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N-nitro-L-arginine methyl ester influence on aluminium toxicity in the brain

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Abstract

We investigated the influence of a non-specific nitric oxide synthase (NOS) inhibitor, N-nitro-L-arginine methyl ester (L-NAME), on brain nitrite concentration and acetylcholine esterase activity in $AlCl_3$-treated Wistar rats. Animals were killed 10 min, three hours, three days and 30 days after the treatment and hippocampus and basal forebrain were removed. The biochemical changes observed in neuronal tissues show the involvement of NO in the $AlCl_3$ toxicity and cholinergic neurotransmission, and that L-NAME may have potential neuroprotective effects. Active avoidance learning was significantly impaired after $AlCl_3$ application, while pretreatment with L-NAME prevented the behavioural deficits caused by $AlCl_3$. We also applied immunohistochemical techniques to identify changes induced by $AlCl_3$, L-NAME+$AlCl_3$, as well as L-NAME injections after survival periods of three days and 30 days. Immunoreactivity of astrocytes and phagocytic microglia based on glial fibrillary acidic protein (GFAP) and a useful marker for rat macrophages (ED1), respectively, revealed a greater inflammatory response in $AlCl_3$-injected animals compared to controls.

Key words: *aluminium, behaviour, L-NAME, nitric oxide.*

Introduction

Aluminium (Al) compounds are neurotoxic and have been shown to induce experimental neurodegeneration, although the mechanism of this effect is unclear [8]. Aluminium has the ability to produce neurotoxicity by many mechanisms, promoting formation and accumulation of insoluble beta-amyloid peptide (Abeta) and hyperphosphorylated tau [16]. Aluminium induces conformational changes of Abeta protein, enhancing its aggregation and leading to progressive neuronal degeneration and death. Tau protein is the major component of paired helical filaments, which form a compact filamentous network described as neurofibrillary tangles [23]. In addition, the acetylcholine (ACh) system has a key role in memory disturbances characteristic of Al intoxica-

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tion, and to some extent, Al mimics the deficit of cortical cholinergic neurotransmission [5,7].

Animals loaded with Al displayed massive cellular depletion in the hippocampal formation, particularly the CA1 field, and also in the temporal and parietal cortex [27]. Prolonged exposure of rats to Al can result in numerous ghost-like neurons with cytoplasmic and nuclear vacuolations, and with Al deposits. The formation of amyloid fibrils is considered to be an important step in the aetiology of various amyloidoses [26]. Evidence has been presented that induction of fibril formation followed the complexation of AlATP by one or more monomers of the respective peptide. However, the formed complex could not be identified directly and it is suggested that AlATP might be acting as a chaperone in the assembly of amyloid fibrils [6].

Aluminium is transported by the iron-carrier protein transferrin (Tf), which enters the brain by binding to Tf receptors. The interaction between Tf and its receptor may function as a general metal ion regulatory system in the central nervous system (CNS). Brain Al entry may involve Tf-receptor mediated endocytosis and a more rapid process transporting small molecular weight Al species. There appears to be Al efflux from the brain, probably as Al citrate [4].

Nitric oxide (NO) is a product of NO synthase (NOS), which exists in three isoforms. Nitric oxide exerts significant neurophysiological functions. However, NO can also be neurotoxic primarily due to its free radical properties, and it has been implicated in neurodegenerative diseases [20]. In the CNS, the activation of N-methyl-D-aspartic acid (NMDA) type of glutamatergic receptor induces $Ca^{(2+)}$-dependent NOS activity and NO release, which then activates soluble guanylate cyclase for the synthesis of cGMP. Aluminium can decrease the ability of rats to learn and memorize, inducing changes of their synaptic configuration, which may be related to synaptic efficacy and may be one of the mechanisms for Al-induced neurodegenerative changes [13].

Our previous results demonstrated beneficial effects of NOS inhibitors on the development of $AlCl_3$-induced neurotoxicity [14,29-32]. In view of the above, the present study was undertaken to examine whether the production of NO, activity of acetylcholine esterase (AChE), as well as the task of active avoidance change after intracerebral injections of $AlCl_3$ into rats and if they are modulated by pretreatment with N-nitro-L-arginine methyl ester (L-NAME), a non-specific NOS inhibitor.

Material and methods

Reagents

All chemicals were of analytical grade or better. Chemicals were purchased from Sigma (St. Louis, MO, USA). All solutions of drugs were prepared on the day of the experiment.

Experimental procedure

Adult male Wistar rats, weighing 500 ± 50 g, were used for the experiments. Two or three animals were housed per cage in an air-conditioned room at a temperature of 23 ± 2°C with 55 ± 10% humidity and with lights on 12 h/day (7 am – 7 pm). The animals were given a commercial rat diet and tap water ad libitum.

Experimental *Wistar* rats were divided into four basic groups (10 rats/time point – 10 min, three hours, three days and 30 days): control group (sham-operated, 40 rats); $AlCl_3$-group (treated with $AlCl_3$ and saline, 40 rats); L-NAME+$AlCl_3$-group (pretreated with L-NAME, 40 rats) and L-NAME-group (treated with L-NAME and saline, 40 rats). All rats were intraperitoneally (*i.p.*) anaesthetized with sodium pentobarbital (45 mg/kg body weight – b.w.) before intrahippocampal administration. Testing substances were administered in a volume of 10 µL, sufficient not to burden nerve tissue, as follows: sham-operated group (10 µL of saline); $AlCl_3$ group (one single dose of 3.7×10^{-4} g/kg b.w./10 µL); L-NAME+$AlCl_3$ group (one single dose of L-NAME, 1×10^{-4} g/5 µL, immediately before $AlCl_3$ administration, 3.7×10^{-4} g/kg b.w./5 µL); and L-NAME group (single dose of L-NAME, 1×10^{-4} g/5 µL before saline administration, 5 µL).

Using a stereotactic instrument for small animals the drugs were administered via a Hamilton microsyringe and injected into the CA1 sector of the hippocampus (coordinates: 2.5 A; 4.2 L; 2.4 V) [17]. In all the treated animals the injected intracerebral volume was 10 µl and it was always injected into the same left side (when two different substances were applied, the volume of each was 5 µl, so the total volume was always 10 µl).

For biochemical analysis the rats were divided into four basic groups; each basic group consisted of

four different subgroups (according to survival times – 10 min, three hours, three days and 30 days). All animals were decapitated and the brains were immediately removed. Ipsi- and contralateral hippocampus and basal forebrain from individual animals were quickly isolated and homogenized in ice-cold buffer containing 0.25 M sucrose, 0.1 mM EDTA, 50 mM K-Na phosphate buffer, pH 7.2. Homogenates were centrifuged twice at 1580 × g for 15 min at 4°C. The supernatant obtained by this procedure was then frozen and stored at –70°C.

For the test of acquisition and expression of active avoidance, on the 26th day after the treatment (saline solution, $AlCl_3$, L-NAME+$AlCl_3$, L-NAME), animals were subjected to behavioural tests (two-way active avoidance) over the five consecutive days. Animals were then sacrificed by decapitation 30 days after surgery; their brains were removed and flash-frozen in liquid nitrogen.

For immunohistochemical analysis the animals were decapitated three days and 30 days after the treatment. Brains were removed from the skull, fixed in 4% paraformaldehyde (TAAB Laboratory Equipment, Aldermaston, UK) for at least 24 h and cryoprotected in graded sucrose at 4°C. Brains were frozen in methylbutane and stored at –70°C until cryosectioning (CRIOCUT-E Reichert-Yung).

Biochemical analyses

Hippocampus and basal forebrain were dissected bilaterally from each frozen brain and a crude mitochondrial fraction was prepared from each region as previously described [10].

After deproteinization the production of NO was evaluated by measuring nitrite and nitrate concentrations. Nitrates were previously transformed into nitrites by cadmium reduction [25]. Nitrites were assayed directly spectrophotometrically at 492 nm, using the colorimetric method of Griess (Griess reagent: 1.5% sulfanilamide in 1 M HCl plus 0.15% N-(1-naphthyl)ethylenediamine dihydrochloride in distilled water).

The determination of AChE activity was based on degradation of acetyl thiocholine iodide by AChE into a product which binds to 5,5-dithiobis-2-nitrobenzoic acid (DTNB), forming a yellow colour [24]. Kinetics of the enzymatic reaction were followed over 3-5 minutes at 412 nm. Values of AChE activity were calculated from the linear part of the reaction curve and were expressed as µmol acetyl thiocholine/min/g prot.

The protein content in the rat brain homogenates (hippocampus and basal forebrain, ipsi- and contralateral) was measured by the method of Lowry using bovine serum albumin (Sigma) as standard [22].

Active avoidance test

Apparatus. Acquisition of two-way active avoidance (AA) was studied in a series of automatically operated commercial shuttle-boxes and programming-recording units (Campden Instruments, USA). Boxes (48 × 21 × 22.5 cm) were used without the central partition.

Procedure. The acquisition of AA responses was achieved using the spaced trials behavioural procedure (20-trial sessions daily for five consecutive days). A conventional two-way AA schedule was used with trials starting at 30 s intervals. Each trial began with a conditioned signal (CS) (broad-band noise of 68 dB lasting seven seconds), followed by an unconditioned stimulus (US) (foot shock of 1.5 mA, three seconds duration) which was delivered through the grid floor. Crossing responses during the conditioned stimulus (AA response) terminated the conditioned stimulus and prevented the onset of the unconditioned stimulus. A response after the onset of the unconditioned stimulus (escape response) terminated both conditioned and unconditioned stimuli. Inter-trial crossings were not punished.

Processing of brain tissue and immunohistochemistry

Frozen, 8 µm thick sections were deposited on poly-L-lysine coated slides and allowed to air dry. DakoCytomation EnVision + System-HRP kit was used in a two-step IHC staining technique. Tissue sections were fixed in acetone and endogenous peroxidase activity was blocked by peroxidase block (0.03% hydrogen peroxide containing sodium azide) (Dako Cytomation) for 15 min. Slides were incubated with appropriate dilutions of mouse monoclonal antibody: ED1 antibody 1 : 10 (AbD Serotec), raised against rat lysosomal membrane antigen of activated macrophage/microglia and [GF5] to GFAP antibody 1 : 1000 (abcam plc Cambridge), raised against glial fibrillary acidic protein, for 60 min. After that

slides were incubated with the labelled polymer (DakoCytomation) conjugated to goat anti-mouse immunoglobulins in Tris-HCl buffer containing stabilizing protein and an anti-microbial agent with addition of 5% normal rat serum for 30 min. Staining was completed by 5-10 min incubation with 3,3'-diaminobenzidine (DAB)+substrate-chromogen (DakoCytomation), which resulted in a brown-coloured precipitate at the antigen site. Finally, slides were counterstained with haematoxylin and mounted with Kaiser gel (Merck). Control slides were incubated in the same way, using mouse isotype-matched irrelevant monoclonal antibody (produced in MMA, Belgrade).

Statistical analysis

Data are expressed as means ± S.D. Statistical significance was determined as $p < 0.05$ using either Student's t-test or ANOVA followed by Tukey's *t*-test.

Results

Nitrite concentration in the rat hippocampus and basal forebrain

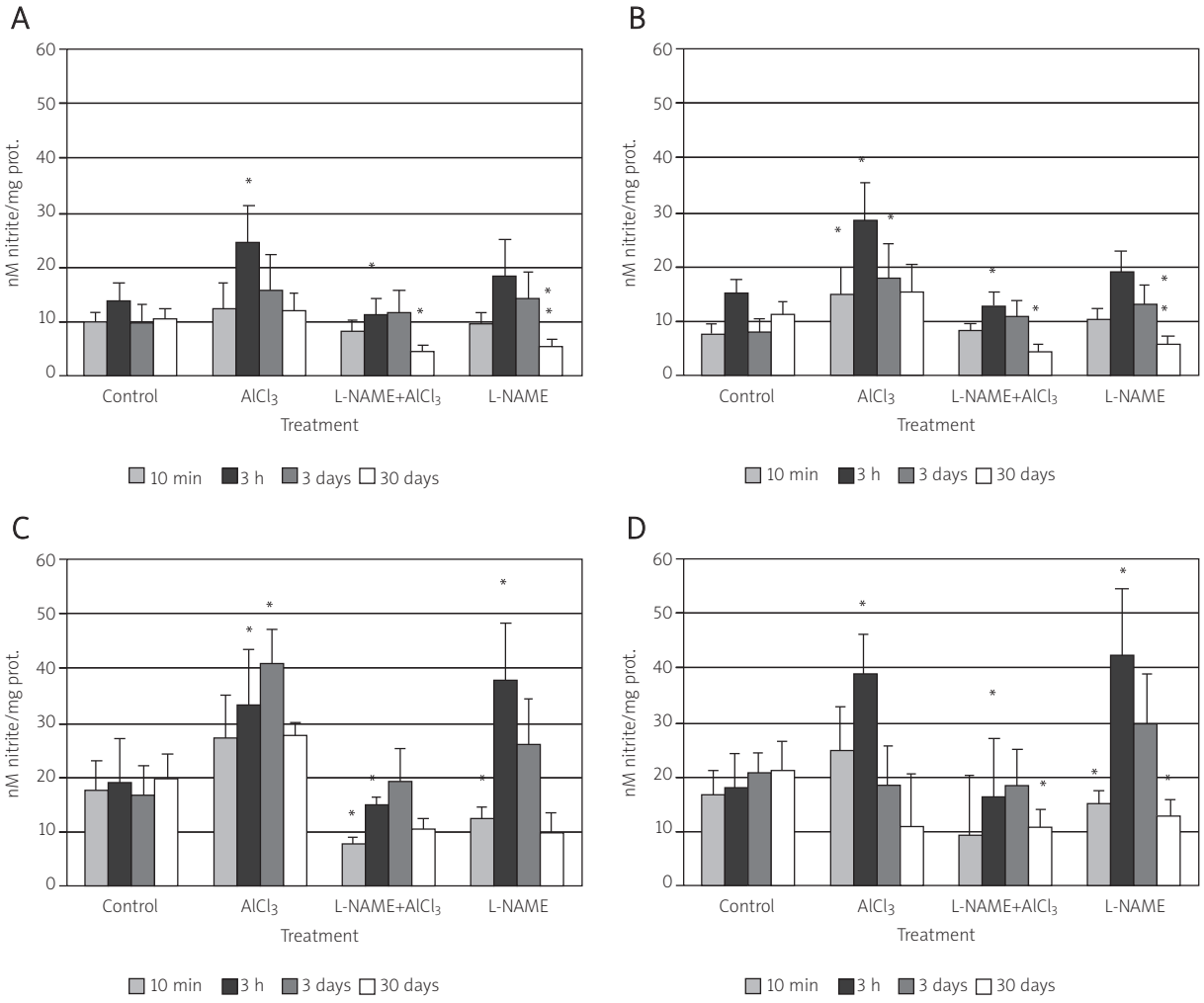
The results presented in Fig. 1 show the nitrite concentration (nM/mg proteins) in hippocampus (ipsilateral – A and contralateral – B) and basal forebrain (ipsilateral – C and contralateral – D), respectively. $AlCl_3$ injection resulted in increase of nitrite production only in the ipsilateral hippocampus after three hours, as well as after 10 min, three hours and three days in the contralateral hippocampus, compared to controls. 30 days after L-NAME application there were significant decreases bilaterally in the hippocampus, compared to controls. Three hours and 30 days after L-NAME+$AlCl_3$ injection and 30 days after L-NAME application, lower nitrite levels were registered bilaterally in the hippocampus, compared to $AlCl_3$-treated animals (Fig. 1A,B). Similar effects were obtained in the basal forebrain (Fig. 1C,D). $AlCl_3$ injection resulted in higher nitrite concentration after three hours and three days in the ipsilateral and only after three hours in the contralateral basal forebrain, compared to controls. After three hours, L-NAME application resulted in higher NO concentration, in both the ipsi- and contralateral basal forebrain, while lower nitrite values were measured in the contralateral basal forebrain after 30 days, compared to controls. At the early tested times (10 min and three hours), L-NAME+$AlCl_3$ injection resulted in diminished nitrite concentration in the ipsilateral basal forebrain (Fig. 1C), while in the contralateral basal forebrain lower values were measured after three hours and 30 days (Fig. 1D), compared to $AlCl_3$-treated animals. 10 min after L-NAME application, nitrite concentration decreased bilaterally in the basal forebrain, compared to $AlCl_3$-treated animals (Fig. 1C,D).

Acetylcholine esterase activity in the rat hippocampus and basal forebrain

Activity of AChE (μM acetylthiocholine/min/g proteins) was significantly lower in the ipsilateral hippocampus after $AlCl_3$, L-NAME+$AlCl_3$, and L-NAME injection, compared to controls (Fig. 2A). Intrahippocampal L-NAME+$AlCl_3$ injection resulted in generally higher AChE activity compared to $AlCl_3$-treated groups at all times tested (Fig. 2A). The results obtained for the ipsi- and contralateral basal forebrain were similar (Fig. 2C,D). Activity of AChE was lower in the ipsilateral basal forebrain after $AlCl_3$, L-NAME+$AlCl_3$, and L-NAME application, compared to controls (Fig. 2C). $AlCl_3$ injection followed with L-NAME clearly increased AChE activity in this brain structure, compared to $AlCl_3$-treated animals at all times tested (Fig. 2C). There was no statistically significant difference in AChE activity between the ipsi- and contralateral sides of hippocampus (Fig. 2B) and basal forebrain (Fig. 2D) in either group.

Behavioural changes after the treatment

We determined the number of AA in response to an aversive unconditioned stimulus during five consecutive days (26-30 days after the treatment), at 20 trials per day, as a measure of acquisition of positive reactions. Difference in the number of correct responses first became evident 28 days after $AlCl_3$ injection (3rd day of examination) and progressively widened over the subsequent three days. At the end of the 30th day (5th day of examination), $AlCl_3$-treated animals showed a two-fold reduction in correct responses compared to the control group. From the 3rd until the 5th day there was a decrease in improvement of reactions in the group treated with $AlCl_3$, compared to the L-NAME and L-NAME+$AlCl_3$ group. L-NAME+$AlCl_3$ and L-NAME injection clearly inhibited the number of correct responses compared to controls achieved from the 3rd day of testing (Fig. 3).



**Indicates a statistically significant difference between treated ($AlCl_3$-, L-NAME+$AlCl_3$- and L-NAME-treated) and control (sham-operated) animals ($P < 0.05$).*

•Indicates a statistically significant difference between treated (L-NAME+$AlCl_3$- and L-NAME-treated) and $AlCl_3$-treated animals ($P < 0.05$).

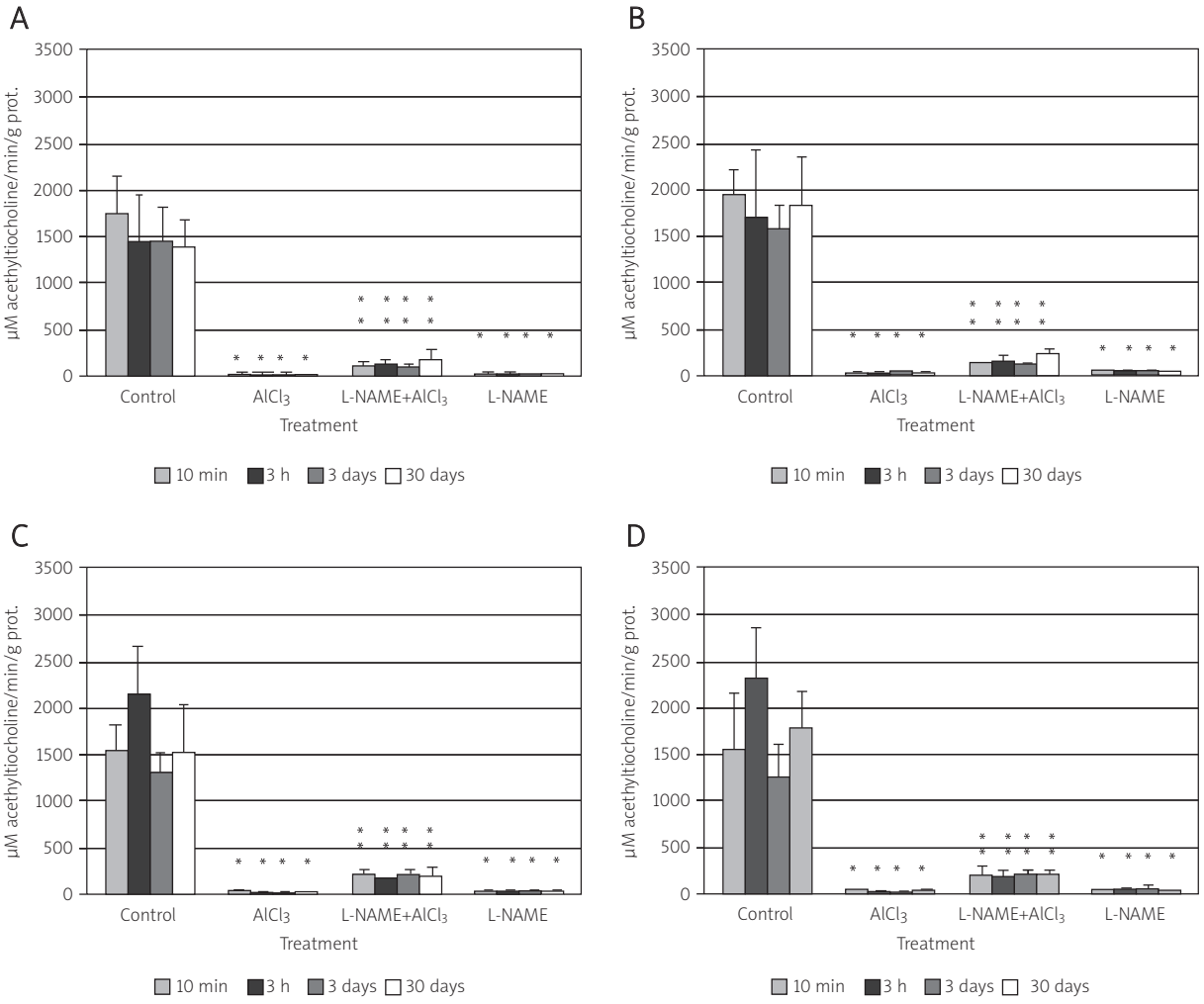
Fig. 1. The effect of intrahippocampal drug injection on the concentration of nitrite (nM nitrite/mg protein) in the rat hippocampus, ipsilateral (**A**) and contralateral (**B**), as well as basal forebrain, ipsilateral (**C**) and contralateral (**D**), at different times post-injection: 10 min, three hours, three days and 30 days. Data are means ± SD of 10 animals.

ED1

No labelling of cells for ED1 was seen 30 days after saline solution injection in rat brain (Fig. 4A). A lot of roundish ED1 positive macrophage/microglia were registered in the hippocampus 30 days after L-NAME+$AlCl_3$ administration (4C). The microglia faint stain ED1 was observed in L-NAME-treated animals (4D). However, maximal expression of ED1 appeared 30 days after $AlCl_3$ application in the hippocampus (Fig. 4B).

GFAP

Glial fibrillary acidic protein (GFAP) labelling was always weak in the hippocampus in control rats after three days (5A) and 30 days (5B) of saline application. GFAP labelling was present three days (5C) and 30 days (5D) after $AlCl_3$ application and enhanced three days (5E) and 30 days (5F) after L-NAME+$AlCl_3$ injection. A faint GFAP stain was observed in L-NAME-treated animals after both three days (5G) and 30 days (5H) of L-NAME application.



**Indicates a statistically significant difference between treated ($AlCl_3$-, L-NAME+$AlCl_3$- and L-NAME-treated) and control (sham-operated) animals ($P < 0.05$).*

•Indicates a statistically significant difference between treated (L-NAME+$AlCl_3$- and L-NAME-treated) and $AlCl_3$- treated animals ($P < 0.05$).

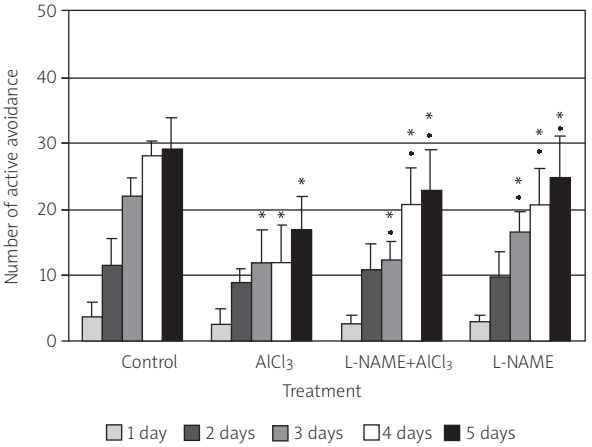
Fig. 2. The effect of intrahippocampal drug administration on AChE activity (µM acetylthiocholine/min/g proteins) in the rat hippocampus, ipsilateral (**A**) and contralateral (**B**), as well as basal forebrain, ipsilateral (**C**) and contralateral (**D**), at different times post-injection: 10 min, three hours, three days and 30 days. Data are means ± SD of 10 animals.

Discussion

The application of $AlCl_3$ to the CA1 sector of the hippocampus resulted in the impairment of cognitive functions accompanied with significant bilateral decrease in AChE activity, as well as increase of NO production in the hippocampus and basal forebrain. Inhibition of NOS by L-NAME generally protects the cells in these regions from $AlCl_3$-induced damage and, therefore, may limit the retrograde and anterograde spread of neurotoxicity.

Aluminium salts have been shown to cause damage to astroglial and neuronal cells in selective brain regions of the associative cortex and hippocampus [1]. Toxicity of Abeta is mediated by excitotoxic cascades, which are associated with increased NOS activity in cortical target areas that may directly lead to the generation of free radicals [3,11]. Iron accumulates in the brain and catalyses formation of superoxide, which reacts with NO to form the very harmful peroxynitrite ($ONOO^-$) [35]. In our study, $AlCl_3$ injection resulted in increased NO production after

three hours bilaterally, in both the hippocampus and basal forebrain (Fig. 1A-D). It has been previously found [33] that production and oxidation of NO in the brain increased in the early stage of the disease, while it decreased with rising loss of neurons. In addition, potential sites for $ONOO^-$-induced cytotoxicity are the tyrosine residues of tyrosine kinase receptors that are crucial for the maintenance of cholinergic neurons, which, unlike other brain cells, utilize acetyl-CoA, not only for energy production but also for ACh synthesis. Aluminium, by potentiating lipid peroxidation, affects the uptake of choline in nerve endings. The enzyme AChE exists in several molecular forms and catalyses degradation of ACh [36]. At all tested times following $AlCl_3$ injection, lower AChE activity was found bilaterally in the hippocampus and basal forebrain, compared to controls (Fig. 2A-D). These results suggest a loss of AChE substrate in cholinergic neurons of the basal forebrain and reduced function and/or damage of its cholinergic neurons. Neurochemical disruption of



**Indicates a statistically significant difference between treated ($AlCl_3$-, L-NAME+$AlCl_3$- and L-NAME-treated) and control (sham-operated) animals ($P < 0.05$).*
•Indicates a statistically significant difference between treated (L-NAME+$AlCl_3$- and L-NAME-treated) and $AlCl_3$-treated animals ($P < 0.05$).

Fig. 3. The influence of intrahippocampal drug injection on the active avoidance behaviour in rats. Data are means ± SD of 10 animals.

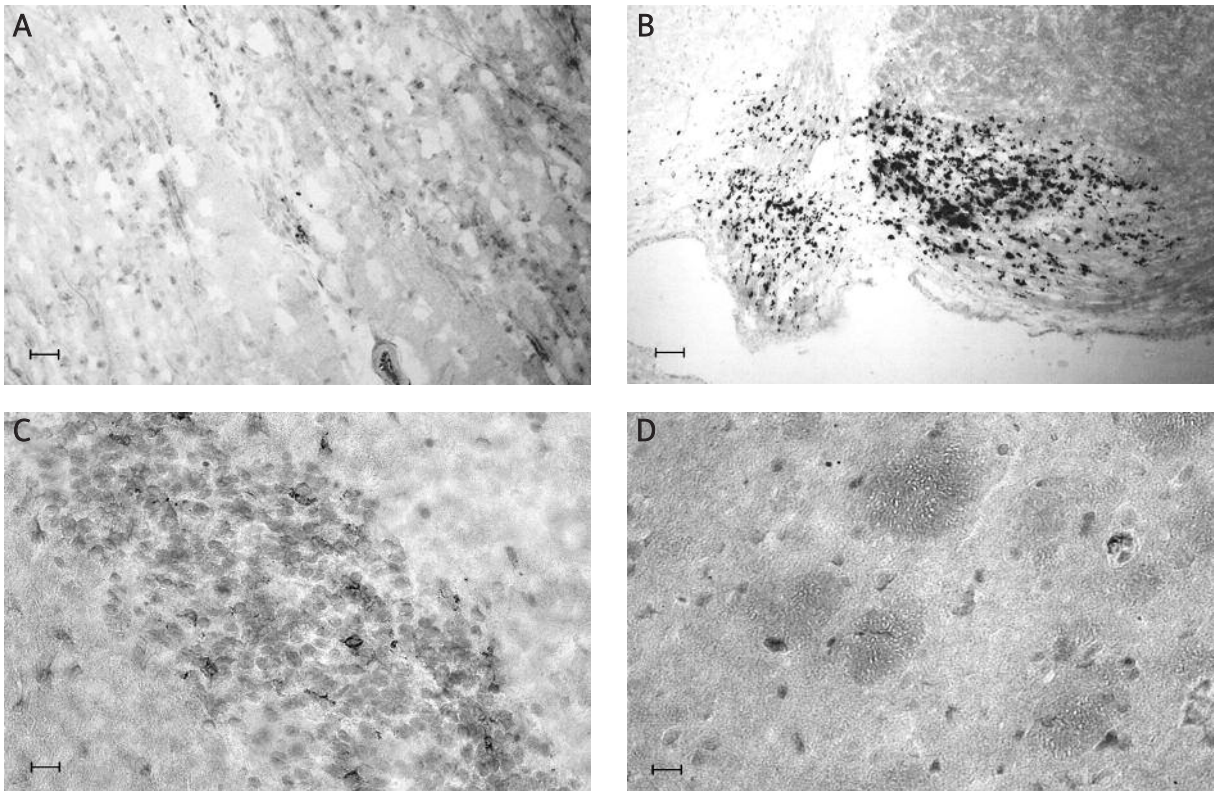


Fig. 4. Glia activation in the hippocampus after 30 days of drug administration. The microglia marker ED1 was not detected in the hippocampus of controls **(A)**, but was obvious in AlCl3-injected rats **(B)**. The microglia stain ED1 was observed in both L-NAME+AlCl3- **(C)** and L-NAME-injected animals **(D)**. Scale bars represent 20 µm.

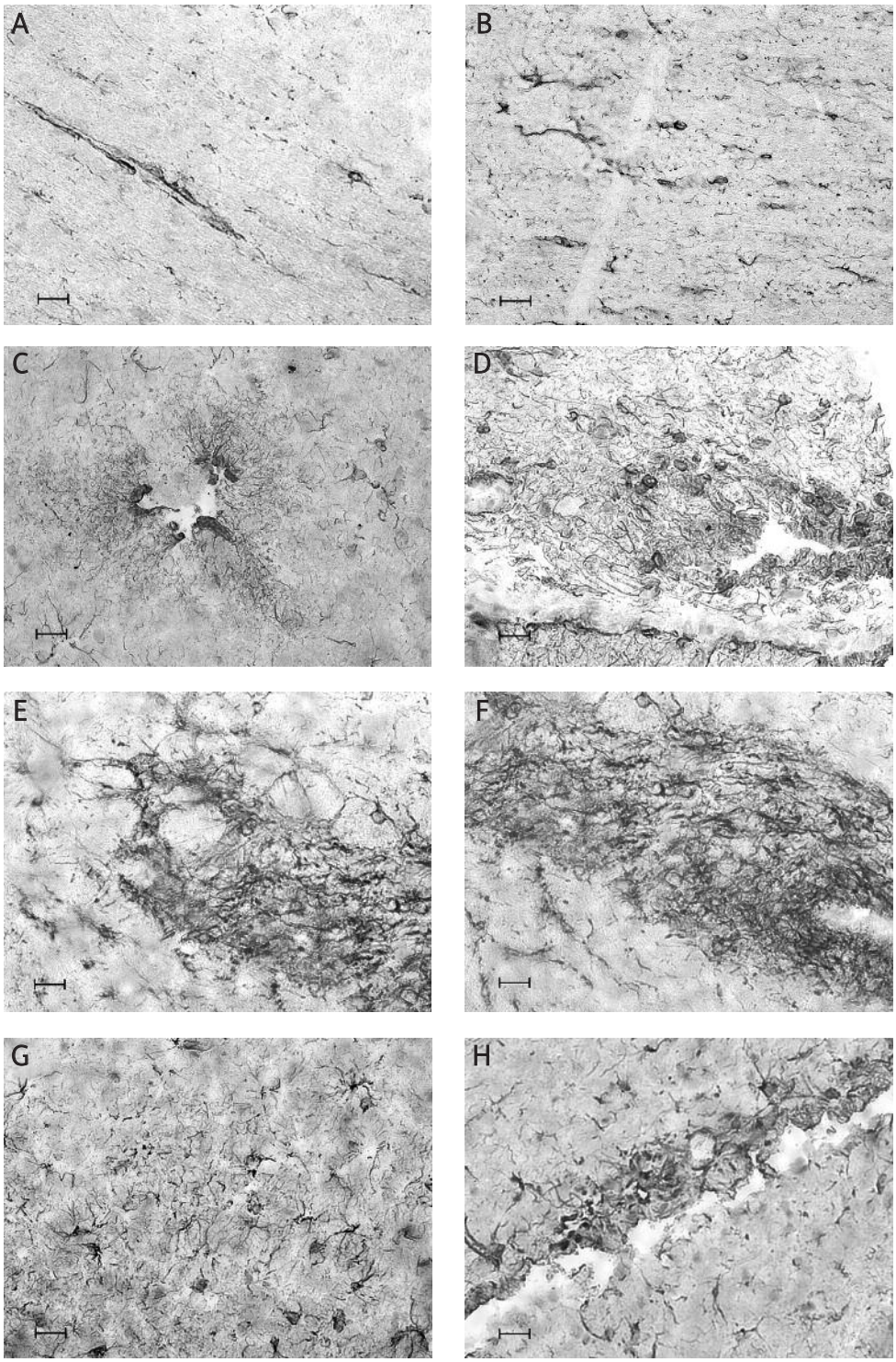


Fig. 5. Glia staining in the hippocampus. Basic glial fibrillary acidic protein (GFAP) labelling for astrocytes present in the hippocampus after three days (**A, C, E, G**) and after 30 days (**B, D, F, H**) of drug administration. GFAP labelling for astrocytes present in the hippocampus of saline-injected controls (**A, B**) was much stronger in $AlCl_3$-treated animals (**C, D**), as well as L-NAME+$AlCl_3$-treated (**E, F**), and L-NAME-treated animals (**G, H**). Scale bars represent 20 µm.

connectivity between the hippocampus and basal forebrain induced by $AlCl_3$ injection could provoke retrograde transneuronal damage of the basal forebrain that could explain the decreased AChE activity. Beside that, some previous studies have shown that attention and spatial learning were disrupted in the Al-treated animals [9]. As part of the AA model, we showed two-fold reduction in correct responses from the 3rd to 5th day of examination in $AlCl_3$-treated animals (Fig. 3). Aluminium induces stable and reproducible depression of the AA reaction, meaning that Al damages rat cognitive function. These results, along with the decreased AChE activity, suggest that Al exerts its toxic effects by altering cholinergic transmission, which is ultimately reflected in neurobehavioral deficits [15].

Also, Zou *et al.* showed that Al could block the induction of long-term potentiation and decrease the amplitude of the population spike potentiated in CA3, as well as that the effect of Al might be antagonized by modulation of the L-arginine-NO pathway [37]. Decreased nitrite concentrations after both three hours and 30 days of L-NAME+$AlCl_3$ application (compared to $AlCl_3$-treated group) suggest an L-NAME suppressing effect on nitrite accumulation as well as its decreased impairment in neuron damage through NMDA receptors (Fig. 1A-D). Furthermore, in the same experimental groups we found approximately 50% increase in AChE activity, which may suggest L-NAME-mediated cholinergic neurotransmission, even though enzyme activity was not restored to control value levels (Fig. 2A-D). Also, increased AChE activity was followed by an increase of correct behavioural responses from the 3rd day of testing in the AA reaction (Fig. 3). This means that application of L-NAME ameliorated memory impairment induced by $AlCl_3$ injection.

An inflammatory reaction of the basal forebrain facilitates GABA release through the production of NO [2]. Inhibition of NOS by L-NAME which decreases NO concentration may prevent the increase in nitrogen intermediates and GABA release, but not glutamate release, which consequently increases NO concentration after three hours bilaterally in the basal forebrain (Fig. 1C,D). In the same group of rats we observed a reduction of AChE activity in the brain structures essentially involved in memory processing (Fig. 2A-D), as well as a progressive decrease in the number of AA reactions, starting from day 28 after treatment (Fig. 3). The results show that L-NAME suppresses spontaneous behavioural rat activity causing cholinergic system functional impairment. Decreased release of ACh from the basal forebrain is caused by suppression of cholinergic basalocortical neurons by nitrergic cortical fibres after L-NAME application [28,34]. Thus, L-NAME can antagonize behavioural function impairment only if given together with $AlCl_3$.

Microglial cells represent the first line of defence against damage in the brain. However, under conditions of neurodegeneration, excessive activation of microglia can contribute to the neurodegenerative process by releasing potentially cytotoxic substances including the cytotoxic free radical NO [21]. ED1 is a pan-macrophage marker and is expressed intracellularly on lysosomes. It detects activated microglia [12]. Immunohistochemical examination of both ED1 and GFAP expression in CNS of old adult rats was performed to demonstrate the occurrence of activated microglia/ED1 positive macrophages in senescence and to find out to what extent this correlates with astrogliosis. The results show a massive region-specific increase in activated microglia and ED1 expressing cell profiles in aged rats (Fig. 4). ED1 is produced when microglia transform into phagocytic cells, thus indicating processes beyond proliferation and migration towards injured tissue [19]. Our data suggest that ED1-positive neurons were present in L-NAME-treated animals (Fig. 4D) and enhanced in L-NAME+$AlCl_3$-treated animals 30 days (Fig. 4C) after drug administration, with maximal ED1 expression 30 days after $AlCl_3$ application in the hippocampus (Fig. 4B). In addition, reactive astrogliosis could be evidenced by the upregulation of GFAP, an indicator of reactive astroglial changes to neuronal damage [18]. Immunostaining for GFAP showed that this was mainly located in peri- and paraventricular regions of the third and lateral ventricles, for instance in the septum, caudate putamen and hippocampus [12]. In our experiment, there was a significant increase in GFAP-positive cells in the hippocampus at the 3rd day (Fig. 5C) and 30th day (Fig. 5D) post-$AlCl_3$ injection, as well as three days (Fig. 5E) and 30 days (Fig. 5F) after L-NAME+$AlCl_3$ application in the hippocampus.

In conclusion, our data indicated that NO was included in $AlCl_3$-induced neurotoxicity, resulting in both temporal and spatial spread of damage to the basal forebrain, with impairment of cholinergic trans-

mission, so that NOS inhibitors, such as L-NAME, could have a potential neuroprotective effect.

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