place preference. *Behavioural Brain Research* **129**, 93–100 (2002).

43. Paton, J. J., Belova, M. A., Morrison, S. E. & Salzman, C. D. The primate amygdala represents the positive and negative value of visual stimuli during learning. *Nature* **439**, 865–870 (2006).

44. Dwyer, D. M. Lesions of the basolateral, but not central, amygdala impair flavour-taste learning based on fructose or quinine reinforcers. *Behavioural Brain Research* **220**, 349–353 (2011).

45. Garavan, H., Pendergrass, J. C., Ross, T. J., Stein, E. A. & Risinger, R. C. Amygdala response to both positively and negatively valenced stimuli. *NeuroReport* **12**, 2779–2783 (2001).

46. Hamann, S. & Mao, H. Positive and negative emotional verbal stimuli elicit activity in the left amygdala. *NeuroReport* **13**, 15–19 (2002).

47. Roozendaal, B., Nguyen, B. T., Power, A. E. & McGaugh, J. L. Basolateral amygdala noradrenergic influence enables enhancement of memory consolidation induced by hippocampal glucocorticoid receptor activation. *Proceedings of the National Academy of Sciences* **96**, 11642–11647 (1999).

48. LaLumiere, R. T., Nawar, E. M. & McGaugh, J. L. Modulation of memory consolidation by the basolateral amygdala or nucleus accumbens shell requires concurrent dopamine receptor activation in both brain regions. *Learning & Memory* **12**, 296–301 (2005).

49. Miranda, M. I. & McGaugh, J. L. Enhancement of inhibitory avoidance and conditioned taste aversion memory with insular cortex infusions of 8-Br-cAMP: involvement of the basolateral amygdala. *Learning & Memory* **11**, 312–317 (2004).

50. Roesler, R., Roozendaal, B. & McGaugh, J. L. Basolateral amygdala lesions block the memory-enhancing effect of 8-Br-cAMP infused into the entorhinal cortex of rats after training. *Eur. J. Neurosci.* **15**, 905–910 (2002).

51. Roozendaal, B., McReynolds, J. & McGaugh, J. The Basolateral Amygdala Interacts with the Medial Prefrontal Cortex in Regulating Glucocorticoid Effects on Working Memory Impairment. *Journal of Neuroscience* **24**, 1385–1392 (2004).

52. Kilpatrick, L. & Cahill, L. Amygdala modulation of parahippocampal and frontal regions during emotionally influenced memory storage. *NeuroImage* **20**, 2091–2099 (2003).

53. Dolcos, F., LaBar, K. S. & Cabeza, R. Dissociable effects of arousal and valence on prefrontal activity indexing emotional evaluation and subsequent memory: an event-related fMRI study. *NeuroImage* **23**, 64–74 (2004).

54. Kensinger, E. A. & Corkin, S. Two routes to emotional memory: distinct neural processes for valence and arousal. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 3310–3315 (2004).

55. Nathan, S. V., Griffith, Q. K., McReynolds, J. R., Hahn, E. L. & Roozendaal, B. Basolateral Amygdala Interacts with Other Brain Regions in Regulating Glucocorticoid Effects on Different Memory Functions. *Annals of the New York Academy of Sciences* **1032**, 179–182 (2004).

56. Hamann, S. B., Ely, T. D., Hoffman, J. M. & Kilts, C. D. Ecstasy and agony: activation of the human amygdala in positive and negative emotion. *Psychol Sci* **13**, 135–141 (2002).

57. Mickley Steinmetz, K. R., Addis, D. R. & Kensinger, E. A. The effect of arousal on the emotional memory network depends on valence. *NeuroImage* **53**, 318–324 (2010).

6

SEX DIFFERENCES IN THE EFFECTS OF ACUTE AND CHRONIC STRESS AND RECOVERY AFTER LONG-TERM STRESS ON STRESS-RELATED BRAIN REGIONS IN RATS

Yanhua Lin[1]

Gert J ter Horst[1]

Romy Wichmann[1]

Petra Bakker[1]

Aibua Liu[3]

Xuejun Li[2]

Christel Westenbroek[1]

[1] Department of Neuroscience, University Medical Center Groningen, University of Groningen
[2] Department of Pharmacology, School of Basic Medical Sciences and State Key Laboratory of Natural and Biomimetic Drugs, Peking University
[3] The State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University

Cerebral Cortex, September 2009; 19: 1978 - 1989

ABSTRACT

Studies show that sex plays a role in stress-related depression, with women experiencing a higher vulnerability to its effect. Two major targets of antidepressants are brain-derived neurotrophic factor (BDNF) and cyclic adenosine monophosphate response element-binding protein (CREB). The aim of this study was to investigate the levels of CREB, phosphorylation of CREB (pCREB), and BDNF in stress-related brain regions of male and female rats after stress and recovery. CREB and pCREB levels were examined in CA1, CA2, CA3, paraventricular nucleus of the thalamus (PVT), amygdala, anterior cingulate area, dorsal part (ACAd), and infralimbic area of prefrontal cortex (PFC), whereas dentate gyrus (DG) and prelimbic area (PL) of PFC were examined for BDNF levels. Our results demonstrate that levels of CREB and pCREB in male CA1, CA2 and CA3, PVT, amygdala, and ACAd were reduced by stress, whereas the same brain regions of female rats exhibited no change. BDNF levels were decreased by chronic stress in female PL but were increased by acute stress in female DG. BDNF levels in male DG and PL were found not to undergo change in response to stress. Abnormalities in morphology occurred after chronic stress in males but not in females. In all cases, the levels of CREB, pCREB, and BDNF in recovery animals were comparable to the levels of these proteins in control animals. These findings demonstrate a sexual dimorphism in the molecular response to stress and suggest that these differences may have important implications for potential therapeutic treatment of depression.

INTRODUCTION

Depression is one of the leading causes of disability in the world when measured by the number of years lived with a disabling condition [1]. Although depression is highly heritable, the onset of depression appears to be triggered by environmental factors such as stress [2,3]. Epidemiological studies demonstrate that women are more vulnerable than men to stress-related psychopathologies and that depression occurs twice as frequently in women as in men [4-7]. However, few studies have been conducted in females, and the mechanisms underlying these sex differences have not been clarified. Results from studies in males indicate that brain-derived neurotrophic factor (BDNF) and cyclic adenosine monophosphate response element-binding protein (CREB) are key mediators of the therapeutic response to antidepressants [8]. CREB and CREB-dependent gene expression are activated in response to many signal transduction cascades implicated in neuronal plasticity [9]. In male rats, CREB overexpression causes a behavioral phenotype similar to that seen with antidepressant therapy [10]. Furthermore, enhancing cortical and hippocampal phosphorylation of CREB (pCREB) has been proposed as a common antidepressant mechanism, based largely on studies of antidepressant action in the naive male rodent [1,11]. pCREB at serine 133 leads to transcription of genes including BDNF [12] that is required for activity-dependent survival of neurons [13,14] and plays a pivotal role in the action of antidepressants [15]. In contrast to the effects of antidepressant treatment, stress decreases BDNF levels [16,17] and suppresses local CREB phosphorylation [18]. Therefore, CREB, pCREB, and CREB-regulated genes such as BDNF are major targets of antidepressant drug action. Our previous study showed that stress decreases pCREB in dentate gyrus (DG) and prelimbic area (PL) of prefrontal cortex (PFC) in male rats whereas not in female rats. However, open-field tests showed no behavioral difference between male and female rats following stress [19]. Clearly, these 2 findings are contradictory and fail to explain the sex differences seen in human depression. As such, other proteins like BDNF should be further investigated. More brain regions related to stress besides DG and PL should also be examined. Multiple brain regions are likely involved in the organization of responses to stressful stimuli, including regions of prefrontal and cingulate cortex, hippocampus, amygdala, and thalamus [20,21]. Until now, studies have primarily focused on PFC and hippocampus because these are the key regions responsible for glucocorticoid feedback in the hypothalamic-pituitary-adrenocortical axis [22-24]. For example, shortly after a stressful event, corticosteroids increase cellular excitability in subfields of the hippocampus [25]. However, stress responses are not limited to PFC and hippocampus. For example, the paraventricular nucleus of the thalamus (PVT) may be an important target for antidepressant therapy because this midline thalamic nucleus responds strongly to various stressors [26]. Also, many of the projection targets of PVT neurons, including PFC and amygdala, show strong stress responses [27]. Certain nuclei of the amygdala are critical in responses to rewarding and aversive stimuli, and these nuclei show

abnormalities in depressed human subjects [28]. The amygdala is also considered central in mediating stress-related changes in hippocampal functions [29], and this observation is consistent with studies reporting abnormalities in hippocampus and PFC in depressed human subjects [20,30,31]. Finally, recent compelling evidence suggests that estrogen is involved in depression, a factor that may help to explain sex differences observed in human depression. Estrogen receptors are found in multiple brain regions, including amygdala where estrogen receptor alpha (ERα) dominates and thalamus where ERß dominates [32], so these brain regions may be important in the study of sex differences in stress-related psychopathologies.

To further explore the mechanisms underlying the sex differences in stress-related depression, we exposed male and female rats to inescapable footshock to mimic depression. Chronic footshock exposure has been proposed as a valid animal model for affective disorders [33]. Using this model, we investigated the effects of footshock (acute and chronic) and recovery (chronic footshock for 3 weeks followed by 3 weeks of recovery) on CREB, pCREB, and BDNF levels. CREB and pCREB levels were examined in CA1, CA2, CA3, PVT, amygdala, anterior cingulate area, dorsal part (ACAd), and infralimbic area (ILA), whereas DG and PL were examined for BDNF levels. Prior to stress induction, males and females showed no differences in protein levels or brain morphology. During the stress response, male rats showed significant changes in pCREB levels and in brain morphology, whereas females showed no such response. However, females showed changes in BDNF levels during the stress response, whereas males did not. Both sexes returned to baseline protein levels and morphology following recovery. These results suggest that sex may play a role in the response to stress and that future targets for clinical antidepressants may be sex dependent.

MATERIALS AND METHODS

Animals

Each experimental group consisted of (8) 6- to 7-week-old male or female Wistar rats, individually housed with ad libitum access to food and water. A plastic tube (diameter 8 3 17 cm) was placed in each cage as a shelter. The light--dark cycle was reversed (lights on 19:00--7:00). All experiments were designed to minimize the number of animals required, and all procedures were approved by the Animals Ethics Committee of the University of Groningen. The effect of the estrous cycle on the stress response was not specifically investigated in the current study. Male and female rats were randomly assigned to 4 experimental groups: 1) control group: subjected to no footshock throughout the experiment; 2) acute stress group: received 6 footshocks on day 42 and exposed to footshock box with light stimulus only on day 43; 3) recovery group: received footshocks daily for 3 weeks followed by a 3-week period with no footshock and on day 43 exposure to footshock box with only light but no shock; 4) chronic stress group: received footshocks daily for 3 weeks followed by 3 weeks of alternating days

of exposure to footshock box with footshocks and without receiving footshock and on day 43 exposure to footshock box only with light.

Stress Procedure

The "footshock chamber" consists of a box containing an animal space positioned on a metallic grid floor connected to a shock generator and scrambler. Rats in the stress group were placed in a box and received variable (2-6) inescapable footshocks with randomized starting time (between 9:00 AM and 5:00 PM) and intervals during a 30-120 min session (0.8 mA as maximum intensity and 8 s in duration) in order to make the procedure as unpredictable as possible. A light signal (10 s) preceded each footshock adding a "psychological" component to the stressor. On the last day, the stress-exposed rats were subjected to light stimulus only, which was crucial as it provided a way to create a stress condition without unwanted side effects of direct physical or painful stimuli.

On day 43, rats were sacrificed using isoflurane anesthesia. Three rats from each group were transcardially perfused with 50 mL of heparinized saline and 300 mL of a 4% paraformaldehyde solution in 0.1 M sodium phosphate buffer (pH 7.4), 2 h after the start of the last exposure to stress box. These 3 rat brains were postfixed in the same fixative overnight at 4 °C and were used for immunohistochemistry analysis. The other 5 rats from each group were decapitated 30 min after the start of the last exposure to stress box, and these brains were removed immediately and put on dry ice and stored at -80 °C to be used for enzyme-linked immunosorbent assay (ELISA) and western blot analysis. In our experiment, 3-5 rats per group (3 rats per group for immunohistochemistry analysis, 5 rats per group for ELISA and western blot analysis) were sufficient to attain statistical significance, as 4-8 slices from each brain region of each rat were used for immunohistochemistry analysis.

Immunohistochemistry

Following an overnight cryoprotection in a 30% sucrose solution, serial 30-μm coronal sections of the brains were made with a cryostat microtome and collected in 0.02 M potassium phosphate saline buffer. CREB- and pCREB-immunoreactivity (IR) in different brain regions was performed on free-floating sections. Sections were rinsed with 0.3% H_2O_2 for 10 min to reduce endogenous peroxidase activity, thoroughly washed with 0.1 M PBS and incubated with rabbit anti-CREB antibody (1:300, Cell Signaling) or anti-pCREB antibody (1:1000, Upstate) diluted in 0.1 M PBS with 0.1% Triton X-100 and 3% normal goat serum for 72 h at 4 °C. After thorough washing, the sections were subsequently incubated for 2 h with biotinylated goat-anti-rabbit IgG (1:1000 in 0.1 M PBS with 0.1% Triton X-100 and 3% normal goat serum) and avidin-biotin-peroxidase complex (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA). After thorough washing, the peroxidase reaction was developed with a diaminobenzidine-nickel solution and 1% H_2O_2. Sections were washed for 15 min

in buffer and mounted with a gelatine solution and air dried, dehydrated in graded alcohol solutions and finally in Histoclear, and then coverslipped with DePeX mounting medium (BDH).To reduce staining artifacts or intensity differences, the sections from all groups were processed simultaneously. CREB- or pCREB-positive cells in CA1, CA2, CA3, PVT, amygdala (basolateral nucleus amygdala, anterior part [BLAa]; basolateral nucleus amygdala, posterior part [BLAp]; basomedial nucleus amygdala, poste- rior part [BMAp]; and lateral nucleus amygdala [LA]) (4 slices for each rat, bregma −2.45 to −2.85), ACAd, and ILA (8 slices for each rat, bregma +3.20 to +2.15) (Fig. 1) were blindly quantified using a computerized imaging analysis system [34]. The selected areas were digitized by using a Sony CCD camera mounted on a LEICA Leitz DMRB microscope (Leica, Wetzlar, Germany) at x100 magnification. Regions of interest were outlined with a light pen, measured, and CREB or pCREB positive nuclei were counted using a computer-based image analysis system LEICA (LEICA Imaging System Ltd., Cambridge, England). Each digitized image was individually set at a threshold to subtract the background optical density. Only cell nuclei that exceeded a defined threshold were detected by the image analysis system. The resulting data were reported as number of positive cells/0.1 mm^2. All brain regions were quantified bilaterally [19]. In amygdala, CREB and pCREB positive nuclei were counted in BLAa, BLAp, BMAp, and LA separately, then different subnucleus measures were combined to make an average that is comparable to the results from western blot in a time course.

BDNF ELISA Analysis

Serial 300-lm coronal sections of the cerebrum were made with a cryostat microtome (−15 °C) and kept frozen on dry ice. Tissue samples were dissected from DG (bregma −2.45 to −2.85) and PL (bregma +3.20 to +2.15) (Fig. 1) by using the "Palkovits Punch" technique (needle diameter 0.94 mm, Stoelting Co., Wood Dale, IL). Two punches per animal (left and right per area, were taken and diluted in 100 µl buffer (50 mM Tris pH 7.0, 500 mM NaCl, 0.2% Triton X-100, 0.1% NaN_3, 2 mM ethylenediaminetetraacetic acid, and 1x complete protease inhibitors [Roche, Basel, Switzerland]). The material was sonicated twice for 5 s followed by 30 min centrifugation at 16 000 x g at 4 °C. The supernatant was removed and saved at −20 °C until use. BDNF levels were measured with the BDNF Emax ImmunoAssay System of Promega (G7611).

Statistical Analysis

Data were expressed as means ± standard error of the mean and analyzed with SPSS (SPSS Inc., Chicago, IL) (version 12.0). $P < 0.05$ was defined as the level of significance. CREB, pCREB, and BDNF levels were analyzed separately in each brain region with an analysis of variance (ANOVA) with treatment group (control or stress) and sex (male or female) as between-subject variables. Bonferroni tests were used to do mean contrasts following ANOVA.

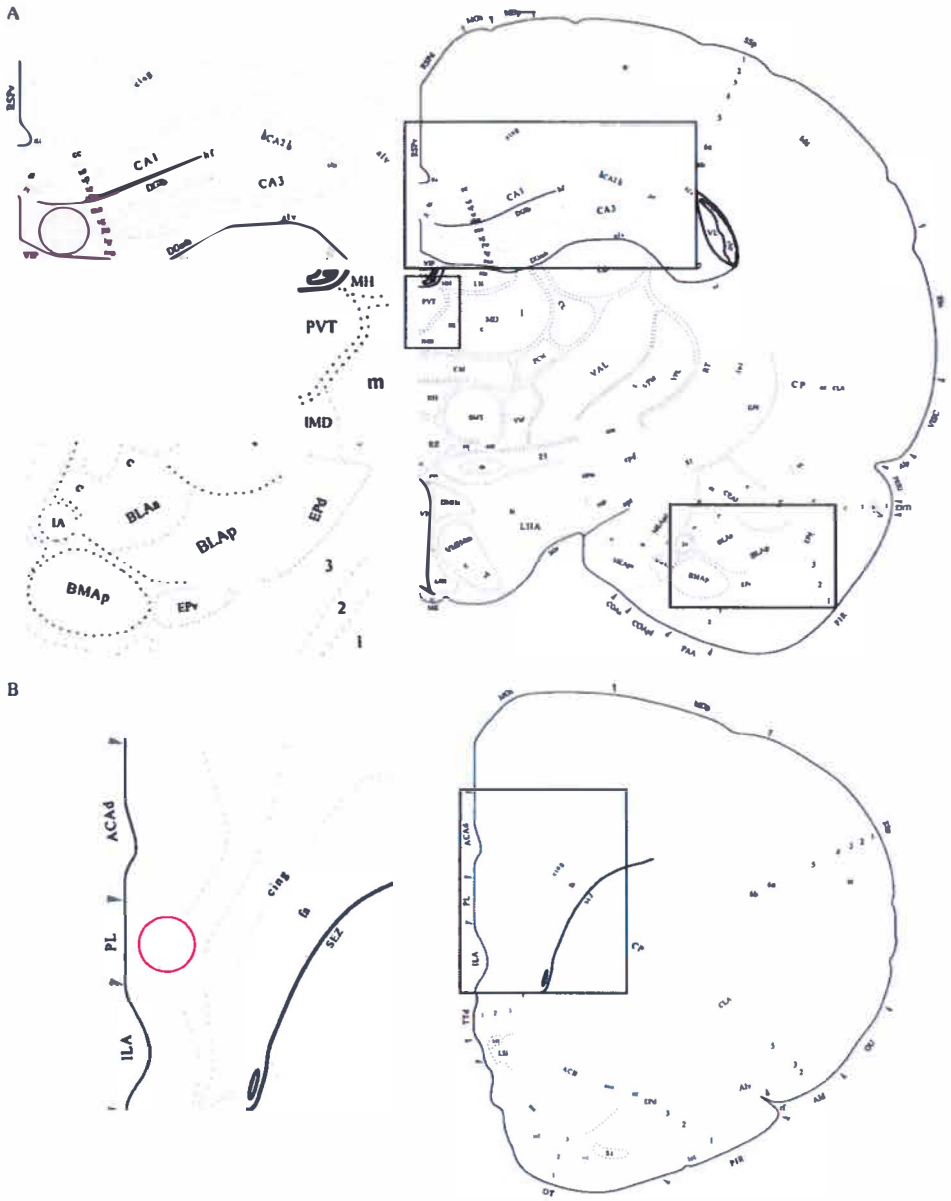


Figure 1. Atlas (Swanson 1992) image represents the approximate brain level where proteins levels were analyzed. CREB and pCREB positive nuclei measured by immunohistochemistry were counted in (A) CA1, CA2, CA3, PVT, and amygdala (BLAa, BLAp, BMAp, and LA); (B) ACAd and ILA. BDNF levels in DG (A) and PL (B) (red circle) were measured by ELISA analysis.

RESULTS

CREB and pCREB Levels in Male and Female CA1, CA2, and CA3 of Hippocampus

In male rats, acute and chronic stress caused a significant change in the number of pCREB-positive cells in hippocampal areas CA1, CA2, and CA3. The number of pCREB-positive cells was significantly decreased in males from the acute group ($F_{1,8} = 98.473$, $P < 0.001$, Fig. 2A; $F_{1,8} = 112.152$, $P < 0.001$, Fig. 2C; $F_{1,8} = 97.523$, $P < 0.001$, Fig. 2E) and from the chronic group ($F_{1,8} = 106.613$, $P < 0.001$, Fig. 2A; $F_{1,8} = 72.698$, $P < 0.001$, Fig. 2C; $F_{1,8} = 112.745$, $P < 0.001$, Fig. 2E). Patches of morphological abnormalities (Fig. 2I,L; black arrow) were seen in CA1, CA2, and CA3 of male rats from the chronic stress group but not in male rats from the acute stress group (Fig. 2H,K). The size of the patches varied, but in comparison to the surrounding tissue, these patches all appeared very lightly stained and showed no CREB- or pCREB-positive cells. In addition to the effects observed in pCREB levels, CREB levels were reduced by acute stress ($F_{1,8} = 15.562$, $P < 0.01$, Fig. 2C,K) and chronic stress ($F_{1,8} = 4.851$, $P < 0.05$, Fig. 2C,L) in male CA2 but not in male CA1 or CA3. Male rats from the recovery group showed no significant change from the control group in the number of pCREB- or CREB-positive cells on day 43 (Fig. 2A,C,E) and also showed none of the morphologically distinct patches seen in the chronic stress group (data not shown). In female rats, neither acute nor chronic stress had a significant effect on CREB or pCREB levels in CA1, CA2, or CA3 (Fig. 2B,D,F), and neither stress group showed any of the morphologically distinct patches observed in chronically stressed males (data not shown). Male and female control rats did not differ on any measures reported above (Fig. 2A-F).

CREB and pCREB Levels in Male and Female ILA and ACAd

In male rats, stress had a significant effect on the number of cells expressing pCREB in ACAd. Chronic stress significantly decreased the number of cells expressing pCREB in male ACAd compared with control male rats ($F_{1,8} = 2.942$, $P < 0.05$, Fig. 3A). After recovery, there was no significant change in the number of cells expressing pCREB in male ACAd (Fig. 3A). There was no significant change in the number of CREB- positive cells in male ACAd. Numbers of pCREB- and CREB- positive cells were not changed significantly in male ILA (Fig. 3A,C) after chronic stress. Chronic stress also induced morphological abnormalities and irregularities in male ACAd which were reflected by appearance of patches (black arrow) (Fig. 3F). There was no positive staining in these patches, and the background of these patches was bright, which is different from the tissue around. Acute stress exerted no significant effects on CREB and pCREB levels in male ILA and ACAd (Fig. 3A,C). In female ILA and ACAd, no significant change in the number of cells expressing CREB and pCREB was found (Fig. 3B,D) after chronic and acute stress, also no patches were present (data not shown). Male and female control rats did not differ on any measures reported above (Fig. 3A-D).

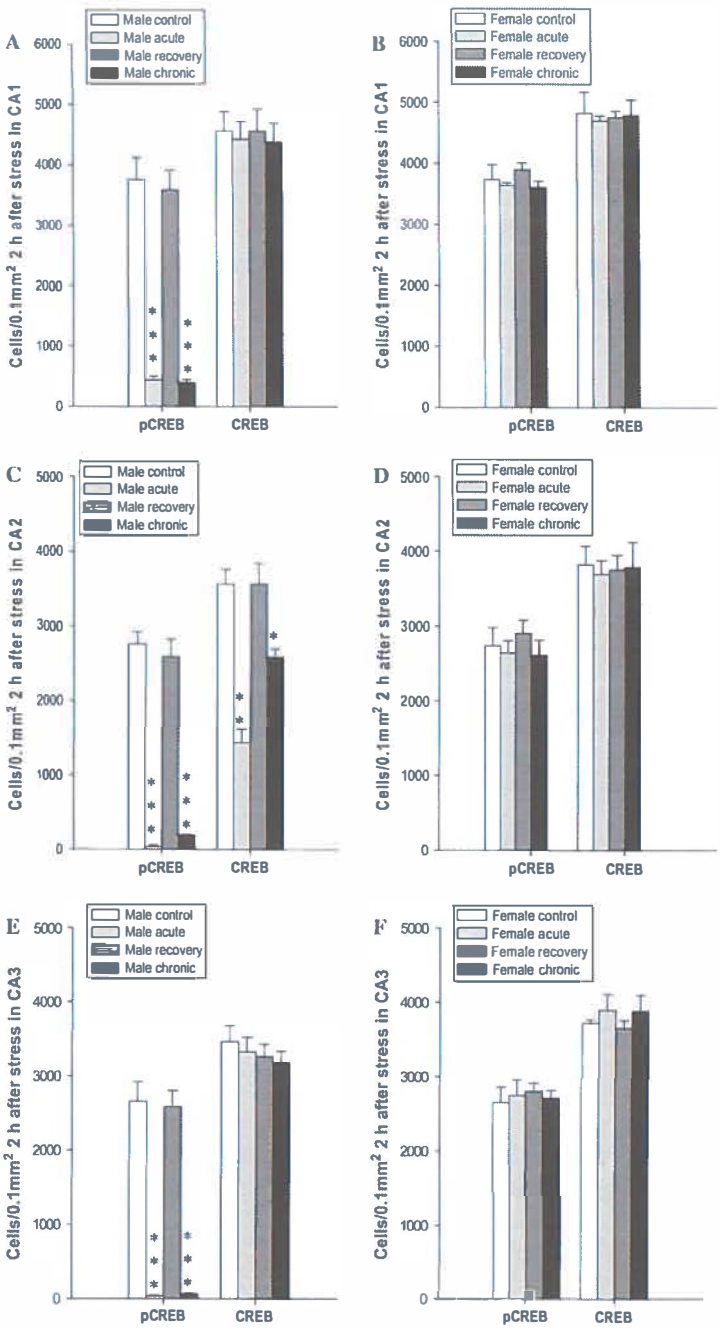
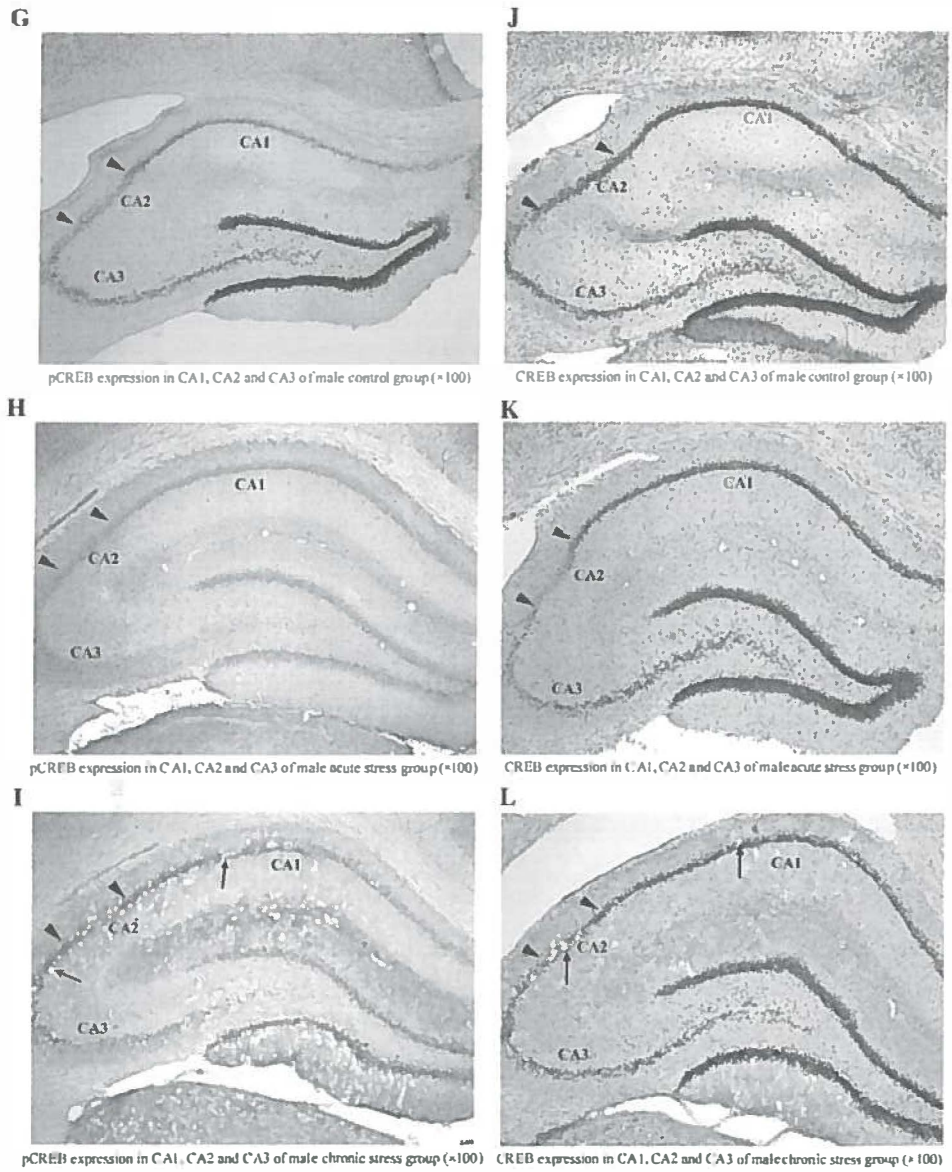


Figure 2. Levels of pCREB and CREB in CA1, CA2, and CA3 of hippocampus. (A) Number of pCREB- and CREB-positive cells in male CA1; (B) Number of pCREB- and CREB- positive cells in female CA1; (C) Number of pCREB- and CREB-positive cells in male CA2; (D) Number of pCREB- and CREB-positive cells in female CA2; (E) Number of pCREB- and CREB-positive cells in male CA3; (F)



Number of pCREB- and CREB-positive cells in female CA3; (G--I) Representative photomicrographs of pCREB-IR in CA1, CA2, and CA3 of male rat after acute and chronic stress; (J-L) Representative photomicrographs of CREB-IR in CA1, CA2, and CA3 of male rats after acute and chronic stress. Patches were clearly observed (black arrow) in CA1, CA2, and CA3 of male rats exposed to chronic stress. Data were expressed as mean ± standard error of the mean, n 5 3. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group.

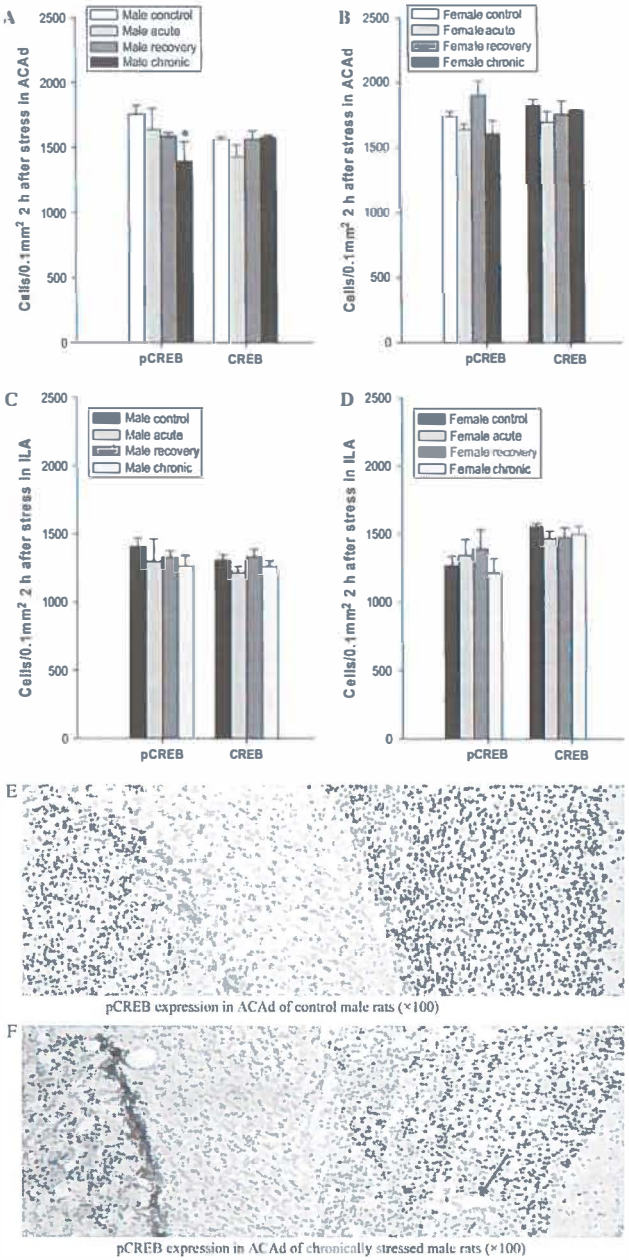


Figure 3. Number of cells expressing pCREB and CREB in ILA and ACAd of PFC. (A) Number of pCREB- and CREB-positive cells in male ACAd; (B) Number of pCREB- and CREB-positive cells in female ACAd; (C) Number of pCREB- and CREB-positive cells in male ILA; (D) Number of pCREB- and CREB-positive cells in female ILA; (E) Representative photomicrograph of pCREB-IR in ACAd in control male rats; (F) Representative photomicrograph of pCREB-IR in ACAd in chronically stressed male rats, patches were clearly observed in male ACAd (black arrow) after chronic stress. Data were expressed as mean ± standard error of the mean, n = 3. *P < 0.05 versus chronically stressed group.

CREB and pCREB Levels in Male and Female Amygdala

In male rats, acute and chronic stress caused a significant change in the number of pCREB-positive cells in amygdala. The amygdala of male rats showed a significant reduction in the number of pCREB-positive cells following acute ($F1,8 = 11.251$, $P < 0.01$, Fig. 4A) and chronic stress ($F1,8 = 7.821$, $P < 0.05$, Fig. 4A), but these levels returned to normal in the recovery group (Fig. 4A). However, amygdala from acutely and chronically stressed males showed no significant change in CREB-IR nor did they show any of the morphological abnormalities seen in the hippocampus and ACAd from the same exposure groups (data not shown). Amygdala from acutely and chronically stressed female rats showed neither morphological abnormalities nor any change in pCREB- or CREB-IR. Male and female control rats did not differ on any of the measures reported above (Fig. 4A,B).

CREB and pCREB Levels in Male and Female PVT

PVT from acutely stressed male rats showed a significant decrease in the number of pCREB-positive cells relative to controls ($F1,8 = 6.594$, $P < 0.05$), but the number of CREB- positive cells was not significantly changed (Fig. 5A). Chronic stress, on the other hand, had no significant effect on the number of pCREB- or CREB-positive cells in male PVT (Fig. 5A). PVT from female rats showed no changes in CREB or pCREB levels in either stress group, and neither sex showed any stress- induced morphological changes in the PVT. Male and female control rats did not differ on any of the measures reported above (Fig. 5A,B).

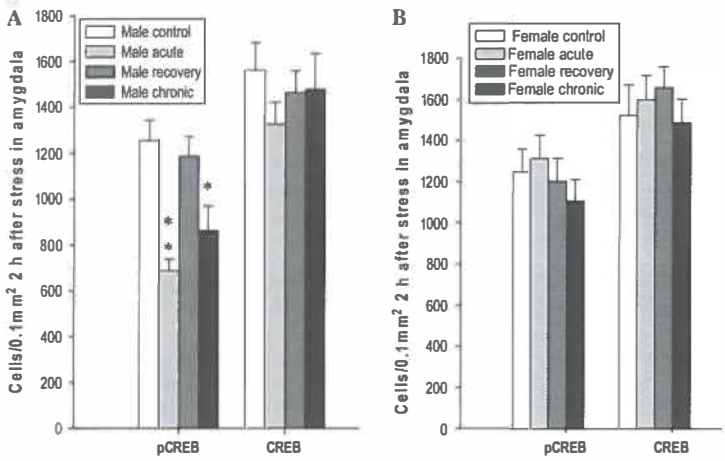


Figure 4. Number of cells expressing pCREB and CREB in amygdala. (A) Number of pCREB- and CREB-positive cells in male amygdala; (B) Number of pCREB- and CREB-positive cells in female amygdala. Data were expressed as mean ± standard error of the mean, n = 3. *P < 0.05, **P < 0.01 versus control group.

BDNF Levels in Male and Female PL and DG

In male rats, neither the D G nor the PL region of PFC showed any significant change in BDNF levels in either acute or chronic stress groups when measured by ELISA (Fig. 6). However, in female rats, the DG showed increased BDNF in the acute stress group only ($F1,16 = 24.451$, $P < 0.01$, Fig. 6A), and the PL region of PFC showed decreased BDNF in the chronic stress group only ($F1,16 = 5.564$, $P < 0.05$, Fig. 6B). Male control rats showed a higher baseline BDNF level in the DG than did females, but no other sex-related differences in BDNF levels were noted in the control groups.

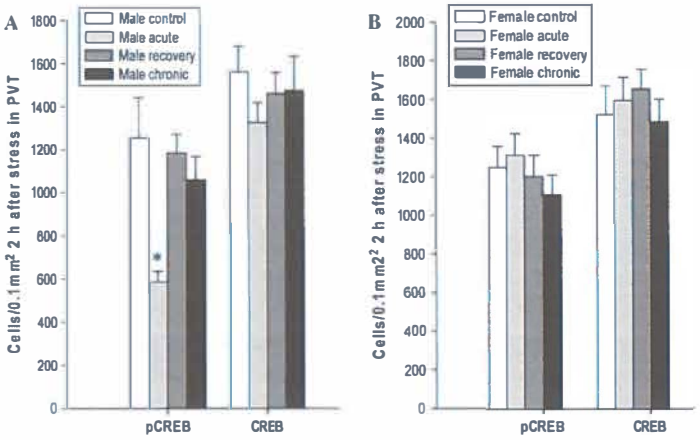


Figure 5. Number of cells expressing pCREB and CREB in PVT. (A) Number of pCREB- and CREB-positive cells in male PVT; (B) Number of pCREB- and CREB-positive cells in female PVT. Data were expressed as mean ± standard error of the mean, n = 3. *P < 0.05 versus control group

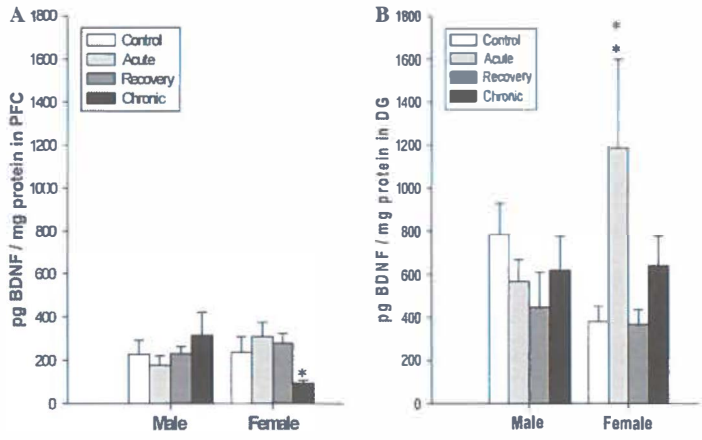


Figure 6. Levels of BDNF measured by ELISA in PFC (A) and DG (B) of male and female rats. Data were expressed as mean ± standard error of the mean, n = 5. *P < 0.05, **P < 0.01 versus control group.

DISCUSSION

Sex-specific analysis has much potential to further our understanding of mental health disorders and to improve the treatment of affected patients [35]. For this reason, we investigated sex differences in response to stress, a risk factor for developing depression. Here we show that the brains of male and female rats respond to stress in different ways.

The main results of this study can be summarized with 4 points: First, in male rats, stress reduces pCREB levels in ACAd, CA1, CA2, CA3, PVT, and amygdala and CREB levels in CA2 but has no similar effect in females; Second, stress exerts no effect on BDNF levels in male PFC and DG, but chronic stress decreases BDNF levels in female FC and acute stress increases BDNF levels in female DG; Third, stress induces morphological alterations in male CA1, CA2, CA3, and ACAd, whereas these changes are not found in females; Fourth, recovery following stress restores these parameters to baseline in both male and female rats.

Hippocampus and PFC are implicated as the key sites of neuropathology in depression [36 38]. Our results reveal morphological alterations (patches) in CA1, CA2, CA3, and ACAd following stress in male rats but not in females, and similar changes are also found in male DG and PL [19]. The patches observed in male rats may be due to necrosis and/or apoptosis [39] and those seen in ACAd may also represent a loss of glial cells [40]. Stress not only induces morphological alterations but also decreases levels of pCREB and CREB in hippocampus and ACAd. Similarly, chronic stress suppresses hippocampal neurogenesis in rats, as indicated by decreased numbers of BrdU-labeled and pCREB-positive cells [41]. In view of the implications of CREB and pCREB in neuronal plasticity, these findings may provide a biological explanation for clinically observed atrophy of neuronal and/or glial cells in depressive patients, particularly in the hippocampus and ACAd [42].

Our results indicate that the male rat hippocampus is more susceptible to stress-induced changes in morphology and in pCREB and CREB levels than is the female rat hippocampus. Despite these findings, men are still less likely than women to experience depression. In fact, increasing evidence from animal studies suggests that females are relatively resistant to neurobiological effects of acute and chronic stress [19]. For example, chronic stress over 21 days produces reversible atrophy of apical dendrites of hippocampal pyramidal neurons in males but not in females [43,44]. Similarly, repeated swim stress over 30 days decreases CA3 and CA4 pyramidal cell numbers in gonadectomized male rats but not in females [45]. The modulation of neurogenesis by controllability is evident in males but not in females [46]. Furthermore, neurogenesis in adult females is reportedly not affected by predator odors, whereas it is significantly decreased in males [47].

Hippocampus and ACAd are implicated in several higher brain functions, including learning, memory, attention, conflict monitoring, pain, pleasure, and decision making [48,49]. The reduction in pCREB and CREB levels and the morphological alterations seen in hippocampus and ACAd suggest dysfunction of neurons in these areas, which may contribute to cognitive deficits in mental disorders [48,50]. Increasing evidence indicates

that there is a strong correlation between depression and cognitive impairments [51], so dysfunction caused by damage to these areas may significantly contribute to depression.

Although chronic stress decreases pCREB levels and induces morphological abnormalities in male hippocampus and ACAd, no such alterations are found in male ILA of PFC. These findings suggest that the effects of stress on the brain are likely to be region specific. However, there is evidence that the ILA has extensive projections to central autonomic nuclei that modulate visceral responses to stress, and ILA has been reported to be activated following stress [52,53]. As such, the evidence presented here should not be considered an argument against the involvement of ILA in depression but rather an elimination of one possible mechanism of its involvement. We also found an interesting pattern of stress- related changes in the amygdala. Although chronic stress decreases pCREB levels in male amygdala, no concurrent abnormalities in morphology are seen. The mechanisms underlying the discrepancy between amygdala and hippocampus or PFC remain unclear. The amygdala is considered to be a primary site of synaptic plasticity and could be involved in anhedonia, a key symptom of depression [54], and chronic antidepressant administration has been shown to increase CREB and pCREB levels in amygdala [11,54]. The reduction in pCREB in the present study suggests a role for amygdala dysfunction following chronic stress.

Contrary to the effects of stress on pCREB and CREB levels, stress has no effect on BDNF, a CREB-regulated target gene [55] in male rats but impacts BDNF levels in female rats. Consistent with these findings, artificial rearing decreases BDNF levels in mPFC in comparison to mother- reared groups in female rats [56]. These results suggest that BDNF may be regulated by factors other than CREB and pCREB. Changes in BDNF protein levels and CREB activation are not necessarily correlated [57,58], and other studies have suggested that nuclear factor of activated T cells may be a more critical regulator of BDNF in the brain [59]. The transcriptional activity of the CREB/pCREB system is known to be enhanced when the ratio of pCREB to CREB increases [60], but our data failed to show any correlation between such a ratio and the measured outcomes reported here. However, it should be noted that the activation of CREB phosphorylation exhibits a biphasic pattern following stress [61,62], and our previous study showed that pCREB levels are normal 30 min after stress but significantly decreased after 2 h [19]. Furthermore, BDNF mRNA shows a biphasic time course following stress [63,64], whereas BDNF protein levels show a monophasic response [60,63]. This could arise from the fact that 4 different RNA transcripts, differentially transcribed in the central nervous system, encode for the same BDNF protein [65] suggesting a possible uncoupling of BDNF transcriptional and translational mechanisms; Further complicating the role of CREB and pCREB in regulation of BDNF responses to stress are findings that CREB-mediated gene expression might occur in the absence of Ser^{133} phosphorylation [66,67]. Clearly, more studies are needed to clarify the signal transduction pathways between CREB/pCREB and BDNF. Nevertheless, our results suggest that the changes in BDNF, CREB, and pCREB cannot fully explain the sex differences and morphologic alterations observed in the stress response.

This study did not attempt to address the role of gonadal steroids in the stress response, but such steroids have been implicated as a modulating factor in some of the sex differences [68 70]. For example, clinical evidence indicates that estrogen may con- tribute to the sex differences [71 73]. Further investigation is needed to determine if sex hormones contribute to the differential regulation of CREB, pCREB, and BDNF in response to stress. It should be noted that in this study 3 weeks of recovery after stress restored all the affected parameters to control levels in both male and female rats, suggesting that the changes in morphology and chemistry seen in hippocampus, PFC, PVT, and amygdala in response to acute and chronic stress are largely reversible. However, other studies have demonstrated that permanent damage may occur in the brain under extreme stress conditions [74]. In our experiment, rats reexposed to the adverse environment after 3 weeks of recovery and were expected to show attenuated versions of the responses seen in acutely stressed animals. However, recovery male rats show pCREB and CREB levels and morphology similar to controls. Corticosteroid levels are increased after reexposure in male and female rats, implying that the reexposure to the adverse environment is still stressful [19], but the stressful reexposure does not induce changes in pCREB and CREB levels and morphology. One possible explanation is that male rats cannot retrieve the previous adverse event because the neuronal circuitry somehow has adapted, which may help male rats to circumvent negative effects of stress [75,76]. Female rats do not show changes in pCREB and CREB levels and morphology after acute and chronic stress, and thus, it is not surprising that we do not find such changes after reexposure. It remains to be seen whether other signal transduction systems are more critical in stress coping in female rats.

Although sex differences are observed at the biochemical level (CREB, pCREB, and BDNF), no sex differences are seen in behavioral responses to stress as measured with the open-field tests [19]. This nonsynchronousness may be explained by the evidence that loss of hippocampal BDNF per se is not sufficient to alter locomotor activity, anxiety-like behavior, fear conditioning or depression-related behaviors [77], and conditional or partial deletion of BDNF is not sufficient to produce a depressive phenotype [78]. Even so, BDNF in DG may be essential in mediating the therapeutic effect of antidepressants. Franklin and Perrot-Sinal (2006) [79] report lower levels of BDNF protein in DG of intact females relative to intact males, which is confirmed in control rats in our study. However, in our experiment, no sex differences in behavior are found in control rats. Interestingly, levels of BDNF in DG of male and female rats are at the same level at the end of our experiment, which may partially explain the lack of sex differences seen in behavior. Although CREB is critical to target gene regulation after chronic drug administration, behavioral responses to antidepressants may occur by CREB-independent mechanisms [80]. This hypothesis is supported by the evidence that CREBad-homozygous deficient mice exhibit normal activity in a familiar environment [81] and indicates that the changes in CREB and BDNF levels are not necessary to induce behavior alteration. Furthermore, the different behavioral effects of CREB levels in

different brain regions, such as hippocampus [10], nucleus accumbens [82], amygdala [83], or CREB-deficient mice [84], make it possible to counteract the behavior induced by stress-related changes in CREB levels in these brain regions. Accordingly, no sex differences in behavior are found despite the sex differences in CREB levels. Therefore, it is of interest to identify other potential effector genes that could influence behavior [54], and consideration of sex should be emphasized in future studies of the neurobiological mechanisms of depression [85]. Furthermore, our findings showing that chronic stress is not different from acute stress in terms of changes in CREB and pCREB suggest that CREB may be critical for a stress response but not for stress-induced depression which is presumed to occur as a result of chronic but not acute stress as the molecular changes do not correspond to the presumed behavioral changes.

Based on our results, it can be concluded that male and female rats respond to stress in different ways. CREB and pCREB levels and morphology are more sensitive to stress in male rats than in female rats, whereas BDNF is more sensitive to stress in female rats than in male rats. The levels of CREB/ pCREB and BDNF are not necessarily correlated. These sex differences may have important implications for development of novel therapeutics for depression.

REFERENCES

1. Blendy, J. A. The Role of CREB in Depression and Antidepressant Treatment. *Biological Psychiatry* **59**, 1144–1150 (2006).
2. Baler, R. D. & Volkow, N. D. Drug addiction: the neurobiology of disrupted self-control. *Trends in Molecular Medicine* **12**, 559–566 (2006).
3. Berton, O. & Nestler, E. J. New approaches to antidepressant drug discovery: beyond monoamines. *Nature Reviews Neuroscience* 7, 137–151 (2006).
4. Kessler, R. C., McGonagle, K. A., Swartz, M., Blazer, D. G. & Nelson, C. B. Sex and depression in the National Comorbidity Survey. I: Lifetime prevalence, chronicity and recurrence. *Journal of Affective Disorders* **29**, 85–96 (1993).
5. Carter-Snell, C. & Hegadoren, K. Stress disorders and gender: implications for theory and research. *Can J Nurs Res* **35**, 34–55 (2003).
6. Kessler, R. C. Epidemiology of women and depression. *Journal of Affective Disorders* **74**, 5–13 (2003).
7. Sun, C. *et al.* Changes of learning, memory and levels of CaMKII, CaM mRNA, CREB mRNA in the hippocampus of chronic multiple-stressed rats. *CHINESE MEDICAL ...* (2006).
8. D'Sa, C. & Duman, R. S. Antidepressants and neuroplasticity. *Bipolar Disord* **4**, 183–194 (2002).
9. Lonze, B. E. & Ginty, D. D. Function and regulation of CREB family transcription factors in the nervous system. *Neuron* **35**, 605–623 (2002).
10. Chen, A. C., Shirayama, Y., Shin, K. H., Neve, R. L. & Duman, R. S. Expression of the cAMP response element binding protein (CREB) in hippocampus produces an antidepressant effect. *BPS* **49**, 753–762 (2001).
11. Thome, J. *et al.* cAMP response element-mediated gene transcription is upregulated by chronic antidepressant treatment. *Journal of Neuroscience* **20**, 4030–4036 (2000).
12. Carlezon, W., Jr, Duman, R. & Nestler, E. The many faces of CREB. *Trends in Neurosciences* **28**, 436–445 (2005).
13. Ghosh, A., Ginty, D. D., Bading, H. & Greenberg, M. E. Calcium regulation of gene expression in neuronal cells. *J. Neurobiol.* **25**, 294–303 (1994).
14. Shieh, P. B., Hu, S. C., Bobb, K., Timmusk, T. & Ghosh, A. Identification of a signaling pathway involved in calcium regulation of BDNF expression. *Neuron* **20**, 727–740 (1998).
15. Wang, X. Y., Zhao, M., Ghitza, U. E., Li, Y. Q. & Lu, L. Stress Impairs Reconsolidation of Drug Memory via Glucocorticoid Receptors in the Basolateral Amygdala. *Journal of Neuroscience* **28**, 5602–5610 (2008).
16. Smith, M. A., Makino, S., Kvetnansky, R. & Post, R. M. Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J. Neurosci.* **15**, 1768–1777 (1995).
17. Nibuya, M., Takahashi, M., Russell, D. S. & Duman, R. S. Repeated stress increases catalytic TrkB mRNA in rat hippocampus. *Neurosci. Lett.* **267**, 81–84 (1999).
18. Laifenfeld, D., Karry, R., Grauer, E., Klein, E. & Ben-Shachar, D. Antidepressants and prolonged stress in rats modulate CAM-L1, laminin, and pCREB, implicated in neuronal plasticity. *Neurobiology of Disease* **20**, 432–441 (2005).
19. Lin, Y. *et al.* Effects of Long-Term Stress and Recovery on the Prefrontal Cortex and Dentate Gyrus in Male and Female Rats. *Cereb. Cortex* **18**, 2762–2774 (2008).
20. Drevets, W. C. Neuroimaging and neuropathological studies of depression: implications for the cognitive-emotional features of mood disorders. *Current Opinion in Neurobiology* **11**, 240–249 (2001).
21. Liotti, M. & Mayberg, H. S. The role of functional neuroimaging in the neuropsychology of depression. *J Clin Exp Neuropsychol* **23**, 121–136 (2001).
22. Popoli, M., Gennarelli, M. & Racagni, G. Modulation of synaptic plasticity by stress and antidepressants. *Bipolar Disord* **4**, 166–182 (2002).
23. Sheline, Y. I. Neuroimaging studies of mood disorder effects on the brain. *BPS* **54**, 338–352 (2003).
24. Sairanen, M., O'Leary, O. F., Knuuttila, J. E. & Castrén, E. Chronic antidepressant treatment selectively increases expression of plasticity-related proteins in the hippocampus and medial prefrontal cortex of the rat. *NSC* **144**, 368–374 (2007).
25. Joëls, M. Functional actions of corticosteroids in the hippocampus. *European Journal of Pharmacology* **583**, 312–321 (2008).
26. Beck, C. H. & Fibiger, H. C. Chronic desipramine alters stress-induced behaviors and regional expression of the immediate early gene, c-fos. *Pharmacology, Biochemistry and Behavior* **51**, 331–338 (1995).
27. Bubser, M. & Deutch, A. Y. Stress induces Fos expression in neurons of the thalamic

paraventricular nucleus that innervate limbic forebrain sites. *Synapse* **32**, 13–22 (1999).

28. Talarovicova, A., Krskova, L. & Kiss, A. Some assessments of the amygdala role in suprahypothalamic neuroendocrine regulation: a minireview. *Endocr Regul* **41**, 155–162 (2007).
29. Yang, C.-H., Huang, C.-C. & Hsu, K.-S. Differential roles of basolateral and central amygdala on the effects of uncontrollable stress on hippocampal synaptic plasticity. *Hippocampus* **18**, 548–563 (2008).
30. Bissette, G., Klimek, V., Pan, J., Stockmeier, C. & Ordway, G. Elevated concentrations of CRF in the locus coeruleus of depressed subjects. *Neuropsychopharmacology* **28**, 1328–1335 (2003).
31. Rajkowska, G. Depression: what we can learn from postmortem studies. *neuroscientist* **9**, 273–284 (2003).
32. Ostlund, H., Keller, E. & Hurd, Y. L. Estrogen receptor gene expression in relation to neuropsychiatric disorders. *Annals of the New York Academy of Sciences* **1007**, 54–63 (2003).
33. Westenbroek, C. *et al.* Gender-specific effects of social housing in rats after chronic mild stress exposure. *Progress in Neuro-Psychopharmacology & Biological Psychiatry* **27**, 21–30 (2003).
34. Westenbroek, C., Boer, Den, J. A. & Horst, Ter, G. J. Gender-specific effects of social housing on chronic stress-induced limbic FOS expression. *Neuroscience* **121**, 189–199 (2003).
35. Vlassoff, C. & Garcia Moreno, C. Placing gender at the centre of health programming: challenges and limitations. *Social Science & Medicine* **54**, 1713–1723 (2002).
36. Rajkowska, G. Postmortem studies in mood disorders indicate altered numbers of neurons and glial cells. *BPS* **48**, 766–777 (2000).
37. McEwen, B. S. Plasticity of the hippocampus: adaptation to chronic stress and allostatic load. *Annals of the New York Academy of Sciences* **933**, 265–277 (2001).
38. Shansky, R. M. *et al.* Estrogen mediates sex differences in stress-induced prefrontal cortex dysfunction. *Molecular Psychiatry* **9**, 531–538 (2004).
39. Sapolsky, R. M. Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. *Arch. Gen. Psychiatry* **57**, 925–935 (2000).
40. Cotter, D., Mackay, D., Landau, S., Kerwin, R. & Everall, I. Reduced glial cell density and neuronal size in the anterior cingulate cortex in major depressive disorder. *Arch. Gen. Psychiatry* **58**, 545–553 (2001).
41. Luo, C., Xu, H. & Li, X.-M. Quetiapine reverses the suppression of hippocampal neurogenesis caused by repeated restraint stress. *Brain Research* **1063**, 32–39 (2005).
42. Rantamäki, T., Knuuttila, J. E. A., Hokkanen, M.-E. & Castrén, E. The effects of acute and long-term lithium treatments on trkB neurotrophin receptor activation in the mouse hippocampus and anterior cingulate cortex. *Neuropharmacology* **50**, 421–427 (2006).
43. Galea, L. A. *et al.* Sex differences in dendritic atrophy of CA3 pyramidal neurons in response to chronic restraint stress. *NSC* **81**, 689–697 (1997).
44. Conrad, C. D., LeDoux, J. E., Magariños, A. M. & McEwen, B. S. Repeated restraint stress facilitates fear conditioning independently of causing hippocampal CA3 dendritic atrophy. *Behavioral Neuroscience* **113**, 902–913 (1999).
45. Mizoguchi, K., Kunishita, T., Chui, D. H. & Tabira, T. Stress induces neuronal death in the hippocampus of castrated rats. *Neurosci. Lett.* **138**, 157–160 (1992).
46. Shors, T. J. *et al.* Neurogenesis and helplessness are mediated by controllability in males but not in females. *BPS* **62**, 487–495 (2007).
47. Falconer, E. M. & Galea, L. A. M. Sex differences in cell proliferation, cell death and defensive behavior following acute predator odor stress in adult rats. *Brain Research* **975**, 22–36 (2003).
48. Genoux, D. *et al.* Protein phosphatase 1 is a molecular constraint on learning and memory. *nature* **418**, 970–975 (2002).
49. Vogt, B. A. & Laureys, S. Posterior cingulate, precuneal and retrosplenial cortices: cytology and components of the neural network correlates of consciousness. *Progress in Brain Research* **150**, 205–217 (2005).
50. Sanders, G. S., Gallup, G. G., Heinsen, H., Hof, P. R. & Schmitz, C. Cognitive deficits, schizophrenia, and the anterior cingulate cortex. *Trends in Cognitive Sciences* **6**, 190–192 (2002).
51. Song, L., Che, W., Min-wei, W., Murakami, Y. & Matsumoto, K. Impairment of the spatial learning and memory induced by learned helplessness and chronic mild stress. *Pharmacology, Biochemistry and Behavior* **83**, 186–193 (2006).
52. Hurley, K. M., Herbert, H., Moga, M. M. & Saper, C. B. Efferent projections of the infralimbic cortex of the rat. *J. Comp. Neurol.* **308**, 249–276 (1991).
53. Nikulina, E. M., Covington, H. E., Ganschow, L., Hammer, R. P. & Miczek, K. A. Long-term behavioral and neuronal cross-sensitization to

amphetamine induced by repeated brief social defeat stress: Fos in the ventral tegmental area and amygdala. *NSC* **123**, 857–865 (2004).

54. Wallace, T. L., Stellitano, K. E., Neve, R. L. & Duman, R. S. Effects of cyclic adenosine monophosphate response element binding protein overexpression in the basolateral amygdala on behavioral models of depression and anxiety. *BPS* **56**, 151–160 (2004).
55. Nibuya, M., Morinobu, S. & Duman, R. S. Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. *J. Neurosci.* **15**, 7539–7547 (1995).
56. Burton, C. L. *et al.* Prenatal restraint stress and motherless rearing disrupts expression of plasticity markers and stress-induced corticosterone release in adult female Sprague-Dawley rats. *Brain Research* **1158**, 28–38 (2007).
57. Miyata, K. *et al.* Involvement of the brain-derived neurotrophic factor/TrkB pathway in neuroprotecive effect of cyclosporin A in forebrain ischemia. *NSC* **105**, 571–578 (2001).
58. Aso, E. *et al.* BDNF impairment in the hippocampus is related to enhanced despair behavior in CB1 knockout mice. *J Neurochem* **105**, 565–572 (2008).
59. Graef, I. A. *et al.* Neurotrophins and netrins require calcineurin/NFAT signaling to stimulate outgrowth of embryonic axons. *Cell* **113**, 657–670 (2003).
60. Pardon, M. C. *et al.* Social threat and novel cage stress-induced sustained extracellular-regulated kinase1/2 (ERK1/2) phosphorylation but differential modulation of brain-derived neurotrophic factor (BDNF) expression in the hippocampus of NMRI mice. *NSC* **132**, 561–574 (2005).
61. Stanciu, M., Radulovic, J. & Spiess, J. Phosphorylated cAMP response element binding protein in the mouse brain after fear conditioning: relationship to Fos production. *Brain Res. Mol. Brain Res.* **94**, 15–24 (2001).
62. Bilang-Bleuel, A., Rech, J., De Carli, S., Holsboer, F. & Reul, J. M. H. M. Forced swimming evokes a biphasic response in CREB phosphorylation in extrahypothalamic limbic and neocortical brain structures in the rat. *Eur. J. Neurosci.* **15**, 1048–1060 (2002).
63. Nanda, S. A. & Mack, K. J. Seizures and sensory stimulation result in different patterns of brain derived neurotrophic factor protein expression in the barrel cortex and hippocampus. *Brain Res. Mol. Brain Res.* **78**, 1–14 (2000).
64. Pizarro, J. M. *et al.* Acute social defeat reduces neurotrophin expression in brain cortical and subcortical areas in mice. *Brain Research* **1025**, 10–20 (2004).
65. Timmusk, T. *et al.* Multiple promoters direct tissue-specific expression of the rat BDNF gene. *Neuron* **10**, 475–489 (1993).
66. Conkright, M. D. *et al.* TORCs: transducers of regulated CREB activity. *Mol. Cell* **12**, 413–423 (2003).
67. Iourgenko, V. *et al.* Identification of a family of cAMP response element-binding protein coactivators by genome-scale functional analysis in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 12147–12152 (2003).
68. Simerly, R. B., Chang, C., Muramatsu, M. & Swanson, L. W. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. *J. Comp. Neurol.* **294**, 76–95 (1990).
69. Hagihara, K., Hirata, S., Osada, T., Hirai, M. & Kato, J. Distribution of cells containing progesterone receptor mRNA in the female rat di- and telencephalon: an in situ hybridization study. *Brain Res. Mol. Brain Res.* **14**, 239–249 (1992).
70. Young, E. A., Midgley, A. R., Carlson, N. E. & Brown, M. B. Alteration in the hypothalamic-pituitary-ovarian axis in depressed women. *Arch. Gen. Psychiatry* **57**, 1157–1162 (2000).
71. Bebbington, P. E. *et al.* The influence of age and sex on the prevalence of depressive conditions: report from the National Survey of Psychiatric Morbidity . *Psychological Medicine* **28**, 9–19 (1998).
72. Born, L., Shea, A. & Steiner, M. The roots of depression in adolescent girls: is menarche the key? *Curr Psychiatry Rep* **4**, 449–460 (2002).
73. Hayward, C. & Sanborn, K. Puberty and the emergence of gender differences in psychopathology. *J Adolesc Health* **30**, 49–58 (2002).
74. McEwen, B. S. Effects of adverse experiences for brain structure and function. *BPS* **48**, 721–731 (2000).
75. Shors, T. J. Acute stress and re-exposure to the stressful context suppress spontaneous unit activity in the basolateral amygdala via NMDA receptor activation. *NeuroReport* **10**, 2811–2815 (1999).
76. Rasmusson, A. M., Shi, L. & Duman, R. Downregulation of BDNF mRNA in the hippocampal dentate gyrus after re-exposure to cues previously associated with footshock. *Neuropsychopharmacology* **27**, 133–142 (2002).
77. Adachi, M., Barrot, M., Autry, A. E., Theobald, D. & Monteggia, L. M. Selective loss of brain-

derived neurotrophic factor in the dentate gyrus attenuates antidepressant efficacy. *Biological Psychiatry* **63**, 642–649 (2008).

78. Duman, R. S. & Monteggia, L. M. A Neurotrophic Model for Stress-Related Mood Disorders. *Biological Psychiatry* **59**, 1116–1127 (2006).
79. Franklin, T. B. & Perrot-Sinal, T. S. Sex and ovarian steroids modulate brain-derived neurotrophic factor (BDNF) protein levels in rat hippocampus under stressful and non-stressful conditions. *Psychoneuroendocrinology* **31**, 38–48 (2006).
80. Conti, A. C., Cryan, J. F., Dalvi, A., Lucki, I. & Blendy, J. A. cAMP response element-binding protein is essential for the upregulation of brain-derived neurotrophic factor transcription, but not the behavioral or endocrine responses to antidepressant drugs. *Journal of Neuroscience* **22**, 3262–3268 (2002).
81. Hebda-Bauer, E. K., Watson, S. J. & Akil, H. CREB deficient mice show inhibition and low activity in novel environments without changes in stress reactivity. *Eur. J. Neurosci.* **20**, 503–513 (2004).
82. Barrot, M. *et al.* CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 11435–11440 (2002).
83. Pandey, S. C., Roy, A. & Zhang, H. The decreased phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding (CREB) protein in the central amygdala acts as a molecular substrate for anxiety related to ethanol withdrawal in rats. *Alcohol. Clin. Exp. Res.* **27**, 396–409 (2003).
84. Valverde, O. *et al.* Modulation of anxiety-like behavior and morphine dependence in CREB-deficient mice. *Neuropsychopharmacology* **29**, 1122–1133 (2004).
85. Monteggia, L. M. *et al.* Brain-Derived Neurotrophic Factor Conditional Knockouts Show Gender Differences in Depression-Related Behaviors. *Biological Psychiatry* **61**, 187–197 (2007).

7

SEX-SPECIFIC ALTERATIONS IN cAMP RESPONSE ELEMENT-BINDING PROTEIN IN THE NUCLEUS ACCUMBENS SHELL AFTER ACUTE AND CHRONIC STRESS

Romy Wichmann [1]
Yanhua Lin [1,2]
Petra L Bakker [1]
Gert J ter Horst [1,3]
Christel Westenbroek [1,4]

[1]Department of Neuroscience, Section Anatomy, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
[2]Department of Ophthalmology, John A. Moran Eye Center, University of Utah School of Medicine, Salt Lake City, Utah, USA
[3]Department of Neuroscience, Neuroimaging Center, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
[4]Molecular and Behavioral Neuroscience Institute, University of Michigan, Ann Arbor, Michigan, USA

Submitted, under review

ABSTRACT

Depression is one of the leading causes of disability worldwide with a twofold higher lifetime prevalence in women than men. Chronic stress has been identified as a major factor in the etiology of depression and is widely used as an animal model to mimic aspects of depression. The cAMP response element-binding (CREB) protein in the nucleus accumbens (NAc) might play an essential role in the molecular mechanism of anhedonia, a hallmark feature of depression. Yet, little is known about sex-specific alterations of CREB in response to stress. Therefore we investigated expression levels of total CREB and its transcriptionally active phosphorylated form (pCREB) in the NAc shell (NAcS) and core (NAcC) of both male and female rats after acute (1d) and chronic (42d) stress exposure using western blot analysis. We not only observed clear differences dependent on the neuroanatomical subregions of the NAc, but importantly, we found significant differences when sex was compared. Male rats exposed to acute stress had elevated levels of pCREB in the NAcS, whereas females show increased levels of total CREB. However, pCREB in the NAcS in female rats was reduced after both, acute and chronic stress. In the NAcC no stress-related changes of CREB or pCREB were found. Together, our findings indicate that cellular responses to stress are highly sex- and region- specific. These results might be instrumental for future studies on treatments of depression, which to date are predominantly investigated in male subjects.

INTRODUCTION

Depression is one of the leading causes of disability worldwide and the prevalence for this debilitating disorder is at least twice as high in women than in men [1]. Prolonged stress has been identified as a major factor in the etiology of depression [2]. There is accumulating evidence that men and women activate different brain regions and neuronal circuits in response to stressful experiences [3,4] which might contribute to the sex difference in the prevalence of depression. However, animal studies designed to model depression have relied almost exclusively on examination of males or ovariectomized females in order to avoid the potential confound of hormone fluctuations.

Stressful events markedly impact the brain's reward system, which is thought to be related to the development of anhedonia, i.e., the loss of motivation and diminished ability to experience pleasure and reward, a hallmark feature of depression [5]. The nucleus accumbens (NAc) is a vital component of the brains' reward system, thus stress-induced modulation in this region could contribute to depressive symptoms like anhedonia [6]. Abnormalities in morphology [7] as well as in neural activity [8] were found in the NAc of depressed patients and stimulation of this region can ameliorate anhedonic symptoms [9].

Recently, research into the pathophysiology and treatment of depression focused on disturbances of intracellular signaling pathways linked to neuroplasticity [10]. The transcription factor cAMP response element-binding protein (CREB) is a key regulator of neuroplasticity within brain reward circuits and thereby regulates individual sensitivity to emotional stimuli [11]. Expression levels of the transcriptionally active, phosphorylated form of CREB (pCREB) are changed in response to stress or antidepressant treatment in several brain areas, including the NAc [10,12,13]. Thus, sex-specific change in CREB activity and neuronal activation may play an important role in the sex specificity of the stress response and therefore the susceptibility to depression. However, despite the importance of CREB and pCREB expression in neuroplasticity and depression few studies were conducted to elucidate sex differences in the expression induced by stress [14,15].

Thus, in the present study, we investigated whether acute and chronic stress exposure differentially affect CREB expression and activity levels in the NAc shell (NAcS) and core (NAcC) of male and female rats.

EXPERIMENTAL PROCEDURES

Subjects

Age matched male and female Wistar rats (186 – 204 g at the start of the experiment) were purchased from Harlan Laboratories (Horst, The Netherlands) and individually housed (reversed 12-h light/dark cycle, food and water ad libitum). All experimental procedures followed ethical guidelines (European Communities Council Directive

86/609/EEC) and were approved by the Institutional Animal Care and Use Committee of the University of Groningen, The Netherlands.

Stress Procedure

Rats were either handled daily (control group) or exposed to two different stress protocols. Rats in the chronic stress group received a variable number (2-6) of inescapable footshocks (0.8 mA, 8 s) with randomized starting times (between 9:00 and 17:00 h) during a 30-120-min session daily for 3 weeks. A 10-s light stimulus preceded each footshock, adding a 'psychological' component to the noxious effects. In the following 3 weeks, rats in the chronic stress group were subjected to alternating days of exposure to the footshock chamber with or without receiving footshocks. Rats in the acute stress group received six footshocks on the last day only. Twenty-four hours later, rats of the two stress groups were re-exposed to the footshock chamber and subjected to the light stimulus only. After a 30-min exposure to the footshock chamber, rats were decapitated, brains rapidly removed, put on dry ice and stored at -80°C. Trunk blood was collected, centrifuged (1800g; 5 min) and plasma was stored at -20°C for corticosterone radioimmunoassay.

Open-Field Test

Animals were subjected to an 8-min open-field (OF) test on day 40 of the experiment. OF testing took place under red-light conditions between 9:00 and 13:00 h, at least 16 h after the last stress session and before the stress procedure of that day. Rats' behavior was recorded with a videotracking system (EthoVision 3.0, Noldus Information Technology, Wageningen, the Netherlands). Due to this design the acute stress group was not yet exposed to the stress procedure and animals were added to the control group.

Western Blot Analysis

Coronal sections (300 µm) were cut on a cryostat microtome and kept frozen on dry ice. Tissue samples were dissected from the NAcC and NAcS (+ 1.4 - 1.6 mm from Bregma) by using the "Palkovits Punch" technique (needle diameter 1.22 mm, Stoelting Co., IL). Bilateral punches were taken from each brain region and processed for western blot analysis as previously described by Lin et al [14]. Primary polyclonal antibodies for CREB or pCREB (Upstate Biotechnology, Lake Placid, NY, 1:1000) and secondary antibodies (peroxidase-coupled anti-rabbit for CREB and pCREB, anti-mouse for ß-actin, Amersham Bioscience; 1:5000) were used. Immunostaining was revealed by the enhanced chemiluminescence Western blot analysis system (Syngene, Westburg, The Netherlands). The intensity of the bands was quantified by image analysis. Membranes were washed and reprobed with the antibody to ß-actin (Abcam, Cambridge, UK; 1:10.000) for loading control. The ratio of CREB/ß-actin or pCREB/ß-actin were determined for each sample and normalized to the corresponding control group to yield a -fold induction.

Corticosterone radioimmunoassay

Plasma samples for corticosterone measurements were processed as previously described by Lin et al (2008)[14] using standard radioimmunoassay techniques.

Statistical Analysis

Data are expressed as mean ± SEM. Two-way ANOVAs for treatment and sex as factors, were used to analyze CREB and pCREB protein levels, rearing behavior and plasma corticosterone levels, followed by Fisher's post-hoc tests when appropriate.

RESULTS

CREB expression

Baseline levels of total CREB did not significantly differ in the NAcS or NAcC between male and female rats.

Total CREB expression in the NAcS showed a significant treatment [$F(2,12) = 5.81$, $p = 0.02$], but no effect of sex [$F(1,12) = 2.90$, $p = 0.12$] or their interaction [$F(2,12) = 1.45$, $p = 0.27$]. As is shown in Figure 1A, post hoc analysis revealed that total CREB was not significantly altered in males. On the other hand, females exhibited significantly higher levels of total CREB ($p = 0.03$) only in the acute stress group.

Total CREB expression levels in the NAcC showed a significant effect of sex [$F(1,12) = 6.74$, $p = 0.02$], but no effect of treatment [$F(2,12) = 2.27$, $p = 0.15$], or their interaction [$F(2,12) = 2.36$; $p = 0.14$] (Figure 1B).

pCREB expression

Since transcriptional activity of CREB mainly depends on its phosphorylation at SER^{133}, this was measured in the same samples. Baseline levels of pCREB did not differ in the NAcC ($p = 0.14$), but significantly differed in the NAcS ($p = 0.04$) between male and female rats.

For pCREB expression in the NAcS significant effects of sex [$F(1,12) = 41.60$, $p = 0.0001$], treatment [$F(2,12) = 4.91$, $p = 0.03$] and their interaction [$F(2,12) = 10.40$, $p = 0.002$] were found. As illustrated in Figure 1C, post hoc analysis revealed an increase in pCREB of males in the acute stress group ($p = 0.011$). On the other hand, female rats showed reduced pCREB in the acute ($p = 0.02$) and chronic stress group ($p = 0.001$).

Analysis of pCREB expression levels in the core showed a treatment by sex interaction effect [$F(2,12) = 5.01$, $p = 0.03$] but no effect of sex [$F(1,12) = 0.01$, $p = 0.93$] or treatment [$F(1,12) = 0.04$, $p = 0.96$]. Post hoc analysis revealed no significant difference of treatment within the two sexes compared to their respective control groups (Figure 1D).

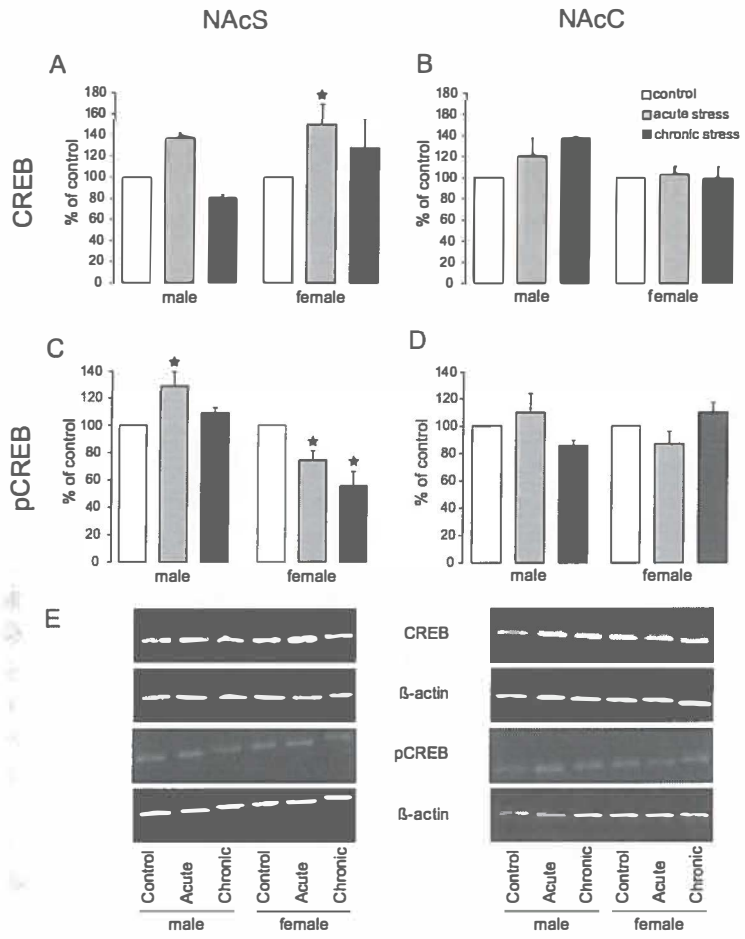


Figure 1. Effects of acute and chronic stress on CREB and pCREB expression levels in the NAcS (left) and NAcC (right) of male and female rats. ß-actin was used to normalize protein loading in each lane; data are expressed as percent of control group mean ± SEM. *$p \leq 0.05$ versus same gender control.

Plasma Corticosterone levels

Significant effects of sex [$F(1,24) = 30.74$, $p = 0.0001$] and treatment [$F(2,24) = 11.85$, $p = 0.0001$] but no interaction effect [$F(2,24) = 0.29$, $p = 0.75$] were found on plasma corticosterone levels (Table 1). Female rats had higher plasma corticosterone levels than male rats in the control as well as in all experimental groups. Exposure to the footshock chamber significantly increased corticosterone levels in both male and female rats in all experimental groups compared to control groups but values did not significantly differ between different stress treatments.

Table 1. Corticosterone levels (nmol/l) 30 min after exposure to the footshock box after acute (1d) or chronic (42d) stress in male and female rats.

Treatment	Male	Female
Control	527.2 ± 180.9	1154.7 ± 210.3''
Acute stress	1198.6 ± 53.3**	1755.0 ± 137.1**''
Chronic stress	1027.9 ± 66.7**	1799.0 ± 144.7**''

Data are presented as mean + SEM. Significant stress effects: *, $p \leq 0.05$; **, $p \leq 0.01$. Significant sex differences: '', $p \leq 0.01$

OF test

Rearing frequency, a measure of exploratory behavior, showed a clear sex [$F(1,26) = 11.84$, $p = 0.002$] and sex by treatment interaction effect [$F(1,26) = 4.95$, $p = 0.03$], but no treatment effect [$F(1,26) = 1.51$, $p = 0.23$]. As shown in Figure 2, post hoc analysis revealed that female rats had higher baseline levels of rearing than male rats ($p = 0.0001$). Rearing behavior in female rats was significantly reduced after chronic stress ($p = 0.02$, compared to female control). Male rats did not show a change in rearing frequency in any of the treatment groups compared to control rats.

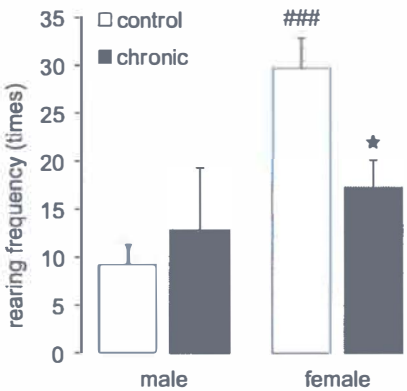


Figure 2. Exploratory behavior in open field test. Females have higher rearing frequency under control conditions. Chronic stress exposure reduces rearing frequency in females but does not influence rearing frequency in males. Data are presented as mean ± SEM. *, $p < 0.05$ compared with female control group; ''' $p < 0.0001$ compared to male control group.

DISCUSSION

The aim of this study was to investigate sex differences of acute and chronic stress exposure on the expression of CREB and pCREB in the NAc.

Our data indicate that acute stress significantly increases total CREB expression only in the NAcS of females. This adaptation of total CREB expression does not persist, since CREB levels are not significantly different from control after chronic stress. Acute stress increased pCREB levels in males, but decreased them in females. After chronic stress, pCREB levels are restored to baseline in males, but showed a persistent decrease in females. In the NAcC no significant changes were found in the expression levels of CREB or pCREB in either sex. All animals still showed a pronounced corticosterone response to the stress-exposure, indicating an enduring effectiveness of the stressor. These findings provide evidence that especially the NAcS pCREB is differently regulated in acute and chronic stress of males and females. This might contribute to the sex differences seen in the prevalence of depression.

The NAc plays a crucial role in reward and motivation [6]. Specifically the shell subregion seems to be responsive for emotional effects [11,16]. Therefore, stress-induced dysfunction especially in the NAcS could contribute to depressive symptoms, like anhedonia – the loss of motivation and inability to experience pleasure and reward. Most studies investigating changes in CREB expression in depression focused on different forebrain structures. For one, viral vector-mediated increases in CREB activity in the hippocampus exert antidepressant-like effects in diverse behavioral assays [17,18] and chronic antidepressant treatment increases CREB expression in this region [19]. Exposure to chronic stress, on the other hand, decreases pCREB levels in different cortical and limbic regions [14,15,20]. However, in the NAc the action of CREB seems to be in utter contrast to that of the aforementioned regions. Elevated CREB activity in the NAc produces depressive-like effects, whereas reductions in CREB activity has antidepressant-like effects in males [11,13,21,22]. While our finding that acute stress increased pCREB in the NAcS in male rats is in line with several other studies [11,12,21], the decrease in pCREB expression in the NAcS seen in females, after acute and chronic stress, was not demonstrated before. Different results from our laboratory showed similar gender-specific behavioral and associated neuronal activity changes after chronic stress in other limbic and cortical regions [14,15]. In these studies, CREB phosphorylation was sensitive to stress in the PFC and hippocampus of male rats but was resistant in females, both to acute and chronic stress. This is opposite to our results, showing decreased pCREB expression in the NAcS after chronic stress in females but no change in males. Taken together, this points to a fundamentally different reaction of females to exposure to acute as well as chronic stress in different regions of the brain and indicates that the female brain uses different strategies and underlying neuronal circuitry activity to cope with stress.

The sex differences observed at the biochemical level are accompanied by differential behavioral responses in the open field test. Only females showed reduced exploratory

behavior, measured as rearing frequency, after 6 weeks of stress. One could argue that males habituated to the prolonged stress paradigm, whereas females were unable to adapt and developed behavioral symptoms accompanied by changes in protein expression levels, indicating an anxious/depressed state. Interestingly, although male and female rats exhibited mainly opposite cellular adaptations after chronic stress, both sexes showed increased corticosteroid levels comparable to that of acutely stressed animals albeit that the basal plasma levels in females were higher (see Lin et al 2008[14] and Table 1). This implies that the exposure to the adverse environment is still stressful, however does not lead to the same behavioral or cellular adaptations in males and females. Chronic stress-induced decrease in pCREB in NAcS and the accompanying changes in exploratory behavior in females are strong evidence that stress coping behavior and cellular adaptational responses are different in males and females. This could play a role in the mechanisms behind the higher incidence of depression in women. Additionally, the results could also show a behavioral correlate to the expression of different subtypes of depression in males and females [6,13]. Determining the nature of this mechanism is beyond the scope of the present study, but it may be relevant to study whether the presently found sex-and region-specific effects of chronic stress on neuronal activity, reflect, disturbed activity of this region, as such disturbances of brain activity have been presumed to mediate stressor-induced brain disorders like depression.

In conclusion, our data contribute to accumulating evidence that stress responses may be fundamentally different in each sex [23-25]. The knowledge why the female brain is more vulnerable to stress and how it copes with extended periods of stress is very limited. Using the same chronic stress paradigm for males and females we could demonstrate that the reaction to chronic stress, in terms of changes in CREB and pCREB, is different in both sexes. The female brain apparently has different innate strategies to process stress, which consequently make it more susceptible for stress-related pathology. This emphasizes the importance of examining both sexes in animal models for stress-related psychiatric disorders, since underlying neurobiological mechanisms, including CREB signaling appear to be sex dependent. Therefore, one cannot justify using the male brain or its physiology to explain neurobiological mechanisms underlying stress-related diseases in women.

REFERENCES

1. Blendy, J. A. The Role of CREB in Depression and Antidepressant Treatment. *Biological Psychiatry* **59**, 1144–1150 (2006).
2. Bale, T. L. Stress sensitivity and the development of affective disorders. *Hormones and Behavior* **50**, 529–533 (2006).
3. Kajantie, E. & Phillips, D. The effects of sex and hormonal status on the physiological response to acute psychosocial stress. *Psychoneuroendocrinology* **31**, 151–178 (2006).
4. Whittle, S., Yücel, M., Yap, M. B. H. & Allen, N. B. Sex differences in the neural correlates of emotion: Evidence from neuroimaging. *Biological Psychology* **87**, 319–333 (2011).
5. Dichter, G. S. Anhedonia in unipolar major depressive disorder: a review. *Open Psychiatry Journal* **4**, 1–9 (2010).
6. Nestler, E. J. & Carlezon, W. A., Jr The Mesolimbic Dopamine Reward Circuit in Depression. *Biological Psychiatry* **59**, 1151–1159 (2006).
7. Baumann, B. *et al.* Reduced volume of limbic system-affiliated basal ganglia in mood disorders: preliminary data from a postmortem study. *J Neuropsychiatry Clin Neurosci* **11**, 71–78 (1999).
8. Epstein, J. *et al.* Lack of ventral striatal response to positive stimuli in depressed versus normal subjects. *Am J Psychiatry* **163**, 1784–1790 (2006).
9. Schlaepfer, T. E. *et al.* Deep Brain Stimulation to Reward Circuitry Alleviates Anhedonia in Refractory Major Depression. *Neuropsychopharmacology* **33**, 368–377 (2007).
10. Manji, H. K., Drevets, W. C. & Charney, D. S. The cellular neurobiology of depression. *Nat. Med.* **7**, 541–547 (2001).
11. Barrot, M. *et al.* CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 11435–11440 (2002).
12. Muschamp, J. W. *et al.* Activation of CREB in the nucleus accumbens shell produces anhedonia and resistance to extinction of fear in rats. *Journal of Neuroscience* **31**, 3095–3103 (2011).
13. Carlezon, W., Jr, Duman, R. & Nestler, E. The many faces of CREB. *Trends in Neurosciences* **28**, 436–445 (2005).
14. Lin, Y. *et al.* Effects of Long-Term Stress and Recovery on the Prefrontal Cortex and Dentate Gyrus in Male and Female Rats. *Cereb. Cortex* **18**, 2762–2774 (2008).
15. Lin, Y. *et al.* Sex differences in the effects of acute and chronic stress and recovery after long-term stress on stress-related brain regions of rats. *Cereb. Cortex* **19**, 1978–1989 (2009).
16. Sellings, L. H. L. & Clarke, P. B. S. Segregation of amphetamine reward and locomotor stimulation between nucleus accumbens medial shell and core. *Journal of Neuroscience* **23**, 6295–6303 (2003).
17. Newton, S. S. *et al.* Inhibition of cAMP response element-binding protein or dynorphin in the nucleus accumbens produces an antidepressant-like effect. *J. Neurosci.* **22**, 10883–10890 (2002).
18. Chen, A. C., Shirayama, Y., Shin, K. H., Neve, R. L. & Duman, R. S. Expression of the cAMP response element binding protein (CREB) in hippocampus produces an antidepressant effect. *BPS* **49**, 753–762 (2001).
19. Nibuya, M., Nestler, E. J. & Duman, R. S. Chronic antidepressant administration increases the expression of cAMP response element binding protein (CREB) in rat hippocampus. *J. Neurosci.* **16**, 2365–2372 (1996).
20. Trentani, A., Kuipers, S. D., Horst, Ter, G. J. & Boer, Den, J. A. Selective chronic stress-induced in vivo ERK1/2 hyperphosphorylation in medial prefrontocortical dendrites: implications for stress-related cortical pathology? *European Journal of Neuroscience* **15**, 1681–1691 (2002).
21. Pliakas, A. M. *et al.* Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. *Journal of Neuroscience* **21**, 7397–7403 (2001).
22. Nestler, E. J. *et al.* Neurobiology of depression. *Neuron* **34**, 13–25 (2002).
23. Dalla, C. *et al.* Chronic mild stress impact: Are females more vulnerable? *Neuroscience* **135**, 703–714 (2005).
24. Drossopoulou, G. Sex differences in behavioral, neurochemical and neuroendocrine effects induced by the forced swim test in rats. *Neuroscience* **126**, 849–857 (2004).
25. Palanza, P. Animal models of anxiety and depression: how are females different? *Neuroscience and Biobehavioral Reviews* **25**, 219–233 (2001).

SUMMARY & GENERAL DISCUSSION

8

Stress plays a growing role in our modern society. Everybody is confronted and has to deal with it at one point in their life. However, stress does not necessarily have to be a bad thing. In the short run, stress, or the physiological response to stress, can help us to perform better in times of pressure and increases our cognitive abilities. Every situation perceived as stressful leads to a series of highly coordinated reactions and results in the enhanced release of the stress hormones adrenaline, noradrenaline and glucocorticoids from the adrenal glands and the brain. This brings about physiological alterations that make us feel stressed and at the same time enable us to react effectively to the situation and help us to prepare for future events [1,2].

In **Chapter 1** of this thesis, we introduced the concept of stress and elaborated on emotional learning and memory, an aspect positively influenced by stress and stress hormones. We further introduced the effects often seen after chronic stress that can lead to several deleterious disorders, including depression.

The relation between stress, emotion and learning and memory is the foundation of the first part of this thesis. We all know that not all experiences are equally well transferred into long-term memory. Significant and emotional moments are especially well remembered. Extensive evidence indicates that the stress hormones released during and after such experiences modulate the neurobiological processes responsible for the facilitated consolidation and storage of emotionally arousing experiences into long-term memory. The first part (**Part I**) of this thesis was aimed at further dissecting the neurobiological circuits and mechanisms associated with emotional memory formation. The experimental approach is based on behavioral experiments and pharmacological manipulations employed to investigate the glucocorticoid-induced modulation of memory consolidation processes, implicated in the storage of emotionally arousing experiences more closely.

In **Chapter 2** we first introduced the main technique used throughout Part I, stereotaxic surgery. Due to a few modifications we were able to refine this long-standing procedure to significantly increase animal welfare and survival. By utilizing this technique in **Chapter 3,** we implanted cannulas into the insular cortex (IC) and showed its involvement in the effects of glucocorticoids on memory consolidation. Previous studies investigating the role of the IC in learning and memory focused primarily on the influence of this brain region in taste learning [3-5]. By using the classical inhibitory avoidance paradigm, we showed that the administration of the specific glucocorticoid receptor (GR) agonist RU 28362 into the IC immediately (but not 3 hours) after training dose-dependently enhanced 48-hour retention performance, resulting in longer delays before entering the dark compartment, on this aversively motivated learning task.

With the aid of a modified version of the inhibitory avoidance task, in which the presentation of the context and footshock were separated, we further discovered that the IC mediates glucocorticoid effects on the consolidation of memory of both components of the task. Increased retention latencies were found with administration of the GR agonist after either context or footshock exposure. Additionally, we show

that training increased neuronal activity of the IC, assessed by an increased number of phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) expressing cells. A memory-enhancing dose of systemically administered corticosterone, however, reduced IC activity. On the basis of these findings, we hypothesize that the IC is much more broadly involved in the consolidation of emotional memory and probably is part of the greater network for the detection of salient information distributed throughout the brain.

Although it is known that the stress response is also activated in reaction to appetitive and rewarding stimuli and that enhanced memory occurs for aversive as well as appetitive/rewarding experiences, research investigating the underlying mechanisms of emotional memory consolidation has almost exclusively focused on aversively motivated learning tasks. Not much attention has been given to the differentiation between emotional positively and negatively valenced experiences. Hence, in **Chapter 4** we investigated whether the effects of glucocorticoids on memory consolidation for differentially valenced emotionally arousing learning experiences are similar. We could show that the GR agonist RU 28362 given immediately (but not several hours) after the training experience into the nucleus accumbens (NAc) enhanced the consolidation of memory of an aversive as well as an appetitive taste learning paradigm. This was indicated by a respective decreased or increased liquid intake compared to the first encounter of the taste. Glucocorticoids are known to interact with the noradrenergic system to promote the consolidation of aversive memories. Therefore, in a separate set of rats we investigated whether an interaction with the arousal-induced noradrenergic system is necessary for the observed enhancement of memory consolidation in both learning tasks. Co-administration of the ß-adrenoceptor antagonist propranolol, in a dose ineffective by itself, blocked the memory-enhancing effect of the GR agonist. This suggests that the glucocorticoid-noradrenergic interaction is independent of the valence of the learning experience and enhances memory consolidation in both cases.

The brain region mostly studied in respect to emotional learning and memory is the basolateral complex of the amygdala (BLA). Extensive evidence indicates its crucial involvement in enabling the facilitatory effects of stress hormones on emotional learning and memory. However, most studies investigating the role of the BLA so far utilized aversive or fear-motivated learning tasks. Hence, whether the BLA is also involved in facilitating the effects of glucocorticoids on appetitive memory consolidation is not yet known. Therefore, in **Chapter 5** we examined whether the BLA is also involved in regulating glucocorticoid effects on appetitive memory consolidation. Administration of the GR agonist RU 28362 into the BLA immediately after drinking an appetitive novel saccharin solution enhanced retention performance 24 hours later, as indicated by an increased intake of the same saccharin solution compared to vehicle-treated rats. This indicates a broad role for the BLA in the mediation of glucocorticoid effects on learning and memory independent of the valence of the stimulus.

Taken together, the investigations of glucocorticoid effects on memory consolidation processes presented here reveal different new insights on a number of questions

regarding the consolidation of emotional memories that did not receive much attention until now. The topic of glucocorticoid-induced memory modulation is part of a greater, captivating question of how emotions can influence learning and memory. Even though an extensive literature already exists regarding this topic, many questions remain unanswered. Additionally, the picture sometimes seems confusing due to different outcomes provoked by glucocorticoids, depending on the duration (acute versus chronic) and time point of manipulation (acquisition, consolidation or retrieval). The experiments presented here deal exclusively with the acute effects of glucocorticoids on memory consolidation. A plethora of studies already investigated this topic and it is generally accepted that acute increases in glucocorticoid levels can enhance memory consolidation. Nonetheless, several questions remained uninvestigated. Some of these questions were addressed in the presented experiments. The present results extend previous findings in different aspects which will be presented in the following sections.

THE BRAIN REGIONS INVOLVED IN EMOTIONAL MEMORY CONSOLIDATION

In the first place, the presented findings expand the number of brain regions influenced by glucocorticoids. Although human imaging studies point to a key role of the IC in emotionally influenced learning and memory [6-8], until now the investigation of the IC in emotional learning and memory was largely underrepresented in animal studies. Only two studies so far investigated the influence of stress hormone treatment on memory function in this region [9,10]. With the data presented in Chapter 3 we could extend the previous investigations and show that the IC is importantly involved in regulating the effects of glucocorticoids on memory consolidation of emotionally arousing (aversive) learning experiences. This shows that the IC is a brain region with extensive influence in emotional memory consolidation and an important part of the network involved in emotional regulation of learning and memory. In Chapter 3 we also report that a memory-enhancing dose of corticosterone reduces neuronal activity within the IC. This has important implications, because it suggests that inactivation, rather than activation, of the IC is responsible for prompting glucocorticoid-induced memory facilitation. Several human imaging studies have shown a similar decrease in activity of the IC along with other regions, which highly correlated with higher cortisol levels during emotional stimuli and enhanced recollection [11,12]. These findings could be interpreted in different ways. First of all, deactivation of these areas could lead to disinhibition (activation) of other subcortical regions. This loss of "top-down control" could enhance for example the speed of decision making [13]. It was shown that individuals that respond to challenges with a strong cortisol increase are more sensitive to immediate rewards [14,15] associated with a loss of top-down control of prefrontal over subcortical areas [14,16-18]. However, it has also been connected with several psychiatric disorders, such as post-traumatic stress disorder (PTSD) and memory deficits as well as intrusions [12,19,20]. A second interpretation

could be a shift in attentional processing from a peripheral to central focused view. This would fit well with the hypothesis that stress specifically improves memory of the gist of information, the so called 'weapon focus phenomenon' [12,21].

The NAc, investigated in Chapter 4, has mostly been studied indirectly for its involvement in stress hormone-induced facilitation of memory consolidation of emotionally arousing experiences [22-24]. Only one study so far investigated the direct effects of glucocorticoids within the NAc [25]. In this study the role of GRs in the formation of morphine-induced conditioned place preference was investigated. Intra-NAc administration of the GR antagonist RU 38486, 30 min before morphine conditioning, prevented the formation of place preference seen in vehicle-treated rats. Although this study shows an involvement of GR activation within the NAc in mnemonic processes, it has several disadvantages compared to our present study. First, due to the pre-training administration of the drug, it cannot be excluded that it affected attentional, motivational or sensory-perceptual processes in addition to mnemonic processes. In our studies, treatments were given only after conditioning. This constitutes a more elegant technique and ensures that animals are drug-free at the time of training (acquisition) as well as during retention testing. This guarantees that any effect can be solely associated to consolidation processes and is unaffected by possible effects on attentional, motivational or perceptual mechanisms and additionally excludes the possibility of state-dependent effects [26]. A second disadvantage of the study by Dong and colleagues are the coordinates used to implant guide cannulas and the selected volume of 0.5 µl per side. Due to this, it is impossible to distinguish properly between the two subregions of the NAc. In contrast, the small infusion volume (0.3 µl per side) and the coordinates used in our study were specifically selected to successfully distinguish between the two subregions of the NAc. Thereby we were able to show that only the shell is involved in regulating the modulatory effects of GR activation during emotional memory consolidation. Thus, the findings presented in Chapter 4 constitute the first demonstration of an involvement of glucocorticoid-mediated memory consolidation within the NAc.

As already stated above, the amygdala has been strongly implicated in regulating emotion and stress effects on memory and appears to be crucial in mediating the neuromodulatory influences of glucocorticoids [27,28]. However, to my knowledge, the findings shown in Chapter 5 are the first demonstration of an involvement of glucocorticoids within the BLA in modulating memory of appetitive learning. This has further implications for the role of how the amygdala is viewed. Next to its well-established function in the processing and consolidation of unpleasant and fear-related stimuli [29,30], different studies found comparable effects in the processing of appetitive and rewarding stimuli [31-33]. Perhaps, the BLA might be more a detector for arousing/ salient information rather than necessarily only for fearful or anxious stimuli.

APPETITIVE VERSUS AVERSIVE EXPERIENCES

Next to the conclusion that glucocorticoids influence a wide range of brain regions in modulating memory representations, the data in Chapter 4 and 5 bring us to a second important conclusion: Memory modulation by stress hormones is not restricted to aversive conditioning. Selye, who´s definition of stress is described in the Introduction, focused his work on responses to diverse noxious and adverse stimuli. This was an important rational behind his definition of stress and ever since the stress response has generally been connected to challenges of aversive nature [34]. As a consequence, the presence of stress mediators has often been regarded as an indicator of exposure to aversive events [34]. However, as stated in the Introduction, rewarding/positive situations like food, sex or drugs of abuse also elicit an activation of the stress response and trigger the release of glucocorticoids to the same extent as do aversive experiences [14,34,35]. For example, Buwalda and colleagues found that the corticosterone response is not different between animals actively involved in a negative (social defeat) or a positive (copulatory behavior) social stimulus [34]. However, as indicated earlier, only a handful of studies actually examined the effect of glucocorticoid administration on appetitive learning [36-39]. However, the involvement of different brain regions and the cellular mechanism of glucocorticoid-induced memory enhancement were not taken into account and the investigation remained mostly systemic and shallow.

The findings presented in Chapter 4 and 5 provide the first evidence of memory modulation by intracranial post-training GR activation in an appetitive conditioning paradigm. This has some interesting implications. First, if the effects of post-training glucocorticoids were limited to aversive conditioning, it could be argued that the administration of glucocorticoids could be aversive in itself. However, the fact that the GR agonist RU 28362 had no effect if rats were pseudo-conditioned to saline instead of the malaise-inducing substance lithium chloride (see Figure 2B, Chapter 5) as well as the fact that the administration of RU 28362 enhanced memory consolidation in the appetitive learning paradigm allows me to discard this possibility. This appears logical when looked at from an evolutionary point of view. Improved recollection of stimuli representing a threat (e.g. a predator) or pleasant events (e.g. food) enhances the ability to predict significant events in the environment and allows a quick, appropriate response (either approach or avoidance), and thus might be equally important for survival. The findings reported here are in line with those of human studies investigating the formation of emotionally influenced learning and memory. Buchanan and Lovallo (2001) as well as Kuhlmann and Wolf (2006) showed that elevated cortisol levels during the encoding of emotionally arousing pictures (positive and negative) enhance long-term recall performance assessed in a surprise delayed-recall task[40,41].

Upon emotional arousal, not only glucocorticoids but also other stress-related hormones such as adrenaline and noradrenaline are released[2]. As already mentioned before, it has been extensively shown that arousal-induced noradrenergic activity

is indispensable for mediating the enhancing effect of glucocorticoids on memory consolidation in aversively motivated learning tasks. However, this mechanism has never been investigated for appetitive learning experiences. The data presented in Chapter 4, are the first to indicate that glucocorticoids exert their effects by interacting with the noradrenergic system in an appetitive learning paradigm. Thus, a sufficient arousal-induced increase in noradrenaline levels is necessary for mediating the enhancing effects of glucocorticoids on memory consolidation of both appetitive as well as aversive learning experiences.

In summary, based on the presented results we can assume that stress hormones facilitate memory consolidation and thus the storage of motivationally significant information in the environment regardless of the valence of the experience by a common system in a network of different brain regions. An interaction with the noradrenergic arousal system seems crucial for memory of experiences of both valences to be enhanced. This supports previous claims that the degree of general arousal rather than valence is responsible for enhanced memory retention [42-44]. Different reports in human literature suggest that glucocorticoids interact with the degree of emotional arousal, independent of valence, at initial encoding to modulate memory processes [40,41,45] [but see Tops et al. (2003)[46]]. Other studies, without manipulation of cortisol, additionally highlight the effect of arousal on recall. Here, pleasant as well as unpleasant pictures or sounds were remembered better than neutral ones and memory was stronger if the stimulus was highly arousing [42,47-50]. Together, these studies and our present findings indicate that the release of glucocorticoids leads to a prioritized storage of emotional arousing information into long-term memory independent of the valence of the stimulus [51].

EFFECT OF AROUSAL VERSUS VALENCE ON THE BRAIN NETWORK INVOLVED

Although the intracellular mechanisms might be similar for the consolidation of memory of appetitive and aversive learning experiences, different brain circuits might be involved in these two processes [52]. At present, it is highly debated whether the role of arousal within the larger emotional memory network may differ depending on valence [32,52]. Kensinger and Corkin (2004) argued that distinct neurocognitive processes contribute to the encoding and successful storage of (negative) arousing information compared to that of non-arousing valenced information [53]. An amygdala-hippocampus network is activated during the successful encoding of highly arousing (negative) words, whereas the encoding of non-arousing, but negatively valenced, words engages a prefrontal-hippocampal circuit. This is in agreement with the interpretation of Dolcos and Cabeza (2002)'s event related potential (ERP) results of an amygdala network related to arousal and a prefrontal-hippocampus network related to valence, and lends further support for a dissociation of valence- and arousal-related brain systems [49]. The effect of glucocorticoids and noradrenaline, however, seems to be valence independent and enhances any process

taking place in the respective brain regions studied. Kensinger and Corkin (2004) further claimed that memory for valenced, low-arousing information relies on controlled processes and has to be more elaborated and rehearsed to be encoded and consolidated into long-term memory compared to high-arousing stimuli. Enhancement of memory of arousing words seems to occur automatically [53]. Thus valence alone can lead to an enhanced storage of information. However, this process seems to involve different brain regions and additional attentional and higher cognitive processes than the storage of arousing information. Nonetheless, even if memories are arousing and well remembered, we are still able to attach a certain valence to the memories and distinguish between appetitive and aversive experiences. This indicates that arousal enhances memory, but that valence is still an important factor and is linked to the memory stored. Different brain areas are proposed to attach the valence to a certain stimulus. A recent study by Mickley Steinmetz and colleagues (2010) indicated that arousal strengthens amygdala connections to frontal regions for negative information while these efferents were decreased for positive information [52]. Furthermore, arousal seems to involve a more widespread, global network of brain regions when associated with negative items, enhancing connectivity between different nodes of the emotional memory network. The effects of arousal on memory for positive information were restricted to amygdala efferents. This emphasizes that, although the global effects of arousal are independent of the valence of the stimulus, the activation and connectivity within and between the individual areas of the emotional memory network depend on the valence of the stimulus. The direction of the modulation of the network seems actually to be opposite depending on the valence.

Based on our current data, we cannot make any conclusion concerning the interaction between the different brain regions investigated that would be more than theoretical speculation. In order to investigate the role of different brain structures, it would be necessary to employ studies locating different brain regions for pharmacological manipulations simultaneously. This would constitute a possible interesting future direction in the field of glucocorticoid-induced memory modulation. Additionally we cannot make any statement about the difference between arousing and valenced information. All training tasks utilized in the present studies are highly emotionally arousing. However, we can say that arousing information, no matter if positive or negative, are preferentially stored and that glucocorticoids acting in different brain regions play a fundamental role in this process.

In conclusion, in Part I of this thesis, we could demonstrate that glucocorticoids, in collaboration with the noradrenergic system, modulate a wide range of types of memory representations of both appetitive and aversive nature. This suggests a common general arousal mechanism mediating this modulation. The findings reported here not only make important additions regarding the role of glucocorticoids in emotional learning and memory, they also are of direct interest to the study of different brain structures implicated in emotional learning. This might also have some far-reaching implications for the understanding of certain psychopathological conditions related to emotional

learning and memory, such as PTSD, drug addiction or depression. To get an even deeper insight into the mechanisms by which emotional arousal modulates memory consolidation, further research needs to be pursued in several lines. First and very generally, it seems of utmost importance to use test stimuli comprising valence and arousal, thus ranging from low to high arousing and positive to negative. Additionally, studies to further dissect the endogenous mechanism of glucocorticoid-noradrenergic interaction, as well as interactions with other neurotransmitter and peptide systems, should be broadened to entail appetitive learning tasks to verify whether the pathway dissected for aversive memories and partly touched in this thesis is a general phenomenon or comprises differences depending on the valence of a stimulus.

The data presented in Part I of this thesis clearly indicate that acute stressful events and the release of stress hormones have positive effects on cognitive functions and are protective and adaptive in nature. However, although the stress response is an adaptive and necessary reaction in the short run, long-term stress, going on for weeks, months or even years, can lead to chronic alterations in the stress response and can have deleterious effects on body and mind. The continuously increased production of stress hormones can lead to long-term alterations in neurochemical, neuroanatomical and cellular functions and cause the development of psychopathologies like for example depression [54-57]. Due to the direct correlation between (chronic) stress and the development of depression, preclinical research has focused on behavioral and neuropathological effects of stress in rodents to investigate the neurobiological mechanisms underlying depression [58].

As stated in the Introduction, the prevalence of depression is twice as high in women than in men [58 60]. There is accumulating evidence that men and women activate different brain regions and neuronal circuits in response to stressful experiences and apply different coping strategies [58,60]. This could be attributed to anatomical differences, hormonal influences as well as environmental factors. However, the underlying mechanism of these sex differences remains virtually unknown [59,61] and most studies on depression are still performed in male animals.

Thus, while the previous chapters investigated the effects of acute glucocorticoid administration on cognitive functions in different brain regions, the aim of the second part (**Part II**) of this thesis was to further investigate sex differences in the response to especially chronic stress. The experimental approach is again based on behavioral experiments employed to investigate the sex-specific changes induced by a chronic stress paradigm, implicated in the development of depression. **Chapter 6 and 7** describe some of the alterations induced by acute and chronic stress on the expression and phosphorylation pattern of the transcription factor cAMP response element binding protein (CREB) in different stress-related brain regions and compares these changes between males and females. CREB is involved in the stress response as well as antidepressant treatment effects and part of one of the major theories explaining the underlying neurobiology of depression.

Our findings of **Chapter 6** indicate that the different subregions of the hippocampus and prefrontal cortex (PFC) in male rats show a reduction in pCREB expression and modifications in the morphology of these brain regions. This is in contrast to females who failed to demonstrate any indication of such processes in these regions. In contrast to this, levels of brain derived neurotrophic factor (BDNF) were only affected in female rats exhibiting an increase after acute stress in the hippocampus and a decrease after chronic stress in the PFC. When investigating the NAc (**Chapter 7**) the picture reversed. Within this region, the most pronounced effects were visible in female rats which showed an increase in CREB levels after acute stress but a decrease in pCREB levels after acute and chronic stress exposure. In this region, CREB and pCREB expression remained largely unaffected in male rats. Overall, our findings indicate that male and female rats respond with different, even opposite cellular reactions to stress. Thus, sex-specific analysis has much potential to further our understanding of the stress response and the development of mental health disorders [62] and sex differences should be emphasized in future studies of the neurobiological mechanisms of the response to stress and depression [63].

EFFECTS OF STRESS AND SEX ON CREB

CREB phosphorylation is crucial for its ability to bind DNA and modulate gene expression. Reduced pCREB availability might result in reduced availability of proteins involved in neuronal plasticity and cell survival (e.g. BDNF). Sustained stressful experiences have already been associated with reduced neurotrophin expression, a condition that might disturb the dynamic equilibrium between intracellular signaling cascades [64,65]. As described in the Introduction, CREB is usually phosphorylated at a specific serine residue, serine 133 (Ser^{133}). This phosphorylation promotes the association of CREB with the CREB-binding protein and forms an active transcription complex enabling target gene activation [66]. This activation can occur via different signal transduction pathways, including the cAMP-dependent protein kinase A (PKA) pathway, the Ca2+/calmodulin-dependent protein kinase IV (CaMK IV) as well as the ERK/Mitogen-activated protein kinase (MAPK) cascade (see figure 4, Chapter 1). It was previously shown that ERK1/2 is hyper-phosphorylated in the PFC of male rats [67,68]. However, female rats did not show an alteration in pERK1/2 levels after chronic stress treatment in this brain region [68]. Preliminary findings from our study investigating different signal transduction cascades, found a similarly enhanced phosphorylation of ERK1/2 in male rats. We additionally could show a comparable increase in phosphorylation in response to chronic stress in female rats. This seems counterintuitive given the decrease of CREB phosphorylation despite increased activation of the ERK/MAPK cascade. However, Wang et al (2003) could show that prolonged ERK phosphorylation negatively regulates CREB activity and phosphorylation and is associated with pathological mechanisms [69,70]. Thus the enhanced activation of signal transduction cascades leads to down-regulated CREB phosphorylation which might play a fundamental role in the effects of stress on the mechanisms of neuroplasticity. Sustained levels of stress or glucocorticoids have been shown

to damage several brain regions at the level of morphological neuroplasticity [71-73]. Excess stress leads to atrophy and retraction of apical dendrites of hippocampal and prefrontal cortical neurons [71,74-76] and can even result in the death of pyramidal cells [77]. Likewise, stress has been shown to reduce neurogenesis in the rodent hippocampus [73,78]. In conclusion, chronic stress-induced pERK and pCREB abnormalities may limit BDNF availability, disrupting neuronal plasticity and facilitating the development of neuronal defects.

A sex-specific impact of stress on neuronal plasticity may be explained by differential mechanisms either potentiating or suppressing the influences of stressful events. The striking fact that the higher prevalence of depression in women arises at puberty and then declines again after menopause, contributes to the idea that ovarian hormones (estrogen and progesterone) play an important factor in the sensitivity to the effects of stress [71,79]. Ovarian hormones have different effects on affective state as well as cognition [80]. Different evidence indicates that the influence of estrogens varies by brain region and reaches from counteracting cognitive impairments [81] and dendritic remodeling [82] in some to enhanced sensitivity to the stress response and greater stress-related cognitive impairments in other regions [83]. The ovarian hormones present in females can stimulate specific intracellular cascades underlying neuronal plasticity and prevent/counteract the reduced CREB phosphorylation observed in different brain regions of chronically stressed male animals [84-86]. It is thus possible that the influences of repeated footshock exposure on ERK activation and CREB phosphorylation seen especially in male rats might have been attenuated by the presence of ovarian hormones in females. Ovarian steroids thus seem to exert different influences on critical neuronal processes. This can lead to the assumption that these hormones prevent the changes in response to stress observed in male rats and thus have a protective function in regard to the effects of stress on the brain. However, keeping in mind that females are more vulnerable to depression especially during their productive years, this hypothesis is misleading. Therefore, the stress-induced neurobiological changes seen in males could be considered as adaptive and active coping mechanisms, potentially preventing subsequent development of depression. The retraction of dendrites, mostly seen as a maladaptive response to stress in especially the hippocampus, could also be interpreted as a protective response. By retracting connections, neurons are shielding themselves from too much excitation or input, thereby preventing overstimulation and potential cell death in times of great demands. The consequences of these adaptations seen in different cognitive functions, like memory, could also be interpreted as adaptive. Relatively impaired memory, seen in male but not female rats after prolonged periods of stress [87,88], is possibly beneficial and may enable them to forget the stress and its associations more quickly [59]. Therefore, maybe the reduced response of females to stress and chronic stress make them in the end more vulnerable for psychopathological disorders like depression.

To conclude, each gender should be evaluated independently to determine the stress and/or treatment response and explore new avenues of therapeutic approach.

CONCLUDING REMARKS

The concept of stress, as introduced in the Introduction has long been subject of scientific discussion. Stress has long carried a negative connotation, especially in laymen terms. People automatically associate the word "stress" with bad feelings and a multitude of other physical, emotional and mental discomforts. However, from the data presented in this thesis and other research in the field of stress it is clear that the pure presence of a neuroendocrine response is not sufficient anymore to label a stimulus as stress [31,35]. The powerful chemicals released are there, first and foremost, to help us survive. However, it remains a matter of timing and dose. Under circumstances of prolonged or severe stress we can suffer negative consequences and develop deleterious disorders and pathologies. Nonetheless, not all changes in the brain seen in response to, even chronic, stress should be immediately labeled as aversive and maladaptive.

REFERENCES

1. de Kloet, E. R., Joëls, M. & Holsboer, F. Stress and the brain: from adaptation to disease. *Nature Reviews Neuroscience* 6, 463-475 (2005).
2. Joëls, M. & Baram, T. Z. The neuro-symphony of stress. *Nature Reviews Neuroscience* (2009). doi:10.1038/nrn2632
3. Bermudez-Rattoni, F. Molecular mechanisms of taste-recognition memory. *Nature Reviews Neuroscience* **5**, 209–217 (2004).
4. Nuñez-Jaramillo, L., rez-Lugo, L. R., Herrera-Morales, W. & Miranda, M. A. I. Taste memory formation: Latest advances and challenges. *Behavioural Brain Research* 1–17 (2009). doi:10.1016/j.bbr.2009.10.040
5. Stehberg, J., Moraga-Amaro, R. & Simon, F. The role of the insular cortex in taste function. *Neurobiology of Learning and Memory* 1–6 (2011).doi:10.1016/j.nlm.2011.03.005
6. Craig, A. D. B. How do you feel--now? The anterior insula and human awareness. *Nature Reviews Neuroscience* **10**, 59–70 (2009).
7. Menon, V. & Uddin, L. Q. Saliency, switching, attention and control: a network model of insula function. *Brain Struct Funct* **214**, 655-667 (2010).
8. Ille, R. *et al.* Emotion recognition and experience in Huntington disease: a voxel-based morphometry study. *J Psychiatry Neurosci* **36**, 383–390 (2011).
9. Miranda, M. I., Quirarte, G. L., Rodriguez-Garcia, G., McGaugh, J. L. & Roozendaal, B. Glucocorticoids enhance taste aversion memory via actions in the insular cortex and basolateral amygdala. *Learning & Memory* **15**, 468–476 (2008).
10. Roozendaal, B. *et al.* Membrane-Associated Glucocorticoid Activity Is Necessary for Modulation of Long-Term Memory via Chromatin Modification. *Journal of Neuroscience* **30**, 5037–5046 (2010).
11. Pruessner, J. C. *et al.* Deactivation of the Limbic System During Acute Psychosocial Stress: Evidence from Positron Emission Tomography and Functional Magnetic Resonance Imaging Studies. *Biological Psychiatry* **63**, 234–240 (2008).
12. van Stegeren, A. H., Roozendaal, B., Kindt, M., Wolf, O. T. & Joëls, M. Interacting noradrenergic and corticosteroid systems shift human brain activation patterns during encoding. *Neurobiology of Learning and Memory* **93**, 56–65 (2010).
13. Porcelli, A. J. & Delgado, M. R. Acute stress modulates risk taking in financial decision making. *Psychol Sci* **20**, 278–283 (2009).
14. Piazza, P. V. & Le Moal, M. Glucocorticoids as a biological substrate of reward: physiological and pathophysiological implications. *Brain Research Reviews* **25**, 359–372 (1997).
15. Newman, E., O'Connor, D. B. & Conner, M. Daily hassles and eating behaviour: the role of cortisol reactivity status. *Psychoneuroendocrinology* **32**, 125–132 (2007).
16. Arnsten, A. F. The biology of being frazzled. *Science* **280**, 1711–1712 (1998).
17. Erickson, K., Drevets, W. & Schulkin, J. Glucocorticoid regulation of diverse cognitive functions in normal and pathological emotional states. *Neuroscience and Biobehavioral Reviews* **27**, 233–246 (2003).
18. van den Bos, R., Harteveld, M. & Stoop, H. Stress and decision-making in humans: Performance is related to cortisol reactivity, albeit differently in men and women. *Psychoneuroendocrinology* **34**, 1449–1458 (2009).
19. Bremner, J. D. Traumatic stress: effects on the brain. *Dialogues Clin Neurosci* **8**, 445–461 (2006).
20. Shin, L. M., Rauch, S. L. & Pitman, R. K. Amygdala, medial prefrontal cortex, and hippocampal function in PTSD. *Annals of the New York Academy of Sciences* **1071**, 67–79 (2006).
21. Cahill, L. & van Stegeren, A. Sex-related impairment of memory for emotional events with beta-adrenergic blockade. *Neurobiology of Learning and Memory* **79**, 81–88 (2003).
22. Kerfoot, E. C. & Williams, C. L. Interactions between brainstem noradrenergic neurons and the nucleus accumbens shell in modulating memory for emotionally arousing events. *Learning & Memory* **18**, 405–413 (2011).
23. Setlow, B., Roozendaal, B. & McGaugh, J. L. Involvement of a basolateral amygdala complex–nucleus accumbens pathway in glucocorticoid-induced modulation of memory consolidation. *European Journal of Neuroscience* **12**, 367–375 (2000).
24. Roozendaal, B., de Quervain, D. J., Ferry, B., Setlow, B. & McGaugh, J. L. Basolateral amygdala-nucleus accumbens interactions in mediating glucocorticoid enhancement of memory consolidation. *Journal of Neuroscience* **21**, 2518–2525 (2001).
25. Dong, Z. *et al.* Morphine conditioned place preference depends on glucocorticoid receptors in both hippocampus and nucleus accumbens. *Hippocampus* **16**, 809–813 (2006).

26. McGaugh, J. L. Time-dependent processes in memory storage. *Science* **153**, 1351–1358 (1966).
27. McGaugh, J. L. Memory--a Century of Consolidation. *Science* **287**, 248–251 (2000).
28. Roozendaal, B. & McGaugh, J. L. Memory modulation. *Behavioral Neuroscience* **125**, 797–824 (2011).
29. Phelps, E. A. & LeDoux, J. E. Contributions of the Amygdala to Emotion Processing: From Animal Models to Human Behavior. *Neuron* **48**, 175–187 (2005).
30. Gerdes, A. Brain activations to emotional pictures are differentially associated with valence and arousal ratings. *Front. Hum. Neurosci.* **4**, (2010).
31. Hsu, E. H., Schroeder, J. P. & Packard, M. G. The amygdala mediates memory consolidation for an amphetamine conditioned place preference. *Behavioural Brain Research* **129**, 93–100 (2002).
32. Hamann, S. & Mao, H. Positive and negative emotional verbal stimuli elicit activity in the left amygdala. *NeuroReport* **13**, 15–19 (2002).
33. Dwyer, D. M. Lesions of the basolateral, but not central, amygdala impair flavour-taste learning based on fructose or quinine reinforcers. *Behavioural Brain Research* **220**, 349–353 (2011).
34. Buwalda, B., Scholte, J., de Boer, S. F., Coppens, C. M. & Koolbaas, J. M. The acute glucocorticoid stress response does not differentiate between rewarding and aversive social stimuli in rats. *Hormones and Behavior* **61**, 218–226 (2012).
35. Koolhaas, J. M. *et al.* Stress revisited: A critical evaluation of the stress concept. *Neuroscience and Biobehavioral Reviews* **35**, 1291–1301 (2011).
36. Micheau, J., Destrade, C. & Soumireu-Mourat, B. Intraventricular corticosterone injection facilitates memory of an appetitive discriminative task in mice. *Behavioral and Neural Biology* **31**, 100–104 (1981).
37. Micheau, J., Destrade, C. & Soumireu-Mourat, B. Time-dependent effects of posttraining intrahippocampal injections of corticosterone on retention of appetitive learning tasks in mice. *European Journal of Pharmacology* **106**, 39–46 (1985).
38. Zorawski, M. & Killcross, S. Posttraining glucocorticoid receptor agonist enhances memory in appetitive and aversive Pavlovian discrete-cue conditioning paradigms. *Neurobiology of Learning and Memory* **78**, 458–464 (2002).
39. Zorawski, M. & Killcross, S. Glucocorticoid Receptor Agonist Enhances Pavlovian Appetitive Conditioning but Disrupts Outcome-Specific Associations. *Behavioral Neuroscience* **117**, 1453–1457 (2003).
40. Buchanan, T. W. & Lovallo, W. R. Enhanced memory for emotional material following stress-level cortisol treatment in humans. *Psychoneuroendocrinology* **26**, 307–317 (2001).
41. Kuhlmann, S. & Wolf, O. T. Arousal and cortisol interact in modulating memory consolidation in healthy young men. *Behavioral Neuroscience* **120**, 217–223 (2006).
42. Bradley, M. M., Greenwald, M. K., Petry, M. C. & Lang, P. J. Remembering pictures: pleasure and arousal in memory. *Journal of Experimental Psychology: Learning, Memory, and Cognition* **18**, 379–390 (1992).
43. Cahill, L. *et al.* Amygdala activity at encoding correlated with long-term, free recall of emotional information. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8016–8021 (1996).
44. van Stegeren, A. H., Everaerd, W., Cahill, L., McGaugh, J. L. & Gooren, L. J. Memory for emotional events: differential effects of centrally versus peripherally acting beta-blocking agents. *Psychopharmacology* **138**, 305–310 (1998).
45. Putman, P., van Honk, J., Kessels, R. P. C., Mulder, M. & Koppeschaar, H. P. F. Salivary cortisol and short and long-term memory for emotional faces in healthy young women. *Psychoneuroendocrinology* **29**, 953–960 (2004).
46. Tops, M. *et al.* Acute cortisol effects on immediate free recall and recognition of nouns depend on stimulus valence. *Psychophysiology* **40**, 167–173 (2003).
47. Bradley, M. M. & Lang, P. J. Affective reactions to acoustic stimuli. *Psychophysiology* **37**, 204–215 (2000).
48. Versace, F., Bradley, M. M. & Lang, P. J. Memory and event-related potentials for rapidly presented emotional pictures. *Exp Brain Res* **205**, 223–233 (2010).
49. Dolcos, F. & Cabeza, R. Event-related potentials of emotional memory: encoding pleasant, unpleasant, and neutral pictures. *Cogn Affect Behav Neurosci* **2**, 252–263 (2002).
50. Liu, D. L. J., Graham, S. & Zorawski, M. Enhanced selective memory consolidation following post-learning pleasant and aversive arousal. *Neurobiology of Learning and Memory* **89**, 36–46 (2008).
51. Wolf, O. T. Stress and memory in humans: twelve years of progress? *Brain Research* **1293**, 142–154 (2009).
52. Mickley Steinmetz, K. R., Addis, D. R. & Kensinger, E. A. The effect of arousal on the

emotional memory network depends on valence. *NeuroImage* **53**, 318–324 (2010).

53. Kensinger, E. A. & Corkin, S. Two routes to emotional memory: distinct neural processes for valence and arousal. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 3310–3315 (2004).
54. Nestler, E. J. *et al.* Neurobiology of depression. *Neuron* **34**, 13–25 (2002).
55. Reagan, L. P., Grillo, C. A. & Piroli, G. G. The As and Ds of stress: metabolic, morphological and behavioral consequences. *European Journal of Pharmacology* **585**, 64–75 (2008).
56. Belmaker, R. H. & Agam, G. Major depressive disorder. *N. Engl. J. Med.* **358**, 55–68 (2008).
57. Pollak, D. D., Rey, C. E. & Monje, F. J. Rodent models in depression research: Classical strategies and new directions. *Ann Med* **42**, 252–264 (2010).
58. Dalla, C., Pitychoutis, P. M., Kokras, N. & Papadopoulou-Daifoti, Z. Sex differences in animal models of depression and antidepressant response. *Basic Clin. Pharmacol. Toxicol.* **106**, 226–233 (2010).
59. Altemus, M. Sex differences in depression and anxiety disorders: Potential biological determinants. *Hormones and Behavior* **50**, 534–538 (2006).
60. Horst, Ter, G. J., Wichmann, R., Gerrits, M., Westenbroek, C. & Lin, Y. Sex differences in stress responses: Focus on ovarian hormones. *Physiology & Behavior* 97, 239–249 (2009).
61. Baler, R. D. & Volkow, N. D. Drug addiction: the neurobiology of disrupted self-control. *Trends in Molecular Medicine* **12**, 559–566 (2006).
62. Vlassoff, C. & Garcia Moreno, C. Placing gender at the centre of health programming: challenges and limitations. *Social Science & Medicine* **54**, 1713–1723 (2002).
63. Monteggia, L. M. *et al.* Brain-Derived Neurotrophic Factor Conditional Knockouts Show Gender Differences in Depression-Related Behaviors. *Biological Psychiatry* **61**, 187–197 (2007).
64. Smith, M. A., Makino, S., Kvetnansky, R. & Post, R. M. Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J. Neurosci.* **15**, 1768–1777 (1995).
65. Duman, R. S. Novel therapeutic approaches beyond the serotonin receptor. *BPS* **44**, 324–335 (1998).
66. Blendy, J. A. The Role of CREB in Depression and Antidepressant Treatment. *Biological Psychiatry* **59**, 1144–1150 (2006).
67. Trentani, A., Kuipers, S. D., Horst, Ter, G. J. & Boer, Den, J. A. Selective chronic stress-induced in vivo ERK1/2 hyperphosphorylation in medial prefrontocortical dendrites: implications for stress-related cortical pathology? *European Journal of Neuroscience* **15**, 1681–1691 (2002).
68. Trentani, A. *et al.* Immunohistochemical changes induced by repeated footshock stress: revelations of gender-based differences. *Neurobiology of Disease* **14**, 602–618 (2003).
69. Wang, Z. Persistent ERK Phosphorylation Negatively Regulates cAMP Response Element-binding Protein (CREB) Activity via Recruitment of CREB-binding Protein to pp90RSK. *Journal of Biological Chemistry* **278**, 11138–11144 (2003).
70. Colucci D'Amato, L. & Perrone Capano, C. Chronic activation of ERK and neurodegenerative diseases. *Bioessays* (2003).
71. Shansky, R. M. & Morrison, J. H. Stress-induced dendritic remodeling in the medial prefrontal cortex: Effects of circuit, hormones and rest. *Brain Research* **1293**, 108–113 (2009).
72. Sapolsky, R. M. Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. *Arch. Gen. Psychiatry* **57**, 925–935 (2000).
73. Pittenger, C. & Duman, R. S. Stress, Depression, and Neuroplasticity: A Convergence of Mechanisms. *Neuropsychopharmacology* **33**, 88–109 (2007).
74. Watanabe, Y., Gould, E. & McEwen, B. S. Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons. *Brain Research* **588**, 341–345 (1992).
75. Magariños, A. M., McEwen, B. S., Flügge, G. & Fuchs, E. Chronic psychosocial stress causes apical dendritic atrophy of hippocampal CA3 pyramidal neurons in subordinate tree shrews. *J. Neurosci.* **16**, 3534–3540 (1996).
76. Radley, J. J. *et al.* Chronic behavioral stress induces apical dendritic reorganization in pyramidal neurons of the medial prefrontal cortex. *NSC* **125**, 1–6 (2004).
77. Sapolsky, R. M. Glucocorticoid toxicity in the hippocampus: temporal aspects of neuronal vulnerability. *Brain Research* **359**, 300–305 (1985).
78. Duman, R. S. Depression: a case of neuronal life and death? *BPS* **56**, 140–145 (2004).
79. Shors, T. J. & Leuner, B. Estrogen-mediated effects on depression and memory formation in females. *Journal of Affective Disorders* **74**, 85–96 (2003).
80. McEwen, B. Estrogen actions throughout the brain. *Recent Prog. Horm. Res.* **57**, 357–384 (2002).

81. Conrad, C., Jackson, J. & Wieczorek, L. Acute stress impairs spatial memory in male but not female rats: influence of estrous cycle. *Pharmacology* ... (2004).

82. Galea, L. A. *et al*. Sex differences in dendritic atrophy of CA3 pyramidal neurons in response to chronic restraint stress. *NSC* **81**, 689–697 (1997).

83. Shansky, R. M. *et al*. Estrogen mediates sex differences in stress-induced prefrontal cortex dysfunction. *Molecular Psychiatry* **9**, 531–538 (2004).

84. Gu, G., Rojo, A. A., Zee, M. C., Yu, J. & Simerly, R. B. Hormonal regulation of CREB phosphorylation in the anteroventral periventricular nucleus. *J. Neurosci.* **16**, 3035–3044 (1996).

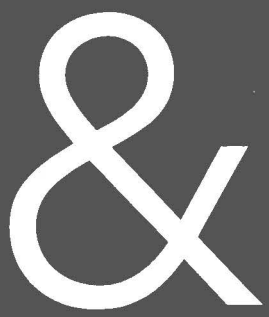
85. Zhou, Y., Watters, J. J. & Dorsa, D. M. Estrogen rapidly induces the phosphorylation of the cAMP response element binding protein in rat brain. *Endocrinology* **137**, 2163–2166 (1996).

86. Wade, C. B. & Dorsa, D. M. Estrogen activation of cyclic adenosine 5'-monophosphate response element-mediated transcription requires the extracellularly regulated kinase/mitogen-activated protein kinase pathway. *Endocrinology* **144**, 832–838 (2003).

87. Luine, V. Sex Differences in Chronic Stress Effects on Memory in Rats. *Stress* **5**, 205–216 (2002).

88. Bianchin, M. & Angrilli, A. Gender differences in emotional responses: A psychophysiological study. *Physiology & Behavior* **105**, 925–932 (2012).

APPENDIX



NEDERLANDSE SAMENVATTING

Stress speelt een steeds grotere rol in onze moderne samenleving. Iedereen is er ooit mee geconfronteerd en moet ermee omgaan. Echter hoeft stress niet noodzakelijk slecht te zijn. Op de korte termijn kan stress, of de lichamelijke reactie op stress, ons helpen om in tijden van druk beter te presteren en ons cognitieve mogelijkheden te vergroten. Elke situatie beschouwt als stress leidt tot een serie gecoördineerde reacties en resulteert in een verhoogde afgifte van de stresshormonen adrenaline, noradrenaline en glucocorticoïden uit de bijnieren en de hersenen. Al deze hormonen dragen samen bij aan de fysiologische veranderingen die ons gespannen laten voelen en op hetzelfde moment in staat brengen om en effectief te reageren op een bedreigende situatie en ons helpen voorbereid te zijn op toekomstige gebeurtenissen.

In hoofdstuk 1 van dit proefschrift hebben we het concept van stress geïntroduceerd en emotionele leren en geheugen, een aspect positief beïnvloed door stress en stresshormonen. We hebben verder de effecten die vaak gezien worden na chronische stress geïntroduceerd, die kunnen leiden tot verschillende schadelijke aandoeningen, waaronder depressie.

We weten allemaal dat niet alle ervaringen even goed worden opgeslagen in ons geheugen. Significante en emotionele gebeurtenissen zijn vooral goed te onthouden. Uit uitgebreide gegevens blijkt dat de stress hormonen die vrijkomen tijdens en na zulke ervaringen, de neurobiologisch processen die verantwoordelijk zijn voor de verbeterde consolidatie en opslag van emotionele ervaringen in lange-termijn geheugen moduleren. Het eerste deel van dit proefschrift was gericht op het verder ontleden van de neurobiologisch circuits en mechanismen geassocieerd met emotionele geheugenvorming. De experimentele aanpak is gebaseerd op gedragsexperimenten en farmacologische manipulaties gebruikt om de glucocorticoïd-geïnduceerde modulatie van de consolidatie processen, die betrokken zijn bij de opslag van emotionele ervaringen nader te onderzoeken.

In hoofdstuk 2 wordt eerst de belangrijkste techniek die wordt gebruikt in Deel 1, stereotactische operatiesgeïntroduceerd. Door een aantal aanpassingen konden we deze al lang bestaande procedure verfijnen en het welzijn en overleving van de dieren aanzienlijk verhogen. Door gebruik te maken van deze techniek in hoofdstuk 3, hebben we canules in de insulaire cortex (IC) geïmplanteerd en konden we de betrokkenheid van de insulaire cortex (IC) bij de effecten van glucocorticoïden op de consolidatie van aversieve gebeurtenissen bestuderen. Door het gebruik van het "inhibitory avoidance" paradigma konden wij aantonen dat administratie van de specifieke glucocorticoid receptor (GR) agonist RU 28362 direct (maar niet 3 uur) na de training dosis-afhankelijk de 48-uurs retentie prestaties verbeterde. Met behulp van een gemodificeerde versie van de "inhibitory avoidance" taak, hebben we verder ontdekt dat de IC de glucocorticoïde werking van de versterking van het geheugen van beiden componenten van de taak bemiddelt/verbetert. Op basis van deze bevindingen veronderstellen we dat de IC veel breder bij de versterking van emotioneel geheugen betrokken is en waarschijnlijk deel van een groter netwerk voor de detectie van opvallende informatie uitmaakt.

Hoewel het bekend is dat de stress reactie ook wordt geactiveerd in reactie op positieve prikkels en dat het geheugen ook verbetert is voor appetitieve ervaringen, is onderzoek naar de onderliggende mechanismen van emotioneel geheugen bijna uitsluitend gericht op negatieve gemotiveerd leertaken. Niet veel aandacht is besteed aan het onderscheid tussen emotionele positieve en negatieve ervaringen. Vandaar dat wij in hoofdstuk 4 onderzocht hebben of de effecten van glucocorticoïden op het geheugen voor negatieve en positieve leerervaringen vergelijkbaar zijn. We konden laten zien dat de GR-agonist RU 28362 onmiddellijk (ipv enkele uren) na de training in de nucleus accumbens (Nac) de consolidatie van het geheugen van een aversieve en een appetitieve smaak leerparadigma versterkt. Dit is aangegeven door een respectieve verlaagd of verhoogd vloeistofinname ten opzichte van het eerste contact met de smaak.

Het is bekend dat glucocorticoïde interageeren met het noradrenerge systeem om de consolidatie van negatief herinneringen te bevorderen. Daarom zijn wij in een apart experiment nagegaan of een interactie met het noradrenerge system ook noodzakelijk is voor de waargenomen verhoging van het geheugen in positief leertaak. Gelijktijdige toediening van propranolol, een ß-adrenerge receptor antagonist, in een dosis ineffectief op zichzelf, blokkeerde de geheugen-stimulerende effect van de GR agonist. Dit suggereert dat de glucocorticoïd-noradrenerge interactie onafhankelijk van de motivatie van de leerervaring is en het geheugen in beide gevallen verbetert.

Het gebied van de hersenen vooral bestudeerd met betrekking tot emotioneel leren en geheugen is de basolateral complex van de amygdala (BLA). Uitgebreide gegevens tonen de cruciale rol bij de faciliterende effecten van stress hormonen op emotioneel leren en geheugen aan. Echter, de meeste onderzoeken naar de rol van de BLA hebben tot nu toe angstgemotiveerde leertaken benut. Het is echter niet bekend of de BLA ook betrokken is bij de regulering van glucocorticoïd effecten op positiefgemotiveerde leertaken. Daarom hebben we dit in hoofdstuk 5 nader onderzocht. Toediening van de GR agonist RU 28362 in de BLA na het drinken van een nieuwe smaak oplossing verbeterde het geheugen 24 uur later, zoals aangegeven door een verhoogde opname van dezelfde oplossing in vergelijking met vehicle behandelde ratten. Dit geeft een brede rol voor de BLA in de bemiddeling van glucocorticoïde werking op het geheugen onafhankelijk van de motivatie van de stimulus.

Al met al kan dit hier beschreven onderzoek van glucocorticoïde effecten op geheugen processen verschillende nieuwe inzichten onthullen op een aantal vragen die tot nu toe geen aandacht hebben gekregen.

De gegevens van het eerste gedeelte van dit proefschrift tonen duidelijk aan dat acute stressvolle gebeurtenissen en het vrijkomen van stresshormonen positieve effecten kan hebben op cognitieve functies en beschermend en adaptief kan zijn. In tegenstelling tot de adaptieve respons op acute stress, kan de reactie op langdurige, oncontroleerbare stress ontsporen. De continu verhoogde productie van stress hormonen kan leiden tot lange-termijn veranderingen in de neurochemische, neuroanatomische en cellulaire functies, waardoor. Psychiatrische aandoening, zoals depressie, kunnen ontstaan.

Door de directe correlatie tussen chronische stress en het ontstaan van depressie, kan stress gebruikt worden als een waardevol hulpmiddel bij dierexperimenteel onderzoek naar de onderliggende neurobiologische mechanismen van depressie.

Affectieve stoornissen zoals depressie zijn een grote oorzaak van morbiditeit, herhalen zich vaak en kunnen levensbedreigend zijn. De kans voor depressie is twee tot drie keer hoger voor vrouwen dan voor mannen. Ondanks dit feit wordt het merendeel van het biomedische onderzoek nog steeds gedaan bij mannetjesratten. Dit is opmerkelijk, gezien het bewijs dat zich begint op te stapelen dat veel processen bij vrouwen anders verlopen dan bij mannen. Het tweede gedeelte van dit proefschrift gaat daarom in op effecten van langdurige stress op verschillende gebieden van het limbische systeem in vrouwtjes- en mannetjesratten. Hoofdstuk 6 en 7 beschrijven een aantal van de veranderingen als gevolg vanacute en chronische stress op de expressie en fosforylering van de transcriptiefactor cAMP response element binding protein (CREB) in verschillende stressgerelateerde gebieden van de hersenen. CREB is betrokken bij de reactie op stress en antidepressiva effecten en is onderdeel van een van de belangrijkste theorieën van de onderliggende neurobiologie van depressie. Onze bevindingen in hoofdstuk 6 geven aan dat verschillende subregios van de hippocampus en de prefrontale cortex (PFC) bij mannelijke ratten een vermindering van de pCREB expressie en wijzigingen in de morfologie van deze gebieden laten zien. Dit in tegenstelling to vrouwen die geen enkele aanwijzing van deze processen in deze gebieden tonen. Bij het onderzoek naar de Nac (hoofdstuk 7) is het beeld omgekeerd. Binnen deze regio zijn de meest uitgesproken effecten zichtbaar bij vrouwelijke ratten. Zij tonden een toename van CREB-spiegels na acute stress aan, maar een daling van pCREB na acute en chronische stress. De eiwitniveaus bleven grotendeels onaangetast bij mannelijke ratten. Al met al geven onze bevindingen aan dat mannelijke en vrouwelijke ratten met verschillende, zelfs tegengestelde reacties op stress reageren. Dus, geslachtspecifieke analyse blijkt veel potentieel te hebben om ons begrip van de ontwikkeling van psychische stoornissen te verbeteren. Sekseverschillen zouden moeten worden benadrukt in toekomstige studies van de neurobiologische mechanismen van de reactie op stress en depressie.

In deze proefschrift hebben wij beide kanten van de stress proces even belicht en zowel positieve als ook negatieve effecten laten zien.

Stress draagt al lang een negatieve connotatie, vooral in leken termen. Mensen associëren het woord 'stress' automatisch met slechte gevoelens en een veelheid van andere fysieke, emotionele en mentale ongemakken. Echter wordt uit de gegevens in dit proefschrift en andere onderzoek op het gebied van stress duidelijk dat de pure aanwezigheid van een 'stress-response' niet voldoende is om een stimulus te labelen als stress. De vrijkomende krachtige hormonen zijn er in de eerste plaats, om ons te helpen overleven. Het blijft echter een kwestie van timing en dosering. Onder omstandigheden van langdurige of ernstige stress kunnen we last krijgen van negatieve gevolgen en de ontwikkeling van schadelijke aandoeningen en pahtologieën. Toch moeten niet alle veranderingen in de hersenen onmiddellijk worden bestempeld als aversieve en onaangepast.

DEUTSCHE ZUSAMMENFASSUNG

Stress spielt eine zunehmend wichtige Rolle in unserer modernen Gesellschaft. Alle werden damit konfrontiert und müssen lernen damit umzugehen. Allerdings muss Stress nicht unbedingt schlecht sein. Kurze Stress-Episoden oder körperliche Reaktionen auf Stress können uns in Zeiten von großem Leistungsdruck helfen und unsere kognitiven Fähigkeiten erhöhen.

Jede Stresssituation führt zu einer Abfolge genau koordinierter Reaktionen und einer erhöhten Freisetzung der Stresshormone Adrenalin, Noradrenalin und Glucocorticoide aus den Nebennieren und dem Gehirn. Die durch diese Hormone ausgelösten physiologischen Veränderungen tragen gemeinsam dazu bei, dass wir uns gestresst fühlen. Gleichzeitig ermöglichen sie uns aber auch, effektiv auf eine bedrohliche Situation zu reagieren und auf zukünftige Ereignisse besser vorbereitet zu sein.

In Kapitel 1 dieser Arbeit haben wir das Konzept von Stress und emotionalem Lernen und Gedächtnis eingeführt. Dieser Aspekt wird positiv durch Stress und Stresshormone beeinflusst. Des Weiteren haben wir auch die Effekte beleuchtet, die oft als Folge von chronischem Stress eintreten und zu verschiedenen Krankheitsbildern, wie zum Beispiel Depressionen, führen können.

Wie jeder weiß, werden nicht alle Erlebnisse gleich gut im Gedächtnis gespeichert. Unser Gehirn ist besonders gut im Speichern von bedeutsamen, emotionalen Ereignissen. Umfangreiche Daten zeigen, dass Stresshormone die während und nach diesen bedeutsamen Erfahrungen abgegeben werden, die für eine verbesserte Speicherung von emotionalen Erfahrungen ins Langzeit-Gedächtnis verantwortlichen Prozesse modulieren. Der erste Teil dieser Arbeit befasst sich mit der genaueren Erforschung des mit emotionaler Gedächtnisformung assoziierten neurobiologischen Mechanismus.

Mit Hilfe von Verhaltensexperimenten und pharmakologischen Manipulationen des Gehirns haben wir die glucocorticoid-induzierte Modulierung von Speicherprozessen emotionaler Erfahrungen genauer unter die Lupe genommen.

In Kapitel 2 wird zuerst die wichtigste Methode für die Durchführung dieser Experimente vorgestellt. Durch einige Veränderungen konnten wir das lang bekannte Verfahren stereotaktischer Operationen an die neue Gesetzgebung anpassen und das Wohlergehen und Überleben der Tiere maßgeblich verbessern.

Mit Hilfe dieser Technik wurden, wie in Kapitel 3 beschrieben, Kanülen in den insulären Cortex (IC) implantiert. Dadurch war es uns möglich die Beteiligung des IC bei den Effekten der Glucocorticoide auf die Speicherung aversiver Ereignisse zu untersuchen. Mit Hilfe des „inhibitory avoidance" Paradigma konnten wir zeigen, dass die Verabreichung des Glucocorticoid Rezeptor (GR) Agonisten RU 28362 direkt nach dem Training das Erinnerungsvermögen verbesserte.

Mittels einer modifizierten Variante des ‚inhibitory avoidance' Tasks konnten wir des Weiteren feststellen, dass der IC die erinnerungsverstärkende Wirkung von Glucocorticoiden von beiden Komponenten des Tests verbessert. Auf der Grundlage dieser Erkenntnisse gehen wir davon aus, dass der IC weitläufiger bei der Speicherung

emotionaler Erinnerungen beteiligt ist und wahrscheinlich Teil eines größeren Netzwerkes zur Erkennung von auffälligen/prägnant Ereignissen ist.

Obwohl lange bekannt ist, dass die Stress-Reaktion auch nach positiven Stimuli aktiviert wird und dass das Erinnerungsvermögen für positive Erfahrungen verbessert ist, hat sich die Forschung nach den zugrunde liegenden Mechanismen des emotionalen Gedächtnis' fast ausschließlich auf aversive Lernaufgaben konzentriert. Nicht viel Augenmerk wurde auf den Unterschied zwischen positiven und negativen Erfahrungen gelegt. Daher haben wir in Kapitel 4 untersucht, ob die Wirkung von Glucocorticoiden auf emotional unterschiedlich gewertete Ereignisse vergleichbar ist. Wir konnten zeigen, dass Infusion des GR-Agonist RU 28362 sofort (allerdings nicht mehrere Stunden) nach der Trainingserfahrung in den Nucleus accumbens zu einer Verbesserung des Gedächtnisses an ein aversives sowie ein appetitives Geschmackserlebnis führt. Dies wurde durch eine jeweils verminderte oder erhöhte Flüssigkeitsaufnahme im Vergleich zum ersten Kontakt mit dem Geschmack angedeutet.

Glucocorticoide sind dafür bekannt, mit dem noradrenergen System zusammen zu arbeiten um die Speicherung negativer Erfahrungen zu verbessern. Deshalb haben wir in einem separaten Experiment untersucht, ob eine Wechselwirkung mit dem noradrenergen System auch für eine Gedächtnisverbesserung positiver Erfahrungen notwendig ist. Die gleichzeitige Verabreichung des ß-adrenozeptor Antagonisten Propranol zusammen mit dem GR Agonisten blockierte die erinnerungssteigernde Wirkung des GR Agonisten. Dies legt nahe, dass das Zusammenspiel beider Stresshormone unabhängig von der Wertigkeit des zu lernenden Ereignisses ist und die Gedächtnisleistung in beiden Fällen erhöht.

Die, im Hinblick auf emotionales Lernen und Gedächtnis, am meisten untersuchte Hirnregion ist der basolaterale Komplex der Amygdala (BLA). Umfangreiches Beweismaterial zeigt deutlich die kritische Beteiligung dieser Region an der gedächtnissteigernden Wirkung von Stresshormonen. Allerdings verwenden die meisten Studien hauptsächlich aversive und angst-motivierte Lernerfahrungen. Daher ist bisher nicht bekannt, ob der BLA auch bei den Auswirkungen der Glucocorticoide auf positive Lernerfahrungen beteiligt ist. Deshalb wurde dies in Kapitel 5 detaillierter untersucht. Infusion des GR-Agonisten RU 28362 in den BLA sofort anschließend an ein neues Geschmackserlebnis verbesserte das Gedächtnis 24 Stunden später, gezeigt durch eine erhöhte Aufnahme der gleichen Geschmackslösung im Vergleich zur Kontrollgruppe. Dies deutet auf eine umfangreiche Rolle des BLA bei der Vermittlung der Glucocorticoid-Wirkung auf Gedächtnisprozesse unabhängig von der Wertigkeit des Stimulus hin.

Zusammenfassend zeigen die hier präsentierten Untersuchungen von Glucocorticoid-Effekten auf Gedächtnisprozesse unterschiedliche neue Erkenntnisse über eine Reihe von Fragen im Bezug auf Speicherprozesse, die bis jetzt wenig Aufmerksamkeit erhalten haben.

Die Ergebnisse aus Teil 1 dieser Arbeit zeigen deutlich, dass akute Stresssituationen und die daraus resultierende Freisetzung von Stresshormonen positive Auswirkungen auf kognitive Fähigkeiten haben können. Doch obwohl die Stress-Reaktion eine adaptive und notwendige Reaktion auf akute Ereignisse darstellt, kann langanhaltender

Stress, über Wochen, Monate oder sogar Jahre, zu chronischen Veränderungen führen, die schädliche Auswirkungen auf Körper und Geist haben. Die kontinuierlich erhöhte Produktion von Stresshormonen kann zu schädlichen Langzeitveränderungen in neurochemischen, neuroanatomischen und zellulären Funktionen führen, wodurch psychiatrische Krankheiten wie z.B. Depressionen entstehen können. Durch den direkten Zusammenhang zwischen chronischem Stress und Depressionen kann Stress als wertvolles Hilfsmittel für Tierexperimente genutzt werden, um die zugrundeliegenden neurobiologischen Mechanismen gründlicher zu untersuchen. Affektive Krankheiten, wie Depressionen, sind eine der weltweit größten Verursacher von Erkrankungen. Sie kehren häufig wieder und können lebensbedrohlich werden. Frauen haben eine zwei- bis dreimal höhere Wahrscheinlichkeit an Depressionen zu erkranken als Männer. Trotz dieses Wissens wird der Großteil der biomedizinischen Forschung weiterhin hauptsächlich in männlichen Versuchstieren durchgeführt. Dies ist beachtenswert, angesichts der sich anhäufenden Beweise, dass viele biologischen Prozesse bei Frauen anders ablaufen als bei Männern. Der zweite Teil dieser Doktorarbeit beschäftigt sich daher mit Effekten von Langzeitstress auf verschiedene Regionen im limbischen System in weiblichen und männlichen Ratten. Kapitel 6 und 7 beschreiben einige der geschlechtsspezifischen Veränderungen im Expressions- und Phosphorylierungsmuster des Transkriptionsfaktors cAMP response element binding protein (CREB) als Folge von akutem oder chronischem Stress. CREB ist sowohl in die Stressreaktion als auch in Behandlungseffekte von Antidepressiva involviert und Bestandteil einer der wichtigsten Theorien der neurobiologischen Grundlage von Depressionen.

Unsere Erkenntnisse aus Kapitel 6 zeigen, dass verschiedene Teilbereiche des Hippocampus und des präfrontalen Cortex (PFC) bei männlichen Ratten eine reduzierte Expression von phosphoryliertem CREB (pCREB) sowie Veränderungen in der Morphologie dieser Hirnregionen aufweisen. Dies steht im Gegensatz zu den weiblichen Ratten, die keinerlei Hinweise auf derartige Prozesse zeigten. Im Kontrast dazu zeigte sich bei Untersuchungen im Nucleus Accumbens (Kapitel 7) ein entgegengesetztes Bild. In dieser Hirnregion wurden die größten Veränderungen bei weiblichen Ratten gefunden. Diese zeigten eine Zunahme der CREB Expression nach akutem Stress. Allerdings war pCREB sowohl nach akutem als auch chronischem Stress signifikant verringert. Die Proteinniveaus blieben in männlichen Ratten größtenteils unverändert. Insgesamt zeigen unsere Ergebnisse, dass männliche und weibliche Ratten auf unterschiedliche, teilweise sogar entgegengesetzte, Weise auf Stress reagieren. Daher hat eine geschlechtsspezifische Analyse enormes Potential um unser Verständnis über die Entwicklung psychischer Störungen zu verbessern. Geschlechtsunterschiede sollten hervorgehoben und in zukünftigen Studien über die neurobiologischen Mechanismen der Stressreaktionen und Depressionen einbezogen werden.

In dieser Arbeit werden beide Seiten der Stressreaktion beleuchtet und sowohl positive als auch negative Effekte entdeckt. Stress trägt, vor allem unter Laien, seit

langem einen negativen Beigeschmack. Die meisten Menschen assoziieren mit dem Wort 'Stress' eine Vielzahl schlechter Gefühle und verschiedene andere physische, emotionale und mentale Beschwerden. Die Daten dieser und anderer Arbeiten zeigen allerdings deutlich, dass die pure Anwesenheit einer 'Stress-Reaktion' nicht mehr ausreicht, um einen Reiz stressvoll zu nennen. Die ausgeschütteten kraftvollen Hormone helfen uns in erster Linie zu überleben. Dennoch bleibt es eine Frage des Timings und der Dosierung. Durch längeren oder besonders starken Stress können negative Folgen entstehen und sich schädliche Störungen und Erkrankungen entwickeln. Trotzdem sollten nicht alle Veränderungen im Gehirn sofort als aversiv und unangepasst angesehen werden.

Geschafft!!! I would never have been able to finish this dissertation without the help and guidance of all the kind people around me. This not only includes scientific and academic staff but also colleagues, friends and family who have been part of the ultimately 6 year long journey of my PhD.

First and foremost, my deepest gratitude goes to my promoters Benno Roozendaal and Gert ter Horst for their excellent support, caring, and patience. Gert, you originally offered me a PhD position and welcomed me to your laboratory at the Department of Biological Psychiatry in 2006. Although not all the things worked out as probably both of us had hoped I will always be very grateful that you brought me to Groningen. Thank you for all your help and mentorship throughout the whole process. Benno, you 'adopted' me at a time point I was not sure if I really want to finish my PhD. Through your guidance and enthusiasm for science I plucked up new courage and went on my way again to finish the path I had started. Thanks for pushing me all the time I felt frustrated or disappointed, to patiently correct my writing and support my research with your infinite knowledge about learning and memory. I am glad to have gotten the opportunity to work with you.

A thanks goes to the reading committee for their time and effort to read and approve my thesis: Prof. dr. Karin Roelofs, Prof. dr. Jaap Koolhaas and Prof. dr. Paul Luiten.

My gratitude also to my paranymphs Heike Eekhoff and Areg Barsegyan. Heike, ich bin super froh, dass du zurück nach Groningen gekommen bist. Was hatten wir es schön die letzten 2 Jahre; unter dem Weihnachtsbaum, beim Ponycamp, und bei vielen anderen Gelegenheiten. Vielen Dank für all die aufbauenden und teilweise sehr erleuchtenden Gespräche auf unseren Autofahrten. Danke, dass du meine 'Paradingens' bist und mich auch in diesen Stunden wieder einnorden kannst.

Areg, 3 years in the Netherlands and you are still working on LA-time (o; Despite our shifted working hours you have become an important friend. Your infinite patience and the positive energy you project are very inspiring to me. Thank you for all your pep talks and valuable advice about 'ze american' lifestyle. Thanks for agreeing to be my paranymph and accompanying me at this important moment.

A special place in these acknowledgements goes to my fellow companions in the lab. Piray, I still remember us sitting on the bench in front of the NIC after your interview. Look at us now! We went through so much together, good, bad, very bad and good again, but we always had each other for social support, empathy, stress coping and a lot of memories. Hassiba, thanks for saving my life at lunch (although it felt more like you were trying to hit me unconscious). Fany, mon coeur, I hope we will ride bikes together on Schiermonnikoog one day. Raquel, although Daniela and I tried our best, we could never completely implement all the 'german genes' into your Brazilian body. Thanks for your friendship and support within and outside of the lab. Without you this book

would really not exist!!! Grande abraços e beijos. Erika (ey hombre), thanks for letting me stay with your parents in Zarautz. It was an amazing vacation (and thanks for finally clarifying why these two strange guys were following us around). Daniela (oans, zwoa, drei, g'suffa!), Du warst eine fantastische Stütze in einer schweren Zeit und ich werde Dir dafür immer dankbar sein. Thanks also to my wonderful master students, Maurien and Magda, for their indispensable help and commitment to this project.

This work would not have been done without the skillful help of our technicians. Thanks a lot everybody for showing me all the ropes in the lab. Petra, thanks for teaching western blots at the beginning of my PhD, helping me with my dutch skills and always being open and straight forward. Tineke, thanks for staying with me in the darkness of the microscopy room to count endless amounts of black dots. Ellie, thanks for all the cutting and staining and taking such good care of the lab. Angelika, thanks for all the help in the CDP; und die tollen Saxophon Stunden und Auftritte. Anneke, thanks for your help with all the brains of the chronic stress experiments. It was a pleasure to work with all of you!

Without administrative help, one would not get that far either. Thus, a big thanks to Gerry, Ineke, Diana and the whole BCN office for organizing contracts, prolongations, money, courses, retreats, GPS-tours, lab uitjes, dinners and so many more things over the years.

I also want to thank all the rest of the people in the Department of Anatomy. I learned a lot from all of you. Janniko (thanks for explaining the world of science from your perspective), Sussan (thanks for all the snoep always available at your desk), Christel, Peter Gerrits, Esther Marije, Ruud, Peter Room, and 'the guys from the basement' Klaas, Peter Veldman and Jan.

I cannot (and also don't want to) count the endless hours I spend in the CDP. When people start asking you if you live there something is seriously wrong. Alex, Flip, Catriene, Miriam, Hester, Linda, Annet, Silvia, Natascha, Hilda, Ar, Annemiek, Ramon, thanks for fixing all my problems, making exceptions with ordering animals, the loving care for all of my little furry friends and providing great soup during lunch time. Arie, I think from now on you have to get a new supplier for your german chocolate treats. An extra big thanks goes to the microsurgery team. Michel, Annemieke, André and Bianca, I would probably still be 'living' in the surgery room trying to finish all my experiments if it wasn't for you guys. Thanks for keeping me company and taking some load (actually a lot) of my shoulders the last couple of months.

To all my friends here in Groningen. Tim, Doro, Isabel, Nils, you made my first year here in Groningen a blast. Ulli, I was desperately waiting for the 'new german girl' to arrive and I am happy we became such good friends. I enjoyed you coming by my office to drink coffee and talk, your support and friendship and all the moments we

shared together. Carlos, we still have to go skydiving! It´s great that you guys will at least live on the same continent, although just coming by for coffee or freeloading off of us for computer cables will involve a great deal of traveling from now on. The rest of the Pintelier Crew: Arne, Maxi (der Puls geht ganz normal), Golnar, Sham, Marcin (the tough guy from Eastern Europe), Kasia: there are some nice pubs in Boston for our next round of beers.

Petra en Frank. Jullie zijn ongelofelijk. Het was altijd heerlijk op de manege en de laatste maanden voor de promotie de enige tijd die ik kon doorbrengen zonder aan mijn werk te denken. Bedankt daarvoor en ik kom zeker terug voor een paard.

I also do not want to forget all my friends outside of Groningen. Beate, seit der Schulzeit sind wir nun verbunden und selbst einen Urlaub haben wir zusammen überstanden (o; Danke für das tolle Cover! Nora, ohne Dich wären meine Erinnerungen an Marburg um einige herrliche Geschichten ärmer. Anna, vielen Dank, dass Du damals über meinen Witz gelacht hast und die tolle Zeit die wir zusammen hatten und immer wieder haben. Anca (aka Schantal), ohne Dich hätte ich niemals den Weihnachtsmann geküsst und wäre nicht heil zusammen mit meinen Sachen nach Groningen gekommen. Vöni, vielen Dank für all die tollen Urlaube und Erlebnisse! Danke für Eure Freundschaft und ich hoffe es kommt in den nächsten Jahren noch ein Haufen toller Erinnerungen dazu!

Ein besonderer Platz in dieser Danksagung gehört auch meinen Eltern und dem Rest meiner Familie. Mutti, Papa, ohne Eure grenzenlose Liebe und Unterstützung wäre ich nicht die Person geworden, die ich heute bin. Ihr habt immer versucht mir alles zu ermöglichen und mein Leben so einfach wie möglich zu gestalten. Ich wünsche mir noch viele schöne Zeiten mit Euch in Schwerin, Boston oder wo auch immer wir dann sein werden. Egal wo, ihr kommt ja sicher öfter mal vorbei...

Reinhard, wo wäre ich ohne Dich? Ich kann nicht mit Worten ausdrücken wie sehr Deine Unterstützung, Zuneigung und Geduld mich gestärkt und mir immer wieder die Kraft gegeben haben weiter zu machen. Du bist mein Fels in der Brandung. Dicker Kuss!

Thanks again to everybody for making this adventure so enjoyable and memorable. For those of you whose names I did not mention here, I offer my apologies but assure you that those that have made this experience so special will never been forgotten!!

&