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Antisense to the glucocorticoid receptor in hippocampal dentate gyrus reduces immobility in forced swim test

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Abstract

Immobility time of rats in the forced swim test was reduced after bilateral infusion of an 18-mer antisense phosphorothioate oligodeoxynucleotide targeted to the glucocorticoid receptor mRNA into the dentate gyrus of the hippocampus. Vehicle-, sense- and scrambled sequence-treated animals spent significantly more time immobile than antisense-treated animals during the initial test. Immunolabeling of the glucocorticoid receptor in brain sections demonstrated a reduced expression of glucocorticoid receptor proteins in antisense-treated dentate gyrus compared to the contralateral sense-treated dentate gyrus or contralateral scrambled sequence-treated dentate gyrus. During the initial test the time spent on immobility was also reduced when rats were treated with the glucocorticoid receptor antagonist RU38486 (17 β -hydroxy-11 β -(4-dimethylamino-phenyl)17 α -(1-propynyl)estra-4,9-diene-3-one) 6 h (but not 1 h) earlier. These results demonstrate the participation of glucocorticoid receptors in the expression of immobility in a forced swim test during the initial test.

Keywords: Immobility behaviour; Forced swim test; Antisense; S-Oligodeoxynucleotide; Glucocorticoid receptor; RU38486; Central nervous system

1. Introduction

Corticosteroids from the adrenal cortex influence a variety of behaviours (e.g., De Kloet, 1991). These hormones act via two intracellular receptor systems, i.e., the mineralocorticoid receptor and the glucocorticoid receptor (Reul and De Kloet, 1985). The two receptor types differ in their neuroanatomical distribution, their affinity for corticosterone, their regulation and their function (for review, De Kloet, 1991). Both mineralocorticoid receptors and glucocorticoid receptors appear in high concentrations in the hippocampal formation (Fuxe et al., 1985; Reul and De Kloet, 1985; Van Eekelen et al., 1987). Low circulating levels of corticosterone predominantly occupy mineralocorticoid receptors, whereas after stress and during the circadian peak both mineralocorticoid receptors and glu-

corticoid receptors are activated (Reul et al., 1987). Behavioural effects of the synthetic glucocorticoid receptor antagonist and the mineralocorticoid receptor antagonist have been measured in animal models designed to examine anxiety (Korte et al., 1995), learning and memory (De Kloet et al., 1988; Jefferys et al., 1983; Jefferys and Funder, 1987; Oitzl and De Kloet, 1992; Oitzl et al., 1994) and learned helplessness (Papolos et al., 1993). One of the behaviours sensitive to corticosterone (the principle glucocorticoid in the rat) is immobility in the Porsolt forced swim test. When rats are forced to swim in a confined space from which they cannot escape they will, after an initial period of vigorous activity, stay afloat in a characteristic immobile position. Acquisition of this immobility is usually measured during an initial 15-min test period. When subjected 24 h later to a 5-min retest, the rats spend most of the time immobile. Jefferys and colleagues (Jefferys et al., 1983; Jefferys and Funder, 1987) were the first to show that the presence of glucocorticoids and opioid peptides after the initial test is necessary for the retention

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of the acquired immobility response in the retest. This finding was confirmed by De Kloet et al. (1988) who have shown that local administration of the glucocorticoid receptor antagonist, RU38486, into the dentate gyrus of the hippocampus directly after the initial test disrupted this retention. Recently, however, it has been shown that inhibition of the synthesis of corticosterone with metyrapone reduced the immobility time during both initial test and retest (Báez and Volosin, 1994).

The aim of the present investigation was to assess the potential glucocorticoid receptor participation in the expression of behavioural immobility in the forced swim test using a new approach to influence glucocorticoid receptor function. Antisense to the glucocorticoid receptor mRNA was administered locally into the dentate gyrus to prevent translation. It is assumed that antisense oligodeoxynucleotides to specific mRNAs block the translation and thereby the synthesis of proteins (Wahlestedt et al., 1993).

2. Materials and methods

2.1. Animals

Male Wistar rats, weighing 300–400 g at the start of the experiment, were used. They were housed 6 per cage. After surgery the rats were housed individually and were allowed 10 days for recovery. Housing conditions were a constant temperature of 21°C on a 12-h light-dark regimen (light on between 08:00–20:00 h) with free access to standard rat chow and tap water.

2.2. Procedure

As demonstrated earlier, specific inhibition of neurotransmitter receptor expression in the living brain can be accomplished by the use of antisense oligodeoxynucleotides (Wahlestedt et al., 1993). For local administration of the antisense oligodeoxynucleotide to glucocorticoid receptor mRNA, scrambled sequence, sense or vehicle, cannulae were placed (under ether anaesthesia) bilaterally into the dentate gyrus of the hippocampus; AP = –3.6 mm from the bregma; L = +1.4; DV = –3.3 from the dura mater (according to the Paxinos stereotaxic rat brain map). Antisense (5'-GGA-TTC-TTT-GGA-GTC-CAT-3') was targeted at the codons immediately downstream of the initiation codon (Miesfeld et al., 1986). All oligodeoxynucleotides were phosphorothioated in all positions, except the 3' and 5' base positions. A scrambled sequence of the antisense oligodeoxynucleotide (5'-AAT-GCT-CGC-TTG-ATG-TTG-3'), sense oligodeoxynucleotide (5'-ATG-GAC-TCC-AAA-GAA-TCC-3') and vehicle (distilled water) were used as controls. The antisense, the scrambled sequence and the sense (2 nmol) or vehicle were bilaterally infused through injectors (0.29 mm o.d.) in a volume of 1 µl/side into the dentate gyrus over a period of 7.5 min; 1

additional min delay was allowed before the injectors were retracted. The possible consequence of inhibition of genetic glucocorticoid receptor expression on immobility behaviour in the forced swim test and retest was studied 6 and 30 h later, respectively. The synthetic glucocorticoid receptor antagonist RU38486 (Gaillard et al., 1984; Moguilewski and Philibert, 1984) (17β-hydroxy-11β-(4-dimethylamino-phenyl)17α-(1-propynyl)estra-4,9-diene-3-one) (2.5 mg/100 g body weight), was freshly prepared before the experiment by dissolving the crystalline steroid in polyethylene glycol for subcutaneous (s.c.) administration 6 h and 1 h before the initial forced swim test. Control animals received the vehicle (polyethylene glycol) only. The following day, 24 h after the initial test, time spent immobile was measured during a 5-min retest period.

The rats were placed in a narrow Plexiglas cylinder (height 50 cm, diameter 19 cm), containing 23-cm-deep water for 15 min (initial test, behaviour was scored in 3 periods of 5 min), followed by a 5-min retest 24 h later. The temperature of the water, i.e. 25°C, has proved to be essential to detect glucocorticoid effects (Peeters et al., 1992). Fresh water was used for each animal (Abel, 1993). Typically, rats paddle vigorously when first immersed, then become relatively immobile and adopt a characteristic vertical floating response (Abel, 1993, 1994; Porsolt et al., 1977). The duration of absence of hindleg movement was recorded as a measure of immobility. The rats were dried after they had swum. All tests were performed between 16:00–19:00 h. After the experiments, localization of the tip of the injectors was verified in cryostat brain sections. Four animals had cannula misplacement and were excluded.

2.3. Immunocytochemistry

The expression of glucocorticoid receptors was studied immunocytochemically in 8 animals which were infused with glucocorticoid receptor antisense in the left dentate gyrus, and with sense in the contralateral dentate gyrus as described above and in another group of 7 animals, with glucocorticoid receptor antisense in the left dentate gyrus, and with scrambled sequence in the contralateral dentate gyrus. Six hours later the rats were perfused transcardially under deep pentobarbital anaesthesia with heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH = 7.4). The intracellular localization of glucocorticoid receptor was studied by means of a polyclonal (Rabbit) anti-human glucocorticoid receptor antibody (GR57; Affinity Bioreagents) as described earlier (McGimsey et al., 1991). Briefly, brain sections of 40 µm were cut on a cryostat microtome and incubated in primary antibody (1 : 100) in phosphate-buffered saline (PBS; 0.01 M, pH 7.4), containing 2% normal goat serum for 72 h at 4°C. After rinsing, the sections were incubated with secondary antibody (biotinylated goat anti-rabbit immunoglobulin G, 1 : 200) for 2 h, followed by a 2 h exposure to

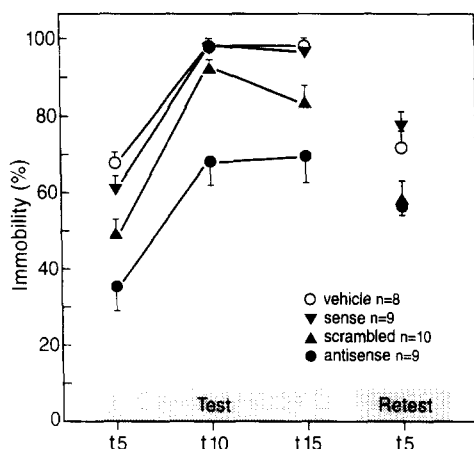


Fig. 1. Immobility behaviour (mean \pm S.E.M.; $n = 8-10$) in the forced swim test during the first day ($T = 5, 10,$ and 15 min) and during retest ($T = 5$ min), 6 h and 30 h following bilateral intra-dentate gyrus infusion of vehicle (\circ), a control oligodeoxynucleotide ('sense'; \blacktriangledown), an 18-mer phosphorothioate oligodeoxynucleotide complementary to the glucocorticoid receptor mRNA ('antisense'; \bullet) or a scrambled sequence ('scrambled'; \blacktriangle).

horseradish-peroxidase-streptavidin (1:200). Finally, the tissue-bound peroxidase was visualized with diaminobenzidine (50 mg/100 ml PBS) and 0.01% H_2O_2 . Between incubations the sections were rinsed several times in PBS over 1 h.

The density of glucocorticoid receptor immunolabeling in the nuclear compartment of the granular cell layer in the dentate gyrus and the pyramidal CA1 cell layer was determined by measuring the optical density using an image-analysing system (Zeiss, IBAS). The optical density was expressed in arbitrary units corresponding to grey levels.

2.4. Statistics

Durations of immobility were tested for overall treatment effect using the Kruskal-Wallis analysis of variance (ANOVA), while individual comparisons were tested using the Mann-Whitney test. The optical density of glucocorticoid receptor immunolabeling in the antisense-treated site was compared with the sense-treated or scrambled se-

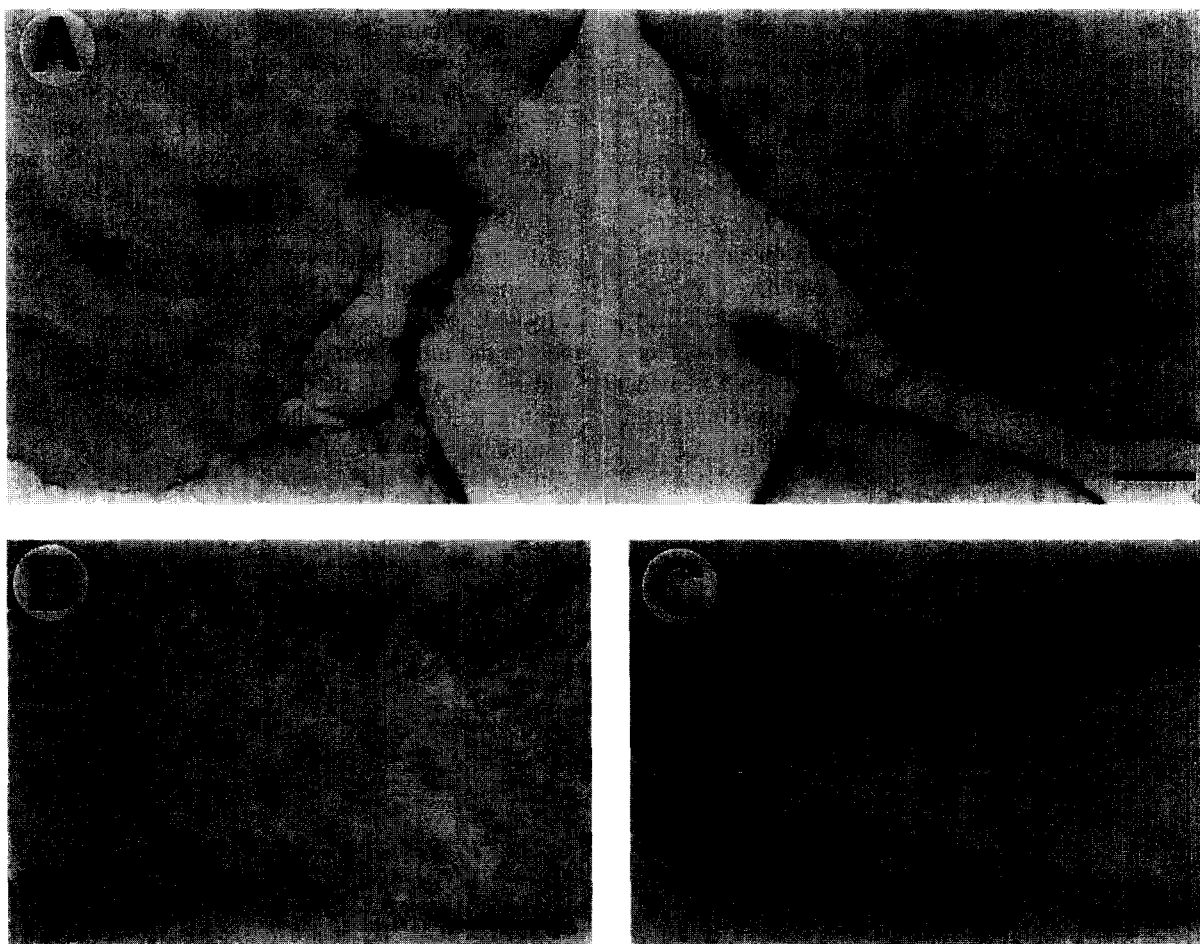


Fig. 2. Photomicrographs of glucocorticoid receptor immunolabeling of the dentate gyrus 6 h after antisense (left, B) and sense treatment (right, C). A decrease of glucocorticoid receptor immunolabeling was mainly observed in the granule cells of the glucocorticoid receptor antisense-treated side (2 nmol/ μ l) compared to the sense-treated contralateral dentate gyrus in hippocampus (bar = 200 μ m; gr = granular cell layer of dentate gyrus; hil = hilus) and in more detail (B, C; bar = 50 μ m).

quence-treated contralateral site in the brain with the aid of paired *t*-tests. A probability level of $P < 0.05$ was taken as significant.

3. Results

Fig. 1 shows that high scores for immobility time were observed in vehicle-, sense- and scrambled sequence-treated animals during exposure to the initial forced swim test. The immobility behaviour was reduced in glucocorticoid receptor antisense-treated animals during both test and retest. Kruskal-Wallis ANOVA revealed a significant overall treatment effect for all periods during test and retest; $P = 0.0002$; $P < 0.0001$; $P = 0.0002$ and $P = 0.017$, respectively. Antisense-treated animals showed significantly lower immobility levels compared to vehicle- and sense-treated animals during the initial test (T_5 , both $P < 0.01$; T_{10} , both $P < 0.01$ and T_{15} , $P < 0.05$, $P < 0.01$ respectively) and during the retest (both $P < 0.01$). Compared to scrambled sequence-treated animals, antisense-treated rats were significantly less immobile during the initial test (T_{10} ; $P < 0.01$), but not in the retest.

Fig. 2 shows the less intense glucocorticoid receptor immunolabeling of the granule cells of the antisense-treated dentate gyrus (left) compared to the contralateral sense-treated dentate gyrus (right) of the same rat. This was also the case for the antisense-treated dentate gyrus compared to the contralateral scrambled sequence-treated dentate gyrus. The optical density of glucocorticoid receptor immunolabeling was significantly decreased in the antisense-treated dentate gyrus ($-18.0 \pm 2.5\%$) compared to the contralateral sense-treated dentate gyrus ($P = 0.0017$). The optical density of glucocorticoid receptor immunolabeling also was significantly decreased in the antisense-treated dentate gyrus ($-15.1 \pm 3.2\%$) compared to the contralateral scrambled sequence-treated dentate gyrus ($P =$

0.0046). No significant differences in optical density were measured in the pyramidal CA1 neurons.

Fig. 3 (left) shows that if the glucocorticoid receptor antagonist was administered 6 h before the initial test, the time spent on immobility was significantly reduced during the T_5 and the T_{15} periods of the initial test ($P = 0.0089$ and $P = 0.026$, respectively) and during the retest ($P = 0.013$). Fig. 3 (right) shows reduced immobility scores for the glucocorticoid receptor antagonist-treated (1 h before initial test) animals compared to vehicle-treated animals ($P = 0.035$) during the 5-min retest, without affecting immobility scores during the 15-min initial test.

4. Discussion

Our results suggest that hippocampal glucocorticoid receptors in the dentate gyrus are involved in the display of immobility behaviour in the forced swim test. Rats spent less time on immobility behaviour in both the initial test and the retest after bilateral infusion of antisense phosphorothioate oligodeoxynucleotide, targeted to the glucocorticoid receptor mRNA, into the dentate gyrus of the hippocampus compared to vehicle- and sense-treated controls. Compared to the scrambled sequence-treated rats, antisense-treated animals showed reduced immobility levels during the initial test. The decreased density of the glucocorticoid receptor immunolabeling in the antisense-treated dentate gyrus compared to that in both the sense-treated side and the scrambled sequence-treated side suggests that antisense treatment produced a decrease in the amount of target glucocorticoid receptor protein translated. Blockade of glucocorticoid receptors with RU38486 6 h before the initial test reduced immobility during both test and retest, whereas the glucocorticoid receptor antagonist treatment 1 h before the initial test only reduced the immobility behaviour in the retest.

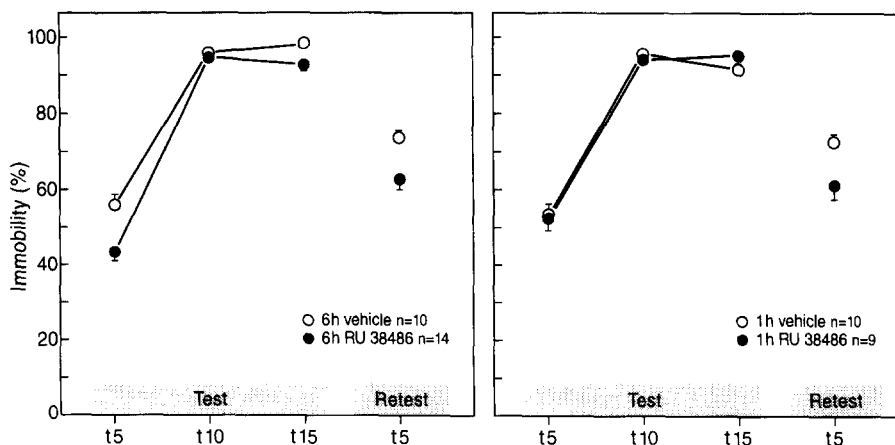


Fig. 3. Immobility behaviour (mean \pm S.E.M.) in vehicle-treated animals (○) compared to that in systemic RU38486-treated (2.5 mg/100 g; 1 h and 6 h prior to initial test) animals (●) during the forced swim test on the first day (i.e., initial test; $T = 5, 10$, and 15 min) and during the 5-min retest 24 h later.

The decreased intensity of the glucocorticoid receptor immunolabeling in the antisense-treated dentate gyrus, with no difference in glucocorticoid receptor immunolabeling in the CA1, reflects a local decrease in the target glucocorticoid receptor protein. The effect of antisense treatment on glucocorticoid receptor immunocytochemistry suggests a partially inhibited expression of glucocorticoid receptors in the dentate gyrus (minus 15–18%). Furthermore a reduction in the display of immobility behaviour in the *initial* test was seen after treatment with both glucocorticoid receptor antisense and glucocorticoid receptor antagonist RU38486 (both given 6 h before testing). Therefore it is suggested that glucocorticoid receptors are involved in the display of immobility during the initial test. This conclusion is supported by the recent indirect finding of Báez and Volosin (1994) who showed that the corticosterone synthesis inhibitor, metyrapone (given 3 h earlier), also reduced immobility, in a comparable way to antisense, during the initial forced swim test but also in the retest. Corticosterone administration reversed this effect in a dose-dependent fashion. In addition, transgenic mice with glucocorticoid receptor function impaired by partial 'knock out' of the genes encoding for glucocorticoid receptor proteins show decreased immobility time, in the forced swim test, during the initial test and the retest compared to the controls (Beaulieu et al., 1994). In contrast to the above-mentioned results, we found that 1 h earlier treatment with the glucocorticoid receptor antagonist failed to have an effect during the initial test. Therefore we postulate that the time interval between treatment (by which binding of corticosterone to glucocorticoid receptors is disturbed) and testing is crucial for the final behavioural outcome.

There is a large body of literature which describes effects of glucocorticoids on the retention of the immobile behaviour only during a 5-min *retest* session, with no mention of effects on the initial 15-min test 24 h earlier (Jefferys et al., 1983; Jefferys and Funder, 1987; Veldhuis et al., 1985; De Kloet et al., 1988; De Kloet, 1991). Jefferys et al. (1983) discovered that adrenalectomy impaired retention of the acquired immobility response. The effect of adrenalectomy was reversed by dexamethasone (microgram dose, subcutaneous) or corticosterone (milligram dose), given within 1 h after the initial test, but not by the mineralocorticoid deoxycorticosterone. Treatment of adrenalectomized rats with the glucocorticoid receptor antagonist RU38486 prior to dexamethasone administration dose dependently blocked the effect of the glucocorticoid (Veldhuis et al., 1985). Jefferys et al. (1983) and Jefferys and Funder (1987) postulated that glucocorticoids (and an additional component, e.g., opioid peptides) are involved in the retention of information post-stress and that these hormones act on the central nervous system directly rather than via the pituitary, since the response to adrenalectomy is not dependent on the presence of the pituitary gland. De Kloet et al. (1988) found that 10 ng of the glucocorticoid receptor antagonist, RU38486, given

intracerebroventricularly or 1 ng into the dorsal hippocampus blocked this retention of acquired immobility.

In the present study we replicated the finding that the glucocorticoid receptor antagonist, RU38486 (1 h before initial test, subcutaneous), reduced the time spent on immobility in the retest, but not in the initial test. Although the reduction in immobility was less robust than reported earlier (probably due to use of another route of administration) (De Kloet et al., 1988), the difference was highly significant. One should keep in mind that, following systemic RU38486 treatment, disinhibition of the pituitary-adrenal system occurs which leads to a rise in circulating corticosterone (Ratka et al., 1989). These higher corticosterone levels may interact with the action of the glucocorticoid receptor antagonist in the brain. Furthermore it must be noted that the immobility scores in the present study reached higher values than in the above-mentioned studies (Jefferys et al., 1983; De Kloet et al., 1988). This may be explained partly by the use of adult rats (± 350 g) in the present study compared to that of younger rats (± 200 g) in the earlier studies. It was reported that the duration of immobility behaviour depends on the age of the animals (Abel, 1993). Surprisingly we found no difference in time spent on immobility between the antisense-treated and scrambled sequence-treated animals during the retest. This discrepancy might be explained by artifactual effects of the scrambled sequence. There was, however, a reduction in immobility in the glucocorticoid receptor antisense-treated animals as compared to vehicle- or sense-treated controls in the retest.

The immobile behaviour in the Porsolt forced swim test is often taken as a state of lowered mood in rats, and this immobility time is reduced by antidepressants (Porsolt et al., 1977). Therefore, one might argue that the antisense treatment or glucocorticoid receptor blockade simulates an antidepressant-like action. But this alternative explanation is not supported by the finding that antidepressants stimulate glucocorticoid receptor gene expression and the fact that the time course of antidepressant actions on glucocorticoid receptors follows that of clinical improvement of depression (Barden et al., 1995). Alternatively, the immobile posture can be viewed as an energy-conserving strategy (Hawkins et al., 1978; West, 1990). According to this line of reasoning immobility behaviour is a successful passive behavioural strategy. The reduction in immobility time during all periods (most prominent during *initial* test) by the glucocorticoid receptor antisense suggests a shift to a behavioural activation. It is proposed that the hippocampus, which contains high concentrations of glucocorticoid receptors and mineralocorticoid receptors, is a target of actions of corticosteroids and thereby promotes behavioural adaptation (i.e., behavioural immobility). It is plausible that glucocorticoid receptor antisense, by reducing the number of glucocorticoid receptors in the dorsal hippocampus, changes the balance of mineralocorticoid receptor and glucocorticoid receptor number, which is

proposed to be critical for the set-point of cellular homeostasis, behavioural adaptation and disease susceptibility (De Kloet, 1991; Joëls and De Kloet, 1991, 1992; Joëls et al., 1991). The hippocampal formation is most likely required to process the kinds of complex stimulus representations that make up the context. The contextual factors are an essential part of what gives a stimulus its meaning (LeDoux, 1993, 1995). Accordingly, it has been reported that the ability to integrate incoming stimuli was decreased in patients lacking cortisol and was normalized by glucocorticoid replacement (Henkin, 1970). Therefore it is suggested that the changed mineralocorticoid receptor/glucocorticoid receptor balance due to glucocorticoid receptor antisense or glucocorticoid receptor antagonist treatment leads to an altered interpretation of the given environment, i.e., inescapability in the forced swim test.

In summary, we find that blockade of glucocorticoid receptor synthesis in the hippocampal dentate gyrus reduces the time spent on immobility behaviour in the forced swim test. Furthermore, the data for glucocorticoid receptor blockade suggest that the time interval between glucocorticoid receptor blockade and testing is crucial for the final behavioural outcome.

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