Stress, emotion and cognition : role of mineralo- and glucocorticoid receptors

Brinks, V.

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"The more emotional an event, the better it will be remembered". Stress hormones such as cortisol (man) and corticosterone (rodent) are crucial for this intricate link between emotion and cognition. The hormones enhance motivation, mood and emotions, and have a profound influence on cognitive processes. This action exerted by the steroids is of evolutionary advantage and promotes health, but if dysregulated the cognitive-emotional changes become detrimental eventually precipitating stress-related diseases like Post-Traumatic Stress Disorder (PTSD). Why only some individuals experience the detrimental effects of stress, while and others remain healthy under similar conditions is a key question in cognitive neurobiology

The objective of this thesis is to identify the contribution of corticosteroids and their receptors to the integration of emotional and cognitive processes.

Corticosteroids are secreted from the adrenals in response to stress, and act in the brain via mineralo- (MR) and glucocorticoid receptors (GR). Emotional and cognitive performance of mice with genetically different MR and GR or pharmacologically-induced differential activation of these receptors was assessed in a variety of behavioural paradigms specifically designed to study the integration between emotional and cognitive domains. In this thesis I describe studies performed with two strains: the stress-susceptible BALB/c mouse strain and the stress-resistant C57BL/6J. We found that:

- Emotional arousal and cognitive performance are optimally integrated in mice with predominant MR- and additional moderate GR activation.
- The stress-susceptible BALB/c mice have an emotionally biased superior memory performance as compared to the resistant C57BL/6J mice; cognitive performance correlates with MR and GR expression in limbic brain areas.
- BALB/c mice generalize their fear responses to context and cue while C57BL/6J mice discriminate between context and cue.
- Injection of corticosterone before or after fear conditioning destabilizes the memory consolidation and facilitates extinction in BALB/c mice; C57BL/6J respond with augmented fear memory and lack of extinction.
- Mutant $MR^{CaMKCre}$ mice with forebrain-specific ablation of the MR gene display increased fear responses during all phases of memory formation and retrieval.

In conclusion, corticosteroids modulate the integration of emotional arousal and cognitive performance via a combined MR- and GR-mediated central action. It is proposed that C57BL/6J mice provide an animal model for PTSD and that the MR is a novel target for treatment of anxiety-related symptomatology.

Chapter 1

General introduction

Outline

1.1 Molecular and cellular mechanisms of corticosteroid action
1.2 Brain areas vulnerable for corticosteroid action
1.3 Corticosteroid action on emotion and cognition
1.4 Interaction between emotion and cognition
1.5 (Behavioural) tasks and animal models used to measure emotion and cognition
1.6 Translational approach: from animal model to stress-related pathology
1.7 Scope of the thesis

Stress is a potent modulator of emotional and cognitive functioning. When exposed to stress during a short period, it influences emotion, learning and memory of the stressful event in such a manner that is beneficial for adaptation and avoidance of similar stressful situations in future.
Stress is generally described as any disturbance to the body, either real or imagined, that interferes with homeostasis. These disturbances or stressors elicit a cascade of neuroendocrine events including the fast activation of the sympatho-adrenomedullary stress system and the slower activation of the hypothalamus-pituitary-adrenal (HPA) axis. Corticosteroids are secreted from the adrenals as a result of HPA-axis activation and subsequently facilitate recovery from stress via negative feedback. Corticosteroids (cortisol in man, corticosterone in rats and mice) bind to two types of nuclear receptors which then modulate gene transcription; the high affinity mineralo- (MR) and tenfold lower affinity glucocorticoid receptor (GR). Both MR and GR are located in brain areas involved in emotion, learning and memory, and correspondingly influence emotional and cognitive functioning.

Besides exerting positive effects on emotion and cognition, stress is mostly known for its negative effects. When being exposed to stress for a prolonged period (chronic) or when exposed to severe stress, some individuals develop stress-related diseases such as depression or post traumatic stress disorder (PTSD). These disorders are characterized by altered emotional and cognitive processing together with disrupted glucocorticoid function [6].
This raises the following questions: (1) general: Why are some individuals more prone to the development of stress-related diseases? And (2) more specific: Are the glucocorticoid stress system, emotion and cognition interdependent? And what is the role of MR and GR in emotional and cognitive processes?
The assessment of the interaction between emotion, cognition and the glucocorticoid stress system will be helpful in understanding the pathogenesis of stress-related diseases and perhaps offers new opportunities for treatment.

The main objective of this thesis is therefore to study the interaction between the glucocorticoid stress system, emotion and cognition by focussing on MR and GR functions.

In section 1.1 of this thesis, the molecular and cellular mechanisms of corticosteroid action are described, followed by an overview of the brain areas that are target for corticosteroid action (1.2), corticosteroid effects on emotion and cognition (1.3), the interaction between emotion and cognition (1.4), (behavioural) mouse models to measure corticosteroid action on emotion and

cognition (1.5), translational approach (1.6), culminating in presenting the scope and outline of this thesis (1.7).

1.1 Molecular and cellular mechanisms of corticosteroid action

Knowledge of the stress system, including the neurobiological and anatomical background, is fundamental for understanding its role in emotion and cognition. The designs used for behavioural experiments are based on this knowledge. Next sections discuss the stress system including molecular and cellular mechanisms.

1.1.1 The main players of the hypothalamus-pituitary-adrenal (HPA) axis

During basal conditions, the neurons of the paraventricular nucleus (PVN) of the hypothalamus stimulate the secretion of corticotropin releasing hormone (CRH), vasopressin (VP) and other neuropeptides. CRH and VP together activate the release of adrenocorticotropin (ACTH) from corticotrope cells in the pituitary glands. ACTH is transported by the blood to the adrenal cortex, which in turn secretes corticosteroids including cortisol (in man, corticosterone in rodents). Due to their lipophilicity, corticosteroids enter the brain and bind to two distinct types of receptors; the mineralocorticoid (MR) and glucocorticoid (GR) receptor.
Corticosterone is secreted in hourly pulses that increase in amplitude towards the circadian activity period. Superimposed on this ultradian rhythm is the response to a stressor, in which the neurons of the PVN enhance CRH and VP secretion, leading to increased ACTH and corticosteroid levels in the blood. A subsequent negative feedback circuitry reduces corticosteroid secretion from "stress-induced" to basal levels (figure 1) [12].

1.1.2 The corticosteroid receptors

Corticosteroids bind to two types of central steroid receptors; the high affinity mineralocorticoid receptor (MR) and tenfold lower affinity glucocorticoid receptor (GR) [13-15]. As a consequence, the MR is extensively occupied due to hourly corticosterone pulses, while substantial GR occupation occurs at ultradian peak levels and following a stressor. MR and GR mediate corticosteroid action as transcription factors and influence HPA axis activity with distinct functions. MR suppresses basal corticosterone pulsatility and the HPA response to a stressor. The latter is due to interference with fast feedback of HPA activity [16]. GR in contrast facilitates the termination of a stress response via a negative feedback loop [17;18].
The MR (116 kD) and GR (97 kD) genes are ancestrally related [19] and show similarities in gene structure; the ligand binding domain has a 57 % amino acid identity and the DNA binding domain is 94% similar between the MR and GR gene [20]. Both genes can be translated to multiple mRNA isoforms due to

alternative splicing and various polymorphisms [21;22]. In addition, post translational modifications such as phosphorylation can result in multiple MR and GR proteins, which might differentially affect metabolism, neuroendocrinology, behaviour and contribute to stress-related diseases [22-26].

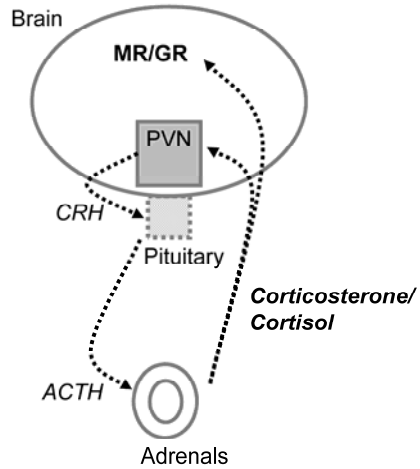

Figure 1. A schematic overview of the hypothalamus-pituitary-adrenal axis. Arrows indicate feedforeward and feedback regulation of hormone secretion.

1.1.3 Genomic effects of corticosteroids

After binding corticosterone, MR and GR dimerize to form mono- and dimers [27-29] These dimers, which mediate corticosteroid action as transcription factors, bind to glucocorticoid response elements (GRE) and result in transactivation or transrepression of gene expression [30]. Transactivation follows GRE binding in the vicinity of gene promoters. In this case, gene expression can be either enhanced or lowered by increasing or decreasing the frequency of transcription. Transrepression takes place when monomers bind to transcription factors (TF) and inhibit transcriptional activity of the target gene (figure 2).

A large part of the corticosterone responsive genes in the hippocampus is regulated by either activated MR or GR [31]. However, MR and GR heterodimers are thought to express an additional functionality in transcriptional regulation of corticosteroid responsive genes [27;29]. This shows the complexity and diversity of MR and GR dependent mechanisms to evoke changes in gene transcription.

The changes in gene transcription due to MR and GR activation follow a distinct time course [32]. Morsink and colleagues have shown that one hour after GR activation (in addition to MR) all affected genes are down regulated, presumably

via transrepression, while at three hours the affected genes are both up and down regulated. At 5 hrs, gene expression is almost back to baseline [32]. These corticosteroid regulated genes include immediate early genes [33] and MR [34], and are related to signal transduction, G-protein coupled receptor protein signalling pathway and protein biosynthesis [32].

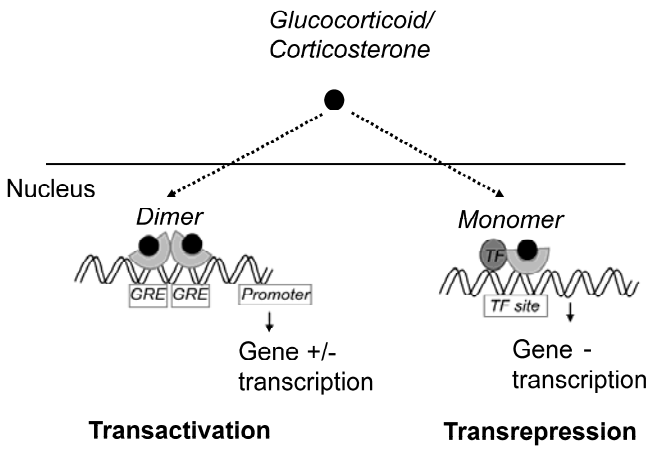

Figure 2. Schematic view of transactivation and transrepression of target genes due to corticosterone binding to its receptors, the mineralo- (MR) and glucocorticoid receptor (GR). MR and GR homo- and dimers bind to glucocorticoid responsive elements (GREs) on the DNA and inhibit or increase gene transcription.

1.1.4 Long term potentiation

Corticosteroids also influence the cellular mechanism which models learning and memory processes *ex-vivo*; long term potentiation (LTP) [35]. LTP is defined as a long lasting strengthening of neuronal connections following (high frequency) stimulation. It is divided into early, protein synthesis independent LTP directly following stimulation (min-hours), and late protein synthesis dependent LTP, which can last from hours up to months [36-38].

LTP is most investigated in the hippocampus. Here, as in other brain areas glutamate is the major excitatory neurotransmitter and its receptors, N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) are critical in inducing LTP.

Corticosterone can exert fast effects on LTP. It quickly increases the release probability of glutamate containing vesicles in a non-genomic manner [39] and increases the chance that alterations in glutamate release results into enhanced firing rates [40]. Karst and colleagues have shown that such non-genomic effects involve membrane located MR [39].

However, the majority of studies present focus on the slow gene-mediated effects of a stressor and corticosteroids. Overall, these experiments show that corticosteroid effects on LTP in the cornu ammonis 1 (CA1) area of the hippocampus (section 1.2.1) follow an inverted U-shape. Low levels of corticosterone sufficient to activate part of the mineralocorticoid receptors are associated with efficient LTP [41-44], whereas periods of stress impair LTP induction [for review: 45]. This clearly shows the suppressing effect of GR activation on LTP induction. Interestingly, corticosteroid effects on synaptic transmission in another part of the hippocampus, the dentate gyrus (DG), do not follow an inverted U-shape [46].
Recently, Olof Wiegert demonstrated that timing of corticosterone is also crucial for its effects on LTP. Corticosterone has fast facilitating effects on LTP when given simultaneous with a high frequency stimulation, however this effect is absent when given before or after repetitive stimulation [47].

In summary, corticosteroids act via distinct receptors, MR and GR, inducing slow genomic actions via transcriptional regulation but also exerting fast non-genomic effects on LTP. These molecular and cellular mechanisms provide the basis for the corticosteroid effects on emotion and cognition.

1.2 Brain areas sensitive for corticosteroid action

The hippocampus, amygdala and prefrontal cortex (PFC) are brain areas involved in emotion and cognitive functions (figure 3). These areas are very connected and sensitive to corticosteroid action due to abundant MR and GR expression. Since the experiments described in this thesis address the function of these brain areas, this section will focus on their role in emotion and cognition.

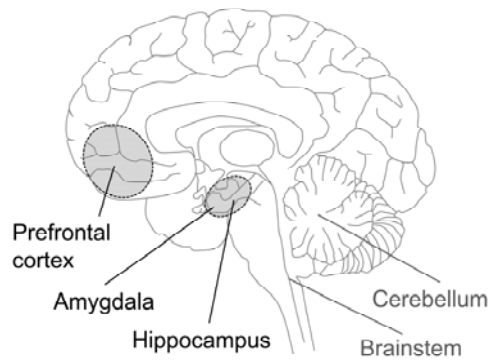

Figure 3. Location of the hippocampus, amygdala and prefrontal cortex in the human brain.

1.2.1 The hippocampus

The main function of the hippocampus is the processing of contextual information [48] which includes spatial learning and memory, but is also involved in fear-related behaviour through connections with the amygdala [49;50].

The hippocampus is part of the limbic system and is situated in the temporal lobe. It consists of a heterogeneous population of neurons and glia cells which form distinct subfields: the dentate gyrus (DG) and cornu ammonis areas (CA1, CA2 and CA3). The DG, CA3 and CA1 areas are connected by a trisynaptic circuit [51;51]. The DG is connected to the entorhinal cortex via the perforant path, and sends information to the CA3 via mossy fibers. The CA3 in its turn is connected to the CA1 via the Schaffer collaterals. This trisynaptic circuit is often used to measure LTP (section 1.1.4).

Most areas contain cells which are characterised by place specific firing patterns and are believed to play a role in navigation and formation of a spatial map, however the naming "space cells" is restricted for the principal cells of the CA1 and CA3 area [52]. The hippocampal subfields express distinct functionality in information processing. While the DG and CA3 areas are involved in encoding of spatial information [53;54], the CA1 is involved in temporal information processing [55]. MR is highly expressed in all hippocampal areas, GR is predominantly expressed in the DG, CA1 and CA2 [56;57].

Box 1. Hippocampal volume of people suffering from stress-related diseases.

Hippocampal volume of patients with depression or PTSD

Several studies have shown that patients suffering from stress related diseases such as major depression or post traumatic stress disorder have a smaller hippocampal volume compared to healthy subjects [4;5], often correlated with impaired memory performance [10]. Interestingly, when PTSD patients undergo treatment with the antidepressant paroxetine (a selective serotonin reuptake inhibitor), hippocampal volume and memory performance recover in parallel [10]. Although small hippocampal volume may be a pre-existing risk factor for stress related diseases, (traumatic) stress could also reduce hippocampal volume.

1.2.2 The amygdala

The amygdala affects the processing of positive and negative stimuli including the autonomic response to emotional stimuli [58-63]. It is predominantly studied for its role in (auditory) fear conditioning, which uses aversive stimuli to measure emotional learning and memory [64-67].

The amygdala, as the hippocampus, is located in the temporal lobe and consists of several nuclei with specific functions. In these sub-nuclei, corticosteroid

receptor expression differs: GR is most expressed in the central and lateral areas, while the MR, which is less abundantly present, is mainly expressed in the corticomedial areas [68;69].

Several hormones beside corticosteroids influence amygdala functioning. One of them is CRH. This hormone facilitates attention to external events, sustains fear-related memory and when increased for an extended period, possibly even contributes to anxious depression [70-72].
The amygdala is also strongly under influence of catecholamines. These hormones are released from the adrenal medulla as a part of the fast sympatho-adrenomedullary stress response and indirectly affect amygdala processing [73-75]. Even more, catecholaminergic activation of the basolateral amygdala is necessary for correct corticosteroid functioning in hippocampal memory [75-77]. This implies that an event has to activate the amygdala, having an emotional "load", for optimal learning and memory of that event. Correspondingly, many studies have shown that emotional stimuli are better learned and remembered than neutral ones [78-80].

Box 2. Examples of amygdala functioning in humans.

Emotion and amygdala function: activation, stimulation and disruption

Presentation of faces with fearful or happy expressions changes the activity of the amygdala. This response is increasing with fearfulness, while it decreases with increasing happiness [3]. Electrical stimulation of the amygdala evokes both negative and positive emotions, accompanied by physiological responses, e.g. skin conductance [7]. Patients with amygdala damage are unable to correctly address emotional value to fearful and happy faces. Even more surprising, these patients give positively biased evaluations for negative facial expressions [11].

1.2.3 The prefrontal cortex

The prefrontal cortex (PFC) also influences emotional and cognitive functioning. The functions of the prefrontal cortex involve decision making, inhibition, behavioural flexibility, capacity to deal with novelty and goal directed behaviour. Overall, these functions allow to selectively respond to relevant external stimuli [81;82].
The PFC is located in the anterior part of the brain just above the orbit of the eyes. Strictly it is not part of the limbic system, but has strong connectivity with limbic structures. The PFC consists of several areas (medial, orbital and lateral) with distinct functions. The infra- and prelimbic areas of the FPC have been associated with diverse emotional and cognitive processes such as flexibility

during novel situations [83] and through connections with the amygdala they can also affect anxiety-related behaviour [84-86].
GR is expressed in all PFC areas, while MR expression is restricted to the infralimbic and prelimbic areas [69].

Box 3. An early case report of frontal lobe damage.

Phineas Cage

The case of Phineas Cage is one of the earliest descriptions of personality and behavioural changes following frontal lobe damage [2]. He was a railway worker in the USA around 1850 that became famous after surviving an explosion resulting in an iron bar planted in the front part of his head. After recovery, Phineas displayed impaired (irrational) decision making and a change in emotional processing. He was unable to keep his job as foreman of railway workers.

1.2.4 Connectivity between the hippocampus amygdala and PFC

The hippocampus, amygdala and PFC are extensively connected. To elaborate on this connectivity without presenting the enormous wealth on studies, the next section shows a schematic overview of some important connections (figure 4) and discusses several interacting connections between these brain areas.

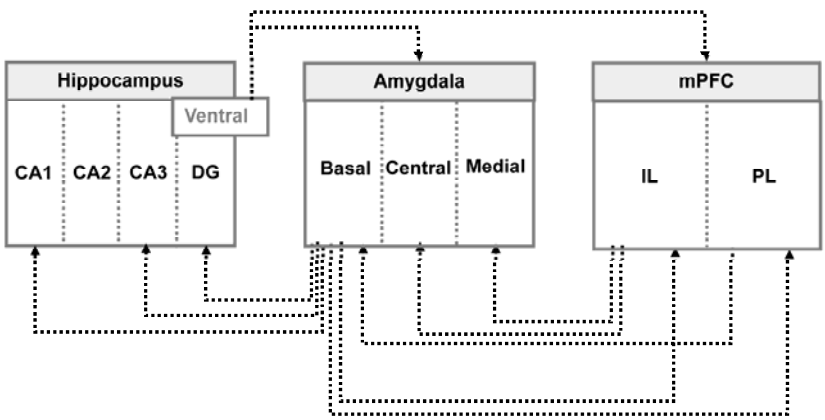

Figure 4. Schematic presentation of some important neural connections between the hippocampus, amygdala and PFC. References: [85;87-91].

Yaniv and colleagues have shown that neural activity in the entorhinal cortex, simultaneously influences LTP in both the hippocampus and amygdala [92], indicating interacting connectivity between these two areas. This is supported by high frequency stimulation in the basolateral part of the amygdala, which evokes LPT in the hippocampus [93;94]. Ishikawa and colleagues even more

showed that connection between the hippocampus and amygdala converge and interact in neural activity of the PFC. This leads to the believe that simultaneous activation of hippocampus and amygdala neurons may be important for enhancing medial PFC activity [90].

In summary, the hippocampus, amygdala and PFC have distinct functionality including contextual (time and place) and emotional processing and selective responses to relevant stimuli. They are sensitive to corticosteroids, are heavily connected and interact in several behavioural processes. This provides a base for interdependent actions of the stress system, emotion and cognition.

1.3 Corticosteroid action on emotion and cognition

Stress and corticosteroids activate MR and GR in the brain and influence different aspects of emotion and cognition. The next section discusses stressor and corticosteroid induced behavioural effects measured in rodents, first focussing on emotional and cognitive processes that are addressed in this thesis and second concluding with the specific role of MR and GR.

1.3.1 General note on behavioural observation in rodents

Before discussing which behaviours are under influence of corticosteroids, it should be realised that cognitive and emotional processes of mice are deduced from activity patterns. While techniques in molecular research have advanced, behavioural analysis is still often performed with limited behavioural data on these activity patterns. In this thesis we will extend behavioural analysis by performing in depth behavioural observation. In this case, conclusions are drawn from a broader behavioural spectrum.

1.3.2 Behavioural reactivity: Unconditioned response

Stressors and corticosteroids modulate exploration and locomotor activity. Exploration, which can be divided into general and directed exploration, is measured by total movement in the setup, walking patterns and rearing behaviour (figure 5); general exploration [95;96], or by the specific exploration of an object; directed exploration [97;98]. Locomotor activity is the total amount of horizontal movement in the setup.

Corticosteroids can have enhancing and suppressing effects on general exploration and locomotor activity [99]. Acute corticosteroid treatment increases locomotor activity [100-104]. This possibly reflects an active coping strategy [104], or anti-depressive actions when using an animal model for behavioural despair such as forced swim [105;106]. In contrast, extremely high corticosterone concentrations or chronic stressors are associated with suppressed locomotion and increased immobility [105-107]. Interestingly, when

using acute stressors instead of pharmacological corticosteroid manipulation, also suppressing effects on locomotor activity are observed. These effects are dependent on the type of stressor [108-110]. Whether reduced locomotor activity is an expression of high emotionality, as suggested in several studies, will be addressed in **chapter 3**.

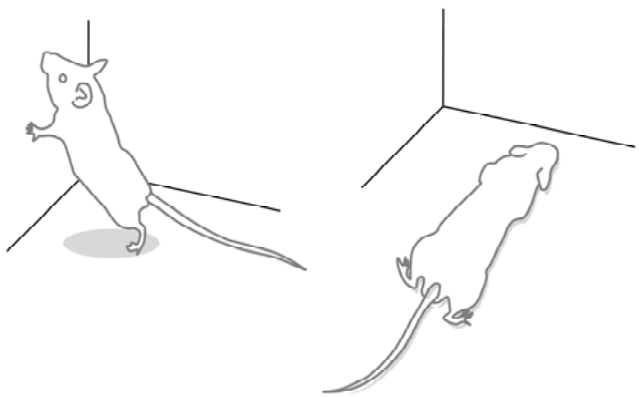

Figure 5. Rearing behaviour (left). The mouse stands on its hind legs, usually with the front legs against a wall, although rearing in an open area is also possible. Stretched attend posture (right). The mouse stretches it body horizontally while keeping its hind legs at the same position.

Unfortunately, data on how corticosteroids or stressors affect directed exploration as part of unconditioned response are very sparse. This is mainly due to the lack of environmental enrichment in the behavioural setups used to measure exploration and locomotor activity. However, directed exploration is often measured in the context of a learning task, e.g. conditioned response, by object recognition or platform finding in the watermaze.

The expression of negative emotions of anxiety and fear form another part of unconditioned behaviour. Anxiety-related behaviour is measured by avoidance of unprotected zones in a setting such as the open field, elevated plus maze (section 1.5.1) and light/dark box [111-115], meaning that locomotor activity and exploration can be confounding factors in measuring anxiety-related behaviour.

Fear-related behaviour is most commonly measured in learning tasks, expressed as freezing, scanning and startle response both after and in expectation of an aversive stimulus. Freezing is defined as total immobility of the animal and scanning is defined as total immobility except for head movement. Both are measures of immobility, however freezing is more severe due to the complete lack of environmental interaction.

At the psychological level, anxiety-related behaviour belongs to trait and state anxiety. Trait anxiety is a basal expression of innate anxiety and depends on

epigenetic influences [116;117], while state anxiety is measured after an exposure to or expectation of a mild aversive stimulus. Unfortunately, most anxiety-related tests in rodents involve placing the animal in a novel environment, which by itself acts as an anxiety enhancing stimulus and thus makes testing for trait anxiety very difficult. For this reason, the next section will only refer to state anxiety. Stressors and corticosteroid treatment enhance anxiety-related behaviour in different behavioural tasks [118-122]. Correspondingly, prolonged exposure to stressors increases fear-related behaviour [123;124]. Interestingly, a study by Skorzewska shows that an acute stressor lowers fear-related behaviour, although exploratory behaviour is increased [124]. This might be interpreted as active fear coping. Besides unconditioned responses to a one-time aversive stimulus, fear-related behaviour is also measured in the context of a learning task such as fear conditioning (section 1.5.1).
Corticosteroids and stressors can also influence risk assessment. This is defined in the mouse as stretched attend posture (figure 5). In general, risk assessment is enhanced by acute stressors and corticosteroid treatment [103;118;125].

1.3.3 Learning and memory: Conditioned response

When addressing the range of corticosteroids and stress effects on cognitive processes of learning and memory such as acquisition, consolidation, retrieval and extinction (short definition in box 4), three major influencing factors can be distinguished.

Box 4. Cognitive processes discussed in this thesis.

Cognitive processes (in short)

Cognitive processes of learning and memory discussed in this thesis (acquisition, consolidation, retrieval and extinction) take place in different time periods during and after an event:

1. During: Acquisition, gain of information about the event (learning)
2. Directly after - hours: Consolidation, memory formation about the event
3. Short/long term: Retrieval, recalling information that is stored
4. Short/long term: Extinction, decrease of memory-related behaviour due to repeated exposure (new learning)

Processes 1, 3, and 4, but not 2, can be deduced from the behaviour of the animal.

The first major factor is the **timing/duration** of the stress hormone action. Stress and corticosteroids facilitate memory formation, but only when the stressor or corticosteroid modulation is closely linked to the learning context [126]. For example, when given directly before a learning task, corticosterone

facilitates consolidation [127]. However, corticosteroid treatment in the period prior to memory testing impairs subsequent performance [128;129]. This impairment is often discussed as a corticosterone effect on memory retrieval [129;130], but another attractive possibility is that under the influence of the hormone an attention shift occurs towards the novel, distracting stimulus, thereby facilitating the processing of this "other" information.
The duration of corticosterone treatment or stressor is another important modulator of conditioned response. While an acute stressor can enhance acquisition, chronic stressors or corticosterone treatment impair memory formation and retrieval [131-133].
The second major factor is the **corticosteroid receptor mechanism**. Differential expression patterns and binding properties of MR and GR in the brain have consequences for cognitive processing. When using a spatial orientation task which depends on hippocampal functioning, corticosterone- and stressor mediated effects follow an inverted U-shape dependency [134-137]. Extremely low or high corticosteroid concentration, indicating relative high MR or GR function, impairs memory, while intermediate corticosteroid doses result in optimal memory performance. If the task used includes a large emotional component and thus heavily relies on amygdala functioning, stressors and corticosteroids affect memory following a linear relationship[138].
The third factor is **gender**. Cognitive (and emotional) functions of female and male rodents are differentially affected by corticosteroids and stressors [139;140]. Sex hormones like estrogens, strongly affect cognitive functioning [141;142] and these effects most likely interact with corticosteroids.

1.3.5. Specific MR and GR function in emotion and cognition

MR and GR are potent modulators of emotion and cognition with partly overlapping but also distinct functionality. MR, having high affinity for corticosteroids, is continuously occupied but can apparently also exert fast non-genomic influences on behaviour during high corticosterone concentrations. MR controls the initial behavioural response (behavioural reactivity) which is then, due to slow activation of the low affinity GR, processed during the consolidation period to facilitate memory for that event.
MR and GR also function in balance. This means that dysfunction of either receptor results in enhanced functioning of the other, hampering the interpretation of such effects. Is the effect due to relative increase of one receptor or due to relative decrease of the other?

MR modulates the behavioural response towards novelty. Novelty is represented by exposure to an unfamiliar environment, but can also be defined

as introducing or removing an object in a familiar setting. Behaviour in both novelty conditions is influenced by MR.
When introducing an unknown object into a familiar environment, MR knockout or overexpression alters exploration of this object [143;144]. Likewise, when removing a familiar object in the watermaze, MR antagonism and overexpression changes swimming patterns and escape strategy [143;145;146].
When placed in a novel experimental setting, MR antagonism lowers corticosterone induced locomotion, changes object recognition [100] and lowers anxiety-related behaviour [147-149]. In contrast, MR overexpression can also lower anxiety-related behaviour [144;150;151]. These findings illustrate the U-shaped dose-dependency of MR-mediated effects, in which complementary GR-mediated actions also seem to participate.
It would be expected that MR modulation and therefore changing behavioural reactivity towards novelty would affect subsequent learning and memory. A change in behavioural reactivity towards novelty likely reflects different perception and focus of attention. This difference in perception and attention could alter the information that is gained about the novelty, leading to consolidation of different information and thus different memory. Indeed, several studies show that MR overexpression and pharmacological activation is associated with enhanced memory consolidation [144;151;152], while less MR activity diminishes spatial learning [146] and memory [143;153].

GR influences cognitive processes by facilitation of consolidation. This is shown by diminished spatial memory in mice with chronic inactivation of whole brain GR (knockout), GR dim/dim mice, mice with less GR expression and acute intracerebroventricular injections of a GR antagonist [127;145;154;155]. GR function has also been extensively studied in fear associated learning and memory. Here, pharmacological blockade of GR in non stressed and chronic stressed animals attenuates the expression of contextual fear response [123;156]. The GR mediated effects on fear (memory) are area specific; acute pharmacological blockade of the GR in the dorsal hippocampus of rats facilitates spatial learning [157], while GR blockade in the ventral hippocampus decreases long term contextual/spatial fear memory [158]. GR in the amygdala is necessary for auditory fear consolidation [67]. This corresponds to the role of the hippocampus in context dependent fear conditioning and the role of the amygdala in cue-related fear conditioning (section 1.5.2).
GR can also influence anxiety-related behaviour. Overall, less GR function lowers anxiety-related behaviour [159-162], while increased GR activation correlates with high anxiety-related behaviour [159].

In summary, stressors and corticosteroids affect emotion, learning and memory depending on duration, dose and gender and are only effective in the context of a learning task. The effects exerted by the steroid are mediated by MR and GR. Via MR, corticosterone influences the behavioural response towards a new or changing situation, while additional activation of GR facilitates memory consolidation.

1.4 Interaction between emotion and cognition

Central to cognitive emotional interactions are the above mentioned brain areas, i.e. hippocampus, amygdala and PFC, which have a high degree of connectivity (section 1.2). Emotional and cognitive processes often interact and contribute together to behaviour. Examples of such interactions in humans and rodents are discussed in the following section.

1.4.1 Human

For a long time, emotion and cognition have been examined as separate entities. Just lately more and more studies have focussed on the specific interaction between emotion and cognition.

For example, exposure to an emotional picture impairs ongoing working memory processes more than exposure to a neutral picture. Furthermore, besides ongoing cognitive functions, also long term cognitive processes such as declarative and procedural memory are sensitive to emotional modulation [163]. In turn, cognitive processes change the response to emotional stimuli [164].

Studies on this interaction between emotion and cognition in stress-related psychopathology have just started. Results show that patients suffering from depression [165] and PTSD often have a memory bias for emotional information [166]. Furthermore, emotional arousal in PTSD patients hampers cognitive functions (see section 1.6). It is expected that behavioural studies will further specify how emotion and cognition are integrated in these diseases.

Besides behavioural research, also brain imaging studies support the interaction between emotional and cognitive functioning. For example, an fMRI study in healthy subjects shows that altered communication between limbic areas (amygdala), prefrontal cortex and cingulated cortex impairs cognitive processing of emotions [167]. Several fMRI studies on interacting emotional and cognitive processes focus on the prefrontal cortex [168]. These studies demonstrate that emotional states can selectively influence working memory-related neural activity in the lateral PFC [169;170].

1.4.2 Rodent

Interacting emotional and cognitive functions are also observed in mouse behaviour [171-174]. For example, pharmacologically increased anxiety

decreases working memory performance of mice in the watermaze [175], while the reduced anxiety observed after deletion of the corticotropin-releasing factor receptor 1 impairs spatial recognition memory [172]. In addition, low anxiety and good cognitive performance correlate in DBA mice, while exploration and cognitive functioning is correlated in C57BL/6J mice [171].

These findings underline the interaction between emotion and cognition, however, the involvement of the glucocorticoid stress system needs to be elucidated.

1.5. (Behavioural) tasks and animal models to measure emotion and cognition

A variety of tasks is available for measuring specific aspects of emotion and cognition in rats and mice. Some tasks focus on behavioural reactivity, while others relate to learning and memory processes. The test paradigms discussed in this thesis include "classic" tasks which are adapted and refined for (i) simultaneous measurements of emotional and cognitive parameters and (ii) discrimination of context and cue-related fear memory and its extinction. Next, the mouse models used in this thesis are described, i.e. mice of distinct strains, as well as mice with genetically manipulated MR. The last section will address the statistical approach used to handle the large amount of behavioural data.

1.5.1. Behavioural tasks

Experiments described in this thesis are based on three behavioural tasks, (i) the elevated plus maze (EPM), a "classic" test to measure unconditioned behaviour including emotional expression related to anxiety, (ii) the modified holeboard (MHB) to measure unconditioned behaviour but also simultaneously emotion and reward-related cognition and (iii) a refined fear conditioning task for testing of alternating context and cue fear memories and their extinction.

The EPM is used to measure unconditioned behaviours by estimation of the balance between anxiety-related behaviour and exploration (figure 6). This test uses the mouse's innate avoidance of open spaces, which is interpreted as anxiety behaviour. As there are no complex features in the test apparatus, aspects of behavioural reactivity such as directed exploration cannot be assessed.

The MHB provides a complex environment and therefore can be used to measure all aspects of behavioural reactivity, exploration and emotional expressions (figure 6). Introducing treats at certain locations modifies the task for additional assessment of reward stimulated learning and memory. Thus, we can simultaneously test emotional and cognitive functioning. The EPM and MHB depend on the voluntary exploration of protected and unprotected areas.

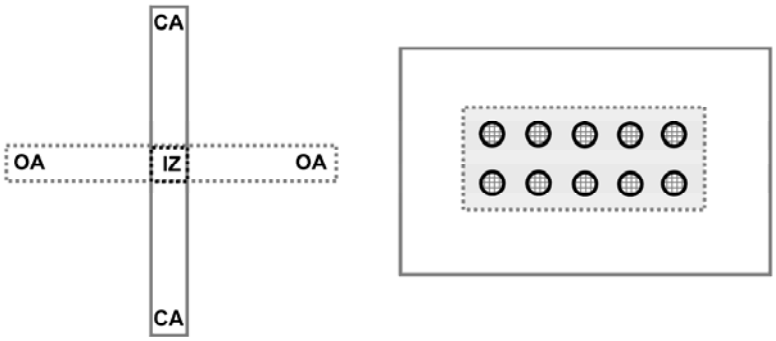

Figure 6. The elevated plus maze (EPM: left) and modified holeboard (MHB: right) seen from above. EPM: The EPM consists of an elevated platform on which four Perspex arms in the shape of a cross, and an intermediate zone are present: two arms with side walls (closed arms; CA; "safe, protected") and two arms without side walls (open arms; OA; "unsafe, unprotected") are separated by an intermediate zone (IZ). The EPM is mainly used as a one trial, short test (5 min) to measure anxiety-related, escape and explorative behaviour [176-179].
MHB: A board containing 10 cylinders is located in the centre of an open field. Thus, the board is in an unprotected unsafe zone, while the areas near the walls provide protection. Moreover, the cylinders represent objects to explore. This task can be used as a short one trial test (5 min) for anxiety-related and explorative behaviour, including both general exploration and directed exploration towards the cylinders. Baiting the cylinders with treats (bait), the MHB can be used as appetitive learning task to measure cognitive parameters such as reference and working memory [171;180]. In this case, the animals undergo a multiple trial protocol in which they have to find these baits. Visual markers of the baited cylinders can be used to assess visual-discrimination learning.

Fear conditioning is based on the classical Pavlovian conditioning paradigm and allows studying the development of fear memories and their extinction (figure 7). Fear conditioning can be used to determine the contribution of two brain systems to fear memory; the hippocampus which processes context-related fear memory and the amygdala which processes cue-related fear memory [181].

1.5.2. Mouse models

In addition to the behavioural tasks described above, this thesis describes several mouse models to study the corticosteroid action on emotion and cognition: (i) pharmacological activation or blockade of MR and GR, (ii) naturally occurring genetic variation of MR and GR in inbred mouse strains and (iii) genetic modification by MR knockout in the forebrain. Next section discusses these mouse models.

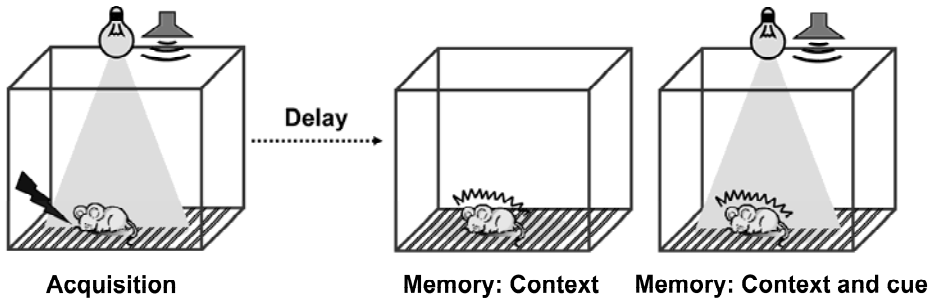

Figure 7. Schematic representation of the fear conditioning setup and protocol that are used for experiments described in this thesis. During acquisition, an unexpected aversive stimulus (electric foot-shock: unconditioned stimulus US), is given several times in association with a neutral stimulus such as a light and tone (cue; conditioned stimulus CS), in a distinct environment (context). The animal will form an association between the announcing cue and aversive stimulus but also the surrounding in which the aversive stimulus was given, i.e., formation of fear memory. After a delay the animal is placed in the same context and additionally the cue (light/ tone) is turned on in the same sequence as during conditioning, but without electric shock. This should evoke a fear response (conditioned response, CR) that is predominantly expressed as freezing (fear memory). Due to repeated exposure to context and cue without electric shock immobility behaviour is expected to decrease, i.e., extinction. Two main types of fear behaviour can be distinguished; immobility and escape behaviour. Immobility includes scanning and freezing. Scanning is defined as immobility of the body, while the head is moving horizontally from side to side. Freezing is defined as immobility of the body and head. Depending on the type of behavioural observation, either immobility (automatic; infrared/light beams) or freezing and scanning (manual) can be registered. Escape behaviour can be observed by the number of attempts to jump out of the setting.

Next to the well known use of MR and GR antagonists, a common method to differentially activate MR and GR is **replacement with corticosteroids** in animals with (almost) no endogenous corticosteroid production **(chapter 2)**. The adrenals are surgically removed and a pellet containing different corticosterone concentrations is subcutaneously implanted. In contrast to rats, mice that undergo adrenalectomy remain to produce low concentrations of corticosterone from scattered cell groups in the vicinity of the adrenals [182]. Therefore, adrenalectomized mice provide an excellent model for predominant MR activation. Different degrees of continuous GR activation can be achieved via corticosterone released from implanted pellets, while an injection results in a phasic activation of GR on the background of continuous MR activation.

Naturally occurring variances in MR and GR expression as present in selected inbred strains provide another possibility to measure MR and GR

function. For example, the Lewis and Fisher rat strains are known for their differences in stress sensitivity [183;184]. Mouse lines selected for short and long attack latency (SAL and LAL respectively) also demonstrate distinct stress system regulation [185;186].
This thesis describes a study in which BALB/c and C57BL/6J mice are characterized for stress system markers, emotion and cognition **(chapter 3)**. These strains have been originally used in immunology research to determine their resistance and immunological response to various infectious agents [187;188]. BALB/c mice have been described in the literature to be more stress reactive during mild subchronic stress compared to C57BL/6J mice [189]. BALB/c and C57BL/6J mice show different exploration patterns [180] and BALB/c mice display higher anxiety-related behaviour [189-192]. As briefly discussed later on, a proposed explanation for the difference in anxiety-related behaviour between these strains is the distinct maternal care given by the dams [193].
Data on cognitive performance of BALB/c and C57BL/6J mice is sometimes contradictive. Some studies report poor spatial learning abilities of BALB/c mice in the water maze [194-196]. However, BALB/c mice do not show inferior cognitive performance when tested in a dry maze or when multiple cognitive parameters for learning and memory are included [195;197]. Fear conditioning studies have shown that C57BL/6J mice freeze more often and display generalised freezing compared to BALB/c mice [198;199].
These strains also have distinct corticosteroid-related molecular determinants that can influence emotion and cognition. For example, BALB/c mice have lower GABA(A) receptor expression compared to C57BL/6J mice [200;201]. GABA(A) is influenced by maternal care and negatively correlates to anxiety-related behaviour [202]. BALB/c and C57BL/6J mice also differ in NMDA mediated cognitive processes in the amygdala [203]. The NMDA receptor, which is more expressed in BALB/c mice, specifically facilitates the magnitude of contextual fear acquisition [204;205]. In addition, beta-adrenoceptor expression also differs between these strains. BALB/c mice exhibit higher amygdala beta-adrenoceptor expression compared to C57BL/6J mice [206]. This receptor binds hormones which are released during the fast sympatho-adrenomedullary stress response, and therefore might suggest that BALB/c mice are more susceptible to fast stress effects on behaviour.
Overall, BALB/c and C57BL/6J mouse strains likely differ in glucocorticoid stress system (and related molecular determinants), emotion and cognition. However, also differences in the fast sympatho-adrenomedullary stress response seem to be present.

Experiments described in this thesis also include genetically altered mice with **MR ablation** in the forebrain. The advantage of these mice is the huge change

in MR function compared to naturally occurring differences in expression, so more pronounced behavioural effects are expected. Another advantage is the neuro-anatomically defined location of the genetic alteration. Although both peripheral and central targets can be selected, these MR ablated mice have forebrain-specific inactivation of the MR gene ($MR^{CaMKCre}$). This allows studying the specific function of limbic MR. The third advantage is the inducibility of gene modulation. These $MR^{CaMKCre}$ have reduced MR at postnatal day 0 and complete loss of MR at postnatal day 12 and during adulthood. They do not show any visual (appearance), acoustic and motor abnormalities compared to controls [143].
This thesis describes experiments using the $MR^{CaMKCre}$ mice to determine specific MR contribution to emotion and cognition tested in the described fear conditioning task **(chapter 6)**.

1.5.3. Statistical analysis

As a result of extended analysis of behavioural parameters of emotion and cognition, a large amount of data is generated. Besides "standard" statistical analysis, principal component analysis (PCA) will be used in order to structure the behavioural data. PCA is a statistical data reduction method that minimises multidimensional data sets and is used to explain variability among behaviours. It determines correlations between behavioural parameters which allow "clustering" of behaviours into so called factors. These correlations and factors can be used to determine which emotional and cognitive parameters interact (or are independent), and with the use of further ANOVA testing determines group or strain differences in this interaction.

1.6. Translational approach: from animal model to stress-related pathology

Nowadays, the long time separated areas of human and animal research on cognition and affect start to merge. Also the study of the implication of this research for psychopathology has just begun..
Animal models provide an opportunity to study the genetic determinants that underlie the endocrine and behavioural stress responses. They allow to determine which factors could play a role in the susceptibility or resistance to stress-related diseases, which involve emotional and cognitive disturbances.
In humans, post traumatic stress disorder (PTSD) is characterized by persistent intrusive fear memories of a stressful event, with concomitant strong emotions. Why these strong emotional memories are present; due to enhanced acquisition, stronger consolidation or impaired extinction, is unknown. In this thesis an animal model for PTSD is described using a dedicated fear conditioning design in mice with distinct MR and GR background. This design allows the study of the development, memory and extinction of strong

emotional memories in mice. Furthermore, it is possible to simultaneously assess if fear memory is generalized or specific for a predictive stimulus.
To clarify the translational approach, characteristics of PTSD with a specific focus on the changes in circulating glucocorticoids, emotion and cognition are described below.

1.6.1. Post traumatic stress disorder (PTSD)

An estimated 8 % of the world population will experience PTSD at some point in their lives. War, sexual or physical abuse, witnessing or being in a life threatening situation, like surgery, accidents or terrorist attacks, but also natural disasters like the tsunami are traumatic experiences that can result in PTSD. Prevalence of PTSD was as high as e.g., 24.4% in relief workers after the tsunami in Asia [207] and 20.9% in Israeli Yom Kippur War veterans [208]. Also psychiatric disorders like depression and anxiety disorders increase the risk for comorbid PTSD. Methods to diagnose PTSD involve measures of symptomatology as can be found in the Clinician-Administered PTSD scale (CAPS), Impact of Events Scale (IES) and PTSD symptoms checklist (PCL).
Neurobiological approaches to understand PTSD are developing [209]. Increased activation of the sympathetic nervous system and hypocortisolism are described as features of autonomic and endocrine dysregulation [210;211]. Indeed, adrenergic activation in the face of low corticosterone has been shown to facilitate learning in animals [212]. Although basal cortisol levels appear to be low, PTSD patients are more sensitive to stress and glucocorticoid negative feedback. However, Baker and colleagues [213;214] have reported increased cortisol, noradrenalin and interleukin 6 in the cerebrospinal fluid, but not in blood plasma of PTSD patients. This shows the complexity of cortisol involvement in PTSD. In addition, the hippocampus has a smaller volume in PTSD patients compared to healthy controls [215;216]. This is often discussed as consequence of high corticosteroid exposure and thus contradicts hypocortisolism, but corresponds to high cortisol levels in cerebrospinal fluid during PTSD.
Often, PTSD is diagnosed together with generalized anxiety disorder, depression or chronic fatigue syndrome [217;218], suggesting that next to stressful live events that contribute to the onset of the disorder there might be common molecular nominators. Indeed, twin studies (like in Vietnam veterans [219]) suggest that genes contribute for an important part in vulnerability to PTSD. Thus, the current point of view is that the risk for PTSD is the product of multiple genes and non-genetic (environmental) factors such as stress [220].
Treatment of PTSD can involve eye movement desensitization and reprocessing therapy or a combination of psychotherapy and medications such as antidepressants and antipsychotic drugs.

Recent clinical trials suggest however that administration of corticosteroids may have a beneficial effect on established PTSD and specific (fear-related) phobia [221-223]. In patients with PTSD, low-dose cortisol treatment for one month reduced symptoms of traumatic memories without causing adverse side effects.

Box 5. PTSD

Post traumatic stress disorder (PTSD)

PTSD is defined as "a normal response to extreme stress resulting in chronic anxiety"[1]. It is characterized by intrusive persistent memories of the trauma, avoidance of stimuli associated with the trauma, numbing of general responsiveness and hyperarousal. Intrusions of a traumatic memory occur as "flashback". Patients avoid social contacts, places and thoughts; have feelings of detachment and an increased risk for drug abuse. Hyperarousal is described as feeling irritable, with problems to concentrate, but also sudden outbursts of anger. Other symptoms include sleep disturbances, including nightmares, insomnia, sleep movement disorders and daytime fatigue. The onset of PTSD follows the trauma with a latency period that may range from a few weeks to months. In a small proportion of cases the condition may follow a chronic course over many years, with eventual transition to an enduring personality change [8;9].

1.7. Scope of the thesis

1.7.1 Objective

Corticosteroids display a large individual variation in effects on emotional processes and cognitive performance. These central effects exerted by the steroids can be facilitating under normal conditions, but become impairing if the action of the stress hormone is excessive, prolonged or inadequate. Such dysregulated corticosteroid action is thought to compromise information processing underlying the proper integration of emotional and cognitive processes which may enhance the vulnerability to stress-related disorders such as depression and PTSD. In this thesis I will focus on the role of the two distinct receptor types that mediate the action of the corticosteroids on specific domains of emotion and cognition, that are considered separately as well as in interaction.

The following questions are addressed:

1. Do corticosteroids affect emotion and cognition via differential MR and GR **activation**? Are emotion and cognition correlated? (chapter 2)

2. Do emotion and cognition correspond to distinct MR, GR **expression** and stress sensitivity as it is expressed in two mouse strains? (chapters 3,4)
3. Does exogenous corticosterone eliminate strain differences in emotion and cognition for a negative event? (chapter 5)
4. Does the time of treatment (before or directly after the negative event) differentially influence memory formation and extinction? (chapter 5)
5. What is the specific function of MR during memory formation and extinction of a stressful emotional experience? (chapter 6)

1.7.2 Experimental approach and outline

In order to study how differential MR and GR activation influences emotion and cognition, plasma corticosterone concentration of C57BL/6J mice was clamped to different levels followed by extensive testing for emotional and cognitive functioning in the modified holeboard. We expected that both emotion and cognition would be affected by variations in corticosterone concentrations, showing a differential and coordinated contribution of MR and GR **(chapter 2)**.
Next we assessed if naturally occurring differences in MR and GR expression would correspond to endocrine and behavioural stress sensitivity, emotional and cognitive functioning. Two inbred mouse strains (BALB/c and C57BL/6J) were characterised for MR and GR protein and mRNA expression in the hippocampus, amygdala and PFC and further tested for emotional and cognitive behavioural patterns in the elevated plus maze and modified holeboard. We expected that BALB/c mice would display glucocorticoid stress system markers indicative for a stress susceptible phenotype; high stress induced corticosterone concentrations and an altered MR/GR balance compared to C57BL/6J mice. In addition, we expected that emotional expressions would differentially contribute to learning and memory in BALB/c and C57BL/6J mice **(chapter 3)**.
These mouse strains (BALB/c and C57BL/6J), which indeed exhibited distinct differences in susceptibility to stress, were subjected to a specifically designed fear conditioning paradigm. We expected that combined, but alternating cue-context exposure would identify either generalized or stimulus-specific fear-responses, and thus determine the influence of the strain-dependent susceptibility to stress on emotion and cognition for an emotionally negative event **(chapter 4)**. In order to assess the impact of corticosteroids on the acquisition and consolidation phase of fear memory BALB/c and C57BL/6J mice were injected with corticosterone directly before or after acquisition of fear conditioning and the retrieval and extinction of context- and cue-related fear memories were observed **(chapter 5)**.
To further specify the role of MR in emotion and cognition, forebrain $MR^{CaMKCre}$ knockout mice were studied for behavioural and corticosterone response,

emotion and cognition in a one trial modified hole board test followed by a fear conditioning paradigm **(chapter 6)**.

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Chapter 2

Differential MR/GR activation in mice results in emotional states beneficial or impairing for cognition

Brinks V, van der Mark MH, de Kloet ER, Oitzl MS
Gorlaeus Lab, LACDR/LUMC, Division of Medical Pharmacology, Einsteinweg 55, 2300 RA Leiden, Leiden University, the Netherlands

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ABSTRACT

Corticosteroids regulate stress response and influence emotion, learning and memory via two receptors in the brain, the high affinity mineralocorticoid (MR) and low affinity glucocorticoid receptor (GR). We test the hypothesis that MR- and GR-mediated effects interact in emotion and cognition when a novel situation is encountered that is relevant for a learning process. By adrenalectomy and additional constant corticosterone supplement we obtained four groups of male C57BL/6J mice with differential chronic MR and GR activation. Using a holeboard task, we found that mice with continuous predominant MR and moderate GR activation were fast learners that displayed low anxiety and arousal together with high directed explorative behaviour. Progressive corticosterone concentrations with predominant action via GR induced strong emotional arousal at the expense of cognitive performance. These findings underline the importance of a balanced MR/GR system for emotional and cognitive functioning that is critical for mental health.

INTRODUCTION

Stress and emotions facilitate or impair learning and memory processes [1]. Glucocorticoids are the stress hormones secreted from the adrenals after activation of the hypothalamus-pituitary-adrenal (HPA) axis; i.e., corticosterone in rats and mice, cortisol in humans. The effect on synaptic plasticity and memory formation is mediated by two types of nuclear receptors: MR (mineralocorticoid receptor) and GR (glucocorticoid receptor) which are located in areas involved in emotion, learning and memory. While MR is present in the hippocampus and to lesser extent in the prefrontal cortex, amygdala and paraventricular nucleus [2-5], GR can be found throughout the brain with high levels in the hippocampus and paraventricular nucleus [6]. Other characteristics are the differential affinities for corticosterone: MR has a tenfold higher affinity than GR, resulting in predominant MR occupation during low basal levels and additional GR activation during increased corticosterone concentration due to stress or circadian peak activity of the hypothalamic-pituitary-adrenal (HPA) axis [7]. The precise involvement of MR and GR in emotion and cognition is still debated.

Animal studies have shown that activation or blockade of either receptor influences behaviour related to anxiety, exploration and memory. These behaviours are linked to the limbic system and are part of the behavioural repertoire tested in spatial memory tasks but also in fear conditioning [8]. With respect to unconditioned fear-related behaviour, Smythe [9] has described that MR modulates anxiety-like behaviour of rats in the light/dark box. Oitzl et al., have shown that intracerebroventricular injection of a rather selective MR antagonist in rats influenced corticosterone-induced behavioural reactivity to spatial novelty [10]. Recent findings in mutant mice with inactivated MR in the forebrain (Cre-loxP recombination [11]), support the pharmacologically detected role of MR on the modulation of behavioural strategies. Loss of the limbic MR impaired behavioural plasticity, evidenced by a differential performance during the first exposure to learning tasks, i.e. their behavioural reactivity to novelty. In contrast, learning slopes in the Water and Radial Arm maze were not affected. This increased behavioural reactivity to novel objects was observed in the face of normal anxiety-like behaviour in the open field and elevated-O-maze [12]. Indeed, it should be clarified whether MR affects anxiety or appropriate context-dependent behavioural reactivity. Others suggest that adaptive behaviour is modulated by a combined MR/GR mediated action. An example is the inhibition of corticosterone production and thus prevention of GR activation in the face of full MR activation: this led to decreased fear-induced immobility and fear-related anxiety in rats [13]. Complementary, exogenous corticosterone application or prior social defeat increased anxiogenic behaviour in rats tested

in the elevated plus maze 24 hrs later. Antagonism of the GR in the lateral septum eliminated the anxiogenic effect [14]. Interesting in this study is the 24 hrs delay, indicating involvement of memory. Indeed, GR is implicated in memory consolidation processes, demonstrated by using GR-agonists and -antagonists in rats, chickens as well as GR mutant mice [15-20]. Calvo et al., have shown that corticosterone-induced effects on anxiety after restraint stress require both MR and GR [21]. Taken together, MR appears to be responsible for the immediate facilitative effects of corticosterone on memory acquisition, while the modulation of spatial and fear memory relies on the presence of a functional GR [22]. To disentangle the combined contribution of MR and GR to most adequate performance, we will study the functions of these receptors in a task that allows simultaneous registration of emotional and memory parameters.

How emotion and cognition affect each other is still relatively unknown. Forgas and George suggested that a stimulus first needs to be identified before the appropriate emotional response will follow [23]. Others focus more on the neurobiological process of emotion and cognition, which can be functionally, anatomically and even pharmacologically separated [24]. We hypothesise that emotion and cognition are interdependent and both will be affected by differential MR and GR activation: we propose that the two corticosteroid receptors MR and GR contribute differentially but in a coordinated way to information processing.

The aim of this study was to examine how MR and GR interact in information processing presented by emotional and learning/memory elements of a task. Next to the well known use of MR and GR antagonists, MR/GR activation ratios can be endocrinologically and pharmacologically adjusted by removal of the adrenals (adrenalectomy -ADX) and additional subcutaneous corticosterone pellet implantation. In contrast to rats, mice that undergo adrenalectomy remain to produce low concentrations of corticosterone from scattered cell groups in the vicinity of the adrenals [25-27]. Therefore, ADXed mice provide an excellent model for predominant MR activation. Different degrees of continuous GR activation can be achieved via corticosterone released from implanted pellets. We used this approach and tested mice in the modified holeboard [28] measuring behaviours that define general activity, emotions, motivation and learning and memory. Subsequent Principal Component Analysis will allow to determine the correlation between emotions and cognition.

MATERIALS AND METHODS

Animals

Forty-eight 12 weeks old male C57BL/6 mice were obtained from Charles River (Maastricht, The Netherlands). After arrival, the mice were housed individually in the experimental room with sawdust bedding, water and food *ad libitum*, at 20^0C with controlled humidity under a 12 h: 12 h light/dark cycle (lights on at 08.00 a.m.) for at least one week. To familiarize with the bait used in the modified holeboard task, all mice received a few pieces of almonds daily in the week before surgery. All experiments were approved by the committee on Animal Health and Care from the Leiden University, The Netherlands and performed in strict compliance with the EEC recommendations for the care and use of laboratory animals.

Endocrine manipulation of MR/GR activation

Mice were randomly selected for one of the following groups and operated accordingly: (i) Sham operated (Sham), (ii) adrenalectomized mice (ADX), (iii) adrenalectomized mice with an additional low corticosterone pellet (ALC) or (iv) adrenalectomized mice with an additional high corticosterone pellet (AHC).

Surgery

Mice were gas anaesthetised with a mixture of isoflurane/nitrous oxide (4% isoflurane bolus followed by 2% isoflurane). Body temperature was kept constant at 37^0C by a heating pad. Adrenals were removed (ADX) using the dorsal approach followed by subcutaneous pellet implantation on the flank of the animal. While in rats ADX removes the endogenous source of corticosterone, in mice it clamps corticosterone to low concentrations comparable to the circadian trough of adrenally-intact mice. Accessory adrenocortical cells secrete stable amounts of corticosterone [25-27;29] that maintains extensive occupation of MR. Stress or circadian rhythm does not lead to a rise in corticosterone in ADX mice. High circulating levels of ACTH indicate the lack of GR activation; i.e., no negative feedback.

Sham operation involved the same procedures as adrenalectomy except for the removal of the adrenals. Surgery was performed between 10.00 and 12.00 a.m. and lasted maximal 10 minutes per mouse. Adrenals were removed within 5 minutes. After surgery, all mice received an additional bottle containing 0.9% salt solution. Behavioural testing started 3 days after surgery. To confirm effectiveness of the adrenalectomy and pellet implantation, plasma corticosterone levels were measured 2 days after surgery, on day 0 of the experiment and one day after the last behavioural test on day 11. Mice with

abnormal corticosterone concentrations in the blood were excluded from further analysis. This resulted in seven mice per group.

Pellet preparation

Two types of pellets were made for subcutaneous implantation: (i) a 5% corticosterone (ICN Biomedicals INC) 95% cholesterol pellet for moderate MR/GR activation and (ii) a 20% corticosterone 80% cholesterol pellet for strong MR/GR activation. All pellets weighed 100 mg, with a diameter of 7 mm and thickness of 2 mm and were home-made. Corticosterone dose was chosen following a pilot experiment in which plasma corticosterone concentrations of about 100 and 150 ng/ml for the 5 % and 20 % pellet respectively, were measured two days after implantation.

Modified holeboard testing

Setup

The modified holeboard consisted of a opaque grey PVC box (50x50x50cm) with a centerboard (37x20cm) on which 10 grey cylinders (4 cm height) were staggered in two lines [30]. Always the same three cylinders were baited with a small piece of almond on top of a grid, and marked with a white ring. Seven other cylinders contained a non-obtainable almond underneath the grid and were marked with a black ring. The mice were placed in the modified holeboard for 3 trials per day with changing start positions. One trial lasted maximally 5 min, or until the mouse had found the three baits. All testing was performed between 9.00-12.00 a.m.

Behavioural observation

The behaviour of the mice was observed, recorded and analyzed with a semi automatic scoring system (The Observer Mobile 4.1, Noldus Information Technology, Wageningen, The Netherlands). All measured behavioural parameters are represented in table 1. As indication for (i) working memory, the number of repeated holevisits was calculated and (ii) reference memory, the number of visits to non-baited holes was taken. In addition, a camera was installed above the setup to measure distance moved and velocity of the mice with an automatic tracking system (Ethovision 1.95, Noldus Information Technology, Wageningen, the Netherlands).

General experimental procedure

Mice were tested in the modified holeboard over 10 days. On days 1 to 5 and 8, the three baited cylinders were marked with a white ring as visual cue while the remaining cylinders were marked with a black ring. This allowed visuo-spatial discrimination. On days 6 and 7, mice were not tested. On days 9 and 10, all

rings were removed from the cylinders, but the bait remained in the same cylinders. This allowed to estimate if the mice used a spatial strategy or visual discrimination to solve the task.
A trial lasted maximally 5 minutes and was ended when the mouse had eaten all three baits.
On days 0 and 11, blood was collected via a tail incision or after decapitation. Blood plasma was used to measure corticosterone concentrations (ICN Biomedicals, Inc). Because exposure to high concentrations of corticosterone results in shrinkage of the thymus, thymusweight was estimated as well.

Total number	Sit
	Rearing
	Stretched attend
	Grooming
	Centerboard-entries
	Holevisits
	Baited holes visited
	Non-baited holes visited
	Repeated holevisits
	Baits obtained
Latency	First centerboard-entry
	First holevisit
	Eat bait
Time	Sit
	Grooming
	On centerboard
	To finish task

Table 1. Behavioural parameters measured in the modified holeboard.

Statistical analysis
Differences in corticosterone concentrations between groups and days were analysed by two-way ANOVA (SPSS 11.5.0) with Tukeys post hoc analysis. To analyse thymus and bodyweight differences, a one-way ANOVA was performed.
The behavioural data are presented as mean of 3 trials per day $\pm$ SEM. Data were subjected to General Linear Model (GLM) -repeated measures with Tukey as post-hoc test to analyse progression over days and group differences per day. Furthermore, factor analysis (Principal Component Analysis PCA) was performed over groups and days to obtain a more comprehensive analysis of emotional and cognitive parameters. This analysis uses cross-mouse comparisons to distinguish the relation between behavioural parameters. It includes as much data as possible in each factor to minimize residual variance from the original dataset. The PCA was performed with a Varimax rotation on

variables with communalities over 0.7, that is, of which 70 % of the variance is explained by the Factors extracted. The number of extracted Factors was not pre-defined; Factors with an Eigenvalue > 1 were accepted. Factor scores were subjected to a two-way ANOVA to determine differences between groups and days. $P < 0.05$ was accepted as level of significance.

RESULTS

Behaviour

Emotion and Exploration

Figure 1 shows the results for some of the emotional and explorative parameters during all days of testing in the modified holeboard. Figure 1A illustrates that ADX followed by ALC mice have a high percentage of time spent on the centerboard, indicative for low anxiety [31-34] during the first few days. In contrast, AHC and sham mice spent little time on the centerboard during this period. From day 4 on, few significant differences were found between groups. GLM from day 1 to 10 revealed a significant group/day interaction $F(21, 588)$ 2.355, $p=0.001$.

Figure 1B shows that AHC mice display twofold more defecation compared to other groups, indicating high arousal. With repeated testing, ALC mice display less defecation compared to ADX and AHC mice. GLM revealed a significant progressive decrease over days $F(21,588)$ 7.629 $p< 0.0001$, just passing statistical significance between groups ($F(21,588)$ 1.524 $p=0.063$).

The number of rearings was taken as measure for general exploration (figure 1C). Comparing the first and the last day of testing, no differences were found between groups while on days 2, 3 and 4 ADX mice display the lowest number of rearings. GLM showed a significant change over days ($F(21,588)$ 11,439 $p< 0.0001$) although not significant between groups ($F(21,588)$ 1.25 $p=0.203$).

ADX mice display highly directed exploration/behavioural reactivity on all days of testing, reaching statistical significance on days 1 and 2 as indicated by the number of holevisits (figure 1D). Sham, AHC and ALC mice start off with few holevisits which increase over time. GLM supported this by significant group/day interaction $F(21,588)$ 1.983, $p=0.006$.

Total distance moved and velocity was comparable between groups over all days of testing (data not shown).

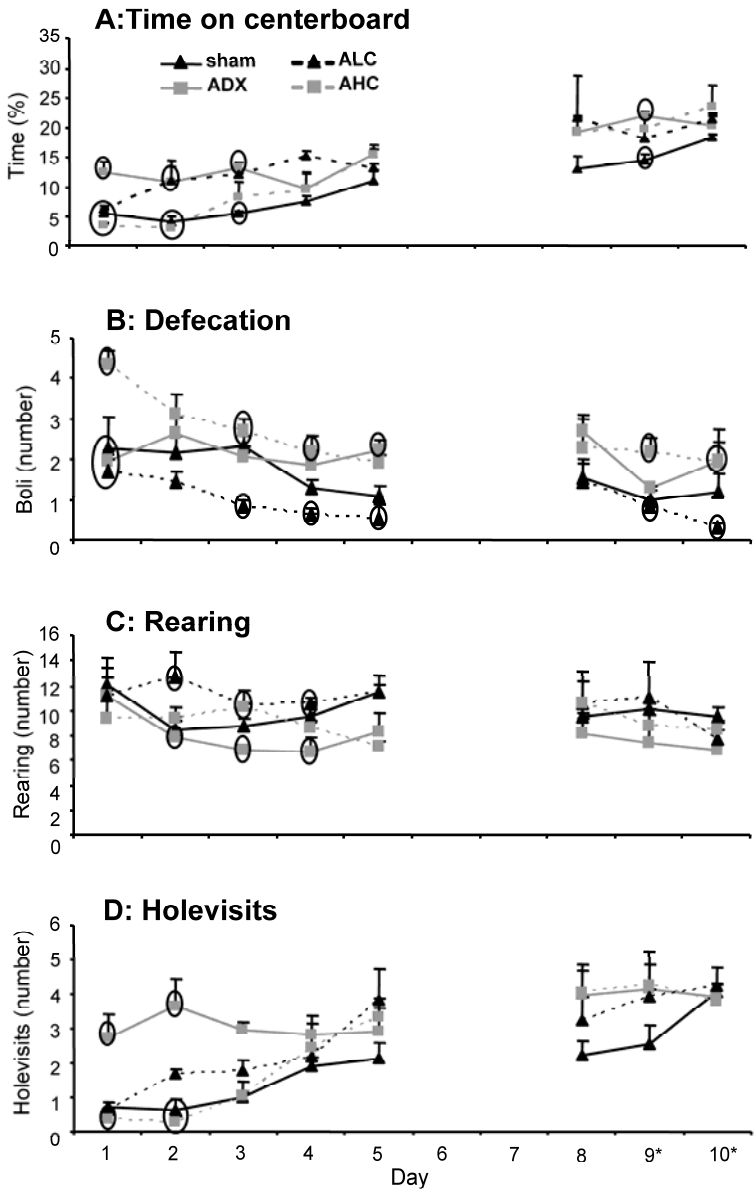

Figure 1. Behaviour of mice in the modified holeboard. (A) Percentage of time spent on centerboard (B) Number of defecation. (C) Number of rearings. (D) Number of holevisits, including revisits of Sham (black line), ADX (grey line), ALC (striped black line) and AHC mice (striped grey line). * at days 9 and 10 on the X-axis indicate removal of rings from all cylinders, while the bait remained in the same cylinders as before. Data present the mean of the three trials per day $\pm$ SEM. Ovals mark data points with significant differences $p < 0.05$ between groups within days.

Cognition

Figure 2 shows the results for three cognitive parameters on all days of testing in the modified holeboard. Figure 2A illustrates increased repeated holevisits (working memory) in ADX mice on day 8 of testing compared to Sham mice. We consider the low repeated holevisits on days 1 and 2 of Sham, ALC and AHC mice as not reliable, because the total number of holevisits is also very low on these days. Over time, Sham, ALC and AHC mice show increased repeats in parallel with increased total holevisits. GLM showed a significant group/day interaction ($F(21, 532)$ 2.029, $p=0.005$).

Figure 2B shows no significant differences in non-baited holevisits (reference memory) between Sham, ADX, ALC and AHC mice during all days of testing.

The time to finish the task is an additional learning parameter (Fig 2C). ADX and ALC mice were fast learners compared to Sham and AHC mice. Removal of the rings on days 9 and 10 did not influence the time to finish the task, indicating the use of a spatial learning strategy at that time of training. At the last day of testing, performance of Sham mice was still poor although progression over days proved to be significant ($F(21,532)$ 18.327, $p=0.000$).

Factor Analysis

Principal component analysis (PCA) over all behavioural data resulted in the extraction of four factors (Table 2) which explain 81% of total variance.

Factor 1 (41%) combines behavioural parameters that can be classified as anxiety, motivation and good learning, Factor 2 (19%) represents directed exploration, behavioural reactivity and working memory, Factor 3 (11%) represents general activity and Factor 4 (10%) includes behavioural parameters that can be classified as impaired learning.

One-way ANOVA between groups on factor loadings for Factor 1 (anxiety, motivation, good learning) revealed significant differences between Sham mice compared to ADX, ALC and AHC mice ($F(3,279)$ 11.562, $p=0.000$). Significant group differences were also found between ADX mice compared to Sham, ALC and AHC mice for Factor 3 (general activity; $F(3,279)$ 8.362, $p=0.000$).

Furthermore, when comparing the factor loadings over days, significant differences were found for Factor 1 between days 3 and 4 compared to days 9 and 10, ($F(7,279)$ 4.460, $p= 0.000$). This indicates low anxiety, more motivation and better learning at the end of testing in all groups. Factor 3 was significantly different between day 2 and days 1, 8 and 9 ($F(7,279)$ 2.522, $p= 0.016$), which indicates that general activity was decreased at the end of testing.

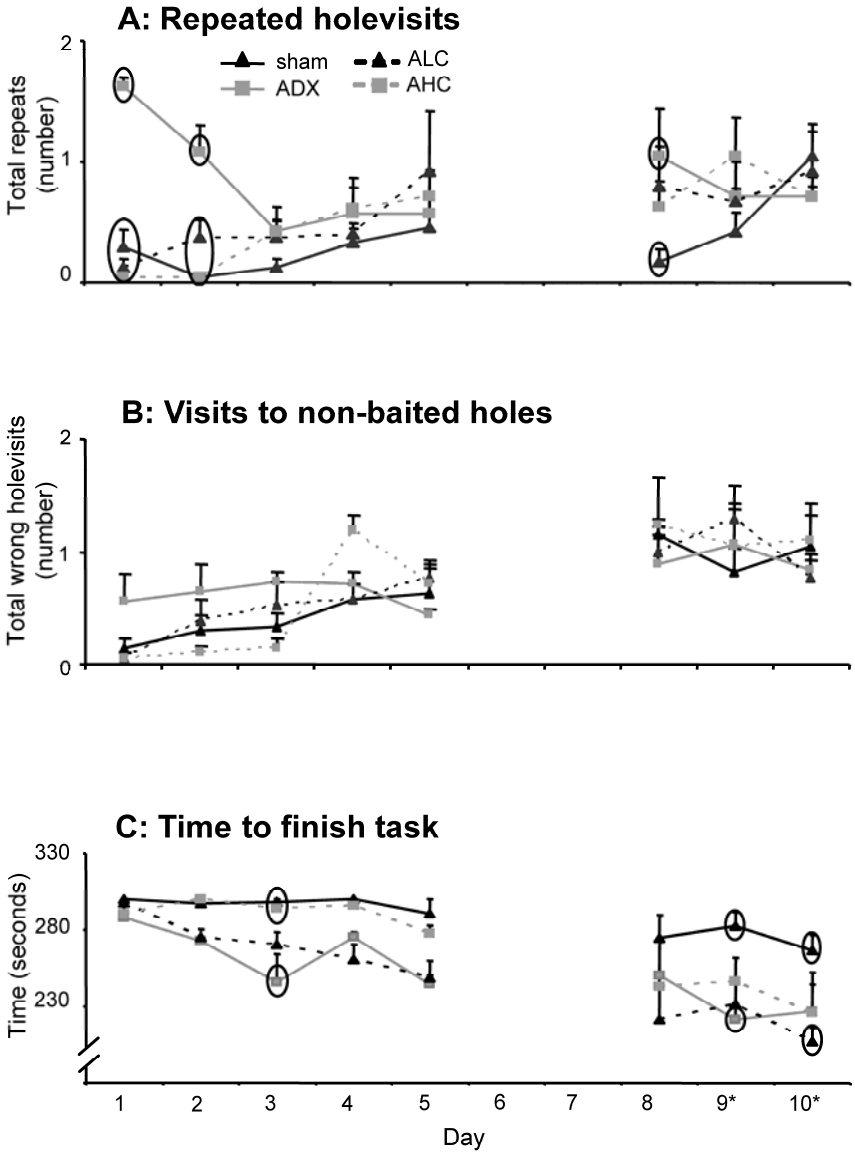

Figure 2. (A) Working memory expressed as number of holes revisited. (B) Reference memory expressed as visits to non-baited holes. (C) Time to finish the task, i.e., to obtain all three baits or 5 min, of Sham (black line), ADX (grey line), ALC (striped black line) and AHC mice (striped grey line). * at days 9 and 10 on the X-axis indicate removal of rings from all cylinders, while the bait remained in the same cylinders as before. Data present the mean of the three trials per day $\pm$ SEM. Ovals mark data points with significant differences $p < 0.05$ between groups within days.

	FACTOR			
	1 anxiety, motivation, good learning	**2 directed exploration/behavioural reactivity, working memory**	**3 general activity**	**4 impaired learning**
Latency to eat bait	-.887			
No. of baits obtained	.862			
Latency to first holevisit	-.792			
No. baited holes visited	.781			
Time on centerboard	.678			
No. repeated holevisits		.927		
No. holevisits		.807		
Time sitting			.840	
No. rearing			-.810	
No. non-baited holes visited				.911
Ratio of % right holevisits/ % wrong holevisits				-.723

Table 2. Principal component analysis over all data, with Varimax rotation and Kaiser normalisation. Behavioural parameters are represented as factor loading per factor. Factor loadings with equal value are positively correlated, while loadings with opposing values are negatively correlated. Loadings < 0.6 are not included in this table. Eleven of the seventeen measured parameters (Table 1) have communalities > 0.7 and are included in the factor analysis. Abbreviation, No. indicates number of.

Corticosterone and Thymus weight

Plasma corticosterone and thymus weights are presented in Table 3. Both low and high corticosterone pellet groups, ALC and AHC, had higher plasma corticosterone concentrations on day 0 ($F(3,31)$ 29.540, $p=0.0001$) than the SHAM and ADX mice. On day 11 of the experiment, only AHC mice showed

significantly increased corticosterone levels (F(3,31) 28.977, p= 0.0001), compared to Sham, ADX and ALC mice. Plasma corticosterone in sham and ADX mice remained at the same low basal morning level throughout the experiment, while corticosterone concentrations of ALC and AHC mice decreased in the course of the study (F(1,15) 7.835, p= 0.014 and F(1,15) 13,344, p= 0.003).
Thymus weights on day 11 supported the exposure to elevated corticosterone during the experiment with significantly lower thymus weights for ALC and AHC mice compared to Sham and ADX mice (F(3,31) 22.332, p=0.000). In fact, ADX mice had an enlarged thymus. ALC mice had a less shrunken thymus than AHC mice, indicating exposure to lower corticosterone concentrations than AHC. Bodyweight on day 11 was comparable between groups F(24, 27) 1.731, p=0.187.

	Plasma Corticosterone (ng/ml)		**Thymus weight (mg)**	**Bodyweight (g)**
Group	**Day 0**	**Day 11**	**Day 11**	**Day 11**
Sham	13.78 ± 2.37	17.96 ± 4.10	49.3 ± 0.9	25.1 ± 0.8
ADX	12.39 ± 1.50	15.24 ± 8.81	64.2 ± 2.5 *	27.4 ± 0.7
ALC	88.67 ± 19.26 *	33.18 ± 4.87	38.9 ± 0.5 *	24.7 ± 0.7
AHC	168.00 ± 19.23 *	88.63 + 10.58 *	21.2 ± 1.2 *	25.3 ± 1.2

Table 3. Plasma corticosterone, thymus and bodyweight. Corticosterone was measured before the first day of testing (day 0) and 24 hrs after the last testing day (day 11). Data are presented as mean ± SEM. * p< 0.05 compared to all other groups.

DISCUSSION

Four groups of mice were generated by endocrine manipulation, resulting in different amounts of circulating corticosterone concentrations in the blood. Given the different affinity of the receptors for the hormone, we expect a differential MR/GR activation in these groups: (i) Sham mice with an intact HPA axis, (ii) ADX mice with residual stable low corticosterone levels and thus continuous MR activation, (iii) ALC mice with moderate elevated circulating corticosterone concentrations allowing extensive MR and moderate GR activation and (iv) AHC mice with a full MR and a substantial GR activation due to high circulating levels of corticosterone. We found emotional expressions and cognitive performance related to differential corticosteroid receptor activation. Continuous predominant MR activation directed emotional components indicative for less anxiety to the benefit of cognition, while continuous additional GR activation was associated with impaired learning.

Continuous predominant MR activation results in emotions that can be beneficial for learning

Mice with stable predominant MR activation (ADX) show increased directed exploration/ behavioural reactivity towards the cylinders (holevisits) and low anxiety during the first days of testing, i.e., when the setting is novel. This corresponds to the observation that transgenic mice with low GR, and rats with ICV injection of GR antagonist express low anxiety related behaviour [35;36]. However, it contrasts previous findings that GR blockade by single infusion of RU38486 into the hippocampus has no anxiolytic effect in rats in the light/dark box [37]. Of course, the methods to achieve predominant MR activation differ in the history of inactivated GR, species, stressed state of the animals and behavioural task. Also a differentiation between context related behavioural reactivity and anxiety is not possible. However, the design of the present study allows to make this distinction. Factor analysis reveals that the variables time on centerboard (anxiety, motivation, good learning; Factor 1) and holevisits (directed exploration and behavioural reactivity; Factor 2) and are not correlated. Thus, the general idea that mice which are more prone to go to the unprotected center area are likely to display more cylinder directed behaviour is not supported. In contrast, anxiety is correlated with motivation (latency to first holevisit, latency eat bait): mice with a low anxiety approach the unprotected area faster.

Overall, low anxiety and high directed exploration/behavioural reactivity could be beneficial for the onset of learning, especially during the first days of testing. We observed an apparent fast onset of learning in these mice with predominant MR activation. High directed exploration towards the cylinders will eventually result in finding all baits, without any necessary learning of the task. Indeed, mice of this group show an increase in working memory errors (revisits) after the two days break without testing. GR is expected to promote the consolidation of MR-related adaptive behaviour, leaving the lack of GR activation as the most likely explanation for the memory deficit. The results of the Berger study [38] can be interpreted the other way round: the lack of forebrain MR resulted in working memory deficits in the water maze task because a functional GR facilitated the consolidation of non-adaptive behaviour. We conclude that the observed behaviour of animals with differential MR and GR conditions will only be understood in relation to the contribution of both receptors.

For optimal cognitive performance, not only MR but also moderate GR activation is necessary

ALC mice with MR and moderate GR activation display low anxiety during the first days of testing, general low arousal and fast learning. Corticosterone levels

in the ALC mice were continuously elevated in the range of the circadian rise, thus, would not be expected to cause damage to neurons, downregulation of MR and GR or alterations in neurotransmitters implied in cognitive impairments [39]. In fact, ALC mice with MR and moderate GR activation, showed the best cognitive performance.

Part of this improved learning and memory ability could be explained by the emotional state of the mice. Like ADX mice, ALC mice have low anxiety (and arousal) during the first days of learning which is correlated with increased motivation and good learning. Supporting our argument is the most recent finding of Herrero, that rats with low anxiety showed faster spatial learning together with increased hippocampal MR; opposite results were found in high-anxiety rats [40]. Stronger MR availability and activation might underlie the fast onset of learning, while GR are responsible for the consolidation of this context-related information [41-44]. Therefore, it is not surprising that ALC mice with a moderately activated GR display improved or normal cognitive performance compared to ADX mice with little or no GR activation throughout testing. For optimal coordination of cognition and emotion both MR and a moderate activation of GR are necessary [45;46].

Substantial continuous GR activation in addition to MR activation is associated with high emotional arousal and impaired learning

As described by many others, chronic strong GR activation caused by e.g., severe stressors or pharmacological modulation of the HPA axis, results in impaired learning and memory [47-49], reduced synaptic plasticity in the hippocampus [50], increased anxiety [51] and even depression-like symptomatology [44]. In patients suffering from depression or Cushing's disease, elevated levels of cortisol have been associated with poorer cognitive performance in verbal memory, working memory and post encoding tasks [52-54]. Furthermore, an association between cortisol level and increased fear perception has been found in patients suffering from recurring depression [55], which also indicates a modulatory role of glucocorticoids in emotional processes.

We find similar results for emotions and cognition: AHC mice with MR and continuous high GR activation have a slow onset of learning together with increased arousal and anxiety-like behaviours and suppression of directed exploration. It is not surprising that these mice display a slower onset of learning (opposite to low anxiety and fast learning as described above). At first glance, it seems surprising that when learning starts to occur, the magnitude of learning (Figure 2 C: time to finish task, slope of the learning curve) is the same in ALC and AHC mice. The change in corticosterone availability, due to the encapsulation of the pellet, is most likely responsible for the altered behaviour.

Corticosterone levels decreased over the days to concentrations in the "normal" range, i.e. comparable to circadian peak secretion and the amount of corticosterone measured in ALC mice at the beginning of testing. Thus, in AHC mice we deal with memory impairments and high emotional arousal only during specific stages of learning, namely during the first days of testing, that coincide with really high exposure to corticosterone.

The highly anxious Sham-operated control group

We used sham-operated mice that have an intact HPA axis as control group. Unexpectedly, these mice were characterized as highly anxious and little motivation, with high arousal and a slow onset and little progress of learning. Factor 1 was significantly different over time between Sham and all other groups tested: low motivation and high anxiety throughout testing days. We got the impression that the behavioural setting remained anxiogenic to these mice. Lack of exploration of the centreboard might also prevent to learn basic rules, e.g., that cylinders are baited with almonds. This and the possible role of a prolonged effect of surgery on the HPA system resulted in a follow-up experiment. We used three groups of mice (n=6 per group): (1) sham-operated mice and (2) naïve, non-operated mice received almonds in the homecage to familiarize with the bait, like the experimental groups. (3) naïve mice received almonds in the cylinders four times in the week before the modified holebard task. Sham and naïve mice without pre-exposure to the cylinders displayed similar high anxiety and slow learning as we saw before. However, after pretraining with baited cylinders anxiety decreased, motivation increased and learning improved (Figure 3).

Since surgery did not influence behaviour on the modified holeboard, incomplete recovery from the surgery is unlikely to affect performance. Using a somewhat different experimental design, comparably long times to finish the task have been reported for C57BL/6 mice (Ohl 2003; still 280 to 300 sec after eight days of training. In contrast, prior familiarization to items of the test condition reduced anxiety-like behaviour and increased motivation, which could (in part) increase cognitive performance like it was observed in ADX and ALC mice.

It is remarkable that mice without adrenals, dysregulated HPA-axis activity and additional pellet implantation "did better" compared to the relative intact Sham and naïve control groups. These findings even more underscore that (i) high anxiety and arousal has negative consequences for cognition while (ii) less anxiety, increased motivation and goal directed exploration have a positive influence on behaviour (see also [40]). We consider the role of MR in the integration of sensory information and behavioural strategies central for reduced anxiety-related behaviour.

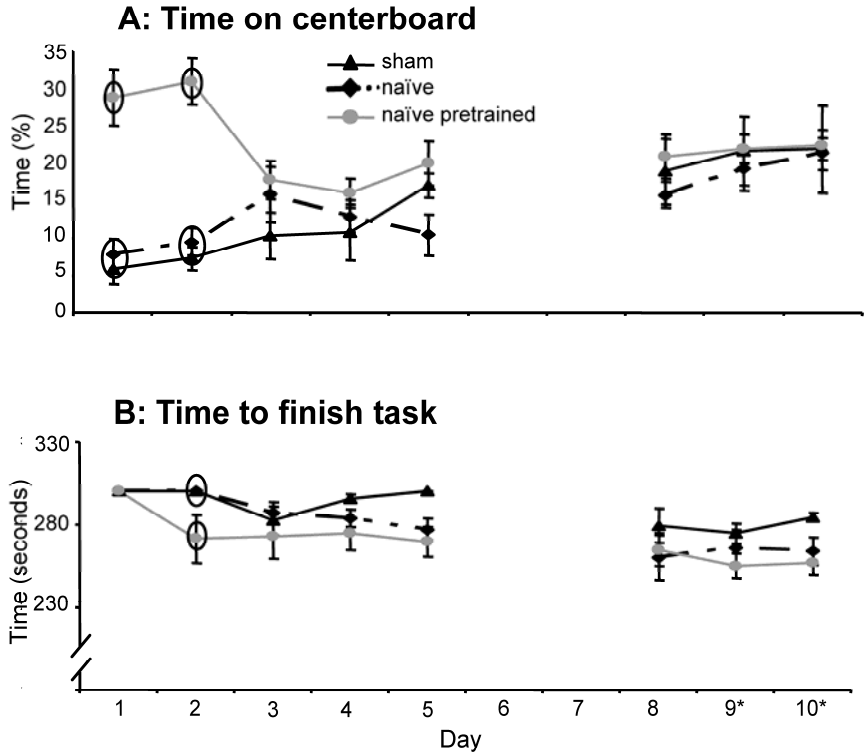

Figure 3. Examples of behaviour of the mice during the follow-up experiment. (A) Percentage of time spent on centerboard. (B) Time to finish the task (5 min or finding all three baits) of Sham (black line), naïve (striped black line) and naïve mice pre-exposed to a bait-containing cylinder in the home-cage (grey line). * at days 9 and 10 on the X-axis indicate removal of rings from all cylinders, while the bait remained in the same cylinders. Data present the mean of the three trials per day $\pm$ SEM. Ovals mark data points with significant differences: $p < 0.05$ between groups within days.

Adrenalectomy - other hormones and anxiety

The adrenalectomy-induced deficit in corticosterone secretion results in the disinhibition of HPA activity and thus enhanced release of corticotrophin-releasing hormone -CRH and vasopressin –AVP from the hypothalamus. Also the adrenal medulla as source of adrenaline is eliminated. CRH, AVP and adrenaline, all might play a role in emotional expressions and cognitive performance [56] of ADX mice, with and without supplementary corticosterone.

Considering the function of the GR in the negative feedback, we may expect that ADX mice (predominant MR activation) and ALC mice (MR and moderate GR) have a deficient suppression of CRH and AVP activity [57;58]. Mice with

elevated levels of CRH that acts predominantly via CRH receptor 1 are expected to display increased anxiety. Mutant mice with a deficient CRH receptor 1 either by genetic deletion or pharmacological blockade are less anxious [59]. Clearly, CRH is involved in anxiety-related behaviour. However in the present study, ADX and ALC mice show low anxiety-related behaviour, while AHC mice (predominant GR activation) are highly anxious. These findings do not support a role of hypothalamus-related CRH activity in anxiety behaviour in the present study. The same argument holds true for AVP.
In response to stress, noradrenalin release increases. This is thought to contribute to the anxiogenic effects of stress [60;61], in which the amygdala plays an important role [62]. AHC and Sham mice showed the strongest arousal (defecation) and were characterized as most anxious: a participation of catecholamines in these responses cannot be excluded. Furthermore, changes in metabolism and food intake have to be considered. Although food was present *ad libitum* throughout the experiment and bodyweight did not differ between the groups, motivation to go for the almond-bait might have been increased in ADX and ALC mice. Factor analysis also underlines the role of motivation in relation to anxiety for the performance.

Less directed exploration: is this anxiety?
Anxiety-related behaviour in rodents is generally deduced from the avoidance of an open, bright and unprotected area. However, tasks characteristics largely influence behaviour. For example, rats that are specifically selected for their avoidance of open arms of the elevated plus maze and thus, classified as high anxiety rats, do not avoid the center (open) area of a holeboard task [63]. Complexity and duration of the task, as well as motivational aspects might overcome state anxiety. Directed exploration or behavioural reactivity is expressed by approach to certain stimuli, e.g. the number of visits to a specific location in the testing area. These opposing behaviours are both related to locomotor activity. Does directed exploration rely on reduced anxiety? In the present study, animals with low directed exploration would spend little time near the cylinders on the centreboard. The interpretation of this behaviour could be: high anxiety. Although it is likely that anxiety interacts with directed exploration, this does not necessarily has to be the case. It could be that our interpretation of high anxiety is characteristic for a more passive exploration strategy [64;65] without a dominant role for anxiety-related behaviour. The setting of our task and subsequent factorial analysis allowed us to differentiate anxiety-like behaviour from directed exploration: they did not coincide into one factor, indicating no correlation between the two.

Conclusion

Anxiety and motivation are important factors for the onset of learning, a process in which MR and GR and their coordinated activation plays a crucial role. Continuous predominant MR activation appears to be beneficial for the emotional state, resulting in low anxiety, high motivation and high directed exploration and behavioural reactivity, but does not result in better learning and memory. Additional moderate GR activation also results in low anxiety and high motivation, with the advantage of improved cognition expressed as a decrease in working memory errors. In contrast, MR with additional substantial GR activation results in a slow onset of learning together with high anxiety, showing similarities with patients suffering from depression and Cushing's disease. We conclude that optimal performance is bound to continuous MR activation together with moderate GR activation. Further increase in corticosterone, and therefore substantial GR activation, will increase emotional arousal with impairing effects for learning and memory.

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Chapter 3

Emotion and cognition in high and low stress susceptible mouse strains: a combined neuroendocrine and behavioural study

Brinks V, van der Mark MH, de Kloet ER, Oitzl MS
Gorlaeus Lab, LACDR/LUMC, Division of Medical Pharmacology, Einsteinweg 55, 2300 RA Leiden, Leiden University, the Netherlands

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ABSTRACT

Emotionally arousing experiences and stress influence cognitive processes and vice versa. Understanding the relations and interactions between these three systems forms the core of this study. We tested two inbred mouse strains (BALB/c, C57BL/6J; male; 3-month-old) for glucocorticoid stress system markers (expression of MR and GR mRNA and protein in hippocampus, amygdala and prefrontal cortex; blood plasma corticosterone), used behavioural tasks for emotions and cognitive performance (elevated plus maze, holeboard) to assess the interdependence of these factors. We hypothesise that BALB/c mice have a stress-susceptible neuroendocrine phenotype and that emotional expressions in BALB/c and C57BL/6J mice will differentially contribute to learning and memory. We applied factor analyses on emotional and cognitive parameters to determine the behavioural structure of BALB/c and C57BL/6J mice. Glucocorticoid stress system markers indeed show that BALB/c mice are more stress-susceptible than C57BL/6J mice. Moreover, emotional and explorative factors differed between naïve BALB/c and C57BL/6J mice. BALB/c mice display high movement in anxiogenic zones and high risk assessment, while C57BL/6J mice show little movement in anxiogenic zones and display high vertical exploration. Furthermore, BALB/c mice are superior learners, showing learning related behaviour which is highly structured and emotionally biased when exposed to a novel or changing situation. In contrast, C57BL/6J mice display a rather "chaotic" behavioural structure during learning in absence of an emotional factor. These results show that stress susceptibility coincides with more emotionality, which drives well orchestrated goal directed behaviour to the benefit of cognition. Both phenotypes have their advantage depending on environmental demands.

INTRODUCTION

Emotion and cognition are two well studied aspects of human and rodent behaviour. While increasing data suggests an interaction between the two [1], a third interacting factor, the glucocorticoid stress system, also becomes more apparent. Emotions profoundly influence ongoing and long term cognitive processes [2-9]. In addition, cognition can also disrupt the response to emotional stimuli [3]. Interestingly, emotion and cognition might also interact in the development of stress related diseases; Hayden and colleagues have shown that cognitive susceptibility to depression can originate from early-emerging differences in the expression of positive emotions [10]. Only few studies have aimed at testing the interaction between emotion, cognition and the glucocorticoid stress system in mice. Recently, we have reported that increasing chronic plasma corticosterone concentrations, and therefore differential mineralo- and glucocorticoid receptor (MR, GR) activation, augments emotional arousal and impairs cognitive performance of C57BL/6J mice [11]. Based on our seminal observations of glucocorticoid actions [12-14] we have developed the concept that both receptor types contribute in complementary fashion to the regulation of ongoing and stress-related behavioural responses: MR in limbic brain facilitates perception and attention and can bias information processing to allow acquisition of a behaviourally adaptive response pattern [15;16]. In contrast, GR promotes memory consolidation and facilitates extinction of responses that are of no more relevance [12;16;17]. We decided to assess the neuroendocrine and behavioural phenotype of two inbred mouse strains, BALB/c and C57BL6J, that are expected to have a differential regulation of the stress system [18-20] in face of emotional expression [21] and cognitive performance [22;23]. This research thus focuses on the interaction between the stress system, emotion and cognition.

We expect that BALB/c and C57BL/6J mice have distinct central and peripheral markers for stress system activity under resting and activated conditions. Therefore, we will first measure MR and GR mRNA expression and protein in limbic brain areas: hippocampus, prefrontal cortex and amygdala and set the time course of corticosterone secretion in response to novelty. In a second series of experiments, we will determine the behavioural phenotype of the mice. Since initial behavioural reactivity towards a novel environment will influence later cognitive processing [24], we will observe naïve BALB/c and C57BL/6J mice in the elevated plus maze and modified holeboard to collect a large amount of behavioural variables related to general activity, exploration and anxiety. To assess if previous stress differentially affects ongoing behaviour, separate groups of mice will be exposed to the elevated plus maze after 60 min sensory

exposure to a rat [25;26]. Next, we will use the modified holeboard for simultaneous emotional and cognitive testing during different stages of task acquisition, retrieval and reversal learning. Moreover, factor analyses on emotional and cognitive parameters will be performed to obtain a more comprehensive insight in the strain-dependent behavioural structure during the learning process. We expect that BALB/c mice will display glucocorticoid stress system markers indicative for a stress susceptible phenotype; high stress induced corticosterone concentrations and an altered MR/GR balance compared to C57BL/6J mice. In addition, we expect that emotional expressions will differentially contribute to learning and memory in BALB/c and C57BL/6J mice.

MATERIAL AND METHODS

Animals

BALB/c and C57BL/6J male mice (a total of n=79 per strain; 12 weeks old) were obtained from Elevage Janvier (Le Genest Saint Isle, France). After arrival, the mice were housed individually in the experimental room with sawdust bedding, water and food *ad libitum*, at 20^{0}C with controlled humidity under a 12 h: 12 h light/dark cycle (lights on at 08.00 hrs.) for one week. Male Long-Evans rats (male n=8) from our own breeding stock were used to activate the stress system of mice. Experiments were performed between 09.00 and 13.30 hours and were approved by the committee on Animal Health and Care from the Leiden University, The Netherlands, in compliance with the EC Council Directive of November 1986 (86/609/EEC) for the care and use of laboratory animals.

Experiment 1: The neuroendocrine phenotype: markers of stress system activity

In situ hybridisation of MR and GR mRNA expression

Eight mice per strain were decapitated between 09.00 and 10.00 hrs, brains were isolated, frozen in isopentane on dry-ice and kept at -80^{0}C until sectioning. For MR and GR mRNA measurements, frozen brains were sectioned at 12 µm using a -20^{0}C cryostat microtome coronal sections on the level of the prefrontal cortex, hippocampus and amygdala (Fig. 1). Sections were thaw-mounted on poly-*L*-lysine-coated slides (0.001%), and kept at -80°C until further use. In situ hybridizations using ^{35}S-labeled ribonucleotide probes (MR, GR,) were performed as described before [27]. In short, sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1 *M* triethanolamine/HCl. followed by dehydration in increasing concentrations of ethanol. The antisense RNA probes were transcribed from linearized plasmids containing exon 2 of mouse MR and GR. On the slides, 100 µl hybridization

buffer was put containing 20 m*M* Tris-HCl (pH 7.4), 50% formamide, 300 m*M* NaCl, 1 m*M* EDTA, 1× Denhardt's, 250 µg/ml yeast transfer RNA, 250 µl/ml total RNA, 10 mg/ml herring sperm DNA, 5% dextran sulfate, 0.1% SDS, 0.1% sodium thiosulfate together with 1.5× 10^6 cpm ^{35}S-labeled riboprobe (MR or GR). A coverslip was placed over the brain sections followed by 55°C overnight incubation. The next day, sections were washed with 2× SSC, treated with RNaseA (20 mg/ml) and washed at room temperature in increasingly concentrations of SSC solutions. Finally, sections were washed in 0.1× SSC at 65°C for 30 min and dehydrated with increasing ethanol concentrations. Kodak Biomax MR films were placed on the slides (Eastman Kodak Co., Rochester, N.Y., USA) for 3 days to measure MR mRNA levels in the hippocampus and prefrontal cortex (PFC) and 6 days for MR mRNA levels in the amygdala. For hippocampal, prefrontal cortex and amygdala GR mRNA measurements, the films were placed on the slides for 6 days.

The autoradiographs (films) were scanned and optical density (OD) of the areas of interest was determined using image analysis computer software (analySIS 3.1, Soft Imaging System GmbH). All optical density measurements for relative mRNA expression were taken bilaterally on two brain slices per mouse, and corrected for aspecific binding by subtracting background and sense signal. For relative hippocampal MR and GR mRNA measurements, greyvalues of the Cornu Ammonis areas (CA1, CA2, CA3) and dentate gyrus of the hippocampus were measured. For prefrontal cortex measurements, the infra- and prelimbic area was chosen because of connections to other limbic areas, and for amygdala measurements, optical densities for the basolateral amygdala were measured.

Western blotting of GR protein

Sixteen mice per strain were decapitated between 09.00 and 10.00 hrs. Eight mouse brains per strain were used for dissection of the complete hippocampus. The prefrontal cortex and amygdala were dissected from the other eight brains. Brain tissue was lysated using 500 µl 1x RIPA lysisbuffer, homogenized (potter apparatus) and centrifuged (20', 4^0C at 15000 rpm). Protein concentration was measured in the supernatant using a Pierce PCA assay. Next, 15 µl samples (containing a total of 30 µg protein, filled up with sample buffer and denaturized at 95^0C for 5 min) were subjected to SDS-PAGE. Blots were blocked in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20 containing 5% nonfat dried milk powder and incubated with the H-300 primary antibody (1:1000, Santa-Cruz Biotechnology, Santa Cruz, CA) followed by incubation of the Goat anti rabbit IgG (1:5000, Sigma, St. Louis, MO) or the monoclonal anti-α-tubulin antibody (Sigma, 1:2500). As negative and positive control respectively, sample buffer and GR transfected cos-1 cells were taken along. After washing, blots were incubated with peroxidase-conjugated antibodies (1:10,000; Jackson

ImmunoResearch Laboratories, West Grove, PA). Blots were washed again and immunoreactive bands were visualized by enhanced chemiluminescence. Finally, the blots were exposed to films for 30 seconds. The autoradiographs (films) were scanned and optical density (OD) of the GR and α-tubulin bands from the hippocampus, prefrontal cortex and amygdala areas were determined using windows Image J software. GR protein measurements were corrected for total protein (α-tubulin).

Corticosterone response to novelty stress

BALB/c and C57BL/6J mice (n=35 per strain) were individually placed in a novel cage containing sawdust bedding. At 5, 30, 60, 120 and 240 minutes mice (n=7 per strain) were decapitated and trunk blood was collected. To estimate basal resting corticosterone, blood was obtained by tail incision one day before the experiment (n=7 per strain, randomly chosen). Corticosterone concentrations were determined from 10 µl isolated plasma using a commercially available radio immune assay kit with a detection limit of 3 ng/ml (MP Biomedicals Inc., Calif., USA).

Experiment 2: The behavioural phenotype: unconditioned behaviour in the modified holeboard and elevated plus maze

Apparatus

The modified holeboard consisted of a grey PVC box (50x50x50cm) with a grey PVC centerboard (37x20cm) on which ten dark grey cylinders (4 cm height) were staggered in two lines of five [7;11]. The bottom of the cylinder is covered by a grid. During testing, the modified holeboard was situated on the floor and a camera placed above the setting allowed later pathway reconstruction from video. Light intensity of the experimental room was set at 80 Lux and a 20 dB background noise originating from a radio was present.

The elevated plus maze included a center area (6 x 6 cm), two open (28 x 6 cm) and two closed arms (28 x 6 cm) with transparent PVC (15 cm high) walls. The floor consisted of grey PVC and the entire setup was elevated on 100 cm high metal bars. Also here, a camera was placed above the setup for later pathway reconstruction from video, light intensity was set at 80 Lux and a 20 dB background noise was present.

Setups were cleaned with tap water and dried before each mouse.

Rat stress

Since rats and mice avoid each other in nature, rat exposure is a powerful stressor for a mouse and will produce a profound activation of the glucocorticoid stress system [26].

Rat stress was performed as described before [25]. Mice were transported to a room which housed the rats and placed individually in a novel cage with sawdust. One rat was placed in a cage with a grid floor and transparent PVC walls on top of two mouse cages. Physical contact was not possible, while mice could see, hear and smell the rat. In this room, no background noise was present, light intensity was set at 80 Lux. The mice were subjected to 1 hr of rat stress immediately followed by behavioural testing in the elevated plus maze in an adjacent room.

General experimental procedure

Behaviour of naïve BALB/c and C57BL/6J mice (n=12 per strain) was studied during a 5 minute exposure to the modified holeboard and elevated plus maze. The interval between the two tasks was 7 days and tasks were counterbalanced. Rat stress induced behaviour on the elevated plus maze was measured in a separate group of mice (n=8 per strain). Behavioural testing took place in the mouse housing room to prevent transport induced activation of the stress system.

All mice were placed (i) in the modified holeboard in the same corner facing the wall and (ii) in the elevated plus maze in the center area facing the closed arm.

In depth behavioural observation during modified holeboard testing was performed using a semi automatic scoring system (Observer, Noldus, Wageningen, The Netherlands). For the modified holeboard, we scored the total number of defecations, sitting, rearing, stretched attends, grooming, centerboard entries and cylinder visits, as well as the time on the centerboard, sitting, grooming and the latency to the first centerboard entry.

The behaviours on the elevated plus maze included the total number of defecations, sitting, walking, stretched attends, grooming, rearings and rim dips. Also the time and entries in the open/closed arms, grooming, sitting and walking were measured.

During both modified holeboard and elevated plus maze exposure, the total distance moved was measured and pathways were reconstructed (Ethovision, Noldus, Wageningen, The Netherlands).

Experiment 3: The cognitive phenotype: simultaneous emotional expression and cognitive performance estimated in the modified holeboard task

Apparatus

The apparatus is described in experiment 1. In addition, visual markers were placed on the walls of the room to support distal visual-spatial orientation and rings were placed on the cylinders for proximal visual discrimination. On day 1, all ten cylinders on the centerboard were baited with a small piece of almond

under and on top of the grid. Placing the almonds under the grid should provide the same odour cue for all cylinders.
On all other days, only three cylinders were baited with a small piece of almond on top of the grid, and marked with a white ring as visual cue. The seven other cylinders contained a non-obtainable almond underneath the grid and were marked with a black ring. This set-up allows visual discrimination as well as spatial location of the baited and non-baited cylinders.

Emotional and cognitive measurements
The behaviour of BALB/c and C57BL/6J mice (n=8 per strain) was observed, video-taped and analyzed with a semi automatic scoring system (The Observer Mobile 4.1, Noldus Information Technology, Wageningen, The Netherlands). The behaviours observed were similar as described for the modified holeboard observations in experiment 2, with the addition of the total number of baited, non-baited and repeated cylinders visited, food rewarded visits and total number of baits eaten. The latency to the first cylinder visit, the latency to eat the first bait and the time to finish the task were also measured.
As indication for (i) reference memory, the number of baited cylinder visits was divided by total cylinder visits, and for (ii) working memory, the number of food rewarded cylinder visits was divided by the number of baited cylinders visited. Cognitive parameters such as time to finish the task and reference and working memory were calculated from day 2 onwards.

General experimental procedure
To familiarize the mice with the bait containing cylinders of the modified holeboard task, a cylinder containing a few pieces of almonds was placed in their homecage daily [11]. Also here, behavioural testing took place in the mouse housing room to prevent transport induced activation of the stress system.
Mice were tested in the modified holeboard over 10 days. On day 1 (all cylinders baited), the mice were allowed to explore the setting for 10 minutes. On all other days, mice were tested for 3 x 5 minutes or until eating all baits, with an intertrial interval of 20 to 30 minutes.
On days 2 to 5, three cylinders were baited and visually marked. On days 6 and 7, mice were not tested. On day 8, the same settings as on days 2-5 were used. On day 9, a reversal was introduced: the three baited cylinders, including the white ring, shifted one position. This allowed (i) to determine the effect of a "novel" situation on emotional and cognitive processes and (ii) to estimate if the mice used a spatial or visual discrimination strategy to solve the task. On day 10, the same settings as on day 9 were used.

Statistical analysis

For experiment 1, MR and GR mRNA expression and corticosterone concentrations are represented as mean ± SEM. Student's T-test was used to determine strain effects on MR and GR mRNA. Between strain, time and strain x time interaction for corticosterone concentrations were determined by General Linear Model (GLM) –general factorial measurement. Student's T-test was used to determine differences in basal and novelty induced corticosterone concentrations.

For experiment 2, the behavioural data are represented as mean ± SEM. Because the order of testing did not influence outcome, modified holeboard and elevated plus maze data of the two testing days was pooled. Between strain differences were determined with a GLM -multivariate measurement. Stress-induced and strain x stress interaction for elevated plus maze testing were also measured with GLM -multivariate analysis. When appropriate, Tukey's post-hoc test was used. Furthermore, a factor analysis (Principal Component Analysis: PCA) was performed over the behavioural data from both naïve modified holeboard and elevated plus maze testing, followed by an one-way ANOVA to determine strain differences in naïve behaviour.

For experiment 3, data are presented as mean of 3 trials per day ± SEM (except day 1; behaviour over 10 minutes with all cylinders baited). Data from days 2 to 10 were subjected to GLM -repeated measures to analyse progression (between strains) over days. Between strain differences on days 1 to 10 and within strain differences from days 8 to 9 (i.e., introducing the reversal) were tested by two-way-ANOVA. Also here, factor analysis was performed over the behavioural data per strain to obtain relevant behavioural parameters for either BALB/c or C57BL/6J mice. These behavioural parameters were used to perform additional factor analyses for each testing day. In this case, behavioural structure over days was obtained with strain specific parameters.

PCA uses cross-mouse comparisons to distinguish the relation between behavioural parameters. It includes as much data as possible in each factor to minimize residual variance from the original dataset. The PCA was performed with a Varimax rotation on variables with communalities over 0.7, that is, of which 70 % of the variance is explained by the Factors extracted. The number of extracted Factors was not pre-defined; Factors with an Eigenvalue over 1 were accepted. Factor scores were subjected to a two-way ANOVA to determine differences between groups and days. $P < 0.05$ was accepted as level of significance for all statistical testing.

RESULTS

Experiment 1: The neuroendocrine phenotype: markers of stress system activity

This experiment was performed to characterize central and peripheral markers of stress system activity of BALB/c and C57BL6J mouse strains

MR and GR mRNA expression

MR and GR mRNA expression differed significantly between BALB/c and C57Bl/6J mice ($F(7,5)$ 7.170, $p=0.023$; table 1, figure 1.). C57BL/6J mice expressed significantly higher MR mRNA in the hippocampus and prefrontal cortex, and higher GR mRNA in the hippocampus compared to BALB/c mice. In contrast, BALB/c mice expressed significantly more GR mRNA in the prefrontal cortex. Interestingly, BALB/c mice had strong MR mRNA expression in the indusium griseum, compared to C57BL/6J mice. The function of the indusium griseum is not known [28].

		BALB/c	C57BL/6J
MR mRNA	Hippocampus	20.84 ± 0.86	23.54 ± 0.75*
	Prefrontal cortex	1.20 ± 0.20	3.15 ± 0.47**
	Amygdala	32.81 ± 3.45	37.56 ± 2.52
	Indusium griseum	12.44 ± 1.85	2.17 ± 1.08 **
GR mRNA	Hippocampus	26.88 ± 1.95	33.49 ± 2.16*
	Prefrontal cortex	11.13 ± 1.48	2.71 ± 1.20**
	Indusium griseum	23.56 ± 1.94	23.71 ± 2.71

Table 1. MR and GR mRNA expression as grey value of optical densities (mean ± SEM) in hippocampus, prefrontal cortex, amygdala and induseum griseum of BALB/c and C57BL/6J mice. * $p < 0.05$, ** $p \leq 0.001$ between strains.

GR protein expression by Western blotting

GR protein expression was significantly different between BALB/c and C57BL/6J mice ($F(3,11)$ 3.114, $p=0.030$). C57BL/6J mice displayed higher GR protein in the hippocampus compared to BALB/c mice, while BALB/c mice showed higher GR protein expression in the amygdala (table 2). In addition, all the GR positive bands of C57BL/6J mice appeared at a slightly higher location on the blot compared to GR positive bands of BALB/c mice.

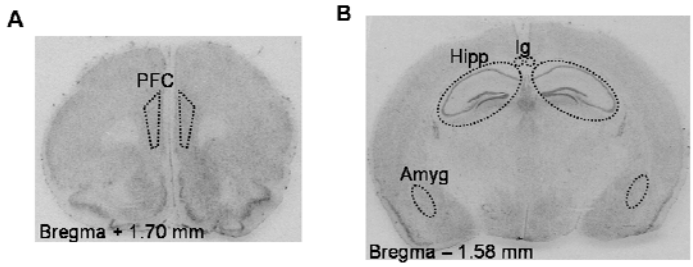

Figure 1. Coronal sections of the mouse brain, stained with cresylviolet. Dotted lines indicate the areas of interest for image analysis of optical density for MR and GR mRNA expression. A: the infra- and prelimbic areas of the prefrontal cortex (PFC), B: the hippocampus (Hipp), amygdala (Amyg) and indusium griseum (Ig).

	BALB/c	**C57BL/6J**
Hippocampus	0.36 ± 0.03	0.64 ± 0.10*
Prefrontal cortex	0.37 ± 0.05	0.32 ± 0.03
Amygdala	2.21± 0.56	0.84 ± 0.14*

Table 2. GR protein expression corrected for total protein (grey values mean ± SEM) in hippocampus, prefrontal cortex and amygdala of BALB/c and C57BL/6J mice. * $p < 0.05$ between strains.

Corticosterone response to novelty stress

Corticosterone responses to novelty were strain dependent (Fig. 2) with a significant main effect of strain ($F(1,79)$ 30.064, $p=0.000$), time ($F(5,79)$ 13.104, $p=0.000$) and interaction between strain and time ($F(5,79)$ 6.169, $p=0.000$).

At 5 and 30 minutes of novelty exposure, BALB/c mice displayed 2 and 3 fold higher plasma corticosterone concentrations compared to C57BL/6J mice ($p<0.05$). At 60 and 120 minutes of novelty exposure, corticosterone levels were still increased compared to basal, but not different between strains. At 240 minutes, C57BL/6J mice displayed lower corticosterone concentrations than BALB/c mice ($p<0.05$).

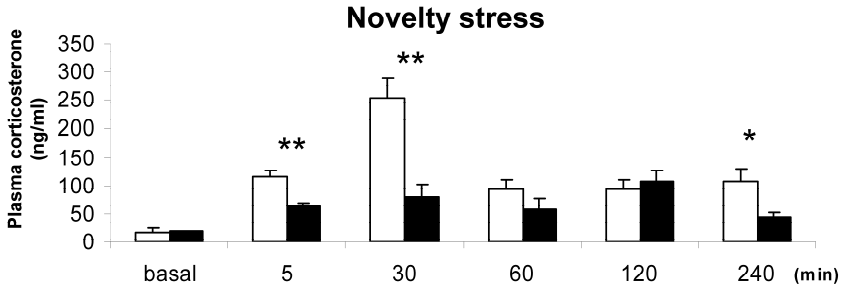

Figure 2. Corticosterone concentrations in ng/ml, basal resting and novelty induced responses at 5, 30, 60, 120 and 240 minutes of novel cage exposure. BALB/c: white bars; C57BL/6J: black bars * $p < 0.05$, ** $p \leq 0.001$.

Experiment 2: The behavioural phenotype: unconditioned behaviour in the modified holeboard and elevated plus maze

Modified holeboard

Multivariate analysis over all scored behaviours revealed a significant strain effect ($F(7,16)$ 2.949, $p= 0.035$). Table 3 lists the significantly different behaviours during modified holeboard exposure (at least $p <0.05$). BALB/c mice spent almost 2-fold more time in the unprotected area, i.e., on the centerboard, have a 7-fold shorter latency to enter the centerboard and have a high number of stretched attends compared to the C57BL/6J mice. In contrast, C57BL/6J mice displayed a high number of rearings. Although C57BL/6J mice walked longer distances than BALB/c, it is the BALB/c mice that moved around more on the "unprotected" area of the centerboard, while C57BL/6J mice showed more movement in proximity of the walls (thigmotaxis). Figure 3 shows representative walking patterns of the BALB/c and C57BL/6J mice.

Modified holeboard		
	BALB/c	**C57BL/6J**
Rearings (no)	24.4 ± 4.9	47.8 ± 3.6*
Stretched attends (no)	5.2 ± 1.3	0.6 ± 0.2*
Lat. first centerboard entry (s)	17.3 ± 3.7	112.1 ± 19.3**
Time on centerboard (s)	124.7 ± 16.6	73.2 ± 9.0*
Distance moved (m)	32.2 ± 2.7	39.4 ± 1.8*

Table 3. Behavioural parameters of naïve BALB/c and C57BL/6J mice in the modified holeboard. Only behaviours with a statistical significance of $p<0.05$ are listed. Abbreviations and symbols: no – number; s – seconds; * $p < 0.05$; ** $p \leq 0.001$ between strains.

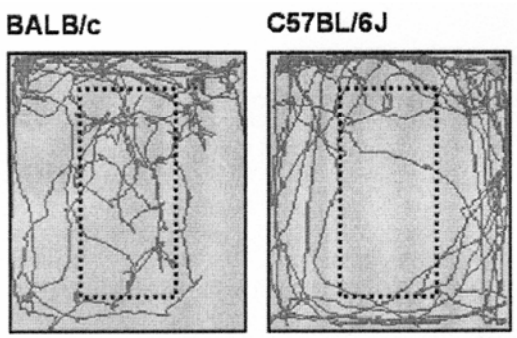

Figure 3. Representative walking patterns of BALB/c (left) and C57BL/6J mice (right) in the modified holeboard. The dotted square indicates the location of the centerboard.

Stress-induced behavioural changes in the elevated plus maze

Multivariate analysis over all behaviours revealed significant effects for strain, condition (naïve/stress) and interaction between strain and condition ($F(9,27)$ 2.764, $p= 0.020$, $F(9,27)$ 96.626, $p< 0.0001$, $F(9,27)$ 3.951, $p= 0.003$). Table 4 shows all significantly different behaviours during basal and stress induced elevated plus maze testing (at least $p<0.05$).

Strain differences in naïve mice: BALB/c mice spent significantly more time in the open arm and correspondingly less time in the closed arm compared to C57BL/6J mice. BALB/c mice also displayed a higher number of rim dips and stretched attends, while C57BL/6J mice displayed a high number of rearings, grooming and closed arm entries. The number of open arm entries and number of defecations did not differ between strains. Moreover, total arm entries and total distance moved were comparable between strains.

Effect of rat stress: During rat stress, BALB/c mice showed more defecations ($p< 0.001$) compared to C57BL/6J mice. Exposure to 1 hr of rat stress significantly altered the behavioural pattern of mice during subsequent testing. The number of stretched attends, rim dips, open arm entries and time in open arm increased in C57Bl/6J mice, while time in closed arms was less in this strain. Numbers of open arm entries in C57BL/6J mice were increased compared to BALB/c mice. Number of rearings, stretched attends, grooming and rim dips were increased in BALB/c mice. Also after rat stress, the distance moved was not significantly different between strains, although stress did increase the distance moved compared to naive in both BALB/c and C57BL/6J mice.

Walking patterns (figure 4) show that naive BALB/c mice displayed more movement in the open arms compared to naïve C57BL/6J mice. After rat stress, C57BL/6J mice increase open arm exploration.

	Elevated plus maze			
	Naive		Stress induced	
	BALB/c	**C57BL/6J**	**BALB/c**	**C57BL/6J**
Rearings (no)	3.2 ± 1.0	13.8 ± 2.4**	11.1 ± 1.8##	12.4 ± 2.8##
Stretched attends (no)	3.8 ± 0.7	1.4 ± 0.3*	24.3 ± 1.9##	28.0 ± 2.4
Grooming (no)	0.9 ± 0.2	2.5 ± 0.3**	4.4 ± 1.3#	3.1 ± 1.0
Rim dips (no)	10.9 ± 1.8	4.6 ± 1.0*	33.0 ± 6.3##	21.5 ± 3.0##
Open arm entries (no)	5.3 ± 0.8	3.6 ± 0.9	5.9 ± 0.6	9.4 ± 0.8* ##
Closed arm entries (no)	5.3 ± 0.7	9.5 ± 1.6*	7.0 ± 1.4	10.3 ± 0.6*
Open arm (s)	142.9 ± 24.8	41.3 ± 24.4*	157.3 ± 25.7	109.6 ± 14.2#
Closed arm (s)	157.1 ± 24.8	258.7 ± 24.4*	119.4 ± 23.1	172.4 ± 12.3#
Distance moved (m)	10.9 ± 0.9	10.0 ± 0.9	30.6 ± 1.1##	27.5 ± 1.3##

Table 4. Behavioural parameters of naïve and rat-stressed BALB/c and C57BL/6J mice in the elevated plus maze. Only behaviours with a statistical significance of $p<0.05$ are listed. Abbreviations and symbols: no – number; s – seconds; * $p < 0.05$; ** $p \leq 0.001$ between strains; # $p < 0.05$ and ## $p \leq 0.001$ within strain naïve vs. Stress.

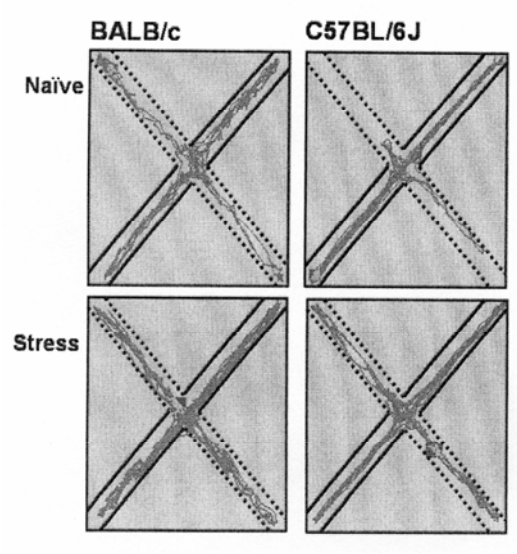

Figure 4. Representative walking patterns of BALB/c (left) and C57BL/6J mice (right) in the elevated plus maze. The dotted lines show the open arms, the straight lines the closed arms.

Principal Component Analysis (PCA)

A PCA performed over behaviour of naïve mice during modified holeboard and elevated plus maze testing resulted in the extraction of 4 factors explaining 83% of total variance. Two factors were significantly different between strains (Factor 1; F(1,22) 6.657, p=0.017, Factor 2; F(1,22) 6.809, p=0.016). These factors include variables of exploration and emotions (table 5).

	Behaviour	**Factor loading**
Factor 1. Exploration	Number of closed arm entries (EPM)	0.88
	Duration in open arm (EPM)	-0.85
	Time in closed arm (EPM)	0.85
	Rearings (EPM)	0.78
Factor 2. Emotion	Grooming (MHB)	0.84
	Stretched attends (EPM)	-0.83
	Grooming (EPM)	0.70

Table 5. Factors extracted from naïve modified holeboard (MHB) and elevated plus maze (EPM) data.

Experiment 3: The cognitive phenotype: simultaneous emotional expression and cognitive performance estimated in the modified holeboard task

Based on the previous data of stress-markers/ stress responses and emotional behaviour we expect differential contribution of emotion on cognitive performance in BALB/c and C57BL/6J mice.

Emotion and exploration

Multivariate analysis over all (e)motional and explorative behaviours and days revealed a significant strain difference (F(10,326) 32.018, p<0.0001). Multivariate analysis over data on day 1 also showed a significant strain difference (F(10,7)26.219, p<0.0001). Behaviours that showed most clear strain difference over all testing days were selected and are presented in figure 5.

BALB/c mice spent twofold more time on the centerboard compared to C57BL/6J mice when first exposed to the setting on day 1 (p=0.011, Fig. 5A). General linear model showed significant progression over days 2 to 10 (F(6,184)6.004, p< 0.0001) with a significant strain difference (F(6,184)6.462, p<0.0001). BALB/c mice spent more time on the centerboard on almost all days. After introducing the reversal on day 9, BALB/c mice increased the time spent on the centerboard with 10 % (p= 0.003), while the C57BL/6J mice did not.

BALB/c mice displayed 3 and 2-fold more cylinder visits on days 1 and 2 compared to C57BL/6J mice (day1:p< 0.0001; day2:p= 0.013). This difference was absent on days 3 to 10. General linear model also showed significantly

different progression in cylinder visits over days 2 to 10 ($F(6,184)6.208$, $p<0.0001$), with a significant strain difference ($F(6,184)$ 4.299, $p= 0.002$). BALB/c mice decreased the number of cylinder visits while visits of C57Bl/6J mice remained stable (Fig. 5B). Reversal did not influence the number of cylinders visited.

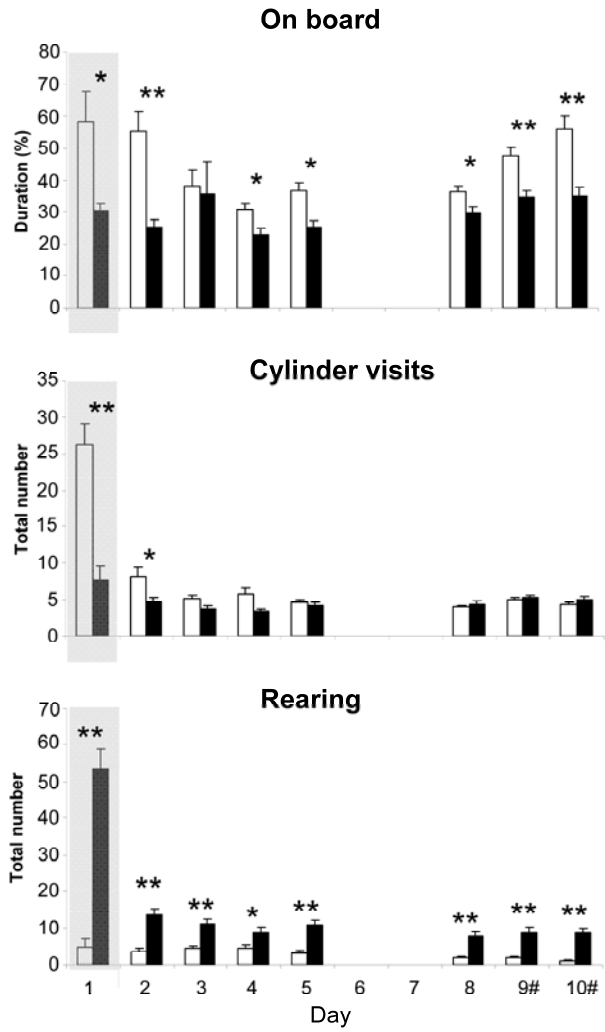

Figure 5. Emotional and explorative behavioural parameters measured on days 1 to 10 of holeboard testing of BALB/c (white bars) and C57BL/6J mice (black bars). (A) Percentage of time spent on the centerboard, (B) number of cylinder visits and (C) number of rearings. The grey background on day 1 indicates data during the 10 minutes exposure; data on days 2 to 10 present the mean values (± SEM) of 3 times 5 minutes exposure. # baited cylinders were relocated on days 9 and 10. * $p < 0.05$ and ** $p \leq 0.001$ between strains.

C57BL/6J mice displayed an 11-fold higher number of rearings during the 10 minute trial on day 1 ($p < 0.0001$) and 2 to 5 fold more rearing than BALB/c mice on days 2 to 10. General Linear model showed a significant progression over days 2 to 10 ($F(6,184)3.900$, $p=0.005$), although passing statistical significance between strains ($F(6,184)$ 2.294, $p= 0.061$). The reversal did not influence the number of rearings for both strains (Fig. 5C).

Cognition

Multivariate analysis over all cognitive behaviours revealed a significant strain difference ($F(9,320)$ 27.744, $p<0.0001$). Selected behaviours that showed most clear strain difference over all testing days are represented in figure 6.

BALB/c mice finished the task much faster compared to the C57BL/6J mice on days 2 to 10 (Fig. 6A). Both BALB/c and C57BL/6J mice showed a progressive decline in time to finish the task over days 2 to 10 ($F(6,184)$ 30.551, $p= 0.000$). Interestingly the course of this decline differed between strains (interaction strain x days ($F(6,184)$ 5.144, $p= 0.001$). On the last testing day (day 10), BALB/c mice finished the task after approximately 60 seconds, while the C57BL/6J mice needed approximately 200 seconds ($p= 0.000$). The reversal did not influence the time to finish the task in both mouse strains.

BALB/c mice showed higher reference memory ratio (i.e. the number of baited cylinder visits divided by total cylinder visits; 1.0 means no mistakes) on testing days 5 and 6 compared to C57BL/6J mice ($p< 0.05$). Introducing the reversal on day 9 abolished the strain differences, which reappeared on day 10; here the reference memory ratio of the BALB/c mice was again higher compared to the C57BL/6J mice (Fig. 6B, $p< 0.05$). Both strains showed a progressive increase in reference memory ratio over days 2 to 10 ($F(6,246)$ 9.882, $p< 0.0001$), although it did not differ between BALB/c and C57BL/6J mice.

Also the working memory of BALB/c mice (i.e. the number of food rewarded cylinder visits divided by the number of baited cylinders visited; 1 means no mistakes) was increased compared to the C57BL/6J mice on days 3, 4 and 8 to 10 (Fig. 6C, $p < 0.05$). Introducing the reversal did not influence working memory ratio for either mouse strain. Both strains showed a progressive increase in working memory ratio over days 2 to 10 ($F(6,246)$ 6.951, $p< 0.0001$), again not different between strains.

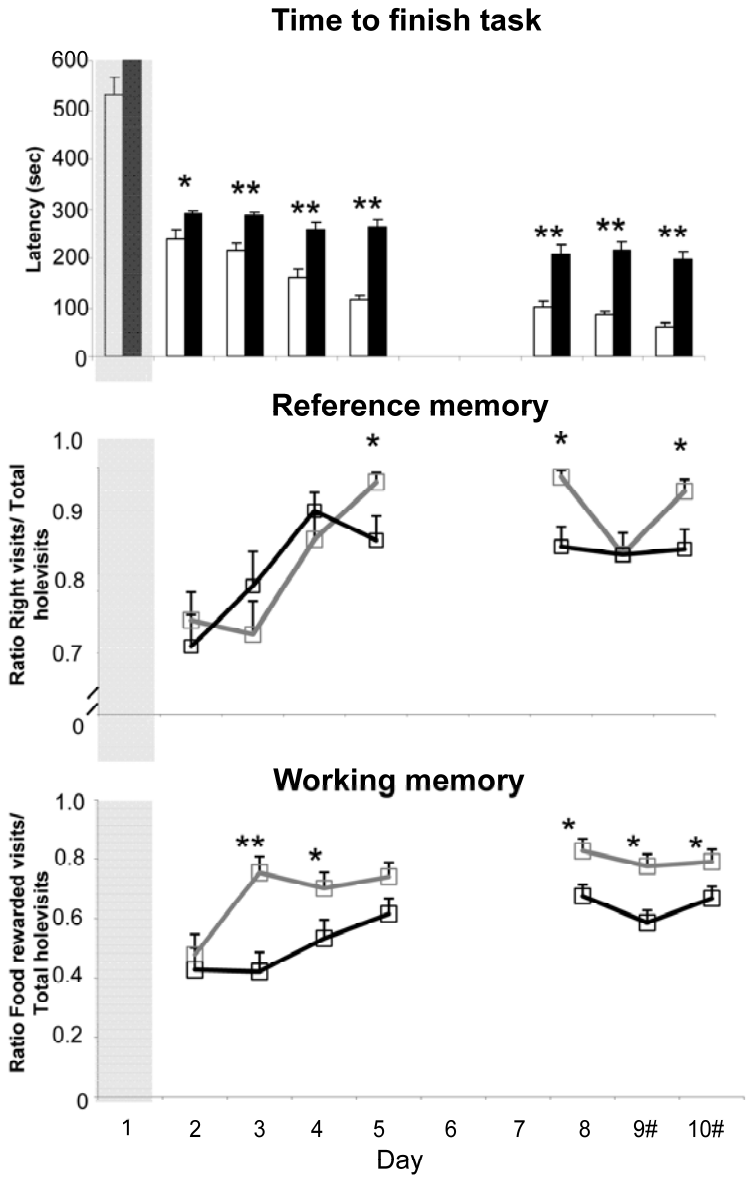

Figure 6. Cognitive parameters of modified holeboard performance of BALB/c (white bars, open squares and grey lines) and C57BL/6J mice (black bars, filled squares and black lines). (A) time to finish the task, (B) reference memory ratio, i.e., the number of baited cylinder visits divided by total cylinder visits and (C) working memory ratio, i.e., the number of food rewarded cylinder visits divided by the number of baited cylinders visited. #: baited cylinders were relocated on days 9 and 10. * $p < 0.05$ and ** $p \leq 0.001$ between strains.

Principal Component Analysis (PCA)

A PCA performed over all behavioural data per strain resulted in the selection of 4 factors for BALB/c and 3 factors for C57BL/6J mice, explaining respectively 86% and 83% of total variance. The behavioural parameters that were included in these factors were subsequently used in further PCA analyses (table 6). These PCA analyses were performed per testing day (on selected behavioural parameters with a factor loading > 0.7).

	BALB/c	C57BL/6J
Defecation (no)	X	
Baits eaten (no)	X	X
Cylinder visits (no)	X	X
Centerboard entries (no)	X	X
Rearings (no)	X	X
Baited cylinders visited (no)	X	X
Stretched attends (no)	X	
Repeated cylinder visits (no)	X	X
Eat bait (lat)	X	X
Time to finish task (s)	X	X
First cylinder visit (lat)	X	X

Table 6. Behavioural parameters included in the extracted factors of BALB/c and C57BL/6J mice (X). Factor values > 0.7 were selected. Abbreviations and symbols: no – number; s – seconds; lat – latency.

To allow interpretation, correlated behavioural parameters were grouped into distinct behavioural classes. We chose the following terms: motivation (latency to first cylinder visit), general exploration (number of entries on board and number of rearings), directed exploration (total number of cylinder visits), learning (time to finish task, total baited cylinders visited), and emotion (stretched attends and defecation). Distribution of these behavioural classes per factor on days 2 to 10 is shown in figure 7. The total number of extracted factors per testing day explained at least 77 % of the total variance for BALB/c and 80 % for C57BL/6J mice.

The pattern of behavioural classes clearly differed between BALB/c and C57BL/6J mice. The behaviour of BALB/c mice appeared to be well structured. On days 2 and 3 learning was correlated with motivation and from day 4 on learning was correlated with directed and general exploration. Furthermore, on days with more involvement of novelty, i.e., on the first day of testing, after the two day break and after introducing the reversal, an additional emotional class

was present in BALB/c, but not C57BL/6J mice. The behavioural classes of the C57BL/6J mice appeared to be randomly distributed over the factors. On day 2, the behavioural class learning was correlated with directed exploration, on day 3 it was not correlated with any other class, on day 4 learning was correlated with directed exploration and motivation and on day 5 it was correlated to general exploration. From day 6 on, distribution of the behavioural classes remained similar.

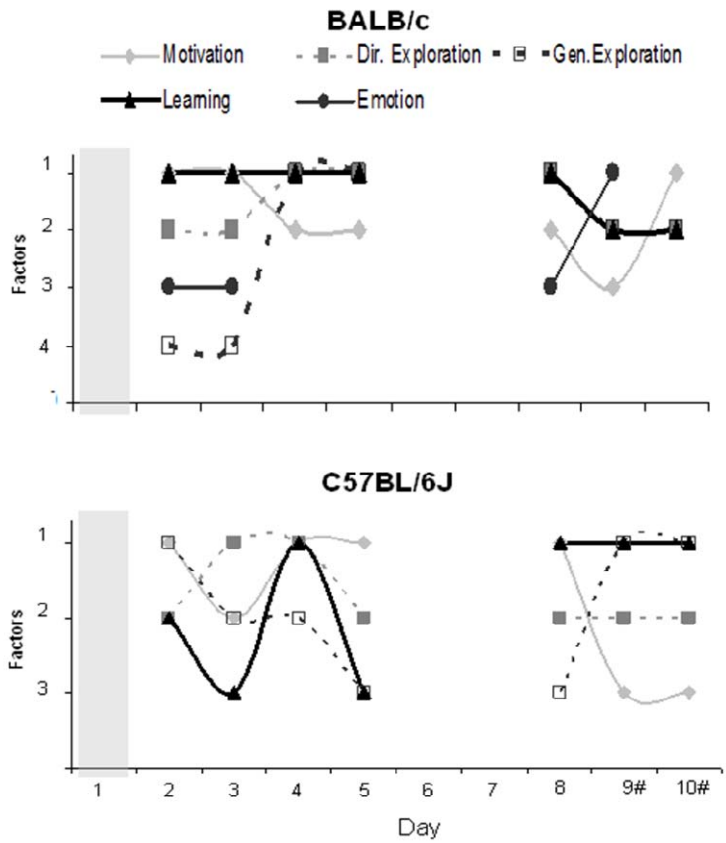

Figure 7. Distribution of behavioural classes per testing day in the extracted factors of the Principal Component Analysis. A; BALB/c mice and B; C57BL/6J mice. Symbols: Closed grey diamonds - motivation; open squares - general exploration; closed circles - emotion; closed squares - directed exploration; closed triangles - learning -. # baited cylinders were relocated on days 9 and 10

DISCUSSION

BALB/c and C57BL/6J mice have characteristic emotional and cognitive behavioural patterns and a distinct regulation and responsiveness of the glucocorticoid stress system. First, we found that BALB/c mice display high risk assessment, intense exploration of the environment in the face of a higher corticosterone responsiveness to stress than C57BL/6J mice. The latter avoid anxiogenic areas, display high general exploration, and are less corticosterone responsive to novelty stress. Interestingly, prior psychosocial stress (rat exposure) dramatically changes the behavioural pattern and eliminates the strain difference in the elevated plus maze. Second, cognitive performance in a visuo-spatial learning task was superior in BALB/c compared to C57BL/6J mice. Third, "Principal Component Analysis" (PCA) compressed the large number of behavioural parameters by extracting factors that signify the differential contribution of exploration and emotion in basic unconditioned behaviour as well as learning and memory. Moreover, this analysis demonstrates that in BALB/c mice initially independent factors of motivation and directed exploration convey during the learning process, while emotions positively contribute to learning. Here, C57BL/6J mice display less structured and a rather random behavioural organisation with no emotional class. Fourth, the strain-dependent MR and GR mRNA and protein expression in limbic brain areas corroborates the function of these receptors in the regulation of corticosterone secretion as well as behaviour. We show not only elevated levels of GR protein in the hippocampus of C57BL/6J mice, but also different post translational modification or splice variants of GR between strains. Although we did not manipulate discrete parameters of the stress system, the two mouse strains provide a vast amount of neuroendocrine and behavioural data that, together with PCA strongly indicates that higher stress-susceptibility and emotions related to risk-assessment contribute positively to cognitive performance.

Strain-dependent neuroendocrine regulation

When placed in a novel environment, corticosterone increase of BALB/c mice is more rapid and higher compared to C57BL/6 mice, which corresponds to the lower MR and GR mRNA expression of BALB/c mice. There is ample evidence from the literature that MR and GR differentially regulate corticosterone response and feedback to stress as well as the diurnal corticosterone rhythm. For example, blockade of brain MR results in a stronger increase in the corticosterone stress response during a mild stressor [29;30], and enhances corticosterone levels during circadian peak in rats [31]. Likewise, comparable neuroendocrine response patterns in rats and mice during aging or with a

specific genetic background coincide with altered MR function in the forebrain. [32-34].
Besides MR function in the proactive phase of hormonal responses, the reactive phase is controlled by GR via the negative feedback loop [30;35]. Lower GR function either induced by pharmacological GR antagonism or mutations of the GR show prolonged elevated secretion of corticosterone [36-38], while increased GR mRNA, either by transgene overexpression or early-life handling results in blunted initial response to acute stress and an enhanced feedback regulation [39;40]. Elevated GR mRNA expression in the hippocampus of C57BL/6J mice might therefore contribute to lower initial corticosterone response as well as faster inhibition of secretion due to negative feedback activity. Based on previous results of our group and others we may conclude that the higher hippocampal MR and GR mRNA expression of C57BL/6J mice, possibly the post translational modification or a different splice variant of the GR protein is responsible for the lower glucocorticoid response to novelty stress, while the lower level of hippocampal receptor expression in BALB/c mice coincides with increased stress sensitivity towards novelty.
Interestingly, GR protein expression correlates with GR mRNA expression in the hippocampus, but not amygdala. BALB/c and C57BL/6J mice have similar amygdaloid GR mRNA expression, while GR protein is elevated in BALB/c mice. This discrepancy suggests that in addition to different MR and GR mRNA contribution to hormone concentrations and behaviour, also pathways related to translation and protein stabilisation [41] are different between BALB/c and C57BL/6J mouse strains. Furthermore, post translational modification or expression of different splice variants of the GR protein also seems to be different between these mice, although the consequence is yet unknown. Naturally we are aware of the fact that more factors than MR and GR contribute to the excitability of the glucocorticoid stress system. However, based on the strain-dependent differential expression of MR and GR and our knowledge of the behavioural role of MR and GR we may predict stronger emotional behaviour of BALB/c than C57BL/6J mice.

Unconditioned behaviour: Strain-dependent patterns of exploration and emotion in relation to MR and GR function

Indeed, behaviour of naïve BALB/c and C57BL/6J mice in the holeboard and elevated plus maze analyzed by PCA shows strain differences for exploration and emotion factors. The preferential areas of activity of BALB/c mice are the "unprotected" (anxiogenic) parts of the test boxes, i.e., open arms of the elevated plus maze and centreboard in the holeboard task. In accordance with other studies, BALB/C mice show higher risk assessment (stretched attends) than C57BL/6J mice [42]. C57BL6J mice avoid the open arms and centreboard and

show high rearing. Before we address the interpretation of this behaviour in relation to anxiety, we will highlight the strain-differences in emotional/explorative behaviours in relation to characteristics of MR and GR.
Numerous studies demonstrated the role of MR in behavioural responses to novel environments [43]. Latest findings are derived from forebrain MR-knockout mice with altered behaviour during their first exposure to a learning task and reactivity to a novel object [28]. Anxiety-related behaviour in the elevated plus maze, open field and defensive buying test are decreased after pharmacological blockade of MR [44;45]. This corresponds to our data in which BALB/c mice with less MR mRNA, show increased directed exploration of their environment compared to C57BL/6J mice. However, the literature is controversial as some studies show that increased MR is related to less anxiety-like behaviour [33;46;47]. Next to the differential interpretation of behaviour as anxiety-like, one possible explanation could be a (dis)balanced contribution of GR [11]. Low GR mRNA expression and protein as we see in the hippocampus of BALB/c mice, has been correlated with less anxiety-related behaviour in rats and mice [48;49], while high GR mRNA or GR activation is implied in high anxiety-like behaviour in C57BL/6J mice [11;49]. In contrast to the lower GR mRNA and protein in hippocampus, BALB/c mice show higher GR mRNA in the prefrontal cortex and GR protein in the amygdala, which could be important for the higher emotionality of this strain.
An alternative explanation for the distinct behavioural patterns of C57BL/6J and BALB/c mice might lie in the initial stress response to novelty, when BALB/c mice increase their corticosterone secretion 10-fold within 5 minutes. Recently, a novel molecular mechanism for a fast non-genomic action of corticosterone has been described [50]. MR, however with a low affinity for the hormone and thus activated by fast rising and high corticosterone concentrations is thought to orchestrate behaviour. Behavioural consequences of short term non-genomic corticosteroid effects like increased risk assessment and altered search strategies have been reported in rats [51;52]. During the last years, interest in gene x environment interaction increased. That maternal care as environmental factor in early life can program the stress system and behaviour has been shown for both strains: C57BL/6J dams display high maternal care compared to BALB/c dams. GABA(A) receptor expression that is involved in anxiety [53], is altered by maternal influences [54;55]. Moreover, cross-fostering demonstrated a change in MR and GR function and anxiety-like behaviour [56].

We conclude that MR and GR via their genomic and most likely also MR non-genomic effects are central molecular mechanism for behavioural regulation.

Anxiety and escape behaviour: stress increases the movement in the anxiogenic zones and risk assessment

During rat stress, BALB/c mice are much more aroused than C57BL/6J mice (increased defecation). When exposed directly thereafter to the elevated plus maze, behavioural changes regarding risk assessment and arousal further increase, while time and entries into the open arms remain as high as in naïve mice. In contrast, stressed C57BL/6J mice specifically increase their risk assessment behaviour together with the number of entries and time spent in the unprotected open arms to the level of BALB/c mice. Is this an anxiolytic effect of acute stress?

Most interpretations of behaviour as anxiety-like are based on the measurement of a few parameters related to the avoidance of unprotected, open, so-called "anxiogenic" zones. By this definition, naïve C57BL/6J mice would be highly anxious, while naïve and stressed BALB/c and stressed C57BL/6J would be labelled as mice with low anxiety. An anxiolytic effect of stress is unlikely as we and others showed that stress or corticosteroids increase anxiety-like behaviour [11;49;57]. However, when anxiety becomes expressed as freezing (passive coping), exploration of the environment is prevented and cognitive performance impaired [47]. The detailed registration of behaviour as suggested by Rogers et al [58] and subjecting the many behavioural parameters to advanced statistical analyses revealed a more refined picture of interacting emotions, exploration and general activity patterns of the mice.

Freezing, exploration of safe areas and exploration for escape possibilities can all be expressions of anxiety. Escape behaviour and stretched attends are important, often forgotten anxiety variables in maze testing [58;59]. When including these behaviours, BALB/c mice are more emotional than C57BL/6J mice. Supported also by the dramatic increase in distance moved and stretched-attends after acute stress, we consider escape behaviour, thus an active coping style as an expression of the underlying emotion of anxiety.

Strain-dependent cognitive performance: structure of behaviour and response to reversal

In this food-rewarded task, BALB/c mice are faster learners with superior reference and working memory compared to C57BL/6J mice. The behavioural pattern of BALB/c mice during learning includes an emotionality factor. Even more, finding bait in the cylinders increases the time spent on the centreboard with high directed exploration towards the cylinders, crystallizing as additional motivation factor. Our findings seem to contradict several reports of poor spatial learning abilities of BALB/c mice in the water maze [60-62]. However, BALB/c mice did not show inferior cognitive performance when tested in a dry

maze or including multiple cognitive parameters for learning and memory [61;63]. The water maze is regarded as a highly stressful, aversive task for mice which prefer dry-land over wet mazes [64]. Already Yoshida et al [61] suggested that the motivation and stress stemming from tasks are likely factors that differentially affect water maze and dry maze learning. Indeed, the apparently contradicting results underline the strain-dependent impact of stress and emotions for cognitive performance as well as the relevance for using multiple tasks with an elaborate behavioural analysis before labelling cognitive capacities of a mouse strain.

Which factors contribute to learning? PCA revealed motivation as correlate of learning in BALB/c mice during the first testing days. Goal directed behaviour gains relevance for performance during later stages of learning. An emotion factor is present only in BALB/c mice at times of relative novelty: at the first days of leaning, after a break of two days and during reversal. Here, behaviour that is susceptible to stress becomes part of the behavioural structure, i.e., risk assessment and arousal. Although this emotion factor does not correlate with learning (or any other behavioural class) we consider it likely that these initial responses to the setting contribute to subsequent learning [24]. In our test conditions, C57BL/6J mice lack this emotion factor. A correlation between directed exploration and learning is initially present, but disappears later on. Learning and other factors do not convey, but alternate rather randomly. We may conclude that the conveyance of behavioural factors supports the superior cognitive performance of BALB/c mice, while the lack of orchestrated behaviour leaves the C57BL/6J mice at a more inferior level of performance.

The design of the task allows spatial (fixed location of the baited cylinders) but also stimulus-response learning (white rings around the baited cylinders). How do mice respond to a reversal, i.e., re-location of the cylinders? A preferential use of a spatial learning strategy will be accompanied by errors and a drop in reference memory, as we see in BALB/c mice. However, the new locations are acquired very quickly and reference memory recovers to its superior level, showing only a short-lasting drop in performance. No such effect is found in C57BL/6J mice, which apparently use the visual discriminating stimulus to locate the bait: a stimulus-response learning strategy. Different memory systems contribute to these strategies: nucleus caudate is related to stimulus-response and the hippocampus to spatial learning strategies [9;65]. Spatial learning requires a more complex organisation and processing of information, implying a higher degree of flexibility. Stimulus-response learning is rather rigid. Both strategies allow to solve the task, albeit the spatial solution appears to be the most efficient one.

Distinct MR and GR characteristics are not only modulating specific phases of memory [15;66], but might be also related to spatial and stimulus-response

strategies. MR plays a role in the appraisal of the situation as well as the flexibility of the behavioural response as evidenced by genetic and pharmacological manipulation of MR functions [28;40;46;52]. These studies suggest that less MR, as seen in BALB/c mice, would allow rather flexible behaviour and together with the increased context-related corticosterone surge, also would allow activation of GR in context to facilitate memory consolidation [67-70]. Overexpressing hippocampal MR results in more intense responses towards novel objects and in an enhancement in the consolidation of non-spatial memory [71]. Thus, higher levels of MR are linked to a stimulus-bound response strategy. This is what we observe in C57BL/6J mice which are characterized by elevated hippocampal MR and GR expression. The stimulus-response strategy is of advantage as long as the visual stimulus predicts the location of the bait, as it happens to be in the current task.

Considering the cognitive performance of BALB/c and C57BL/6J at large, stress-susceptible behaviours of risk-assessment and arousal (both in BALB/c mice only) in the face of an active coping style imply interacting systems of stress, emotion and cognition to the benefit of superior cognitive performance. C57BL/6J mice as rather emotionless and less stress-susceptible phenotype demonstrate less hippocampus-guided behaviour and thus, cognitive performance at a different level. The advantage of either style will be closely related with the demands of the task. Since acute stress activates emotional responses in C57BL/6J mice, increasing the emotional characteristics of the task (e.g., fear conditioning) will reveal more active coping behaviour and clear stimulus-bound responses in this mouse strain. Indeed, C57BL/6J mice show an active coping style, characterized by more scanning than freezing behaviour, while BALB/c mice show more freezing that scanning. So likewise, the acquisition and consolidation of fear memories was predominantly stimulus-bound in C57BL/6J mice compared to BALB/c mice [72].

Conclusion

This study demonstrates that distinct stress system regulation by MR mRNA and GR mRNA and protein expression correlates with emotional behaviour, cognitive performance and behavioural structure in BALB/c and C57BL/6J mice. Lower hippocampal MR and GR mRNA expression, but elevated GR mRNA in prefrontal cortex and GR protein in the amygdala of BALB/c mice coincides with increased stress susceptibility, high emotional expression and superior spatially orientated cognitive performance. High MR and GR in C57BL/6J mice corresponds to lower stress susceptibility and cognitive performance which is stimulus-response driven. Our data contributes to the understanding how the stress system, emotion and cognition interact under basal and stress conditions.

Disclosure/Conflict-of-Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 4

Strain specific fear behaviour and glucocorticoid response to aversive events: modelling PTSD in mice

Brinks V, de Kloet ER, Oitzl MS
Gorlaeus Lab, LACDR/LUMC, Division of Medical Pharmacology, Einsteinweg 55, 2300 RA Leiden, Leiden University, the Netherlands

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ABSTRACT

Post Traumatic Stress Disorder (PTSD) is a stress related disease that has large individual vulnerability. It can develop weeks or even months after a traumatic stressful experience and is characterised by intrusive persistent memories of the traumatic event and changes in the glucocorticoid stress system. Here, we works towards an animal model for PTSD using a modified fear conditioning paradigm in which we can (i) follow learning/acquisition of the negative event by measuring scanning and freezing behaviour, (ii) test memory/retrieval processes for both context and cue after a delay of 24 hrs, (iii) measure corticosterone as endocrine stress parameters before and in response to conditioning and (iv) show the influence of the genetic background on acquisition and retrieval of the negative event. By using two mouse strains, with distinct stress system markers (BALB/c and C57BL/6J) we expect our results to be more representative for the individual vulnerability to stress-related disorders.

BALB/c mice have high fear behaviour with corresponding corticosterone response indicative for a generalised fear response. They display strong fear acquisition/learning, but also strong memory for the negative event. In contrast, C57BL/6J mice display lower fear behaviour during learning, but very strong memory for the cue. Concerning "PTSD like" symptomatology, C57BL/6J mice seem to be more vulnerable to cue specific "flashbacks", while BALB/c mice are suitable for studying generalised fear memory. Fear-extinction paradigms should reveal the capacity to extinguish fear memory.

INTRODUCTION

Post Traumatic Stress Disorder (PTSD) has a clear-cut stress-related onset and genetic components that determine its occurrence. PTSD can develop in the course of weeks or even months after the traumatic event has taken place. Behavioural characteristics are intrusive persistent memories of the trauma, avoidance behaviour and hyperarousal. Besides behavioural symptomatology, also changes in endocrine systems of HPA-axis activity, the glucocorticoid related part of the stress system are present. People suffering from PTSD are reported to have low basal cortisol levels, increased sensitivity to stress and glucocorticoid negative feedback [1]. Furthermore, the volume of the hippocampus, a brain target of glucocorticoid stress hormones, is lower in PTSD patients, indicating a period of strong or prolonged exposure to high stress-hormone concentrations [2;3]. This makes the stress system / HPA-axis activity a key player in PTSD research.

More and more biological data on PTSD patients is published [4-7]. At present, only animal research can provide clues to uncover the molecular mechanisms. But, like all other psychiatric disorders, animal models will never cover all aspects of PTSD. The parameter (component) of strengthened memory for the adverse event offers a central access for animal research focussing on fear conditioning and its molecular mechanisms.

In fear conditioning, an unexpected, for the mouse even of unknown quality, aversive stimulus such as an electric shock (unconditioned stimulus UCS), is given once or several times in association with a non aversive stimulus (cue; conditioned stimulus CS) such as a light and/or tone, in a distinct environment (context). This is the well-known Pavlovian conditioning paradigm. The animal will remember the association between the announcing cue and aversive stimulus but also the surrounding in which the aversive stimulus was given. Thus, placing the animal in the same context and/or turning on the light/tone that was previously associated with an electric foot shock, will evoke a fear response expressed in mice and rats as scanning and freezing behaviour (conditioned response, CR). We defined scanning as immobility of the body, while the head is moving horizontally from side to side. The animal is still actively interacting with its environment. We defined freezing as immobility of the body and head and is devoid of interaction with the environment. Although scanning and freezing are interdependent, they express a different quality of fear. With automatic scoring, both scanning and freezing are measured as immobility behaviour.

The present experiment works towards an animal model for PTSD in which we can (i) follow learning/acquisition of the negative event by measuring scanning and freezing behaviour, (ii) test memory/retrieval processes for both context

and cue after a delay of 24 hrs, (iii) measure corticosterone as endocrine stress parameters before and in response to conditioning and (iv) show the influence of the genetic background on acquisition and retrieval of the negative event. By using two mouse strains, we expect our results to be more representative for the individual vulnerability to stress-related disorders.

MATERIAL AND METHODS

Male BALB/c and C57BL/6J mice (n=8 per group; 3-month-old) were subjected to a specific fear conditioning paradigm that allowed to differentiate context and context/cue related responses in the same setting. This included 10 light/tone+shock pairings with a one minute interval on day 1. Pairings were as follows; light (260 lux) and tone (70dB) were given simultaneously for 20 seconds of which an additional shock (0.4 mA) was administrated at the last two seconds. Scanning and freezing was measured when the animals were placed in the setting (Figure 1, point 1) and during the 1-min intervals after light/tone+shock pairings (Figure 1, points 2-11). Memory of this association, expressed as scanning and freezing was estimated 24 hrs later on day 2. Then, mice were returned to the same box: 3 min exposure to the setting (context only) was followed by 2 min of light/tone exposure (context/cue) and ended with 2 min exposure to the setting (context only). Plasma corticosterone was estimated at several time points: on the day before conditioning, after conditioning and after retention testing (see Table 1). General Linear Model-Repeated Measures was used to test for significant progression of scanning and freezing over conditioning intervals on day 1. Students T-test for independent variables was used to compare percentage scanning and freezing for context and context/cue between strains on day 2. Furthermore, Students t-test statistics were used to compare corticosterone concentrations of the two strains at each time point and to basal corticosterone values before the experiment started.

RESULTS

Figure 1A and B presents the percentages of freezing and scanning in both strains on days 1 and 2.

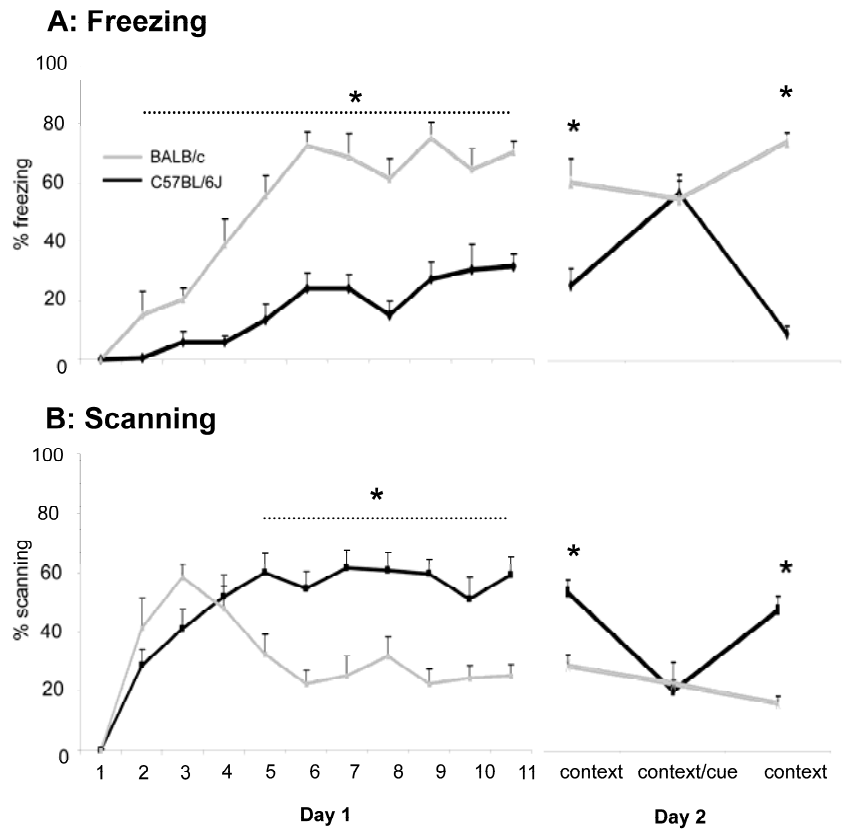

Figure 1. A. Percentage freezing of C57BL/6 (black line) and BALB/c mice (grey line). B. Percentage scanning of C57BL/6 (black line) and BALB/c mice (grey line). Day 1: Acquisition; time point 1 represents scanning and freezing during the first minute in the setting; time points 2-11 represent scanning and freezing in the 1-min intervals between the 10 light/tone+shock pairings. Day 2: memory/retrieval; scanning and freezing during context (3min), context/cue (2 min) and context (2 min) exposure is presented. Data are presented as mean (± SEM) percentage of behaviour. Horizontal lines and asterisks indicate significant differences between groups. Significance was accepted at $p < 0.05$.

On day 1, when the light/tone+shock pairings took place (conditioning), the percentage of freezing progressively increased for both C57BL/6J and BALB/c mice ($F(10,140)$ 25.710, $p=0.000$), albeit to a different degree ($F(10,140)$ 4.860, $p=0.000$). BALB/c mice displayed faster increase in freezing resulting in a plateau at approximately 70%, while freezing in C57BL/6J mice reached approximately 30-40%. Also scanning behaviour increased in both mouse strains ($F(10,140)$ 12.279, $p=0.000$) to different degrees ($F(10,140)$6.662 $p=0.000$). Group differences for scanning and freezing appear at separate time points: distinct

scanning behaviour starts at interval 5, while freezing percentages differ from interval 2 onwards. Also, there is a strain-dependent main effect for freezing and scanning: C57BL/6J mice display high scanning and lower freezing behaviour, while BALB/c mice have high freezing and low scanning behaviour. Interestingly, total immobility measured by scanning and freezing together is the same for C57BL/6J and BALB/c mice.

On day 2, BALB/c mice displayed more freezing compared to C57BL/6J mice when first exposed to the context ($F(1,14)$ 10.551, $p=0.001$). For both strains, this percentage of freezing is comparable to the last freezing response on day 1. Next, the light/tone cue was presented. This resulted in a comparable amount of freezing in C57BL/6J and BALB/c mice ($F(1,14)$ 12.921, $p= 0.857$). Subsequently switching off the cue (and thus only exposure to the context) again separated the strains ($F(1,14)$ 12.988, $p=0.000$). C57BL/6J mice reduced their freezing while BALB/c mice even increased their freezing response to the context.

Scanning of BALB/c mice was lower when first exposed to the setting compared to C57BL/6J ($F(1,14)$ 9.873, $p=0.008$), however in both strains comparable to the last scanning data on day 1. When presenting the light/tone cue, C57BL/6J and BALB/c mice displayed similar low percentage of scanning ($F(1,14)$ 13.688, $p=0.689$). Differentiation between strains occurred again when the cue was turned off ($F(1,14)$ 9.930, $p=0.000$).

Plasma corticosterone concentrations mirrored the behavioural response of C57BL/6J and BALB/c mice for acquisition and retrieval of fear memories (see table 1). At 30 and 60 minutes after onset of testing on day 1, corticosterone concentrations were twofold higher in BALB/c compared to C57BL/6J mice (30 min: $F(1,6)$ 5.761, $p= 0.000$, 60 min: $F(1,6)$ 5.111, $p= 0.002$). On day 2, corticosterone concentrations of BALB/c mice were increased compared to C57BL/6J mice at 30 min ($F(1,6)$ 4.972, $p= 0.027$), but returned to comparable low levels at 60 min. Undisturbed basal morning resting corticosterone concentrations were comparable between strains ($F(1,14)$ 10.589, $p= 0.483$) and significantly lower than all 30 and 60 min data.

		Day 1. Acquisition		**Day 2. Retrieval**	
	Basal	**30 min**	**60 min**	**30 min**	**60 min**
C57BL/6J	11.2 ± 2.4	172.5 ± 10.0*	94.9 ± 15.5*	84.0 ± 9.6*	50.4 ± 12.5
BALB/c	9.0 ± 1.3	370.4 ± 12.6*	189.0 ± 10.0*	137.4 ± 15.7*	54.3 ± 8.8

Table 1. Plasma concentrations of corticosterone in ng/ml (mean ± SEM) of C57Bl/6J and BALB/c mice measured on the day before conditioning (basal morning values), day 1 (30 and 60 min after the start of conditioning) and day 2 (30 and 60 min after start of retention test). Corticosterone assay was performed with the use of a commercially available radio immune assay kits (MP Biomedicals Inc., Calif., USA). Data is represented as mean ± SEM. Significant differences: all 30 and 60 min samples compared to basal concentrations, * C57BL/6 versus BALB/c mice. Significance was accepted at $p < 0.05$.

DISCUSSION

The two mouse strains used in this study show distinctly different fear responses during conditioning. BALB/c mice display the fastest increase and highest plateau in freezing, while scanning is the main fear response in C57BL/6J mice. During retrieval on day 2, both strains have the same anticipatory amount of freezing and scanning to the cue, but differ in their response to context. BALB/c mice lack the discrimination between context and cue, showing a comparable amount of freezing to context and cue, which indicates a generalised and even potentiated fear response. C57BL/6J mice clearly differentiate between context and cue by adapting the ratio of scanning and freezing accordingly. Only few fear conditioning studies have been performed with C57BL/6 and BALB/c mice together. In one study, mice were subjected to two cue-shock pairings and tested for context and cued fear memory expressed as immobility only. With this method, generalised freezing was observed in C57BL/6J, but not BALB/c mice [8]. Another study showed that BALB/c mice have a memory impairment for the aversive event when exposed to the cue only [9]. Apparently, severity of the conditioning protocol (number of shocks) and type of memory testing (separate or combined context and cue-retrieval) are important factors influencing fear behaviour. Our study is the first to measure scanning and freezing behaviour separately, and has of advantage that differences between strains (or treatments) are more pronounced. Measuring total immobility (scanning and freezing), which was similar for C57BL/6J and BALB/c mice, would not have been effective in showing differences in fear behaviour this experiment.

Our model therefore allows a differentiation between different qualities of fear: the more active fear behaviour expressed by scanning and the rather passive fear behaviour indicated by freezing. Recognizing the light/tone stimulus as threat and freezing in anticipation of the negative event can be considered as an adaptive response. Increased scanning in the context indicates a more active coping strategy that might allow possibilities to escape the expected aversive event [10].

The endocrine data after acquisition and retention test shows that BALB/c mice are more corticosterone responsive to our fear conditioning paradigm compared to C57BL/6J mice. Here, corticosterone concentrations might be indicative for behavioural responses to and in anticipation of the negative event. Furthermore, the increased corticosterone concentration after conditioning likely facilitates the consolidation process [11].

Different brain areas are involved in context and cue fear conditioning. The role of the hippocampus is more in the spatial aspect of conditioning, i.e., associating the environment/context with the shock. The amygdala is involved

in the association between cue and shock [12]. Knowing this, we may conclude that for C57BL/6J and BALB/c mice, the hippocampus and amygdala differentially contribute to learning and memory processes involved in fear conditioning. Furthermore, the amygdala and hippocampus are both targets for corticosterone action, so their functionality in this fear conditioning paradigm could be modulated by different corticosterone concentrations [13].

Our data shows that consolidation and possibly retrieval processes are different for C57BL/6J and BALB/c mice. While C57BL/6J mice display distinct fear responses to context and additional cue, BALB/c mice show generalised fear to both. This raises the question whether extinction processes would also be different between these strains. People with PTSD have recurring intrusive memories for the negative event that do not extinguish. So studying this process in C57BL/6J and BALB/c mice should be the next step in modelling PTSD in mice.

The different fear behaviours, possibly influenced by differential contributions of the amygdala and hippocampus, and corticosterone response in the C57BL/6J and BALB/c mice can be explained by genetic make-up but also by differences in environmental factors such as maternal care [14]. BALB/c dams display low maternal care behaviour (nursing, licking and grooming) compared to C57BL/6 dams. Cross fostering BALB/c pups with C57BL/6J dams resulted in reduced anxiety behaviour and basal corticosterone concentrations of the BALB/c mice in later life, showing the role of early life events and a differentially organized stress system for the phenotype. This also corresponds to the fact that vulnerability for PTSD in humans is influenced by traumatic early life events [15].

The behavioural and endocrine data shows that our fear conditioning model can be used to follow acquisition of a negative event, but also to test context and context/cue retrieval processes. A distinct genetic background and early life programming seem to be important for the acquisition and memory of fear. With this model we can therefore mimic some PTSD symptoms in mice.

Conclusion

The BALB/c mice show high fear behaviour with corresponding corticosterone response indicative for a generalised fear response. They display strong fear acquisition/learning, but also strong memory for the negative event. C57BL/6J mice have lower fear behaviour during learning, but very strong memory for the cue. Concerning “PTSD like” symptomatology, C57Bl/6J mice seem to be more vulnerable to cue specific “flashbacks”, while BALB/c mice are suitable for studying generalised fear memory. Fear-extinction paradigms should reveal the capacity to extinguish.

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Chapter 5

Corticosterone facilitates extinction of fear memory in BALB/c mice but strengthens cue related fear in C57BL/6 mice

Brinks V, de Kloet ER, Oitzl MS
Gorlaeus Lab, LACDR/LUMC, Division of Medical Pharmacology, Einsteinweg 55, 2300 RA Leiden, Leiden University, the Netherlands

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ABSTRACT

Corticosterone, the naturally occurring glucocorticoid of rodents is secreted in response to stressors and is known for its facilitating, but also detrimental effects on emotional learning and memory. The large variability in the action of the stress hormone on processing of emotional memories is postulated to depend on genetic background and the spatio-temporal domain in which the hormone operates. To address this hypothesis, mice of two strains with distinct corticosterone secretory patterns and behavioural phenotype (BALB/c and C57BL/6J mice) were treated with corticosterone (250 µg/kg, i.p.), either 5 minutes before or directly after acquisition in a fear conditioning task. As the paradigm allowed assessing in one experimental procedure both context- and cue-related fear behaviour, we were able to detect generalization and specificity of fear. BALB/c showed generalized strong fear memory, while C57BL/6J mice discriminated between freezing during context-and cue episodes. Corticosterone had opposite effects on fear memory depending on the mouse strain and time of injection. Corticosterone *after* acquisition did not affect C57BL/6J mice, but destabilized consolidation and facilitated extinction in BALB/c. Corticosterone *5 min before* acquisition strengthened stress-associated signals: BALB/c no longer showed lower fear memory, while C57BL/6J mice displayed increased fear memory and impaired extinction in cue episodes. We propose that corticosterone-induced facilitation of fear memory in C57BL/6J mice can be used to study the development of fear memories, corticosterone administration in BALB/c mice rather presents a model to examine treatment. We conclude that genetic background and time of corticosterone action are modifiers of fear memory with interesting translational implications for anxiety-related diseases.

INTRODUCTION

Emotional experiences are remembered very well. However, the strength of emotional memory varies between individuals. Good memory of a salient experience has the advantage to facilitate adaptation to similar situations in the future. However, when memory for emotional events becomes too strong and also unpredictable, pathologies such as post traumatic stress disorder (PTSD), panic and anxiety disorders might develop.

Individuals suffering from PTSD show abnormal cognitive-emotional interactions. This implies that specific situations may lead to re-emergence (retrieval) of intrusive, unwanted memory of a traumatic event together with extreme emotions related to fear. Recent clinical trials have shown that treatment with glucocorticoids can have a beneficial effect on established PTSD [1] and specific phobias [2]. It is known for decades that glucocorticoids modulate fear memories [3-10]. For a rational treatment of anxiety disorders it is therefore essential to understand how glucocorticoids contribute to the formation and extinction of emotional memories.

The present study is focused on the interplay of glucocorticoids with memory formation and extinction of a traumatic event. BALB/c and C57BL/6J mice have a distinctly different stress neuroendocrinological and behavioural phenotype. During fear conditioning BALB/c mice display a much higher stress responsivity and emotionality than C57BL/6J mice [11;12]. Hence these two mouse strains will be used to examine the role of corticosterone in individual differences in processing of fearful information.

Pavlovian fear conditioning provides one of the best rodent models to study cognitive processes related to fear. Fear conditioning studies classically consist of the pairing of a conditioned stimulus (CS) with an aversive unconditioned stimulus (US; mostly electric footshock), which mainly induces freezing as a conditioned fear response. Different neural mechanisms seem to be involved depending on whether the CS is a relatively simple stimulus or cue, such as a tone or light (unimodal), or the context (multimodal) in which the US is delivered. Lesion experiments showed that the amygdala is necessary for both types of conditioning, whereas the hippocampus is predominantly required for contextual conditioning [13;14].

Our recently developed fear conditioning paradigm allows the assessment of both context and cue related fear-memory processes in one experimental procedure. Using this paradigm we recently found that BALB/c mice show strong fear-responses to context and cue (i.e., generalization), while C57BL/6J mice display specific fear memory towards the predictive conditioned stimulus, the cue [11]. Remarkably, BALB/c mice have a twofold higher corticosterone response after conditioning and retrieval of fear memory than C57BL/6J mice

[11]. Based on these results, we hypothesized that additional corticosterone treatment prior to acquisition and consolidation of fear memories will result in altered fear-related memory formation and thus, retrieval and extinction patterns of fear behaviour. For this purpose corticosterone was administered either 5 minutes before or directly after acquisition. We expect that the timing of the corticosterone treatment in relation to acquisition and consolidation will affect subsequent retention of behaviour in a strain dependent fashion.

MATERIAL AND METHODS

Animals

Twelve week old male BALB/c (n=40) and C57BL/6J mice (n=36) from Charles River (Maastricht, The Netherlands) were housed individually with sawdust bedding, water and food *ad libitum*, at 20^0C with controlled humidity under a 12 h: 12 h light/dark cycle (lights on at 07.00 a.m.) for at least one week. All experiments were approved by the committee on Animal Health and Care from Leiden University, The Netherlands and performed in strict compliance with the EEC recommendations for the care and use of laboratory animals.

Pain sensitivity

We included an experiment to determine possible differences in the pain threshold between BALB/c and C57BL/6J mice. A separate group of mice (n=8/strain) were subjected to a tail flick protocol that included placing the last two cm the tail in water with a constant temperature of 55^0C [15]. Tailflick latencies of three subsequent trials per mouse were determined with a cut-off latency of 12 sec. The experiment was performed between 09.00 and 10.00 hrs. Tailflick latencies were in the range of 1.32 to 4.18 sec and similar in BALB/c and C57BL/6J mice (data not shown, $F(1,47)1.192$, $p=0.281$), indicative for comparable pain thresholds between strains.

Corticosterone dose and time of injection

Corticosterone (corticosterone-HBC complex, Sigma, The Netherlands) was dissolved in physiological saline on the day of the experiment and injected intraperitoneally (i.p.) with a dose of 250 µg/kg bodyweight in a volume of 0.2 ml. The vehicle (saline) was injected in a corresponding volume of 0.2 ml. A pilot experiment (data not shown) using several corticosterone doses showed that the 250 µg/kg bodyweight dose increases corticosterone concentration of C57BL/6J mice to the level of BALB/c mice when exposed to our fear conditioning procedure [11].

BALB/c (n=16) and C57BL/6J mice (n=14) were injected with corticosterone or vehicle at 5 minutes before the start of the acquisition on testing day 1. We expected that this treatment would affect both acquisition and consolidation processes. To selectively influence the consolidation process, BALB/c (n=16) and C57BL/6J mice (n=14) were injected with corticosterone or vehicle directly after acquisition on testing day 1.

Fear conditioning

Apparatus

The fear conditioning chamber was made of black Plexiglas (25x 25 x 35 cm high) covered by a transparent rim (3 cm width). A speaker was fixed into one wall (25 cm high) and connected to a tone generator (70 dB). The floor consisted of stainless steel bars (5 mm in diameter, spaced 0.5 cm apart) connected to a shock generator. Hereunder was a tray with paper tissues to collect faeces and urine of the mice. A white light source (260 lux) and a camera connected to a video recorder were fixed 20 cm above the conditioning chamber.

A radio produced 20 dB of background noise and the light intensity of the experimental room was 90 lux. After each animal, the chamber was cleaned with tap water and the tissues were replaced.

Procedure

The fear conditioning paradigm allowed differentiating between context and context/cue related behavioural responses in the same setting. Training (day 1) involved 3 minutes of baseline recording, followed by 6 light/tone (CS) + shock (US) pairings with an episode of one minute. Pairings consisted of the cue (i.e., a combined light (260 lux) and tone exposure (70dB)) for 20 seconds and an electric footshock (0.4 mA) during the last two seconds of the cue. Mice were returned to their homecage 2 minutes after the last pairing. At 48 and 72 hrs after conditioning (days 3 and 4, respectively), the same experimental procedure was repeated in absence of shocks to test for memory and extinction of the conditioned fear response. The procedure lasted 12 minutes per mouse/day and was performed between 8.00 a.m. and 13.00 p.m. in an experimental room adjacent to the housing room.

Behavioural assessment

Freezing behaviour was recorded as parameter of fear behaviour. Freezing is defined as immobility of the body including the head devoid of any interaction with the environment. According to Morgan and colleagues, we started and finished behavioural registration with the first and sixth cue presentation during memory and extinction testing [16]. To determine the behavioural structure,

freezing and behaviours such as scanning, grooming, sitting, rearing, stretched attends, jumping and walking were subjected to a Principal Component Analysis (PCA). All behaviours were scored with a semi automatic scoring program (The Observer 4.1, Noldus, Wageningen, The Netherlands) from the video tape.

Statistical analysis

Differences in tailflick latency between BALB/c and C57BL/6J mice were determined by one-way-ANOVA.

Fear conditioning data are presented as mean $\pm$ SEM percentage of freezing during context and cue episodes of the whole session and for each context and cue episode. For acquisition, pre- and post acquisition treatment groups were analysed using General Linear Model (GLM) to determine treatment (naive, saline, corticosterone), strain (BALB/c and C57BL/6J) and time (progression over separate episodes) effects over context or cue episodes. GLM analyses per treatment group (pre- or post acquisition) was used to determine main effects of treatment (corticosterone, saline), strain (BALB/c, C57BL/6J) and day (days 1, 3 and 4) for averaged freezing behaviour in context and cue episodes. If main effects were present, subsequent GLM analyses on context ***or*** cue induced freezing behaviour were performed to determine treatment, strain and day effects. Progression of context ***or*** cue induced freezing behaviour per testing day was also determined with GLM, if adequate, followed by post-hoc LSD test.

Principal Component Analysis (PCA) was performed over all behavioural data. Kaiser normalisation was used on behaviours with communalities over 0.68, i.e., more than 68% of variation is explained by the factors extracted. Factors with an Eigenvalue over 1 were included in the results. A subsequent two-way ANOVA on factor loadings was performed to determine the significance of treatment and strain differences. $P \leq 0.05$ was accepted as level of significance.

RESULTS

BALB/c and C57BL/6J mice were trained in a fear conditioning paradigm in which a novel environment (context) and a light-tone stimulus (cue) were paired with a footshock. Corticosterone had been injected either 5 min before or directly after acquisition. Forty-eight hours later (day 3), re-exposure to the context and cue paradigm (without shock) elicited significant fear responses indicating retrieval of a learned association between this environment and the aversive footshock stimulus. Another 24 hrs later (day 4), mice were re-exposed to the same conditions to study extinction of the conditioned fear responses. Data are presented in the sequence of the phases of memory: acquisition, memory retrieval and extinction in relation to corticosterone treatment. We

found strain, treatment and time of treatment dependent effects on freezing behaviour.

Fear conditioning: Acquisition (Day 1, figures 1-4)

Comparing the percentage of freezing during the alternating cue and context episodes revealed that treatment prior to fear conditioning changed the freezing responses depending on the mouse strain (interaction: strain x treatment, $F(2,53)$ 4.77, $p=0.012$; Figures 1 and 2). Both strains increased freezing over time although with different patterns of freezing in cue and context episodes ($F(11,583)$ 4.613, $p=0.0001$) and treatment ($F(22,583)$ 2.125, $p=0.002$). Strain effects (no injection, Figures 3 and 4): Already naïve BALB/c and C57BL/6J mice responded with a different freezing pattern to fear conditioning (strain $F(1.27)$ 11.846, $p=0.002$). Freezing increased in both strains during consecutive cue/shock pairings and intermittent context periods, albeit with a different pattern ($F(11,297)$ 4.083, $p=0.0001$). While freezing during context was comparable between strains, BALB/c mice were more active during cue periods than the C57BL/6J mice (i.e, more freezing in C57BL/6J mice during cue episodes $F(1,27)$ 31.321, $p=0.0001$). Treatment effects within strains (compare Day 1, Figures 1 and 3 and Figures 2 and 4): Injection of either vehicle or corticosterone before conditioning increased freezing in BALB/c mice ($F(2,29)$ 6.467, $p=0.005$; steeper increase $F(22,319)$ 2.725, $p=0.0001$; and more freezing during cue and context episodes (cue $F(2,29)$ 6.994 $p=0.003$; context $F(2,29)$ 3.571, $p=0.041$)). Injections prior to conditioning did not affect freezing in C57BL/6J mice. Treatment effects between strains (Figures 1A ,2A vs 1B, 2B): Due to the injection procedure BALB/c mice displayed more freezing to context than C57BL/6J mice ($F(1,26)$ 4.753, $p=0.038$). Total amount of freezing during cue episodes was comparable between strains, but showed a different time course ($F(5,130)$ 3.016, $p=0.013$).

Memory retrieval and extinction overall: strain dependency and time of corticosterone treatment

Corticosterone treatment resulted in a strain dependent effect (interaction strain x treatment $F(1,51)$ 8.120, $p=0.006$). In addition, time of treatment (before or after acquisition) differentially influenced the freezing to cue and context during the retrieval and extinction tests on days 3 and 4 (interaction time x treatment $F(1,51)$ 8.220, $p=0.006$). In both strains, freezing responses were altered by corticosterone (BALB/c: treatment $F(1,28)$ 7.304, $p=0.012$; interaction time x treatment $F(1,28)$ 4.531, $p=0.042$; C57BL/6J: interaction time x treatment $F(1,23)$ 3.850, $p=0.05$).

Memory retrieval and extinction: Treatment prior to acquisition (figures 1 and 2)

Overall analysis of freezing on days 3 and 4 revealed an interaction of strain x treatment (F(1,56) 4.178, p=0.046). Increased total amount of freezing on day 3 indicated the retrieval of fear memory.

Overall, BALB/c displayed more freezing during context than C57BL/6J mice (F(2,25) 7.127, p=0.004), while C57BL/6J mice froze more during cue episodes (F(2,25) 13.147, p<0.0001, Figure 1). Depending on the strain, vehicle and corticosterone differentially altered cue-related freezing (strain x treatment F(2,25) 6.056, p=0.007): cue freezing was initially not affected and later on decreased in BALB/c mice, while it was increased in C57BL/6J mice (Figure 1).

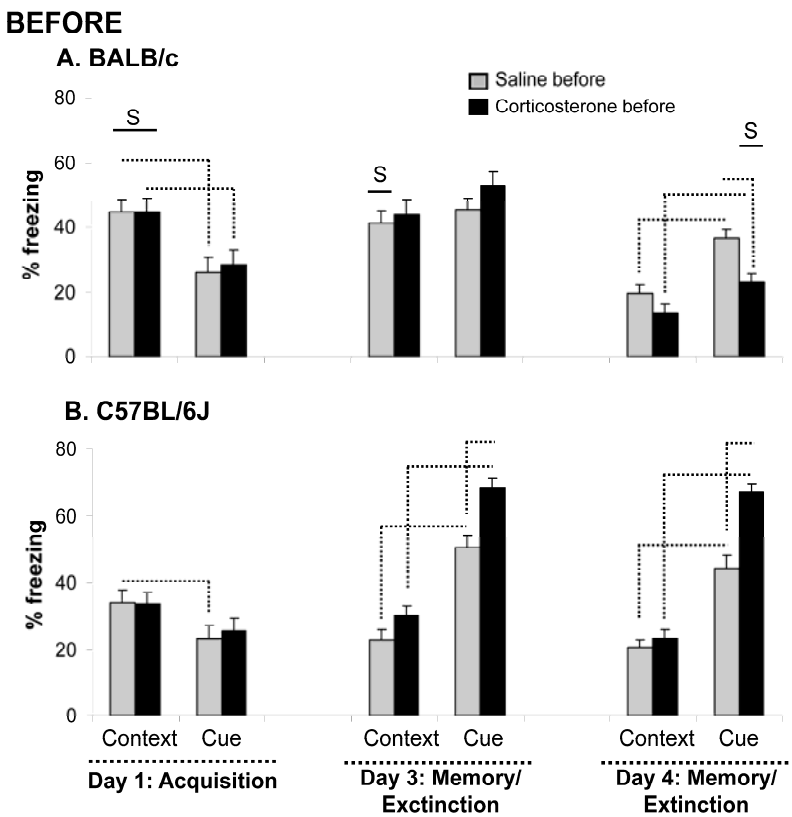

Figure 1. Treatment before acquisition. Percentage of freezing for context only or additional cue intervals of BALB/c mice (A) and C57BL/6J mice (B) injected i.p. with corticosterone (black bars) or saline (grey bars). P<0.05, dotted lines: within strain effects and, S: between strain effect determined with ANOVA.

Vehicle injection: Context and cue induced freezing progressed differently in BALB/c and C57BL/6J mice over days (context: F(2,52) 7.392, p=0.0001, cue: F(2,52) 12.023, p<0.0001, Figure 1). BALB/c mice decreased their freezing during context from day 3 to 4 (Figure 1), while freezing was generally lower and did not differ between days in C57BL/6J mice. Freezing during cue increased in both strains from day 1 to 3, remained high in C57BL/6J mice on day 4, but decreased in BALB/c mice.

Corticosterone had distinct effects on freezing during cue, but not during context episodes, in both strains (interaction of strain x treatment (F(2,52) 5.081, p=0.01). Compared to the vehicle treated C57BL/6J mice, C57BL/6J mice of the corticosterone group had increased freezing during cue episodes on days 3 and 4 (Figure 1). In contrast, cue induced freezing of BALB/c mice did not differ on day 3, but dropped significantly on day 4.

Freezing to alternating context and cue conditions within a session (figure 2)

Analyzing the freezing pattern of alternating cue and context episodes provides additional information on the progression of distinct strain specific behavioural responses: are mice able to show different degrees of freezing to context and cue? These data were analyzed for days 3 and 4.

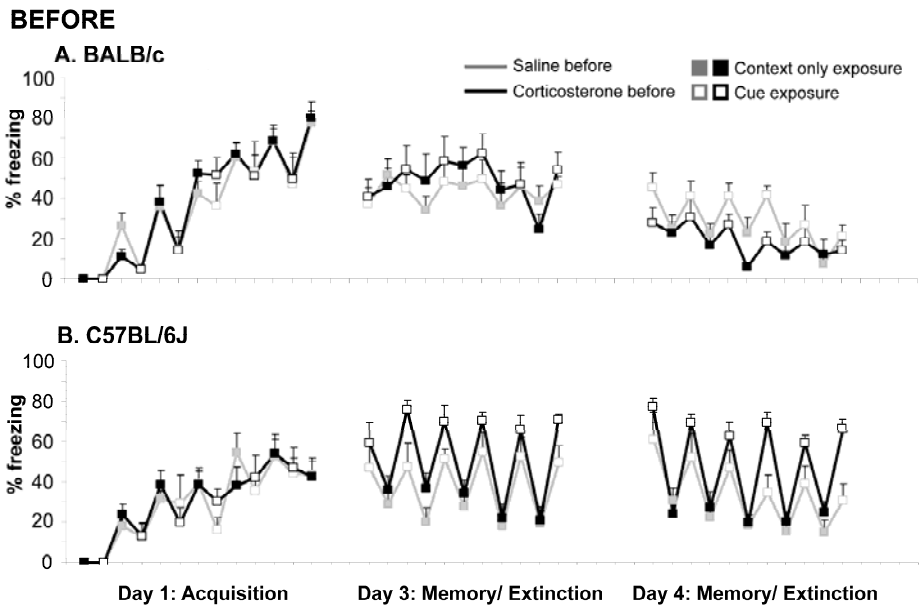

Figure 2. Treatment before acquisition. Freezing behaviour of BALB/c (A) and C57BL6J mice (B) during the three testing days injected i.p. with saline (gray) or 250 µg/kg corticosterone (black). Closed markers indicate context intervals alternating with open markers representing cue intervals. Note that C57BL/6J mice distinctly switch between freezing during context to cue intervals.

Day 3: ANOVA revealed a significant interaction of cue-context x strain (F(10,260) 3.492, p=0.0001). BALB/c mice did not discriminate between freezing to context and cue episodes throughout the session, independent of treatment. In contrast, C57BL/6J mice showed a strong alternating pattern of cue-context freezing (F(10,120) 12.865, p=0.0001), also independent of treatment.
Day 4: The significant interaction of cue-context x strain F(10,260) 4,194, p=0.0001, was complemented by an interaction cue-context x strain x treatment F(10,260) 2.470, p=0.008.
While the differentiation between freezing to context and cue was rather small in BALB/c mice (independent of treatment), it was clearly expressed in C57BL/6J mice and distinctly different in the corticosterone group (interaction cue-context x treatment F(10,120) 3.144, p=0.001).

Memory retrieval and extinction: Treatment immediately after acquisition (figures 3 and 4)
Overall analysis of freezing on days 3 and 4 revealed main effects of strain (F(1,54) 14.615, p=0.0001), treatment (F(1,54) 7.105, p=0.010) and an interaction of strain x treatment (F(1,54) 4.314 p=0.043). Both mouse strains freeze more on day 3 than day 1, indicating the retrieval of fear memory.
Vehicle injection: From day 3 to 4, freezing during context decreased more in BALB/c than in C57BL/6J mice (F(2,50) 4.956, p=0.011, figure 3). BALB/c froze less during cue episodes than C57BL/6J mice, already on day 3 (cue F(1,27) 5.696, p=0.025). Cue-related freezing further decreased in BALB/c on day 4, but remained at the same high level in C57BL/6J on both days (F(2,50) 3.744, p=0.031).
Corticosterone resulted in less freezing to context and cue in BALB/c mice on day 3 (F(1,25) 6.596 p=0.017, figure 3), which further decreased on day 4 (strain (F(1,25) 31.622, p=0.0001). Corticosterone and vehicle-treated C57BL/6J mice showed comparably strong freezing responses to context and cue (interaction strain x treatment F(1,25) 4.346, p= 0.047).
Freezing to alternating context and cue conditions within a session (figure 4)
BALB/c and C57BL/6J mice showed different responses to the alternating cue and context conditions.
Day 3: ANOVA revealed a significant main effect of treatment (F(1,25) 6.596, p=0.017) and interaction of cue-context x strain F(10,250) 2.439, p=0.009 and cue-context x treatment F(10,250) 2.056, p=0.029. BALB/c mice did not discriminate between freezing to context and cue throughout the session; however, when treated with corticosterone, freezing declined in the course of the session. In contrast, C57BL/6J mice showed an alternating pattern of more

cue than context freezing (F(10,110) 6.330, p=0.009) at the end of the session, which was independent of treatment.
Day 4: The significant interaction of cue-context x strain F(10,250) 3.711, p=0.0001 indicated the little differentiation of BALB/c mice between freezing to context or cue episodes and the fast decrease of freezing during the session. C57BL/6J mice again differentiate between freezing to cue (more) and context (less) episodes (F(10,110) 16.000, p=0.0001). Corticosterone treated C57BL/6J mice remain responding with high freezing to the cue throughout the session, while freezing decreases in vehicle-injected mice (interaction cue-context x treatment (F(1,110) 2.361, p=0.041).

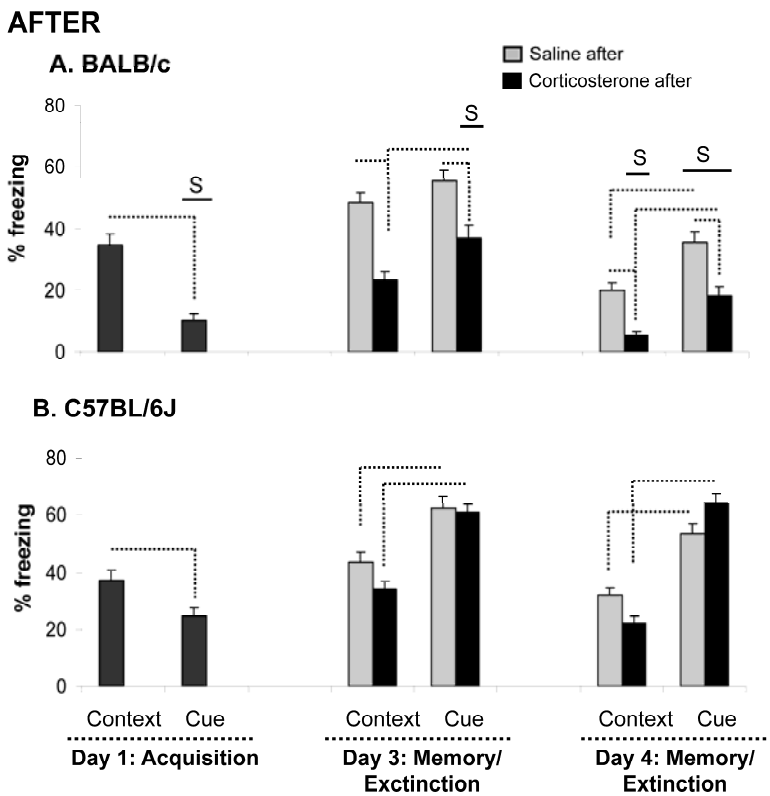

Figure 3. Treatment after acquisition. Percentage of freezing for context only or additional cue intervals of BALB/c mice (A) and C57BL/6J mice (B) injected i.p. with corticosterone (black bars) or saline (grey bars). P<0.05, dotted lines: within strain effects and, S: between strain effect determined with ANOVA.

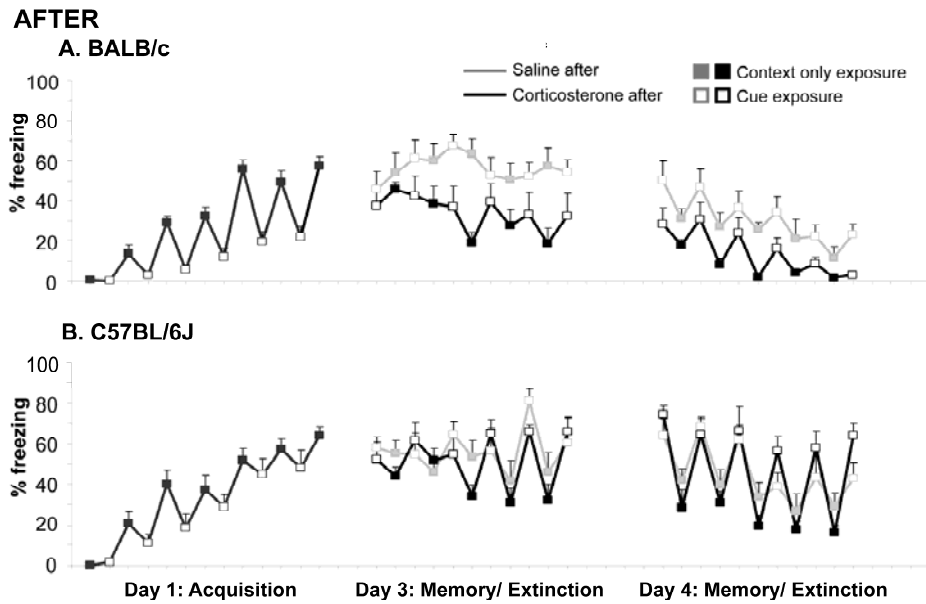

Figure 4. Treatment after acquisition. Freezing behaviour of BALB/c (A) and C57BL/6J mice (B) during the three testing days when injected with saline (grey line) or corticosterone (black line). The dark grey line on day 1 represents pooled data of mice that received treatment later on during the experiment. Closed boxes indicates context intervals, open boxes indicates additional cue intervals.

PCA analysis

PCA analysis resulted in the extraction of one factor explaining 76.3% of the variance. This factor included the behaviours freezing (factor loading: -0.917), sitting (factor loading: 0.877) and walking (factor loading: 0.825), indicating fear or immobility behaviour during all testing days. Further ANOVA's on factor loadings revealed a significant treatment effect in post-acquisition treated BALB/c mice ($F(1,527)$ 63.126, $p<0.0001$) and pre-acquisition treated C57BL/6J mice ($F(1,461)$ 7.936, $p=0.005$).

In addition, PCA analysis showed distinct strain specific fear behaviour when treated with saline post-acquisition ($F(1,461)$ 9.348, $p=0.002$), or pre- and post-acquisition corticosterone ($F(1,527)$ 17.102, $p<0.0001$ and $F(1,494)$ 87.563, $p<0.0001$ respectively). However, pre-treatment of saline diminished strain differences between BALB/c and C57BL/6J mice ($F(1,461)$ 0.127, $p=0.721$), likely reflecting the injection effect during acquisition.

DISCUSSION

Our results demonstrate distinct strain-dependent differences in the acquisition, consolidation, retrieval and extinction of fear memories. The highly stress sensitive and emotional BALB/c mice generalize their fear memory, which is expressed by similar amounts of freezing during context and cue episodes (Figures 1 and 3, day 3: saline). In contrast, the less stress sensitive and less emotional C57BL/6J mice exhibit more freezing during cue than context episodes. C57BL/6J mice specifically identified the cue as predictor of the aversive experience. Corticosterone has opposite effects on fear memory depending on the mouse strain and the time of injection. In C57BL/6J mice, pre-acquisition corticosterone enhances cue fear memory and prevents cue extinction. In BALB/c mice however, post-acquisition corticosterone destabilizes consolidation of fear memory, allowing faster extinction. Remarkably, pre-acquisition corticosterone counteracts this weak retrieval on day 3, while showing similar fast extinction as post-acquisition treated BALB/c mice one day later. These data identify genetic background and time of corticosterone application as modifiers of fear memory, a finding with interesting translational implications for PTSD and other anxiety disorders.

BALB/c and C57BL/6J mice show different context vs. cue related fear acquisition and memory

The fear conditioning paradigm uses six pairings of combined auditory and visual stimulus stimuli (i.e, the cue) with aversive shocks alternating with "context only" episodes. Freezing as fear response to the environment where the aversive shock has been received (i.e., the context) is related to hippocampal information processing while the cue-related fear response is controlled by the amygdala [13;14]. In support and extension of our previous findings [11], BALB/c and C57BL/6J mice display different patterns of fear acquisition and memory in the alternating context and cue episodes.

During acquisition, C57BL/6J mice display more freezing during cue episodes that precede and predict the shock than BALB/c mice. BALB/c mice are more active during this cue and freeze relatively more during the intermittent context episodes. Thus, C57BL/6J mice respond rather to discrete (cue) than more complex stimuli. In line with this reasoning, we have previously shown in an appetitive learning task that C57BL/6J mice preferentially use a visual stimulus driven learning strategy compared to the predominant spatial and emotionally biased learning, which is favoured by BALB/c mice [12]. These findings indicate a remarkable strain-dependent behavioural performance and cognitive processing. It possibly reflects the active (increased activity, escape) coping style

displayed by BALB/c as opposed to the passive coping style (freezing) of C57BL/6J related to fear. These distinct behavioural strategies are likely to affect later consolidation and thus contribute to memory formation.
Indeed, BALB/c and C57BL/6J mice also show distinct fear memory. While C57BL/6J mice display higher cue (70%) than context (about 20-30%) related freezing during memory testing, BALB/c mice generalize freezing over context and cue during memory testing on day 3. These differences in cue and context related fear memory between BALB/c and C57BL/6J mice forward strain-specific abilities of identifying the cue as predictive stimulus for the aversive experience and most likely represent the strain-specific contribution of hippocampus and amygdala to fear memory.
Besides generalized fear memory, BALB/c mice display a strong extinction of contextual and cued fear memory on day 4. Similar improved extinction of fear memory of BALB/c mice compared to four inbred mouse strains has been reported in another paradigm [17]. This facilitated extinction of fear behaviour has been ascribed to corticosterone [18;19]. We propose that the high post-retrieval corticosterone concentrations we observed in BALB/c mice [11] are causally related to the facilitated extinction of fear memory. Indeed, Cai et al [3] also reported the same results with post-retrieval injections of corticosterone, which will be discussed below.

Fast non-genomic effects of corticosterone during acquisition
Injections *before* and *after* acquisition further differentiate subsequent fear conditioning effects between strains as well as the spatio-temporal action of corticosterone. Pre- and postacquisition treatments are expected to influence the consolidation, but corticosterone treatment *before* is the only one to have an effect on acquisition. An important observation is the apparent absence of corticosterone-induced behavioural effects during acquisition. This might lead to the idea that corticosterone treatment is ineffective and thus, devoid of fast non-genomic effects [20;21]. However, when comparing the effects of corticosterone treatment *before* and *after* acquisition on later memory/extinction testing on day 3, another argument becomes more likely. For BALB/c mice, corticosterone treatment *before* has little effect on fear memory while corticosterone treatment *after* acquisition has a strong impairing effect on fear memory. For C57BL/6J mice, corticosterone *before* clearly increases fear memory, specifically for the cue, while corticosterone after acquisition does not have such clear effect. In fact, the timing of corticosterone action just differs by 17 minutes. It is therefore (1) more likely that the difference in fear memory / extinction between corticosterone treatment *before* and *after* originates from a difference in corticosterone levels and its action during acquisition, and thus, (2) to conclude that corticosterone treatment before

acquisition does have fast non-genomic effects on the acquisition process, most likely via the low affinity membrane bound mineralocorticoïd receptor (Karst et al 2005; Joels et al. 2008). In the case of BALB/c mice, these fast effects seem to diminish the effect of later high corticosterone levels during consolidation, i.e., counteract the destabilized consolidation and weak retrieval. In C57BL/6J mice, high corticosterone during acquisition potentiates fear memory for the cue.

Long-term corticosterone actions differ between strains: corticosterone treatment increases cue memory in C57BL/6J mice, but decreases cue and context fear memory in BALB/c mice

There is an intriguing dual action of corticosteroids: they facilitate memory consolidation, but behavioural responses that are of no more relevance are extinguished [8;9;22]. Using a forced extinction paradigm in a one step-through inhibitory avoidance test, this effect appeared to be specific for corticosterone [8]. In the present study, we report the strain-dependency of this dual action of corticosterone: the already shortly discussed augmented cue fear memory in C57BL/6J mice and less fear memory in BALB/c mice. The observed increase in cued fear memory in C57BL/6J mice likely reflects a well known facilitating effect of increased glucocorticoid receptor (GR) activation seen in other tasks using this mouse strain [12;23;24]. In BALB/c mice, post-acquisition corticosterone treatment does not affect freezing in the first cue and context episodes on day 3, but reduces freezing in the later episodes of retention testing on that day, suggesting that consolidation is less stable due to corticosterone treatment. The observed corticosterone-induced decrease in fear memory and thus improved extinction corresponds to other studies in which corticosterone facilitates extinction in an appetitive operant conditioning task [9]. Interestingly, post-retrieval injections of corticosterone in C57BL/6J mice [3] also results in enhanced extinction of freezing. We may assume that the high endogenous post-retrieval corticosterone concentrations, as reported by Brinks et al., 2008, modify subsequent memory reconsolidation and extinction processes.

Studying strain- and time-dependent effects, we did not address the issue of possible dose-dependent effects of corticosterone. Fear of BALB/c and C57BL/6J mice, with and without corticosterone, does not reflect a linear gradient that is characteristic for fear memories (Sandi, Pinelo-Nava, 2007). Post-retrieval injected corticosterone, that supposedly modifies re-consolidation of fear memory also nicely follows a linear dose-response relationship and impairs extinctions (0.3 mg up to 10 mg/kg corticosterone; Cai et al 2006). In the present study, corticosterone underlined the strain-dependent fear behaviour: it strengthened the already existing strong distinction between context- and cue-related fear in C57BL/6J mice and destabilized memory and facilitated extinction in BALB/c mice. It seems unlikely, that further increasing the dose of

corticosterone in C57BL/6J mice would result in processing of fear comparable to the high stress sensitive, high corticosterone secreting BALB/c mouse.
Rey and colleagues [25] provide mechanistic data on how corticosterone could decrease fear memory in BALB/c mice. While corticosterone is known to enhance LTP, the cellular mechanism believed to underlie learning and memory, in the hippocampal CA1 area of C57BL/6J mice [26], moderate and high doses of corticosterone decrease the spike amplitude in hippocampal slices of BALB/c mice. This decrease could lower the number of action potentials, therefore impair LTP and in parallel decrease (fear) memory [17;27;28].

Molecular mechanisms contributing to fear acquisition and memory
Distinct HPA reactivity of BALB/c and C57BL/6J mice and thus corticosterone levels, would likely contribute to distinct corticosterone related molecular mechanisms in the hippocampus and amygdala. For example, Yilmazer-Hanke and colleagues [29] found strain differences in corticosterone related NMDA and GABA receptor expression in the amygdala. The NMDA receptor in the amygdala, which facilitates the magnitude of contextual fear [30] seems to be higher expressed in BALB/c than C57BL/6J mice. In addition, GABA receptors, which are more abundant in the amygdala of C57BL/6J mice, specifically affect fear expression to conditioning stimuli during acquisition [31] and memory testing [32].
The sympathic nervous system, in collaboration with the glucocorticoid stress system, is also involved in fear related memory formation [33]. Hu and collegues [34] have shown that increased norepinephrine function in the amygdala lowers threshold for LTP and thus providing a molecular mechanism for the well known enhancing effect of emotion on learning and memory. In contrast, Maroun and Akirav [35] reported that increased arousal via activation of noradrenergic receptors in the amygdala is detrimental for the consolidation processes. This discrepancy suggests that emotional load or norandrenergic activity can both facilitate and impair cognitive functioning.
BALB/c mice, which are highly emotional and display higher amygdala beta-adrenoceptor expression [36] and noradrenergic activity [37] compared to C57BL/6J mice, display less stable consolidation or earlier onset of extinction than C57BL/6J mice. This might suggest that very high emotional or noradrenergic involvement in the BALB/c mice, and less emotional and noradrenergic involvement in C57BL/6J mice contributes to distinct fear related memory and extinction processes.

Timing of corticosterone treatment is important in revealing its effects on fear behaviour

As underlined by principal component analysis (PCA), pre-acquisition corticosterone mainly affects retention of fear behaviour in C57BL/6J mice, while post acquisition corticosterone predominantly affects retention of fear behaviour in BALB/c mice. Relevance of timing has been shown for the corticosterone effects on LTP [26;38]. In these studies, corticosterone facilitated LTP when given in the same time domain as the tetanus and then, even regulated beta-adrenergic modulation of LTP. We not only show that this timing effect has a cognitive functionality, but that it also differs between mouse strains.

Why corticosterone would affect fear behaviour in different time domains in BALB/c and C57BL/6J relies most likely on the background of the neuroendocrine and behavioural phenotype [12], and thus the aforementioned differences in fast corticosterone actions.

Conclusion

Conditioning of fear and testing of fear memory in an alternating cue and context set-up proves to be a promising approach towards a mouse model for PTSD and anxiety disorders. Strain-specific formation and extinction of fear memories, the importance of timing of corticosterone actions in BALB/c and C57BL/6J mice present a tool to study specific aspects of stress-related psychiatric disorders. (1) C57BL/6J mice might serve to address the strengthening of emotional memory related to certain cues under influence of stress and stress hormones, and thus the development of PTSD, while (2) BALB/c mice might serve as model to study strong context-related, rather generalized fear and the process of how stress hormones decrease fear memories, as also observed in successful treatment of PTSD and patients with phobias. (3) The neuroendocrine and behavioural phenotype of both strains (Brinks et al. 2007) is promising for the identification of biomarkers that are predictive for vulnerability or resilience to stress-related anxiety disorders.

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Chapter 6

Mineralocorticoid receptors control emotional arousal and fear extinction

Brinks V*, Berger S[#], Gass P[¥], de Kloet E.R*, Oitzl M.S*

* Division of Medical Pharmacology, LACDR/LUMC, Gorlaeus Laboratories, P.O. Box 9502, Leiden University, 2300 RA Leiden, The Netherlands.
[#] German Cancer Research Center, Division Molecular Biology of the Cell I, Im Neuenheimer Feld 280, Heidelberg, Germany.
[¥] Central Institute of Mental Health, J 5, D-68159, Mannheim, Germany

Submitted at hormones and behavior

ABSTRACT

Glucocorticoids, such as corticosterone, are well known modulators of emotion and cognition. Corticosterone binds to two nuclear receptor types, the high affinity mineralocorticoid receptor (MR) and the tenfold lower affinity glucocorticoid receptor (GR). Both receptor types coordinate the action of corticosteroids in endocrine and behavioral functioning and have established roles in emotion and cognition. Here we studied how changing the MR/GR balance due to MR ablation will affect unconditioned and conditioned behaviour under stress. Behavioural response towards novelty was tested in female mice with forebrain-specific inactivation of MR gene ($MR^{CaMKCre}$, 4 months old) and control littermates: after 5 minutes of restraint stress mice were subjected to modified holeboard testing. After a one-week interval, the same mice performed a fear conditioning procedure to study the development and extinction of fear memories. Plasma corticosterone was measured at different time points during both experiments. Only when pre-stressed, $MR^{CaMKCre}$ mice displayed higher arousal and less locomotor activity in a novel environment than control mice. The MR ablation furthermore enhanced cue-related fear acquisition and persistently increased fear memory specific for the context, resulting in a lack of extinction. Interestingly, during this time period corticosterone levels of $MR^{CaMKCre}$ mice were 40% higher than controls exposed to the same conditions.

We conclude that under stress, deletion of forebrain MR function increases emotional arousal resulting in increased anxiety-related responses. Fear memories appear to be enhanced due to stronger consolidation and resistance to extinction probably caused by the higher corticosterone concentrations acting via GR in the absence of forebrain MR.

INTRODUCTION

The involvement of the glucocorticoid stress system in control of emotional arousal and cognitive performance has been well established. The major glucocorticoid hormone, corticosterone in rodents and cortisol in humans, binds to two steroid receptor types in the brain: the high affinity mineralocorticoid (MR) and the tenfold lower affinity glucocorticoid receptor (GR). Both receptors are located in brain areas involved in emotional regulation, learning and memory processes.

GR and MR mediate complementary and in part overlapping actions of corticosterone in endocrine and behavioural functioning. Corticosterone facilitates the recovery from stress by a negative feedback action via GR [1-3] and also facilitates memory consolidation [4-6]. MR mediates the regulation of pulsatile corticosterone secretion during the basal ultradian rhythm and has an important function in the control of the onset of the stress response [3;7-9]. MR is involved in the control of behavioural reactivity in a novel situation [5;10-12] and coordinates most likely together with GR, subsequent memory processes [11-13]. Interestingly, both MR and GR have been shown to facilitate anxiety-like responses induced by restraint [14].

Previously we have demonstrated that distinct pharmacological activation of MR and/or GR differentially affects emotional and cognitive processes in mice. This underlines the importance of a concerted MR- and GR-mediated action of corticosterone in behavioural expressions [15;16]. However, the individual contribution of both receptor types in emotional and cognitive functioning under stressful conditions needs to be further elucidated. The recently generated mice with brain-specific MR ablation [MRCaMKCre, 12] provide a unique opportunity. In these mice, the MR gene is inactivated in the limbic forebrain using the Cre/loxP-recombination system. Berger and colleagues [12] have previously shown that $MR^{CaMKCre}$ mice are impaired in learning the water-maze task, show deficits in working memory on the radial maze, are hyperreactive towards a novel object but appear to display normal anxiety-like behavior.

Here we elaborate on these results by extensively testing for unconditioned behaviour in acutely stressed $MR^{CaMKCre}$ and control mice using the modified holeboard [16]. In a second experiment we study the influence of limbic MR inactivation on conditioned fear behaviour and memory. Apparently, performance in a standard fear conditioning task was not affected by the lack of forebrain MR (Berger et al., 2006). However, based on the proposed function of MR, we expect specific changes in the acquisition and extinction of fear memories. We use a fear conditioning protocol that allows testing acquisition, consolidation, retrieval and extinction of fear memories for both context and

cue in one procedure. For both experiments, continuous in depth behavioural analysis is combined with the determination of plasma corticosterone concentrations at different time points.
We expect that the behavioural response to novelty is altered in $MR^{CaMKCre}$ mice. Extensive behavioural testing will allow to specify the affected behaviours. We furthermore hypothesise that such altered unconditioned behaviour extends its influence to conditioned behaviour, e.g., cognitive processes involved in different phases of fear memory, and that an altered endocrine regulation of plasma corticosterone concentrations in $MR^{CaMKCre}$ mice might strengthen GR function.

MATERIAL AND METHOD

Animals

$MR^{CaMKCre}$ mice (female, 4 months) were generated as described before [12, supporting information on PNAS website] and together with female control littermates of the C57BL/6j strain (n=8) obtained from the German Cancer Research Center (Heidelberg, Germany). After arrival, the mice were housed individually in the experimental room with sawdust bedding, water and food *ad libitum*, at 20^0C with controlled humidity under a 12 h: 12 h light/dark cycle (lights on at 08.00 hrs.) for one week. Experiments were performed between 09.00 and 13.30 hours (during resting phase) and were approved by the committee on Animal Health and Care from the Leiden University, The Netherlands, in compliance with the EC Council Directive of November 1986 (86/609/EEC) for the care and use of laboratory animals.

Experiment 1: Stress-induced unconditioned behavioural response in the modified holeboard

Apparatus:

The modified holeboard consisted of a grey PVC box (50x50x50cm) with a grey PVC centerboard (37x20cm) on which ten dark grey cylinders (4 cm height) with a bottom grid were staggered in two lines of five [15;17]. During testing, a camera was placed above the setting to allow later pathway reconstruction from video. Light intensity of the experimental room was set at 80 Lux and a 20 dB background noise originating from a radio was present.

General experimental procedure

To induce a stress response, mice were subjected to 5 minutes of restraint, which involved placing them in a narrow container that still allowed breathing but no further movement. This method has been shown to activate the HPA-axis

and enhance corticosterone concentrations in mice [18;19]. Immediately after restraint, the mice were tested for unconditioned behaviour in the modified holeboard for 5 minutes. All mice were placed in the same corner facing the wall and tested individually. The setup was cleaned with normal tap water between trials.

Behavioural observation

In depth behavioural observation during modified holeboard testing was performed using a semi-automatic scoring system (Observer, Noldus, Wageningen, The Netherlands). The total number of rearing, sitting and walking, as well as the time on the centerboard, sitting, walking and grooming were determined. Walking patterns were later reconstructed from videos (Ethovision, Noldus, Wageningen, The Netherlands).

Experiment 2: Conditioned response- Fear conditioning

Fear conditioning apparatus:

Combined auditory and contextual fear conditioning was performed in a conditioning chamber (25 cm x 25 cm) with black Plexiglas walls (35 cm high) fitted with a 3 cm transparent rim. A speaker was fixed into one of the walls (25 cm high) connected to a tone generator (70dB). Stainless steel bars on the bottom of the chamber (n=37, 5 mm diameter, spaced 5 mm) were connected to a shock (0.4mA). Tissues were placed in a drawer under the bars to collect faeces and urine during testing. A white light source (260 lux) was placed 20 cm above the conditioning chamber together with a camera for later behavioural analysis from video tape. A radio on the other side of the experimental room produced 20 dB of background noise and the light intensity of the experimental room was 90 lux. After each animal, the chamber was cleaned with normal tap water and allowed to dry, and the tissues in the container were replaced by new clean ones.

Fear conditioning procedure:

The fear conditioning experiment started one week after holeboard testing. The fear conditioning paradigm was used to differentiate between context and cue-related behavioural responses in the same setting [20]. Conditioning (day 1) included three minutes of baseline recording followed by 6 light/tone (CS) + shock (US) pairings with a one minute interval. Light and tone were paired for 20 seconds and an electric footshock was administrered during the last two seconds. Two minutes after the last pairing, mice returned to their homecage. At 48 (day 3) and 72 hrs (day 4) after the initial conditioning, the same procedure was repeated without shocks to test memory and extinction resulting from repeated context and additional cue exposure. The procedure involved 12

minutes of behavioural testing for each mouse per day and was performed between 9.00 a.m. and 13.00 p.m.

Behavioural assessment

Freezing behaviour was recorded as parameter of fear behaviour. Freezing was defined as immobility of the body including the head without any interaction with the environment. We also measured the total number of rearing, sitting and walking, the time sitting, walking and grooming to determine (i) differences in unconditioned response to the fear conditioning apparatus between $MR^{CaMKCre}$ and control mice, and (ii) differences in behavioural structure by principal component analysis (PCA).

All behaviours were scored from video tape using a semi automatic scoring program (The Observer 4.1, Noldus, Wageningen, The Netherlands). Walking patterns during first exposure to the fear conditioning apparatus were reconstructed from videotape using Ethovision (Noldus, Wageningen, The Netherlands).

Corticosterone measurements

Plasma corticosterone concentrations were determined at 5 different time points during the experiments. For experiment 1, basal levels were measured 1 day before the modified holeboard testing (between 9.00 and 10.00 a.m.) and stress-induced levels were determined 30 minutes after the start of the restraint (i.e., 20 minutes after modified holeboard testing). For experiment 2, basal levels were re-examined one day before the conditioning took place (between 9.00 and 10.00 a.m.). In addition, conditioning-induced corticosterone concentration was determined 30 minutes after the start of conditioning on day 1, and memory testing-induced corticosterone levels were measured 30 minutes after the start of the last day of memory testing (day 4). Blood samples were obtained by a small incision at the base of the tail, plasma was isolated and corticosterone concentrations were measured using a commercially available radio immune assay (MP Biomedicals Inc., CA, USA).

Statistical analysis

Data are represented as mean $\pm$ SEM. For experiment 1, a multivariate analysis was performed to determine group differences in unconditioned behaviour when exposed to the holeboard. Post-hoc tests with Bonferroni correction specified the statistically significant behaviours. For experiment 2, similar statistics as described above were used to measure group differences in unconditioned behaviour during the first exposure to the fear conditioning setup on day 1. Main effects of group ($MR^{CaMKCre}$, control) and day (day 1, 3 and 4) on freezing behaviour were determined with a general linear model-repeated

measures (GLM) over average freezing per testing day. Further GLM analyses determined group and day effects over context only or additional cue exposure. Progression of freezing behaviour over the different intervals per testing day was also determined using GLM.
To measure group differences in overall behavioural structure, all behavioural parameters of experiments 1 and 2 were subjected to a Principal Component Analysis (PCA) with Varimax rotation and Kaiser normalisation. Variables with a communality over 0.7, that is of which at least 70% variance was explained by the extracted factors, were included. Factors with an eigenvalue over 1 were accepted, making the number of extracted factors not pre-defined. Further ANOVA's on factor loadings were performed to determine group differences.
Group differences in plasma corticosterone concentrations were determined with a Two-way ANOVA. Significance for all statistical testing was accepted at $p \leq 0.05$.

RESULTS

Experiment 1: Stress-induced unconditioned behavioural response in the modified holeboard

Unconditioned behaviour

Following 5 min of acute restraint-stress, multivariate analysis revealed a significant difference in behavioural parameters between $MR^{CaMKCre}$ and wild-type control mice during modified holeboard exposure ($F(6,8)$ 3.991, $p=0.038$, table 1 for significant behaviours). $MR^{CaMKCre}$ mice displayed twofold more time grooming and sitting, and twofold less time walking compared to controls.

	$MR^{CaMKCre}$, stressed	**control, stressed**
Time grooming (%)	47.68 ± 6.7 **	23.42 ± 4.2
Time sitting (%)	2.48 ± 0.3 **	1.23 ± 0.2
Time walking (%)	49.83 ± 7.1 **	75.34 ± 4.4

Table 1. Behaviour of $MR^{CaMKCre}$ and control littermates during 5 minutes of modified holeboard exposure following acute restraint stress. ** $p \leq 0.01$ compared to control.

Walking patterns

Walking patterns (fig 1) during modified holeboard testing supported behavioural data showing less movement in $MR^{CaMKCre}$ than wild-type mice. Both genotypes predominantly walked along the walls (thigmotaxis).

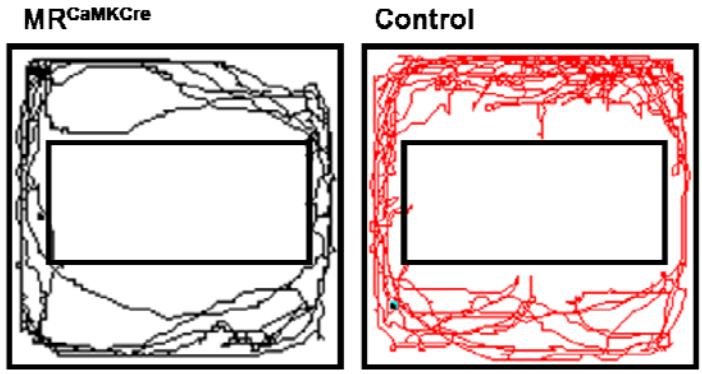

Figure 1. Representative walking patterns of $MR^{CaMKCre}$ (left) and control mice (right) during 5 minutes of modified holeboard exposure following acute restraint stress. The outer square indicates the walls of the setting, the inner square shows the position of the holeboard.

Follow-up experiment: comparing behaviour of stressed and non-stressed mice

C57BL/6J mice are the backcross strain of the $MR^{CaMKCre}$ mice, and of the control littermates. To determine to what extent the restraint stressor itself influenced unconditioned behaviour in the modified holeboard, we performed an additional experiment. Naive and stressed C57BL/6J mice (female, n=8/group) were tested for unconditioned behaviour during 5 minutes of modified holeboard exposure. Naive mice were directly taken from their homecage and placed into the setup. Restraint stress was done as described above.

Multivariate analysis revealed significant differences in unconditioned behaviour of naive and restraint-stressed C57BL/6J mice ($F(6,8)$ 5.258, $p=0.016$, table 2).

C57BL/6J	Stressed	Naive
Time grooming (%)	22.86 ± 7.5*	3.02 ± 0.4
Time walking (%)	68.45 ± 10.4*	93.37 ± 1.6
No. walking	8.25 ± 1.3*	4.50 ± 0.5
Sitting (%)	8.69 ± 3.8	3.60 ± 1.7

Table 2. Behavioural parameters of naive and stressed C57BL/6J mice during 5 minutes of holeboard exposure. * $p \leq 0.05$ compared to naive.

Applying a stressor prior to behavioural testing in the modified holeboard increased the time grooming seven fold, walking by 25% and the number of walking bouts two fold. There was a trend towards more sitting in stressed mice. In conclusion, stressed C57BL/6J mice of this experiment and the stressed controls of the previous experiment show time grooming and walking during the holeboard procedure to a similar extent.

Unconditioned behaviour in the fear conditioning box

Before starting the fear conditioning paradigm, unconditioned behaviour in the conditioning setup was examined in $MR^{CaMKCre}$ and wild-type control mice. During the first three minutes exposure to the fear conditioning apparatus, multivariate analysis showed similar behaviour for $MR^{CaMKCre}$ and control mice ($F(6,9)$ 1.790, $p=0.209$), with comparable walking patterns (fig 2).

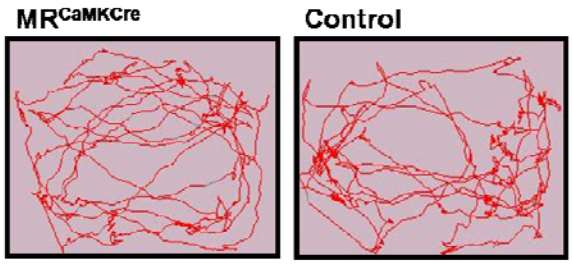

Figure 2. Representative walking patterns of $MR^{CaMKCre}$ (left) and wild-type control mice (right) during three minutes of exposure to the fear conditioning setup.

Experiment 2: Conditioned response - Fear conditioning

Freezing behaviour during acquisition and fear memory / extinction testing

Fear expression and fear memory is inferred from the freezing response during the subsequent context and cue episodes on the three testing days. Comparing the percentage of freezing responses over all days of testing revealed that $MR^{CaMKCre}$ mice displayed more freezing compared to controls (main effect of genotype $F(1,42)$ 24.412, $p<0.0001$) and that freezing behaviour differed between $MR^{CaMKCre}$ and controls depending on the day of testing ($F(2,42)$ 78.246, $p<0.0001$).

In addition, freezing behaviour significantly progressed over days ($F(14,588)$ 35.437, $p<0.0001$). This progression depended on the genotype ($F(14,588)$ 1.961, $p=0.019$), as well as the day of testing ($F(28,588)$ 11.993, $p<0.0001$), and differed significantly between $MR^{CaMKCre}$ and controls on testing days (genotype*day $F(28,588)$ 2.793, $p<0.0001$).

Context- and cue- induced freezing responses:

During acquisition, freezing of $MR^{CaMKCre}$ and control mice increases over time ($F(11,154)$ 2.924 $p=0.002$, figure 3). This increase is due to the progression in freezing behaviour during cue exposures ($F(5,70)$ 2.492 $p=0.039$) and significantly differs between genotypes ($F(1,14)$ 15.187 $p=0.002$; table 3). Amount and progression of context-induced freezing was similar between genotypes.

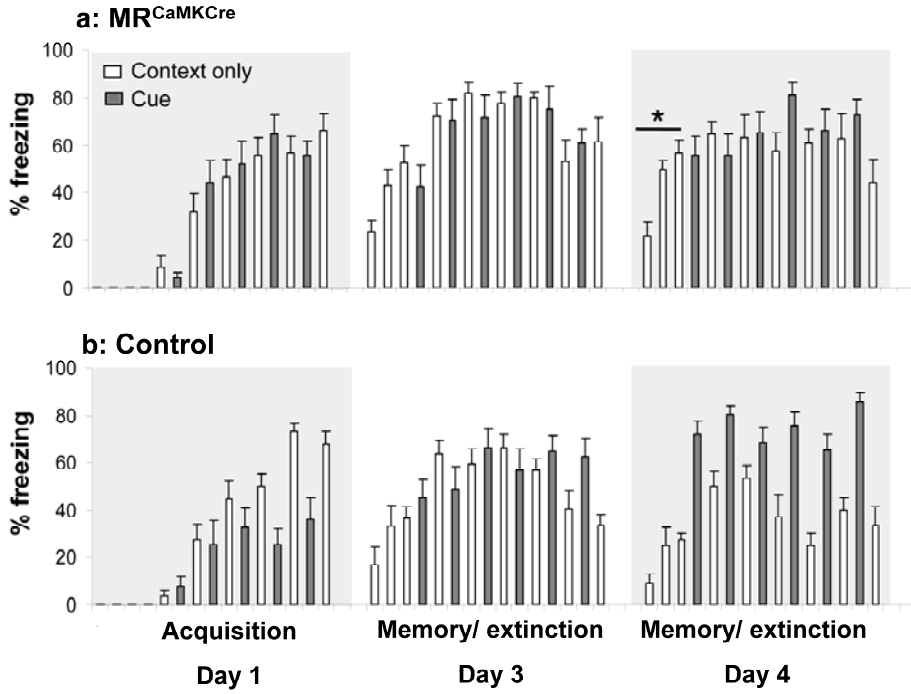

Figure 3. Percentage of freezing over the 15 intervals of context and cue exposures per testing day of $MR^{CaMKCre}$ (A) and wild-type control mice (B). White bars: during context exposure, dark grey bars: additional cue on. ** $p<0.01$ compared to control.

		$MR^{CaMKCre}$	Control
Day 1: acquisition (without first 3 intervals)	Context	44.43 ± 3.88	44.39 ± 4.06
	Cue	36.90 ± 4.53 **	21.24 ± 3.35
Day 3: memory/ extinction	Context	61.85 ± 2.86 **	45.37 ± 2.68
	Cue	70.02 ± 3.12*	57.46 ± 3.40
Day 4: memory/ extinction	Context	55.45 ± 2.58 **	33.51 ± 2.47
	Cue	66.16 ± 3.35	74.54 ± 2.38

Table 3. Average freezing behaviour (percentage of time) for $MR^{CaMKCre}$ and control mice per testing day during context only or additional cue episodes. * $p< 0.05$ and ** $p< 0.01$compared to controls.

During memory testing on day 3, $MR^{CaMKCre}$ and control mice showed similar freezing during initial context exposure and similar freezing during the first cue exposure. No significant difference was present in the time course of freezing behavior over the intervals, however overall, $MR^{CaMKCre}$ mice froze more than

controls (F(1,14) 24.908 p=0.000). This increase was mainly due to more freezing to context (F(1,14) 22.54 p=0.000) and to lesser extent to more cue-induced freezing (F(1,14) 6.729 p=0.021).
During memory testing on day 4, $MR^{CaMKCre}$ mice displayed more context-induced freezing behaviour compared to controls both during initial exposure (first three intervals: F(1,14)15.829 p=0.001) and later context intervals (F(1,14)16.147 p=0.001). Over time, freezing behaviour progressed significantly (F(14,196) 4.002 p = 0.000), however differently between strains (F(14,196) 4.002 p = 0.000).

Principal component analysis
Principal Component Analysis on behavioural data of experiments 1 and 2 was performed to determine differences in behavioural structure between $MR^{CaMKCre}$ and control mice. This analysis resulted in the extraction of two factors explaining 89% of total variance (table 4). Factor 1 included variables measured in stressed mice during modified holeboard exposure and represents arousal and locomotor activity. Factor 2 included behaviours measured during the stressful procedure of fear conditioning and represents fear behaviour. Further ANOVAs revealed group differences for both factors (factor 1: F(1,623) 9.262, p=0.002, factor 2: F(1,623) 16.908, p<0.0001), indicating high arousal, low locomotor activity and high fear behaviour in $MR^{CaMKCre}$ mice under stress.

	Variables / factor loading
Factor 1: Arousal and locomotor activity (modified holeboard)	Walking / -0.999 Grooming / 0.945 Sitting / 0.928
Factor 2: Fear behaviour (fear conditioning)	Walking / 0.924 Freezing / -0.922

Table 4. Factors extracted from behavioural data of experiment 1 and 2 using principal component analysis with Varimax rotation and Kaiser normalisation.

Corticosterone concentrations
Plasma corticosterone concentrations were determined at different time points during the experiment to determine if MR depletion would affect endocrine regulation of the glucocorticoid stress system. $MR^{CaMKCre}$ mice did not differ in basal morning corticosterone concentrations compared to controls, independent of prior stress one week earlier (fig 4). In addition, $MR^{CaMKCre}$ mice also did not differ in stress-induced corticosterone concentrations, either due to restraint or exposure to shocks during the fear conditioning procedure on day 1. However, $MR^{CaMKCre}$ mice did show a 40% higher corticosterone concentration when tested for fear memory on day 4 of fear conditioning (F(1,14) 8.133, p<0.0001).

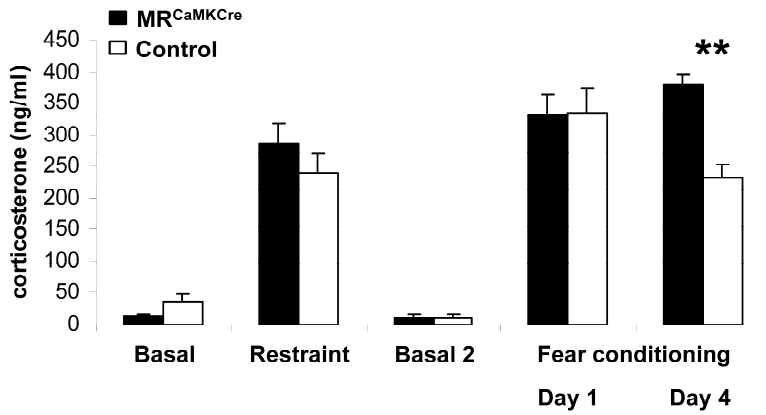

Figure 4. Plasma corticosterone concentrations measured one day before modified holeboard testing (basal), 30 minutes after restraint stress (i.e.; 20 min after modified holeboard testing), one day before fear conditioning (basal 2) and 30 minutes after the start of conditioning on day 1 and memory testing on day 4. Black bars: $MR^{CaMKCre}$, white bars: control. ** $p<0.010$.

DISCUSSION

Concerted MR and GR mediated actions are essential for correct behavioural functioning. Using recently generated mice with brain-specific MR ablation, we specify which unconditioned and conditioned behavioural aspects are vulnerable to long-term MR ablation in the limbic forebrain. $MR^{CaMKCre}$ mice displayed increased emotional arousal (grooming) and decreased their locomotor activity when exposed to a novel environment, although only when stressed prior to the test. Limbic MR ablation furthermore enhances cue related fear acquisition and persistently increases fear memory that is specific for the context. Principal component analysis confirms these behavioural differences between $MR^{CaMKCre}$ and control mice. Interestingly, plasma corticosterone concentration of $MR^{CaMKCre}$ mice was increased compared to controls after fear memory / extinction testing. We consider this as indication that corticosterone strengthens the action of GR on memory consolidation, especially since $MR^{CaMKCre}$ mice show GR upregulation [12].

MR mediates corticosterone action in unconditioned behaviour only under stimulated conditions

$MR^{CaMKCre}$ mice showed increased emotional arousal and less locomotion in a novel environment, although only when pre-stressed. Naive $MR^{CaMKCre}$ mice

placed in the novel environment of the fear conditioning setup behaved comparable to controls. Indeed, Berger and colleagues had reported no difference in unconditioned behaviour between naive $MR^{CaMKCre}$ and control mice when tested in the open field [12]. MR overexpression also did not affect this parameter in naive mice tested in the open field [13]. Increasing the challenge, reveals the involvement of MR in behavioural reactivity. When introducing an unknown object into the familiar environment, both $MR^{CaMKCre}$ and MR overexpressing mice differed in exploration of this object compared to controls [11;12]. In addition, when extending the number of exposures to the open field, or when using the elevated plus maze and light dark box, MR overexpressing mice displayed less anxiety compared to controls [11;13]. It therefore appears that under relative unstimulated conditions MR does not influence unconditioned behaviour. However, when applying novelty to an already habituated setting or increasing the aversiveness of a task, and thus, stimulating the stress system, MR does influence anxiety and exploration parameters [11;21].

The observed stress dependency in MR mediated behavioural effects had also been demonstrated by Oitzl and colleagues (1994). While MR antagonism in non-stressed rats produced rather a trend for different behavioural reactivity towards a novel environment, it significantly inhibited behavioural reactivity when corticosterone levels were elevated [10]. This strengthens our conclusion that MR mediates behavioural response predominantly under stimulated conditions.

Given the MR characteristics of high affinity and thus, already activation of MR at low concentrations of corticosterone, these results might seem puzzling. High GR function, possibly due to the shift in MR:GR balance or in relation to GR upregulation in $MR^{CaMKCre}$ mice [12], could explain part of the behavioural differences between $MR^{CaMKCre}$ and control mice. The contribution of GR has been correlated with less exploration in rats when exposed to a novel environment [22], and increased emotional arousal in mice [23]. In addition, non-genomic MR mediated effects might also contribute to the observed behavioural differences between $MR^{CaMKCre}$ and control mice [24]. Previous exposure to a stressor in our experiments could activate the low affinity membrane located MR and thus affect behavioural response. We conclude that the functionality of the balanced MR:GR receptor system reveals itself in conditions of stress.

MR knockout facilitates cue learning, enhances contextual memory and impairs endocrine and behavioural adaptation to the safe situation

A distinct behavioural response of $MR^{CaMKCre}$ mice was absent when introduced to the novel environment of the fear conditioning apparatus, however, they had

not been pre-stressed in this task. During acquisition of fear memory $MR^{CaMKCre}$ mice quickly developed a stronger and faster increase of freezing behaviour to the cue than controls. This could imply that the MR ablation facilitates stimulus specific learning of a stressful event. On the other hand, the high percentage of fear behaviour and thus inhibition of locomotion is similar to the stress-induced behavioural response in the modified holeboard. Thus, in both conditions, $MR^{CaMKCre}$ mice show high passive coping in response to a stressful event. The increased tendency of $MR^{CaMKCre}$ mice for passive behaviour had been observed previously (Berger et al [12]. These data support the idea that loss of brain MR function increases passive coping or immobility during a stressful situation. Since our data do not point to a general effect on acquisition, but rather to the specificity of freezing towards the cue, an additional cognitive component, perhaps due to GR activation/overexpression seems likely.

Besides distinct expression of fear during acquisition, $MR^{CaMKCre}$ mice displayed a persistent increase in contextual fear memory throughout testing. Since freezing during context episodes of fear-acquisition did not differ between genotypes, it seems likely that increased contextual memory reflects enhanced consolidation or retrieval of spatial stimuli. In literature, increased MR function has been related to improved (spatial) memory [11;25;26], while less MR function had been correlated with impaired spatial memory [12;27]. This seems contradicting our present data. However, specifically in learning tasks, behaviour has to be discussed in relation to the functionality of both receptors, MR and GR. Task-dependent intensity of stress, together with the endocrine corticosterone response can strongly affect cognitive performance [15;28]. Indeed, $MR^{CaMKCre}$ mice have increased plasma corticosterone concentration during the later stages of memory testing, and they show increased fear memory. Enhanced corticosterone levels imply different onset, amplitude and offset of the endocrine stress response. The expected cognitive effect is strengthening of GR function, and thus, facilitation of memory consolidation [6].

In addition to high contextual fear expression during initial memory testing, we also show that $MR^{CaMKCre}$ mice did not decrease context-related freezing over time compared to controls. $MR^{CaMKCre}$ mice still showed very high levels of contextual fear behaviour on testing day 4, while control mice had less contextual freezing behaviour and clearly differentiated between context and cue stimuli. This finding is in line with several studies which have shown that less MR function influences behavioural adaptation to changes within the task, e.g. removing the escape platform from the watermaze [5;12;29]. Furthermore, MR was implied in the extinction of passive avoidance behaviour [30], supporting the role of MR in fear-related extinction.

Together, our data shows that limbic MR ablation interferes with behavioural and endocrine adaptation to a changing situation. $MR^{CaMKCre}$ mice are less able

or slower to adapt to the "new and safe" situation in which light and tone cues do not longer predict the aversive consequence of an electric shock. Thus, in the absence of forebrain MR functions, individuals appear to be less capable in assessing the safe from unsafe condition. This cannot automatically be extrapolated to similar effects due to acute MR blockade.

Conclusion

We show here that loss of MR in the forebrain of mice enhanced emotional arousal and supported a passive coping strategy during or after stress. $MR^{CaMKCre}$ mice showed enhanced fear to cue during acquisition, increased contextual fear memory and impaired behavioural and endocrine adaptation to changing demands of the task. Increased GR function appears to be contributing to the consolidation of fear behaviour, and thereby, supporting the conclusions drawn in previous literature on the relevance of a coordinated MR/GR action.

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Chapter 7

General discussion

Outline

7.1 Do corticosteroids affect emotion and cognition via differential MR and GR activation? Are emotion and cognition correlated?
7.2 Do emotion and cognition correspond to distinct MR, GR expression and stress susceptibility as it is expressed in two mouse strains?
7.3 Can strain differences in emotion and cognition for a negative event be eliminated by manipulating endogenous corticosterone levels?
7.4 Does the time of treatment (before or directly after the negative event) differentially influence memory formation and extinction?
7.5 What is the specific function of MR during memory formation and extinction of a stressful emotional experience?
7.6 Proposed model of integrating glucocorticoid stress system, emotion and cognition
7.7 Translational approach: from mouse to man
7.8 Perspectives
7.9 Conclusions

The objective of this thesis was to identify the distinct contribution of corticosteroids and their receptors to the integration of emotional and cognitive processes. I focussed on the following questions:

1. Do corticosteroids affect emotion and cognition via differential MR and GR **activation**? Are emotion and cognition correlated?
2. Do emotion and cognition correspond to distinct MR, GR **expression** and stress susceptibility as it is expressed in two mouse strains?
3. Can strain differences in emotion and cognition for a negative event be eliminated by manipulating endogenous corticosterone levels?
4. Does the time of treatment (before or directly after the negative event) differentially influence memory formation and extinction?
5. What is the specific function of MR during memory formation and extinction of a stressful emotional experience?

Below I will discuss the results of this thesis by addressing these main questions **(sections 7.1** to **7.5)**, propose a model that describes the integration between the glucocorticoid stress system, emotion and cognition **(section 7.6)**, and address the implications for the development and possible treatment of stress-related diseases like post-traumatic stress disorder (PTSD, **section 7.7**). I will give perspectives for future research **(section 7.8)** and finalize with the general conclusions **(section 7.9)**.

7.1 Do corticosteroids affect emotion and cognition via differential MR and GR activation? Are emotion and cognition correlated?

In **chapter 2**, the correlation between emotion and cognition, and the influence of differential MR and GR activation on this correlation are examined. Combined emotional and cognitive testing was performed in a positively stimulated spatial task using C57BL/6J mice with distinct MR and GR activation. Results show that emotion and cognition are indeed correlated. With the help of principal component analysis I demonstrate that anxiety and motivation are correlated to learning, and that both emotions are especially important during the early phase of learning.

In addition, the results show that distinct MR and GR activation differentially affects emotional and cognitive processes. When confronted with a novel situation, continuous predominant MR activation is beneficial for the emotional state. This state is expressed by low anxiety, high motivation and high directed exploration, which allows to gain detailed knowledge of the environment. Remarkably, this condition of predominant MR activation does not result in better learning and memory. To gain profit from this adaptive behaviour when

confronted with the same situation again, additional consolidation is required. This could be achieved by the concurrent activation of the GR [1-4]. Indeed, mice with continuous predominant MR and additional moderate GR activation are fast learners. They display low anxiety and arousal together with high directed explorative behaviour as well as improvement of cognitive performance. Thus, moderate GR activation contributes to the facilitation of memory. Further increase to continuous GR activation, however, induces strong emotional arousal at the expense of cognitive performance. This has also been found in previous research [5-7], however the present study has the advantage of combining such emotional and cognitive effects into a clear correlation.
Several studies have addressed the issue that increasing corticosterone levels affect cognitive functioning in complex spatial tasks in a dose-dependent inverted-U-shaped fashion, with MR and GR as molecular candidates for this effect [8-12]. The results of this thesis add novel information to the inverted U-shaped function of corticosterone, by demonstrating for the first time how the integration of corresponding emotional parameters affects cognitive processes of learning and memory.

7.2 Do emotion and cognition correspond to distinct MR, GR expression and stress susceptibility as it is expressed in two mouse strains?

To answer this question, glucocorticoid stress system markers together with emotional expression, learning and memory were studied in two distinct mouse strains **(chapter 3)**. Results indeed show corresponding MR, GR expression, stress susceptibility, emotion and cognition in BALB/c and C57BL/6J mice. Lower hippocampal MR and GR mRNA expression, but elevated GR mRNA in prefrontal cortex and GR protein in the amygdala of BALB/c mice coincides with increased stress susceptibility, high emotional expression and superior cognitive performance in a spatial test. High hippocampal MR and GR mRNA expression and high GR protein in hippocampus of C57BL/6J mice corresponds with less stress susceptibility and inferior cognitive performance. The latter is stimulus-response driven and lacks emotional contribution.
This data corresponds to literature which describes that similar differences in MR and GR expression coincide with distinct stress dependent neuroendocrine regulation [13-16], emotion [17-21] and cognition [1;3;17].
However, it adds novel insights on how genetic variation of the glucocorticoid stress system could affect the correlation between emotion and cognition.

In summary, **chapters 2** and **3** show a clear contribution of the glucocorticoid stress system acting via MR and GR on the integration of emotion and cognition; **chapter 2** shows that moderate levels of corticosterone coincide with

optimal emotional state and cognitive performance, and **chapter 3** shows that in highly stress sensitive mice emotions positively contribute to optimal cognitive performance. MR and GR may play a coordinating role for these emotional and cognitive processes [2;22].

7.3 Can strain differences in emotion and cognition for a negative event be eliminated by manipulating endogenous corticosterone levels?

In **chapter 4,** the development of fear behaviour and the expression of fear memories are examined in naive BALB/c and C57BL/6J mice. A paradigm was designed in which several aspects of possible strain dependent fear responses could be tested. First, the developed setup allows assessing both context- and cue-related fear behaviour in one experimental procedure. This enables the detection of generalized and specific fear responses. Second, applying in depth behavioural observation allows to differentiate between qualities of fear behaviour. While scanning expresses active fear behaviour, freezing indicates passive, more intense fear expression. It was expected that due to their distinct stress susceptibility, emotional expression and cognitive functioning described in **chapter 3,** learning and memory of fearful events would also differ.

Chapter 4 supports the findings of **chapter 3,** demonstrating that BALB/c mice are the more stress susceptible strain displaying twofold higher corticosterone levels after fear conditioning and fear memory testing than C57BL/6J mice. In addition, a clear strain dependent (i) expression of fear behaviour by scanning and freezing and (ii) differentiation between context and cue related fear is observed. BALB/c mice display higher freezing than scanning behaviour during acquisition and memory testing, while C57BL/6J mice show more scanning than freezing behaviour. This reflects high passive coping behaviour in BALB/c mice and increased active coping behaviour in C57BL/6J mice. The latter has been suggested to reflect escape behaviour in expectance of the aversive event [23]. Interestingly, MR expression in these strains could contribute to the distinct coping behaviour: less MR function, as observed in BALB/c mice, appears to facilitate fear induced freezing [17;24].

BALB/c and C57BL/6J mice also display different acquisition of fear behaviour and fear memory. BALB/c mice display high levels of extreme fear (freezing) during context episodes of the acquisition compared to C57BL/6J mice. Strain differences are also present during fear memory testing: C57BL/6J mice very quickly change their fear behaviour between context and cue episodes, showing low freezing during context and high freezing when the cue is switched on. In contrast, BALB/c mice display a generalized high fear response independent of context or cue episodes. This stimulus driven cognitive performance of C57BL/6J mice and strong contribution of spatial (contextual) stimuli in BALB/c mice

reflects the strain specific cognitive performance described in **chapter 3.** Thus, the cognitive performance keeps the strain-specific characteristics independent of the motivational aspects of the task (adverse for fear conditioning; appetitive for the hole board)
Also here, distinct expression of MR and GR in the brain (hippocampus for context and amygdala for specific stimuli [25]), and stress susceptibility could underline strain dependent cognitive performance. Furthermore, the data presented emphasize the distinct emotional and cognitive functioning of these mice.

The results presented in **chapter 4** lead to the question whether the distinct stress susceptibility of BALB/c and C57BL/6J mice as expressed by different endogenous corticosterone levels, would underlie the strain specific emotion and cognition for a negative event. Thus will changing endogenous corticosterone levels either potentiate or diminish their distinct fear behaviour and fear memory? Since corticosterone effects are known to be facilitating as well as impairing for memory formation and extinction [2;26;27], therapeutic effects of the hormone might be unveiled. To address this question, corticosterone was given to BALB/c and C57BL/6J mice before or after acquisition **(chapter 5)**.
Interestingly, during this follow-up experiment **(chapter 5)** it appears that the kind of fear conditioning apparatus specifically affects the behaviour of C57BL/6J mice during the conditioning phase. Experiments presented in **chapter 4** used a transparent box, while a black, non-transparant box was used **chapter 5.** C57BL/6J mice showed less freezing during context episodes in the transparent box **(chapter 4)**. This finding is in line with the stimulus-driven behaviour of this mouse strain. However, the difference in expression of freezing as fear behaviour is bound to the acquisition phase, as naïve mice of both strains do display similar strain specific memory in both experiments **(chapters 4 and 5)**. BALB/c mice show generalized strong fear memory, while C57BL/6J mice clearly discriminate alternating context-and cue episodes.
Remarkably, corticosterone treatment strengthens the strain-dependent fear behaviour. The existing strong distinction between context- and cue-related fear in C57BL/6J mice becomes even more prominent. In BALB/c mice, corticosterone destabilizes fear memory to the benefit of facilitated extinction. It seems unlikely, that further increasing the dose of corticosterone in C57BL/6J mice would result in processing of fear comparable to the high stress sensitive, high corticosterone secreting BALB/c mouse.

In summary, data from **chapter 3** clearly shows a strain specific anxiety-like behaviour in novel environments which could be the consequence of distinct

MR and GR expression and stress susceptibility of BALB/c and C57BL/6J mice **(chapter 4)**. Modulation of endogenous corticosterone levels does not eliminate the strain specific fear behaviour **(chapter 5)**.

7.4 Does the time of treatment (before or directly after the negative event) differentially influence memory formation and extinction?

Besides determining the possible diminishing or potentiating effect of corticosterone treatment on the strain specific fear memory, the experiment discussed in **chapter 5** also reveals the influence of timing of corticosteroid action on cognition [3;12;26;28;29]. It is expected that corticosterone treatment before training (pre-acquisition) influences behaviour during acquisition *and* consolidation, while treatment after the aversive event (i.e., the fear conditioning procedure; post-acquisition) will affect solely memory consolidation.
Indeed, **chapter 5** shows that timing of corticosterone treatment does influence fear memory differently, with strain-dependent characteristics. For BALB/c mice, corticosterone treatment *before* acquisition hardly affects fear memory, while corticosterone treatment *after* acquisition apparently destabilizes consolidation and thereby facilitates extinction. In C57BL/6J mice, corticosterone treatment *before* the acquisition results in increased fear memory and impaired extinction of cue related fear, while corticosterone treatment *after* acquisition does not clearly affect fear memory. The presence of additional corticosterone during acquisition has opposite effects on fear memory. This is a novel and unexpected result. The general idea is that corticosterone facilitates fear memory consolidation, when given in *context* with the fear conditioning (pre- and post-acquisition). The present fear conditioning paradigm allowed to demonstrate the profound differences of pre- and post-acquisition treatment of corticosterone. On top of that, the effect of corticosterone is strain-dependent. It will be a challenge to unravel the underlying molecular mechanisms. At present, I may speculate that these time-of-treatment and strain-dependent effects corticosterone point to fast non-genomic actions of corticosterone mediated by membrane located low affinity MR [30].

In summary, as observed in **chapters 2** and **3**, **chapters 4** and **5** demonstrate distinct strain-dependent corticosterone levels and other markers of a differentially regulated glucocorticoid stress system, as well as behavioural patterns to spatial (context) and more specific stimuli (cue). Emotional expressions and memory performance show large individual differences. Distinct MR and GR expression in the brain areas specific for these memory processes could be contributing to the strain dependent memory processes **(section 7.6)**.

Additional exogenous corticosterone treatment influences memory for the adverse emotional event depending on time of administration (i.e. either before or after acquisition) and mouse strain. We conclude that genetic background and time of corticosterone action during processing of stressful information are modifiers of fear memory with interesting translational implications for anxiety-related diseases. How these results can be used in the translational research involving modelling and treating stress-related diseases such as PTSD will be discussed in **section 7.7**.

7.5 What is the specific function of MR during memory formation and extinction of a stressful emotional experience?

Previous research has shown that corticosterone action via MR influences behavioural reactivity and possibly also cognitive functioning [2;17;31-35]. To determine the specific contribution of MR to these behaviours, (female) mice with ablated MR in the forebrain ($MR^{CaMKCre}$ mice [17]) were tested for behavioural responses towards novelty and cognitive processing **(chapter 6)**.
$MR^{CaMKCre}$ mice show higher arousal and less locomotion in a novel environment compared to control mice, although only when the $MR^{CaMKCre}$ mice were pre-stressed. This increase in passive coping corresponds to previous findings using these mice [17], and to other experiments using mice with less MR function [24]. It appears that absence of MR function over time changes stress-induced behaviour. In addition, the timeframe of this behavioural effect could imply the involvement of fast-non genomic corticosterone actions via membrane MR in control mice [30].
Results also show that conditioned behaviour is affected by the absence of forebrain MR function. $MR^{CaMKCre}$ mice display enhanced cue-related fear behaviour during acquisition and persistently increased fear memory for the context. Besides the MR mediated effect, the importance of GR contribution to conditioned behaviour has to be considered as well [20;21], especially since these $MR^{CaMKCre}$ mice have higher GR expression compared to controls.
Chapter 6 presents very relevant data on the contribution of the MR to anxiety-like behaviour and the fear-related learning and memory processes with a high potential for translational research **(section 7.7).** In contrast, animal models in which the MR is overexpressed in the brain should be considered with caution due to the use of different promoters and MR expression in areas where normally no MR expression is found [18;32;33].
Interestingly, fear responses during acquisition and memory tests of the female control mice for the $MR^{CaMKCre}$ ablation are rather comparable to the fear responses observed in naïve male C57BL/6J mice in **chapters 4** and **5**. Both female control mice and male C57BL/6J mice discriminate between context- and

cue-related fear responses. This finding is relevant, since the genetic background of the $MR^{CaMKCre}$ control mice is predominantly C57BL/6J. It appears that the similarity in background has more influence on fear related behavioural response than a possible effect of gender.

In conclusion, **chapter 6** clearly specifies which unconditioned behaviours are under modulating influence of MR and how disrupted MR function influences different stages of learning and memory. Since the balance of MR/GR is shifted towards a larger contribution of GR-mediated effects, these results furthermore stress the importance of coordinated glucocorticoid receptor actions [2;22].

7.6 Proposed model of integrating glucocorticoid stress system, emotion and cognition

This thesis presents several experiments addressing the interaction between the glucocorticoid stress system with emotion and cognition. The experiments focus on pharmacological activation of MR and GR, naturally occurring variances in MR and GR expression and genetic modification of MR. To gain insight in the complete behavioural spectrum, both low and high emotional behavioural test conditions are used [25;36-39] .

The obtained results lead to several conclusions (see **section 7.9**), but the following two conclusions are relevant for the proposed model:

1. Emotion and cognition interact strongly.
2. Expression and activation of the corticosteroid receptors MR and GR clearly influence the contribution of emotional components to cognitive functioning.

How emotion and cognition interact and how the glucocorticoid stress system influences this interaction is presented in the proposed model in figure 1. BALB/c and C57BL/6J mice, with distinct MR and GR expression in the hippocampus, amygdala and PFC already exhibit different behaviour during unstressed, non-activated conditions. Indeed, the experiments performed in this thesis support this. The behavioural pattern of the mice in unstressed conditions, their cognitive performance and response to acute corticosterone treatment will allow to use both strains to model different aspects of stress-related disorders, like PTSD and generalized anxiety disorder **(section 7.5)**. For reasons of clarity, BALB/c and C57BL/6J mice are addressed separately in the proposed model.

In naive BALB/c mice, emotions contribute strongly to unconditioned and conditioned behaviour. This large emotional component parallels and could even directly enhance cognitive performance in this mouse strain. The learning strategy of BALB/c mice depends on complex (spatial) stimuli and is well orchestrated. When introducing a stressor or corticosterone treatment, the emotional component increases and cognitive performance becomes impaired. In naive C57BL/6J mice, the contribution of emotions to unconditioned and conditioned behaviour is much less expressed and no clear interaction is observed*. Their learning strategy focuses on the processing of specific stimuli, and performance in complex, spatial tasks is inferior compared to BALB/c mice. When introducing a stressor or injecting additional corticosterone, the emotional component becomes stronger, accompanied by facilitation in cognitive functioning.

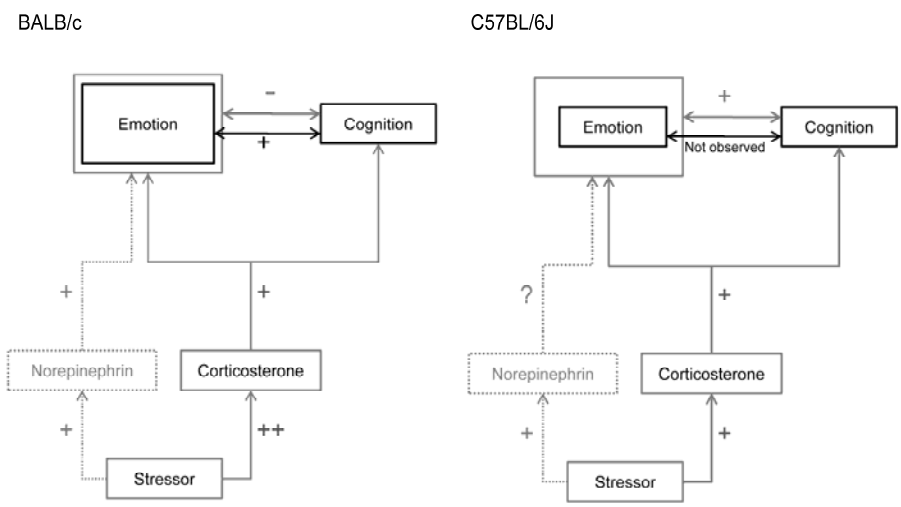

Figure 1. Schematic view of proposed model that describes the interaction between the glucocorticoid stress system, emotion and cognition. **Unstressed conditions**: the black connection line between the emotion and cognition box indicates the relationship between emotion and cognition in naive BALB/c and C57BL/6J mice. **The effect of a stressor or corticosterone**: the grey connection line and boxes indicate the effect of a stressor or corticosterone injection + (facilitation) and – (impairment) on emotion and cognitive functioning. This thesis does not experimentally address the response of the sympathetic nervous system to stress. However, due to a possible contribution of the noradrenergic system to the behaviour of BALB/c mice (see discussion **chapter 5**), this is included in the proposed model (dotted light grey boxes and lines).

*Note: **Chapter 2** does describe a clear interaction between emotion and cognition in C57BL/6J mice. However, most of these mice have surgically removed adrenals and are subjected to continuous corticosterone exposure via implanted pellets. The absence of the endogenous glucocorticoid stress response makes this ADX (mouse) model appropriate to determine the effect of differential MR and GR activation on interacting emotion and cognition. The C57BL/6J mice in this model are not considered as naive C57BL/6J mice depicted in figure 1. Sham operated mice with an intact adrenocortical (glucocorticoid) stress and adrenal medullary response differ significantly from the surgically manipulated mice in the factor describing the interaction between emotion and cognition.

7.7 Translational approach: from mouse to man

PTSD is a well known stress related disease characterised by disrupted glucocorticoid stress system regulation and altered cognition for the emotional event [40;41]. One of the major behavioural symptoms is the intrusive uncontrollable reoccurrence of traumatic memory [42].
This thesis describes several experiments that study the relation of the glucocorticoid stress system and memories for an adverse, most likely traumatic event **(chapters 4, 5 and 6)**. Results obtained with these experiments may provide information on how fear memories develop and perhaps even point out new possibilities for the therapy of pathological fear memories. First, I will discuss the translational value of the models presented in this thesis, followed by a comparison with existing models.

Translational value of presented models

It is of relevance to note that patients suffering from PTSD often display symptoms in the presence or imagination of particular stimuli [42], whereas in generalized anxiety disorder, behavioural and emotional reactions often emerge in the absence of a particular stimulus, or are interconnected with a more complex environment [43]. BALB/c and C57BL/6J mice were used to determine to what extend differences in genetic background related to the glucocorticoid stress system contribute to the formation and extinction of fear memories. We carefully studied the separate phases of acquisition, consolidation, retrieval and extinction of fear to complex (*context:* the environment) and simple stimuli (*cues*: light and tone). The highly emotional and stress sensitive BALB/c mice acquire fear and remember fear-related conditions differently from the less emotional and less stress sensitive C57BL/6J mice. With respect to "PTSD like" symptomatology, naive C57BL/6J mice seem to be more vulnerable to cue-specific fear memories, easily to trigger and most likely expressing "flashback" memories. Naive BALB/c mice present more the phenotype with higher anxiety-related behaviour and generalization of fear memory to discrete stimuli and context.

Using glucocorticoids as therapeutics to restrict the consolidation and facilitate the extinction of fear [44;45], BALB/c mice treated with corticosterone appear to be a good model. BALB/c mice respond with alleviated fear memory preferentially when treated *after* the adverse experience. Similar results have been described in PTSD patients after glucocorticoid treatment [45;46]. On the other hand, BALB/c mice do not show the cue-related specificity of fear memories that is characteristic for PTSD. This is characteristic for C57BL/6J mice that show stimulus specific fear memories. Based on results of C57BL/6J mice, I may predict that increased levels of glucocorticoids present during an adverse, stressful event will be a risk factor for PTSD. C57BL/6J mice present an animal model for studying the strong acquisition and consolidation of fear memories for a specific stimulus as seen in PTSD patients.
The strain-specific effects of corticosterone on fear memories highlight the relevance of the genetic background related to the glucocorticoid stress system for therapeutic efficacy.

Comparison with existing models

Many studies described in literature focus on modelling PTSD related fear memory in rodents using fear conditioning paradigms. I will compare some of these models with the ones presented in this thesis, focussing on glucocorticoid stress system activation/ modulation and genetic variance.
A common approach to acquire rodent models to study PTSD related fear involves (repeated) exposure to a stressor or corticosteroid treatment. Fear conditioning uses the same approach since it involves the acquisition of fear memory due to the repeated exposure to stressful events (shocks). However, several studies have shown that additional single or repeated exposures to a stressor enhances fear memory [47-50], possibly due to a GR-mediated effect [51]. The model described in this thesis (treating C57BL/6J mice with corticosterone before acquisition) agrees with these models. In addition, the present paradigm allows a more specific analysis of cognitive processes such as the contribution of context and cue to fear memory formation and extinction. These models are very valuable for translational research as they allow to study how environmental stressors can contribute to the formation of fear memories in humans.
Besides facilitating effects of stressors and corticosteroids on the formation of fear memory, and thus PTSD symptomatology, corticosteroid treatment in rodents can also be used to model possible intervention of established fear memories. This has been shown in **chapter 5** using BALB/c mice, but also by the proposed model of Cai and colleagues (2006). In their model, treatment with corticosterone after reactivation of established fear impairs later recall of that

fear memory [52], and even could improve extinction [26]. Others elaborate on this, showing with their models that the corticosteroid effect depends on the strength of fear memory [53;54] .
As glucocorticoid treatment in recent human studies confirmed [44;45], these rodent models are well suited to further study the mechanisms of traumatic fear memories.
Other rodent models focus on the genetic factors that underlie development and disruption of fear memory. Some of these models propose transcription factors or amygdala functioning [55;56]. However only few rodent models include genetic modulation of glucocorticoid associated genes. Chourbaji and colleagues propose that mice with GR overexpression might be suitable as model for increased fear memory, arguing that increased GR activation contributes to strengthening of consolidation of fear memories [57]. **Chapter 6** of this thesis describes how MR ablation can model the impairment in adjusting fear responses to a safe situation, as observed in PTSD patients. Also in this MR dysfunctional mice, increased GR activation might contribute to the strong fear-memories, resistant to extinction. Thus, mouse models focussing on the glucocorticoid stress system might indeed be very helpful in determining which genes are involved in the establishment and disruption of fear memories.
Another option is to use animal models that rely on naturally occurring genetic variance. Some of them involve the use of different inbred mouse strains [58] or rely on the crossbreeding of strains [59;60]. The experiments in this thesis demonstrate that C57BL/6J and BALB/c mice are very suitable in determining how naturally occurring genetic differences in the glucocorticoid stress system correlate with anxiety-related behaviour, fear memories and other cognitive abilities.

7.8 Perspectives

The chapters in his thesis present new insights on how the glucocorticoid stress system affects the integration between emotion and cognition. Knowledge on this interaction is sparse, but very much needed when addressing vulnerability and treatment of stress related diseases such as depression and PTSD. The tools developed here include animal models, detailed behavioural and statistical analysis, and can be used to further study various aspect of the integration between the glucocorticoid stress system, emotion and cognition. Below, I will present several ideas for future experiments regarding the (i) underlying mechanisms of glucocortiocoid stress system interaction on emotion and cognition and (ii) translational research.

Mechanisms of glucocorticoid action

An interesting line of research is to determine in more detail how and which of the brain structures involved in the glucocorticoid stress system modulate the effects on emotion and cognition. It would be very interesting to study the contribution of amygdala versus hippocampus in BALB/c and C57BL/6J mouse strains. This can be achieved by fMRI studies using mice with distinct glucocorticoid stress system activation due to knockout or pharmacological modulation tested in either positively or negatively stimulated cognitive tasks.

Additional to these experiments, mouse studies regarding the smaller hippocampal volume after traumatic events in humans should be performed. Such studies, would give more insight in the question whether smaller hippocampal volume reflects higher vulnerability to strong fear related memory formation or if less volume is a result of experiencing a negative event. Interestingly, Penet and colleagues have found a relative small hippocampus in C57BL/6J mice [61]. So this strain, possibly in combination with others, would be very suitable for such research.

Another very promising tool is the use of siRNA to specifically knockdown a gene of interest in a very limited spatial domain. This means that MR and GR function can be determined in specific sub-areas of the hippocampus, amygdala and PFC, and that therefore their contribution to strain specific behaviour can be elucidated. Experiments performed in collaboration with L. van Hooijdonk, E. Vreugdenhil, C. Fitzsimons and colleagues have already revealed first results, showing that the GR in the DG of the hippocampus affects context and cue-related fear memories and extinction processes (unpublished data).

In addition to this, further experiments using inducible ablation of GR and MR should be performed. A start has already been made in **chapter 6**, using mice with MR ablation. However, a similar study on the specific role of limbic GR would complement this data. BALB/c and C57BL/6J mice are very suitable for such research as it appears that these strains have distinct GR protein **(chapter 3)**.

When addressing MR function, non-genomic actions of glucocorticoids via membrane bound MR should not be overlooked [30]. This thesis suggests that it is the fast non-genomic MR mediated action of glucocorticoids that affects behaviour during acquisition of fear memory. Which emotional and cognitive processes are under influence of these effects is yet unknown.

Translational research

One of the next steps concerning translational research is to study fear related cognitive processes and their modification over an extended time interval. The delay between the traumatic event and recurrence of fear memory in PTSD

patients can be up to months or even years [62;63]. In this thesis, relatively short time spans (days) of occurrence and extinction of fear memories were used.
Another promising observation described in this thesis is the influence of disrupted MR function on formation of fear memory and its extinction. Ablation of MR in the forebrain increases fear memory, but also impairs adaptation to the relative safe situation, i.e. when the cue is not followed by shock anymore. These $MR^{CaMKCre}$ mice do not extinguish their fear. As this is one of the hallmarks of PTSD, a study in PTSD patients screened the genetic differences in the structure of the MR would be very interesting. In support, loss of function single nucleotide polymorphisms (SNP's) of the MR have shown enhanced susceptibility to a psychosocial challenge [64]. Studies on the influence of MR and GR on (catecholaminergic) stress responses and stress related pathologies have shown an correlation between genetic variance of MR and GR and the increased occurrence of PTSD and depression [64-67].

I expect that on the long term, the proposed lines of research will provide more insight in the development and treatment of PTSD. In parallel, we will gain more knowledge on how the glucocorticoid stress system affects the integration of emotion and cognition.

7.9 Conclusions
The following conclusions can be drawn:

1. Emotional contribution improves cognitive performance.

2. Both MR and GR activation influence the contribution of emotion to cognition.

3. Corticosterone treatment can have impairing and facilitating effects on emotional memory depending on the genetic background of the mice and the time of administration.

4. BALB/c and C57BL/6J mice are good models to study the role of the glucocorticoid stress system on stress related disorders such as PTSD.

5. The MR is a promising drug target that can be used for treating PTSD related pathology.

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Chapter 8

Summary
Samenvatting

SUMMARY

Stress hormones such as corticosteroids are potent modulators of emotional and cognitive functioning. Their effects can be beneficial under normal conditions, but become impairing if the corticosteroid action is excessive, prolonged or inadequate. Such dysregulated corticosteroid function is thought to compromise information processing underlying the integration of emotional and cognitive processes. As a consequence, some individuals develop stress-related disorders such as depression and PTSD. These disorders are characterized by altered emotional and cognitive processing together with disrupted corticosteroid function.

This raises the following questions: (1) why are some individuals more prone to the development of stress-related diseases? And (2), are the glucocorticoid stress system, emotion and cognition interdependent?

Determining genetic factors contributing to the vulnerability of stress related diseases in addition to assessing the interaction between emotion, cognition and glucocorticoid stress system will help to understand the development, resilience to and even treatment of stress related diseases. Experiments described in this thesis focus on the role of two distinct receptor types in the limbic brain areas, i.e. the mineralo- (MR) and glucocorticoid receptors (GR). These receptors control stress system activity, facilitate recovery from stress and mediate the actions of corticosteroids on emotion and cognition.

The main objective of this thesis is therefore to study the interaction between the glucocorticoid stress system, emotion and cognition, focussing on MR and GR functions.

In **chapter 2**, the influence of the differential activation of brain MR and GR on emotional responses and cognitive processes is described. This differential activation of both receptor types is achieved by substituting adrenalectomized mice with different amounts of corticosterone released from subcutaneously implanted pellets that result in different degrees of MR and GR occupation with the ligand in the brain. My results show that changes in emotion are correlated with cognitive performance. Moreover, learning and memory performance is correlated with both anxiety and motivation as revealed by principal component analysis. These two domains of emotion appear especially important in the early phase of the learning process.
The results also show that distinct MR and GR activation in the limbic brain affects emotional and cognitive processes in a differential manner. When

confronted with a novel situation, continuous predominant MR activation achieved with the constant release of the steroid from the pellet is beneficial for the emotional state. This state is expressed by low anxiety, high motivation and high directed exploration, which allows to gain detailed knowledge of the environment. Remarkably, this condition of predominant MR activation does not result in better learning and memory. For this purpose concurrent activation of the GR colocalized with MR is required that facilitates consolidation of the experience, so the individual is prepared for the same confrontation in the future. Indeed, mice with continuous predominant MR and additional moderate GR activation are fast learners in a positively motivated spatial learning task. They display low anxiety and arousal together with high directed explorative behaviour as well as improvement of memory performance. Thus, moderate GR activation contributes to the facilitation of memory. Further increase to continuous GR activation by higher circulating corticosterone concentrations, however, induces strong emotional arousal at the expense of cognitive performance. This increase in emotional arousal (anxiety) and impaired cognitive functioning has been shown by others, but it has to our knowledge never been tested in one experimental design.
The findings reported in **chapter 2** show a clear correlation between emotion and cognition and underline the importance of a balanced MR/GR activation in the limbic brain for emotional and cognitive functioning that is critical for optimal performance in a complex environment and thus, is beneficial for mental health.

Next we assessed if naturally occurring differences in MR and GR expression would correspond to endocrine and behavioural stress sensitivity, emotional and cognitive functioning. Two inbred mouse strains (BALB/c and C57BL/6J) were characterised for MR and GR protein and mRNA expression in the hippocampus, amygdala and PFC and further tested for emotional and cognitive behavioural patterns in the elevated plus maze and modified holeboard **(chapter 3)**. The results show that lower hippocampal MR and GR mRNA expression, but elevated GR mRNA in prefrontal cortex and GR protein in the amygdala of BALB/c mice coincides with increased stress susceptibility, high emotional expression and contribution of emotions to superior cognitive performance in a positively motivated spatial learning task. High MR and GR expression in C57BL/6J mice corresponds with lower stress susceptibility and reduced cognitive performance. Learning is stimulus-response driven and lacks emotional contribution in this strain. Therefore, high susceptibility to stress and an enhanced emotional contribution seems to be beneficial for cognitive functioning.

In summary, **chapters 2** and **3** demonstrate a clear contribution of the glucocorticoid stress system to the integration of emotion and cognition. The steroid replacement experiments in **chapter 2** show that moderate levels of corticosterone suggesting predominant MR and moderate GR occupancy coincide with an emotional state optimal for cognitive performance expressed as fast acquisition of the task. **In chapter 3** the comparative study between two mouse strains shows that in highly stress susceptible mice emotions positively contribute to optimal cognitive performance. In this comparison, the differential expression of MR and GR in hippocampus, prefrontal cortex and amygdala likely plays a coordinating role for these emotional and cognitive processes.

The research in **chapters 4** and **5** aims to determine how the glucocorticoid stress system of BALB/c and C57BL/6J mice would influence learning and memory of a negative event. Mice of both strains were subjected to a specifically designed fear conditioning paradigm in which formation and extinction of memory of an adverse, most likely traumatic experience could be measured **(chapter 4)**. Generalized and stimulus-specific fear-responses expressed as scanning and freezing behaviour were assessed in alternating episodes of context and cue. C57BL/6J and BALB/c mice display a distinctly different acquisition of contextual fear, indicative of active and passive coping respectively. In addition, they display very distinct fear memory. C57BL/6J clearly differentiates between context (low freezing) and cue (high freezing). BALB/c mice show a generalized fear response, a similar strong freezing response to context and cue.
These results lead to the question whether stress sensitivity including the corticosterone response to this stressful task contributes to the distinct fear memory pattern of BALB/c and C57BL/6J mice. We therefore assessed the impact of corticosteroids in the acquisition and consolidation phase of fear memory in both strains. BALB/c and C57BL/6J mice were injected with corticosterone shortly before or directly after fear conditioning. The retrieval and extinction of context- and cue related fear memories were observed on later days **(chapter 5)**.
During acquisition, naïve mice of both strains show similar fear responses during context, but not cue episodes. The strain-specific behavioural response pattern during memory testing is similar in **chapters 4** and **5.** BALB/c mice show generalized strong fear memory, while C57BL/6J mice discriminate between freezing during context-and cue episodes. In BALB/c mice corticosterone treatment *after* acquisition results in less stable consolidation, reduced fear memory and thus facilitated extinction, while it hardly affects fear memory of C57BL/6J mice. In contrast, corticosterone treatment *before* acquisition facilitates specifically cue-related fear memory of C57BL/6J mice, while BALB/c

mice are relatively unaffected. Since the differential action of corticosterone *before* and *after* acquisition takes place within a very short time frame, I assume that a rise corticosterone *before* the adverse event affected the acquisition process via the recently described fast (non-genomic) membrane MR.

In **Chapter 6** the role of MR in emotion and cognition is further specified using $MR^{CaMKCre}$ mutant C57BL/6J mice which have ablated forebrain MR and increased GR expression. These $MR^{CaMKCre}$ mice, together with control C57BL/6J mice, were studied for behavioural responses and learning and memory performance in a one trial holeboard test followed by the aforementioned fear conditioning paradigm. Ablation of limbic MR leads to higher emotional arousal and less locomotion immediately after exposure to a novel environment, although only when stressed. This finding suggests that forebrain MR ablation may have abolished fast non-genomic corticosterone actions. If indeed these membrane MRs are involved, these receptors can be activated by the stress-induced high level of corticosterone, which is present in the control mice. However, it cannot be excluded that also the increased expression of GR contributes to the altered behavioural reactivity.
MR ablation furthermore enhances cue-related freezing during fear acquisition and persistently augments fear memory specific for the context. Also here, the increased number of GR might be involved in the facilitation of context-related fear memories. In addition, extinction of fear is delayed or prevented. Thus, ablation of forebrain MR shifts emotional and cognitive processing towards stronger memories for fearful events. The lack of MR function and thus the larger contribution of GR in this model is an excellent example of the relevance of a balanced and coordinated action of corticosterone by MR and GR.

From this thesis the following conclusions can be made:

1. Emotional contribution improves cognitive performance.

2. The extent of GR relative to MR activation modulates the contribution of emotion to cognition.

3. Corticosterone treatment can have impairing and facilitating effects on emotional memory depending on the genetic background of the mice and the time of administration.

4. BALB/c and C57BL/6J mice are a good model to study the role of the glucocorticoid stress system on stress related disorders such as PTSD.

5. The MR is a promising drug target that can be used for treating PTSD related pathology.

In conclusion, **corticosteroids modulate the integration of emotion and cognition via a combined MR and GR-mediated action**. The findings suggest that BALB/c and C57BL/6J mice provide animal models for specific aspects of PTSD and other stress-related diseases.

SAMENVATTING

Stress leidt tot de afgifte van corticosteroïden door de bijnierschors. De corticosteroïd hormonen regelen energiemetabolisme en hebben een sterke ontstekingsremmende en immunosuppressieve werking. De hormonen dringen gemakkelijk de hersenen binnen en beïnvloeden met name de functie van hippocampus, amygdala en prefrontale cortex in emotie, cognitie en de aanpassing aan stress. Emotionele en cognitieve processen worden bevorderd door corticosteroïden, maar verstoord door een te sterk ofwel een te zwak werkend hormoon. Hoe het corticosteroïd signaal kan veranderen van beschermend naar beschadigend is niet precies bekend. Recent onderzoek heeft laten zien dat bij een ontregelde corticosteroïdwerking neuronale functies worden verstoord die ten grondslag liggen aan de integratie van emotie en cognitie. Bij sommige individuen leidt dit vervolgens tot stressgerelateerde aandoeningen zoals depressie of posttraumatische stress-stoornis (PTSS).

Waarom de een wel en de ander niet ziek wordt van stress is niet bekend. Men denkt dat corticosteroïden hiervoor van belang zijn, gezien hun rol in de wisselwerking tussen genetische factoren en de invloed van eerdere stressvolle ervaringen. De experimenten beschreven in dit proefschrift richten zich op de betekenis van corticosteroïden voor stress-gerelateerde psychopathologie onderzocht in diermodellen. Corticosteroïden werken door binding aan de mineralo- (MR) en glucocorticoid receptoren (GR), waarna gentranscriptie beïnvloed kan worden. Recentelijk zijn ook snelle membraaneffecten gevonden die door deze receptortypen tot stand komen, hoewel ze klassiek als intracellulaire (nucleaire) receptoren te boek staan. MR en GR komen overvloedig tot expressie in limbische hersengebieden met een belangrijke functie in emotionele en cognitieve processen.

Het doel van het onderzoek beschreven in dit proefschrift is om vast te stellen welke bijdrage MR- en GR leveren aan de corticosteroïd effecten op de integratie van emotionele en cognitieve processen.

Hoofdstuk 1 bevat een gedetailleerde beschrijving van de achtergrond van het onderzoek. Hier wordt ook de vraagstelling en de hypothese geformuleerd en onderbouwd.

In **hoofdstuk 2** wordt de invloed van differentiële MR- en GR-activatie op emotioneel en cognitief functioneren bestudeerd aan de hand van een apetitief gestimuleerde taak die het geheugen voor plaats en ruimte test. Differentiële activatie van de receptortypen kan worden verkregen door in bijnierloze muizen

tabletten met verschillende corticosteronconcentraties subcutaan te implanteren. Een toenemende corticosteronconcentratie in het bloed leidt geleidelijk tot een grotere bezetting van GR ten opzichte van MR, omdat de GR een lagere affiniteit voor het ligand heeft. De resultaten laten zien dat emotie en cognitie gecorreleerd zijn. Zo beschrijft de componentenanalyse dat leren en geheugen afhankelijk is van zowel angstgerelateerd gedrag als van motivatie. Deze twee emotionele componenten zijn vooral relevant in de eerste leerfase.

Een belangrijke vondst was dat differentiële MR- en GR-activatie een verandering tot stand brengt in emotionele reactie en cognitieve processen. Overwegende MR activatie gaat gepaard met een mate van emotionaliteit die bevorderlijk is voor de verwerking van gedetaïlleerde informatie uit de omgeving. Er is dan weinig angstgerelateerd gedrag, maar de dieren zijn zeer gemotiveerd, zoals afgeleid kan worden uit de hoge mate van exploratief gedrag. Tezamen hebben hoge motivatie en geringe angst een gunstige invloed op cognitieve prestaties. Opvallend is dat de MR-afhankelijke emotionaliteit in deze muizen niet per se resulteert in beter leren en geheugen. Hiervoor is een hogere corticosteronspiegel nodig die, naast activatie van de MR, ook de GR activeert. Muizen die aldus in de limbische structuren met een hogere MR- en GR-activatie zijn toegerust, vertonen ook weinig angstgerelateerd en veel exploratief gedrag, maar leren en onthouden de taak juist opmerkelijk goed. Echter, verdere limbische GR-activatie door nog hogere corticosteron-concentraties in het bloed, leidt tot een dermate hoge emotionele reactiviteit dat cognitieve prestaties belemmerd worden. Deze combinatie van extreme emotionele reactiviteit en verminderd cognitief presteren is eerder aangetoond, maar - naar mijn beste weten - nog nooit dosisafhankelijk in één experimentele opstelling vastgesteld.

Vervolgens is in **hoofdstuk 3** onderzoek beschreven waarin is nagegaan of natuurlijke variatie in de mate van MR- en GR-expressie overeenkomt met zowel stressgevoeligheid op endocrien en gedragsniveau als op het niveau van emotie en cognitie. Hiervoor is het gedrag van twee *inbred* muizenstammen (BALB/c en C57BL/6J) geobserveerd, waarna MR- en GR-expressie in de hippocampus, amygdala en prefrontale cortex is gemeten. Een patroon van lagere hippocampale MR- en GR mRNA-expressie, maar hoger GR mRNA in de PFC en meer GR eiwit in de amygdala van BALB/c muizen dan die van de C57BL/6J muizen blijkt gecorreleerd met een verhoogde stressgevoeligheid, met meer emotionele reacties en met betere cognitieve prestaties. In deze muizenstammen blijkt dus hogere stressgevoeligheid en emotioneel gedrag positief bij te dragen aan het cognitief functioneren.

Samenvattend benadrukken de bevindingen in **hoofdstuk 2** het belang van een gebalanceerde MR/GR-activatie in de limbische hersengebieden voor emotioneel en cognitief functioneren in een complexe taak. Verder wijzen de resultaten in **hoofdstukken 2 en 3** erop dat corticosteroïden bepalend zijn voor de mate van interactie tussen emotie en cognitie. Het experiment met corticosteroïd-substitutie en differentiële MR-en GR-activatie in **hoofdstuk 2** laat zien dat een gemiddeld voorkomende corticosteronconcentratie, en dus hoge MR- en matige GR-activatie, leidt tot een emotionele toestand die optimaal is voor cognitief presteren gezien de snelle acquisitie van de taak. De vergelijking van twee *inbred* muizenstammen in **hoofdstuk 3** laat zien dat in muizen met hoge stress- en emotie-reactiviteit positief correleert met cognitief presteren. Het differentiële expressiepatroon van MR en GR in de hippocampus, amygdala en prefrontale cortex lijkt hierbij een belangrijke rol te spelen.

De experimenten in **hoofdstuk 4 en 5** richten zich op de vraag hoe corticosteron in BALB/c- en C57BL/6J-muizen het leren en onthouden van een negatieve ervaring beïnvloedt. Muizen van beide stammen zijn blootgesteld aan een angstconditioneringsprocedure waarin het ontstaan en de uitdoving van de herinnering aan een negatieve, waarschijnlijk traumatiserende ervaring bepaald kan worden **(hoofdstuk 4)**. Gegeneraliseerd, maar ook stimulus-specifiek angstgeheugen is gemeten door herhaaldelijk de gedragsrespons op de omgeving (context) en op de geconditioneerde stimulus te meten. Angstgedrag wordt gekenmerkt door immobiliteit en kan zowel door *freeze*- als *scan*-gedrag tot expressie worden gebracht.

C57BL/6J- en BALB/c-muizen vertonen in verschillende mate angstgedrag tijdens context-leren, hetgeen indicatief is voor een actieve of juist passieve omgang met de stressvolle, angstige situatie. Ook het angstgeheugen is erg verschillend tussen deze stammen. C57BL/6J muizen maken duidelijk onderscheid tussen context (veel immobiliteit) en stimulus (minder immobiliteit), terwijl BALB/c muizen geen onderscheid maken tussen context en stimulus. De BALB/c stam vertoont dus meer gegeneraliseerd angstgedrag in vergelijking tot de C57BL/6J- stam.

Deze resultaten roepen de vraag op of de verschillen in stressgevoeligheid voor deze taak, inclusief de corticosteronrespons, een bijdrage leveren aan het verschil in angstgeheugen tussen BALB/c- en C57BL/6J-muizen. Om deze vraag te beantwoorden is de invloed van corticosteron op de acquisitie- en consolidatie-fase van het angstgeheugen in beide stammen bepaald **(hoofdstuk 5)**. BALB/c- en C57BL/6J-muizen zijn met corticosteron geïnjecteerd, net voor of direct na acquisitie van de angstconditioneringtaak. Op

de daarop volgende dagen is gekeken naar het ophalen en de uitdoving van het angstgeheugen.
Gedurende acquisitie vertonen beide stammen hetzelfde gedrag tijdens de context-intervallen, maar een verschillende stimulusrespons. Het stamspecifieke gedrag tijdens de geheugentesten in **hoofdstuk 4** is ook hier herkenbaar: BALB/c muizen hebben een gegeneraliseerd, sterk angstgeheugen, terwijl C57BL/6J muizen differentiëren tussen context- en stimulus-gerelateerd geheugen. In BALB/c muizen resulteert corticosteron-injectie ***na*** acquisitie in een minder stabiele consolidatie, in verminderd angstgeheugen en dus in gefaciliteerde uitdoving. In C57BL/6J muizen daarentegen, heeft corticosteron-injectie ***na*** acquisitie geen effect. Aan de andere kant, corticosteronbehandeling ***voor*** acquisitie heeft een versterkend effect op het angstgeheugen van C57BL/6J muizen, terwijl het bij BALB/c muizen bijna geen effect heeft. Omdat het verschil in corticosteron effect door behandeling voor en na acquisitie plaatsvindt in korte tijd, neem ik aan dat verhoging in corticosteron vóór de negatieve ervaring de acquisitie ervan heeft beïnvloed door middel van de snelle, niet genomische, membraan MR effecten, die recent zijn ontdekt.

In Hoofdstuk 6 wordt de specifieke rol van MR in emotie en cognitie beschreven door in de experimenten gebruik te maken van $MR^{CaMKCre}$ C57BL/6 muizen. Deze muizen hebben in de voorhersenen geen MR, maar wel een verhoogde GR expressie. In deze mutanten en in de controlemuizen is emotionele expressie, en leren en geheugen van een negatieve ervaring gemeten. Afwezige MR functie leidt tot hogere "emotional arousal" en minder locomotie bij blootstelling aan een nieuwe omgeving, maar alleen na acute stress. Dit zou een aanwijzing kunnen zijn dat snelle, niet genomische effecten de verschillen in gedrag veroorzaken tussen $MR^{CaMKCre}$- en controle muizen. Echter, een mogelijk invloed van de verhoogde GR expressie op emotionele gedragingen kan niet worden uitgesloten.

Tevens leidt afwezige MR-functie tot verhoogd stimulus-specifiek angstgedrag tijdens acquisitie, tot een aanhoudend sterk angstgeheugen voor de context en tot verminderde uitdoving. De relatief verhoogde GR-expressie in $MR^{CaMKCre}$ muizen zou ook hier een bijdrage hebben kunnen leveren door bevordering van context-gerelateerd angstgeheugen. Over het algemeen lijkt verstoring van MR-functie gepaard te gaan met emotionele en cognitieve processen die leiden tot een sterker geheugen voor een angstige gebeurtenis. De verhoogde bijdrage van GR in de $MR^{CaMKCre}$ muizen is een goed voorbeeld van de relevantie van een gebalanceerde werking van corticosteroïden via MR en GR.

De experimenten in dit proefschrift leiden tot de volgende conclusies:

1. Emotie verbetert de cognitieve prestatie.

2. De mate van GR- versus MR activatie beïnvloedt de integratie van emotie en cognitie.

3. Corticosteronbehandeling kan de herinnering aan een emotione gebeurtenis verminderen *of* verbeteren afhankelijk van de genetische achtergrond van de muizen en het tijdstip van toediening van het hormoon.

4. BALB/c en C57Bl/6J muizen zijn uitstekende diermodellen om de rol van corticosteron in de pathogenese en behandeling van stress gerelateerde aandoeningen zoals PTSS te bestuderen.

5. De resultaten geven een aanwijzing dat de MR een aangrijpingspunt van farmaca kan zijn om PTSS gerelateerde symptomen te behandelen.

De conclusie van dit proefschrift is dat **corticosteroïden de integratie van emotie en cognitie beïnvloeden door middel van een complementaire MR- en GR-werking**. Bovendien blijken BALB/c- en C57BL/6J-muizen goede diermodellen te zijn om specifieke psycho-biologische aspecten van PTSS en andere stressgerelateerde ziekten.

Chapter 9

List of abbreviations
Curriculum Vitae
Publications

LIST OF ABBREVIATIONS

ACTH	adrenocorticotropin
ADX	adrenalectomy
AHC	ADX with high corticosterone substitution
ALC	ADX with low corticosterone substitution
ANOVA	analysis of variance
CA	closed arms
CA-	cornu ammonis area
CAPS	clinician administered PTSD scale
CORT	corticosterone
CR	conditioned response
CRFR	corticotropin-releasing factor receptor
CRH	corticotropin releasing hormone
CS	conditioned stimulus
DG	dentate gyrus
EPM	elevated plus maze
GABA	gamma-aminobutyric acid
GLM	general linear model
GR	glucocorticoid receptor
GRE	glucocorticoid response elements
HPA	hypothalamus-pituitary-adrenal
IES	impact of events scale
IL	infralimbic
IZ	intermediate zone
KO	knockout
LAL	long attack latency
LTP	long term potentiation
MHB	modified holeboard
MR	mineralocorticoid receptor
MRCaMKCre	MRflox/floxCaMKCre mice
NMDA	N-methyl-D-aspartate
OA	open arms
OD	optical density
PCA	principal component analysis
PCL	PTSD symptoms checklist
PET	positron emission tomography
PFC	prefrontal cortex
PL	prelimbic
PTSD	post traumatic stress disorder

PVN	paraventricular nucleus
SAL	short attack latency
SEM	standard error of means
US	unconditioned stimulus
VP	vasopressin

CURRICULUM VITAE

Vera Brinks werd op 11 september 1980 geboren te Rotterdam. Zij behaalde in 1998 haar VWO diploma aan het Citycollege St. Fransiscus te Rotterdam. Aansluitend begon zij met de studie biofarmaceutische wetenschappen aan de Universiteit van Leiden. Als onderdeel van deze studie heeft zij zich gespecialiseerd in de neurobiologie door middel van een stage bij de afdeling Medische Farmacologie van het LACDR. Tijdens deze stage heeft zij onder begeleiding van Prof. Dr. M.S. Oitzl en dr. I. de Jong het gedrag van muizen en de neuronale expressie van stressgerelateerde genen in de hersenen bepaald na stimulatie met cocaïne. In februari 2003 heeft zij haar doctoraalexamen behaald. Aansluitend is zij begonnen aan haar promotieonderzoek genaamd "Stress hormone effects on cognitive performance" bij de vakgroep Medische Farmacologie (LACDR, Universiteit Leiden). Dit onderzoek werd uitgevoerd onder begeleiding van Prof. Dr. M.S. Oitzl en Prof. Dr. E.R. de Kloet. Sinds 1 februari 2008 is Vera Brinks werkzaam als postdoc bij de afdeling Biofarmacie en Farmaceutische Technologie (UIPS, Universiteit Utrecht), waar zij werkt aan de immunogeniciteit van therapeutische eiwitten onder leiding van Prof. Dr. H. Schellekens en Prof. Dr. W. Jiskoot.

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Poster presentations

Corticosterone effects on fear memory depend on time of injection and genetic background of mice

- Endo-Neuro-Psycho Meeting, Doorwerth, June 2006.

Molecular and behavioural characterisation of male BALB/c and C57BL/6J mice
- LACDR spring symposium, April 2005, Amsterdam.
- Endo-Neuro-Psycho Meeting, June 2005, Doorwerth.
- ULLA summerschool, July 2005, Uppsala.
- Published in Acta Neurobiologiae Experimentalis, vol 65, suppl 2005, p72-73.

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- FENS, July 2004, Lissabon.
- Endo-Neuro-Psycho Meeting, June 2004, Doorwerth.
- NWO cognitiedag, December 2004, Den Haag.

Cognitive processes: a synergy of glucocorticoid action between mineralo-MR and glucocorticoid-GR receptors
- LACDR, Spring symposium, April 2003, Amsterdam.

Invited oral presentations
Glucocorticoid effects on learning and memory. Seminar, January 6, 2009, Bochum University, Germany.

Veteran mice: How do Traumatic memories develop and can we disrupt them? LACDR Spring Symposium, April 4, 2007, Amsterdam and Figon Dutch Medicine Days, Oktober 3, 2007, Lunteren.

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MR/GR balance in emotion and cognition: C57BL/6 and BALB/c mouse strains as model for MR/GR mediated differences in emotion and cognition. Workshop "Animal models in cognitive neuroscience", October 25, 2005, Oud Poelgeest, Leiden.

Emotional and cognitive processes: a synergy of glucocorticoid action via the mineralocorticoid (MR) and glucocorticoid (GR) receptors. Endo-Neuro-Psycho Meeting, June 2, 2005, Doorwerth.

Stress, emotions and cognition. NWO cognitiedag, December 22, 2004, Den Haag.

Awards

Poster presentations

- 1st prize at the Endo-Neuro-Psycho meeting, June 2005. Title: Molecular and behavioural characterisation of male BALB/c and C57BL/6J mice.
- 2nd prize at the NWO cognitiedag, December 2004, Den Haag. Title: Emotional and cognitive performance in response to Mineralo-(MR) and Glucocorticoid receptor (GR) activation in male C57BL/6J mice.

Oral presentations

- 2nd prize at the Figon Dutch Medicine Days, Oktober 3, 2007, Lunteren. Title: Veteran mice: How do Traumatic memories develop and can we disrupt them?
- 1st prize at the LACDR Spring Symposium, April 4, 2007, Amsterdam. Title: Veteran mice: How do traumatic memories develop and can we disrupt them?

Scholarships:

Dr. J.L. Dobberke Stichting voor Vergelijkende Psychologie, Title: Lange termijn angstgeheugen en uitdoving van een negatieve ervaring, 2008.

Hamilton Kinder Scholarship for the 2nd Annual Experimental Neurogenetics of the Mouse, University of Tennessee, Memphis 2005, USA.