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Spatial learning and the hippocampal corticosterone receptor system of old rats: effect of the $ACTH_{4-9}$ analogue ORG 2766

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Old (26 months) and young (6 months) male Wistar rats were treated chronically for 2 weeks with ORG 2766 or with vehicle, delivered via subcutaneously implanted minipumps (0.5 μg peptide/0.5 μl/h). Learning of a spatial task was not impaired in the old animals, except for one measure, i.e. the latency to find the goal box. In neither age group did ORG 2766 influence behavioral performance. The number of corticosterone receptor sites was decreased in the hippocampus of senescent rats, but restored to the level observed in young rats following ORG 2766 treatment. It is concluded that the number of hippocampal corticosterone receptor sites is a sensitive index of brain aging and effectiveness of ORG 2766.

ORG 2766 (H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH) is a behaviorally active $ACTH_{4-9}$ analogue. The peptide increases responsiveness of rats to motivationally relevant stimuli[14,20,31], speeds regeneration of injured peripheral nerves[8] and enhances mood, when given chronically to elderly persons[31]. In rats age-associated changes in hippocampus morphology can be influenced by chronic treatment with ORG 2766. The $ACTH_{4-9}$ analogue delayed the development of astrocyte hypertrophy in the hippocampus of aging rats[21,22].

The hippocampus is involved in spatial orientation[30]. Senescent rats have been found to display a deficit in learning the lay-out of a circular platform[2,37]. Chronic treatment with ORG 2766 restored performance of old female rats to the level of young control animals[37].

Thus, the effect of chronic treatment with ORG 2766 is expressed in changes in brain morphology, learning, mood and nerve regeneration. We assume that some, if not all of these expressions of the activity of the peptide have a common denominator, e.g. some trophic effect on neural tissue, which may slow down the process of brain aging. It is of great importance to localize and characterize such an effect of ACTH-related peptides in the brain and to find sensitive indices for its operation.

In this study we examined the susceptibility of spatial learning and of hippocampal corticosterone receptors to aging and to ORG 2766 treatment. As a follow-up of a previous study[37], we used male rats of the same strain. The choice of hippocampal corticosterone receptors as a measure for a possible trophic effect of ORG 2766 was based on the following considerations. The corticosterone receptor system has its predominant localization in the hippocampal neurons[13,17,23,24,42,43]. In rats the action of corticosterone on extinction of certain learned responses[4,23,26] and on exploration of a novel environment[40,41] displays the same stringent specificity as the corticosterone binding to the hippocampal corticosterone receptor system[42]. Moreover, an age-associated decline in receptor capacity for glucocorticoids has been reported in neurons of rat forebrain[34] and of hippocampus[35]. Recent studies have shown, that vasopressin- and ACTH-related peptides are involved in regulating the number of corticosterone receptor sites in the hippocampus[11,38,39].

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Our results show that the number of hippocampal corticosterone receptor sites is a sensitive index of brain aging and effectiveness of ORG 2766.

Twenty young (4 months) and 18 old (24 months) male Wistar rats were supplied by TNO, Zeist, The Netherlands. After a 2-month period of acclimatization they were trained on a spatial learning task. The task has been described by Barnes et al.[2]. Briefly, a circular platform was used, 1.22 m in diameter, with 18 circular holes, 9.5 cm in diameter, lining the circumference. The rotable platform was placed on a 6 cm high pole. Underneath the surface a goal box was kept in a fixed position. The holes on the platform surface were numbered near the edge (1–18). Before each trial for each rat the surface of the maze was rotated so that a hole with a randomly chosen number was placed above the goal box. Thus, odor trials could not be followed to the goal box from trial to trial. On day 1 of the experiment, each rat was placed next to the hole over the goal box and allowed to step down and remain in the box for a 2-min adaptation period. Subsequently, the rat was given a regular trial (see below).

All regular trials were started by placing an animal into the start box which rested in the middle of the platform. After 10 s, the start box was raised with a rope pulley, leaving the rat free to move on the platform for 4 min. If the rat located and entered the goal box before the time limit, it was left in the box for 1 min before being returned to its home cage. If the rat did not find the goal box by the end of the 4-min period, it was placed next to the hole over the goal box and allowed to step down into the box and remain there for 1 min. The trials were continued once daily for 9 consecutive days.

The performance of each rat was filmed and videotaped. Computer-assisted analysis of the tape[27] yielded 3 measures of performance: total distance run, speed of running, and the time taken by the rat to find the goal box and enter it with all 4 feet (latency). A fourth measure, the number of errors (head-dips in 'empty' holes) was not of much help as the performance of old and young animals was virtually errorless from the beginning of the experiment. Accordingly, the results of this measure will not be discussed. The results of the 3 measures mentioned, were analyzed by means of Student's *t*-test, using the area under the (learning) curve as index of performance.

The animals were divided into 2 groups of 10 young and 2 groups of 9 old rats. One group of young rats and one group of old rats were treated with ORG 2766 and the other groups with placebo (vehicle). Alzet osmotic minipumps (model 2002) were used to deliver the treatments. The minipumps were implanted subcutaneously under anesthesia with Avertine one day before the beginning of the experiment. The rate of release of the peptide was 0.5 μg/0.5 μl/h for 14 days. The vehicle was 0.9% saline.

The results are summarized in Table I. On only one measure, i.e. the latency to find the goal box, was performance of old rats significantly worse than that of young rats. In neither age group did ORG 2766 influence performance.

Two days after the end of the spatial task, the animals were bilaterally adrenalectomized. The rats were killed one day after adrenalectomy (ADX). Prior to sacrifice the animals were anesthetized with Nembutal and then perfused with saline (± 25 ml per rat) via the heart. The hippocampus was dissected on ice[19], frozen and stored at −80 °C until receptor assay.

Tissue was homogenized in 5 mM Tris buffer (Tris(hydroxyl-methyl)aminomethane) containing 1 mM EDTA (ethylene-diamine-tetra-acetate, disodium salt) 1 mM 2-mercapto-ethanol and 5% glycerol adjusted to pH 7.4 with hydrochloric acid. The homogenate was centrifuged for 1 h at 2 °C at 105.000 g_{av} for preparation of cytosol. Cytosol was

TABLE I

Performance of young and old rats on a spatial learning task. Comparison of treatment with placebo or ORG 2766.

Group	*(n)*	*Latency (s)*	*Speed (cm/s)*	*Total distance run (cm)*
Young placebo	(10)	226.1 ± 25.4	123 ± 5	1833 ± 222
Young ORG 2766	(10)	211.6 ± 21.7	120 ± 3	1700 ± 109
Old placebo	(9)	463.7 ± 57.4*	109 ± 5	2232 ± 190
Old ORG 2766	(9)	585.6 ± 99.1*	102 ± 3	2365 ± 135

* $P < 0.01$ compared to corresponding young group; n = number of animals.

added to previously evaporated [^{3}H]corticosterone (spec. act. 50 Ci/mmol, New England Nuclear) solutions (concentration range: 0.5–35 nM) with and without a 500-fold excess of unlabeled steroid to correct for non-specific binding. Incubation for 4 h at 0 °C is sufficient to reach binding equilibrium. Separation of bound and unbound [^{3}H]steroid was performed using Sephadex LH_{20} gel filtration and data were expressed as fmol steroid/mg cytosol protein. The binding data were evaluated using the Scatchard analysis.

Fig. 1 depicts the Scatchard analysis of [^{3}H]corticosterone binding. The apparent maximal binding capacity (B_{max}) in hippocampus cytosol of the young rats was about 20% higher than in the old rats (Table-II). The old animals responded to ORG 2766 treatment with an increase in B_{max} of nearly 30%, while the young rats did not respond. Thus, ORG 2766-treatment of old animals restored B_{max} to the value observed in young animals. The apparent K_d was not different among the 4 experimental groups.

Our data indicate that old rats have less corticosterone receptors in the hippocampus than young rats. This observation extends previous findings[33–35] demonstrating decreased binding capacity of glucocorticoid receptors in cerebral cortex. The present study showed that the $ACTH_{4-9}$ analogue ORG 2766 increased the number of corticosterone receptor sites in old rats to the level observed in young animals, while the peptide did not affect the receptors in the latter age group.

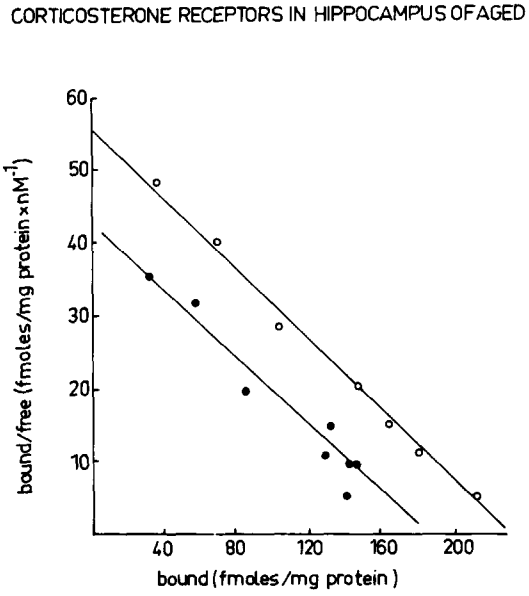

Fig. 1. Effect of ORG 2766 treatment on in vitro [^{3}H]corticosterone binding to receptor sites in hippocampus of old rats treated with placebo of ORG 2766. Data are plotted according to Scatchard. The amount of specifically bound [^{3}H]corticosterone (abcissa) was plotted against the ratio of bound/free (ordinate). ●——● placebo-treated old animals adrenalectomized 24 h before sacrifice B_{max}: 177 fmols/mg protein; K_d: 4.1 nM (r, 0.96). ○——○ peptide-treated old animals. B_{max}: 227 fmols/mg protein; K_d: 4.1 nM (r, 0.98).

TABLE II

Effect of ORG 2766 treatment on hippocampal corticosterone receptor system of old and young rats

Group	*(n)*	B_{max}	K_d
Old placebo	(7)	177 ± 8*	4.1 ± 0.5
Old ORG 2766	(8)	227 ± 9**	4.1 ± 0.4
Young placebo	(7)	214 ± 9	4.0 ± 0.6
Young ORG 2766	(7)	208 ± 12	4.1 ± 0.6

Scatchard analysis of binding data gives apparent maximal number of binding sites (B_{max}) and dissociation constant (K_d) (± S.D.). B_{max} is expressed as fmol/mg protein; K_d is expressed as nM; n = number of hippocampi for binding assay; * $P < 0.05$ vs young placebo (Student's t-test) ** $P < 0.05$ vs old placebo (Student's t-test).

A reduced number of receptor sites in the hippocampus may imply a decreased responsiveness of the target cells to corticosterone. Although reduced responsiveness to hormones is a phenomenon commonly observed in senescence, there have been no data reported as yet on corticosterone action on hippocampal neurons of aged rats. Corticosterone has a specific action on a number of chemical and behavioral parameters associated with the hippocampus[11,23]. For instance, 5-HT turnover[12] and the content of vasoactive intestinal peptide (VIP)[32] are changed after ADX and restored by corticosterone replacement. Deficits in extinction of learned behavior[4,5,23,26] and of exploration of a novel invironment[40,41] develop after ADX and are also restored by corticosterone. The specificity and localization of these corticosterone effects suggest implication of the hippocampal corticosterone receptor system and it would be of interest to study these measures in aged animals as well.

The old male rats in the present study performed worse in only one behavioral paradigm, the latency to find the goal box, while ORG 2766 was not effective. In fact, the deficits noted in previous work[2,37] were not apparent in this study. Since behavior inte-

grates many different processes, it is possible that a deficit in one neural process is compensated for elsewhere in the brain. This may explain the relative intractability of spatial learning in our old male rats to the effect of aging and also the ineffectiveness of the peptide. That ORG 2766 improved spatial learning of female rats[37] and not of males, presents a puzzling problem. A sex difference in the effectiveness of peptides on behavior has been described by Beckwith[3].

Elevated glucocorticoid levels induce astrocyte hypertrophy[21,22]. In senescent rats, corticosterone levels are elevated, while age-associated changes have also been observed in hippocampus morphology, in particular increased astrocyte reactivity and loss of hippocampal neurons[22]. Bilateral ADX or chronic administration of ORG 2766 has been found to delay the development of astrocyte hypertrophy[22]. Bilateral ADX results at long (more than 12 h) intervals after surgery in increased number of receptor sites for corticosterone in the hippocampus[23], which also corroborates the increasing effect of ORG 2766 on receptor number in the present study. Receptors labeled with corticosterone in the brain are heterogenous and occur in glial cells as well as in neurons[11,23,25,42]. It would be of interest to study which population of corticosterone-labeled sites (mineralo-, gluco-, or 'corticosterone preferring'-sites[11],) are affected.

The reduced number of receptor sites in aged animals may be due to down regulation as a consequence of higher circulating levels of glucocorticoid. It is certainly not due to receptor occupancy by endogenous steroids, since the adrenals were removed 24 h prior to sacrifice. This interval is sufficient to remove endogenous corticosteroids; radioimmunoassay of hippocampal extracts of the animals does not reveal the presence of the steroid in detectable (pg) amounts[39]. The decreased corticosterone receptor level at old age may be a consequence of a slower rate of synthesis, an enhanced rate of degradation, the presence of non-functional (non-binding) receptors at old age or just loss of receptor containing cells. ACTH-related peptides have effects on protein synthesis and on phosphorylation of proteins[18]. These effects could lead to increased number of functional receptor sites. Alternatively, the transformation of the receptor associated with the steroid into the DNA binding state may be deficient. Such a defect has recently been observed in the progesterone receptor system of the oviduct of non-laying hens[7]. In recent studies we have defined a number of conditions that produce changes in binding capacity for corticosterone in the rat hippocampus. Reduced receptor number was observed after chronic stress or elevated plasma corticosterone level[36,39], and in diabetes insipidus animals, that have a hereditary lack in vasopressin synthesis[10,38]. Increased receptor number was found after hypophysectomy[39], lesioning of the serotonin input to the hippocampus[1] or damage to the receptor containing hippocampal neurons[28,29]. In the latter case, a compensatory increase in receptor number occurred in remaining hippocampal neurons after unilateral hippocampectomy[28] as well as after partial neurotoxic lesioning of the hippocampus with kainic acid[29]. In the search for chemical mediators of these receptor changes, it was found that chronic treatment of hypophysectomized rats with ACTH related peptides reduced the increased corticosterone binding capacity[39] and that vasopressin-related peptides increased the reduced binding capacity of the diabetes insipidus animals[38]. As shown in this study, the ACTH analog increased the number of corticosterone receptors in the hippocampus to the level observed in young control animals. Receptor number was in all cases normalized with centrally active peptides, virtually devoid of peripheral endocrine activity that also normalized aspects of adaptive behavior[14]. It seems, therefore, that whether the number of corticosterone receptors is increased or decreased, neuropeptides may act to restore the altered receptor number.

Aging of eukaryotic organisms may result from a failure to maintain homeostasis[6,15,16]. The hormones of the pituitary–adrenal system play an important role in maintaining homeostasis, implicating these hormones in some age-related changes. It is conceivable that the corticosterone receptor system not only participates in homeostasis by transmitting the steroid signal to the genome, but also adjusts the number of its functionally active binding sites under the influence of neural and endocrine factors. Thus, the number of receptor sites may represent a sensitive index for aging of the brain and for effectiveness of neurotropic peptides, as is shown in the present study with ORG 2766.

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