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Neuroprotective effect of ACTH (4-9) in degeneration of hippocampal nerve cells caused by dexamethasone: morphological, immunocytochemical and ultrastructural studies

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Abstract. Sustained exposure to glucocorticosteroids (GCs), adrenal hormones secreted during stress, can cause neural degeneration. This is particularly so in the hippocampus, a principal neural target site for GCs. The purpose of this research was an assessment of the neuroprotective effect of ACTH (4-9) in degenerative changes of hippocampal neurons induced by synthetic GC - dexamethasone. Experiments were conducted on male Albino-Swiss mice. We studied the morphology of neurons in the dorsal hippocampus in slides stained with cresyl violet. Immunocytochemical analysis was carried out with the use of monoclonal antibody anti-MAP2 in order to detect alterations in the neuronal cytoskeleton. We also performed ultrastructural examinations of hippocampal neurons. Quantitative analysis of morphological changes was completed using a computer analyser of histological pictures. It was shown that dexamethasone administered in toxic doses evokes neuronal death in layer CA3 of the hippocampus. Results indicate that ACTH (4-9) shows protective effects in that model. Dexamethasone-induced damage to hippocampal pyramidal neurons (assessed by cell counts, immunocytochemical analysis of cytoskeletal alterations and ultrastructural studies) was significantly reduced in animals administered ACTH (4-9).

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INTRODUCTION

In recent years there have been reports showing that prolonged exposure to elevated levels of glucocorticosteroids may cause degenerative changes in the central nervous system. These changes occur both as a result of hypersecretion of endogenous glucocorticosteroids or exogenous glucocorticosteroids administered in high doses for therapeutic purposes (Starkman and Schteingart 1981, Stein--Behrens et al. 1994, Watanabe et al. 1992, Wolkowitz et al. 1990). Neuronal damage induced by these hormones occurs mainly in the hippocampal formation - a structure of the brain containing the highest concentration of glucocorticosteroid receptors (Twardowska and Rybakowski 1996, Uno et al. 1989). It is thought that neurotoxic action of glucocorticosteroids may participate in some forms of dementia, such as posttraumatic dementia, cognitive impairments in patients with depression and Cushing's syndrome, difficulties in memory and concentration in patients treated with exogenous corticosteroids (Seckl and Olson 1995, Wolkowitz et al. 1990). Clinical observations of neurotoxic effects of glucocorticosteroids were confirmed in numerous experiments on animals (Arbel et al. 1994, Elliot et al. 1993, Sekita-Krzak et al. 1999a, Sekita-Krzak et al. 1999b, Uno et al. 1989).

It has been reported that the ACTH (4-9)-analog H-Met (O(2))-Glu-His-Phe-D-Lys-Phe-OH (ORG 2766), which is the adrenocorticotropic hormone (ACTH) fragment centrally active (but not stimulating the release of adrenal hormones), has trophic effects on neuronal tissue (Horvath et al. 2000, Murry et al. 1993, Sporel-Ozakat et al. 1990, Spruijt 1992). Several earlier studies showed its neuroprotective effect in some models of central and peripheral neuronal degeneration (Elliot et al. 1993, Gerritsen van der Hoop et al. 1990, Gispen et al. 1992, Kiburg et al. 1996, Sporel-Ozakat et al. 1990). Mechanisms that mediate such actions of ACTH (4-9) are still not explained.

The purpose of this research was an assessment of the neuroprotective action of ACTH (4-9) in degeneration of hippocampal nerve cells caused by prolonged administration of synthetic glucocorticosteroid-dexamethasone, based on morphological examinations of hippocampal neurons. MAP2 immunocytochemistry was carried out in order to assess the changes in nerve cells' cytoskeleton. The microtubule associated protein 2 (MAP2) is the major microtubule associated protein of brain tissue normally localized in dendrites and nerve cell bodies throughout the hippocampus. Reduction in MAP2 immunoreactivity indicates alterations in neuronal cytoskeleton.

METHODS

The experiments were carried out on adult male Albino-Swiss mice weighing 19-22 g at the beginning of the experiment. Care and treatment of the animals were in accordance with the guidelines for laboratory animals established by the National Institutes of Health as well as by the Local Ethical Committee of the Medical University of Lublin. Mice were housed 20 per cage under standard laboratory conditions, with free access to granular standard diet and tap water. Their weight was monitored daily. The animals were divided into three groups (including 60 animals each). Animals of the control group received distilled water (i.p. 0.2 ml/24 h) for 28 days. Animals in experimental group I received dexamethasone. Experimental group II animals received dexamethasone and ACTH (4-9). Dexamethasone (Dexaven-Jelfa S.A., Poland) was administered i.p. in a single dose 8 mg/kg/24 h for 28 days. ACTH (4-9) (Bachem, Switzerland) was administered s.c. in a dose of 50 μg/kg twice a week 30 min prior to dexamethasone. Twenty four h after the last distilled water or last dexamethasone injection all animals were anesthetized with pentobarbital (Nembutal 180 mg/kg i.p.) and perfused with 0.9% NaCl with heparin, followed by 10% formaldehyde (pH 7.4). Following decapitation, brains were removed from the skull and postfixed in the same fixative solution at 4°C for at least 24 h. Specimens were then dehydrated in graded ethanol solutions and embedded in paraffin. Six- μm thick paraffin slices were serially cut in the frontal plane.

Histological study

For histological analysis selected paraffin-embedded tissue slices were stained with cresyl violet and assessed using a light microscope. We examined morphology of neurons in the dorsal hippocampus of both hemispheres.

Immunocytochemical study

For immunocytochemical analysis, paraffin-embedded tissue sections were processed on glass slides covered with Vectabond reagent (Vector Laboratories). Sections were repeatedly rinsed in phosphate buffered saline (PBS), then pretreated with 1%H_2O_2 to block any

possible endogenous peroxidase. After incubation in 5% normal serum, sections were put into 0.2% Triton X-100. Then sections were rinsed in PBS and incubated in a 1:500 solution of monoclonal antibody anti-MAP2, at 4°C for 72 h. Then, they were incubated in a solution of biotinylated secondary antibody for 24 h at 4°C and in a solution of avidin-biotin peroxidase complexes for 1 h at room temperature. The bound peroxidase was detected by incubating the sections in a medium containing 0.05% 3,3'-diaminobenzidine (DAB, Sigma) and 0.01%H_2O_2 for 10 min at room temperature. Rinsing the sections in H_2O stopped the reaction. Sections were finally counterstained with cresyl violet, dehydrated in graded alcohols and cleared in xylene.

Ultrastructural study in the CA3 region

For ultrastructural studies, brains were perfusion-fixed in 4% glutaraldehyde. Next, tissue material after being dehydrated was embedded in Epon 812. Semi-thin sections were stained with methylene blue and examined in order to dissect the CA3 region of the hippocampal formation using a light microscope. The preparations were observed using a transmission electron microscope TESLA BS 500.

Quantitative analysis and statistics

Quantitative analysis of morphological changes was carried out by counting the number of damaged neurons in the CA3 region (in slides stained with cresyl violet) using a computer analyser of histological pictures (Lobophot 2, Nicon). Cells with round nuclei and visible nucleoli were considered undamaged, while cells with dark shrunken nuclei were considered damaged. Cell counts were made within two adjacent 40 x microscopic fields in the pyramidal cell layer (from the point directly ventral to the most lateral extension of the upper limb of the dentate granule cell layer). The percentage of damaged neurons in the CA3 region was counted in all groups.

All data are presented as means ± SEM. The statistical significance of the differences between groups was assessed by chi-square test. Statistically significant differences were designated by $P<0.05$.

RESULTS

Body weight changes and mortality

Body weight changes of all animals are presented in Fig. 1. Mice from experimental groups I and II showed statistically significant decrease in body weight in comparison with the control group. ACTH (4-9) did not change the body weight reduction induced by dexamethasone.

The mortality in both experimental groups was similar (about 25%).

Histological study

Light microscopy examination of cresyl violet stained sections from the control group revealed the regular structure of the hippocampus. The nuclei of pyram-

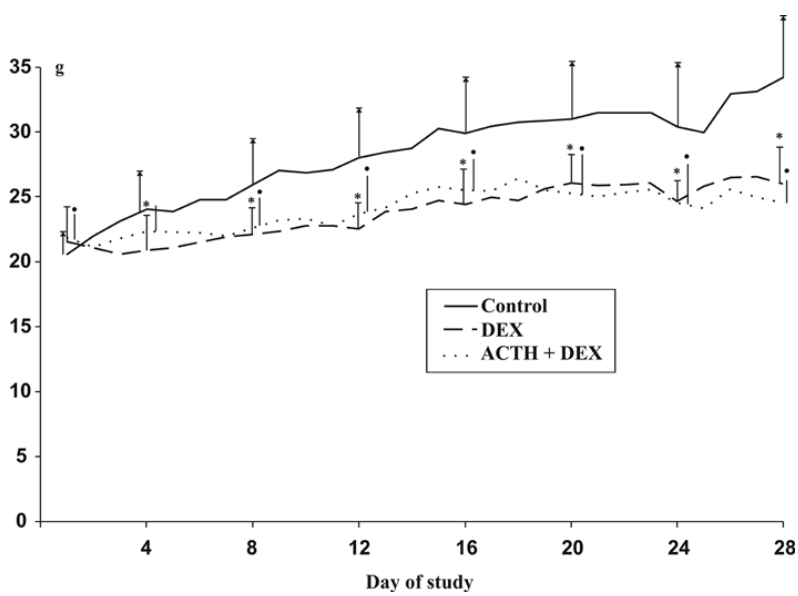

Fig. 1. Body weight changes of animals during experiment. Dexamethasone (DEX) was injected at a dose of 8 mg/kg/day for 4 weeks.ACTH (4-9) was injected at a dose of 50 μg/kg twice a week 30 min before DEX. *, $P<0.05$ DEX vs. Control; •, $P<0.05$ ACTH+DEX vs. Control.

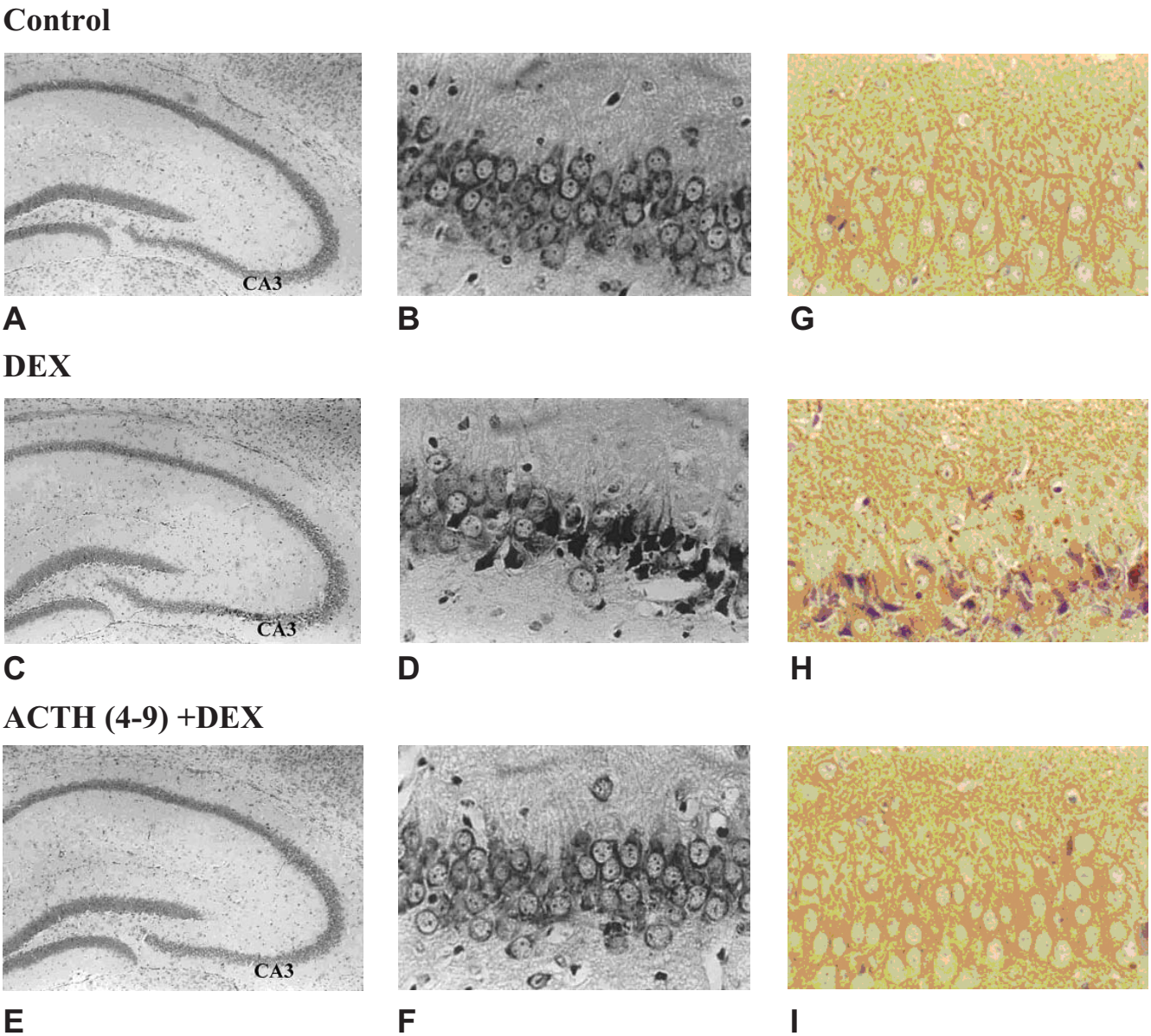

Fig. 2. Coronal sections of the dorsal hippocampus from control animal (Control), dexamethasone treated animal (DEX) and ACTH (4-9)+dexamethasone treated animal (ACTH + DEX). The left photomicrographs (A, C, E) show small magnifications of the dorsal hippocampus in respective groups. Cresyl violet staining. Magnification, x 100. The middle photomicrographs (B, D, F) show high magnifications of the CA3 region in respective groups. Note the evident cell loss and morphological damage of neurons after administration of DEX and significantly smaller damage after administration of ACTH (4-9) with DEX. Cresyl violet staining. Magnification, x 400. The right photomicrographs (G, H, I) show changes in MAP2 immunoreactivity in the CA3 region after administration of dexamethasone (DEX) and ACTH (4-9). Note the evident decrease in MAP2 immunoreactivity after administration of DEX and similarity to ACTH+DEX and control animals. Magnification, x 400. DEX – dexamethasone was injected at a dose of 8 mg/kg/day for 4 weeks. ACTH (4-9)+DEX – ACTH (4-9) was injected at a dose of 50 μg/kg twice a week.

idal neurons in CA1- CA4 regions were clear, round or oval in shape with distinct nucleoli. In the CA3 region they were arranged in 3 to 4 layers (Figs. 2A and 2B).

Significant morphological changes of pyramidal neurons were observed in experimental group I. After 28-day-long administration of dexamethasone, the number of pyramidal neurons especially in the CA3 region decreased. In this region, nerve cells were arranged in 2 to 3 layers in contrast with the higher number in the control group. Numerous neurons in the CA3 region

showed robust morphological changes including the shrinkage of perykarions and an increased intensity of cytoplasm staining. Nuclei of damaged nerve cells were dark, irregular in shape and shrunken in comparison with clear round nuclei of the control group (Figs. 2C and 2D).

In the group of animals receiving ACTH (4-9) along with dexamethasone, examinations revealed a significantly smaller degree of morphological damage to pyramidal neurons. In 40% of animals the total number of neurons in the CA3 region was similar to that of the control group (Figs. 2E and 2F). The changes resembling damage following dexamethasone were present in 10 out of 60 animals.

Quantitative analysis

The mean values of the percentage of damaged neurons in respective groups were: 2.4% in the control group; 46.3% in experimental group I; 15.6% in experimental group II (Fig. 3).

Immunocytochemical study

Immunocytochemical analysis revealed a significant reduction of MAP2 immunoreactivity in experimental group I in comparison with the control group. It was particularly evident in neurons of the CA3 region. Nerve cells of this region showed considerable lowering of MAP2 immunoreactivity after administration of dexamethasone.

Immunoreactivity in 40% of animals from experimental group II was similar to that of the control group. In remaining animals receiving ACTH (4-9) with dexamethasone we observed the decrease in MAP2 immunoreactivity in the CA3 region but its intensity was smaller than in the case of experimental group I (Figs. 2G, 2H and 2I).

Ultrastructural study in the CA3 region

In the control group pyramidal neurons of the CA3 region demonstrated round or oval nuclei. Ribosomes were equally dispersed within the cytoplasm (Fig. 4A).

Experimental group I: the number of pyramidal neurons in the CA3 region was significantly reduced. Pyramidal neurons in the CA3 region demonstrated pathological changes in the shape of a condensation of the nucleus and the cytoplasm. Neurons were dark and irregular in shape (Fig. 4B). They possessed dark, irregular nuclei. The nuclei were electron dense and contained compact chromatin. Nucleoli were enlarged. The cytoplasm became condensed. It consisted of areas of clumped free ribosomes separated by clear spaces representing the dilated *cisternae* of endoplasmic reticulum. Shrunken neurons were surrounded by swollen processes of astrocytes (Fig. 4C). Ultrastructural examination also revealed the swelling of endothelial cells in

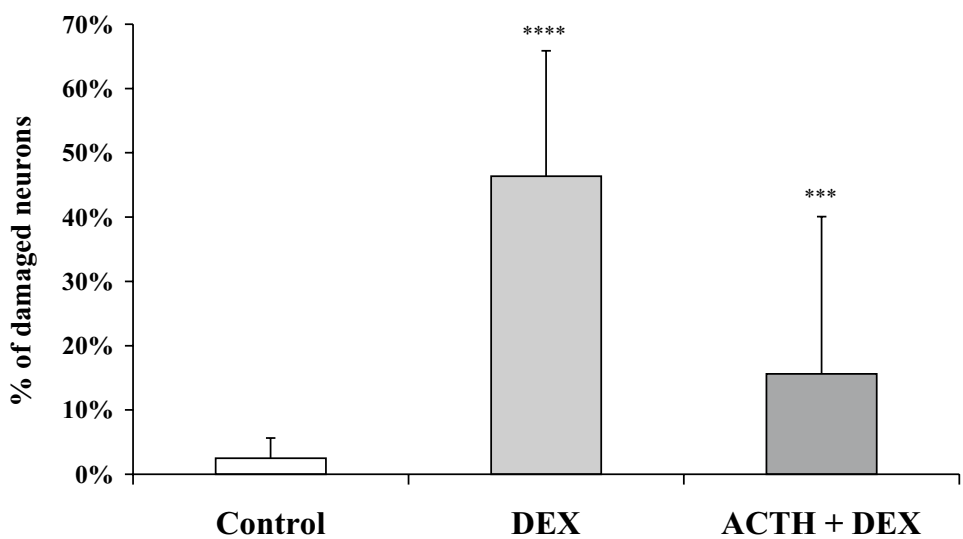

Fig. 3. Percentage of damaged neurons in the CA3 region of hippocampus. Dexamethasone (DEX) was injected at a dose of 8 mg/kg/day for 4 weeks. ACTH (4-9) was injected at a dose of 50 μg/kg twice a week 30 min before DEX. ****, $P<0.0001$ DEX vs. Control; ***, $P<0.01$ ACTH+DEX vs. DEX, $n = 6$ in the control group, $n = 6$ in DEX, $n = 12$ in ACTH+DEX.

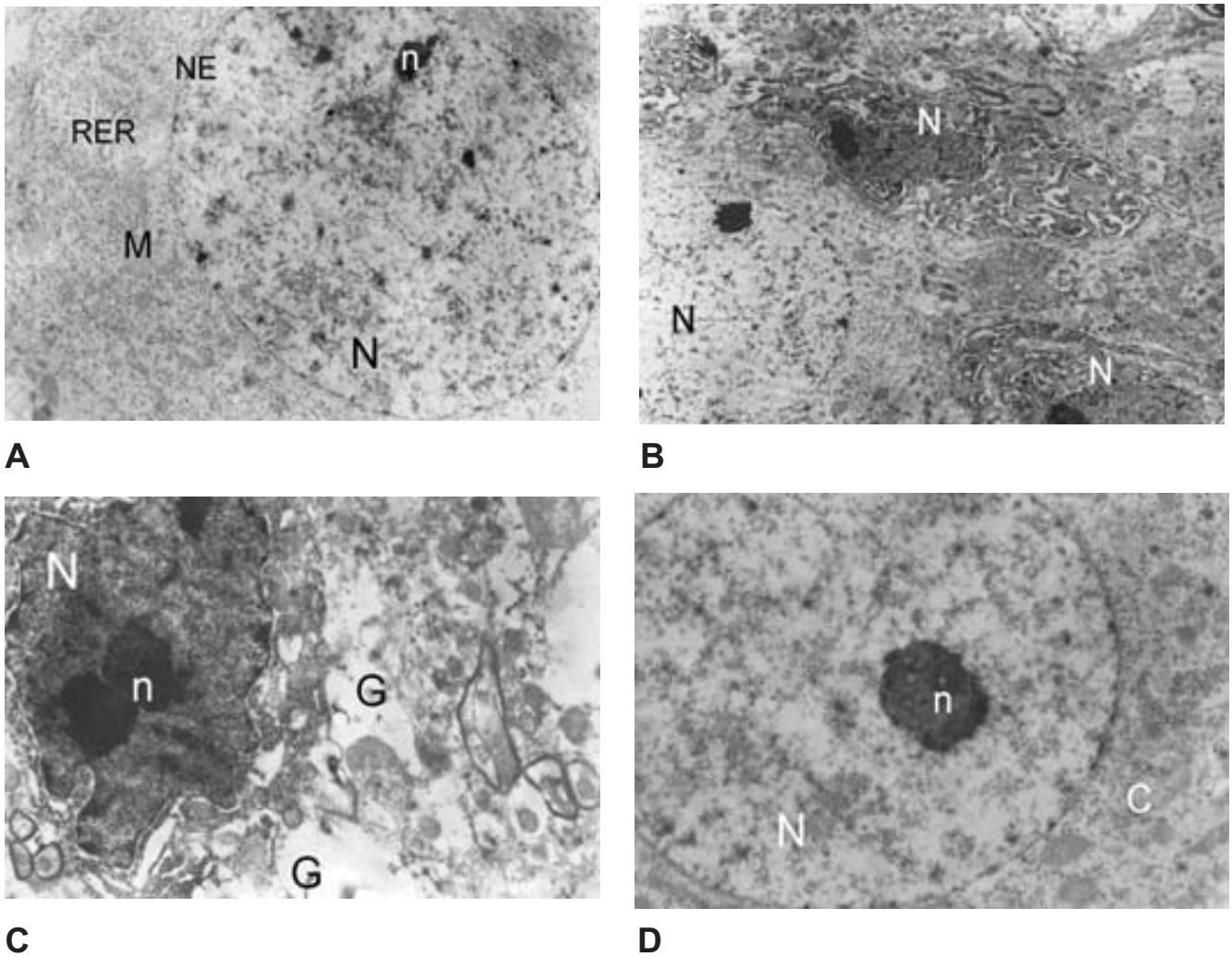

Fig. 4. Electron micrographs. Control (A.) - neuron in the CA3 region of the hippocampus: N, nucleus; n, nucleolus; NE, nuclear envelope; M, mitochondrium; RER, rough endoplasmic reticulum. Magnification x 6,000; DEX (B.) – N, neurons in the CA3 region of the hippocampus. Magnification x 3,000; DEX (C.) - shrunken and dark neuron in the CA3 region: N, nucleus; n, nucleolus; G, swollen processes of glial cells. Magnification x 6,000; ACTH+DEX (D.) - neuron in the CA3 region of the hippocampus: N, nucleus; n, nucleolus; C, cytoplasm. Magnification x 6,000. DEX – dexamethasone was injected at a dose of 8 mg/kg/day for 4 weeks; ACTH (4-9)+DEX – ACTH (4-9) was injected at a dose of 50 μg/kg twice a week.

blood vessels and swelling of glial processes surrounding these vessels.

Experimental group II: ultrastructural examinations revealed that morphological changes in hippocampal neurons following administration of ACTH (4-9) were similar to those observed in experimental group I but the degree of damage in the majority of animals was significantly lower. Some 40% of animals from experimental group II showed no morphological changes in hippocampal neurons (Fig. 4D). In other animals, we observed pathological changes leading to the condensation of neuronal nucleus and cytoplasm, but the amount of damaged nerve cells and intensity of changes were significantly reduced.

DISCUSSION

There is no doubt that glucocorticosteroids are toxic to central nervous system (Elliot et al. 1993, Sapolsky 1996, Seckl and Olson 1995, Stein-Behrens et al. 1994, Uno et al. 1989). This is particularly evident in the hippocampus, a principal neural target tissue for glucocorticosteroids with the highest concentration of glucocorticosteroid receptors (Arbel et al. 1994,

De Kloet et al. 1990, Elliot et al. 1993, Seckl and Olson 1995, Twardowska and Rybakowski 1996, Uno et al. 1989, Van Eckelen et al. 1988, Watanabe et al. 1992). The results of the present study revealed pathological changes in hippocampal neurons caused by exogenous glucocorticosteroid-dexamethasone. The primarily and most severely affected cells in our experiment were pyramidal neurons in the CA3 region. Morphological alterations observed in nerve cells after long-term administration of dexamethasone at the level of light microscopy (shrinkage of perykarions, more intense cytoplasm staining, pyknotic changes of the nucleus, a lack of morphological features characteristic for inflammation) and ultrastructural changes (a shrinkage of the cell body, condensation of the nucleus and cytoplasm, preservation of cell membrane, an enlargement of nucleoli and a large amount of ribosomes) are suggestive of apoptosis (Motyl 1998). In any tissue, cell death may follow two relatively distinct morphological and biochemical patterns: necrosis or apoptosis. Glucocorticosteroids are classical inductors of apoptosis (Motyl 1998). They induce apoptosis in thymocytes and lymphoma cells (Distelhorst and Dubyak 1998). The mechanism by which glucocorticosteroids induce apoptosis in neurons is not completely understood. It is supposed that the mechanism of neurotoxic effects of glucocorticosteroids is connected with the impairment of glucose uptake in neurons and thus induces a state of increased metabolic vulnerability (Phillips 1987, Virgin et al. 1991). This effect is similar to classic glucocorticosteroid inhibition of glucose transport in numerous peripheral tissues. Energy depletion enables the damaging action of glutamate. That is so because the control of glutamate release and, more importantly, glutamate uptake are processes which require a large amount of energy. Glucocorticosteroids increase the concentration of glutamate in the extracellular space (Mc Ewen 1997, Sapolsky 2000, Schasfoort et al. 1988, Venero and Borrell 1999, Virgin et al. 1991). An activation of NMDA receptors by high concentrations of glutamate may be decisive for induction of degenerative changes typical for apoptosis in nerve cells under the influence of glucocorticosteroids (Kamińska and Stańczyk 1998, Mc Ewen 1997, Sapolsky 2000, Schasfoort et al. 1988).

On the basis of results achieved after administration of ACTH (4-9) we can assume that this fragment of ACTH used in our research shows a protective effect against the neurotoxic influence of dexamethasone. Earlier experiments show that ACTH (4-9) prevents acrylamide-, vincristine- and taxol-induced neuropathy (Hamers et al. 1993, Kiburg et al. 1996, Sporel-Ozakat et al. 1990). This fact seems interesting as regards the mechanism of damaging action of both anticancer drugs. Experimental data indicate that vincristine and taxol induce in neurons changes typical for apoptosis (Muzylak and Maślińska 1992, Wakabayashi and Spodnik 2000). Results of our investigations indicate that the protective action of ACTH (4-9) against the neurotoxic effect of dexamethasone is connected with its ability to inhibit apoptotic processes in neurons. The last finding may suggest that the mechanism of neuroprotective action of ACTH (4-9) may be connected with its antagonistic action on NMDA receptors (Horvath et al. 2000).

CONCLUSIONS

Sustained exposure to toxic doses of dexamethasone evokes neuronal death in layer CA3 of the hippocampus. Altered neurons show morphological features suggestive of apoptosis. ACTH (4-9) exerts a protective effect in this model of neurodegeneration. Our results suggest that the mechanism of the neuroprotective action of ACTH (4-9) may be connected with its ability to inhibit apoptotic processes in neurons.

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