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Postnatal treatment with ACTH-(4-9) analog ORG 2766 attenuates *N*-methyl-D-aspartate-induced excitotoxicity in rat nucleus basalis in adulthood

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Abstract

It has been reported that the ACTH-(4-9) analog H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH (ORG 2766) administered in adulthood has trophic effects on neuronal tissue and when given postnatally, it can induce long-lasting changes in brain development. In the present study, we investigated whether early postnatal treatment with ORG 2766 affects adult neuronal vulnerability, i.e. the sensitivity of cholinergic neurons against excitotoxic damage. Wistar rat pups received injections of ORG 2766 or saline on postnatal days 1, 3 and 5 and were then left undisturbed until adulthood. At the age of 6 months, the animals were subjected to unilateral lesion of magnocellular basal nucleus by infusion of high dose of *N*-methyl-D-aspartate (NMDA). The effects of the excitotoxic insult were studied 28 hours and 12 days after the lesion by measuring both the acute cholinergic and glial responses, and the final outcome of the degeneration process. Twenty eight hours after NMDA infusion, postnatally ACTH-(4-9)-treated animals showed stronger suppression of choline-acetyltransferase immunoreactivity and increased reaction of glial fibrillary acidic protein -immunopositive astrocytes in the lesioned nucleus compared to control animals. However, 12 days post-surgery, the NMDA-induced loss of cholinergic neurons, as well as the decrease of their acetylcholinesterase -positive fibre projections in the cortex, were less in ACTH-(4-9) animals. Our data indicate that the early developmental effects of ACTH-(4-9) influence intrinsic neuroprotective mechanisms and reactivity of neuronal and glial cells, thereby resulting in a facilitated rescuing mechanism following excitotoxic injury.

Keywords: Neonatal; ACTH (adrenocorticotropin); Neuroprotection; Magnocellular basal nucleus; Cholinergic neuron; Glia

1. Introduction

Based on the pioneering studies of Greven and De Wied (1973) and De Wied and Jolles (1982), it is well established that the synthetic ACTH-(4-9) analog H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH (ORG 2766) has many of the central nervous system effects of the full-length adrenocorticotrop hormone (ACTH), but it has no peripheral stimulatory effect on the adrenal gland. ACTH-(4-9) has been extensively studied for its effects on cognition, neuronal excitation and neuronal damage. Earlier studies reported that the peptide has a trophic effect on neurons and may enhance neuronal recovery following injuries of the nervous system. It accelerates neuronal outgrowth of peripheral nerves (Van der Hoop et al., 1990) and stimulates compensatory neuronal networks in the central nervous system after experimental brain injury (Nyakas et al., 1985; Pitsikas et al., 1991). Additionally, chronic treatment with ORG 2766 significantly attenuates the age-related impaired plasticity of the hippocampus (Rigter et al., 1984; Spruijt, 1992a,b).

To date, the majority of studies with ACTH-(4-9) were performed on animals and humans in adulthood. Consider-

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ing its possible trophic effects on neuronal tissue, it is of interest to investigate the long-lasting developmental influences of ORG 2766 administered in early postnatal life when brain structures and neuronal pathways are still developing. In this regard, a number of recent studies suggest that ACTH-(4-9) may indeed have an impact on brain development. Long-term upregulation of hippocampal mineralocorticoid receptors (MR) was reported after postnatal treatment with ORG 2766 by Nyakas et al. (1997), concomitant with an improvement of hippocampus-associated spatial learning in adulthood (Horvath et al., 1999). These studies, in fact, strongly suggest that neonatal application of the ACTH-(4-9) analog may result in life-long alterations in brain structures, such as hippocampus, neocortex and basal forebrain cholinergic cell groups, which are implicated in the regulation of cognitive performance.

It is well known that one of the pathological features that is associated with severely impaired cognition is cholinergic hypofunction in cortex and hippocampus (Bartus et al., 1982; Gaykema et al., 1992). Loss of cholinergic forebrain function is primarily due to degeneration of neurons in the magnocellular basal nucleus (MBN), one of the nuclei of the forebrain cholinergic system, which provides extensive cholinergic projections to the entire cortical mantle (Luiten et al., 1985, 1987). Moreover, it was shown that cholinergic neurons of the MBN are particularly susceptible to excitotoxic insults and undergo neurodegeneration both in rat (Luiten et al., 1995; Stuiver et al., 1996; Ábrahám et al., 1997; Harkany et al., 2000) and in cognitively impaired humans, e.g. as is the case in Alzheimer's disease (Bartus et al., 1982).

Taken together, the developmental influence of ACTH-(4-9) on adult cognition and its reported neurotrophic effects in adulthood led us to investigate the effect of postnatal ORG 2766 treatment on adult brain vulnerability by subjecting animals to excitotoxic damage of the cholinergic MBN. Neurotoxic injury was achieved by injecting a high concentration of the specific glutamate receptor agonist *N*-methyl-D-aspartate (NMDA). Activation of NMDA subtype of glutamate receptors has been implicated as an important factor in several excitotoxic injuries, such as cerebral hypoxia, ischemia, epilepsy and neurodegenerative disorders, like Alzheimer's disease (Choi, 1995; Maragos et al., 1987; Meldrum and Garthwaite, 1990; Coyle and Puttfarcken, 1993).

The present study was designed to measure the effects of postnatal ORG 2766 treatment, first, on acute reactivity 28 h after lesion, and second, on the final outcome of neuronal degeneration 12 days after neurotoxic challenge. Reduction of cholinergic cells in the injection area and loss of cholinergic fibre projections in the somatosensory cortex were determined by means of quantitative immunohistochemistry and histochemistry of the two cholinergic marker enzymes, choline-acetyltransferase (ChAT, EC 3.2.1.6) and acetylcholinesterase (AChE, EC 3.1.1.7), respectively. Post-lesion accumulation of activated astrocytes was evaluated with glial fibrillary acidic protein (GFAP) immunohistochemistry.

2. Materials and methods

2.1. Animals and treatment

The study was carried out on Wistar rats, bred in our own facilities. At birth, the pups of different nests were mixed and re-distributed among the mothers to exclude the influence of genetic variation among the nests. The number of pups was reduced to eight (six males and two females) and the pups within each nest were divided in two treatment groups: ACTH-(4-9)-treated animals and saline-treated, control animals. Half of the male pups received three subcutaneous injections of 1 μg/g body weight of the ACTH-(4-9) analog, ORG 2766 (Organon Oss, The Netherlands) on postnatal days 1, 3 and 5. The other pups served as saline-controls and received three injections of saline in a volume of 10 μl/g of bodyweight. Animals were weaned on day 23 and the male rats of each nest remained group-housed. Each nest thus contained three male ORG 2766-treated and three male saline-treated animals. The rats were kept under standard laboratory temperature conditions in an air-conditioned room at 20 ± 1°C and 12 h light/dark cycle (lights on at 0900 h). At an adult age of 6 months, the animals were individually housed, subjected to NMDA infusion, and perfused either 28 h or 12 days post-lesion; each group containing six animals. All animal experiments were conducted in accordance with the regulations of the Committee for Use of Experimental Animals of the University of Groningen (DEC No. 2111).

2.2. Intracerebral NMDA injection

At the age of 6 months, prior to NMDA infusion, animals were deeply anaesthetised by halothane (1.5% v/v; 1,5 l/min flow-rate) and their heads were mounted in a stereotaxic frame (Narishige). Sixty nmol of NMDA (Sigma, St. Louis, USA), dissolved in a total volume of 1 μl phosphate buffer saline (PBS; pH 7.4), was slowly injected (0.1 μl/min) into the right MBN at standard co-ordinates (AP: −1.5 mm, L: 3.2 mm; Paxinos and Watson, 1986), at two dorsoventral positions (7.0 and 6.2 mm from the dura).

2.3. Tissue processing

Twenty-eight hours or 12 days after NMDA infusion, the animals were transcardially perfused with 300 ml ice-cold fixative, composed of 4% paraformaldehyde in

0.1 M phosphate buffer (PB; pH 7.4), which was preceded by a short prerinse of heparinized saline (flow-rate: 21 ml/min). The brains were removed and stored for an additional 3 h in 4% paraformaldehyde, then cryoprotected by 48 h storage in 30% sucrose in 0.1 M PBS at 4°C. Sections were cut on a cryostat microtome at 20 μm thickness and series of sections, spanning the damaged basal nucleus and its cortical target region, were collected in PBS.

2.3.1. ChAT immunohistochemistry

Free-floating sections processed for ChAT immunostaining were rinsed several times in 0.01 M PBS (pH 7.4) and pre-incubated in 0.3% H_2O_2 for 30 min. Thereafter, sections were immersed in 0.01 M PBS containing 0.02% TritonX-100 (TX-PBS) and 5% normal rabbit serum (Zymed, San Francisco, CA, USA), and incubated with the primary antibody, goat anti-ChAT immunoglobulin G (IgG), 1:1000 for 60 h at 37°C, then for 5 h at room temperature kindly donated by Dr. L.B. Hersh (Bruce et al., 1985). Subsequently, sections were thoroughly rinsed in PBS, incubated in normal rabbit serum for 1 h and exposed to the secondary antibody, rabbit anti-goat IgG (1:50, Sigma, 4 h at room temperature). After overnight rinsing in PBS, slices were incubated in goat peroxidase anti-peroxidase complex (1:300; Dakopatts, Glosstrup, Denmark). The staining was visualised by 3,3′diaminobenzidine as chromogen (30 mg in 100 ml Tris–HCl buffer, pH 7.6) with 0.01% H_2O_2. Omission of the primary antibody did not yield any appreciable labelling.

2.3.2. GFAP immunohistochemistry

GFAP immunohistochemistry was performed as described above (ChAT) with slight modification. Briefly, free-floating sections were pre-treated with 0.1% H_2O_2 for 15 min. Subsequently, the sections were pre-incubated in 5% normal sheep serum for 30 min and incubated in mouse anti-GFAP IgG, (1:200, Amersham Bucks, U.K.) overnight at 37°C in 0.01 M PBS to which 0.5% Triton X-100 had been added. Thereafter, sections were rinsed and exposed to biotinylated sheep anti-mouse IgG (1:200, Amersham, 2 h). Following thorough rinsing, the sections were incubated in streptavidin–horseradish-peroxidase (1:200, Zymed, 2 h). Staining was visualized conventionally immunostaining was carried out with 3,3′diaminobenzidine as a chromogen (30 mg/100 ml Tris–HCl buffer, pH 7.6).

2.3.3. AChE histochemistry

Free-floating brain sections were postfixed by immersion in a 2.5% glutardialdehyde solution in 0.1 M PB overnight at 4°C. To visualise cholinergic, AChE histochemistry was carried out according to a previously described protocol (Harkany et al., 1998).

2.4. Quantification

Neurotoxic effects of NMDA infusion were demonstrated by unbiased quantitative determination of the numbers of ChAT-positive cells in the MBN in both ipsi- and contralateral sides of the brain by means of an optical disector initially described by West (1993), with slight modifications. Cell counting was carried out using a transparent disector probe (Jansen et al., 1998). Cells were counted at 400× magnification. The fields of view were systematically sampled using a step size of 0.05 mm along the *x*-axis and 0.05 mm along the *y*-axis with a disector counting frame of 0.0025 mm^2. This way, 25% of the total fields of view was covered by the counting frame. In each animal, three sections (Bregma −1.2, −1.5, −1.8) were determined with a standard cross-sectional distance of 300 μm, spanning the central subdivision of the MBN. Only those cholinergic neurons were counted, which were lying within the rectangular counting frame or touching the two non-forbidden sides of the frame. Subsequently, the counted area of the nucleus was delineated and measured by computer-assisted image analysis (Leica, Quantimet Q-600HR, Rijswijk, The Netherlands). Non-significant difference in the measured surface areas between groups was prerequisite of further data processing. Density of ChAT-immunoreactive (i.r.) cells in the central region of basal nucleus, taking into account the thickness of the section (20 μm) and the Floderus correction factor (0.7368) was calculated by applying the following formula:

$$\text{density of ChAT-i.r. cells}\left(\text{number of ChAT-i.r. cells}/\text{mm}^3\right) = 50 \times \left(\text{number of counted cells} \times 0.7368\right) / 0.25 \times \text{area}$$

Subsequently, values of the three analysed sections were averaged and changes in the density of ChAT-i.r. cells on the injected side were calculated as percentage values of the contralateral, non-injected side.

Quantification of AChE-positive fibre density was performed in layer V of the posterior somatosensory cortex, according to a standard protocol by using a Quantimet Q-600HR computerised image analysis system (Leica) (Harkany et al., 1998, 1999). Surface area density of cortical AChE-positive fibres was measured in four sections (Bregma −0.5, −0.92, −1.4, −2.3) in each animal. Due to the strict unilateral cortical projections of cholinergic cells in the basal nucleus (Luiten et al., 1995), contralateral fibre density values served as controls within each animal and the fibre reduction was calculated as the percentage difference of fibre densities at the injected side and contralateral sides of the brain. Decrease in fibre density was calculated according to the following formula:

$$\text{percentage decrease in fibre density} = 100 - \left(\text{injected side}/\text{control side} \times 100\right)$$

GFAP-i.r. astrocytes were quantified both in forebrain nuclei and in layer V of the somatosensory cortex.

At the level of forebrain nuclei, GFAP-positive astrocytes accumulated abundantly in a rim-like pattern surrounding the core of the neurotoxic lesion, exceeding the target nucleus. Therefore, we aimed at quantifying the numbers of GFAP-i.r. astrocytes not only in the MBN, but also in the predominantly cholinergic substantia innominata (SI), associated ventrally to the basal nucleus. Quantitative determination of individual (GFAP) immunoreactive astroglial cell numbers was not possible and could only be measured by means of optical densitometry. Local changes in GFAP immunoreactivity were therefore quantified by an unbiased, random sampling-based method by applying a computer-assisted (Leica, Quantimet Q-600HR) image analysis protocol in detail detailed by Harkany et al. (2000). Briefly, three coronal sections were measured (starting at −1.4 mm from Bregma; Paxinos and Watson, 1986) with a standard distance of 50 μm. Following manual delineation of the basal nucleus and substantia innominata (without significant differences in the measured surface area between the groups), optical density (OD) values of superposed 80 primary quantification elements were independently measured. To determine the mean ± S.E.M. OD values in each brain, absolute OD values of the individual frames were averaged for both areas and, subsequently, the average of such values was calculated in the three sections analysed.

Density of GFAP-positive astrocytes in layer V of the somatosensory cortex of both hemispheres was measured in a similar way and at the same position as quantifying AChE-positive fibres.

2.5. Statistics

Data were expressed as means ± S.E.M. One way analysis of variance (ANOVA) was used to determine the effects of postnatal ACTH-(4-9) on NMDA-induced changes in the density of ChAT-i.r. cellbodies, the cholinergic (AChE-positive) fibre densities, and the OD of GFAP-immunostained astrocytes in the MBN at 28 h and 12 days post-surgery. Tukey's post-hoc test was employed to determine differences within treatment groups at 28 h and 12 days post-surgery. A P value of < 0.05 was taken as indicative of statistical significance for the tests.

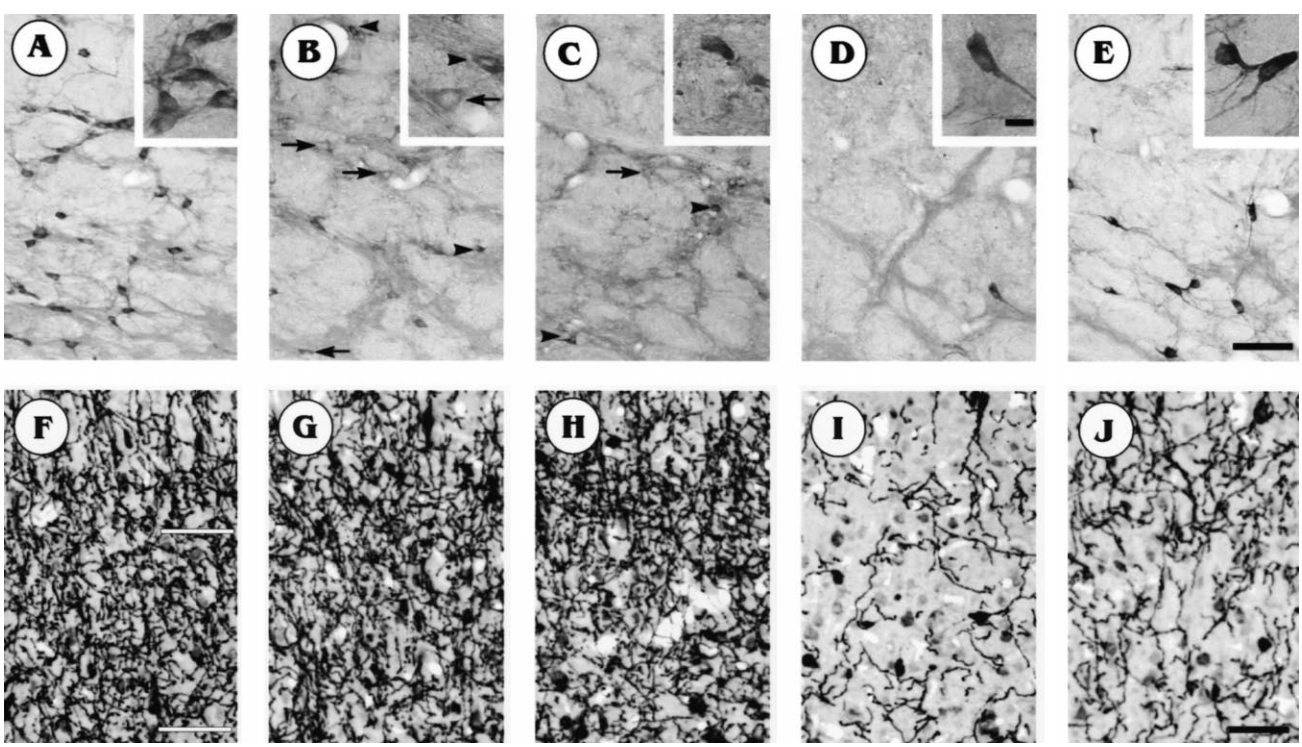

Fig. 1. Photomicrographs of ChAT-positive cells in the magnocellular basal nucleus (A–E) and of AChE-stained fibres in layer V of the parietal cortex (F–J). Compared to the non-lesioned side (A), NMDA infusion in the cholinergic nucleus basalis induced strong decrease in ChAT-i.r. cells 28 h post-lesion in both control (B) and ACTH-(4-9) animals (C). At that time, the cortical AChE positive fibre density had shown a slight decrease in both control (G) and ACTH-(4-9) animals (H), compared to the contralateral side (F); degenerated axons are indicated by black dots among intact fibres. Twelve days after NMDA lesion, control animals displayed very few ChAT-i.r. cells (D), whereas in ACTH-(4-9) animals, a significant amount of somata were preserved (E). Concordant with this effect, AChE fibre loss was more pronounced in control animals (I) than in ACTH-(4-9) rats (J). The white bars in F indicate the border of layer V of the parietal cortex, where quantitative measurements were performed. Arrowheads (in B, C) point to densely-stained and arrows to lightly-stained ChAT-i.r. perikarya, indicating changes in ChAT enzyme content of cellbodies shortly after lesion. Scale bar in (E, J) = 250 μm, whereas in (D, inset) = 25 μm.

3. Results

Data of only properly lesioned animals were processed for further analysis. One animal was removed from the 12 days survival control group because the position of the needle during infusion was not correct.

3.1. Effect of postnatal ACTH-(4-9) treatment on NMDA-induced cholinotoxicity

ChAT-i.r. neurons of the MBN appeared as large, intensely stained multipolar neurons (Fig. 1A, inset). Analysis of ChAT-i.r. cell counts revealed similar data as reported previously by Smith and Booze (1995).

A significant decrease in the number of ChAT-positive neurons in the injected hemisphere was detected 28 hours post-surgery (Fig. 1B,C). Density of ChAT-immunostained cells, expressed in cubic millimeter, on the contralateral side in control and ACTH-(4-9) animals were 1539.29 ± 186.11 and 1840.90 ± 180.46, respectively, versus 711.15 ± 161.21 and 311.63 ± 91.67 detected on the ipsilateral side. At that time the density of ChAT-i.r. cellbodies in the injured basal nucleus was lower in ACTH-(4-9) animals compared to controls ($F(1,11) = 5.57$, $P = 0.040$). Expressing the reduction of ChAT-positive neurons of the injected side as a percentage of the values at the contralateral side revealed pronounced effects of ACTH-(4-9) treatment (52.05 ± 9.52% in controls vs. 82.68 ± 5.13% in ACTH-(4-9) animals, $F(1,11) = 9.62$, $P = 0.011$; Fig. 2A). In contrast with the result 28 hours post-surgery, the amount of remaining ChAT-i.r. somata in the lesioned nucleus was significantly higher in ACTH-(4-9) animals 12 days after lesion (Fig. 1D,E). Density of ChAT-immunostained cells per cubic millimeter on the contralateral side in control and ACTH-(4-9) animals were 1497.76 ± 73.40 and 1894.61 ± 168.59, respectively, versus 157.99 ± 56.49 and 589.82 ± 149.99 detected on the ipsilateral side ($F(1,10) = 7.31$; $P = 0.024$). When cholinergic cell loss was calculated, as the percentage ratio between ipsilateral and contralateral hemispheres, a remarkable beneficial effect of postnatal ACTH-(4-9) treatment became apparent (89.24 ± 3.97% in control versus 66.22 ± 9.13% in ACTH-(4-9) animals, ($F(1,10) = 5.42$, $P = 0.045$; Fig. 2A).

Measurement of AChE-positive fibre density in the somatosensory cortex revealed 17.62 ± 3.79% and 20.80 ± 4.48% reduction in control and ACTH-(4-9) animals, respectively, 28 h after NMDA infusion (Fig. 2B). At that time point, there was no significant difference between the groups. Twelve days after NMDA infusion in both treated groups, the AChE-positive fibre loss had become much stronger (Fig. 1I,J) as compared to the groups 28 h post-lesion 59.36 ± 8.31% in control ($P = 0.000$) and 39.71 ± 3.81% in ACTH-(4-9) animals ($P = 0.056$). However, the fibre loss 12 days after lesion was much smaller in the ACTH-(4-9) animals compared to the control group ($F(1,10) = 5.43$; $P = 0.045$; Fig. 2B). The animals, which were postnatally treated with ORG 2766 exhibited a significant preservation of the cortical AChE-positive cholinergic innervation after excitotoxic lesion of the MBN, as compared to controls.

3.2. Effect of postnatal ACTH-(4-9) treatment on NMDA-induced astrocyte activation

The OD of GFAP-i.r. glial cells was quantified in both the MBN and the substantia innominata. Twenty eight hours post-surgery, ACTH-(4-9) animals generally showed

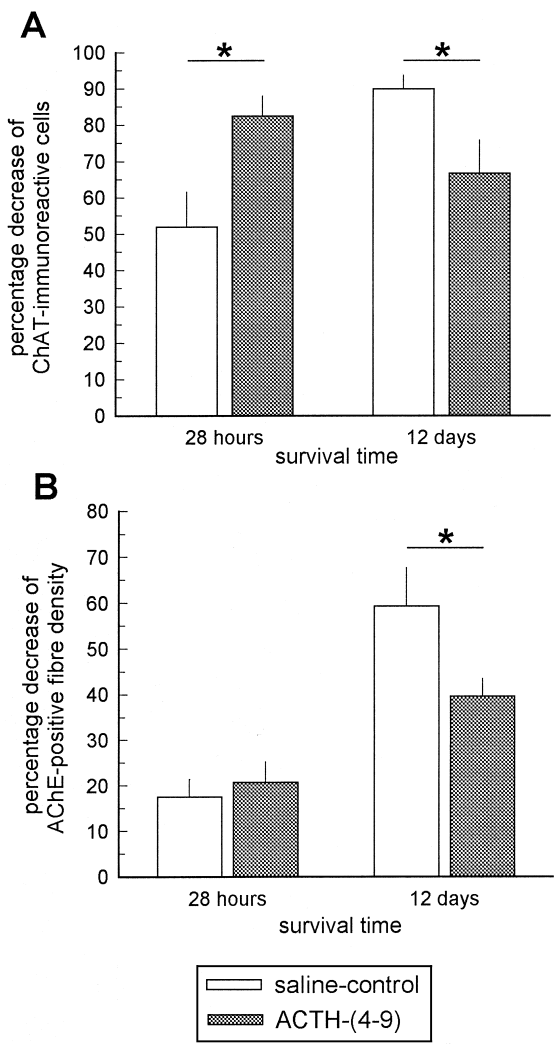

Fig. 2. Effect of postnatal ACTH-(4-9) treatment on decrease in ChAT-i.r. cellbodies in the magnocellular basal nucleus (A) and on cholinergic fibre loss in the somatosensory cortex (B) following infusion of NMDA into the MBN during adulthood. Measurements were taken at 28 h ($n = 6$ in each group) and 12 days post-lesion ($n = 5$ controls and $n = 6$ ACTH-(4-9 animals). Data are expressed as percentages of the value of the non-lesioned side. Data represent means ± S.E.M. $^{*}P < 0.05$.

stronger glial response to the infusion of NMDA compared to controls (Fig. 3A,B). The mean OD of reactive astrocytes in the basal nucleus was significantly stronger in the ACTH-(4-9) animals ($F(1,11) = 5.83$, $P = 0.038$; Fig. 4A). Twelve days after NMDA infusion, the OD of GFAP-positive astrocytes in the basal nucleus further increased significantly only in the control group ($P = 0.014$; Fig. 3C). While in the substantia innominata, both control and ACTH-(4-9) (Fig. 3C,D) animals showed a stronger astrocyte reaction compared to the results of 28 h post-surgery groups (control: $P = 0.000$; ACTH-(4-9) animals: $P = 0.024$). Comparison of the two treatment groups 12 days following NMDA infusion revealed a strong tendency toward a more robust GFAP immunoreactivity in the substantia innominata in control vs. ACTH-(4-9) animals ($F(1,10) = 4.81$, $P = 0.056$; Fig. 4B). The mean OD of GFAP-i.r. astrocytes in the non-lesioned hemispheres did not exhibit significant differences between control and ACTH-(4-9) animals both 28 h and 12 days post-lesion (Fig. 4A,B).

A diffuse, scattered GFAP immunolabelling was visualised in layer V of the somatosensory cortex. Irrespective of postnatal treatment, NMDA infusion resulted in an least twofold increase in GFAP immunoreactivity in the lesioned hemisphere, as compared to control side. Although the OD of GFAP-labelled structures was similar to that found in the basal forebrain, the area coverage did not exceed 0.4–3.0% of the cortical surface subjected to quantitative analysis. Twenty eight hours post-surgery, similar elevations of GFAP immunoreactivity in the lesioned side were visualised in both the ACTH-(4-9)- and saline-injected experimental groups (surface area density $0.54 \pm 0.09\%$ in control vs. $0.74 \pm 0.17\%$ in ACTH-(4-9) animals). At 12 days post-surgery, this initial GFAP activity exhibited significant extensions. However, no significant differences were detected in surface area densities between

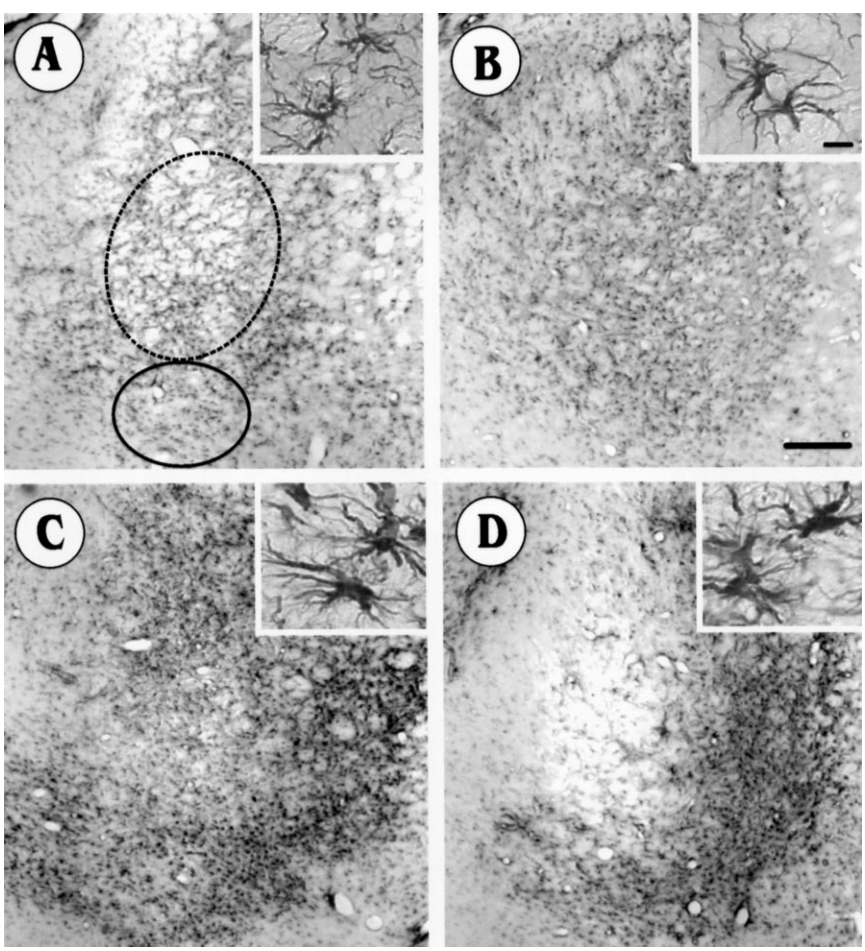

Fig. 3. Photomicrographs of GFAP-positive astrocytes activated by NMDA infusion into the cholinergic basal nucleus during adulthood in a postnatally saline- and ACTH-(4-9)-treated animal, 28 h (A and B, respectively) and 12 days after lesion (C and D, respectively). Dotted circle indicates magno-cellular basal nucleus, while lined circle (in A) shows the measured region of substantia innominata. Note the enlarged, hypertrophic astrocytes (C, D inset) 12 days post-surgery compared to astrocytes detected 28 h after lesion (A, B inset). Scale bar in B (large photograph) = 1000 μm, whereas in B (inset) = 25 μm.

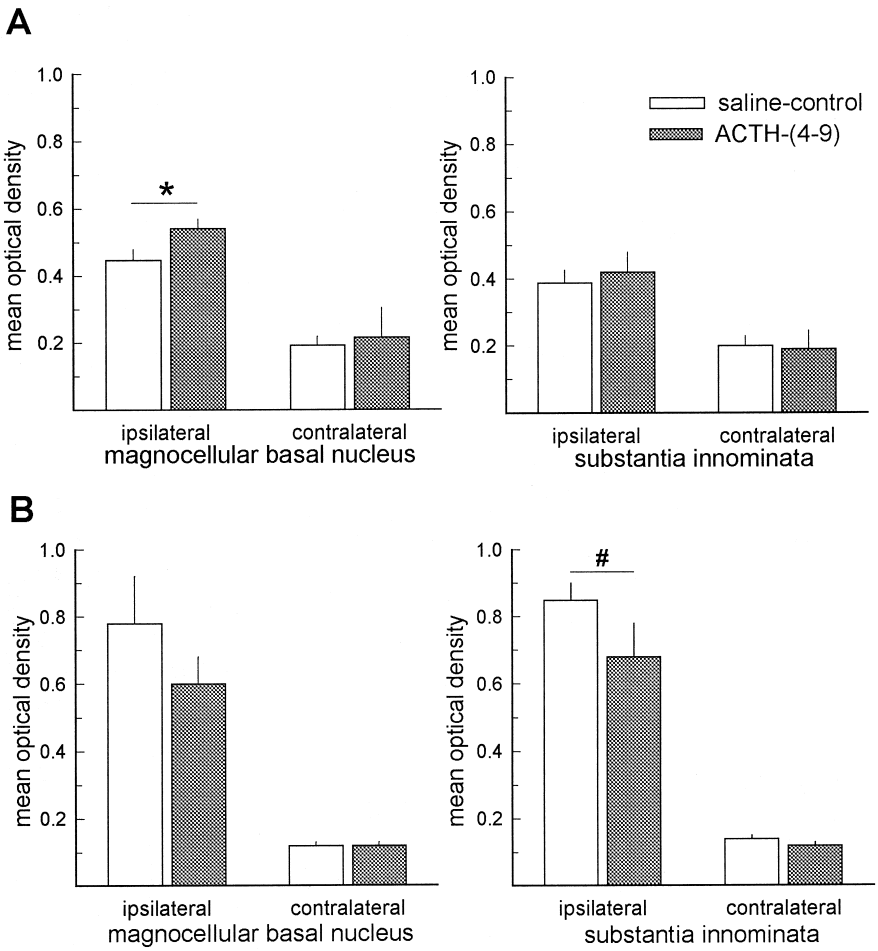

Fig. 4. Effect of postnatal ACTH-(4-9) treatment on mean OD of GFAP-i.r. astrocyte in the MBN and substantia innominata after infusion of NMDA during adulthood. Measurements were taken at (A) 28 h ($n = 6$ in each group) and (B) 12 days post-lesion ($n = 5$ controls and 6 ACTH-(4-9) animals). Data are expressed as the average optical density of the designated areas (Fig. 3A). Data represent means ± S.E.M. $^{*}P < 0.05$.

the groups (2.38 ± 0.50% in controls vs. 1.62 ± 0.84% in ACTH-(4-9) animals).

4. Discussion

The present study shows that neonatal application of the ACTH-(4-9) analog, ORG 2766 induces developmental changes that reduce excitotoxic damage of cholinergic MBN neurons in adulthood. After NMDA infusion, the loss of ChAT-i.r. neurons in the basal nucleus and that of their fibre projections invading the somatosensory cortex was smaller in animals that were postnatally treated with ORG 2766. In addition to its effect on cholinergic neurons, ORG 2766 treatment affected the local accumulation of activated GFAP-i.r. astrocytes in the vicinity of the excitotoxic lesion.

Exposure to NMDA results in the activation of Ca^{2+} permeable NMDA type glutamate receptors. A strong Ca^{2+} influx into the neurons and a sustained high level of $[Ca^{2+}]_i$ are considered as important causes of eventual neuronal damage. Elevations of $[Ca^{2+}]_i$ can result in oxidative stress via generation of free radicals, notably by the mitochondria (Beal et al., 1997; Coyle and Puttfarcken, 1993). Furthermore, it will lead to increased energy demands and — in case of depletion of intracellular energy stores — to bioenergetic failure. Following such a sequence of events, the extent to which ATP decreases may define the subsequent mode of cell death, with a moderate decline leading to apoptotic cell death and a severe depletion resulting in necrosis. Cell death is primarily necrotic in the core of the lesion (Choi et al., 1987; Gwag et al., 1997), whereas with decreasing concentration of diffusing NMDA and glutamate released by neurons, in the so-called penumbra zone, transition to an apoptotic mode of cell death and survival has been reported (Ankarcrona et al., 1995; Tenneti et al., 1998).

Interestingly, 28 h after lesion, the decrease in ChAT-i.r. cell number was much stronger in ACTH-(4-9)-treated than in control animals. However, this change was reversed at 12 days after NMDA infusion when ACTH-(4-9) animals displayed more ChAT-immunopositive cells on

the injured side. The reduction in ChAT-positive cell number and AChE-positive fibre density after NMDA injection could be explained in two ways: first, the neuronal perikarya and axonal projections may be intact but their enzyme content, as a consequence of their decreased production, is below the immunohistochemical detection threshold. The second option is that the neuronal perikarya become subject to NMDA-induced cell death. The reduction of ChAT-i.r. cells seen after 28 h may be the result both of cell loss and of decreased enzyme content of surviving cells. However, the reduced cell number found after 12 days is most likely caused by actual cell death. Accordingly, the data suggest that 28 h after lesion the cholinergic neurons of the basal nucleus in ACTH-(4-9) animals react more actively and effectively by decreasing their enzyme production in an attempt to preserve intracellular energy stores and via this mechanism, rescue the cell from bioenergetic failure. Consequently, more ChAT-positive cells survive and are detectable 12 days after NMDA lesion, with a proportional reduction of cholinergic fibre loss in the cortex. It appears that buffering and counteracting mechanisms that protect cells against damage or help them to recover from it are more effective in postnatally ORG-2766 treated animals.

Our GFAP measurements may support this conclusion. Twenty eight hours after the NMDA lesion, a much stronger GFAP immunoreactivity was found in the vicinity of the lesion in the postnatally ACTH-(4-9) analog-treated animals. GFAP is a common marker for identifying astrocytes in the intact and injured CNS. It is well known that glial cells play an important supporting role in neuronal function. "Damage signals", such as neurotrophins (e.g. ciliary neurotrophic factor), growth factors (e.g. transforming growth factor-β), cytokines (e.g. interleukin-6, tumor necrosis factor-α), emanating from injured neurons, astrocytes and macrophages are reported activators of astrocytes (Ridet et al., 1997). The complex role of reactive astrocytes in post-lesion recovery is not well understood, but data suggest that early astrocyte reactivity has a positive impact on neuronal protection. The mechanisms of this can be the following: (i) protection of neurons from delayed cell death by supporting them with lactate (Schuur et al., 1999; Magistretti et al., 1999), increased production of neurotrophins, such as nerve growth factor (Schwartz and Nishiyama, 1994), other growth factors (e.g. insulin-like growth factor-I; Garcia-Estrada et al., 1992), and oxidoreductive enzymes; (ii) decreasing the magnitude of secondary excitotoxic injury following initial trauma by increased uptake of glutamate (Bergles and Jahr, 1997); (iii) mechanical isolation of the still intact nervous tissue from further lesion. Taken together, a faster and more efficient astrocyte activation in ORG 2766-treated animals could support the neurons in their combat for survival.

Twelve days after the NMDA injection, GFAP immunostaining revealed a reversed glial activity. There was some evidence that 12 days post-surgery, ORG 2766-treated animals developed less abundant glial scar tissue. It is suggested that such a glial scar can be a major impediment to axonal regrowth (Ridet et al., 1997).

Clearly, the exact mechanism along which postnatal ACTH-(4-9) analog affects sensitivity to neurodegenerative processes in later life remains to be defined. This mechanism of action may be highly complex and indirect since the ORG 2766 treatment occurred shortly after birth, while the NMDA lesion was performed during adulthood. The lower neuronal damage in the postnatally ACTH-(4-9)-treated animals may be caused by a reduced sensitivity to NMDA, either through modification of NMDA receptor function or through changes at the level of intracellular cascade mechanisms. We do not have evidence for this, but it is not excluded that neonatal treatment with ORG 2766 directly affects the development of the glutamatergic system and/or NMDA receptors. Studies in adult animals suggest that the analog might have its effect directly through NMDA receptor (Spruijt, 1992a,b). Another route of action may be via the glucocorticoid system. It is well known that glucocorticoids can modulate neurodegenerative processes (Sapolsky and Pulsinelli, 1985; Landfield, 1994; Ábrahám et al., 2000) acting through two receptor types, the mineralocorticoid (MR) and the glucocorticoid receptor (GR) (Reul and De Kloet, 1985). The occupational ratio of these two receptors can strongly influence the excitability of neurons (De Kloet et al., 1998; Joels and De Kloet, 1994). ORG 2766 can selectively increase MR expression not only in adulthood (Rigter et al., 1984; Spruijt, 1992a,b), but also after postnatal administration of the peptide (Nyakas et al., 1997). An increased MR number in the present study could be directly or indirectly responsible for the attenuated neurodegeneration. Recent studies from our laboratory showed that slightly elevated levels of corticosterone, which mainly occupies MR, significantly decrease NMDA neurotoxicity in the MBN (Ábrahám et al., 2000).

In conclusion, the present study shows that postnatal treatment with the ACTH-(4-9) analog ORG 2766 decreases the extent of cholinergic neuronal degeneration after NMDA lesion of the MBN in adulthood. The exact mechanism of the postnatal treatment remains to be clarified, but our data suggest that postnatally administered ORG 2766 potentiates the development of intrinsic neuroprotective mechanisms and the plastic responsivity of neuronal and glial cells, resulting in a facilitated rescuing mechanism following excitotoxic injury.

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