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Spatial Learning Deficits in Mice with a Targeted Glucocorticoid Receptor Gene Disruption

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Abstract

Previous studies in rats using the Morris water maze suggested that the processing of spatial information is modulated by corticosteroid hormones through mineralocorticoid and glucocorticoid receptors in the hippocampus. Mineralocorticoid receptors appear to be involved in the modulation of explorative behaviour, while additional activation of glucocorticoid receptors facilitates the storage of information. In the present study we used the water maze task to examine spatial learning and memory in mice homozygous and heterozygous for a targeted disruption of the glucocorticoid receptor gene. Compared with wild-type controls, homozygous and heterozygous mice were impaired in the processing of spatial but not visual information. Homozygous mutants performed variably during training, without specific platform-directed search strategies. The spatial learning disability was partly compensated for by increased motor activity. The deficits were indicative of a dysfunction of glucocorticoid receptors as well as of mineralocorticoid receptors. Although the heterozygous mice performed similarly to wild-type mice with respect to latency to find the platform, their strategy was more similar to that of the homozygous mice. Glucocorticoid receptor-related long-term spatial memory was impaired. The increased behavioural reactivity of the heterozygous mice in the open field points to a more prominent mineralocorticoid receptor-mediated function. The findings indicate that (i) the glucocorticoid receptor is of critical importance for the control of spatial behavioural functions, and (ii) mineralocorticoid receptor-mediated effects on this behaviour require interaction with functional glucocorticoid receptors. Until the development of site-specific, inducible glucocorticoid receptor mutants, glucocorticoid receptor-knockout mice present the only animal model for the study of corticosteroid-mediated effects in the complete absence of a functional receptor.

Introduction

Corticosteroid hormones modulate behavioural adaptation and cognitive function (McEwen and Sapolsky, 1995). This action occurs through the high-affinity mineralocorticoid receptors (MRs) and lower-affinity glucocorticoid receptors (GRs) which are abundantly coexpressed in hippocampal neurons (Reul and De Kloet, 1985; Van Steensel *et al.*, 1996). The different receptor affinities permit low amounts of corticosterone to occupy predominantly MRs, while higher hormone levels induced by stress progressively occupy GRs (Reul *et al.*, 1987 a, b).

Electrophysiological studies have demonstrated that MR- and GR-mediated genomic effects exert coordinated but differential control over the excitability of hippocampal neurons (Joëls and De Kloet, 1994). Low concentrations of corticosterone maintain excitability in hippocampal neuronal networks, while higher concentrations of the steroid suppress excitability transiently raised by excitatory stimuli. These steroid effects mediated by MRs and GRs are thought to add to the ability to organize and synchronize information processing

(De Kloet, 1991) which is largely ascribed to the hippocampus (Eichenbaum *et al.*, 1994; Gray, 1995; McDonald and White, 1995). Behavioural studies revealed interaction between MR- and GR-mediated effects in the coordinated control of various aspects of spatial learning and avoidance behaviour. It was found that predominant MR activation altered explorative search and escape patterns, while the brief, additional activation of GRs facilitated the consolidation of information (De Kloet *et al.*, 1988, 1994; Oitzl and De Kloet, 1992; Oitzl *et al.*, 1993, 1994; Bohus, 1994; Sandi and Rose 1994a, b; Korte *et al.*, 1995; Roozendaal *et al.*, 1996). In spite of the receptor specificity of the steroid agonists and antagonists used in these studies, the drugs lack the rigid temporal specificity for either MRs or GRs (e.g. the onset and duration of action due to the competitive nature of an antagonist). Thus, a definite conclusion about the role of each receptor type is not yet possible.

Recently, animals with a targeted disruption in the GR gene were obtained by homologous recombination in mouse embryonic stem

cells (Cole *et al.*, 1995). These homozygous GR-deficient (GR-knockout) animals provide a unique model to study the behaviour of animals that have never experienced a GR-mediated effect, and to examine the specific function of MRs in the absence of any GR-related interaction.

In the present study we examined the spatial learning and exploratory behaviour of homozygous and heterozygous GR-deficient mice and their wild-type controls. Based on our earlier findings using specific receptor antagonists to block central corticosteroid receptors, we expected (i) the spatial learning ability of GR mutants to be disturbed, and (ii) behavioural reactivity as an indication for MR-function to become prominent. We developed a special design of the Morris water maze test, which is widely used to study hippocampus-dependent spatial learning and memory in rodents (Morris *et al.*, 1982; Brandeis *et al.*, 1989). Behavioural reactivity towards an object in a large open field was used as an indicator of preferentially MR-mediated effects (Oitzl *et al.*, 1994). General locomotor activity was measured in a small and large open field. The findings demonstrate that GR-deficient mice have a specific deficit in spatial learning.

Materials and methods

Animals

Glucocorticoid receptor-deficient mice were generated as a mixed background strain (C57BL/6 and 129/J) and were outbred as described previously (Cole *et al.*, 1995). Male GR-mutants [six homozygous mice, mean age 7.5 ± 1.3 (SEM) months; 12 heterozygous mice, age 6.8 ± 0.7 months], five sibling wild-type mice from the German Cancer Research Center, Heidelberg, Germany, and eight mice generated from the C57BL/6 and 129/J strains (from the breeding facilities at the University of Leiden, The Netherlands), all aged 4–10 months, were allowed to acclimatize to the new environment for at least 3 weeks. The mice were housed singly, food and water were available *ad libitum*, and the light was on from 07.00 to 19.00 h each day. With respect to the tested parameters, no significant differences were found between the last two groups and their data were pooled (wild-type control group, $n = 13$, age 8.5 ± 0.8 months). After behavioural testing most of the animals were used for electrophysiological and morphological studies (Hesen *et al.*, 1996; Meijer *et al.*, 1997).

Animals were housed and tested in the same room. All animals participated in the following test programme. On day 1 general locomotor activity was determined in a novel environment (small cage; dim, standard light condition); on days 2–4 spatial (and visual) learning and memory were determined in a water maze and free-swimming trials; on day 5 general locomotor activity and behavioural reactivity were determined in a large open field (bright light condition). Because of the number of animals ($n = 31$) and the need to restrict behavioural testing to the period between 08.30 and 14.00 h, the tests were run twice with half of the animals per week. All tests were videotaped and analysed using an image analysis system (EthoVision, Noldus Information Technology, Wageningen, Netherlands). The experimenter was unaware of the genotype of each mouse.

Handling of the mice

Prior to behavioural experiments, rats are usually handled. This is not recommended for mice, which try to avoid being touched by hand. Since the animals had to be placed in and removed from the behavioural apparatus, particular attention was paid to handling the mice gently and quietly. Before behavioural testing the ability of mice to climb onto and retain their hold on a metal grid tilted at 45°

TABLE 1. Schedule of the water maze test. FS: free swim trial; spatial: platform under the water surface in a fixed location; visible: platform above water level in variable positions.

Inter-trial-intervals			
	20 min ▼	<5 min ▼	90 or 120 min ▼
Day 2 FS 1 spatial		trials 1 to 4	trials 5 to 8
Day 3 spatial		trials 9 to 12 + FS 2	trials 13 to 16
Day 4 FS 3 visible		trials 17 to 20	trials 21 to 24 + FS 4

was checked (~ 30 s). All mice performed equally well. From their cages, mice were picked up by the base of the tail and placed in the test apparatus. A sieve (18 cm diameter) was used to guide them to the platform of the water maze if search latency was >60 s, and to remove them after the test. Upon presentation of the sieve, animals climbed onto it and could easily be transported to their home cage. Any unwanted punishment (e.g. for finding the platform or chasing the mouse through the open field) was avoided by this procedure. Animals were always handled by the same person.

Animal care procedures were in accordance with the EC Council Directive of November 1986. All experiments were approved by Local Committee for Animal Health, Ethics and Research of the University of Amsterdam and Leiden, The Netherlands.

Locomotor activity and behavioural reactivity

Locomotor activity was measured under two conditions: a small, dimly lit cage and a large brightly lit open field. Behavioural reactivity, considered to be independent of general locomotor activity (Oitzl *et al.*, 1994; Dai *et al.*, 1995), was defined as any activity involving exploration of the object in the centre of the large open field. Whereas locomotor activity decreased over time, behavioural reactivity increased or remained stable.

Equipment and procedure

Small cage. Four mice were placed at the same time into one of four small cages (Plexiglas, $25 \times 25 \times 30$ cm; one transparent wall) for 30 min. The total walking distance and the walking activity pattern (2 min periods) were determined.

Open field. An object (a glass ball, diameter 5 cm) was placed in the centre of a large open field (diameter 80 cm; white floor and walls 30 cm high). A 150 W lamp was fixed 1.50 m above the open field. The mouse was gently placed somewhere along the side wall and allowed to explore for 10 min. To assess behavioural reactivity and locomotor activity the open field was subdivided into three zones: a 'wall area' (circle of 10 cm along the side wall; 37% of the arena); a 'middle area' (circle of 20 cm; 57% of the arena); and an 'object area' (circle of 10 cm around the centre of the object; 6% of the arena). The indicators of behavioural reactivity used were the walking distance, and the time spent in the object area, the number of entries into the object area, and the distribution of activity over time. General locomotor activity was given by the total walking distance and its change over time.

Spatial learning and memory

Spatial learning requires a functional hippocampus. The most commonly used task to test spatial learning and memory is the Morris water maze (Morris *et al.*, 1982; Sutherland *et al.*, 1983; Morris, 1989). In this apparatus, rats or mice are trained to locate a submerged platform. In the course of training trials the animals use several strategies to reach the platform. Transitions are made from exploratory strategies at the beginning of water maze training to goal-directed escape. The latter, the most efficient spatial strategy, requires the processing of spatial information. In the spatial condition, the underwater platform is kept in a fixed position with variable starting points. A visible condition with the platform above the water level, positioned at different locations and at fixed or variable starting points, is used to control for sensory, motor and motivational abilities in a non-spatial context. In the absence of the platform, free-swimming trials are run at different time points of training to determine search patterns.

Localization of the platform means that the animal climbs and remains for a certain period on the platform. Spatial localization is the ability of the animal to swim directly to the site of the platform. In pilot studies with C57BL/6 mice we observed that mice swam faster than rats, which increased their chance of bumping against the platform. Often they did not climb onto it and continued swimming. Mice also sometimes climbed onto the platform and immediately jumped off, which made it difficult to differentiate between spatial location and accidental finding of the platform during the training trials. The time spent on the platform is required for spatial information processing (Sutherland and Lingard, 1982; Holzhäuer and Bureš, 1986; Bohbot *et al.*, 1996). In contrast to rats, mice often have to be 'trained' to remain on the platform. We found that this can be done by holding a large sieve above the mouse during the first session; the animal will remain on the platform thereafter. Thus, for the training trials we noted if the animal bumped against the platform and continued swimming, or tried to escape from the platform by jumping off and had to be covered by the sieve in order to keep it on the platform.

Equipment

A pool (white; diameter 80 cm) was filled with warm water ($26 \pm 1^\circ\text{C}$), made opaque by the addition of chalk. A platform (diameter 8 cm) was situated 8 mm below the surface of the water, invisible to the animal (spatial condition) or 8 mm above the water level (dark coloured rim; visible condition). The pool was divided into four quadrants with the platform in the middle of one of the quadrants. For each trial, the mouse was placed in the water at a different location. A maximum of 60 s was allowed, during which the mouse had to find the platform and climb onto it. It remained there for 20 s (trials 1–4) or 10 s (other trials). If the animal failed to find the platform, it was guided with a sieve to the platform, where it was allowed to stay for 20 s. Four animals were run sequentially for the same trial in a session of four trials, resulting in an intertrial interval (ITI) of maximally 5 min (except that between trials 1 and 2, which was 10 min). This was done to prevent physical exhaustion of the mice. After each trial mice were placed under a red warming lamp to dry. Free-swimming trials were run at a certain time of training. The platform was removed, and the mouse was placed into the water opposite the previous location of the platform and allowed to swim for 60 s.

Schedule and procedure

An overview of the water maze schedule is presented in Table 1. At the end of day 1 the pool was filled with 2 cm of warm water. This was the mouse's first contact with water and each animal was allowed to move around for 120 s. Day 2 started with a 120 s free swim (FS 1) in the absence of the platform, followed 20 min later by the first spatial training trial. We expected this to motivate the animal to search for escape from the novel 'aversive' environment and accept the underwater platform as a 'safe' place. Moreover, we were able to estimate the swimming ability of the mice, to allow us to determine the pretraining swimming pattern of the animals and indicate any basic preferences for a certain part of the pool.

Two days of spatial training with two sessions of four trials a day

and one day of visible platform training with two sessions of four trials were given to the mice. Multiple components of memory emerge at different times after training. At least three distinct periods of amnesia, corresponding to the appearance of short-, intermediate- and long-term memory are discussed (for review see DeZazzo and Tully, 1995). To dissociate possible differential effects on memory in homozygous and heterozygous GR-deficient and wild-type control mice, different time intervals between the trials were chosen. Between

the trials within a session (e.g. trials 2 and 3) the interval was <5 min and between two sessions the interval was 90 or 120 min (trial 4 to trial 5, 120 min; trial 12 to trial 13 and trial 20 to trial 21, 90 min). These periods lie within the realm of short-term to intermediate memory. Between the last trial of one day and the first trial of the next day (trial 8 to trial 9; trial 16 to trial 17) at least 20 h elapsed. This allowed us to assess long-term memory effects.

With an ITI of <5 min, trial 12 was followed by free-swimming

FIG. 1. Performance of homozygous and heterozygous GR-deficient mice and wild-type controls in the water maze in locating a submerged (invisible) platform in the first 2 days of training and a platform above the water level (visible) on the third day. Time to reach (A) and distance swum (B) to the platform and swimming speed (C) for the 24 trials. Arrows mark the free-swimming trials (FS2, FS3): latencies, distance and speed are given for reaching the platform location the first time. Note that homozygous mice showed variable performance over trials and responded immediately to the first presentation (trial 17) of the visible platform. Data are mean $\pm$ SEM in s, m and cm/s respectively.

TRAINING TRIALS

FIG. 2. Swimming patterns of a homozygous and a heterozygous GR-deficient mouse and a wild-type control mouse in training trials 1, 4, 5, 8 and 12 to locate the underwater platform. Homozygous and heterozygous mutants adopted a circular search path and did not change to the direct spatial approach. The response to the first presentation of the visible platform in trial 17 shows that the GR-knockout mouse directly headed for the platform, in contrast to the wild-type mouse.

trial 2 (FS 2). In fact the time or distance swum to reach the platform should have been similar to or shorter than for trial 12. Of interest was the continuing swimming pattern, which indicated the accuracy of searching and a preference for a certain area of the pool. This also held true for free-swimming trials 3 and 4 (FS 3, FS 4). When trial 16 had been reached, animals were expected to have made the transition from general exploratory to spatial search for the platform. The subsequent FS 3, which was run >20 h after spatial training trial 16, was expected to provide evidence for long-term spatial memory, expressed by the time and distance to reach the position of the platform.

During the first session of visible platform training (trials 17–20) the platform was positioned in one of the other three quadrants with respect to the underwater position. The same starting position was used in these four trials. During the second session (trials 21–24) the platform remained in a fixed position opposite the former location of the underwater platform and the starting positions were varied. FS 4 was given after the last visible training trial (trial 24; ITI <5 min). As the visible platform was never positioned at the location of the underwater platform, it was expected that in the absence of the proximal visual platform cue animals might use the former spatial search pattern.

For the platform training trials we assessed the time needed and distance swum to find and climb onto the platform. From the free-swimming trials the latency and distance to reach the position of the platform were analysed (memory). Search measures were the time and distance spent in the four quadrants, and the number of exact crossings of the platform positions was used as the measure of accuracy. Total distance swum indicated the level of general activity.

To allow comparison of the four free-swimming trials, the first 60 s of the 2 min FS 1 was analysed. The swimming speed (cm/s) during training and free-swimming trials was also calculated.

Statistics

Data (mean $\pm$ SEM) were subjected to one-way analysis of variance (ANOVA, F), when appropriate with repeated measurements, followed by a *post hoc* Tukey test. The free-swimming trials were analysed by Friedman's analysis of variance (within group) and the Wilcoxon test (within group). Values for different treatment groups were compared by ANOVA followed by a *post hoc* Tukey test. Significance was accepted at $P < 0.05$.

Results

Water maze performance

Homozygous and heterozygous mutants had a deficit in the processing of spatial but not visual information, as shown by their performance in training and free-swimming trials.

Training trials

Figure 1 depicts the performance of mice during the 2 days of training with the underwater platform. Homozygous mice performed quite variably from trial to trial [trial $\times$ group interaction: latency, $F_{30,420} = 1.586$, $P < 0.05$ (Fig. 1A); distance, $F = 1.738$, $P < 0.01$ (Fig. 1B)] in comparison with the other groups. Heterozygous mutants and wild-type control mice decreased their time to locate the platform consequently from trial to trial (within group effect over trials:

TABLE 2. The number of bumps (mean $\pm$ SEM) against the platform without climbing onto it in the first eight trials and number of animals per group (n/n), showing that this behaviour did not differ between groups

	Bumps	(n/n)	Jumps (n/n) ¹
Homozygotes	6.6 $\pm$ 0.7	6/6	6/6
Heterozygotes	8.4 $\pm$ 2.1	11/12	10/12
Wild-type	7.8 $\pm$ 1.9	10/13	3/13

¹Number of animals that jumped off the platform more than once within <2 s and had to be restricted to the platform with a sieve. More homozygous and heterozygous mutants jumped off than wild-type mice.

latency, $F_{15,420} = 15.670$, $P < 0.001$; distance, $F = 15.683$, $P < 0.001$). During training trials swimming velocity did not differ significantly between the groups and did not change over trials (Fig. 1C). However, wild-type animals swam more slowly in most of the trials. Typical swimming patterns of six training trials for the three groups of animals are presented in Figure 2. The swimming patterns of the training trials showed that homozygous and also heterozygous mutants developed a more circular explorative or searching pattern. This was very effective for reaching the platform position but does not reflect spatial learning. Regarding the latencies to reach the platform, heterozygous mutants behaved similarly to wild-type controls. That the latency measure was not able to reveal the spatial or non-spatial learning component will be substantiated below in the description of the free-swimming trials. As can be deduced from Figure 1, the different lengths of the ITIs influenced the performance of GR-deficient but not that of wild-type mice.

To shed light on the variable performance of the homozygous mice, their behaviour during the first eight spatial training trials was analysed in more detail. Using their specific circular strategy, homozygous mice performed well in terms of the number of seconds needed to locate the platform during the first four trials. In trial 4 they located the platform significantly faster ($P < 0.05$) than heterozygous and wild-type mice (Fig. 1A, Fig. 2). After the 120 min ITI, the performance of the three experimental groups was similar. However, with respect to their own performance in trial 4, the homozygous animals took significantly longer during trial 5 to locate the platform (Wilcoxon, $P < 0.05$). The performance in trial 7 was similar between the groups, but in trial 8 four of the six homozygous animals did not locate the platform at all ($P < 0.01$). Trials 13–16 showed a similar pattern. Other responses also pointed to altered behaviour of the GR-deficient mice. As shown in Table 2 the homozygous, heterozygous and wild-type mice were similar in the number of times they bumped against the platform. Unlike the wild-type animals, homozygous and heterozygous mutants jumped and/or had to be prevented from jumping more often. Jumps were not related to a particular trial number and were directed not towards the side wall but to some area of the pool. During trials 9–24 mice did not jump.

In the visual condition (Fig. 1) mice had to locate a platform above the water level. Independent of the location of the platform or starting point, mice of all groups approached the visible platform directly. The homozygous mice lost their variable performance. The GR-knockout mice swam to the visible platform without hesitation (trial 17). The significantly longer latencies of heterozygous mutants and wild-type control mice in trial 17 only ($P < 0.05$; Fig. 1A) were due to their search at the previous location of the underwater platform (see also Fig. 2). Homozygous mice swam the shortest distance (Fig. 1B; $P < 0.05$ versus heterozygous and wild-type mice) with the highest speed (Fig. 1C; $P < 0.05$ versus wild-type mice). This was reflected by the number of crossings (mean $\pm$ SEM) of the underwater

platform location, which was significantly higher ($P < 0.05$) in wild-type (2.0 ± 0.8) than in homozygous mice (0.2 ± 0.2), with an intermediate value for heterozygous animals (1.1 ± 0.6). In the visible trials following trial 17, the homozygous and heterozygous mutants did not show the circular swimming pattern that they showed in the underwater training trials.

Free-swimming trials

Inspection of the water maze training data, i.e. time and distance to the platform, might give the impression that the spatial learning abilities of heterozygous mutants are similar to those of wild-type control animals, and that only homozygous mice behave differently. However, analysis of the free-swimming trials uncovered the altered processing of spatial information in the heterozygous GR-deficient mice (Figs 3 and 4, Table 3).

The first free-swimming trial performed before training (FS 1) revealed that all mice had the coordinated motor skills required for swimming. They swam similar distances, mainly close to the side wall. This resulted in a generally low number of crossings of the later platform locations. Incidentally, all groups spent the least time in the future underwater platform quadrant ($P < 0.05$).

The free-swimming trial performed shortly after trial 12 (FS 2) showed that only wild-type mice spent most of the time in the platform quadrant and crossed the exact location of the platform (time in quadrant, Friedman, 12.16, $P < 0.01$; crossings of platform, Friedman, 10.20, $P < 0.01$) significantly more often than in the other three quadrants (Wilcoxon, $P < 0.01$). Only wild-type animals preferentially crossed the submerged platform location (11 of 13 wild-type mice; three of 12 heterozygous mice; one of six homozygous mice). Heterozygous mutants did not focus their search to one quadrant (Friedman not significant), but spent the least time and fewest crossings (Wilcoxon, $P < 0.01$) in the left adjacent quadrant. Also, homozygous mice spread their search (Friedman not significant) over three quadrants, with the opposite quadrant visited least compared with the platform quadrant (time and crossings, Wilcoxon, $P < 0.05$). This differential distribution of time per quadrant and crossings indicates that only wild-type mice expressed a focused search pattern. The circular swimming pattern within the platform distance of the pool (Fig. 4) demonstrated the differences between wild-type and homozygous and heterozygous mice; differences in total swimming distance and speed were statistically significant. Figure 1 and Table 3 depict the latencies to reach the platform position the first time, the swimming velocity and distance. Homozygous mice took the longest time and distance to the former platform ($P < 0.05$ versus wild-type) with no significant difference in swimming speed.

More than 20 h after trial 16, the third free-swimming trial (FS 3) was run. By trial 16 animals were expected to have switched from general exploratory to spatial searching for the platform. Homozygous and heterozygous mutants and wild-type mice differed significantly in their performance in reaching the platform position for the first time. A difference in search strategy and long-term memory between the groups was indicated by the significantly shorter distance to the platform ($F_{2,28} = 3.294$, $P < 0.05$) for wild-type compared with homozygous and heterozygous mutants. As shown in Table 3, homozygous mice compensated for their longer route to the platform by swimming significantly faster. For the total duration of FS 3, swimming velocity differed significantly between the groups, which was also expressed in the significantly longer swimming paths of homozygous and heterozygous mutants compared with wild-type controls ($F = 7.207$, $P < 0.01$). The increased activity of homozygous animals was also reflected in their significantly higher total number of crossings of

FIG. 3. Homozygous and heterozygous GR-deficient mice behaved differently during free-swimming trials when compared with wild-type controls. Number of crossings of (possible) platform locations (left) and seconds spent in the quadrants (right) during the four free-swimming trials represent the two measures of the search pattern. The schematic drawing of the pool (above right) shows the four quadrants. The platform quadrant and the position of the underwater platform are marked with the dark, cross-hatched pattern. Left and right adjacent and opposite (blank, hatched, dotted) quadrants and platform positions provide information on the exploration of the pool (FS 1) and the search pattern for the underwater platform (FS 2, FS 3), which was altered after visible platform training (FS 4). The broken line gives the level of an equal distribution of time per quadrant. Data are mean $\pm$ SEM. * $P < 0.05$ for platform position or platform quadrant versus the other three positions or quadrants after platform training.

(possible) platform locations. With respect to the swimming pattern, again the wild-type animals showed a clear-cut preference for the underwater platform quadrant (time in quadrant, Friedman, 7.62, $P < 0.05$; crossings, Friedman, 15.3 $P < 0.002$; significantly different for both measures from the other three quadrants,

Wilcoxon, $P < 0.05$). In heterozygous mice the number of crossings of the exact location was significantly higher (Friedman, 12.73, $P < 0.002$; significant versus all other locations, Wilcoxon, $P < 0.01$). The time heterozygous mutants spent in the platform quadrant did not differ from all other three locations [platform

FREE SWIM TRIALS

FIG. 4. Swimming patterns during the four free-swimming trials of the same homozygous and heterozygous and wild-type animals as in Figure 2. The small circles indicate the positions of the platform; the underwater platform and its quadrant are in the right lower quadrant of the pool. The swimming paths illustrate the focused search of wild-type animals in the platform quadrant and the random, circular swimming pattern of the homozygous and heterozygous mice.

significantly different from left and opposite quadrants (Wilcoxon, $P < 0.05$) but not from the adjacent right quadrant]. Homozygous mice did not distinguish among the platform, right and left adjacent quadrants. The number of crossings was not focused to the platform location only.

The last free-swimming trial (FS 4) performed after visible platform training revealed that the clear selectivity to search in the underwater platform quadrant was not present in the wild-type mice and was thus similar to that in homozygous and heterozygous animals. The distance swum to reach the platform for the first time was similar between groups (data not shown). However, wild-type animals still crossed the exact location of the original underwater platform significantly more often (Friedman, 7.82, $P < 0.05$) than the three other sites (Wilcoxon, $P < 0.05$). There were still nine out of the 13 wild-type mice that preferentially crossed the position of the underwater platform. This indicates that, in the absence of proximal visual cues, previously successful strategies can be used. Also, heterozygous mutants showed an increased number of crossings of the platform location (Friedman, 8.60, $P = 0.05$), but this was not significantly different from the number for the adjacent right location. Six of 12 heterozygous mutants preferentially crossed the underwater platform location, but none

of the homozygous animals did so. For the homozygous mice we found an even distribution of time and platform crossings over the four quadrants. Again, homozygous animals swam the longest distances ($F_{2,28}$, 3.278, $P < 0.05$; homozygous versus wild-type, $P < 0.05$; heterozygotes not significantly different from homozygotes and wild-types).

The series of free-swimming trial patterns represented in Figure 4 are for the same animals as those for which training trial swimming patterns are presented in Figure 2. The swimming paths present an impression of the animals' search for the platform. It also demonstrates that homozygous as well as heterozygous mutants used a non-spatial strategy to locate and search for the platform. They swam in smaller or larger circles at a certain distance from the side walls. This was on most occasions very effective, as can be seen from the latencies to reach the platform in Figure 1.

It is questionable if the length of the swimming path in the 60 s free-swimming trial is another measure of general activity only. Swimming distances did not differ significantly between the groups in the first free-swimming trials. The later free-swimming trials reflected a combination of activity, learning and other search components. The specific search in the platform quadrant most probably reduced the distance, indicating that the longer path

A locomotor activity **B** behavioral reactivity

FIG. 5. (A) Locomotor activity presented as distance (m) walked in the large open field and (B) behavioural reactivity expressed as seconds per minute spent close to the object in the centre of the large open field (mean $\pm$ SEM per 2 min for the 10 min observation). As in the small cages, homozygous GR-deficient mice showed the highest amount of locomotor activity. Behavioural reactivity was highest in heterozygous GR-deficient animals.

TABLE 3. Different behavioural parameters of the four free-swimming trials in the absence of the platform

General activity	FS 1	FS 2	FS 3	FS 4		
Total crossings						
Homozygous	9.17 ± 1.6	13.16 ± 1.5	14.01 ± 1.5	15.5 ± 1.4		
Heterozygous	10.08 ± 0.9	12.16 ± 1.0	10.75 ± 1.4	13.5 ± 1.7		
Wild-type	9.57 ± 1.0	10.71 ± 0.9	10.00 ± 1.2	11.2 ± 0.7		
Distance swum						
Homozygous	10.59 ± 0.9	11.57 ± 0.9	12.95 ± 0.7	14.48 ± 0.4		
Heterozygous	10.83 ± 0.6	12.46 ± 0.4	11.28 ± 0.6	13.84 ± 0.5		
Wild-type	11.15 ± 0.5	10.87 ± 0.4	9.42 ± 0.5	12.84 ± 0.3		
Spatial learning and memory	FS 2			FS 3		
	Latency	Distance	Speed	Latency	Distance	Speed
Homozygous	18.1 ± 2.4	3.4 ± 0.4	19.3 ± 1.8	11.5 ± 2.4	2.4 ± 0.4	21.5 ± 1.4
Heterozygous	14.8 ± 2.6	2.9 ± 0.5	20.7 ± 0.6	16.2 ± 2.7	3.1 ± 0.9	18.8 ± 1.0
Wild-type	10.0 ± 2.7	1.7 ± 0.5	18.1 ± 0.7	8.8 ± 1.8	1.4 ± 0.3	15.7 ± 0.8

FS 1, before training; FS 2, immediately after trial 12; FS 3, >20 h after trial 16; FS 4, after eight trials with the visible platform. General activity is given as the number of crossings of the four possible platform positions and the distance swum in metres during the 60 s of the free-swimming trials. For spatial learning and memory, the latency (s), the distance swum (m) to reach the platform position and the speed (cm/s) of the animals in FS 2 and FS 3 indicate a spatial learning and memory deficit in homozygous and heterozygous GR-deficient mice. Data are mean $\pm$ SEM.

* $P < 0.05$.

swum by homozygous and also heterozygous mutants might also have been related to their learning deficit.

Behavioural reactivity

For the observation time of 10 min the time spent exploring the object differed significantly between the groups ($F_{2,28}$, 4.548, $P < 0.05$). In contrast to their locomotor activity (Fig. 5A), heterozygous mutants showed a pattern of behavioural reactivity different from that of homozygous and wild-type mice (Fig. 5B, Table 4). Behavioural reactivity, i.e. exploration of the object and its surrounding area, was most strongly affected during the first minutes of exposure to the

large open field. The heterozygous mice spent significantly more time in the object area (first minute, $F_{2,28} = 5.958$, $P < 0.01$; minutes 1–4, $F_{2,28} = 3.247$, $P < 0.05$). The degree of behavioural reactivity did not change significantly over time in homozygous and heterozygous mice, but increased in wild-type animals (time $\times$ group interaction, $F_{4,88} = 4.056$, $P < 0.01$; wild-type, Friedman, 11.492, $P < 0.02$). Elements of behaviour in the first minute (Table 4) showed that the heterozygous mutants entered the object area first, spent more time there and visited this area more often than wild-type mice. As a consequence, the heterozygous mutants walked the least distance in the wall area during this period. Figure 5A, B and Table 4 also

FIG. 6. Homozygous mice walked longer distances than heterozygous GR-deficient and wild-type mice in a small cage. Data are mean $\pm$ SEM metres walked per 2 min period and during the 30 min observation period.

TABLE 4. Behavioural reactivity and locomotor activity of homozygous, heterozygous and wild-type mice in the large open field with an object in its centre

Behavioral reactivity: object area							
	Latency to enter	Seconds spent		Entries		Distance walked (m)	
		First minute	Total	First minute	Total	First minute	Total
Homozygous	27.9 ± 5.7]	6.8 ± 1.1]	121 ± 24	3.0 ± 0.5	44.3 ± 6.3]	0.64 ± 0.24	8.9 ± 1.8]
Heterozygous	11.6 ± 2.0]*	16.9 ± 4.0]*	217 ± 43	4.3 ± 0.8]	27.5 ± 4.0]*	0.65 ± 0.15]	5.2 ± 0.9]*
Wild-type	35.9 ± 8.2]	3.9 ± 1.4]	149 ± 14	1.6 ± 0.4]*	26.6 ± 3.5]	0.23 ± 0.07]*	4.7 ± 0.6]

General locomotor activity					
	Distance in meters				Speed (cm/s)
	Wall area		Whole open field		
	First minute	Total	First minute	Total	
Homozygous	2.7 ± 0.3]	25.3 ± 1.5]	5.3 ± 0.4]	47.0 ± 4.3]	6.2 ± 0.2]
Heterozygous	1.2 ± 0.2]*	18.0 ± 2.7]*	3.4 ± 0.6]*	25.1 ± 2.2]*	4.3 ± 0.4]*
Wild-type	2.2 ± 0.4	15.0 ± 2.2]	3.7 ± 0.5	24.5 ± 2.6]	3.7 ± 0.4]

Data are presented for the first minute and the observation period of 15 min (total); mean $\pm$ SEM.

* $P < 0.05$.

show that locomotor activity is dissociated from behavioural reactivity. For example, the homozygotes entered the object area as often as the heterozygous mutants, ran approximately the same distance but spent less than half of the time exploring the object. Locomotor activity decreased over time; behavioural reactivity did not.

Locomotor activity

The homozygous mice walked significantly more than mice of the other two groups (Fig. 6; small cages, $F_{2,28} = 4.505$, $P < 0.05$; Fig.

5A and Table 4; open field, $F_{2,28} = 5.146$, $P < 0.01$). The degree of locomotor activity was similar between heterozygous mutants and wild-type mice. Although in the small cages the level of activity showed only a slight decrease over time in all three groups (Fig. 6; habituation of locomotor activity), it was significant for the homozygous (Friedman, 26.56, $P < 0.05$) and wild-type mice (Friedman, 43.69, $P < 0.05$). In the large open field, locomotor activity changed significantly over time ($F_{4,112} = 20.523$, $P < 0.001$; Fig. 5A). The course of locomotor activity (time $\times$ group interaction, $F_{8,112} =$

2.335, $P < 0.02$) was similar between homozygous and wild-type mice (a decrease over time; homozygotes, Friedman, 20.933, $P < 0.001$; wild-type, Friedman, 20.615, $P < 0.001$); as in the small cage, the heterozygous mutants displayed a quite constant amount of activity (Friedman analysis not significant). The size of the apparatus determined the amount of locomotor activity. Animals of all groups walked during the 10 min observation period in the large open field (Table 4) about as much as during the 30 min in the small cage (Fig. 5). Walking distance and to some extent also the number of entries into the different parts of the large open field are measures of general locomotor activity, which was higher in the GR-knockout mice in all areas. The wall area was the preferred place for all animals, where they spent 50–60% of their time.

Discussion

Previous studies in rats and chicks demonstrated that transient GR activation by exposure to corticosteroids facilitates the storage of information; by contrast, corticosteroid-induced changes in behavioural reactivity are predominantly determined via an MR-mediated response (Oitzl and De Kloet, 1992; Oitzl *et al.*, 1994; Sandi and Rose, 1994a, b; Korte *et al.*, 1995; Roozendaal *et al.*, 1996). Accordingly, knocking out the GR was expected to eliminate GR function while MR-mediated effects were expected to remain. Thus, it was predicted that the homozygous GR-knockout animals should display (i) deficient processing of spatial information, but (ii) strong behavioural reactivity towards the object in the open field. Similar responses were expected from heterozygous animals, probably to a lesser extent.

This study shows that alterations in the corticosteroid receptor system induced by targeted GR gene disruption indeed have clear-cut and circumscribed effects on spatial learning and behavioural reactivity. The homozygous mutants found the platform by swimming in small or larger circles at a certain distance from the side wall of the pool. This circular and inaccurate search path still proved to be quite efficient in most of the training trials. Moreover, the free-swimming trials revealed that there was also no indication of any spatial bias to the correct platform quadrant and platform location in free-swimming trials. Since these homozygous mutants used other, non-spatial strategies and also learned to locate the visible platform, their learning and memory deficits specifically concerned the spatial component, which is representative of hippocampal functioning.

Typically, GR-knockout mice displayed increased locomotor activity in the open fields, jumped off the platform more often and had a higher swimming velocity in the later free-swimming trials. This increased motor activity might have interfered with the acquisition of spatial capacities, as expressed by the variable performance over trials. On the other hand, increased swimming velocity partly compensated for the spatial learning failure of GR-knockout mice, as shown in FS 3. Increased motor activity might imply disturbed hippocampal function, since mechanical and neurochemical lesions of the hippocampus and connected brain areas are known to increase locomotor activity (Gray, 1982; Sutherland *et al.*, 1983; Schenk and Morris, 1985).

The absence of spatial learning and the random search pattern in the water maze of GR-knockout mice in fact resembled the MR- and GR-related deficits described previously for short-term adrenalectomized and receptor antagonist-treated rats (Oitzl and De Kloet, 1992; Roozendaal *et al.*, 1996). Interestingly, the exploration pattern of GR-knockout mice in the open field also pointed to a loss rather than the expected dominance of MR-mediated functions. These observations may indicate that the GR has an as yet unknown regulating effect on

MR function. Some degree of MR/GR heterodimerization or synergism in MR/GR-mediated action might be required, as has been found in transfected cell systems (Trapp *et al.*, 1994). A similar conclusion regarding GR/MR interaction has been deduced from the electrophysiological responses of hippocampal CA1 neurons of GR-knockout mice (Hesen *et al.*, 1996).

The deficit in spatial learning of the heterozygous GR-deficient mice did not appear from the latencies to find the platform during training trials. Like the GR-knockout mice the heterozygous mutants used the quite efficient, more circular strategy. In the free-swimming trials however, the deficiency of heterozygous mutants in processing spatial stimuli was reflected by the absence of spatial search strategies. During FS 2, neither a preference for the platform quadrant nor the accurate localization of the platform position was observed. This deficiency in spatial processing became more obvious in FS 3. At this time, wild-type animals used spatial strategies. The heterozygous animals, however, had the longest latencies and swam the longest distance to the former platform location. Their search still was not focused to the platform quadrant only. However, as soon as these mice arrived within the platform quadrant, the exact location of the platform could be identified (i.e. being there is knowing where). Thus, in their acuity in being able to exactly cross the platform location, the heterozygous mutants were similar to the wild-types. The dissociation between localized search pattern and acuity, which has also been described by others (Schenk and Morris, 1985), indicates that the heterozygous mutants might be able to process a limited set of spatial information or have used other, non-spatial strategies to locate the platform.

Behavioural reactivity in the open field, a response that was previously found to depend predominantly on MR-mediated effects, was clearly increased in the heterozygous mutants. In line with this, *in situ* hybridization studies performed in the same group of animals showed that MR mRNA expression in CA2 and CA3 hippocampal areas of heterozygous mutants was increased compared with the homozygous mice. In the hippocampus of homozygous mutants GR mRNA was undetectable (Meijer *et al.*, 1997), whereas the heterozygous animals showed a specific reduction of GR mRNA in the CA1 area, the functionality of which is strongly correlated with the performance of spatial tasks in rats and man (Jarrard, 1978; Zola-Morgan *et al.*, 1986; Volpe *et al.*, 1992). The electrophysiological properties of hippocampal CA1 neurons of homozygous mice were found to resemble those of adrenalectomized wild-type mice. On the other hand, the large amplitudes of the calcium currents and transmitter responses of heterozygous mice compared with wild-type controls resembled the responses observed in rats subjected to very high doses of corticosterone (Hesen *et al.*, 1996). It is reasonable to assume that the differential expression of MR and GR mRNA in the hippocampal subregions and the known differential effect of corticosterone, through MRs and GRs, on neuronal activity (Joëls and De Kloet, 1994; Hesen *et al.*, 1996) contribute to altered signal transduction and concurrently to the behavioural expression of altered hippocampal functioning, as we found in these mutants.

Do the GR mutant mice represent an appropriate model for studying the role of corticosteroids in hippocampus-related behaviours? First, the GR deficiency provoked several other deviations in these mice (Cole *et al.*, 1995). As GR activation is an important factor in embryonic lung development, only 5–10% of the GR-knockout but all heterozygous mice survived the first hour after birth. Why these animals survived is not known. The appearance of a new functional receptor molecule reminiscent of GRs can be excluded by the lack of GR mRNA and the very high corticosterone level in these animals (Meijer *et al.*, 1997). All surviving homozygous mice had a non-

isogenic background. They were not retarded in growth, were not fat, and appeared healthy and mobile. Their mortality rate in later life was similar to that of heterozygous and wild-type animals. Still, we cannot exclude the possibility that by studying these surviving GR knockouts an unintended selection of animals may have taken place which was not related only to the GR deficit. This deficiency, and possibly others, which are coinherited with the GR gene disruption, may also have consequences for the development and function of brain circuits.

Secondly, the generalized GR deficiency of the GR knockouts was associated with hypercorticism, an enlarged adrenal cortex and the almost complete absence of cells of the adrenal medulla. The elevated adrenocorticotrophic hormone (ACTH) and corticosterone levels found in GR mutant mice (Cole *et al.*, 1995; Hesén *et al.*, 1996; Meijer *et al.*, 1997) are indicative of impaired negative feedback regulation of the hypothalamic–pituitary–adrenal axis. Hypercorticism and attenuated function of brain GRs are considered to be unfavourable conditions for the acquisition of spatial information (Sapolsky *et al.*, 1986; Meaney *et al.*, 1988; Bodnoff *et al.*, 1995; McEwen and Sapolsky, 1995). If the analysis of the water maze performance in the present study had been restricted to the (first) training trials, the observation would have suggested advanced spatial performance of the GR-knockout mice, and heterozygous mutants would not have appeared different from wild-type controls. Free-swimming trials uncovered the specific deficit of spatial information processing of homozygous and heterozygous GR mutants. Furthermore, one could argue that the sympathetic adrenomedullary hormones, which have long been discussed as modulators of memory (for reviews see McGaugh, 1989; Bohus, 1994), might be involved in the spatial learning deficit of GR-deficient mice. This point of view can be refuted by the fact that in rats the surgical removal of the adrenal medulla (sparing the adrenal cortex) has no effect on spatial learning and search patterns in the Morris water maze (Oitzl and De Kloet, 1992).

Thirdly, the phenotypic abnormalities of transgenic and knockout animals could simply result from the effects of background genes (Gerlai, 1996; Lathe, 1996). Specific behavioural disturbances have been reported for mice of the 129 strain; for example, the 129/SV strain displayed severe impairment in the water maze, without attempting to develop any kind of strategy (Wolfer *et al.*, 1995), and decreased locomotor activity in the open field (Gerlai, 1996). Importantly, none of our mice, which had a partial 129/J background, showed any of these extreme behaviours. GR-knockouts and heterozygous mutants readily developed several modes of performance, but did not use the spatial solution of the water maze task. It therefore seems unlikely that in the present study the genetic background determined the behavioural effects.

Clearly, the use of GR-knockouts in behavioural studies should be considered cautiously, although some of the confounding factors mentioned above can be ruled out in the present study. Notwithstanding this caution, the GR-knockout mice as yet constitute the only animal model in which corticosteroid-mediated effects on behaviour can be studied in the complete absence of functional GRs. This represents a valuable addition to the recently constructed mice bearing a GR antisense transgene, resulting in brain-specific GR underexpression (Pepin *et al.*, 1992). Partial reduction of brain GR expression in these animals was accompanied by increased levels of ACTH and corticosterone, indicative of glucocorticoid feedback resistance. In contrast to the GR-knockout mice, the GR-antisense animals showed signs of Cushing's disease and have been suggested as model for studying neuroendocrine and behavioural aspects of depression (Barden *et al.*, 1995). Striking similarities occurred in the behaviour

of these GR-antisense transgenic mice (Montkowski *et al.*, 1995) and rats treated with hippocampal injections of antisense targeted to the GR mRNA and the GR antagonist RU 38486 (Korte *et al.*, 1996): the immobility of the animals was reduced in the initial phase and the retention test of the Porsolt swimming test. Since the initial response of the animals is different already, these data might indicate the predominant involvement of MRs in addition to the reduced GR effect. If the temporal aspects of GR activation are taken into account, which is possible with receptor antagonist treatment, only the memory component of the behaviour was influenced (Korte *et al.*, 1996). Clearly, mice with a GR-antisense transgene are useful in the study of some aspects of GR-mediated effects while GR-knockout mice make it possible to study the consequences of the complete absence of GRs.

In conclusion, our results demonstrate that homozygous and heterozygous GR-deficient mice both have circumscribed deficits in their capacity to process and organize spatial information. The GRs appear to be critical for the control of spatial behavioural functions. As yet, the GR-knockout mice represent the only available animal model in which the effects mediated by MR can be studied in the complete absence of GR. This allows us to propose that for these hippocampus-related MR functions in behaviour interaction with GR is required.

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Abbreviations

ACTH	adrenocorticotrophic hormone
FS	free-swimming trial
GR	glucocorticoid receptor
ITI	intertrial interval
MR	mineralocorticoid receptor

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