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Downregulation of brain mineralocorticoid and glucocorticoid receptor by antisense oligodeoxynucleotide treatment fails to alter spatial navigation in rats

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Abstract

Adult male Brown Norway rats were long-term intracerebroventricularly (i.c.v.) infused with antisense oligodeoxynucleotides (18-mer, double endcapped phosphorothioate protected) targeting either mineralocorticoid or glucocorticoid receptor mRNA, or received the respective mixed bases sequence or vehicle. Mineralocorticoid receptor-mixed bases and glucocorticoid receptor-mixed bases oligodeoxynucleotide infusion (1 μg/0.5 μl/h) over a time period of seven days did not alter hippocampal mineralocorticoid receptor and glucocorticoid receptor binding when compared to vehicle treatment. In contrast, i.c.v. administration of mineralocorticoid receptor, as well as glucocorticoid receptor-antisense over the same time period resulted in a significantly reduced binding of mineralocorticoid receptor and glucocorticoid receptor in the hippocampus [mineralocorticoid receptor-antisense group approx. 72% of mineralocorticoid receptor-mixed bases and vehicle groups (100%); glucocorticoid receptor antisense group approx. 77% of glucocorticoid receptor-mixed bases and vehicle]. The specificity of these antisense effects is indicated by the finding that rats treated with mineralocorticoid receptor-antisense did not show any changes in glucocorticoid receptor and vice versa. Animals treated according to this infusion protocol and tested in the Morris water maze for their spatial navigation abilities failed to show significant differences among the groups. These data indicate that a reduction of hippocampal mineralocorticoid receptor or glucocorticoid receptor binding capacity by 20–30% does not interfere with spatial navigation.

Keywords: Antisense targeting; Hippocampus; Spatial navigation; Morris water maze

1. Introduction

Appropriate behavioral performance of animals in test procedures which are considered to measure learning and memory is provided by a multitude of interactions between different systems that transfer information to and between neurons at the brain level. Among the chemical substances which take part in this information transfer, glucocorticoids are thought to play a distinguished role for two reasons. Firstly, although their main source is the adrenal glands, glucocorticoids pass readily the blood–brain barrier and reach the brain tissue. Secondly, once at their target neurons, glucocorticoids form complexes with their soluble intracellular receptors and, thus, have the potential to interact with nuclear DNA and to influence gene expression directly without the necessity of activating second messenger systems.

Two different types of receptors have been described via which glucocorticoids may exert their central effects. The mineralocorticoid receptor is located almost exclusively in limbic brain areas like the hippocampus and the lateral septum and is functionally involved in the regulation of hypothalamic–pituitary–adrenocortical axis activity under basal and stress conditions (Reul and de Kloet, 1985). In contrast, glucocorticoid receptors are widely distributed within the brain including the hypothalamus and hippocampus (Reul and de Kloet, 1985, 1986; Fuxe et al., 1985). The latter receptor subtype is thought to play a central role in the mediation of the negative feedback signal of elevated glucocorticoid levels in response to

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stress (Reul and de Kloet, 1985; de Kloet and Reul, 1987; Dallman et al., 1987).

Considering their mode of action and receptor distribution within the brain, an important role of glucocorticoids in behavioral regulation has been hypothesized (de Kloet and Reul, 1987). Indeed, different studies point toward an involvement of central mineralocorticoid receptor and/or glucocorticoid receptor in spatial navigation in the Morris water maze test (Oitzl and de Kloet, 1992; Yau et al., 1995). This behavioral procedure has attracted enormous interest not only because it allows for testing the spatial navigation abilities of the experimental subjects (Morris, 1981) but also because numerous studies have indicated this procedure to be a sensitive indicator of functional hippocampal integrity (Brandeis et al., 1989).

In a recent study, the antisense targeting technique was used successfully to downregulate hippocampal mineralocorticoid receptor capacity (without affecting glucocorticoid receptor) in Wistar rats, an effect which was shown to have a considerable impact on the stress-induced activation of the hypothalamic–pituitary–adrenocortical axis (Reul et al., 1997). Thus, in the present study, we used the same protocol to investigate the effects of antisense treatment targeting the mRNA of mineralocorticoid receptor and glucocorticoid receptor on spatial navigation in the Morris water maze. However, we have chosen Brown Norway rats as experimental subjects because different authors (Tonkiss et al., 1992; Yau et al., 1994) suggest that pigmented rats differ in their spatial navigation abilities from albino strains possibly due to differences in their visual system (see, e.g., Buresova and Bures, 1971).

2. Material and methods

2.1. Animals

Male Brown Norway rats (140–180 g bwt) were obtained from Charles River, Sulzfeld (Germany). The animals were housed 4–6 per cage with free access to food and water under controlled laboratory conditions (10:14 h light:dark cycle with lights on at 0600 h, temperature 21 ± 1°C, humidity 55 ± 5%). During this study two sets of animals were used; one to investigate the effects of oligodeoxynucleotide treatment on receptor binding ($n = 12$), the other for assessing the behavioral effects of oligodeoxynucleotide administration ($n = 44$).

2.2. Oligonucleotides

Double endcapped, gel-filtered and high performance liquid chromatography-purified phosphorothioate oligodeoxynucleotide (18-mer) were custom synthesized at the Genzentrum Martinsried (Munich, Germany). Antisense sequences overlapping the respective initiation codon (Table 1) were designed for targeting the mRNA of mineralocorticoid receptor (mineralocorticoid receptor-antisense; Patel et al., 1989) or glucocorticoid receptor (glucocorticoid receptor-antisense; Miesfeld et al., 1986). Mixed bases oligodeoxynucleotides (mineralocorticoid receptor: mineralocorticoid receptor-mixed bases; glucocorticoid receptor: glucocorticoid receptor-mixed bases) contained a random sequence of the nucleotides used for the respective antisense sequences (Table 1) and showed no complementarity to any known gene sequence according to electronic searches (FASTA).

Oligodeoxynucleotides were dissolved in sterile pyrogen-free 0.9% saline. Four hours before starting surgery for i.c.v. implantation, osmotic minipump-tubing-cannula devices (each consisting of an Alzet® osmotic minipump, model: 1007D and an Alzet® brain infusion kit, Alza, Palo Alto, USA), were filled with the oligodeoxynucleotide solution or vehicle. Then the pumps were placed into sterile pyrogen-free 0.9% saline at 37°C to allow for operating with a constant pumping rate after implantation (0.5 μl/h).

2.3. Implantation of the intraventricular infusion kit

All surgical procedures were performed between 1200 and 1700 h under aseptic conditions.

For implantation of an osmotic minipump-tubing-cannula device into the right lateral ventricle, the animal was anesthetized with Halothane® (Hoechst, Frankfurt, Germany) and fixed in an stereotaxic frame. Coordinates of the implantation side were 0.5 mm rostral to bregma, 1.2 mm lateral and 2.5 mm deep from skull surface. The cannula was fixed to the skull with stainless steel screws and dental acrylic, and the minipump was placed into a

Table 1
Nucleotide sequences of receptor mRNA and oligonucleotides used

mRNA and oligonucleotides	Sequence (5′-3′; initiation codon underlined)
Mineralocorticoid receptor mRNA	GAA AAG CUA UGG AAA CCA
Mineralocorticoid receptor-antisense	TGG TTT CCA TAG CTT TTC
Mineralocorticoid receptor-mixed bases	TGA TCT CCG TAG CTA TTA
Glucocorticoid receptor mRNA	AUU UGC CAA UGG ACU CCA
Glucocorticoid receptor-antisense	TGG AGT CCA TTG GCA AAT
Glucocorticoid receptor-mixed bases	TGA AGT TCA GTG TCA ACT

subcutaneous cavity on the back side of the animal. The skin was closed with surgical silk.

2.4. Adrenalectomy, tissue collection and [^{3}H]steroid binding assay

In a separate set of experiments, the effects of oligodeoxynucleotide treatment on hippocampal mineralocorticoid receptor and glucocorticoid receptor concentration was assessed by a soluble corticosteroid binding assay (Reul et al., 1993, 1994).

For the corticosteroid binding experiments, rats were bilaterally adrenalectomized (via the dorsal approach under halothane anesthesia) on day 6 of the treatment. Adrenalectomy was performed between 0900 and 1100 h. Adrenalectomized animals received 0.9% NaCl in tap water as their drinking solution. Immediately after removal from the body, adrenal glands were carefully cleaned, placed in an eppendorf tube (adrenals of one rat per tube), and frozen on dry ice. The weight of the adrenals was determined on the same day as the day of surgery to prevent unreliable adrenal weight data due to water loss from the tissue during long-term storage at −80°C. In addition, the rats were weighed at the time of adrenalectomy.

One day after adrenalectomy, rats were quickly anesthetized with halothane and next killed by decapitation.

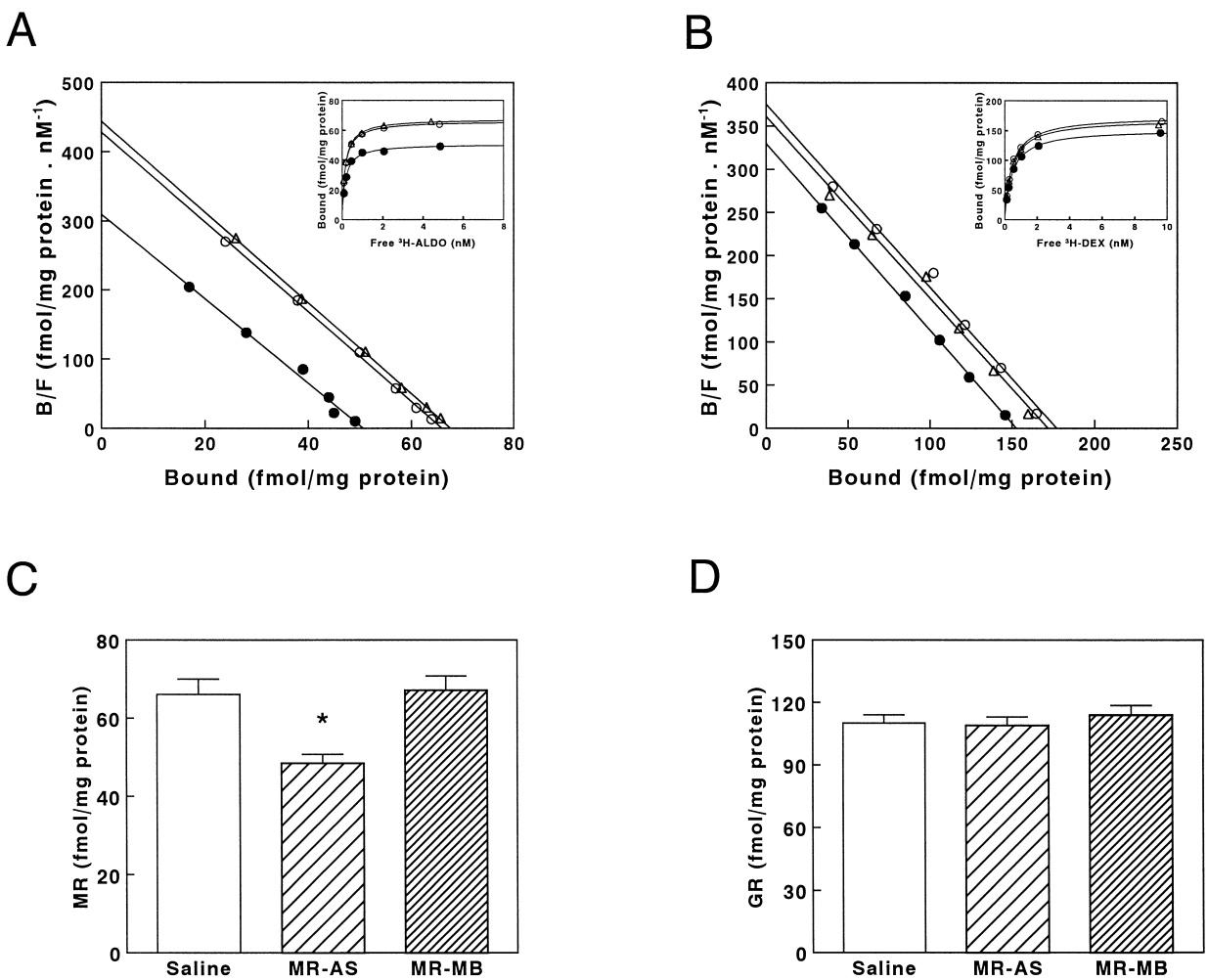

Fig. 1. Saturation binding (inset) and Scatchard analysis of [^{3}H]aldosterone binding of mineralocorticoid receptor (**A**) and [^{3}H]dexamethasone binding to glucocorticoid receptor and mineralocorticoid receptor (**B**) in hippocampus of mineralocorticoid receptor-antisense, mineralocorticoid receptor-mixed bases- and saline (= control)-treated groups (6 to 8 animals per group). One representative experiment of a total of five is depicted. The rats were intracerebroventricularly (i.c.v.) infused with oligodeoxynucleotide (mineralocorticoid receptor-antisense or mineralocorticoid receptor-mixed bases)-containing solution (1 μg/0.5 μl/h) or vehicle (saline) and killed after 1 week of treatment. In addition, all animals were adrenalectomized one day before death. The B_{max} (in fmol/mg protein) and K_d (in nM) of hippocampal mineralocorticoid receptor for binding [^{3}H]aldosterone were estimated to be 68 and 0.15, 66 and 0.15, 51 and 0.16 for the saline (△), mineralocorticoid receptor-mixed bases ○), and mineralocorticoid receptor-antisense (●) groups, respectively. The B_{max} and K_d of hippocampal glucocorticoid receptor and mineralocorticoid receptor for binding [^{3}H]dexamethasone were estimated to be 172 and 0.48, 177 and 0.47, and 153 and 0.46 for the saline (△), mineralocorticoid receptor-mixed bases (○), and mineralocorticoid receptor-antisense (●) groups, respectively. The mean ± SEM values of mineralocorticoid receptor and glucocorticoid receptor measurements of five independent experiments are depicted in **C** and **D**, respectively. Corticosteroid receptors were measured in a soluble binding as outlined in Section 2. $* P < 0.05$, Duncan multiple range test.

Trunk blood was collected in pre-chilled EDTA-coated tubes and the plasma was checked for the absence of any endogenous corticosterone by radioimmunoassay (ICN Biomedicals, Costa Mesa, CA). Animals with detectable levels of corticosterone were excluded. Immediately after decapitation, the brain was rapidly removed from the skull and the hippocampus was dissected. Dissected tissues were instantaneously frozen in liquid N_2 and stored at −80°C until corticosteroid receptor assay.

Pooled tissues of six to eight rats per group were homogenized (100 mg brain tissue/ml; 10 strokes at 900 rpm) in ice-cold 5 mM Tris–HCl (pH 7.4) containing 5% glycerol, 10 mM sodium molybdate, 1 mM EDTA, and 2 mM β-mercaptoethanol using a glass homogenizer with a Teflon pestle milled at a clearance of 0.25 mm on the radius. The homogenate was centrifuged at 100,000 g for 60 min at 0–2°C to obtain cytosol (i.e., supernatant fraction). All reagents used were analytical grade. Aliquots of cytosol (100 μl) were incubated with ^{3}H-labeled steroids over a concentration range of 0.1–10 nM (6–8 concentrations; total volume 150 μl). Total binding to soluble macromolecules was determined with [^{3}H]aldosterone (87–94 Ci/mmol, NEN DuPont, Dreieich, Germany) or with [^{3}H]dexamethasone (85–106 Ci/mmol, Amersham, Braunschweig, Germany). For measurement of mineralocorticoid receptor, total binding was assessed by incubating cytosol with [^{3}H]aldosterone in the presence of a 100-fold excess of the specific glucocorticoid RU 28362 (11β,17β-dihydroxy-6-methyl-17-(1-propionyl)androsta-1,4,6-triene-3-one). Unlabeled RU 28362 was included to prevent [^{3}H]aldosterone from binding to glucocorticoid receptor, so that only binding of this [^{3}H]ligand to miner-

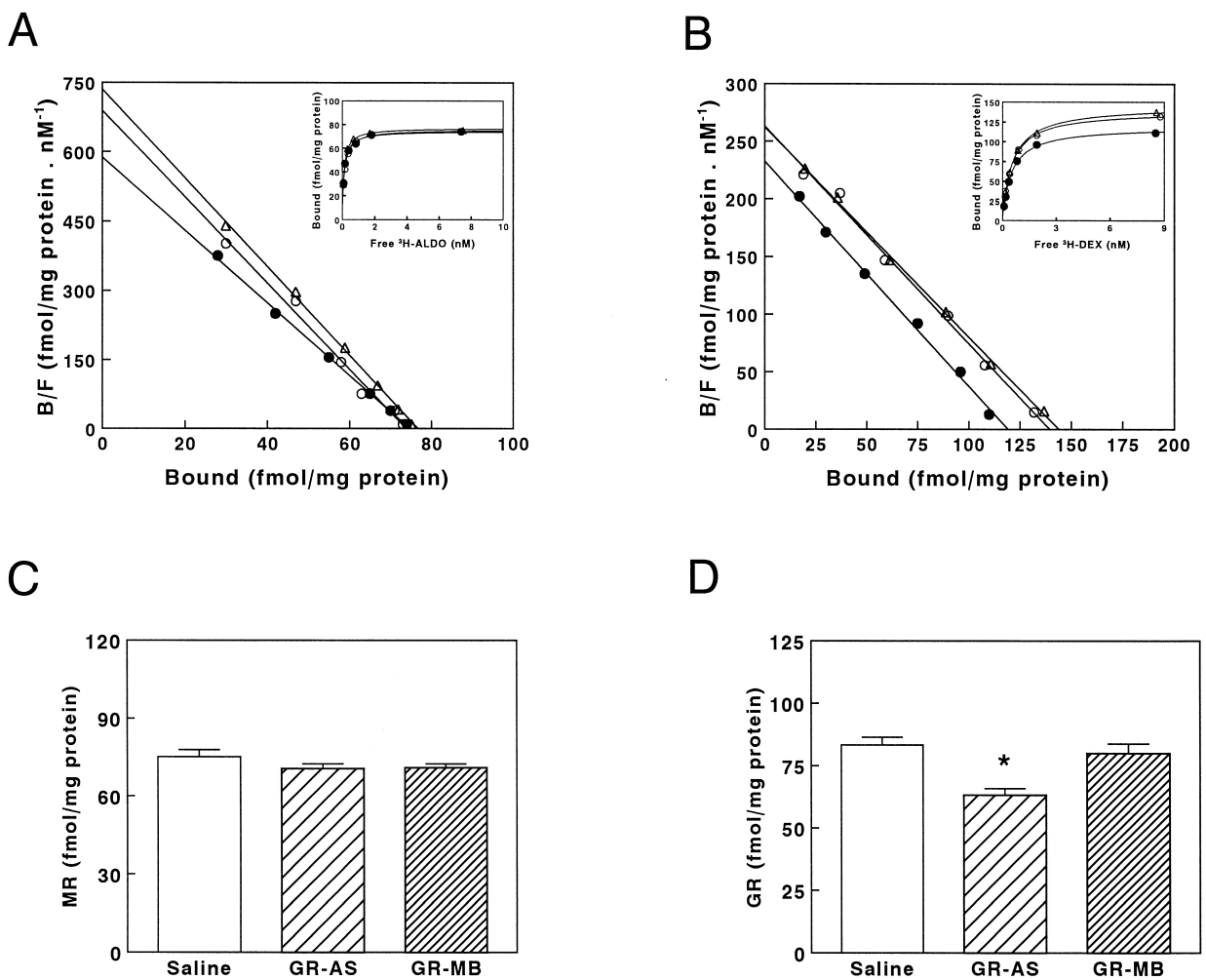

Fig. 2. Saturation binding (inset) and Scatchard analysis of [^{3}H]aldosterone binding of mineralocorticoid receptor (**A**) and [^{3}H]dexamethasone binding to glucocorticoid receptor and mineralocorticoid receptor (**B**) in hippocampus of glucocorticoid receptor-antisense, glucocorticoid receptor-mixed bases- and saline (= control)-treated groups (6 to 8 animals per group). The B_{max} and K_d of hippocampal mineralocorticoid receptor for binding [^{3}H]aldosterone were estimated to be 77 and 0.11, 74 and 0.11,and 75 and 0.13 for the saline (△), mineralocorticoid receptor-mixed bases (○), and mineralocorticoid receptor-antisense (●) groups, respectively. The B_{max} and K_d of hippocampal glucocorticoid receptor and mineralocorticoid receptor for binding [^{3}H]dexamethasone were estimated to be 144 and 0.55, 140 and 0.53, and 119 and 0.51 for the saline (△), mineralocorticoid receptor-mixed bases (○), and mineralocorticoid receptor-antisense (●) groups, respectively. The mean ± SEM values of mineralocorticoid receptor and glucocorticoid receptor measurements of five independent experiments are depicted in **C** and **D**, respectively. For further details see legend to Fig. 1.

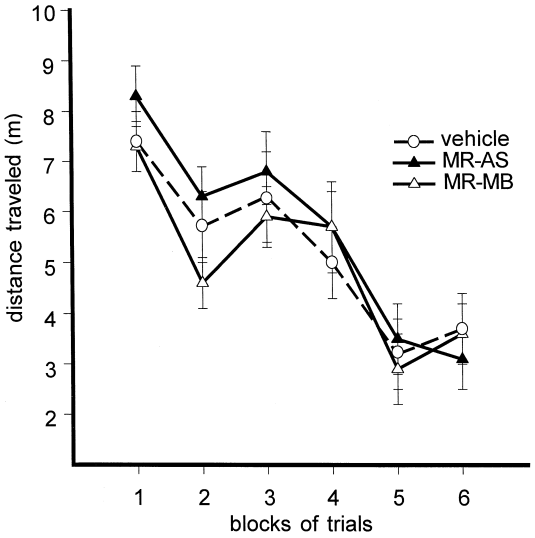

Fig. 3. Behavioral performance of adult male Brown Norway rats during acquisition in the Morris water maze indicated by the average distance traveled to find the hidden platform (±SEM). Testing took place on 3 consecutive days and consisted of one session/day and two blocks of four trials/session. All animals were implanted with chronic i.c.v. cannulas connected to an Alzet® osmotic minipump four days before the first session started. Rats received either mineralocorticoid receptor-antisense (MR-AS, $n = 9$), mineralocorticoid receptor mixed bases sequence (MR-MB, $n = 11$) or vehicle ($n = 9$).

alocorticoid receptor was measured. Nonspecific binding was determined in parallel incubations containing a 1000-fold excess of corticosterone in addition to cytosol and [^{3}H]aldosterone. Total binding for the glucocorticoid receptor was determined by incubating cytosol with [^{3}H]dexamethasone. Since [^{3}H]dexamethasone also displays considerable affinity for mineralocorticoid receptor (Luttge et al., 1989; Reul et al., 1990; Spencer et al., 1990), the fraction of [^{3}H]dexamethasone binding to mineralocorticoid receptor was estimated by including a 100-fold excess of RU 28362 in parallel incubation tubes. Nonspecific binding was determined in parallel incubations containing a 1000-fold excess of dexamethasone in addition to cytosol and [^{3}H]dexamethasone.

After incubation for 20–24 h at 0°C, bound and free [^{3}H]steroid were separated by Sephadex LH-20 (Pharmacia, Sweden) gel filtration (100 μl of the cytosol-[^{3}H]steroid mixture was applied to the LH-20 columns), and radioactivity was measured in a liquid scintillation counter. The protein concentration was determined by the method of Lowry (Lowry et al., 1951) with bovine serum albumin as the standard. The binding data were expressed as femtomoles per mg protein and nonspecific binding was subtracted from total binding to yield specific binding. In this manner, the mineralocorticoid receptor concentration could be directly measured. However, glucocorticoid receptor binding was estimated by subtraction of the specific binding of [^{3}H]dexamethasone + 100 × RU 28362 from the specific binding of [^{3}H]dexamethasone. [^{3}H]dexamethasone + 100 × RU 28362 rather than [^{3}H]aldosterone + 100 × RU 28362 binding data were used to estimate the amount of the specific [^{3}H]dexamethasone binding to mineralocorticoid receptor, because [^{3}H]dexamethasone + 100 × RU 28362 binding to mineralocorticoid receptor was found to be about 30% less than [^{3}H]aldosterone + 100 × RU 28362 binding to this receptor type (J.M.H.M. Reul, unpublished observation).

Binding data were expressed as femtomoles per mg and the maximal number of binding sites (B_{max}) and relative binding affinity (K_d) were determined by Scatchard analysis.

2.5. Morris water maze

The apparatus was an 180-cm diameter circular pool with walls 50 cm high. The pool was filled with water (21°C, 35 cm high) and contained a small (10 cm diameter) platform positioned in one of the imaginary quadrants such that its top was 1 cm beneath the surface of the water. The platform was made of plexiglas to make it invisible to the animals.

Behavioral testing started four days after implantation, took place between 0900 and 1400 h and was done by a trained experimentator who was blind to the treatment of the animals. The rats were tested for two blocks (each consisting of 4 trials) per session, each session on one of three consecutive days. For each trial the animals were released into the water from one randomly chosen out of four fixed starting positions. During each block of trials

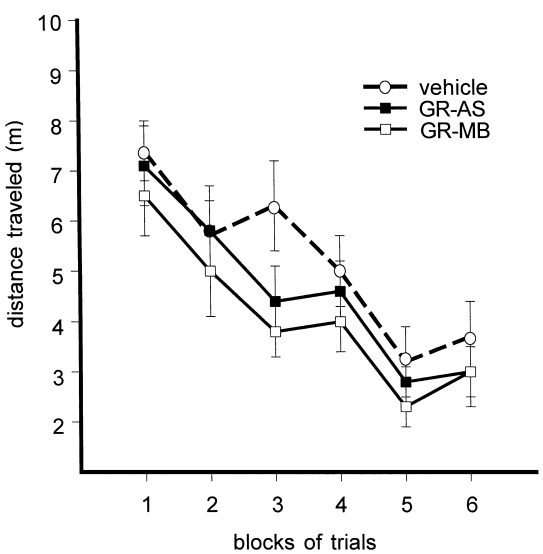

Fig. 4. Performance of adult male Brown Norway rats during acquisition in the Morris water maze indicated by the average distance traveled to find the hidden platform (±SEM). Rats received either glucocorticoid receptor antisense (GR-AS, $n = 8$), glucocorticoid receptor mixed bases sequence (GR-MB, $n = 7$) or vehicle (the vehicle group is the same as in Fig. 3; $n = 9$). For further details see legend to Fig. 3.

the animals were started once from each possible starting position. The rats were allowed to swim either until they found and stayed on the target platform for 5 s or until 60 s elapsed. Time, path length and the ratio of the actual path length and the shortest possible path length (error) of each trial were measured using a computerized tracking system (Kaminsky et al., 1992). The inter-trial interval during which the animals rested on the platform was 20–30 s.

Immediately after the last trial, the target was removed and the rats were allowed to swim free for 60 s during which the duration spent in each quadrant was estimated.

2.6. Statistics

Statistical analysis of binding capacity and physical parameters (body, adrenal and thymus weight, and body weight gain) was performed using one-way analysis of variance (ANOVA). Data (calculated averages of each block) obtained to characterize the performance in the Morris water maze were tested using two-way ANOVA (treatment × blocks of trials) with repeated measures on the second factor. Post-hoc Duncan multiple range test was used if appropriate. Results were considered to be significant if $P < 0.05$.

3. Results

We did not observe any toxic or other adverse 'non-specific' effects in oligodeoxynucleotide-treated rats that could have been expressed as sickness behavior of the rats or loss of their body weight (data not shown).

3.1. Effect of long-term i.c.v. mineralocorticoid receptor-antisense and glucocorticoid receptor-antisense treatment on physical parameters

Both long-term i.c.v. mineralocorticoid receptor-antisense and mineralocorticoid receptor-mixed bases treatment produced no significant changes in body weight, body weight gain, thymus weight and adrenal weight (data not shown). Whereas long-term i.c.v. glucocorticoid receptor-antisense treatment exerted no significant effects on adrenal and thymus weight (data not shown), one-way ANOVA analysis detected a borderline significant decrease in body weight ($F(2,15) = 3.68$, $P = 0.050$; control: 203 ± 3 g, glucocorticoid receptor-antisense: 194 ± 4 g, glucocorticoid receptor-mixed bases: 202 ± 3 g) and a clearly significant reduction in body weight gain ($F(2,15) = 7.40$, $P = 0.006$; control: 11.5 ± 1.2 g, glucocorticoid receptor-antisense: 3.5 ± 1.3 g (post-hoc analysis: $P < 0.05$, as compared to control), glucocorticoid receptor-mixed bases: 8.8 ± 1.9 g).

3.2. Effects of long-term i.c.v. mineralocorticoid receptor-antisense treatment on hippocampal corticosteroid receptor levels

Fig. 1A and C show that i.c.v. administration of mineralocorticoid receptor-antisense for 7 days produced a sig-

Table 2
Results of statistical analysis of behavioral data monitored during acquisition in the Morris water maze [two-way ANOVA (treatment × blocks of trials) with repeated measures on the last factor]

Groups	Parameter	ANOVA factor	Degrees of freedom	*F*-value	*P*
Mineralocorticoid receptor-antisense, mineralocorticoid receptor-mixed bases, vehicle[a]	distance	treatment	2, 26	0.78	0.47
		blocks of trials	5, 130	21.41	0.00
		interaction	10, 130	0.49	0.89
		treatment	2, 26	1.01	0.38
	ratio	blocks of trials	5, 130	16.03	0.00
		interaction	10, 130	0.52	0.87
		treatment	2, 26	0.35	0.71
	time	blocks of trials	5, 130	30.80	0.00
		interaction	10, 130	0.62	0.80
Glucocorticoid receptor-antisense, glucocorticoid receptor-mixed bases, vehicle[a]		treatment	2, 21	1.81	0.19
	distance	blocks of trials	5, 105	17.97	0.00
		interaction	10, 105	0.43	0.93
		treatment	2, 21	1.97	0.16
	ratio	blocks of trials	5, 105	12.74	0.00
		interaction	10, 105	0.62	0.79
		treatment	2, 21	2.22	0.13
	time	blocks of trials	5, 105	25.97	0.00
		interaction	10, 105	0.68	0.74

[a] The same vehicle controls were used for statistical analysis.

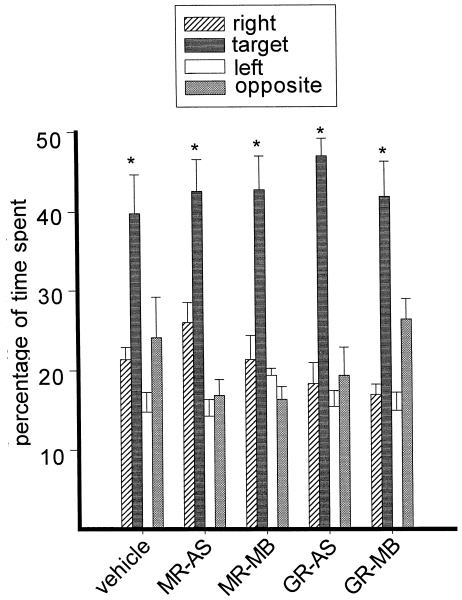

Fig. 5. Percentage of search time (±SEM) in each of the four quadrants of the Morris water maze during the 60-s free swim trial performed immediately after the last acquisition trial (session 3). Rats were chronically infused i.c.v. with either vehicle ($n = 9$), mineralocorticoid receptor-antisense (MR-AS, $n = 9$), mineralocorticoid receptor mixed bases sequence (MR-MB, $n = 11$), glucocorticoid receptor-antisense (GR-AS, $n = 8$) or glucocorticoid receptor mixed bases sequence (GR-MB, $n = 7$). $*$ $p < 0.01$ vs. all other quadrants of the same treatment group.

nificant decrease of 28% ($F(2,12) = 9.82$, $P = 0.003$) in the hippocampal mineralocorticoid receptor concentration. However, no changes in hippocampal glucocorticoid receptor levels were found after this treatment (Fig. 1B and D). Furthermore, no changes in either mineralocorticoid receptor or glucocorticoid receptor were observed after treatment with the mixed bases-control (i.e., mineralocorticoid receptor-mixed bases). As indicated by the Scatchard analyses, neither oligodeoxynucleotide treatment had any effect on the apparent binding affinity (K_d) of both mineralocorticoid receptor and glucocorticoid receptor (Fig. 1A and B). The ranges of K_d estimates for [^{3}H]aldosterone binding to mineralocorticoid receptor and [^{3}H]dexamethasone binding to glucocorticoid receptor + mineralocorticoid receptor were 0.09–0.16 nM and 0.46–0.51 nM, respectively.

3.3. Effects of long-term i.c.v. glucocorticoid receptor-antisense treatment on hippocampal corticosteroid receptor levels

Intraventricular treatment with glucocorticoid receptor-antisense for 7 days resulted in a significant decline of 23% ($F(2,12) = 14.60$, $P = 0.0006$) in glucocorticoid receptor levels in the hippocampus (Fig. 2A and C). No effects on hippocampal mineralocorticoid receptor concentrations were observed after this treatment (Fig. 2B and D). In addition, both mineralocorticoid receptor and glucocorticoid receptor levels were unaltered after treatment with the mixed bases-control (i.e., glucocorticoid receptor-mixed bases). Neither oligodeoxynucleotide treatment had any effect on the apparent binding affinity (K_d) of mineralocorticoid receptor and glucocorticoid receptor (Fig. 2A and B). The ranges of K_d estimates for [^{3}H]aldosterone binding to mineralocorticoid receptor and [^{3}H]dexamethasone binding to glucocorticoid receptor + mineralocorticoid receptor were 0.10–0.15 nM and 0.49–0.55 nM, respectively.

3.4. Morris water maze

The swim distance traveled by antisense oligodeoxynucleotide treated rats compared to that of corresponding controls is shown in Figs. 3 and 4. Statistical analysis of all behavioral parameters monitored indicated that performance improved in all groups over sessions regardless of their treatment (Table 2, ANOVA factor: blocks of trials). However, as shown in Table 2, oligodeoxynucleotide treatment failed to cause any significant effect on the acquisition of target localization in the Morris water maze. Furthermore, the data obtained from the free swim trial performed at the end of the testing period revealed a significant preference of all treatment groups for the quadrant that previously contained the target ($P < 0.01$, Fig. 5). Again, there was no indication of any inter-group differences (Table 3).

Table 3
Results of the statistical analysis of data obtained during the free swim trial [percentage of time spent in each of the four quadrants; two-way ANOVA (treatment × quadrants) with repeated measures on the last factor]

Groups	ANOVA factor	Degrees of freedom	*F*-value	*p*
Mineralocorticoid receptor-antisense, mineralocorticoid receptor-mixed bases, vehicle[a]	treatment	2, 26	0.065	0.53
	quadrants	3, 78	29.80	0.00
	interaction	6, 78	0.89	0.50
Glucocorticoid receptor-antisense, glucocorticoid receptor-mixed bases, vehicle[a]	treatment	2, 21	0.20	0.82
	quadrants	3, 63	31.88	0.00
	interactions	6, 63	0.74	0.62

[a] The same vehicle controls were used for statistical analysis.

4. Discussion

We used antisense targeting as a tool to investigate the role of brain corticosteroid receptors in spatial learning of Brown Norway rats in the Morris water maze. A growing number of publications report on the successful application of antisense oligodeoxynucleotides to selectively downregulate receptor (sub)types and, thus, to interfere with ligand receptor interaction (Wahlestedt et al., 1993; Mani et al., 1994; McCarthy et al., 1994; Landgraf et al., 1995). Although the behavioral effects of the treatment cannot always simply be related to specific molecular and cellular events (Liebsch et al., 1995), this technique has been proven to be a selective and valuable tool in neurobiological research (Pilowsky et al., 1994), in particular if receptor (sub)types are to be investigated for which antagonists are either not available (Wahlestedt et al., 1993) or not discriminative enough (Landgraf et al., 1995). The latter holds also true for antagonists that are commonly used to block mineralocorticoid receptor and glucocorticoid receptor (e.g., RU 486, RU 28318, RU 26752, ZK 91587; Sutanto and de Kloet, 1988; Reul et al., 1990; Rupprecht et al., 1993; J.M.H.M. Reul, unpublished observations).

An important issue in antisense oligodeoxynucleotide technology is the selection and use of appropriate controls such as mixed bases and nonsense oligodeoxynucleotide sequences. In neuroscience, however, inclusion of all possible controls is virtually impossible given the tremendous extra use of animals and the major investment in terms of financial resources and time this would mean. Therefore, in the present study it was decided to make a compromise by including a vehicle group and two mixed bases groups selected on the basis of pilot studies in which also other sequences were tested.

The results of the present study show that chronic infusion of mineralocorticoid receptor antisense produced a significant reduction of mineralocorticoid receptor levels in the hippocampus of Brown Norway rats (Fig. 1). This confirms and extends findings of a recent study using animals of the Wistar strain (Reul et al., 1997). Furthermore, the present investigation parallels other reports (Korte et al., 1996; Sakai et al., 1996) of a successful knock-down of hippocampal glucocorticoid receptor receptors by antisense treatment (Fig. 2). Both mineralocorticoid receptor-antisense and glucocorticoid receptor-antisense administration reduced the binding capacity of the targeted receptor to an amount of approx. 70–80% of that of mixed bases treated animals or untreated controls (Figs. 1 and 2). The concentration of the respective other receptor subtype was not significantly affected, strongly suggesting a selective action of the antisense treatment and/or irrelevant secondary (i.e., indirect) effects on the other receptor subtype (see also Sakai et al., 1996). Moreover, oligodeoxynucleotide treatment proved to have minor effects on physical parameters. The only exception was that glucocorticoid receptor-antisense-treated rats showed a reduced body weight gain. This may amongst others be attributable to reduced food intake, because central glucocorticoid receptors have been shown to be involved in feeding behavior (Dallman et al., 1995).

However, in spite of its influence on the respective receptor levels, mineralocorticoid receptor-antisense as well as glucocorticoid receptor-antisense treatments failed to significantly alter spatial learning of Brown Norway rats in the Morris water maze. This is indicated by the analysis of all parameters obtained during the acquisition (Table 2, Figs. 3 and 4) and confirmed by the data obtained during the free swim trial (Fig. 5). The latter is thought to be a valid measure of the search strategy used by the animals to solve the task (Brandeis et al., 1989). Neither glucocorticoid receptor-antisense nor mineralocorticoid receptor-antisense treated animals differed significantly from their respective mixed bases or vehicle treated controls (Tables 2 and 3). In their study, Oitzl and de Kloet (1992) applied mineralocorticoid receptor and glucocorticoid receptor antagonists i.c.v. before and after training sessions in the Morris water maze. From their results the authors concluded that glucocorticoids act via mineralocorticoid receptor to optimize the specific search strategy in the Morris water maze whereas glucocorticoid–glucocorticoid receptor interaction is a prerequisite for successful consolidation of the acquired information (see also Oitzl et al., 1994). As shown in the present paper, continuous infusion of antisense oligodeoxynucleotides over a duration of seven days resulted in a knock-down of hippocampal glucocorticoid receptor and mineralocorticoid receptor binding of approx. 20–30%. The synthetic antagonists used by Oitzl and de Kloet (1992) were acutely administered in a dosage of 100 ng which probably competes significantly with the endogenous ligand for mineralocorticoid receptor and glucocorticoid receptor binding in the hippocampus and other brain areas such as septum, amygdala or brain stem (Reul and de Kloet, 1985, 1986). Thus, the extent of receptor downregulation in the present study may not be high enough to allow for the detection of this kind of behavioral effects. In this context, it has to be mentioned that probably not all mineralocorticoid receptors and glucocorticoid receptors are equally involved in the behavioral regulation which makes it rather unlikely to find a linear relationship between the receptor level and a defined behavioral performance. It is worth noting that the assessed reduction of 20–30% measured in our binding experiments represents the situation after an antisense infusion period of seven days. However, behavioral testing started already on the fifth day of infusion. A severe degree of hippocampal impairment to significantly change the behavior of rats in the Morris water maze has been suggested from chronic corticosterone and stress treatment studies (Bodnoff et al., 1995). Furthermore, due to the subchronic administration protocol used in the present study, compensatory processes might have taken place. However, as shown by our binding studies (see Figs. 1 and 2), possible compensatory

effects do not involve changes in receptor binding of the respective other receptor subtype.

It may be argued that a decline of 20–30% of the receptor levels by antisense treatment is in principle not sufficient to significantly alter the functional properties of the receptor systems under study. However, a recent investigation using the same sequence and treatment protocol and resulting in a similar reduction of mineralocorticoid receptor within the hippocampus of Wistar rats caused significantly increased plasma corticotropin responses to a combined social and novelty stress exposure (Reul et al., 1997). Furthermore, Korte et al. (1996) reported that local glucocorticoid receptor antisense treatment reduced glucocorticoid receptor immunolabeling by 15% vs. scrambled sequence and caused a significant reduction in floating behavior in Wistar rats as measured in a forced swim test. Thus, although strain differences in brain corticoid receptor characteristics as shown in mice (Patacchioli et al., 1990) and rats (Oitzl et al., 1995) have to be considered, these findings indicate that the amount of reduction in receptor binding should be sufficient to alter the stress response of the hypothalamic–pituitary–adrenocortical axis and other behavioral characteristics in our Brown Norway rats. Nevertheless, further studies are necessary to clarify how antisense treatment alters receptor expression and possible functional consequences over time.

Taken together, the results of the present study demonstrate on the one hand that antisense targeting is a useful tool to selectively downregulate different types of brain corticosteroid receptors. On the other hand a reduction of hippocampal glucocorticoid receptor and mineralocorticoid receptor levels of 20–30% after chronic i.c.v. antisense oligodeoxynucleotide infusion does not affect learning in the Morris water maze. In this context a recent study reported on a dose-dependent effect of blocked corticosterone synthesis by metyrapone on Morris water maze acquisition (Roozendaal et al., 1996). Consequently, additional experiments are required to reveal the degree of receptor downregulation in order to affect ‘normal’ spatial learning. However, such studies may be hampered by the restricted doses of oligodeoxynucleotides to preclude nonspecific side effects such as fever and sickness behavior (Pezeshki et al., 1996). In addition, it should be clarified in which brain area(s) glucocorticoids act to influence Morris water maze performance.

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References

Bodnoff, S.R., Humphreys, A.G., Lehman, J.C., Diamond, D.M., Rose, G.M., Meaney, M.J., 1995. Enduring effects of chronic corticosterone treatment on spatial learning, synaptic plasticity, and hippocampal neuropathology in young and mid-aged rats. J. Neurosci. 15, 61–69.

Brandeis, R., Brandys, Y., Yehuda, S., 1989. The use of the Morris water maze in the study of memory and learning. Intern. J. Neurosci. 48, 29–69.

Buresova, O., Bures, J., 1971. The significance of crossed and uncrossed projections for the acquisition and retrieval of visual engrams in hooded and albino rats. Psychol. Forsch. 35, 46–56.

Dallman, M.F., Akana, S.F., Jacobson, L., Levin, N., Cascio, C.S., Shinsako, J., 1987. Characterization of corticosterone feedback regulation of ACTH secretion. Ann. NY Acad. Sci. 512, 402–414.

Dallman, M.F., Akana, S.F., Strack, A.M., Hanson, E.S., Sebastian, R.J., 1995. The neural network that regulates energy balance is responsive to glucocorticoids and insulin and also regulates HPA axis responsivity at a site proximal to CRF neurons. Ann. NY Acad. Sci. 771, 730–742.

de Kloet, E.R., Reul, J.M.H.M., 1987. Feedback action and tonic influence of corticosteroids on brain function: a concept arising from the heterogeneity of brain receptor systems. Psychoneuroendocrinology 12, 83–105.

Fuxe, K., Wikstrom, A.C., Okret, S., Agnati, L.F., Harfstrand, A., Yu, Z.Y., Granholm, L., Zoli, M., Vale, W., Gustafsson, J.A., 1985. Mapping of glucocorticoid receptor immunoreactive neurons in the rat tel- and diencephalon using a monoclonal antibody against rat liver glucocorticoid receptor. Endocrinology 117, 1803–1812.

Kaminsky, Y., Bureš, J., Nerad, L., Krekule, I., 1992. A new IBM PC based system for automation of the Morris water tank experiments. Physiol. Res. 41, 26P.

Korte, S.M., De Kloet, E.R., Buwalda, B., Bouman, S.D., Bohus, B., 1996. Antisense to the glucocorticoid receptor in hippocampal dentate gyrus reduces immobility in forced swim test. Eur. J. Pharmacol. 301, 19–25.

Landgraf, R., Gerstberger, R., Montkowski, A., Probst, J.C., Wotjak, C.T., Holsboer, F., Engelmann, M., 1995. V1 vasopressin receptor antisense oligodeoxynucleotide administration into septum reduces vasopressin binding, social discrimination abilities and anxiety-related behavior in rats. J. Neurosci. 15, 4250–4258.

Liebsch, G., Landgraf, R., Gerstberger, R., Probst, J.C., Wotjak, C.T., Engelmann, M., Holsboer, F., Montkowski, A., 1995. Chronic infusion of a CRH1 receptor antisense oligodeoxynucleotide into the central nucleus of the amygdala reduced anxiety-related behavior in socially defeated rats. Regul. Pept. 59, 229–239.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, N.J., 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Luttge, W.G., Davda, M.M., Rupp, M.E., Kang, C.G., 1989. High affinity binding and regulatory actions of dexamethasone-type I receptor complexes in mouse brain. Endocrinology 125, 1194–1203.

Mani, S.K., Blaustein, J.D., Allen, J.M., Law, S.W., O’Malley, B.W., Clark, J.H., 1994. Inhibition of rat sexual behavior by antisense oligonucleotides to the progesterone receptor. Endocrinology 135, 1409–1414.

McCarthy, M.M., Kleopoulos, S.P., Mobbs, C.V., Pfaff, D.W., 1994. Infusion of antisense oligonucleotides to the oxytocin receptor in the ventromedial hypothalamus reduces estrogen-induced sexual receptivity and oxytocin receptor binding in the female rat. Neuroendocrinology 59, 432–440.

Miesfeld, R., Rusconi, S., Godowski, P.J., Maler, B.A., Okret, S., Wilkstroem, A.-C., Gustafsson, J.-A., Yamamoto, K.R., 1986. Genetic complementation to a glucocorticoid receptor deficiency by expression of a cloned receptor cDNA. Cell 46, 389–399.

Morris, R.G.M., 1981. Spatial localization does not require the presence of local cues. Learning Motiv. 12, 239–260.

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

Oitzl, M.S., Fluttert, M., de Kloet, E.R., 1994. The effect of cortico-

sterone on reactivity to spatial novelty is mediated by central mineralocorticosteroid receptors. Eur. J. Neurosci. 6, 1072–1979.

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

Patacchioli, F.R., Puglisi-Allegra, S., Maccari, S., Casolini, P., Angelucci, L., Cabib, S., Oliverio, A., 1990. Strain-dependent differences in hippocampal glucocorticoid binding capacity and active avoidance in the mouse. Behav. Brain Res. 37, 185–188.

Patel, P.D., Sherman, T.G., Goldman, D.J., Watson, S.J., 1989. Molecular cloning of a mineralocorticoid (type I) receptor complementary DNA from rat hippocampus. Mol. Endocrinol. 3, 1877–1885.

Pezeshki, G., Schöbitz, B., Pohl, T., Reul, J.M.H.M., 1996. Intracerebroventricular administration of missense oligodeoxynucleotide induces interleukin-6 mRNA expression in brain and spleen of rats. Neurosci. Lett. 217, 97–100.

Pilowsky, P.M., Suzuki, S., Minson, J.B., 1994. Antisense oligonucleotides: a new tool in neuroscience. Clin. Exp. Pharmacol. Physiol. 21, 935–944.

Reul, J.M.H.M., de Kloet, E.R., 1985. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117, 2505–2515.

Reul, J.M.H.M., de Kloet, E.R., 1986. Anatomical resolution of two types of corticosterone receptor sites in rat brain with in vivo autoradiography and computerized image analysis. J. Steroid Biochem. 24, 269–272.

Reul, J.M.H.M., De Kloet, E.R., Van Sluijs, F.J., Rijnberk, A., Rothuizen, J., 1990. Binding characteristics of mineralocorticoid and glucocorticoid receptors in dog brain and pituitary. Endocrinology 127, 907–915.

Reul, J.M.H.M., Probst, C.J., Skutella, T., Stec, I.S.M., Montkowski, A., Landgraf, R., Holsboer, F., 1997. Increased hypothalamic–pituitary–adrenal axis reactivity after long-term intracerebroventricular treatment of rats with antisense mineralocorticoid receptor oligonucleotides. Neuroendocrinology 65, 189–199.

Reul, J.M.H.M., Stec, I., Wiegers, G.J., Labeur, M.S., Linthorst, A.C.E., Arzt, E., Holsboer, F., 1994. Prenatal immune challenge alters the hypothalamic–pituitary–adrenocortical axis in adult rats. J. Clin. Invest. 93, 2600–2607.

Reul, J.M.H.M., Stec, I., Söder, M., Holsboer, F., 1993. Treatment of rats with the antidepressant amitriptyline attenuates the activity of the hypothalamic–pituitary–adrenocortical system. Endocrinology 133, 312–320.

Roozendaal, B., Bohus, B., McGaugh, J.L., 1996. Dose-dependent supression of adrenocortical activity with metyrapone: Effects on emotion and memory. Psychoneuroendocrinology 21, 681–693.

Rupprecht, R., Reul, J.M.H.M., van Steensel, B., Spengler, D., Söder, M., Berning, B., Holsboer, F., Damm, K., 1993. Pharmacological and functional characterization of human mineralocorticoid and glucocorticoid receptor ligands. Eur. J. Pharmacol. Mol. Pharmacol. Sect. 247, 145–154.

Sakai, R.R., Ma, L.Y., Zhang, D.M., McEwen, B., Fluharty, S.J., 1996. Intracerebral administration of mineralocorticoid receptor antisense oligonucleotides attenuate adrenal steroid-induced salt appetite in rats. Neuroendocrinology. 64, 425–429.

Spencer, R.L., Young, E.A., Choo, P.H., Mc Ewen, B.S., 1990. Adrenal steroid type-I and type-II receptor binding—estimates of in vivo receptor number, occupancy, and activation with varying level of steroid. Brain Res. 514, 37–48.

Sutanto, W., de Kloet, E.R., 1988. ZK 91587: a novel synthetic anti mineralocorticoid displays high affinity for corticosterone (type I) receptors in rat hippocampus. Life Sci. 43, 1537–1543.

Tonkiss, J., Shultz, P., Galler, J.R., 1992. Long–Evans and Sprague–Dawley rats differ in their spatial navigation performance during ontogeny and at maturity. Dev. Psychobiol. 25, 567–579.

Wahlestedt, C., Merlo Pich, E., Koob, G.F., Yee, F., Heilig, M., 1993. Modulation of anxiety and neuropeptide Y–Y1 receptors by antisense oligonucleotides. Science 259, 528–530.

Yau, J.L., Morris, R.G.M., Seckl, J.R., 1994. Hippocampal corticosteroid receptor mRNA expression and spatial learning in the aged Wistar rat. Brain Res. 657, 59–64.

Yau, J.L., Olsson, T., Morris, R.G.M., Meaney, M.J., Seckl, J.R., 1995. Glucocorticoids, hippocampal corticosteroid receptor gene expression and antidepressant treatment: relationship with spatial learning in young and aged rats. Neuroscience 66, 571–581.