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

Brinks, V.

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

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 20^0C 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
<u>In situ hybridisation of MR and GR mRNA expression</u>
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 -80^0C until sectioning. For MR and GR mRNA measurements, frozen brains were sectioned at 12 µm using a -20^0C 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 mM Tris-HCl (pH 7.4), 50% formamide, 300 mM NaCl, 1 mM 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.

<u>Western blotting of GR protein</u>
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 $\pm$ 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 $\pm$ 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 $\pm$ 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 $\pm$ 0.86	23.54 $\pm$ 0.75*
	Prefrontal cortex	1.20 $\pm$ 0.20	3.15 $\pm$ 0.47**
	Amygdala	32.81 $\pm$ 3.45	37.56 $\pm$ 2.52
	Indusium griseum	12.44 $\pm$ 1.85	2.17 $\pm$ 1.08 **
GR mRNA	Hippocampus	26.88 $\pm$ 1.95	33.49 $\pm$ 2.16*
	Prefrontal cortex	11.13 $\pm$ 1.48	2.71 $\pm$ 1.20**
	Indusium griseum	23.56 $\pm$ 1.94	23.71 $\pm$ 2.71

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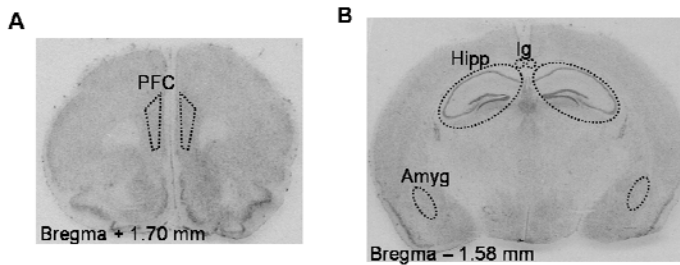
Figure 1. Coronal sections of the mouse brain, stained with cresylviolet. Dotted lines indicate the areas of interest for image analysis of optical density for MR and GR mRNA expression. A: the infra- and prelimbic areas of the prefrontal cortex (PFC), B: the hippocampus (Hipp), amygdala (Amyg) and indusium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

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

Table 2. GR protein expression corrected for total protein (grey values mean $\pm$ 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).
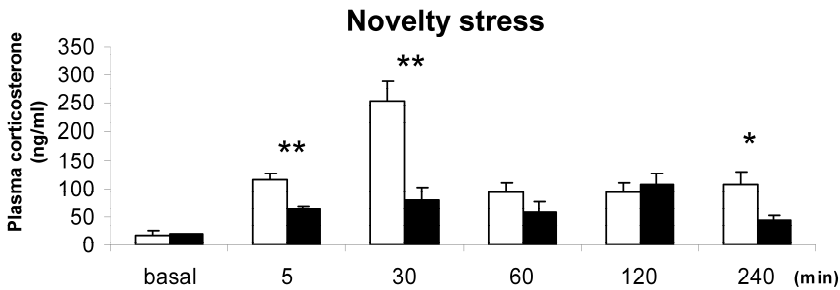
Figure 2. Corticosterone concentrations in ng/ml, basal resting and novelty induced responses at 5, 30, 60, 120 and 240 minutes of novel cage exposure. BALB/c: white bars; C57BL/6J: black bars * p < 0.05, ** p $\leq$ 0.001.

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 $\pm$ 4.9	47.8 $\pm$ 3.6*
Stretched attends (no)	5.2 $\pm$ 1.3	0.6 $\pm$ 0.2*
Lat. first centerboard entry (s)	17.3 $\pm$ 3.7	112.1 $\pm$ 19.3**
Time on centerboard (s)	124.7 $\pm$ 16.6	73.2 $\pm$ 9.0*
Distance moved (m)	32.2 $\pm$ 2.7	39.4 $\pm$ 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.
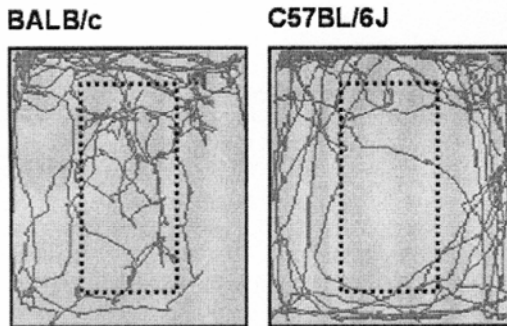
Figure 3. Representative walking patterns of BALB/c (left) and C57BL/6J mice (right) in the modified holeboard. The dotted square indicates the location of the centerboard.

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 naïve 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.
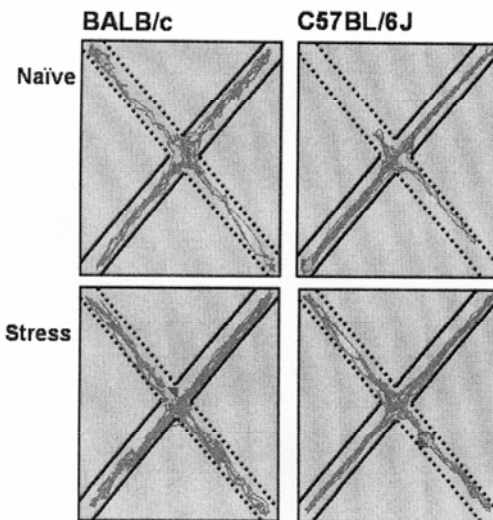

Figure 4. Representative walking patterns of BALB/c (left) and C57BL/6J mice (right) in the elevated plus maze. The dotted lines show the open arms, the straight lines the closed arms.

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.
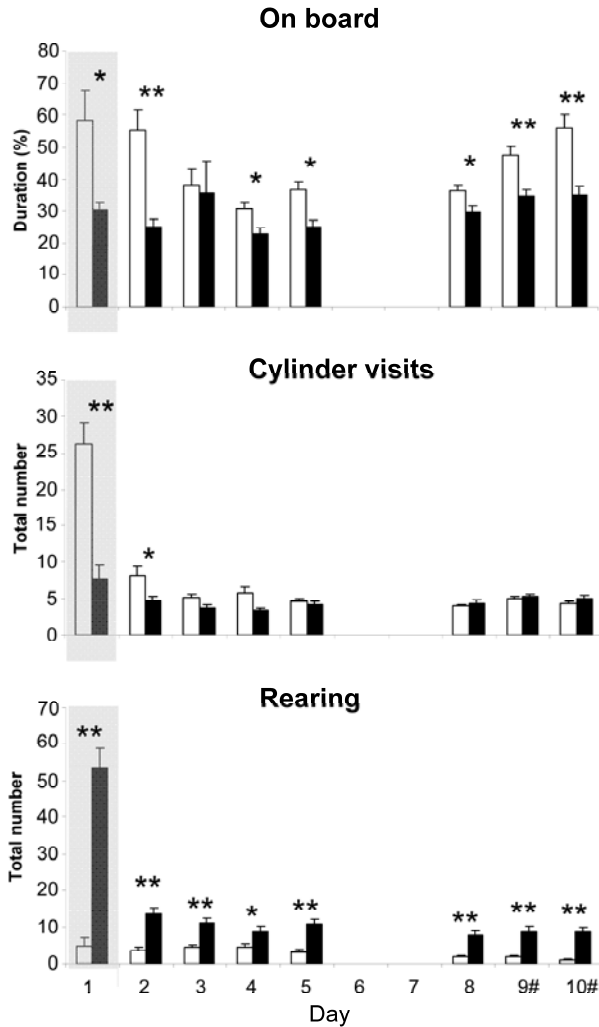

Figure 5. Emotional and explorative behavioural parameters measured on days 1 to 10 of holeboard testing of BALB/c (white bars) and C57BL/6J mice (black bars). (A) Percentage of time spent on the centerboard, (B) number of cylinder visits and (C) number of rearings. The grey background on day 1 indicates 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≤ 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.
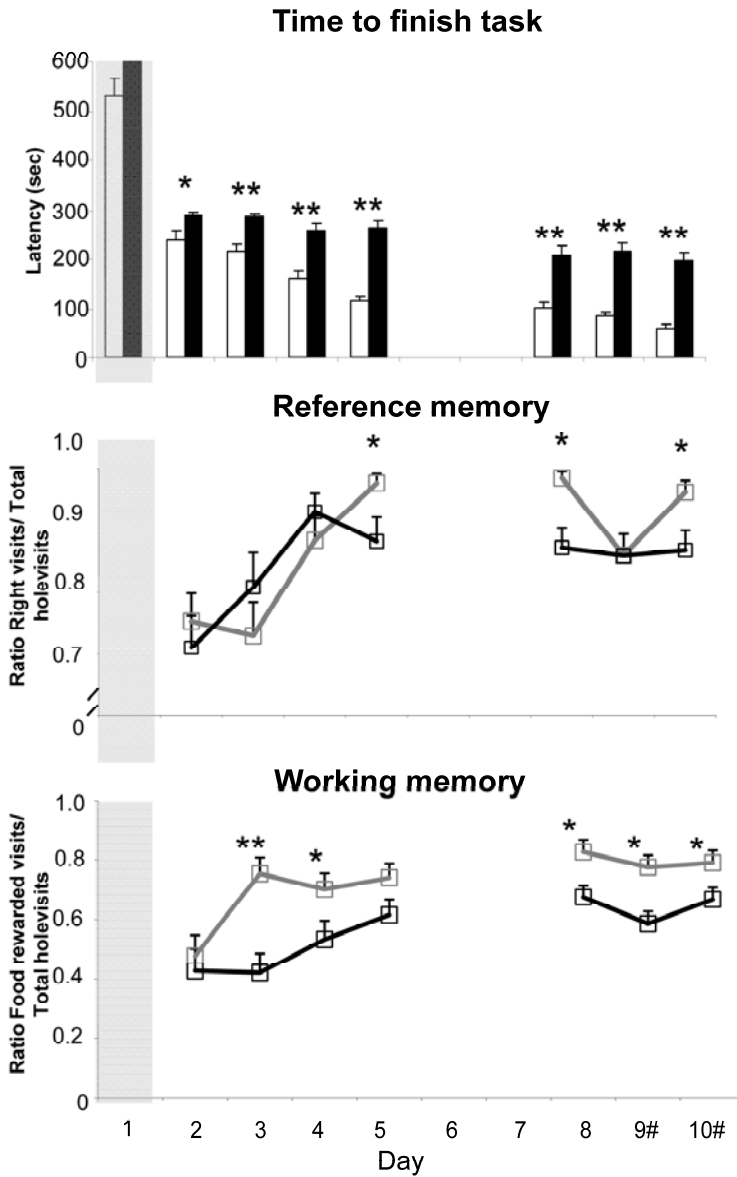
Figure 6. Cognitive parameters of modified holeboard performance of BALB/c (white bars, open squares and grey lines) and C57BL/6J mice (black bars, filled squares and black lines). (A) time to finish the task, (B) reference memory ratio, i.e., the number of baited cylinder visits divided by total cylinder 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.
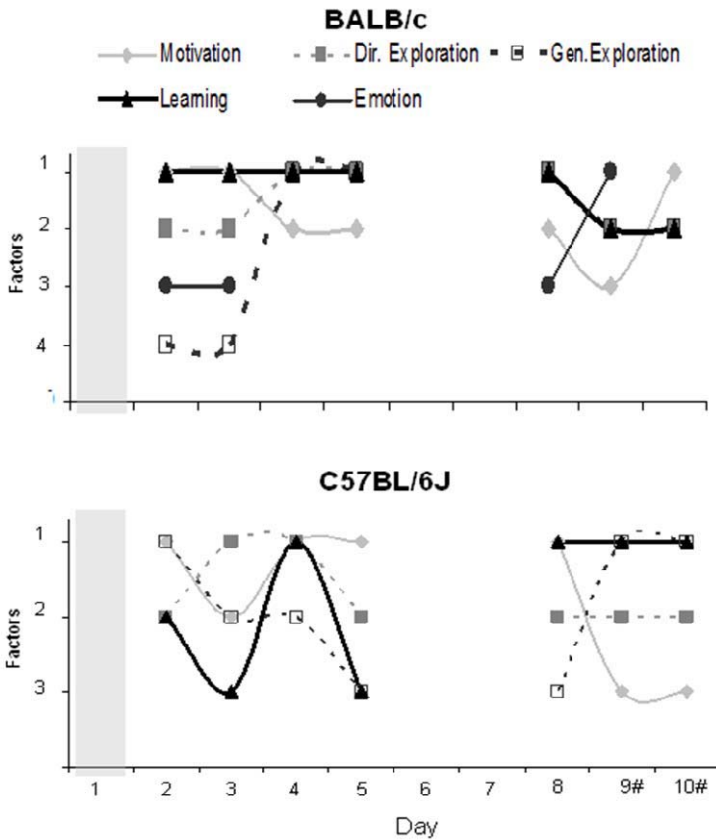

Figure 7. Distribution of behavioural classes per testing day in the extracted factors of the Principal Component Analysis. A; BALB/c mice and B; C57BL/6J mice. Symbols: Closed grey diamonds - motivation; open squares - general exploration; closed circles - emotion; closed squares - directed exploration; 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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