Adult vulnerability to neurodegeneration
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CHAPTER 5

POSTNATAL TREATMENT WITH ACTH-(4-9) ANALOG ORG 2766 ATTENUATES NMDA-INDUCED EXCITOTOXICITY IN RAT NUCLEUS BASALIS IN ADULTHOOD

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Abstract

It has been well reported that ACTH$_{4-9}$ analog ORG 2766 administered in adulthood has a trophic effect on neuronal tissue and by 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 the vulnerability of cholinergic neurons against excitotoxic damage in adulthood. Wistar rat pups received injections of ORG 2766 or saline on postnatal day 1, 3 and 5 and were then left undisturbed until adulthood. At an age of 6 month, the animals were subjected to unilateral lesion of magnocellular basal nucleus neurons by infusion of a high dose of N-methyl-D-aspartate (NMDA). The effect of excitotoxic insult were studied 28 hours and 12 days after lesion to measure both the acute cholinergic and glial responses, and the final outcome of the degeneration process. 28 hours after NMDA infusion, postnatally ACTH$_{4-9}$ treated animals showed stronger suppression of choline-acetyltransferase (ChAT) immunoreactivity in the lesioned nucleus and an increased glial fibrillary acidic protein (GFAP)-ir astrocyte reaction compared to control animals. However, 12 days post-surgery the NMDA-induced loss of ChAT-ir cells as well as the decrease of their acetylcholinesterase (AChE) positive fiber projections in the cortex were less in ACTH$_{4-9}$ animals. Our data indicate that early developmental effects of ACTH$_{4-9}$ influences intrinsic neuroprotective mechanisms and reactivity of neuronal and glial cells, resulting in a facilitated rescuing mechanism following excitotoxic injury.

Key Words: neonatal, ACTH, magnocellular basal nucleus, neurodegeneration, neuroprotection, cholinergic neuron, GFAP
Introduction

Based on the pioneering studies of de Wied and his associates it is well established that the synthetic ACTH$_{4-9}$ analog ORG 2766 has many of the central nervous system (CNS) effects of the full-length adrenocorticotropic hormone (ACTH) molecule but without the stimulatory effect on the peripheral adrenal gland (Greven and De Wied, 1973; De Wied and Jolles, 1982). 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 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 CNS 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; Spruit 1992).

To date, the majority of studies with ACTH$_{4-9}$ was performed on animals and humans in adulthood. Considering 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, few 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, Abraham et al., 1997, Harkany et al., 2000b) 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 neuronal vulnerability by subjecting animals to excitotoxic damage of the cholinergic neurons of the basal forebrain nuclei. Neurotoxic injury of the MBN was achieved by injection of 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 disease (Choi, 1995; Maragos et al., 1987; Meldrum and Garthwaite, 1990; Coyle and Puttfarcken, 1993).

The present study was designed to establish the effects of postnatal ORG 2766 treatment first, on acute reactivity 28 hours after lesion, second on the final outcome of neuronal degeneration 12 days after neurotoxic challenge by NMDA. Reduction of cholinergic cells in the injection area and loss of cholinergic fiber projections in the somatosensory cortex were determined by means of quantitative 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.

Materials and methods

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 8 (6 males and 2 females) and the pups within each nest were divided in two treatment groups: ACTH₄₋₉ treated animals (=A) and saline-treated, control animals (=C). Half of the male pups received 3 subcutaneous injections of 1µg/g body weight of the ACTH₄₋₉ analog, ORG 2766 (Organon, Oss, The Netherlands) on postnatal day 1, 3 and 5. The other half of the pups served as saline-controls and received 3 injections of saline in a final volume 10µl/g of bodyweight. Animals were weaned at day 23 and the male rats of each nest remained group-housed. Each nest thus contained 3 male ORG 2766-treated and 3 male saline-treated animals. The rats were kept under standard laboratory temperature conditions in an air-conditioned room at 20 ± 1°C and 12h light/dark cycle (lights on at 09.00 h). At an adult age of 6 month, animals were individually housed, subjected to NMDA infusion, and perfused either 28h (6C and 6A) or 12d post-lesion (6C and 6A). All animal experiments were conducted in accordance with the regulations of the Committee for Use of Experimental Animals of the University of Groningen (DEC Nr. 2111).
Intracerebral NMDA injection

At the age of 6 month unilateral NMDA injections in the right MBN were performed in the following way. The animals were deeply anesthetized by halothane (1.5 v/v%; 2 l flow-rate) and their heads mounted in a stereotaxic frame (Narishige). Sixty nmol NMDA (Sigma, St. Louis, USA) dissolved in phosphate-buffered saline (PBS, pH 7.4) was slowly injected (0.1 µl /min) in a total volume of 1 µl, 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) in the MBN.

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 (PA) 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 3h in 4% PA, then cryoprotected by 48 h storage in 30% sucrose in 0.1 M phosphate buffer saline (PBS) at 4ºC. Sections were cut on a cryostat microtome at 20 µm thickness and series of sections, spanning the damaged MBN and its cortical target region, were collected in PBS.

ChAT immunohistochemistry

Free-floating sections processed for ChAT immunostaining were rinsed several times in 0.01 M PBS (pH 7.4) and pre-incubated for 30 min in 0.3 % H2O2. Thereafter, sections were immersed in 0.01 M PBS containing 0.02 % TritonX-100 (TX-PBS) and 5 % normal rabbit serum (NRS, Zymed, San Francisco, CA, USA), and incubated with the primary antibody, goat anti-ChAT IgG, 1:1000 for 60h at 37ºC then 5h at room temperature (RT) kindly donated by dr. L.B. Hersh (Bruce et al., 1985). Subsequently, sections were thoroughly rinsed in PBS, incubated in NRS for 1h and exposed to the secondary antibody, rabbit anti-goat IgG (1:50, Sigma, 4h at RT). After overnight rinsing in PBS, slices were incubated in goat peroxidase anti-peroxidase complex (1:300, Dakopatts, Glostrup, Denmark). The staining was visualized by 3,3’diaminobenzidine, DAB as chromogen (15 mg in 100ml Tris-HCl buffer, pH 7.6) with 0.01% H2O2. Omission of the primary antibody did not yield any appreciable labeling.

GFAP immunohistochemistry

GFAP immunocytochemistry was performed as described above with slight modification. Briefly, free-floating sections were rinsed several times in 0.01 M PBS (pH 7.4), pre-treated for 15 min with 0.1 % H2O2. Subsequently, the sections were
pre-incubated in 5% normal sheep serum (NSS) for 30 min and incubated in mouse anti-GFAP IgG, (1:200, Amersham) overnight at 37°C in 0.01M PBS to which 0.5% TritonX-100 had been added. Thereafter sections were rinsed and exposed to biotinylated sheep anti-mouse IgG (1:200, Amersham, 2h, RT). Following thorough rinsing, the sections were incubated in HRP-Streptavidin for 2h (1:200, Zymed). Conventional immunostaining was carried out with DAB as a chromogen (30mg/100ml Tris-HCl buffer, pH 7.6).

**AChE histochemistry**

For the detection of AChE–positive structures in the MBN and cerebral cortex free-floating brain sections were postfixed by immersion in a 2.5 % glutaraldehyde solution in 0.1 M PB overnight at 4°C. AChE histochemistry was carried according to previously described protocol (Harkany et al. 1998).

**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 x 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 mm². That way 40% of the total fields of view was covered by the counting frame. In each animal 3 sections (Bregma 1.2, -1.5, -1.8) were determined with a standard cross-sectional distance of 900 µm spanning the central subdivision of the MBN. Only those cholinergic neurons were counted, which strictly belong to the MBN, positioned between the internal capsule and the globus pallidus and only if they were lying within the rectangular counting frame or touching the two non-forbidden sides of the frame. Subsequently, the counted area of MBN was delineated and measured by computer-assisted image analysis (Leica, Quantimet Q-600HR, Rijswijk, The Netherlands). Non-significant differences in the measured areas between groups were prerequisite of further data processing. Density of ChAT-ir cells in the central region of MBN was calculated applying the following formula taking into account the thickness of the section (20 µm).

Density of ChAT-ir cells (mm³) = 50 x (number of counted cells / 0.04) / measured area.

Changes in the density of ChAT-ir cells on the injected side were calculated as percentage values of the contralateral, non-injected side.
Quantification of AChE fiber density was performed in layer V of the posterior somatosensory cortex according to a standard protocol by using a Quantimet Q-600HR computerized image analysis system (Leica) (Harkany et al., 1998, 1999). Surface area density of cortical AChE-positive fibers 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 MBN (Luiten et al. 1995), contralateral fiber density values served as controls within each animal and the fiber reduction was calculated as the percentage difference of fiber densities at the injected side and contralateral sides of the brain. A decrease in fiber density was calculated according the following formula:

Percentage decrease in fiber density = 100 - (injected side / control side x 100)

GFAP-immunoreactive astrocytes were quantified both in the nucleus basalis (more precisely in the MBN and substantia innominata-SI) and in layer V of the somatosensory cortex. At the level of the nucleus basalis GFAP-ir astrocytes accumulated abundantly in a rim-like pattern surrounding the core of the neurotoxic lesion. As NMDA infusion in the MBN results in widespread neuronal damage - often exceeding the target nucleus (Harkany et al, 2000b)-, astroglial accumulation is not restricted to the MBN. We, therefore, aimed at quantifying the numbers of GFAP-ir astrocytes not only in the MBN, but also in the predominantly cholinergic substantia innominata (SI), associated ventrally to the MBN. Quantitative determination of individual 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 described detailed by Harkany et al. (2000b). Briefly, three coronal sections were measured (starting at -1.4 mm from Bregma – Paxinos et al. 1986) with a standard distance of 50 µm. Following manual delineation of the intermediate MBN and substantia innominata (without significant differences in the measured surface area between the groups) OD values of superposed primary quantification elements were independently measured. OD values ranged from 0.000 - 2.500 based on standardised grey-scale values after logarithmic correction. The densitometrically obtained data were subsequently analyzed to determine the mean ± S.E.M. OD value in each brain. 

Mean OD: Absolute OD values of the individual frames were averaged for each MBN and subsequently, the average of such values was calculated in the three sections analyzed.

Density of GFAP-positive astrocytes in layer V of the somatosensory cortex of both hemispheres were measured similar way as the AChE quantification. Area densities of astrocytes at injection side were expressed as a percentage of area densities at the non-lesioned, control side for each individual.
Statistics

Data were expressed as means ± S.E.M. One way analysis of variance (ANOVA) was used to determine the effects of postnatal ACTH4-9 on NMDA-induced changes in the density of ChAT-ir cellbodies, the cholinergic (AChE-positive) fiber densities, and the OD of GFAP-ir astrocytes in the MBN at 28h and 12d postsurgery. Tukey’s post-hoc test was employed to determine differences within treatment groups at 28h and 12d post-surgery. A \( p \) value of < 0.05 was taken as indicative of statistical significance for the tests.

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.

Effect of postnatal ACTH4-9 treatment on NMDA-induced cholinotoxicity

The ChAT-ir neurons of the MBN appeared as large, intensely-stained multipolar neurons (Fig 1A, inset). Analysis of ChAT-ir 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 28h post-surgery (Fig 1B, C). Density of ChAT-ir cells, expressed in mm\(^3\), on the contralateral side in control and ACTH4-9 animals were 65285.83 ± 7893.43 and 78477.50 ± 7649.55, respectively versus 30151.33 ± 6832.51 and 13328.58 ± 3983.74 detected on the ipsilateral side. At that timepoint the density of ChAT-immunoreactive cellbodies in the injured-MBN was much lower in ACTH4-9 animals compared to controls (\( F_{1,11}=5.429, p=0.042 \)). Expressing the reduction of ChAT-ir MBN neurons as a percentage of the values of the contralateral side revealed pronounced effects of ACTH4-9 treatment (52.07 % ± 9.52 in control vs. 82.5% ± 4.04 in ACTH4-9 animals, \( F_{1,11}=9.202, p=0.013 \); Fig 2A).
Figure 1. Photomicrographs of ChAT-ir cells in the MBN (A-E) and AChE stained fibers in layer V of the parietal cortex (F-J). Compared to the non-lesioned side (A, F), NMDA infusion into the NBM induced strong decrease in ChAT-ir cells 28h post-surgery in both control (B) and ACTH_{4-9} animals (C), and slight decrease in AChE fiber density in the cortex, indicated by black dots among intact fibers in both control (G) and ACTH_{4-9} animals (H). 12 days after the NMDA lesion control animals displayed much less ChAT-ir cells (D) than ACTH_{4-9} animals (E). Concordant with this effect, AChE fiber loss was more pronounced in control animals (I) than in ACTH_{4-9} rats (J). The white bars in F indicate layer V of the parietal cortex, where quantitative measurement was performed. Arrowheads in (B, C) point to densely, while arrows in (B, C) to lightly stained ChAT-ir perikarya indicate changes in ChAT content of cellbodies shortly after lesion. Scale bar in (E, J) = 250 µm, whereas in (D, insert) = 25 µm.
In contrast with the result 28h post-surgery, the amount of remaining ChAT-ir somata in the lesioned MBN was significantly higher in ACTH$_{4-9}$ animals 12 days after lesion (Fig 1D, E). Density of ChAT-ir cells per mm$^3$ on the contralateral side in control and ACTH$_{4-9}$ animals were 63430.30 ± 3119.38 and 81516.75 ± 6361.67, respectively versus 6247.90 ± 2252.13 and 25134.17 ± 6368.03 detected on the ipsilateral side (F$_{1,10}$=7.846; p=0.021). When the loss of the number of ChAT-ir nerve cells were expressed as the percentage ratio between ipsilateral and contralateral hemispheres a remarkable beneficial effect of postnatal ACTH$_{4-9}$ treatment became apparent (89.92 % ± 3.78 in control vs.66.73% ± 8.29 in ACTH$_{4-9}$ animals, (F$_{1,10}$=5.61, p=0.042; Fig 2A).

Measurement of AChE-positive fiber density in the somatosensory cortex revealed 17.6% ± 3.79 and 20.8% ± 4.48 reduction in control and ACTH$_{4-9}$ animals, respectively, 28h after NMDA infusion (Fig. 2B). At that timepoint there was no significant difference between the groups. 12 days after NMDA infusion in both treated groups the AChE-positive fiber loss had become much stronger (Fig 1I, J) as compared to the groups 28h 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, fiber loss 12 days after the 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 postnatally treated with ORG 2766 exhibited a significant preservation of the cortical AChE-positive cholinergic innervation after excitotoxic MBN lesioning as compared to controls.

![Graph A](image1)

**Figure 2.** Effect of postnatal ACTH$_{4-9}$ treatment on adult NMDA-induced decrease in ChAT-ir cellbodies in the MBN (A) and on cholinergic fiber loss in the somatosensory cortex (B). Measurements were taken at 28 hours (n= 6 in each group) and 12 days post-lesion (n= 5 control and 6 ACTH$_{4-9}$ animals). Data are expressed as percentages of the value of the non-lesioned side. Data represent means ± SEM. *p < 0.05.
Effect of postnatal ACTH$_{4-9}$ treatment on NMDA-induced astrocyte activation

The optical density (OD) of GFAP immunoreactive glial cells was quantified in both the MBN and the SI. The ACTH$_{4-9}$ animals generally elicited stronger glial responses to the infusion of NMDA 28h post-surgery when compared to control cases (Fig 3A, B). The mean OD of reactive astrocytes in the MBN 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-ir astrocytes measured in the MBN further increased significantly only in the control group ($p=0.014$; Fig 3C). While both control and ACTH$_{4-9}$ (Fig 3C, D) animals showed a stronger astrocyte reaction in the SI compared to the results of 28h post-surgery groups (control, ACTH$_{4-9}$ animals $p=0.000$, $p=0.024$, respectively). Comparison of the two treatment groups 12d following NMDA infusion revealed a strong tendency toward a more robust GFAP-immunoreactivity in the SI in control vs. ACTH$_{4-9}$ animals ($F_{1,10}=4.81$, $p=0.056$; Fig 4B). The mean OD of GFAP-ir astrocytes in the non-lesioned hemispheres did not exhibit significant differences between control and ACTH$_{4-9}$ animals both 28h and 12d post-lesion (Fig 4A, B).

A diffuse, scattered GFAP immunolabeling was visualized in layer V of the somatosensory cortex of the lesioned hemisphere. Although the optical density of GFAP-labeled structures was similar to that found in the basal forebrain, the area coverage did not exceed 0.4-3% of the cortical surface subjected to quantitative analysis. Irrespective of ACTH$_{4-9}$ or saline treatment, NMDA infusion into the MBN resulted in at least two-fold, increase in GFAP immunoreactivity in the somatosensory cortex. Moreover, an increase of GFAP immunolabeling was demonstrated from 28h up to 12d post-surgery. Similar elevations of GFAP immunoreactivity were visualized in both the ACTH$_{4-9}$ and saline-injected experimental groups 28h post-surgery (area coverage 0.54% ± 0.09 in control vs.0.74% ± 0.17 in ACTH$_{4-9}$ animals). This initial GFAP activity exhibited significant extensions when compared to corresponding experimental groups 12d post-lesion. However, there was no enhanced area coverage in the ACTH$_{4-9}$ rats 12d post-lesion (2.38% ± 0.5 in control vs. 1.62% ± 0.84 in ACTH$_{4-9}$ animals).
Figure 3. Photomicrographs of GFAP-positive astrocytes activated by NMDA infusion into the cholinergic nucleus basalis during adulthood in control animals and in postnatally ACTH₄₋₉ treated rats, 28 hours (A, B respectively) and 12 days post-lesion (C, D respectively). The diffusion pattern of NMDA exceeded the MBN/substantia innominata region and also injured the globus pallidus complex as well as fiber projections in the capsula interna. Dotted circle in (A) shows the area of MBN, while the lined circle in (A) indicates the region of substantia innominata, that were measured. Note enlarged, hypertrophic astrocytes (C, D inset) 12 days post-lesion compared to astrocytes detected 28h after lesion (A, B inset). Scale bar in (B, large picture) = 1000 µm, whereas in (B, inset) = 25 µm.
Figure 4. Effect of postnatal ACTH<sub>4-9</sub> treatment on mean optical density (OD) of GFAP immunoreactive astrocyte in the MBN and SI after injections of NMDA during adulthood. Measurements were taken at A) 28 hours (n= 6 in each group) and B) 12 days post-surgery (n= 5 controls and 6 ACTH<sub>4-9</sub> animals). Data are expressed as the average optical density of the alineated area (Fig 3A). Data represent means ± S.E.M. *p < 0.05.

Discussion

The present study shows that neonatal application of the ACTH<sub>4-9</sub> analog, ORG 2766 induces developmental changes that reduce excitotoxic neuronal damage of cholinergic MBN neurons in adulthood. After NMDA infusion the loss of ChAT-ir neurons in the MBN and that of their fiber projections invading the somatosensory cortex was abated in animals that were postnatally treated with ORG 2766. In concordance with its effect on cholinergic neurons, ORG 2766 treatment affected the local accumulation of GFAP-ir activated astrocytes in the vicinity of the excitotoxic lesion.

Exposure to NMDA results in the activation of Ca<sup>2+</sup> permeable NMDA type glutamate receptors. A strong Ca<sup>2+</sup> influx into the neurons and a sustained high level of [Ca<sup>2+</sup>]i is considered an important cause of eventual neuronal damage.
Elevation of [Ca\(^{2+}\)]\(_i\) can further 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 severe depletion being associated with necrosis and a more moderate decline leading to apoptotic cell death. Cell death is primarily necrotic (Choi et al., 1987; Gwag et al., 1997) in the core of the lesion, while 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, 28h after lesion the decrease in ChAT immunoreactive cell number was much stronger in ACTH\(_{4-9}\) treated than control animals. However, this change reversed at 12 days after NMDA infusion when ACTH\(_{4-9}\) animals displayed more ChAT-ir cells on the injured side. The reduction in ChAT positive cell number and AChE fiber density after an 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-ir cells seen after 28h 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 28h after lesion the cholinergic neurons of the nucleus basalis 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 cell survive and are detectable 12 days after NMDA lesion, with a proportional reduction of cholinergic fiber 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. 28h after the NMDA lesion a much stronger GFAP-ir 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. CNTF), growth factors (e.g. TGF\(\beta\), cytokines (e.g. IL-6, TNF\(\alpha\)), 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 (Schurr et al., 1999; Magistretti et al., 1999), increased production
of neurotrophins, such as NGF (Schwartz et al., 1994), growth factors (e.g. IGF-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 et al., 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 12d 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 ACTH₁₄₋₉ 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. However, the lower neuronal damage in the postnatally
ACTH₁₄₋₉ treated animals may also 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. It is well known that glucocorticoids can
modulate neurodegenerative processes (Sapolsky and Pulsinelli 1985, Landfield
1994; Abraham 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 1992,) 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 on MBN (Abraham et al.,
2000).

However, not excluded, we have no evidence yet whether neonatal treatment
with ORG 2766 affects directly the development of the glutamate system and / or
NMDA receptors. Studies in adult animals suggested that the analog might have
its effect directly through NMDA receptor (Spruit 1992).

In conclusion, the present study shows that postnatal treatment with the
ACTH₁₄₋₉ 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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The two-way active shock avoidance test is an associative learning paradigm based on fear-induced avoidance conditioning.