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Aluminium exposure induces Alzheimer's disease-like histopathological alterations in mouse brain

L.F. Rodella[1], F. Ricci[1], E. Borsani[1], A. Stacchiotti[1], E. Foglio[1], G. Favero[1], R. Rezzani[1], C. Mariani[2] and R. Bianchi[1]

[1]Unit of Human Anatomy, Department of Biomedical Sciences and Biotechnology, University of Brescia and [2]Neurological Clinic, University of Milan, Italy

Summary. Aluminium (Al) is a neurotoxic metal and Al exposure may be a factor in the aetiology of various neurodegenerative diseases such as Alzheimer's disease (AD). The major pathohistological findings in the AD brain are the presence of neuritic plaques containing ß-amyloid (Aß) which may interfere with neuronal communication. Moreover, it has been observed that GRP78, a stress-response protein induced by conditions that adversely affect endoplasmic reticulum (ER) function, is reduced in the brain of AD patients. In this study, we investigated the correlation between the expression of Aß and GRP78 in the brain cortex of mice chronically treated with aluminium sulphate. Chronic exposure over 12 months to aluminium sulphate in drinking water resulted in deposition of Aß similar to that seen in congophilic amyloid angiopathy (CAA) in humans and a reduction in neuronal expression of GRP78 similar to what has previously been observed in Alzheimer's disease. So, we hypothesise that chronic Al administration is responsible for oxidative cell damage that interferes with ER functions inducing Aß accumulation and neurodegenerative damage.

Key words: Beta amyloid, GRP78, neurodegenerative damage, aluminium sulphate, Alzheimer's disease

Introduction

Chronic exposure to aluminium (Al) is a real eventuality due to its presence in food, water, dust, air and medicines. Only a very small fraction of Al becomes available for absorption (0.1-1%) by the gastrointestinal tract (Taylor et al., 1998) and then most is eliminated by the kidney and to a lesser extent in the bile (Exley et al., 1996). The small amount that is absorbed may cause, over several years, significant accumulation in brain, bone and other tissues like hematopoietic organs (Harrington et al., 1994; Exley et al., 1996). Al is a neurotoxic metal (Boegman and Bates, 1984; Jope and Johnson, 1992) and it is implicated as a possible aetiological factor in several neurological diseases such as dialysis syndrome (Alfrey et al., 1976), amyotrophic lateral sclerosis (Gajdusek and Salazar, 1982; Perl et al., 1982; Perl and Moalem, 2006), multiple sclerosis (Exley et al., 2006), Alzheimer's disease (AD) (Good et al., 1992; Exley, 1999, 2001; Lukiw, 1999; Grant et al., 2002; Polizzi et al., 2002) and Parkinson's disease (Hirsch et al., 1991). The molecular mechanism involved in Al neurotoxicity has not yet been clarified, but it has been suggested that Al interferes with glutamatergic and nitrossidergic neurotransmission (Provan and Yokel, 1992; Cuccarella et al., 1998; Hermenegildo et al., 1999; Rodella et al., 2001, 2006). In addiction Al present in drinking water enhanced inflammatory markers (Campbell, 2004) and ß-amyloid levels (Pratico et al., 2002a,b) in the CNS and potentiated oxidative stress (Bondy and Kirstein, 1996; Bondy et al., 1998; Exley, 2004).

Alzheimer's disease (AD) may be caused by the progressive accumulation and deposition of neurotoxic ß-amyloid (Aß) plaques and aggregates in brain with aging. However, ß-amyloid deposition is not the only features in AD: the aggregation of phospho-tau in neurofibrillary tangles (Walton, 2006), synaptic and neuronal loss, and glial and inflammatory responses are other important characteristics (Turner, 2006). Endoplasmic reticulum (ER) dysfunction could be relevant in AD because the accumulation of unfolded or misfolded proteins in ER induces oxidative stress (Nakagawa et al., 2000). GRP78 is a ER resident

Offprint requests to: Prof. L.F. Rodella, Department of Biomedical Sciences and Biotechnology, Unit of Human Anatomy, University of Brescia, Viale Europa, 11, 25123 Brescia, Italy. e-mail: rodella@med.unibs.it

molecular chaperone that seems to be involved in Aß, metabolism and it has been observed that it is reduced in the brain of AD patients (Katayama et al., 1999).

To better understand the relationship between AD and exposure to Al, in this study we investigated the correlation between the expression of Aß and GRP78 in the cortex of mice chronically treated with Al.

Materials and methods

Animal treatment

Forty male (20 days old ; 13.2±2.8 g b.w.) C57BL/6 mice were used in this study. They were housed in a controlled environment, at regular 12-h light/dark cycle in a temperature controlled (20±1°C) room. Food and water were provided ad libitum. Twenty mice were treated with 2.5% aluminium sulphate (Sigma, St. Louis, MO, USA) in tap water (Hermenegildo et al., 1999) for 2, 4, 6 and 12 months (5 animals/group) and 20 mice, used as controls, were treated with tap water alone. All the animals were anaesthetised with sodium pentobarbitone and perfused with 4% paraformaldehyde in phosphate buffer. Every effort was made to minimize animal suffering and the studies were performed according to Italian Law on the protection of laboratory animals. All the experimental procedures were approved by the Italian Ministry of Health.

Tissue processing

The brains were dissected out, post fixed in the same fixative for 12 hours and cryoprotected in 30% sucrose for 24h. Ten serial sections (25 μm thick) from the cortex of each animal were cut by a cryostat between the bregma +1 mm and bregma -3 mm according to Franklin and Paxinos atlas (Franklin and Paxinos, 1997). Some sections were processed for the demonstration of ß-amyloid and adjacent sections were stained for GRP78 immunohistochemistry or with toluidine blue for morphological control.

ß-amyloid immunohistochemistry

The sections were incubated in 70% formic acid for 30 minutes to reduce antigen's denaturation or diffusion from their original position. After a short washing in 10% PBS, the sections were incubated in normal goat serum (10% in PBS containing 0.1% Triton X-100) for 30 min followed by rabbit polyclonal primary antiserum directed against ß-amyloid (Oncogene Research Products, San Diego, CA, USA) 1:200 in PBS containing 3% normal goat serum and 0.1% Triton X-100, for 24 h at 4°C. After incubation in the primary antiserum, the sections were sequentially incubated in biotinylated goat anti-rabbit immunoglobulins and avidin-biotin peroxidase complex (Vector Labs, Burlingame, CA, USA). The reaction product was visualized using hydrogen peroxide (3%) and diaminobenzidine (DAB 5mg/10 ml PBS) (Sigma). The control of immunohistochemistry was performed omitting the primary antibody and incubating the sections with non-immune goat serum.

GRP78 immunohistochemistry

The sections were incubated as described for ß-amyloid immunohistochemistry, but without incubation in formic acid 70%, using the rabbit polyclonal antibody against GRP78 (Stressgene, Victoria, BC, Canada) diluted 1:1000.

Integrated optical density evaluation of GRP78 staining

GRP78 staining intensity was computed as integrated optical density (IOD) and measured in 200 GRP78 positive neurons for each experimental group. The neurons were randomly selected by two different investigators unaware of the animal group assignement and their boundaries were manually traced before being measured. Digitally fixed images of the neurons at 100x magnification were analysed under light microscope (Olympus, Germany) equipped with an image analyser (Image Pro Plus, Milan, Italy) that automatically calculated the IOD. All the data were analysed by ANOVA and Bonferroni's test.

GRP78 positive neurons counting

The GRP78 positive neurons were counted using a light microscope.The analysis was made by two different investigators unaware of the animal group assignement. We count the total number of positive neurons in 10 randomly selected fields for each experimental group. The number was compared with the total number of neurons showed by toluidine blue staining. All the data were analysed by ANOVA and Bonferroni's test.

Results

Animal parameters

The data about the body weights of the animals at the different experimental times, were reported in Table 1. The mortality was not statistically different in control and Al treated mice and was respectively zero and one

Table 1. Body weight of the animals at the different experimental times.

Months of treatment	Control animals Water	Al Treated animals 2.5% $AlSO_4$ in Water
0	13.2±2.8 g b.w.	13.2±2.0 g b.w.
2	23.0±3.9 g b.w.	24.3±3.6 g b.w.
4	26.3±3.6 g b.w.	24.8±5.1 g b.w.
6	29.5±4.2 g b.w.	27.1±4.1 g b.w.
12	33.4±4.7 g b.w.	30.8±4.2 g b.w.

animal.

The daily amount of water was 4±2.5 ml/mouse and of water supplemented with $Al_2(SO_4)3$ was 3.8±3.1 ml/mouse, without any significant differences between control and Al treated animals at 2, 4, 6 or 12 months. Even if in this study we did not make specific behavioural tests, we did not observe particular behavioural differences among the different groups of animals.

ß-amyloid immunohistochemistry

In the cerebral cortex of control animals the ß-amyloid immunostaining was undetected at all experimental times. In these animals we observed neither deposits of Aß around the vessels nor in neurons (Fig. 1a). In the cerebral cortex of the Al treated animals we observed Aß immunoreactivity around the vessels, which appeared brown due to DAB reaction. The immunoreactivity was present mainly at the vessel's bifurcations levels (Fig. 1b-d). Even if in this study we did not quantify the amount of the Aß, we observed that in the majority of the animals the deposits of Aß were found especially at 12 months. The immunoreactive deposits were scattered in all cortical areas, nevertheless they are more evident in the hippocampal cortex. We did not observe positive staining of neurons for Aß at any experimental time.

GRP78 immunohistochemistry

In the cerebral cortex of control animals we observed a large number of GRP78 stained neurons. These neurons showed from moderate to intense

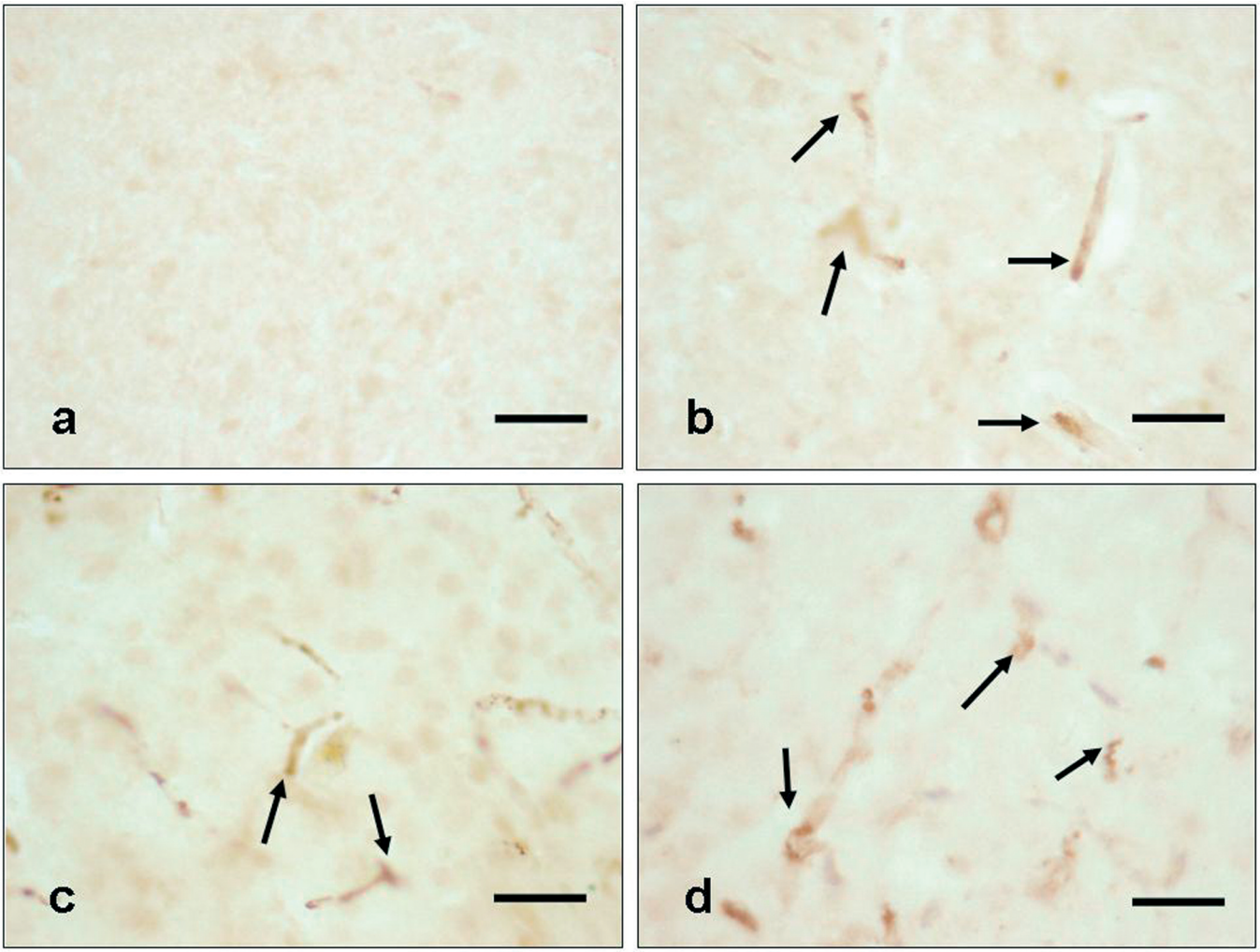

Fig. 1. Immunohistochemical localisation of ß-amyloid in the cerebral cortex of control mice **(a)**, Al treated mice for 4 **(b)**, 6 **(c)** and 12 **(d)** months. Arrows indicate the vascular depositions of ß-amyloid. Bar: 50 μm.

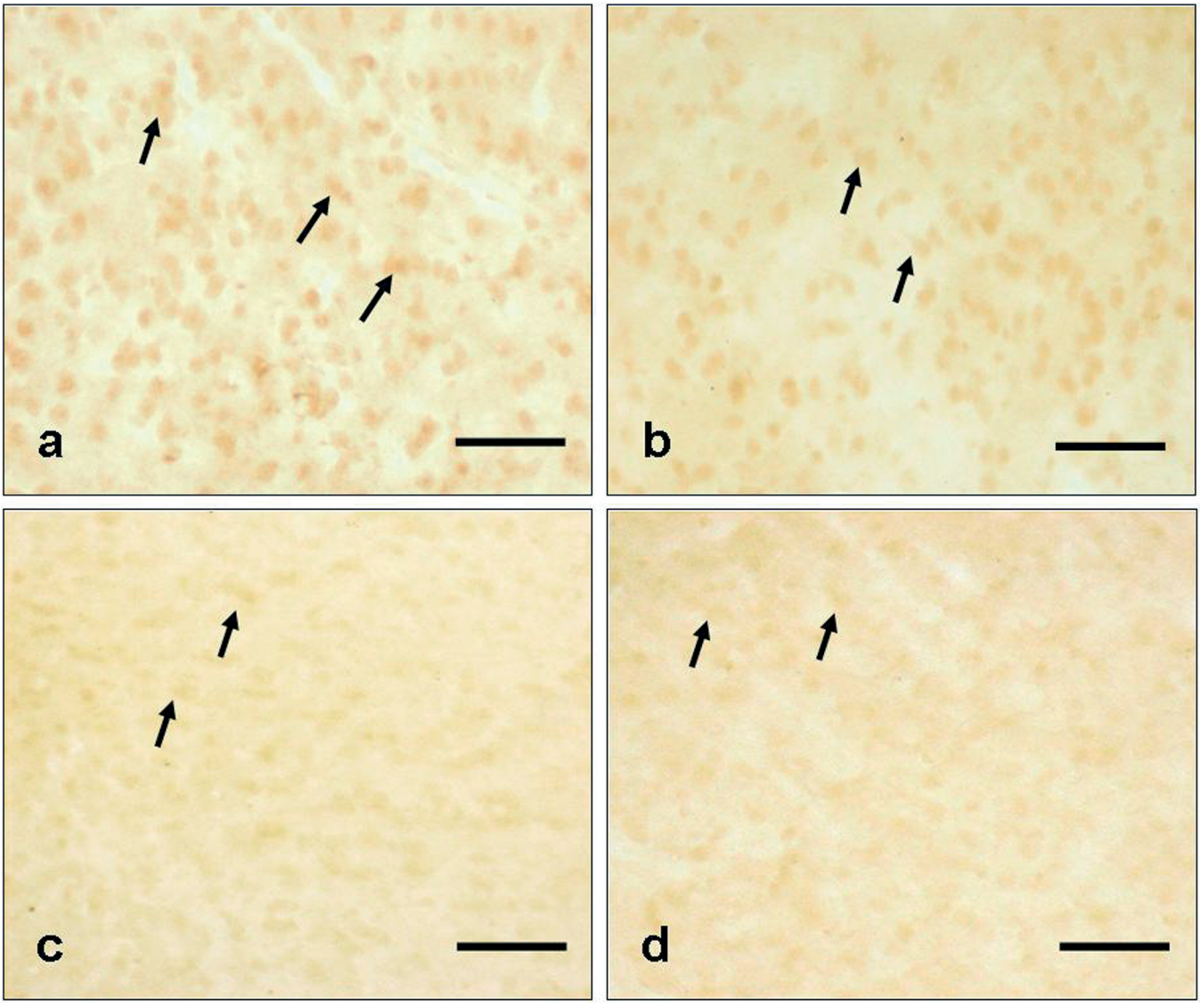

Fig. 2. GRP78 positive neurons in the somatosensory cortex in control mice **(a, b)** after 2 months **(c)** and after 12 months **(d)** of Al administration. Note the faint staining of the neurons in Al-treated animals. Arrows indicate the positive neurons. Bar: 100 μm.

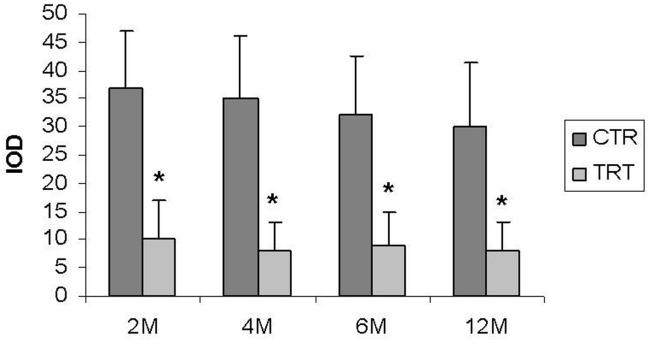

Fig. 3. Analysis of the integrated optical density (IOD) of GRP78 immunostaining in control (CTR) and 2, 4, 6, 12 months Al treated (TRT) mice. **P*<0.05 vs CTR

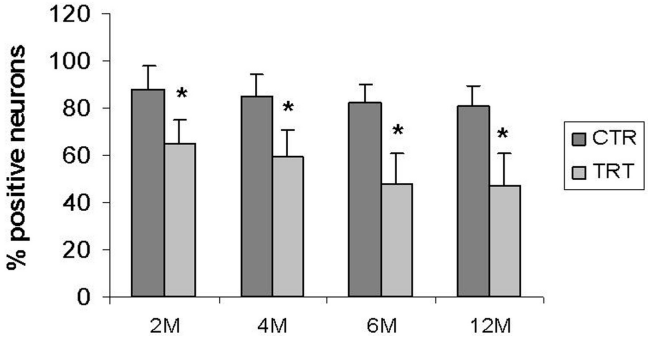

Fig. 4. Quantitative evaluation of positive GRP78 neurons in control (CRT) and 2, 4, 6, 12 months Al treated (TRT) mice. **P*<0.05 vs CTR .

immunostaining (Fig. 2a,b). In the cerebral cortex of the Al treated animals we observed that immunostaining of GRP78 was lower than in control animals. In the Al treated animals, we found about 50% of moderately stained neurons whereas the other neurons appeared only very faintly stained. The intensity of GRP78 immunostaining decreased in the Al treated animals at all experimental times (Fig. 2c,d). The decrease of GRP78 staining was evident at short-time of treatment (2 and 4 months) and did not appear to be incremented significantly after longer exposition (6 and 12 months). The quantitative data about GRP78 immunostaining were summarised in Figs. 3 and 4.

Discussion

There is evidence that Al is a potential neurotoxic agent (Strong et al., 1996; Golub and Tarara, 1999) and there is also an increasing amount of evidence which suggests the involvement of the Al ion in a variety of neurodegenerative disorders, such as Alzheimer's disease (Perl and Brody, 1980; Harrington et al., 1994; Kawahara, 2005). In the present work we proposed to investigate if a chronic aluminium intake (aluminium sulphate 2,5% in drinking water for 2, 4, 6 and 12 months) exerts specific effects in the cerebral cortex of mice. In particular, we wanted to evaluate the possible variations in Aß, and GRP78 expression in the cerebral cortex of Al treated mice. The results obtained in this work showed that exposure to aluminium sulphate in drinking water resulted in deposits of Aß which were largely confined to vascular areas and especially at the bifurcations of the vessels. We found that Aß deposition was already present after 2 months of treatment, but that the highest pattern of immunohistochemical staining was detected following exposure over 12 months suggesting that accumulation of ß-amyloid was time-dependent. Our findings of a vascular associated Aß neuropathology are in agreement with the data reported by Exley and Esiri (2006), who showed that high levels of brain Al were coincident with a severe form of congophilic amyloid angiopathy (CAA) in individuals who were exposed to aluminium sulphate in their drinking water (Exley and Esiri, 2006). Since cerebrovascular deposition of Aß in human cerebral cortex is one of the first steps in the physiopathology of AD (Cummings et al., 1996), it is possible that Al exposure may accelerate or aggravate CAA and our mouse model appears to support such a notion.

Recent papers have shown that endoplasmic reticulum (ER) dysfunction, due to accumulation of unfolded or misfolded proteins in the ER provoked by various cellular stresses (e.g. oxidative stress), is relevant to the pathogenesis of Alzheimer's disease via the amyloid cascade (Nakagawa et al., 2000). Excessive or long-term stress results in apoptotic cell death, while cells respond to short-term stress by increasing transcription of genes encoding ER-resident chaperones (e.g. GRP78/BiP) to facilitate protein folding. GRP78/BiP (glucose-regulated protein) is a stress-response protein, which is induced by conditions that adversely affect endoplasmic reticulum (ER) functions. This protein is essential for the proper glycosylation, folding and assembly of many membrane-bound and secreted proteins (Mote et al., 1998). GRP78 is critical for maintenance of cell homeostasis and prevention of apoptosis (Yang et al., 2000) and its protective function in neurons exposed to glutamate and oxidative stress has been described (Yu et al., 1999). We reported that Al induced a decrease of GRP78 immunostaining in the cerebral cortex. These findings are in agreement with the observations of Katayama et al. (1999), who reported that GRP78 is reduced in the brain of AD patients (Katayama et al., 1999). A possible explanation for the relationship between AD and GRP78 could be that since the interactions between GRP78 and immature unfolded forms of APP in the ER may prevent APP translocation to distal compartments, therefore its cleavage to Aß by the γ-secretase under ER stress (Cupers et al., 2001), after a GRP78 reduction, the APP can be free to translocate in the late compartments and may reach a location suitable for cutting, resulting in Aß generation. This explanation is supported by a recent paper (Hoshino et al., 2006) showing that "in vitro" the over-expression of GRP78 or the inhibition of its basal expression decreased or increased respectively the level of Aß peptides in the culture medium. About the effect of Al on GRP78, several studies indicate that this metal induces stress in the ER of neurons and that this acts as cofactor in neurodegeneration (Ghribi et al., 2001, 2002), so we can suppose that a direct or indirect action of Al on GRP78 reducing its expression in the ER and promoting the accumulation of misfolded proteins. In addition we showed that the decrease of GRP78 is not time dependent and is evident also after short time of Al treatment. This indicates that Al early affects GRP78 and reinforces the evidence about the possible pivotal role of this ER chaperone in preventing amyloid deposition. In conclusion, the results of this study provide a further evidence, in an experimental mouse model, that Al is a neurotoxic metal and that it has an impact related to the development of the vascular component of AD pathology, involving ER chaperones .

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