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REGULAR ARTICLE

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Aluminum impairs gap junctional intercellular communication between astroglial cells in vitro

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Abstract The purpose of the present study was to investigate the effect of aluminum on gap junctional intercellular communication (GJIC) in cultured astrocytes. In the CNS the extracellular environment and metabolic status of neurons is dependent upon astrocytes, which are known to exhibit GJIC. This cell-to-cell communication provides a cytoplasmic continuity between adjacent cells, allowing exchange of diverse ions, second messengers, and metabolites. To study the effects of aluminum intoxication on GJIC in cultured glial cells, astroglial cell cultures obtained from fetal rat brains were exposed to aluminum lactate for 2–6 weeks. To demonstrate the metabolic coupling of neighboring cells, the technique of microinjection of the gap junction permeable substance neurobiotin was performed. Whereas in controls intensive GJIC was observed by dye transfer of neurobiotin from the microinjected cell into the adjacent astrocytes, aluminum treatment significantly impaired this cellular communication. As aluminum is known to affect cytoskeletal elements, additional investigations into the organization of intermediate filaments (glial fibrillary acid protein, GFAP) and microfilaments in control astrocytes and subsequent aluminum exposure were performed with the aid of fluorescence microscopy and rapid-freeze, deep-etch electron microscopy. Aluminum exposure led to an aggregation of GFAP-positive filaments near to the cell nucleus, accompanied by a destruction of the actin cytoskeleton, especially close to the cell membrane. Ultrastructurally these data could be verified as prominent areas without actin filaments contacting the cell membrane detectable in aluminum-treated astrocytes. Immunohistochemical staining of Cx43 revealed an impaired trafficking of this connexin into the cell prolongations following aluminum treatment, although electron-microscopic data revealed that gap junctions between adjacent astrocytes were still present after aluminum incubation for 24 days. In conclusion, in cultured astrocytes the morphological integrity of microfilaments and the intermediate filament network seem to be fundamental for the translocation of connexins from Golgi complex into the cellular prolongation to exhibit proper and extensive cellular communication through gap junctions.

Keywords Cytoskeleton · Connexin 43 · Microinjection · Electron microscopy · Dye transfer · Cell culture

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Introduction

Aluminum is suggested to be involved in the development of neurodegenerative disorders in humans by producing profound alterations of the cytoskeleton of neuronal and glial cells. In animal experiments aluminum intoxication shows similarities to the pathology observed in neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Perl et al. 1982; Troncoso et al. 1985, 1986; Leterrier et al. 1982; Sass et al. 1993). In neuronal cells aluminum affects an aggregation of neurofilaments (DeEstable-Puig et al. 1971; Theiss and Meller 2001), and moreover induces functional impairment of axonal transport processes (Theiss and Meller 2001). DeEstable-Puig and coworkers (1971) have demonstrated that aluminum exposure not only affects an accumulation of fibrillary material in neuronal perikarya, dendrites and axons but also in glial cytoplasm and astrocytic nuclei, termed as gliafibrillary degeneration. Studies on the binding capacity of aluminum in different brain cells showed that aluminum interacts more with astrocytes than with neuronal cells (Rao 1992).

In the nervous system the extracellular environment of neurons is dependent upon astrocytes, which are functionally connected via gap junctions (Dermietzel et al. 2000). Gap junctions are clusters of intercellular channels (Spray et al. 1998), which are permeable to molecules up to 1 kDa and allow a direct exchange of ions be-

tween cells (Rozental et al. 2000). Additionally they provide a conduit for removal of extracellular K^+ and neurotransmitter from the local environment of neurons (Dermietzel and Spray 1993). In addition, gap junctions mediate the delivery of metabolites throughout the network (Giaume et al. 1997) and increase the buffer capacity for dilution of toxic or metabolically active compounds (Blanc et al. 1998).

In correlation with aluminum intoxication the knowledge of the effects is based on morphological investigations, but up to now no data on functional aspects of aluminum intoxication on cultures of rat brain astrocytes have been available. Therefore we performed microinjection of the gap junction permeable substance neurobiotin to quantitatively analyze gap junctional intercellular communication (GJIC) in cultured astrocytes subsequent to aluminum exposure. The presented data demonstrate a relation between aluminum intoxication and impaired GJIC in astrocytes. These observations have been correlated with morphological changes of the distribution of gap junctions, intermediate filaments and microfilaments seen by means of immunohistochemistry and cryo-electron microscopy to unravel new aspects of the close relationship between GJIC and the cytoskeleton.

Materials and methods

Cell cultures

Astrocyte cultures of rat brain were prepared as described previously (Meller and Waelsch 1984). Briefly, forebrains of rat fetuses of the 18th to 20th embryonic day were removed under sterile conditions, cut into small pieces and dissociated with 0.05% trypsin. After the dissociation procedure (Meller et al. 1969), the fetal brain cells were cultured in 25-ml flasks in Eagle's minimum essential medium (MEM; ICN, Germany), supplemented with 10% horse serum, 2% fetal calf serum, 2% chicken embryonic extract, 1% L-glutamine (2 mM), 0.6% glucose and 50 µg/ml gentamycin. The final concentration of the cells was 1×10^6/ml. Cell cultures were kept at 37°C in a modified atmosphere of 5% CO_2 in air. The medium was changed twice a week, and subcultures were made at weekly intervals using 0.05% trypsin and 0.025% ethylenediaminetetraacetic acid (EDTA). During the first and second subcultures the flasks were coated with *l*-polylysine (Yavin and Yavin 1974). Allohydroxyl-*l*-proline (HPA) (100 µg/ml) (Kao and Prockop 1977) was added to the MEM medium for 2–3 days or cytosine arabinoside (10^{-5} M) and uridine (35 µg/ml) for 24 h. For the procedure of microinjection or immunohistochemistry the cell suspension (~1×10^5 cells/ml) was cultured on glass coverslips (∅ 32 mm, Kindler, Germany) covered with rat-tail collagen in a CO_2 incubator (5% CO_2, 37°C, 90% humidity).

Experimental medium contained 340 µM aluminum lactate, 328 µM citric acid and 2 µM L-glutamic acid as described by Jones and Oorschot (1998). Experimental medium that had L-glutamic acid added to it did not have L-glutamine added to the basal medium. The effect of aluminum was investigated by treatment of astrocyte cultures for periods of 13 days, 24 days, 31 days, 37 days, and 41 days. To determine whether aluminum effects were reversible, incubation for 7 days in the nutrient medium without aluminum was carried out in groups that were treated previously for 24 days with aluminum. As controls, astrocytes treated for the same duration with all the media supplements (328 µM citric acid, 2 µM glutamic acid) except aluminum were used.

Immunohistochemistry

The antibodies used were mouse monoclonal IgG (mAb) to connexin 43 (Cx43, MAB3067; Chemicon, USA) and to glial fibrillary acid protein (GFAP, Sigma, Germany). For immunohistochemistry, cell cultures were fixed in 4% paraformaldehyde (PFA) for 30 min followed by a 1% Triton X-100 incubation in phosphate-buffered saline (PBS) for 15 min. Thereafter astrocyte cultures were intensively washed in PBS and incubated with anti-Cx43 (1:100) or anti-GFAP (1:400) in PBS + 0.3% Triton X-100 (PBS-T) overnight at 4°C. Afterwards, to block non-specific binding sites, cell cultures were treated with 10% (w/v) normal goat serum in PBS-T for 30 min. The samples were then reacted with the secondary antibody goat anti-mouse IgG (Alexa 488 conjugated) (1:250) in PBS-T for 2 h at room temperature (Molecular Probes, Germany). To additionally demonstrate the actin cytoskeleton in astrocytes stained with anti-GFAP, a histochemical staining with 0.5 µl/ml phalloidin-rhodamine (Boehringer, Germany) in PBS for 30 min followed. Finally, cell cultures were rinsed in PBS and coverslipped.

Microinjection of neurobiotin

Microinjection of neurobiotin was done by the recently described technique (Meller 1992; 1994). Briefly, microinjection of neurobiotin (neurobiotin hydrochloride, 8% in distilled water, Vector Laboratories, Camon, Germany) into a single astrocyte was achieved under visual control on an inverse microscope with the aid of phase-contrast optics (Zeiss, Germany) and a pressure injection device (Eppendorf, Germany) fitted with sterile glass capillaries (∅ 0.2–0.5 µm, Femtotips, Eppendorf). The pressure microinjector maintained a constant pressure of 80–100 hPa on the tip, and the injection was performed by increasing the pressure to 100–120 hPa for about 0.6 s. Altogether 452 astrocytes were microinjected. After the microinjection, glial cells were incubated for 1 h, which was sufficient to demonstrate cell coupling between adjacent astrocyte cells by neurobiotin transference.

To illustrate that in fact only a single cell was injected with neurobiotin, we performed double injection of neurobiotin together with the gap junction impermeable substance Lucifer yellow-dextran (5% in distilled water, D-1825, Molecular Probes, Germany).

Neurobiotin labeling

Cell cultures were fixed in 4% PFA + 0.02% picric acid in 0.1 M phosphate buffer (PB, pH 7.4) for 1 h at room temperature. After intensive washes, a conventional ABC-peroxidase (Vector Elite-kit, Burlingame, CA) was applied to the cultures for 1 h, before visualization of neurobiotin was done with 3,3'-diaminobenzidine (DAB) (Graham and Karnovsky 1966). In addition, in some probes neurobiotin was fluorescently visualized by extravidin-rhodamine (1:400, L-3642, Sigma).

Analysis of dye spreading

Fluorescent probes, microinjected with neurobiotin and Lucifer yellow-dextran, were evaluated by confocal laser scanning microscopy with the addition of differential interference contrast (DIC) filters (LSM 510, Zeiss). DAB probes of neurobiotin-coupled cells were quantitatively analyzed (MetaMorph 3.3, Visitron, Germany). Here, each condition consisted of at least 18 microinjected cells. Statistical analysis of all data was performed by using the software "Statistica for Windows" (Release 4.5, StatSoft, Inc. 1993, USA). An analysis of variance (ANOVA) with additional post hoc analysis (Newman-Keuls test) was applied to compare cell coupling in controls with that in aluminum-treated cells.

Transmission electron microscopy

Specimens for transmission electron microscopy (TEM) were fixed in 2.5% GA in PB, postfixed in OsO_4, dehydrated and embedded in Araldite or Epon.

Rapid-freeze deep-etch electron microscopy

Rapid-freeze deep-etch electron microscopy was performed as previously reported (Meller 1989). Briefly, cell cultures were frozen using a Balzers' Cryojet (Balzers, Liechtenstein) apparatus. The specimens were then fractured and etched in a Balzers' freeze-etching device. Platinum was applied at an angle of 35° to the rotating specimens. The platinum-carbon replicas were cleaned with household bleach, washed in distilled water, and placed unsupported on mesh grids.

Results

Microinjection of neurobiotin

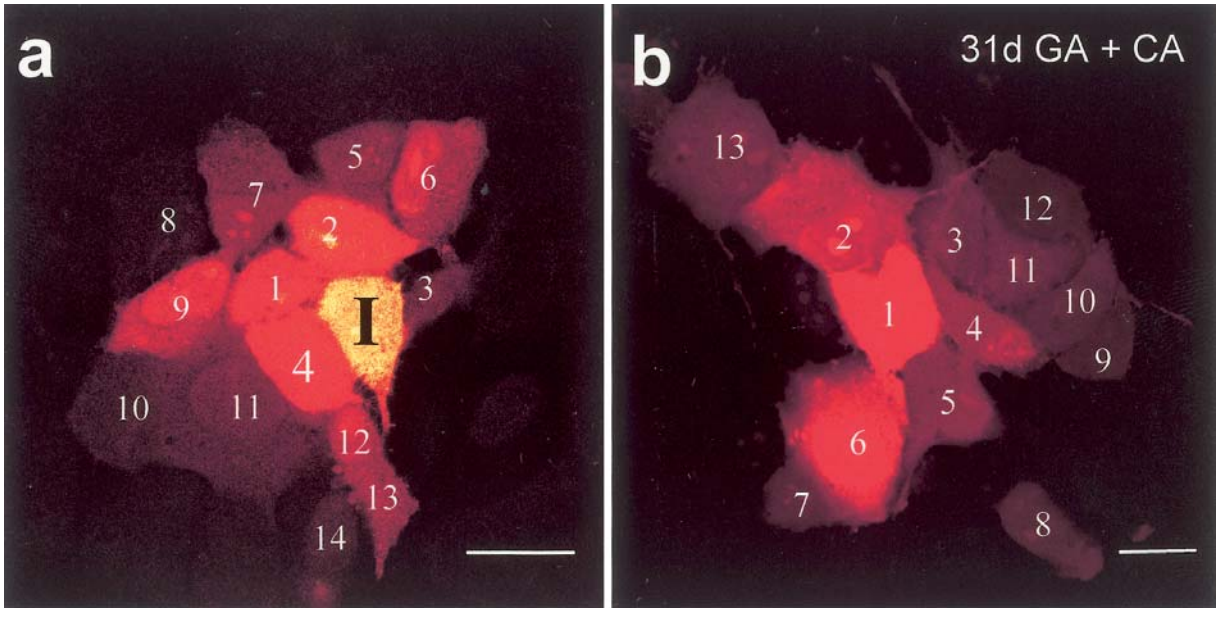
After several passages, astroglial cells were cultivated to exhibit a monolayer of confluent cells. To demonstrate the technique of microinjection as a suitable tool for investigations on GJIC, a mixture of neurobiotin and the gap junction impermeable substance Lucifer yellow-dextran was microinjected (Fig. 1a). Whereas dye spreading of neurobiotin was prominent in numerous adjacent astrocytes, the microinjected astrocyte turned yellow, indicating that Lucifer yellow-dextran remained in the microinjected cell and did not couple within other cells.

GJIC in astrocytes in vitro

In cultured astrocytes dye transfer from the microinjected astrocyte to neighboring cells was observed in radial fashion 1 h after injection (Figs. 1, 2). Here, in the same cell culture, and therefore with exactly the same culture conditions, most cells displayed intensive GJIC, whereas a few cells showed weak dye transfer of neurobiotin. Altogether the quantitative analysis of dye spreading in 156 cells revealed an average of 14.1±9.3 coupled cells in 22 cell cultures. Whereas long-term exposure with medium supplemented with citric acid and glutamic acid had no effect on GJIC (Fig. 1b), medium also containing aluminum for 24 up to 41 days significantly impaired GJIC (n=369, P<0.001). At this juncture longer aluminum exposure times effect a continuous decrease of GJIC (Figs. 2c–f, 3a). In detail, aluminum treatment for 24 days diminished the GJIC to 11.4±6 stained cells (n=134, P<0.05). This decline of GJIC was even more evident after 31 days aluminum incubation (10.8±5.5 coupled cells, n=38, P<0.05). Aluminum exposure for 37 days and 41 days reduced cell communication through gap junctions to 8.8±7.1 (n=23, P<0.05) and 8.7±5.4 (n=18, P<0.05) coupled cells, respectively. Consequently permanent aluminum treatment for periods of 24 days up to 41 days significantly impaired, but did not completely block, GJIC.

The effect of aluminum exposure on the GJIC was shown to be reversible (Figs. 2g, h, 3b). After 24 days of aluminum exposure followed by incubation in nutrient medium for 7 days, the recovered GJIC of microinjected

Fig. 1a, b Microinjection of neurobiotin. **a** Demonstration of dye transfer by simultaneous injection of the gap junction permeable substance neurobiotin (*red*) and the gap junction impermeable substance Lucifer yellow-dextran (*yellow*) into one astrocyte. One hour after microinjection, 14 coupled astrocytes could be detected (*red*), whereas Lucifer yellow-dextran was trapped in the microinjected cell. **b** Representative example of GJIC in cultured astrocytes exposed to glutamic acid (*GA*) and citric acid (*CA*). For instance, cells were treated with GA and CA for 31 days, before GJIC was visualized by microinjection of neurobiotin into a single cell. *Numbers* indicate coupled cells after 1 h postincubation. No impairment of intercellular communication compared to untreated controls was detected. *Scale bar* 20 µm



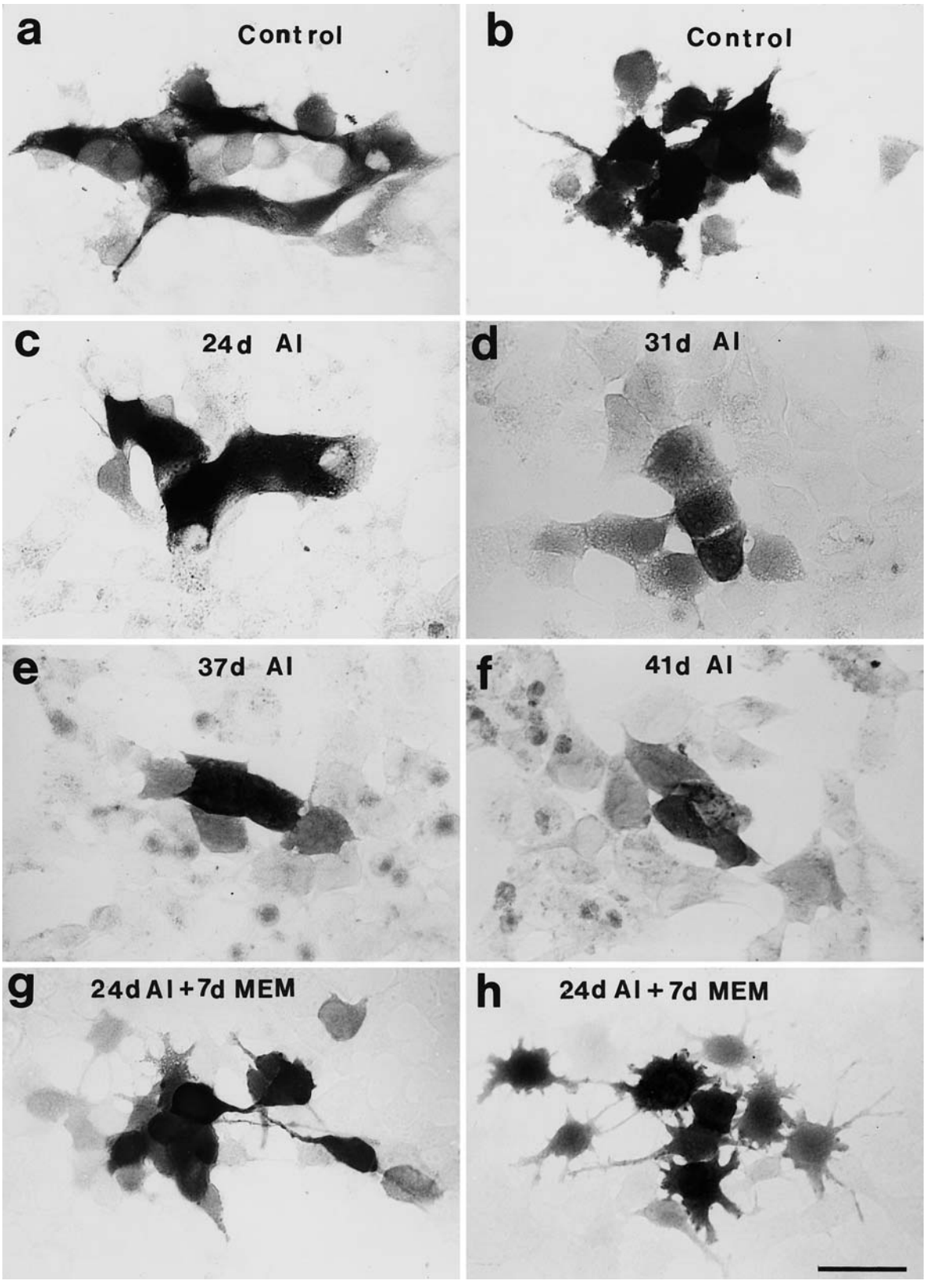


Fig. 2a–h Gap junctional intercellular communication affected by aluminum intoxication. **a, b** Control astrocytes exhibit intensive gap junctional intercellular communication, demonstrated by dye spreading of neurobiotin 1 h after microinjection. In contrast, aluminum intoxication for 24 days **(c)**, 31 days **(d)**, 37 days **(e)** and 41 days **(f)** impaired this cell-to-cell communication subsequent to neurobiotin injection into a single astroglial cell. After aluminum treatment for 24 days followed by incubation in nutrient medium for 7 days, dye transfer of neurobiotin appeared to be normal. *Scale bar* 50 μm

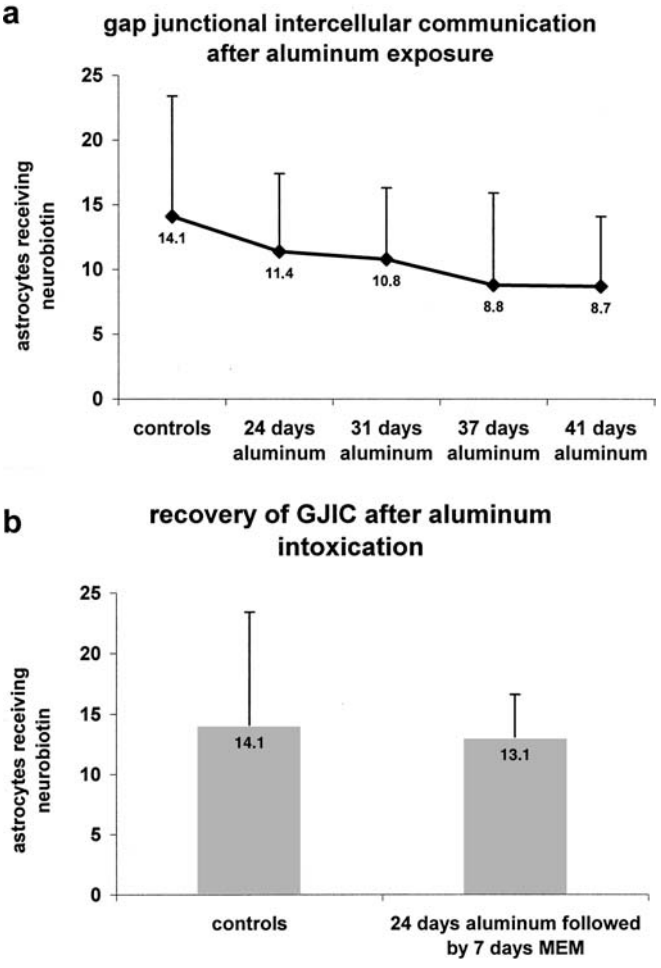


Fig. 3a, b Quantitative analysis of GJIC after aluminum exposure. **a** Quantification of gap junctional intercellular communication in astrocytes subsequent to aluminum exposure for periods of 24 days up to 41 days in vitro. Aluminum treatment for various times significantly impaired gap junctional intercellular communication. In **b** the reversible effect of aluminum intoxication on GJIC was quantified in cultured astrocytes. Values represent means ± SEM

neurobiotin was estimated to be 13.1±3.5. Statistically there was no significant difference between dye transfer in cells after recovery of aluminum intoxication as compared to untreated control cultures (n=83, P=0.50).

Effect of aluminum exposure on the cytoskeleton

As demonstrated by immunohistochemistry, cultured astrocytes were GFAP positive, with intermediate filaments spanning through the whole cell in a regular and organized structure, even reaching up to the small cell prolongations in controls (Fig. 4a, b). In addition, a prominent labeling of actin filaments was observable. In detail dots of actin-positive structures were visible in the cytoplasm, while nearly the whole plasma membrane of astrocytes seemed to be covered by actin filaments in controls. In contrast, aluminum intoxication for 24 days exhibited a toxic action on glial cells in vitro (Fig. 4c, d). In any case the effect on intermediate filaments was not prominent, but an aggregation of GFAP-positive elements could be revealed near to the perikaryon after aluminum treatment for 24 days. Also the staining of actin filaments differed significantly from that in controls. Subsequent aluminum treatment phalloidin labeling was weak in the cytoplasm of cultured astrocytes and discontinuous along their cell membrane, indicating an interference of aluminum on the distribution on actin filaments with regard to the plasma membrane. Additionally we investigated whether the effect of aluminum on the cytoskeleton was reversible or not. Therefore cell cultures were treated with aluminum for 24 days, depleted of the drug by thorough washing and incubated in nutrient medium for 7 days. Following the wash-out treatment, the organization of cytoskeletal elements in the astrocytes appeared to be normal by fluorescence immunohistochemistry (Fig. 4e, f). In addition to the prominent staining of GFAP in the cytoplasm, actin filaments could be detected along the whole plasma membrane. These data confirm the assumption that the organization of the cytoskeleton recovered within a few days after depletion of aluminum from the cell culture medium.

Ultrastructural findings corroborate the fluorescence-microscopic data on aluminum-induced changes in astrocytes. Electron-microscopic observations revealed a highly organized cytoskeleton in cultured astrocytes, with prominent expression of intermediate and actin filaments (Fig. 5a). In these cells thin filaments, which were likely to be actin filaments, got up to the cell membrane, whereas intermediate filaments were localized among them. Aluminum exposure for 13 days seemed to disrupt the cytoskeleton, for instance, numerous globular particles occurred in the cytoplasm, without any obvious filamentous organization (Fig. 5b). Here, globules without any connection of thin filaments and the cell membrane were observable. The same altered cytoskeletal morphology was obvious after 24 days aluminum treatment (not shown).

Distribution of gap junctions in cultured astrocytes

With specific antibodies to Cx43, labeling was prominent in the perikaryon as well as in the cell prolongations

Fig. 4a–f Distribution of actin filaments and GFAP in cultured astrocytes exposed to aluminum. Histochemical staining of actin (*red*) and GFAP (*green*) in controls **(a, b)**, after 24 days of aluminum exposure **(c, d)**, and after reversal of aluminum exposure **(e, f)**. In **a, b** *arrows* indicate actin filaments in contact with the cell membrane, whereas after aluminum treatment the actin filaments seem to be collapsed **(c, d)**. *White arrows* indicate segments of the cell membrane without actin filaments, accompanied by an aggregation of GFAP-positive elements near to the perikaryon (*blue arrows*). After 24 days aluminum treatment, followed by 7 days incubation in nutrient medium, the actin cytoskeleton completely recovered, as moderate actin staining at the cell membrane was detectable (*arrows*). In addition, an aggregation of GFAP was no longer observed in the cytoplasm. *Scale bars* 20 µm ▶

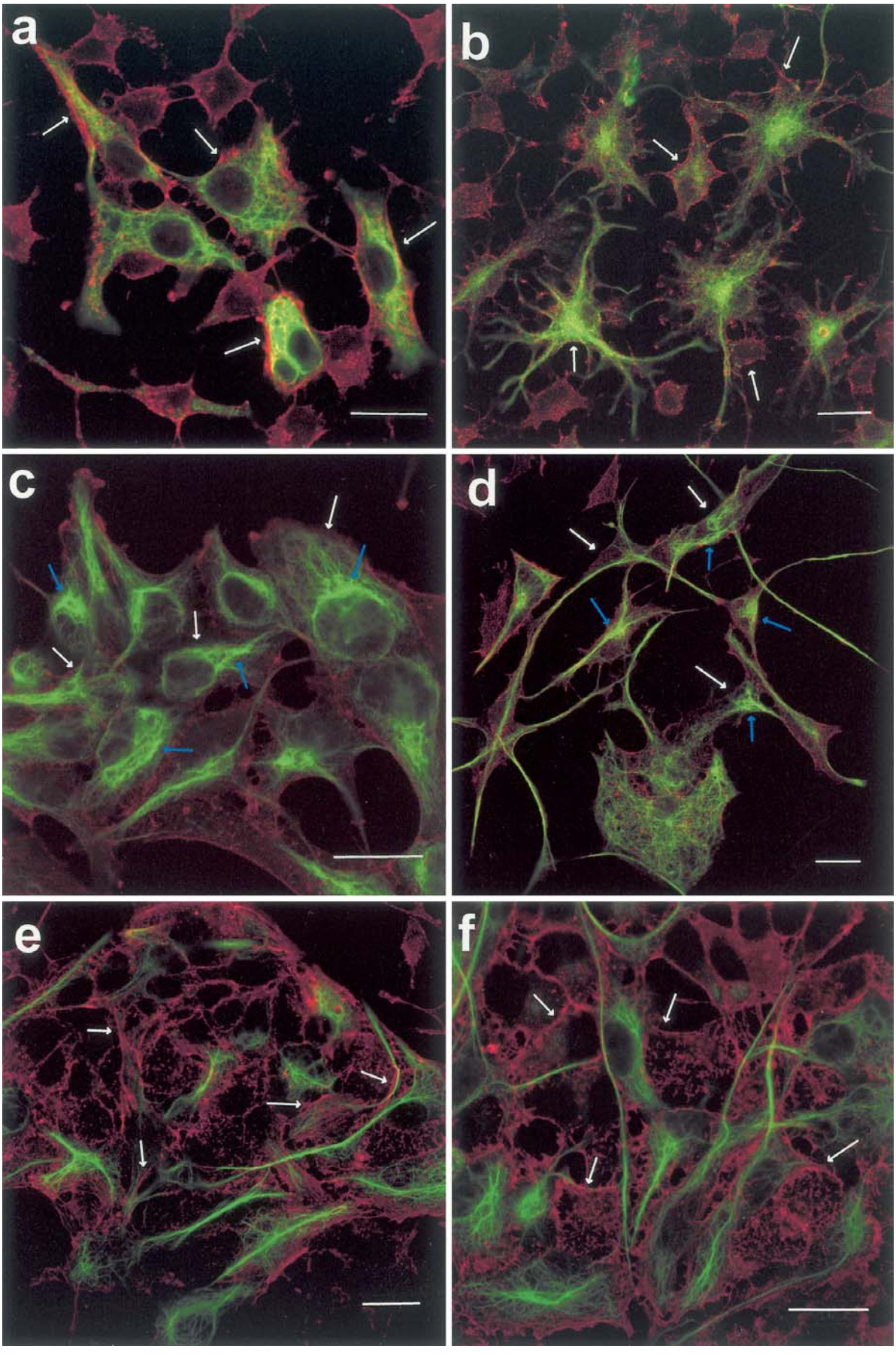


Fig. 4a–f Legend see page 147

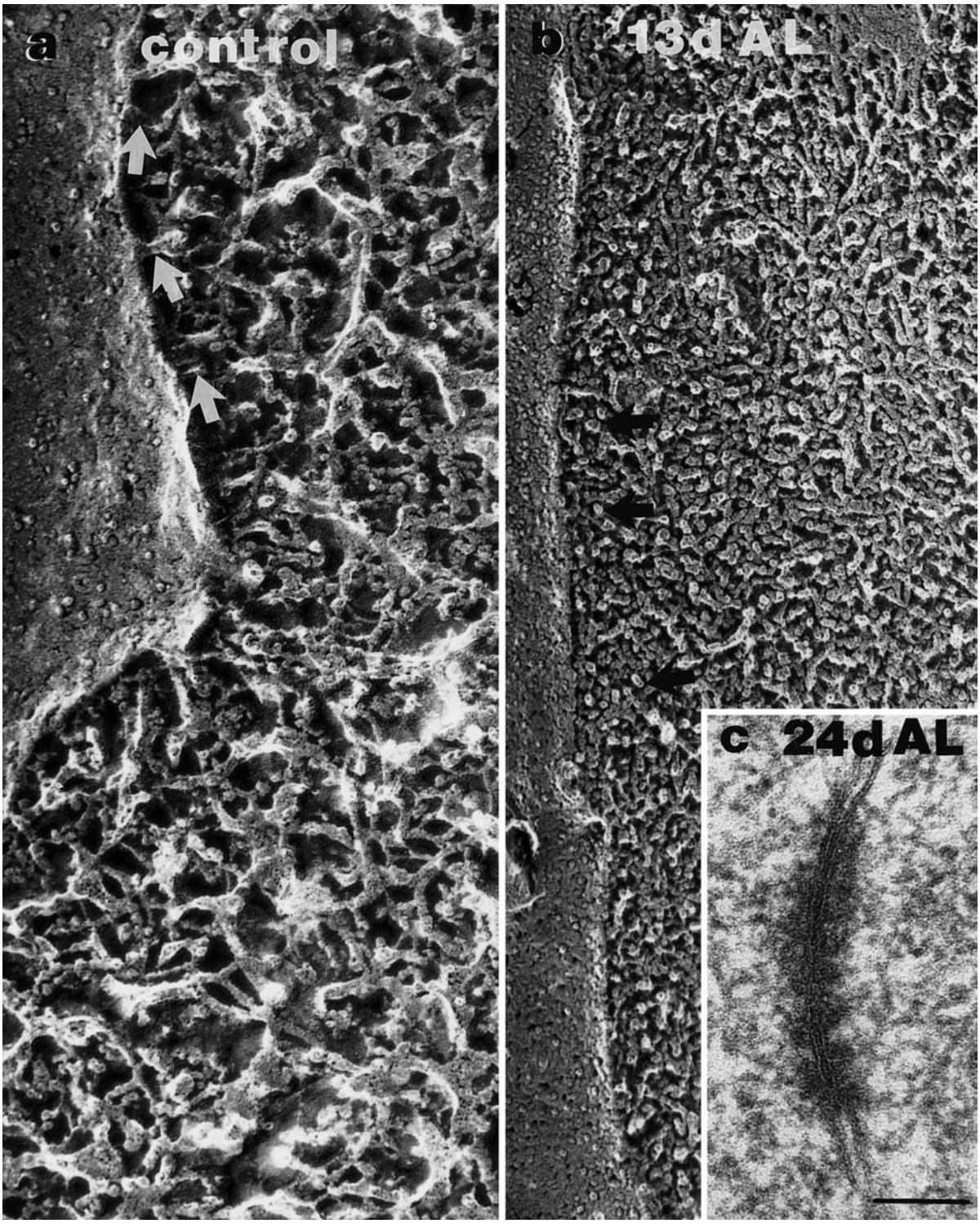


Fig. 5a–c Rapid-freeze deep-etch electron microscopy of cultured astroglial cells. Electron micrographs of astroglial cytoskeleton in controls **(a)** compared with aluminum-exposed cells for 13 days **(b)**. **a** High-magnification rapid-freeze deep-etch electron micrographs demonstrate the normal distribution of cytoskeletal elements, in which cytoskeletal elements, probably actin filaments, insert themselves into the cell membrane (*arrows*). Subsequent to aluminum treatment for 13 days, the cytoskeleton was disturbed, as globular aggregations of cytoskeletal elements (*arrows*), which probably represent GFAP and actin filaments, occurred near to the cell membrane. **c** Transmission electron microscopy of a gap junction after aluminum treatment for 24 days. *Scale bars* 100 nm

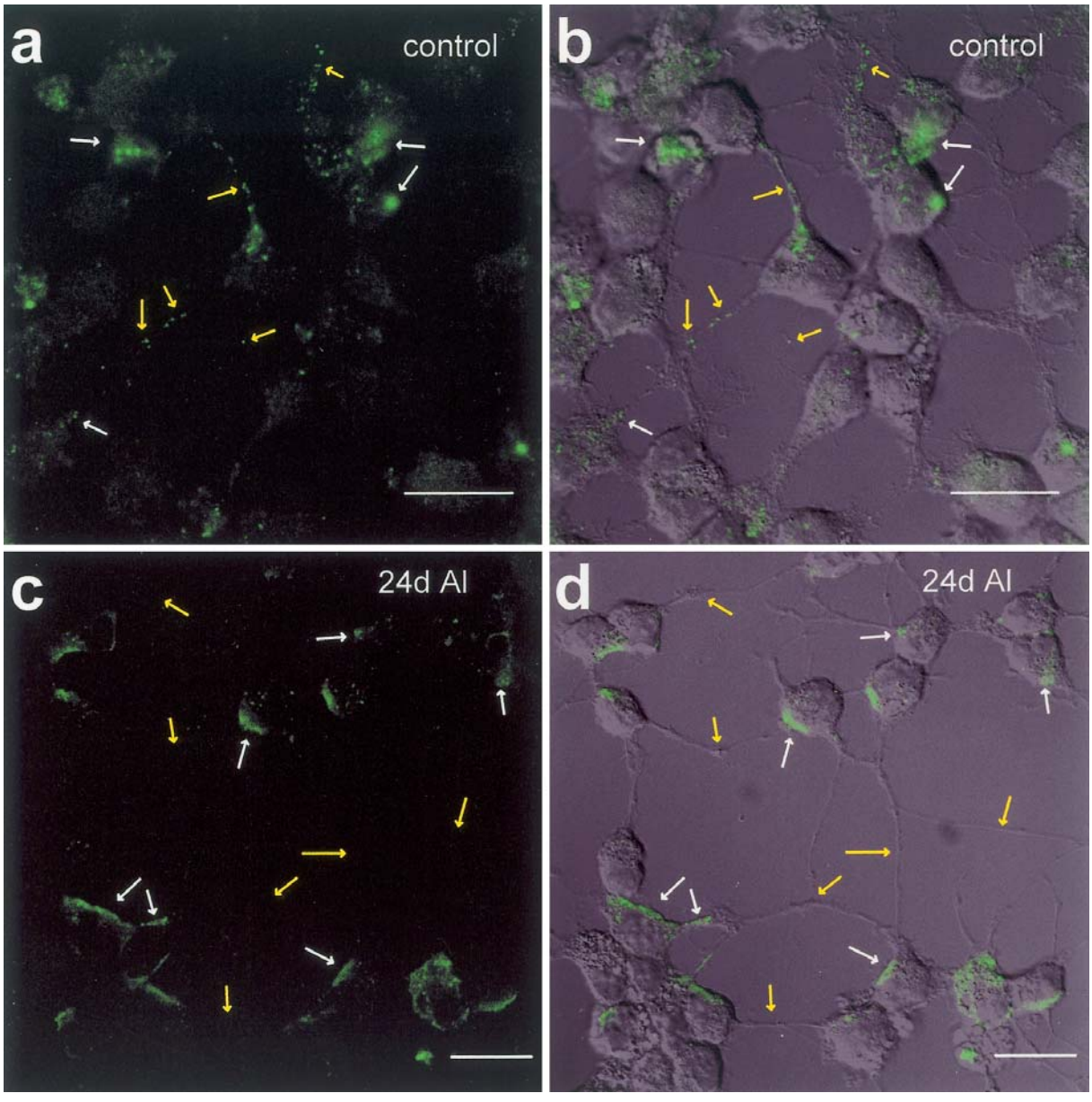


Fig. 6a–d Immunohistochemical staining of Cx43 in controls and aluminum-exposed cultured astrocytes. Distribution of Cx43 in controls **(a, b)** and after 24-day aluminum exposure **(c, d)**. To obtain a better orientation of Cx43 distribution in regard to the cell morphology of astrocytes, DIC pictures were added **(b, d)**. With the aid of confocal laser scanning microscopy, Cx43 (*green*) was detected in the cytoplasm as well as in the cellular prolongations of cultured astrocytes **(a, b)**. *White arrows* indicate intensive Cx43-immunopositive dots in the cytoplasm, probably representing the Golgi complex, and *yellow arrows* indicate moderate Cx43 dots in the cell prolongation. **c, d** After aluminum treatment for 24 days, Cx43 immunoreactivity was restricted to the cytoplasm (*white arrows*), whereas no Cx43 staining was observable in the cell prolongations (*yellow arrows*). *Scale bars* 20 µm

of astrocytes in vitro (Fig. 6a, b). In the perikaryon accumulations of Cx43-positive dots were visible mostly restricted to one side of the cells, probably representing the point of origin. In contrast subsequent aluminum exposure for 24 days Cx43 immunoreactivity was mostly limited to the perikaryon of these astrocytes. At this point aluminum incubation dramatically decreased Cx43 immunoreactivity in the cell prolongations, whereas intensively Cx43-labeled dots could be figured out in the perikaryon (Fig. 6c, d). Therefore, at the level of fluorescent microscopy, translocation of Cx43 from the cell body to the cell prolongations seemed to be impeded in astrocytes by aluminum.

With the aid of electron microscopy, these data could be stated more precisely, as gap junctions were even identified after aluminum treatment for 24 days (Fig. 5c). In conclusion, these results suggest that aluminum treatment did not completely block the translocation of connexins, as indicated by the immunohistochemistry data, but impeded this process.

Discussion

The results of the present study suggest that long-term aluminum exposure impairs the functional integrity of the cytoskeleton in cultured astrocytes, which affect connexin translocation from the point of origin to the plasma membrane. In these glial cells, GJIC was significantly impaired. Therefore cultured astrocytes were exposed to aluminum lactate, supplemented with citric acid and L-glutamic acid, to investigate changes of the cytoskeletal morphology in regard to GJIC, comparable to earlier investigations on the effect of these substances on the cytoskeleton in nervous cells (Jones and Oorschot 1998; Theiss and Meller 2001). The use of aluminum lactate represents an improvement upon administration of aluminum alone, as aluminum is readily soluble in an aluminum lactate solution (Zatta et al. 1993). In addition, astrocytes exhibit a high capacity for lactate utilization, suggesting that lactate may be an important substrate for brain cells (Tabernero et al. 1993; Vicario et al. 1993). Citric acid was supplemented as aluminum is thought to enter cells via the transferrin receptor by complexing with citric acid (Birchall and Chappell 1988), but seem to have no effect on the cytoskeletal morphology of nervous cells (Jones and Oorschot 1998). Substitution of L-glutamine by glutamic acid was performed, as in nervous cells glutamate appeared to have a protective rather than a detrimental effect (Jones and Oorschot 1998). Also incubation of astrocyte cultures with glutamate was followed by a large increase in GJIC (Rouach et al. 2000). In the present study treatment of cultured astrocytes with all the media supplements except aluminum showed no effect on GJIC. Therefore the observed impairment of GJIC after administration of a mixture of citric acid, glutamic acid and aluminum lactate seems to be based on the impact of aluminum lactate on the cytoskeleton, whereas citric acid and glutamic acid support the uptake and the viability of cultured astrocytes in these long-term exposure experiments.

Effects of aluminum exposure on cultured astrocytes

Astrocytes exhibit extensive intercellular communication mediated by gap junctions built of connexin 43 in vivo (Dermietzel et al. 1989; Yamamoto et al. 1989) and in vitro (Dermietzel et al. 1991; Giaume et al. 1991) and later on in development also by connexin 30 (Kunzelmann et al. 1999). In earlier investigations, GJIC was assayed by direct cell-to-cell transfer of Lucifer yellow (Mambetisaeva et al. 1999), whereas in the present investigation neurobiotin was used to visualize GJIC after microinjection into an individual astrocyte. As demonstrated in the retina, the relative ratios of neurobiotin permeability are of the same magnitude as their relative electrical coupling (Bloomfield et al. 1995; Mills and Massey 2000), and therefore neurobiotin seems to be suitable for the display of physiological coupling of astrocytes.

In the present investigation we examined the effects of aluminum exposure on GJIC in cultured astrocytes that formed a functional syncytium-like organization after maturation (Mugnaini 1986). GJIC, demonstrated by neurobiotin transfer, was significantly impaired after aluminum exposure for 24 up to 48 days. However, the target of aluminum toxicity is not known exactly; in neurons bundling of neurofilaments was described in the somata and the neurites (Katsetos et al. 1990; Gilbert et al. 1992; Theiss and Meller 2001). In addition, several investigations indicate that aluminum is preferentially taken up by glial cells of the CNS (Albrecht et al. 1991; Rao 1992; Campbell et al. 1999), where it is found in the outermost flat portions of the cell bodies, rather than in their central, thicker parts (De Stasio et al. 1994). Our fluorescence-microscopic investigations as well as the ultrastructural data of an abnormal distribution and structure of the cytoskeleton in astrocytes exposed to aluminum are in line with these findings. Astrocytes exhibit a differentiated cytoskeleton formed by actin filaments and the intermediate filaments GFAP and vimentin (Chiu and Goldman 1984). Fluorescence immunohistochemistry as well as rapid-freeze, deep-etch electron microscopy in the present investigation revealed a highly organized network of these cytoskeletal elements. While GFAP-stained filaments stretched through the whole cell, probably actin filaments entered the cell membrane. In contrast, aluminum exposure for several days affected this organization. With the aid of fluorescence microscopy, it could be figured out that aluminum treatment for 24 days led to an aggregation of GFAP-positive elements in the cytoplasm, accompanied by a collapse of the actin cytoskeleton near to the cell membrane. On the level of electron microscopy, actin filaments, normally entering the plasma membrane, were displaced by globular aggregates close to the cellular membrane after 13 and 24 days of aluminum treatment. In these cells, areas without cytoskeletal elements reaching up to the cell membrane could be observed. Similar cytoskeletal alterations have been described as a consequence of aluminum toxicity, especially in regard to GFAP-positive filaments (Muller and Bruinink 1994; Yokel and O'Callaghan 1998). The presented data are also in line with investigations of aluminum exposure on cultured astrocytes that induced apoptosis, accompanied by condensation and fragmentation of chromatin, shrinkage of cell bodies and furthermore the retraction of cellular processes (Suarez-Fernandez et al. 1999; Guo and Liang 2001).

Interaction of GJIC and the cytoskeleton

The cytoskeleton of neuronal cells is known to be associated with the physiological functioning of these cells, as dysfunction of cytoskeletal components influences vesicular biogenesis and synaptic signaling (McMurray 2000) and vesicle transport (Meller 1992; Krah and Meller 1999; Theiss and Meller 2000, 2001). The presented fluorescence- and electron-microscopic data of impaired,

but not completely blocked, Cx43 trafficking from intracellular storage sites to the plasma membrane as a result of aluminum treatment verified the importance of an intact cytoskeleton also in glial cells. Since treatments with nocodazole, a microtubule-disrupting drug, were earlier reported to block enhanced but not basal levels of connexin construction (Paulson et al. 2000), a second, microtubule-independent, trafficking pathway may be utilized during connexin assembly. In regard to cell-to-cell communication via gap junctions, especially actin filaments are likely to be involved in regulative mechanisms (Meller 1981). Disruption of the actin cytoskeleton by cytochalasin B exposure inhibited intercellular contact formation in plexus cells (Meller and El-Gammal 1983), and cytochalasin D treatment reduced the number of astrocytes engaged in calcium signaling via GJIC (Cotrina et al. 1998). In neurons also Ca^{2+}-channel-dependent inactivation was demonstrated by cytoskeletal interactions (Johnson and Byerly 1993). In the present study aluminum exposure impaired GJIC, accompanied by alterations of the cytoskeleton. However, aluminum is likely to affect intermediate filaments, and our electron-microscopic data additionally demonstrated an altered distribution of actin filaments, without microfilaments entering the cell membrane in aluminum-exposed cells. Therefore the actin cytoskeleton is influenced by aluminum treatment, either directly or also indirectly. Probably bundling of intermediate filaments or their dissociation into globular particles also disturbs the organization of the actin cytoskeleton. As a consequence, connexin trafficking could be perturbed, which is substantial for the linkage of gap junction formation of two hemichannels in adjacent cells. As Yamane and co-workers (1999) co-localized immunopositive spots for connexin with actin filaments in the tips of cell processes, these authors suggested that actin associated with connexins could play an important role in the early stage of the adhesion of astrocytes. At this point the actin-gap junction associated protein ZO-1 is considered to be important for connexin transport from intracellular storage sites to the cell membrane (Toyofuku et al. 1998).

Based on the present data of an altered distribution of actin filaments in cultured astrocytes after aluminum exposure, an additional correlation between GJIC and cytoskeletal elements is conceivable. Actin-membrane associations have been demonstrated through members of the spectrin superfamily and through proteins at the cytoplasmic surface of focal contacts and adherent junctions (Hitt and Luna 1994). The actin cytoskeleton is associated with the regulation of the cell volume through functional interactions with K^+ and Cl^- channels (Cantiello et al. 1993), and by mediation of extracellular signals (Papakonstanti et al. 2000). In addition, actin filaments are involved in the regulation of Na^+ channel activity (Cantiello et al. 1991; Berdiev et al. 1996). In regard to GJIC, Toyofuku and co-workers (1998) demonstrated a direct association between ZO-1 and the gap junction protein Cx43 in cardiocytes. ZO-1 is a tight junction-associated protein (Itoh et al. 1993) that is closely associated with actin in several tissues (Stevenson and Begg 1994; Giepmans and Moolenaar 1998). Therefore ZO-1 serves as an adapter or anchor between the cytoskeleton and gap junctions. As we noted, aluminum treatment abolished the connection between actin filaments and the plasma membrane. In conclusion it is likely that the association of actin-ZO-1 and gap junctions is also disturbed, with the consequence of an impaired regulation of GJIC by these cytoskeletal elements.

Aluminum intoxication and buffering capacity of astrocytes

The impairment of GJIC between astroglial cells is very likely to have an impact on the performance of neurons and therefore probably constitutes a key factor in the pathology of aluminum-induced neurodegenerative diseases. Aluminum exposure for several days results in degeneration of cultured astrocytes, and this aluminum-induced toxicity is critical in determining neuronal death when co-cultured with astrocytes (Suarez-Fernandez et al. 1999). The present investigation also demonstrates an impaired GJIC between astrocytes in vitro before morphological degeneration is clearly visible. Thus, aluminum might induce neurotoxicity as an indirect effect on glial cells, which are impaired in their capability to buffer ions and transmitters in the extracellular environment. Thereafter, as a consequence of the limited functionality of the astrocytes, aluminum could affect the physiological activity of neurons.

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