Stress, emotional learning and AMPA receptors: from behavior to molecule
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Chapter II

Both Mineralocorticoid and Glucocorticoid Receptors Regulate Emotional Memory in Mice

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Abstract
Corticosteroid hormones are thought to promote optimal behavioral adaptation under fearful conditions, primarily via glucocorticoid receptors (GRs). Here we examined – using pharmacological and genetic approaches in mice - if mineralocorticoid receptors (MRs) also play a role in fearful memory formation. As expected, administration of the GR-antagonist RU38486 prior to training in a fear conditioning paradigm impaired contextual memory when tested 24 (but not when tested 3) hours after training. Tone-cue memory was enhanced by RU38486 when tested at 4 (but not 25) hours after training. Interestingly, pre (but not post)-training administration of MR antagonist spironolactone impaired contextual memory, both at 3 and 24 hours after training. Similar effects were also found in forebrain-specific MR knockout mice. Spironolactone also impaired tone-cue memory, but only at 4 hours after training. These results reveal that – in addition to GRs - MRs also play a critical role in establishing fear memories, particularly in the early phase of memory formation.
Introduction
Exposure to emotionally arousing events activates the Hypothalamo-Pituitary-Adrenal (HPA) axis. As a consequence, enhanced levels of corticosteroid hormones (corticosterone in most rodents and cortisol in humans) are released into the circulation (de Kloet et al., 2005). Corticosteroid hormones enter the brain and bind to two receptor subtypes; the mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). MRs are occupied when hormone levels are low and exert their effects classically via transcriptional regulation of responsive genes (de Kloet et al., 2005). However, recent evidence shows that corticosteroid hormones can also exert rapid non-genomic effects via MRs (Karst et al., 2005; Olijslagers et al., 2008). Compared to MRs, GRs have a 10-fold lower affinity for corticosterone, become activated when hormone levels rise after stress and slowly exert genomic actions (de Kloet et al., 2005; Joels et al., 2006) but also non-genomic effects (Di et al., 2003; Karst et al., 2010b).

Corticosteroid hormones alter neuronal activity in areas that play central roles in attention and selection of appropriate behavioral strategies (i.e. hippocampus, prefrontal cortex and amygdala; (de Kloet et al., 2005; Joels, 2010; Roozendaal et al., 2009c). As part of behavioral adaptation to stressful events, these hormones via activation of MRs and GRs, in interaction with other hormones and neurotransmitters (Joels and Baram, 2009; Roozendaal et al., 2009c), promote the storage of information (McEwen and Gianaros, 2010; Oitzl et al., 2010).

Corticosteroid hormones in vitro rapidly increase neuronal activity in the hippocampus (Karst et al., 2005; Olijslagers et al., 2008) and amygdala (Karst et al., 2010a) via a mechanism that requires MRs activation. Behavioral studies indicate that MRs are involved in appraisal of information and response selection in various tasks (Brinks et al., 2007; Oitzl et al., 2010; Oitzl and de Kloet, 1992; Sandi and Rose, 1994a). Moreover, genetic deletion of MRs in the forebrain led to various cognitive impairments,
including impaired learning in a Morris water-maze task (Berger et al., 2006). In contrast, via the activation of GRs, a gene-mediated cascade is initiated to slowly restore neuronal activity and suppress synaptic plasticity (Joels and de Kloet, 1989; Kerr et al., 1989; Kim and Diamond, 2002). At the behavioral level, activation of GRs promotes consolidation of the acquired stressful information at the later phase of memory formation (Lupien and McEwen, 1997; Oitzl et al., 2001; Pugh et al., 1997a; Pugh et al., 1997b; Roozendaal, 2003).

The existing data on the role of MRs in neuronal activity and cognitive function justifies the question whether MRs also play a role during the early phase of memory formation, in addition to the role of GRs in promoting memory formation at later time points (e.g. up to one day after acquisition of information). We therefore tested this hypothesis by examining whether specific blockade of MRs and GRs interferes with contextual and tone-cue memory formation at two different time points (i.e. contextual memory tested either 3 or 24 hours after training, tone-cue memory tested one hour later, i.e. either 4 or 25 hours after training). These time points (3-4 hours versus 24-25 hours) presumably reflect different learning phases (early encoding versus long-term memory) and cellular events that may underlie the learning process (Zhou et al., 2009). Corticosteroids have been reported to particularly modulate synaptic plasticity evoked by weak stimulation paradigms (Alfarez et al., 2002; Pu et al., 2009) and promote transition from short- to long-term memory in a relatively weak aversive learning paradigm (Cordero and Sandi, 1998; Sandi and Rose, 1994b). We therefore tested the roles of MRs and GRs both on contextual and tone-cue memory formation using mild (less aversive) and relatively strong (more aversive) learning paradigm, with shock intensities of 0.4 and 0.8 mA respectively.
Materials and Methods

Animals
Male C57/BL6 mice (6-8 weeks old, derived from Harlan, The Netherlands) and forebrain specific male MR-deficient animals (MR\textsuperscript{CAMKCre} mice, Berger et al., 2006, 4-5 months old, bred at Leiden University, Leiden) were individually housed 1-2 weeks before the experiment started. Within one experiment, control and experimental animals were trained in a random fashion. All animals were kept at a light/dark cycle of 12 hours (lights on at 8 a.m.; room temperature kept at 22 °C ± 2). Food and water were given without restriction. The experiments were carried out in accordance with and approved by the local Animal Committees of the University of Amsterdam and Leiden University.

Drugs
To examine the roles of GRs and MRs in fear conditioning, we pharmacologically targeted these receptors using the GR antagonist RU38486 (mifepristone, Sigma) and the MR antagonist spironolactone (Sigma) respectively. RU38486 or its vehicle (DMSO) was administered intra-peritoneally (i.p.) one hour before training at a dosage of 10 mg/kg which is sufficient to prevent GR mediated effects (Pugh et al., 1997a). Spironolactone or its vehicle (propylene glycol) was injected subcutaneously (s.c.) one hour before or immediately (within less than 5 minutes) after training at a dosage of 50 mg/kg which blocks MR mediated effects (Herman and Spencer, 1998; Kumar et al., 2007). The dose of spironolactone was taken as an effective and well-tolerated dose with little effects on spontaneous behavior as documented before (Adamec et al., 2007; Koenig and Olive, 2004).

Immunocytochemistry
Brains from MR\textsuperscript{CAMKCre} and wild type mice were immersion-fixed for 24 hours
in 4 % paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) and stored in PB (+ sodium azide) at 4 °C. Before sectioning, the brains were washed and cryoprotected by overnight incubation in 20% sucrose in phosphate buffered saline (PBS, pH 7.4). Frozen coronal sections (30 µm thick) were cut using a sliding microtome and stored in PB (+ sodium azide) until needed. Slices were washed thoroughly with PB to get rid of sodium azide, then rinsed with 1% H_2O_2 in 0.1 M Tris Buffered Saline (TBS, pH 7.4) to block endogenous peroxidase activity, followed by washing with TBS, TBS + Triton-X100 (TBS-TX) and TBS. Then, sections were incubated with the first antibody against MR (1D5, 1:500, Alfarez et al., 2009) in TBS for 48 hours in a cold room, kept for another 30 minutes at room temperature and washed in TBS. No blocking step prior to primary antibody application was applied since background staining was virtually absent. Next, sections were incubated for two hours with the secondary antibody (biotinylated Sheep-anti-Mouse 1:200 in TBS-TX, Amersham Biosciences) at room temperature, followed by washing with TBS and two-hour incubation in ABC-elite (1:800) in TBS-TX at room temperature. Then, sections were washed in TBS and 0.05 M TB (pH 7.6), followed by a DAB reaction. The duration of the reaction was established specifically for this staining; for negative controls always the same duration was kept as for the experimental slices. Specificity of the first antibody was confirmed by omitting the first antibody, which revealed in no staining. The reaction was stopped with three washes in 0.05 M TBS and two washes in TBS before slices were mounted.

_Fear conditioning_

Procedures were comparable to those described before (Zhou et al., 2009). The grid floor of the fear conditioning chamber (30 cm x 24 cm x 26 cm; W x L x H) was made of 37 stainless steel rods and connected to a shock generator (Med-Farm LION-ELD) that was developed in-house. During
training (between 8:30-11:30 am) one mouse at a time was put into the training chamber (cleaned with 1% acetic acid) and was allowed to freely explore the chamber for 3 minutes before 3 tone-foot shock pairs were introduced with an interval of one minute. Each tone (100 dB, 2.8 kHz) lasted 30 seconds and was accompanied by a foot shock of either 0.4 mA or 0.8 mA during the last 2 seconds. Thirty seconds after the end of the last pairing, the mouse was taken back to its home cage. Three hours or 24 hours (in separate experimental groups) later the animal was introduced into the same chamber for 3 minutes to test contextual memory, followed by tone-cue memory test one hour later, in a novel chamber with different contextual background. This cage was cleaned with 70% ethanol. After a free exploration period of 3 minutes the animals were exposed to the same tone (100 dB, 2.8 kHz) only once for 30 seconds. One minute later the animal was placed back into its home cage. Freezing behavior, defined as no body movements except those related to respiration, was determined every 2 seconds throughout the training period and during contextual and tone-cue memory testing. The percentage of freezing time was used for statistical analysis. During the training session, freezing behavior immediately after each of the tone-footshock pairings was recorded to examine effects on training over time. For the contextual memory test, total freezing behavior over the entire 3 minutes was compared between groups. In order to examine possible effects over time (Zhou et al., 2009), the whole 3 minutes was split into two periods, 90 seconds each. Then, data was averaged per period and between- as well as within-group effects over time were then studied. During the tone-cue memory test, freezing behavior during free exploration and the 30 seconds tone presentation was recorded and compared between the experimental groups.

Statistical analysis
Data was analyzed by repeated measures ANOVA or using a two-tailed
independent-samples students't-test. Results are presented as mean ± SEM. P values smaller than 0.05 were considered significantly different.

Results

Pre-training RU38486 treatment alters contextual and tone-cue memories, using mild foot-shock intensity

Earlier studies have reported that GRs are involved in the consolidation of spatial (Oitzl et al., 2001) and aversive memories (Pugh et al., 1997a). Here we examined whether pre-training application of GR antagonist RU 38486 affects contextual and tone-cue memory at two different time points, i.e. at 3 or 24 hours (context) and 4 or 25 hours (cue) after training, using mild foot-shock intensity (0.4 mA). Both RU38486 (n=8) and vehicle (n=8) treated animals displayed similar levels of freezing during training (between-group effect, F_{1, 14} = 2.52, P>0.05). Three hours after training both groups showed comparable amount of freezing during contextual memory test (vehicle versus RU38486: 30.4 ± 3.2 % and 33.3 ± 7.5 % respectively, t_{14}=0.36, P>0.05). As reported before (Zhou et al., 2009), freezing behavior decreased over time (Figure 1A, within-group effect, F_{1, 14}=7.53, P<0.05). No differences between experimental groups were found (between-group effect: F_{1, 14}=0.13, P>0.05). In the tone-cue memory test (one hour later), both vehicle and RU38486 treated animals showed strong freezing behavior during the presence of tone (Figure 1B). Interestingly, animals treated with RU38486 displayed significantly more freezing when compared to vehicle treated animals (between-group effect, F_{1, 14}=5.57, P<0.05).

In a separate experiment, mice were trained with 0.4 mA footshock intensity and contextual memory was tested 24 hours after training. Administration of RU38486 (n=9) did not affect freezing during training when compared to vehicle (n=6) treated animals (between-group effect, F_{1, 13} = 3.35, P>0.05). The average amount of freezing during the 3 minutes context memory test revealed a trend towards less freezing compared to vehicle
treated animals (vehicle versus RU38486: 60.3 ± 7.3 % and 40.3 ± 6.2 %, \( t_{13}=4.32, \ P=0.058 \)). Further analysis of freezing behavior showed that pre-training application of the GR-antagonist significantly reduced freezing behavior during the 1st period of the contextual memory test (Figure 1C, 1st period: \( t_{13}=2.70, \ P<0.05 \)). One hour later, in the tone-cue memory test, both RU38486 and vehicle treated animals showed comparable freezing behavior (Figure 1D, between-group effect, \( F_{1, 13}=0.20, \ P>0.05 \)).

**Figure 1.** Effect of Pre-training administration of RU38486 on fear conditioning using mild footshock intensity. A) Animals that received RU38486 or vehicle before training showed comparable contextual freezing at three hours after training. B) During the presence of the tone (four hours after training) the RU38486 treated animals showed higher freezing behavior when compared to vehicle treated mice. C) Twenty four hours after training, when compared to vehicle treated animals, pre-training administration of RU38486 resulted in less freezing behavior during the 1st period of the contextual memory test. D) Twenty five hours after training, animals that received RU38486 prior to training showed similar freezing behavior.
when compared to vehicle treated animals in tone-cue memory test.

Taken together, these data reveal that GRs are involved in both contextual and tone-cue memory formation, but the direction of the effect depends on the moment at which retention is tested.

**Pre-training spironolactone treatment reduces contextual and tone-cue memories, using mild foot-shock intensity**

We next examined whether pre-training blockade of MRs affected fearful learning at 3 or 24 hrs (context) and 4 or 25 hours (cue) after training using mild foot shock intensity (0.4 mA). Overall, spironolactone administration enhanced freezing behavior when compared to vehicle-treated animals during training (between-group effect, $F_{1, 18} = 4.83$, $P<0.05$), yet freezing behavior was not different between the two groups after the last tone-footshock paring ($t_{18} = 1.34$, $P>0.05$).

Testing contextual memory at 3 hours after training revealed that pre-training application of spironolactone to mice ($n=12$) resulted in significantly less freezing behavior when compared to vehicle-treated animals ($n=8$) (vehicle versus spironolactone: $30.4 \pm 6.0$ % and $2.5 \pm 0.8$ % respectively; Figure 2A, between-group effect: $F_{1, 18} = 32.16$, $P<0.01$). One hour later in the tone-cue memory test, spironolactone-treated mice showed also less freezing behavior when compared to vehicle-treated mice (Figure 2B, between-group effect, $F_{1, 18} = 10.16$, $P<0.01$).
Figure 2. Effect of Pre-training administration of spironolactone on fear conditioning using mild footshock intensity. A) Animals that received spironolactone before training showed less contextual freezing at three hours after training. B) During the presence of the tone (four hours after training) the spironolactone treated animals also showed less freezing behavior when compared to vehicle treated mice. C) Twenty four hours after training, when compared to vehicle treated animals, pre-training administration of spironolactone resulted in less freezing behavior during the contextual memory test. D) Twenty five hours after training, animals that received spironolactone prior to training showed similar freezing behavior when compared to vehicle treated animals in tone-cue memory test.

In order to examine whether the contextual and tone-cue memories were also impaired when tested at 24 and 25 hours after training respectively, a separate group of animals was used and spironolactone or vehicle was given one hour prior to training (n=6 per group). During training, no effect of spironolactone on freezing behavior was observed.
(between-group effect, $F_{1,10} = 0.96, P>0.05$). However twenty-four hours after training we found that pre-training administration of spironolactone significantly reduced freezing behavior during the contextual memory test when compared to vehicle treatment (vehicle versus spironolactone: 58.5 ± 6.5 % and 30.7 ± 9.4 % respectively; Figure 2C; between-group effect over time : $F_{1,10}=5.89, P<0.05$). During the tone-cue memory test, both groups displayed comparable freezing behavior (Figure 2D, $F_{1,10}=0.96, P>0.05$).

Overall, these results show that MRs are critically involved in contextual memory formation, both when animals were tested 3 hours and 24 hours after training. Blocking MRs disturbs tone-cue memory only shortly (4 hours) after training.

**Post-training spironolactone treatment does not affect contextual and tone-cue memories, using mild footshock intensity**

Pre-training administration of spironolactone dramatically affected contextual and tone-cued memories, especially shortly after training. As a first attempt to distinguish between putative MR-involvement in behavioral strategy / appraisal of the situation on one hand and early encoding / consolidation on the other, we examined whether administration of spironolactone immediately after training (i.e. not affecting the acquisition phase) also modulated fear memories when tested at 3 hours after training (using mild foot shock intensity). During the training session, the two groups of mice displayed similar levels of freezing behavior (between-group effect, $F_{1,13}=0.01, P>0.05$). Three hours after training, mice that were treated with spironolactone (n=8) and vehicle (n=7) showed comparable freezing behavior (vehicle versus spironolactone: 37.1 ± 7.8 % and 29.0 ± 7.4% respectively). No between-group effects ($F_{1,13}=0.67, P>0.05$) was present (Figure 3A). One hour later, tone-cue memory was also not different between the two groups (Figure 3B, $F_{1,13}=1.90, P>0.05$). These results show that blocking MRs immediately after training has no effect on
emotional memory processes.

Figure 3. Effect of post-training administration of spironolactone on fear conditioning using mild footshock intensity. Immediate post-training administration of spironolactone did not affect contextual memory (A) or tone-cue memory (B) when tested three and four hours after training respectively.

**MR**\textsuperscript{CAMKCre} mice show impaired contextual memory, using mild-footshock intensity

To further substantiate the pharmacological finding that pre-training blockade of MRs hampers emotional learning, esp. the contextual memory 3 hours after training with mild footshock intensity, we examined contextual and tone-cue memories in MR\textsuperscript{CAMKCre} mice, in which the MR gene in the forebrain was inactivated using the Cre/loxp-recombination system (Berger et al., 2006). In control mice, immunocytochemical staining specific for MRs confirmed the presence of MRs in principal neurons of important limbic areas, including pyramidal cells of Cornu Ammonis, in granular cells of the dentate gyrus (Figure 4A\textsubscript{1}) and in principal neurons of the central and basolateral amygdala (not shown). By contrast, immunocytochemical staining for MRs was absent in principal cells in the hippocampus (Figure 4A\textsubscript{2}), basolateral and central amygdala from MR\textsuperscript{CAMKCre} mice. Berger et al (2006) described that inactivation of the MR gene in the forebrain did not impair survival of the animals and MR\textsuperscript{CaMKCre} mice were visually indistinguishable from their control littermates. Extensive behavioral studies have revealed
that these MR$^{\text{CaMKCre}}$ animals perform normally when tested for sensory and motor function and anxiety-like behavior (Berger et al., 2006). Also, in adult MR$^{\text{CaMKCre}}$ mice, a gross survey of the cornu ammonis and dentate gyrus of the hippocampus by using Nissl staining showed no conspicuous change in cell number and density (Berger et al., 2006). Moreover, corticosterone levels in MR$^{\text{CaMKCre}}$ mice are comparable to those found in littermate control animals, both at diurnal trough and peak as well as after restraint stress (Berger et al., 2006).

Interestingly, we found that MR$^{\text{CAMKCre}}$ mice (n=7) displayed reduced freezing behavior during training when compared to control animals (n=8) (between-group effect, $F_{1, 13}=9.16$, $P<0.05$), yet no difference was found right after the last tone-footshock pairing ($t_{13}=1.30$, $P>0.05$). Three hours later, during the contextual memory test, MR$^{\text{CAMKCre}}$ mice displayed less freezing behavior when compared to control mice (Figure 4B, control versus MR$^{\text{CAMKCre}}$: 54.2 ± 7.9 % and 24.0 ± 7.6% respectively, $t_{13}=2.72$, $P<0.05$), with a significant between-group effect over time (Figure 4C, $F_{1, 13}=7.42$, $P<0.05$). No difference in tone-cue memory was found between MR$^{\text{CAMKCre}}$ animals and controls (Figure 4D, between-group effect, $F_{1, 13}=0.36$, $P>0.05$). These data confirm that MRs are involved in formation of contextual fearful memories.
Figure 4. Contextual and tone-cue memories in MR<sup>CAMKCre</sup> mice. Localization of MRs in wild type (A1) and MR<sup>CAMKCre</sup> mice (A2). While MR positive cells were present in cellular subfields of the hippocampus in wild type littermates, none was present in the same areas of MRCAMKCre mice. B) Three hours after training, MR<sup>CAMKCre</sup> mice displayed less total freezing during contextual memory test when compared to control animals. C) Three hours after training, MR<sup>CAMKCre</sup> mice showed less freezing behavior over time when compared to control animals during contextual memory test. D) Four hours after training, no difference in tone-cue memory was found between MR<sup>CAMKCre</sup> mice and control animals.

**Pre-training effects of spironolactone and RU38486 on fear conditioning, using strong foot shock intensity**

Corticosteroid hormones have been reported to modulate synaptic potentiation mainly under weak synaptic stimulation (Alfarez et al., 2002; Pu et al., 2009) and modulate learning and memory processes in relatively weak aversive learning paradigms (Cordero and Sandi, 1998; Sandi and
Rose, 1997). We therefore examined the effect of MR and GR antagonists separately on contextual and tone-cue memories 3 and 4 hours after training respectively, using stronger foot shock intensity (0.8 mA). Pre-training administration of spironolactone did not affect freezing behavior during training (between-group effect, $F_{1, 14}=0.19$, $P>0.05$). In the contextual memory test, three hours after training, both vehicle and spironolactone treated groups showed comparable freezing behavior (vehicle versus spironolactone: 46.9 ± 8.0 % and 54.9 ± 9.2 % respectively, $t_{14}=0.65$, $P>0.05$) with no between-group effect (Figure 5A, $F_{1, 14}=0.42$, $P>0.05$). One hour later in the tone-cue memory test, spironolactone treatment did not affect freezing behavior (Figure 5B, between-group effect, $F_{1, 14}=0.14$, $P>0.05$).

Pre-training treatment with RU38486 (n=8) did not affect freezing behaviour during training when compared to vehicle treated animals (n=8) (between-group effect, $F_{1, 14} = 0.50$, $P>0.05$). Three hours later, when contextual memory was tested, freezing behavior was comparable for both RU38486 and vehicle treated animals (vehicle versus RU38486: 46.9 ± 6.5 % and 42.1 ± 5.2 % respectively, $t_{14}=0.59$, $P>0.05$) and no between-group difference was found (Figure 5C, $F_{1, 14}=0.35$, $P>0.05$). However, similar to what we found with mild (0.4 mA) foot shocks, RU38486 increased freezing behavior in response to the tone’s presence when compared to vehicle using 0.8 mA foot shock intensity (Figure 5D, between-group effect, $F_{1, 14}=7.59$, $P<0.05$).
Figure 5. Effect of Pre-training administration of spironolactone and RU38486 on fear conditioning using strong footshock intensity. Using strong (0.8mA) learning paradigm, A) Pre-training administration of spironolactone did not affect contextual memory at three hours after training; B) Pre-training administration of spironolactone did not affect tone-cue memory at four hours after training; C) Pre-training administration of RU38486 did not affect contextual memory at three hours after training; D) Pre-training administration of RU38486 enhanced tone-cue memory at four hours after training.

Discussion
In this study we examined the role of MRs and GRs in the formation of emotional memories. In line with earlier findings (Cordero and Sandi, 1998; Oitzl and de Kloet, 1992; Pugh et al., 1997a; Pugh et al., 1997b), we found that pretraining administration of RU38486, which results in blockade of GRs during and after acquisition of a stressful learning task, impaired contextual memory. This effect was only apparent at 24 but not as early as 3
hours after acquisition which indicates that the modulatory role of GRs in emotional memory formation takes time to develop (de Kloet et al., 2005; de Kloet et al., 1999; Oitzl et al., 2001) presumably by inducing genomic cascades to promote long-term memory storage (Oitzl et al., 2001). To our surprise we found that blocking GRs enhanced tone-cue memory shortly after training. A possible explanation could be that blockade of GRs in the context of enhanced corticosterone release results in a predominant activation of MRs. In line with this assumption it has been reported that over-expression of MRs enhances memory formation for both spatial (Lai et al., 2007) and non-spatial information (Ferguson and Sapolsky, 2007). The fact that blocking GRs enhanced tone-cue freezing four hours after training while reducing contextual freezing 24 hours after training might indicate that corticosteroid hormones time-dependently affect different brain areas in tone (amygdala, prefrontal cortex) and context conditioning (hippocampus).

While the effect of GRs on contextual memory became only apparent at 24 hours after training, MR blockade was already very effective in reducing emotional memory at 3 hours after training. This effect was confirmed using forebrain-specific MR$^{\text{CAMKCre}}$ mice. MR blockade also reduced contextual fear conditioning at 24 hours after training. This may indicate that blocking MR prior to and during training might change behavioral strategy and/or appraisal of the situation and thereby interferes with memory formation.

Interestingly, blocking MR immediately after training had no effect on contextual memory nor on tone-cue memory performance, supporting that the effects of spironolactone given prior to training most likely resulted from a specific role on memory formation rather than of sensorimotor effects. The data could indicate that MRs are particularly important for the immediate appraisal of the situation during training as well as for determining an appropriate behavioral strategy (de Kloet et al., 1999; Oitzl and de Kloet, 1992; Schwabe et al., 2010), which might influence all subsequent stages of memory formation. However, we can not exclude that the lack of efficiency
of MR blockade after training to modulate emotional memory may result from full occupancy of MRs by elevated plasma corticosterone levels (induced by the stressful training paradigm) and the inability of spironolactone to prevent MR activation. Overall, these results indicate that activation of MRs during training is critical for emotional memory formation in the early phase as well as at a later stage (i.e. 24 hours) after training. Most likely, these effects are not the result of altered anxiety-related behavior: while over-expression of MRs reduces anxiety (Ferguson and Sapolsky, 2007; Lai et al., 2007), depletion of MRs does not affect anxiety related behavior (Berger et al., 2006).

In the current study we applied both pharmacological and genetic approaches to target corticosteroid receptors in the brain and examined their roles in emotional memory formation. The pharmacological approach benefits from the ability to target MRs acutely (but acts potentially also peripherally), while the genetic approach benefits from its anatomical specificity (but is slow in onset) (Berger et al., 2006). The finding that both the pharmacological approach to target MRs and the genetic approach to ablate MRs lead to impaired freezing behaviour during contextual fear conditioning strengthens the notion that MRs are involved in emotional memory formation.

Taken together, our results suggest that corticosteroid hormones, via activation of MRs and GRs, play a critical role in converting information from short-term memory into long-term memory. Although we did not measure corticosterone levels in this study, it is quite likely that the amount of hormone released under more aversive conditions (0.8 mA) is higher than that under mild conditions (0.4 mA). However, a simple linear correlation between the hormone concentration and the effect on fear learning does not seem to exist (Pugh et al., 1997b). The findings that blocking MRs and GRs affected memory of a weaker (less aversive) task much while leaving memory acquired in a stronger (more aversive) learning task unaffected is in
accordance with studies in which corticosterone potentiated memory for weak training situations, and highlights a modulatory role of corticosteroid hormones in learning and memory processes (Cordero and Sandi, 1998).

Electrophysiological studies also support this behavioral concept. First, the rapid increase in corticosteroid hormone levels after exposure to a stressful event - via a fast non-genomic mode of action - has been reported to promote synaptic transmission by increasing the frequency of mEPSCs in the hippocampus (Karst et al., 2005; Olijslagers et al., 2008) as well as in the amygdala (Karst et al., 2010b). This might contribute to a rapid enhancement of long-term potentiation by corticosterone (Wiegert et al., 2006), reflecting an increased ability to encode information. Meanwhile synaptic insertion of AMPARs which are critically involved in synaptic transmission and synaptic plasticity (Kessels and Malinow, 2009; Malinow and Malenka, 2002) is persistently enhanced via a slow activation of GRs (Groc et al., 2008; Martin et al., 2009). This increase in synaptic strength offers a mechanism by which these hormones can promote consolidation of information. Alternatively (or additionally) MR activation may affect synaptic transmission and/or plasticity via other means. The calcium-dependent K current is small when MRs are activated thereby enhancing cellular activity (Joels and de Kloet, 1990). Moreover, MR activation alters monoaminergic transmission (Joels and de Kloet, 1989). It should also be mentioned, that spironolactone in the current study may have affected peripheral processes which indirectly can change brain function. Although the precise cellular mechanisms thus need to be unraveled, our current behavioral study supports the role of MRs in fearful memory formation and different roles of MRs and GRs in learning and memory processes (Schwabe et al., 2010).

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