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Research report

Nimodipine's protection against corticosterone-induced morphological changes in the hippocampus of young rats

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Abstract

Sustained high levels of corticosterone (CORT), one of the major stress-induced hormones in the rat, were suggested as generating 'accelerated brain aging' and were shown to induce both specific brain changes in the hippocampus and learning impairments in young and middle-aged Fischer-344 rats. Evidence that altered calcium (Ca) homeostasis may play a major role in brain aging has accumulated over the last decade. Recently, new data established a connection between glucocorticoids and voltage-activated Ca influx in aged hippocampal neurons. In the present study, an attempt was made to block the CORT-induced 'accelerated aging' by the simultaneous administration of the L-type Ca channel blocker nimodipine. CORT or placebo sustained-release (SR) pellets were implanted subcutaneously in 3 months old Fischer male rats. Each group was further sub-divided between nimodipine and placebo SR treatments. Characteristic CORT-induced morphological changes were observed in pyramidal hippocampal cells, such as at the CA1 and CA4 sub-regions (22.2% $\pm$ 7.7 and 28.6% $\pm$ 8.4 of pyknotic cells without clear nuclei, respectively). Concomitant treatment with nimodipine conferred full protection against CORT-induced morphological changes (e.g. 3.2% $\pm$ 0.8 and 2.1% $\pm$ 1.9 of pyknotic cells in CA1 and CA4, $n = 7$ rats in each group; $P < 0.04$). The neuroprotective efficacy of nimodipine supports the theory of Ca involvement in CORT related 'accelerated brain aging'.

Keywords: Aging; Alzheimer's disease; Calcium; Cognition; Glucocorticoid; Memory; Morphometry; Stress

1. Introduction

Stress response is a complex series of events, resulting in characteristic behavioral, physiological, endocrine and biochemical processes. Among these, activation of the hypothalamic-pituitary-adrenocortical (HPA) axis, controlled in part by the hippocampus, plays a major role. This activation leads to an increase in the secretion of glucocorticoids (GC), principally cortisol in humans and corticosterone in rats [16]. Prolonged exposure to GC may cause, through a cascade of events, damage to hippocampal neurons. A similar effect was found in rats and monkeys subjected to chronic stress [22,51]. A positive correlation was exhibited between the extent of the morphological damage in the hippocampus and the adrenal activity of rats during the aging process [25]. With the progress of age there is an increase in levels of corticosteroids [7,8]. Moreover, an increase in corticosterone (CORT) concentrations was found to be related to impairment of cognitive functions in aging rats [15]. Changes in HPA axis were also observed during aging in rats. An inhibition of the negative feedback of HPA axis and an increase in ACTH levels were accompanied by an impairment in spatial memory and a decrease in number of cells in the hippocampus. These findings led to the development of the glucocorticoid hypothesis of brain aging and degeneration [27,28].

The activity of CORT in the brain is mediated through two receptor systems: mineralocorticoid receptors (MR's) and glucocorticoid receptors (GR's) [16,41]. MR's play an important role in the basic control of the HPA axis while, both MR's and GR's are responsible for the regulation of the reaction to stress [40,42]. The two also regulate cholinergic, serotonergic and adrenergic responses in complex manners [18].

Sapolsky et al. [43] suggested the glucocorticoid cascade hypothesis assuming that any impairment in control of CORT secretion, due to an injury to the hippocampus,

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will cause additional increase in CORT and an aggravation of the existing damage.

Using subcutaneously sustained-release (SR) CORT pellets at a level corresponding to mild stress, we have recently introduced an animal model for the study of the effects of long term exposure to GC on cognition [5]. Following nine weeks treatment with CORT, subtle behavioral decrements were observed in young Fischer 344 rats. In pre-selected, cognitively 'non-impaired' middle-aged rats, similar treatment resulted in a more prolonged impairment of the learning process in the 8-arm radial maze [2].

Events accompanying aging processes (e.g. increase in GC, decrease in glucose transport and increase in glutamate levels) were shown to lead to an increase in neuronal calcium (Ca) levels, resulting in cell death [7,11,12,21,36,52]. Landfield's group demonstrated that the primary effect of corticosteroids was on Ca conductance and that with age the effect increased [21]. Joels and De Kloet [17] showed that GC enhance the Ca-dependent afterhyperpolarization. Recently, Kerr et al. [23], using a specific agonist (RU 28362), showed that activation of GR's caused an increase in the conductance through voltage-dependent Ca channels and therefore an increase in Ca currents.

In this study, an attempt was made to block the CORT-induced brain changes by using an L-type Ca channel blocker, nimodipine. Nimodipine was found effective in treatment of subarachnoid hemorrhage, global and focal ischemia and in epilepsy [35,38,45] and thus seemed a suitable candidate to test pharmacologically the hypothesis of Ca involvement in CORT-induced hippocampal brain damage.

2. Material and methods

2.1. Animals

Thirty two male Fischer-344 rats, 3 months old upon arrival (obtained from Charles River, UK) were used for this study (average weight $\pm$ S.E.M.: 241.1 $\pm$ 1.5 g). All rats were housed in individual cages in a temperature-controlled environment (22 $\pm$ 1°C) with lights on from 5:00 to 18:00 h, and ad libitum access to food (Altromin, Lage, Germany) and water. Their weight following implantation was monitored daily.

Another identical group of 24 rats (same age and from the same supplier; average weight 277.7 $\pm$ 2.3 g) was used to monitor plasma CORT levels during the drug treatment, in order to eliminate an additional stress factor in the main study experimental groups.

2.2. Drug treatment

Rats were randomly divided into two groups treated either with CORT-sustained release (SR) or placebo pellets (produced by Innovative Research of America, Toledo, OH, USA). Each group was further subdivided into two subgroups treated with nimodipine SR pellets or placebo pellets, produced by the same supplier (4×8 rats in the brain morphometry study, 4×6 rats in the biochemical study of plasma level monitoring). CORT, nimodipine or the equivalent placebo SR pellets were implanted under general anesthesia, induced by a mixture of halothane and oxygen. Four CORT pellets (each containing 200 mg, released over 90 days) or four placebo pellets, were implanted subcutaneously around 4 cm lateral of the median line. Nimodipine treatment, also given via SR pellets (20 mg released over 21 days), were implanted every three weeks at the nape of the neck, 4 consecutive implantations.

To determine hormone plasma concentration in the biochemical study group, samples were collected from the tail vein into heparinized glass capillaries (at 9:00 h for morning levels). Special care was taken to insure that all blood samples were collected within 2 min after approaching the rat's cage, so that stress would not affect the measurement. Plasma samples were stored at -20°C until analyzed for CORT concentration using a radioimmunoassay kit (ICN Biomedicals Inc., Costa Mesa, CA, USA). According to the manufacturer, typical intra-assay variation was around 4–7% and typical inter-assay variation around 7%.

2.3. Histology

Rats were anesthetized (180 mg/kg i.p. of nembutal) and perfused with saline followed by a mixture of 10% formaldehyde, acetic acid and methanol (FAM; 1:1:8). Following decapitation, brains were removed and further fixed in the same FAM fixative at 4°C for at least 24 h. Specimens were then dehydrated in graded alcohols solutions, cleared in methyl-salicylate and embedded in paraffin. Six micron coronal sections were serially cut and selected sections, mainly from the hippocampal level (according to Figs. 31–33 in Paxinos and Watson) [37], were stained with hematoxylin and eosin (H&E).

Quantitative analysis of morphological brain changes was carried out by counting the number of damaged cells, as well as total number of cells, in constant frames in various areas of the hippocampus: CA1, CA3 and CA4. In the dentate gyrus (DG) only damaged cells were counted. For each area, three consecutive sections were monitored, and cells were counted in both hemispheres. Cells were considered normal according to their distinct, bright and large nuclei. Pyknotic cells without clear nuclei which were stained intensely with hematoxylin were considered as damaged cells.

2.4. Statistical analysis

Biochemical and histological data were subjected to statistical analysis using ANOVA software for repeated

measures, in order to determine statistical significance of differences between groups. Simple main effect comparisons or Scheffé's test were applied on data whenever statistical significance was found.

3. Results

3.1. Weight changes

Changes in body weight of all rats following implantation are presented in Fig. 1. CORT-implanted rats lost almost 30% of their body weight during the first ten days following implantation. Later they regained weight at a rate similar to that of placebo rats, however, their weight remained about 30% lower until 90 days following implantation.

Placebo-implanted rats lost only around 2% of their body weight within the first two days following implantation. On day three they started regaining weight.

3.2. Corticosterone plasma levels

Morning values of plasma CORT concentrations for the four different experimental groups at seven sampling times throughout the whole study period are presented in Fig. 2. Plasma CORT levels in CORT-implanted rats were significantly elevated during the whole period, and were always above 15 μg/dl. A gradual decrease in concentrations was observed throughout the experiment, probably due to a continuous decline in the pellet's content. Nimodipine treatment had no effect on CORT concentrations, neither in placebo group nor in the CORT-implanted group ($P = 0.963$).

An additional blood analysis was performed on day 95, and at that time plasma CORT concentrations for all four experimental groups were in the normal range of low morning values, with no detected differences.

3.3. Morphological changes in the hippocampus

Light microscopy examination of brain sections, at hippocampal level, revealed striking differences among the four treatment groups.

Fig. 3 displays representative sections of CA1 area of the four experimental groups. In a placebo-treated rat and a rat treated with placebo and nimodipine cells appeared to be normal and it was possible to distinguish between nucleus and cytoplasm of each cell (Fig. 3A,C). Following CORT treatment (Fig. 3B), large numbers of pyramidal cells were pyknotic and shrunk probably due to a severe process of degeneration. However, following three months of combined treatment with CORT and nimodipine most of pyramidal cells in CA1 area displayed normal morphology (Fig. 3D).

In the CA3 region of CORT-treated rats, severe damage to pyramidal cells was also obvious. In Fig. 4A an example of an exceedingly damaged rat is given. However, parallel sections taken from rats treated with both CORT and nimodipine contained mostly normal cells (Fig. 4B). In the inner dentate gyrus (DG) of rats treated with CORT many damaged cells were found, mainly in its basal layer (Fig. 4C). In rats that received concomitant treatment of nimodipine, cells at the DG region were mostly intact (Fig. 4D).

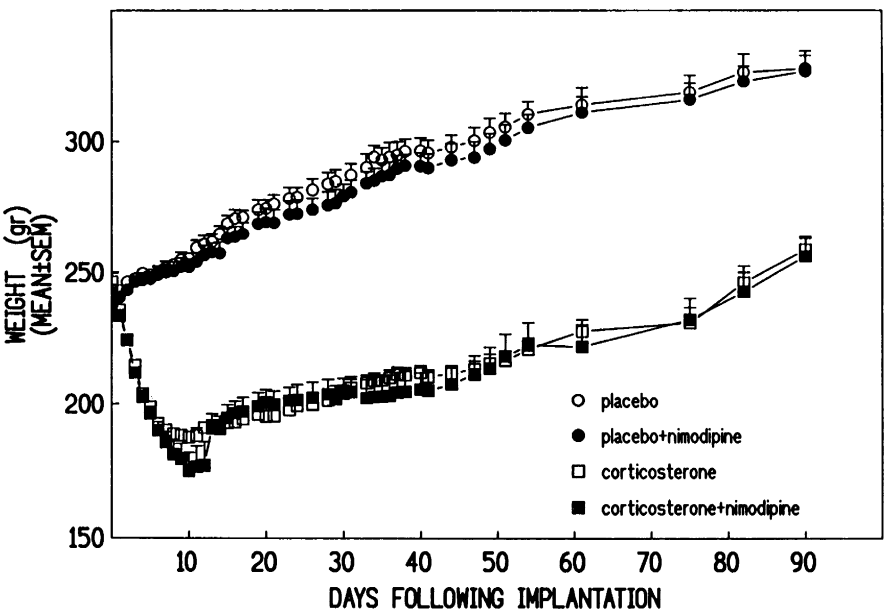

Fig. 1. Body weight changes of young rats following implantation of CORT-, CORT + nimodipine-, placebo- and placebo + nimodipine-sustained release pellets ($n = 8$ in each group).

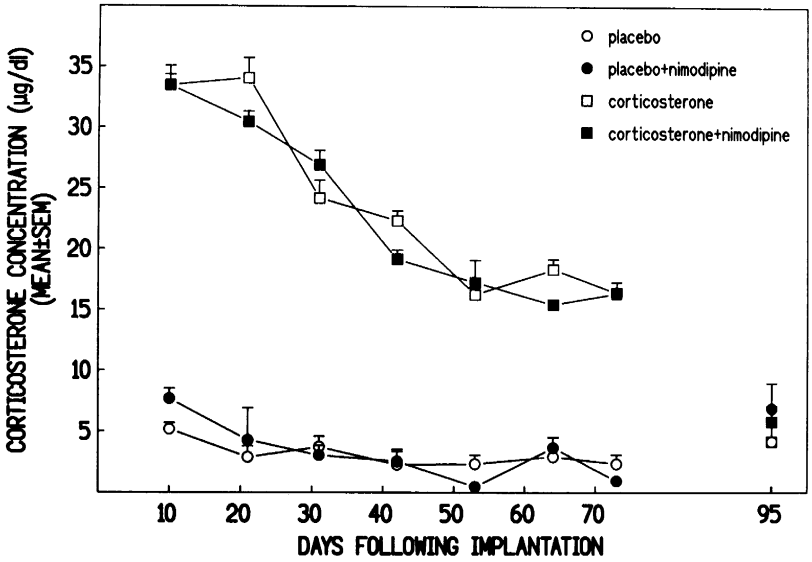

Fig. 2. Plasma CORT concentrations following implantation of CORT-, CORT + nimodipine-, placebo- and placebo + nimodipine-sustained release pellets ($n = 8$ in each group).

In order to quantify these histological results, a morphometric analysis compared the percentage of damaged cells in seven rats of each treatment group (Fig. 5). The percentage of damaged cells in CA1, CA3 and CA4 regions was very low in placebo-treated rats, as well as in rats treated with placebo and nimodipine (in the range of 3–8%). In

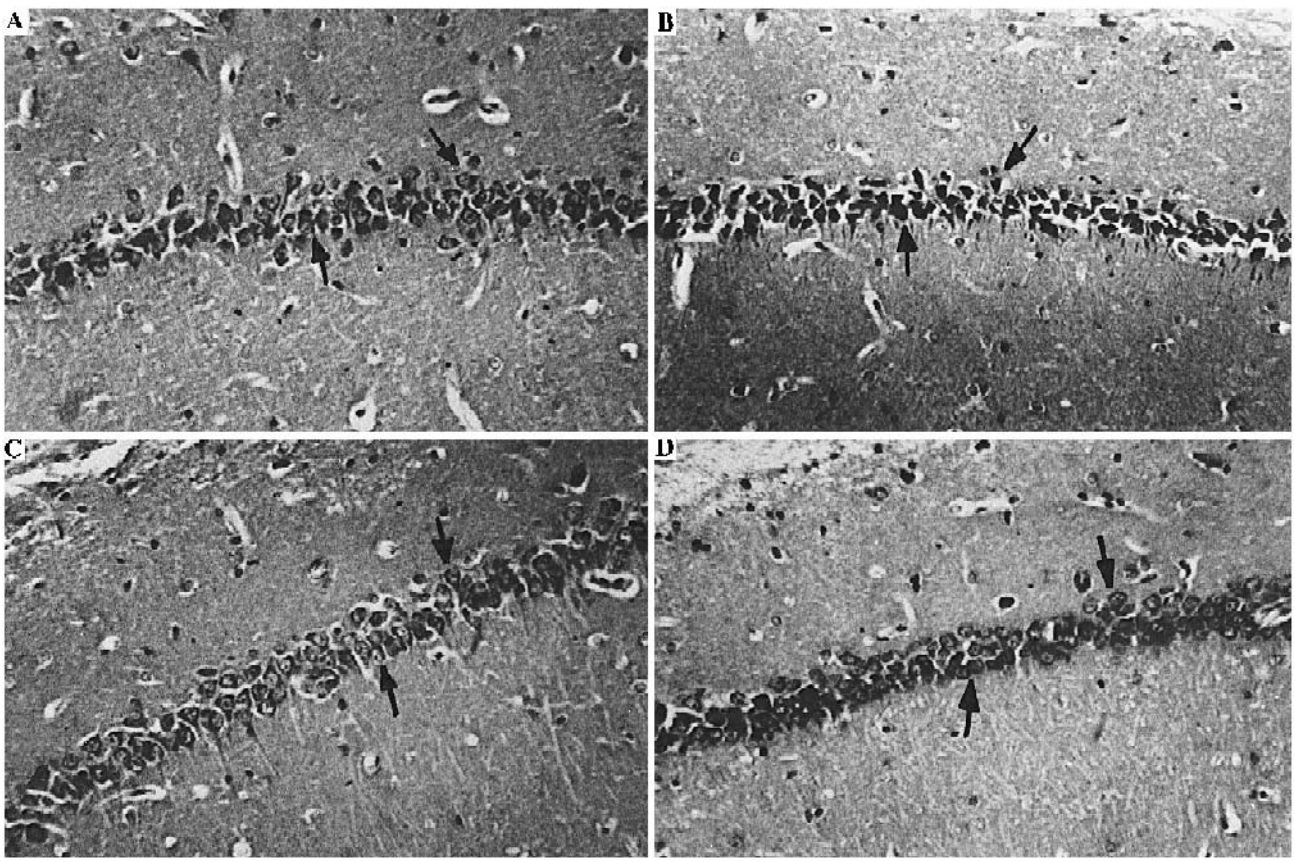

Fig. 3. Sections through the hippocampal CA1 region: a placebo-implanted rat (A) and a rat treated with placebo and nimodipine (C). The pyramidal cells in both sections have normal morphology (arrows), where nuclei are clearly seen. Following CORT implantation (B) most cells are pyknotic and shrunk (arrows). However, following treatment with CORT and nimodipine (D) most cells have normal morphology (arrows) (H&E staining; original magnification × 100).

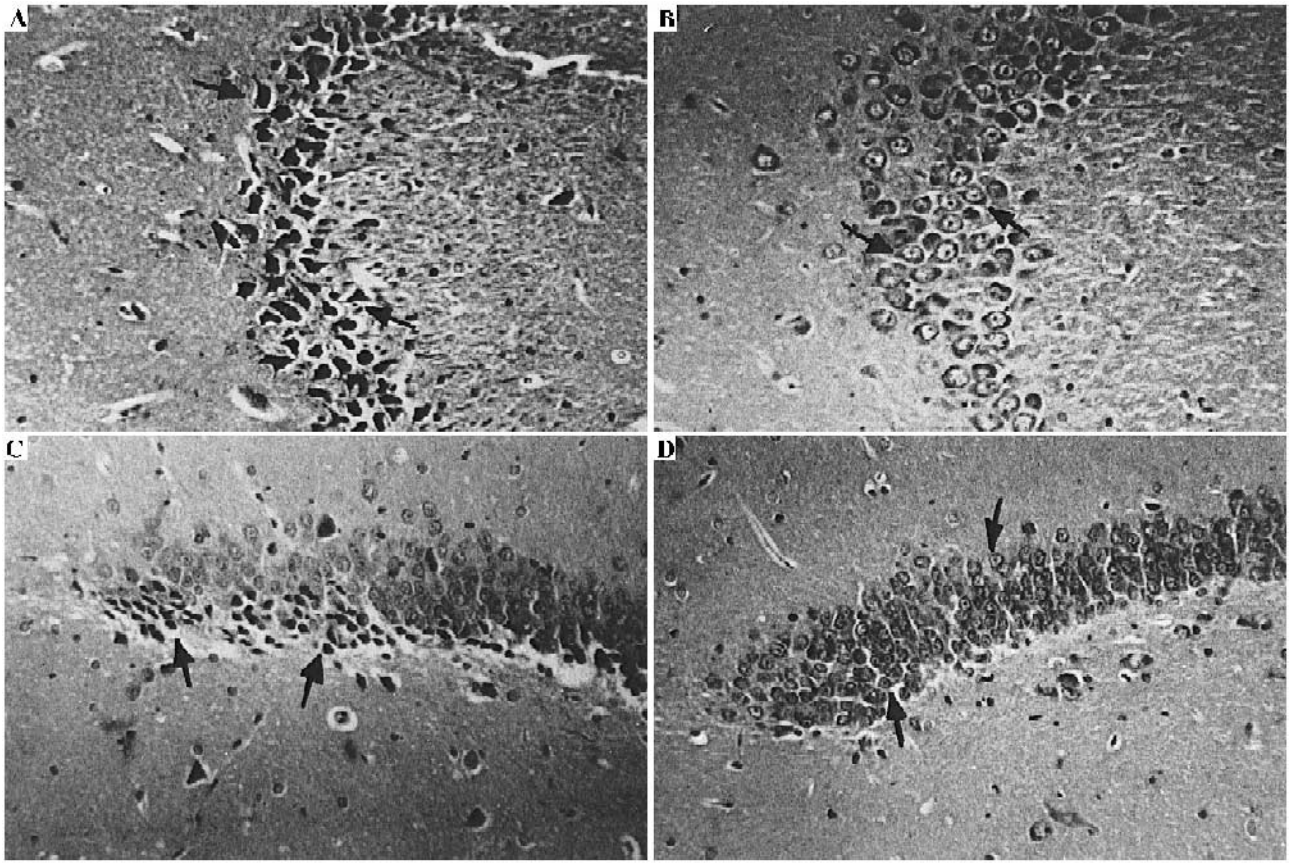

Fig. 4. Sections through the hippocampal CA3 and DG regions. In CORT-treated rats many pyramidal (A) and granular (C) cells are pyknotic and shrunk (arrows). However, following treatment with CORT and nimodipine (B and D) most cells are intact (arrows) (H&E staining; original magnification × 100).

CORT-treated rats, however, the extent of the damage was dramatically increased (23–28% in CA1, CA3 and CA4). Rats treated with both CORT and nimodipine showed a complete protection, as the percentage of damaged cells was in the range of 3–6%.

Statistical analysis of the results revealed statistical

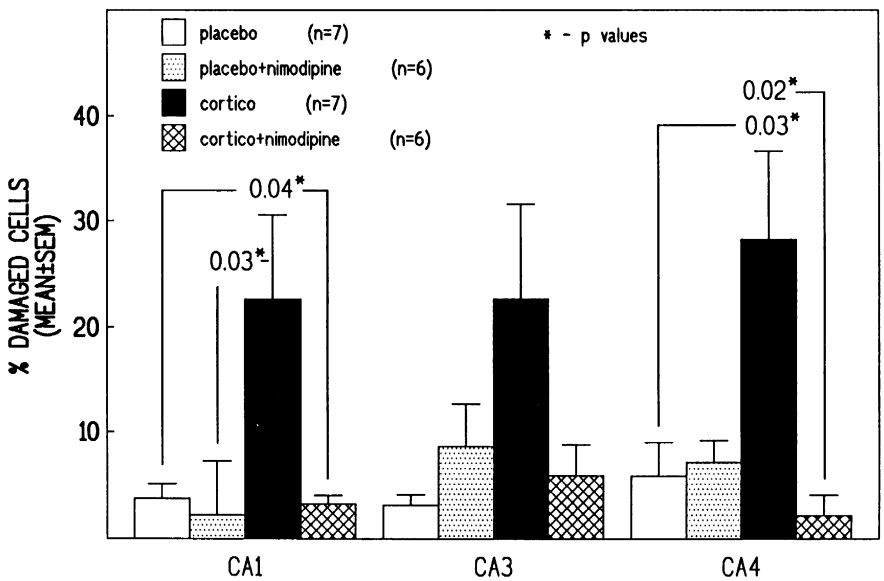

Fig. 5. Percentage damaged cells in CA1, CA3 and CA4 subfields of the hippocampus following three months treatment with, placebo, placebo + nimodipine, CORT and CORT + nimodipine in young rats.

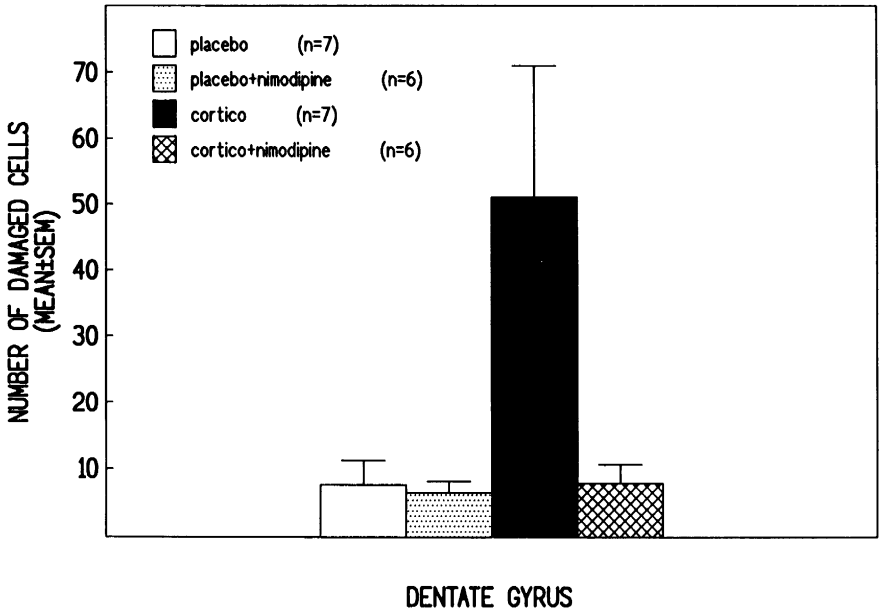

Fig. 6. Number of damaged cells in DG subfield of the hippocampus following three months treatment with placebo, placebo + nimodipine, CORT and CORT + nimodipine in young rats.

significance in CA1 and CA4 (but not in CA3) regions, when the percentage of CORT-induced damaged cells was compared to either the placebo group or to the CORT + nimodipine group ($P = 0.04$ for both in CA1; $P = 0.03$ and $P = 0.02$ in CA4; $P = 0.07$ and $P = 0.15$, respectively in CA3; see Fig. 5).

The total number of damaged cells in the DG region is given in Fig. 6. Comparisons of the data for the four experimental groups in this hippocampal area, as for those in the CA3 region, were not statistically significant ($P = 0.067$ for CORT against placebo; $P = 0.086$ for CORT against CORT + nimodipine), most likely because of a larger variation between rats, as not all rats were affected to the same extent by the CORT treatment in both CA3 and DG.

4. Discussion

4.1. Corticosterone administration and plasma levels

High levels of GC were found to exacerbate the neuronal brain damage caused by other insults and thus endanger primarily hippocampal neurons (for a recent review see [44]). In the present study, an attempt was made to investigate the neuronal deteriorating effect of high GC as the sole abusive factor. This was carried out using SR CORT pellets. To avoid additional stress, bleeding for hormone level determination was carried out using parallel experimental groups. Except for the drug administration, the only apparent concomitant process was normal aging. After termination of the treatment, it was established that hormone levels receded back to normal (see Fig. 2), and rats were killed for histological examination approximately six weeks later.

CORT levels chosen for the present study, although they gradually declined during the treatment period, were generally within the physiological range associated with severe (35 μg/dl) to mild stress (15 μg/dl). In a previous study [5] it was demonstrated that under these conditions the normal circadian rhythm of CORT concentrations was abolished, resulting in nearly constant levels throughout day and night.

The only distinct difference between CORT-treated and placebo rats was the loss of weight (Fig. 1), which is well documented and associated with GC metabolic effect [10]. Nimodipine treatment affected neither plasma CORT levels, nor body weight. Thus, any effect of nimodipine could not be attributed to differences in CORT levels between the groups. As nimodipine treatment did not alter CORT's metabolic features, its neuroprotective mechanism is probably the result of its Ca channel blocker activity.

4.2. Morphological variations within hippocampal subfields

CORT-induced damage to hippocampal neurons is typically found (as also shown in the present study) at CA1, CA3, CA4 and DG subfields. The detrimental effect, as well as nimodipine's neuroprotective action, were statistically significant in CA1 and CA4 but not in CA3 and DG regions. CORT activates both MR's and GR's in the hippocampus, however, due to the higher affinity of MR's, most of these receptors are occupied at normal CORT levels. It is therefore logical that the effect of excessively high CORT concentrations is mediated primarily via GR's.

Studies of the distribution of MR's and GR's within the hippocampus indicated that the formers predominate at CA3 [6], while GR's constitute a major part of CA1 receptors. In previous studies [30], we often found larger variations in CORT-induced damage at CA3, in which some CORT-treated young rats were sometimes barely affected. The higher abundance of GR's in CA1, as compared to CA3, might be one reason why in our previous studies the damage in CA1 was more pronounced at early CORT treatment stages [5,30]. A future temporal study on the progression of the morphological injury, following various CORT treatment intervals, may reveal that neuronal damage appears initially at CA1, is then followed by an increasingly retrograde degeneration at CA3 and finally affects the DG. This hypothesis is in agreement with the hippocampal innervation network via the perforant path (i.e. DG to CA3 to CA1) [1].

A new direct and even more conclusive evidence for the role of GR's in age-related hippocampal impairments was reported recently [50]. In this study, a prolonged chronic treatment with RU486, a specific and potent GR antagonist, from mid-age to senescence attenuated electro-physiological biomarkers of CA1 aging and probably Ca homeostasis.

Hippocampal changes during aging were reported in many studies [e.g. [26,47,20]], mainly in relation to CA3 region but also observed in CA1. The fact that following high CORT treatment we observed predominately CA1 damage might be related to the finding that in human pathological aging, such as in Alzheimer's disease, CA1 was found to be more vulnerable [14]. Thus, CORT treatment may mimic pathological rather than normal aging. With regard to the current controversy on the validity of hippocampal cell-loss evaluation methods [53], it seems to us that species differences rather than different technical methods are responsible for the discrepancies, with Fischer-344 rats being possibly more sensitive to both aging and CORT treatment. Thus, CORT-induced morphological changes in hippocampus, observed in Fischer-344 rats, could not be reproduced in Long–Evans rats [3].

Massive cell death was reported in the granule cell layer of the DG following zero CORT levels, induced in rats by adrenalectomy [13,48,49]. Under these conditions, however, no significant change was observed in the morphology of CA1 and CA3 pyramidal cells. A recent study found in DG an interesting correlation between cells age and vulnerability to CORT levels [4]. Following adrenalectomy, significant numbers of DG mature cells died, while the number of younger cells did not decrease. In the present study, a unique pattern of morphological damage was recorded following high CORT levels, being restricted to a specific layer of the granule cells at the inner DG (see Fig. 4C). This basal layer of cells was shown by immunocytochemical staining to contain high concentration of GABA-positive cells [46]. Further studies are needed to clarify this interesting phenomenon.

4.3. Significance of nimodipine's neuroprotection

The L-type Ca channel blocker nimodipine was mainly considered in the past for its neuroprotective effects in subarachnoid hemorrhage and in cerebral ischemia (e.g. [38]). Some of the recent studies investigated the effect of aging on its efficacy against NMDA-induced cell death [31], demonstrated its efficiency against MPTP-induced neurotoxicity [24] and exhibited an attenuation of age-related neurochemical changes following nimodipine's chronic administration [19]. The present study extended these data by revealing nimodipine's neuroprotective efficiency against prolonged high CORT levels, simulating 'accelerated aging' hippocampal deterioration. This finding is in agreement with recent evidence which showed that activation of GR's increased the conductance of L-type Ca channels and consequently Ca currents [23]. Thus, this pharmacologic activity of nimodipine supports previously published theories on the association between the neurotoxic effects of GC and Ca homeostasis [11,12,29]. The present study indicated an important role for L-type channels, at least with regard to Ca changes induced in vivo by prolonged high CORT exposure. This, however, does not exclude the involvement of an indirect action of CORT or other mechanisms [e.g. [11,12]].

Increasing numbers of recent studies are engaged in investigating the relationships between human aging, cortisol levels and cognitive deficits [32–34,39]. The accumulating information strengthens the hypothesis of GC and Ca involvement in both normal brain aging and Alzheimer's Disease [9,28]. Along these lines, the present findings which established nimodipine's efficacy against brain injuries induced by prolonged high CORT exposure might have significant implications in future efforts to prevent age-related cognitive degeneration.

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