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

Brinks, V.

Citation

Brinks, V. (2009, February 19). *Stress, emotion and cognition : role of mineralo- and glucocorticoid receptors*. Retrieved from https://hdl.handle.net/1887/13503

Version:	Corrected Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/13503

Note: To cite this publication please use the final published version (if applicable).

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).

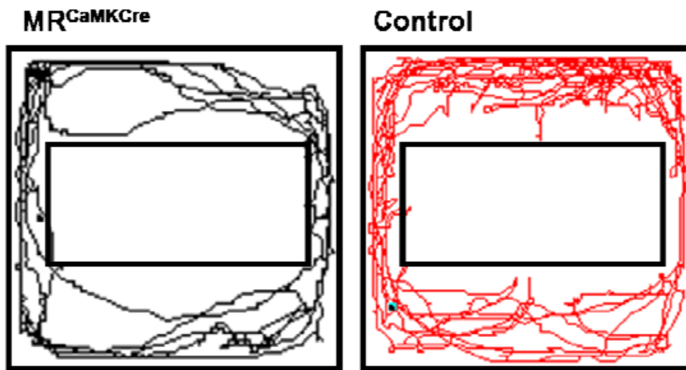

Figure 1. Representative walking patterns of MR^{CaMKCre} (left) and control mice (right) during 5 minutes of modified holeboard exposure following acute restraint stress. The outer square indicates the walls of the setting, the inner square shows the position of the holeboard.

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 $\pm$ 7.5*	3.02 $\pm$ 0.4
Time walking (%)	68.45 $\pm$ 10.4*	93.37 $\pm$ 1.6
No. walking	8.25 $\pm$ 1.3*	4.50 $\pm$ 0.5
Sitting (%)	8.69 $\pm$ 3.8	3.60 $\pm$ 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).

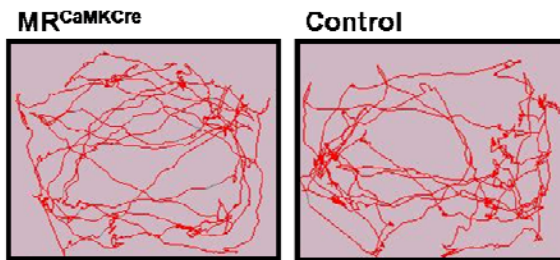

Figure 2. Representative walking patterns of $MR^{CaMKCre}$ (left) and wild-type control mice (right) during three minutes of exposure to the fear conditioning setup.

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.

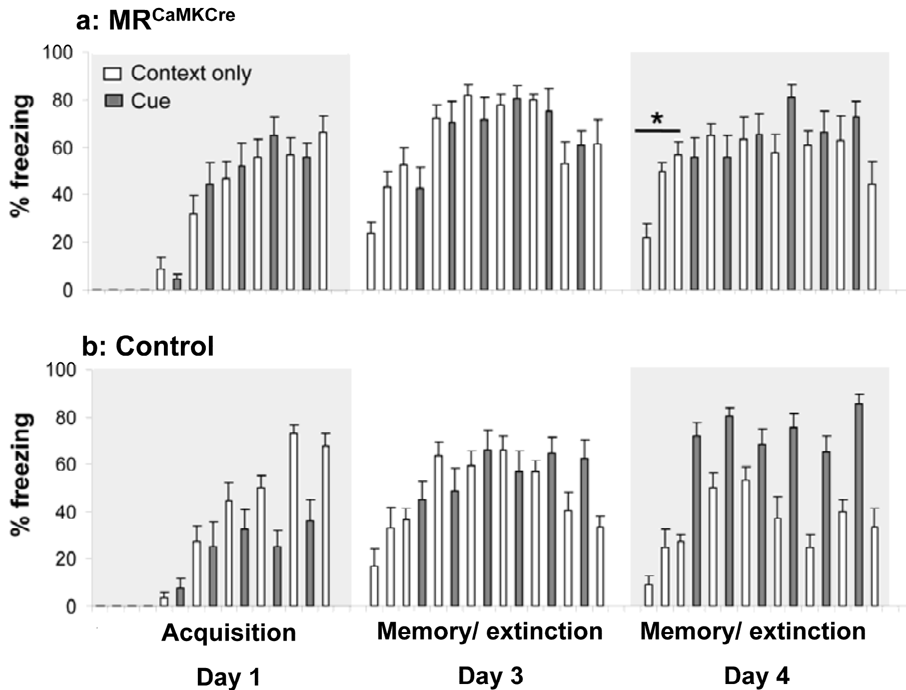

Figure 3. Percentage of freezing over the 15 intervals of context and cue exposures per testing day of MR^{CaMKCre} (A) and wild-type control mice (B). White bars: during context exposure, dark grey bars: additional cue on. ** p<0.01 compared to control.

		MR^{CaMKCre}	**Control**
Day 1: acquisition (without first 3 intervals)	Context	44.43 $\pm$ 3.88	44.39 $\pm$ 4.06
	Cue	36.90 $\pm$ 4.53 **	21.24 $\pm$ 3.35
Day 3: memory/ extinction	Context	61.85 $\pm$ 2.86 **	45.37 $\pm$ 2.68
	Cue	70.02 $\pm$ 3.12*	57.46 $\pm$ 3.40
Day 4: memory/ extinction	Context	55.45 $\pm$ 2.58 **	33.51 $\pm$ 2.47
	Cue	66.16 $\pm$ 3.35	74.54 $\pm$ 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< 0.01compared 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).

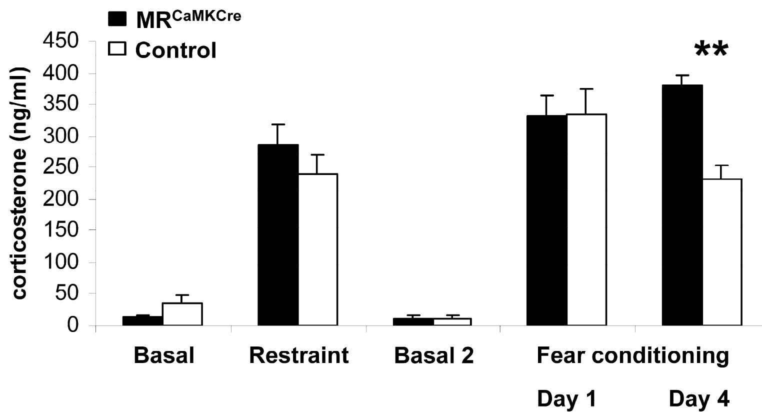

Figure 4. Plasma corticosterone concentrations measured one day before modified holeboard testing (basal), 30 minutes after restraint stress (i.e.; 20 min after modified holeboard testing), one day before fear conditioning (basal 2) and 30 minutes after the start of conditioning on day 1 and memory testing 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.

ACKNOWLEDGEMENTS

This work was supported by the Netherlands Organization of Scientific Research NWO-Cognition 051.02.10, NWO-Aspasia 015.01.076 grant , the Trier-Leiden IRTG program supported by NWO DN 95-429 and the Deutsche Forschungsgemeinschaft (grants SFB636-A3 to S.B. and G.S. and SFB636-B3 to P.G.). We thank Patrycja Wotjak and Maaike van der Mark for practical help, and Harm Krugers (SILS, Amsterdam) for scientific discussions.

Reference List

1. S. Feldman and J.Weidenfeld, Glucocorticoid receptor antagonists in the hippocampus modify the negative feedback following neural stimuli, Brain Res. 821 (1999) 33-37.

2. A.D. van Haarst, M.S.Oitzl, and E.R.de Kloet, Facilitation of feedback inhibition through blockade of glucocorticoid receptors in the hippocampus, Neurochem. Res. 22 (1997) 1323-1328.

3. A. Ratka, W.Sutanto, M.Bloemers, and E.R.de Kloet, On the role of brain mineralocorticoid (type I) and glucocorticoid (type II) receptors in neuroendocrine regulation, Neuroendocrinology 50 (1989) 117-123.

4. M.P. Donley, J.Schulkin, and J.B.Rosen, Glucocorticoid receptor antagonism in the basolateral amygdala and ventral hippocampus interferes with long-term memory of contextual fear, Behav. Brain Res. 164 (2005) 197-205.

5. M.S. Oitzl and E.R.de Kloet, Selective corticosteroid antagonists modulate specific aspects of spatial orientation learning, Behav. Neurosci. 106 (1992) 62-71.

6. M.S. Oitzl, H.M.Reichardt, M.Joels, and E.R.de Kloet, Point mutation in the mouse glucocorticoid receptor preventing DNA binding impairs spatial memory, Proc. Natl. Acad. Sci. U. S. A 98 (2001) 12790-12795.

7. T.W. Pace and R.L.Spencer, Disruption of mineralocorticoid receptor function increases corticosterone responding to a mild, but not moderate, psychological stressor, Am. J. Physiol Endocrinol. Metab 288 (2005) E1082-E1088.

8. M.S. Oitzl, A.D.van Haarst, W.Sutanto, and E.R.de Kloet, Corticosterone, brain mineralocorticoid receptors (MRs) and the activity of the hypothalamic-pituitary-adrenal (HPA) axis: the Lewis rat as an example of increased central MR capacity and a hyporesponsive HPA axis, Psychoneuroendocrinology 20 (1995) 655-675.

9. H.C. Atkinson, S.A.Wood, E.S.Castrique, Y.M.Kershaw, C.C.Wiles, and S.L.Lightman, Corticosteroids mediate fast feedback of the rat hypothalamic-pituitary-adrenal axis via the mineralocorticoid receptor, Am. J. Physiol Endocrinol. Metab 294 (2008) E1011-E1022.

10. M.S. Oitzl, M.Fluttert, and E.R.de Kloet, The effect of corticosterone on reactivity to spatial novelty is mediated by central mineralocorticosteroid receptors, Eur. J. Neurosci. 6 (1994) 1072-1079.

11. M. Lai, K.Horsburgh, S.E.Bae, R.N.Carter, D.J.Stenvers, J.H.Fowler, J.L.Yau, C.E.Gomez-Sanchez, M.C.Holmes, C.J.Kenyon, J.R.Seckl, and M.R.Macleod, Forebrain mineralocorticoid receptor overexpression enhances memory, reduces anxiety and attenuates neuronal loss in cerebral ischaemia, Eur. J. Neurosci 25 (2007) 1832-1842.

12. S. Berger, D.P.Wolfer, O.Selbach, H.Alter, G.Erdmann, H.M.Reichardt, A.N.Chepkova, H.Welzl, H.L.Haas, H.P.Lipp, and G.Schutz, Loss of the limbic mineralocorticoid receptor impairs behavioral plasticity, Proc. Natl. Acad. Sci. U. S. A 103 (2006) 195-200.

13. A.M. Rozeboom, H.Akil, and A.F.Seasholtz, Mineralocorticoid receptor overexpression in forebrain decreases anxiety-like behavior and alters the stress response in mice, Proc. Natl. Acad. Sci. U. S. A 104 (2007) 4688-4693.

14. N. Calvo and M.Volosin, Glucocorticoid and mineralocorticoid receptors are involved in the facilitation of anxiety-like response induced by restraint, Neuroendocrinology 73 (2001) 261-271.

15. V. Brinks, M.H.van der Mark, E.R.de Kloet, and M.S.Oitzl, Differential MR/GR activation in mice results in emotional states beneficial or impairing for cognition, Neural Plast. (2007) 90163.

16. V. Brinks, M.Van der Mark, E.R.de Kloet, and M.S.Oitzl, Emotion and cognition in high and low stress sensitive mouse strains: a combined neuroendocrine and behavioral study in BALB/c and C57BL/6J mice, Front. Behav. Neurosci (2007).

17. F. Ohl, A.Roedel, E.Binder, and F.Holsboer, Impact of high and low anxiety on cognitive performance in a modified hole board test in C57BL/6 and DBA/2 mice, Eur. J. Neurosci. 17 (2003) 128-136.

18. Q. Dong, A.Salva, C.M.Sottas, E.Niu, M.Holmes, and M.P.Hardy, Rapid glucocorticoid mediation of suppressed testosterone biosynthesis in male mice subjected to immobilization stress, J. Androl 25 (2004) 973-981.

19. S.L. Bowers, S.D.Bilbo, F.S.Dhabhar, and R.J.Nelson, Stressor-specific alterations in corticosterone and immune responses in mice, Brain Behav. Immun. 22 (2008) 105-113.

20. V. Brinks, E.R.de Kloet, and M.S.Oitzl, Strain specific fear behaviour and glucocorticoid response to aversive events: modelling PTSD in mice, Prog. Brain Res. 167 (2008) 257-261.

21. J.W. Smythe, D.Murphy, C.Timothy, and B.Costall, Hippocampal mineralocorticoid, but not glucocorticoid, receptors modulate anxiety-like behavior in rats, Pharmacol. Biochem. Behav. 56 (1997) 507-513.

22. M. Kabbaj, D.P.Devine, V.R.Savage, and H.Akil, Neurobiological correlates of individual differences in novelty-seeking behavior in the rat: differential expression of stress-related molecules, J. Neurosci 20 (2000) 6983-6988.

23. V. Brinks, M.H.van der Mark, E.R.de Kloet, and M.S.Oitzl, Differential MR/GR activation in mice results in emotional states beneficial or impairing for cognition, Neural Plast. 2007 (2007) 90163.

24. H. Karst, S.Berger, M.Turiault, F.Tronche, G.Schutz, and M.Joels, Mineralocorticoid receptors are indispensable for nongenomic modulation of hippocampal glutamate transmission by corticosterone, Proc. Natl. Acad. Sci. U. S. A 102 (2005) 19204-19207.

25. A.I. Herrero, C.Sandi, and C.Venero, Individual differences in anxiety trait are related to spatial learning abilities and hippocampal expression of mineralocorticoid receptors, Neurobiol. Learn. Mem. 86 (2006) 150-159.

26. E. Kitraki, O.Kremmyda, D.Youlatos, M.Alexis, and C.Kittas, Spatial performance and corticosteroid receptor status in the 21-day restraint stress paradigm, Ann. N. Y. Acad. Sci. 1018 (2004) 323-327.

27. Z.G. Hodgson, S.L.Meddle, M.L.Roberts, K.L.Buchanan, M.R.Evans, R.Metzdorf, M.Gahr, and S.D.Healy, Spatial ability is impaired and hippocampal mineralocorticoid receptor mRNA expression reduced in zebra finches (Taeniopygia guttata) selected for acute high corticosterone response to stress, Proc. Biol. Sci. 274 (2007) 239-245.

28. R.L. Wright and C.D.Conrad, Enriched environment prevents chronic stress-induced spatial learning and memory deficits, Behav. Brain Res. 187 (2008) 41-47.

29. J.L. Yau, J.Noble, and J.R.Seckl, Continuous blockade of brain mineralocorticoid receptors impairs spatial learning in rats, Neurosci Lett. 277 (1999) 45-48.

30. S.M. Korte, Corticosteroids in relation to fear, anxiety and psychopathology, Neurosci Biobehav. Rev. 25 (2001) 117-142.