unclear or controversial. Nevertheless, it is known that as over 95% of cases with AD are sporadic, some environmental factors are expected to be etiological [47]. Aluminum is an environmental factor, which is a ubiquitous element used extensively in contemporary life. Until the controversial role of aluminum as an environmental factor in the pathogenesis of neurodegenerative diseases is well resolved, therefore, it will continue to be a subject of man's curiosity.

Recent findings have implicated astrocytes as the principal target for aluminum toxic action [13,31,72,74]. Thus, the primary culture of astrocytes would provide a good model for evaluating neurotoxic injury. Unfortunately, the majority of works on the toxic effects of aluminum has involved an examination of the direct effects of aluminum on neuronal cells, while works on the toxic effects of aluminum on astrocytes are lagging behind. However, the astroglial environment of neurons provides metabolic and trophic support, and contributes to local modulation of synaptic efficacy at excitatory inputs by controlling glutamate clearance and represents an important regulator of glutamatergic communication between dependent synapses by setting the parameters of diffusion in the extracellular space. Defects in these functions may lead to neurodegeneration [53,56,61,73]. Thus, when astroglia are in a compromised state, this may secondarily impact the neuronal population and thus eventually lead to neurodegeneration and/or loss of neuronal functions. Aluminum pretreatment has been shown to impair the ability of astrocytes to protect neurons from glutamate toxicity [62], but the mechanism of action was only speculative. Smale et al. [69] have also demonstrated direct evidence for astrocytes undergoing an active process of apoptosis in AD brain in a postmortem study. In this vein, there is accumulated evidence for astrocytes to have a role in a number of neurodegenerative disorders of which AD is the most prevalent [1,7,8,33,48,71]. In spite of the implications of aluminum in AD, apoptotic effect of aluminum on primary culture of astrocyte is not well documented [75].

Moreover, the form by which aluminum enters brain cells as well as the intracellular consequences of aluminum in relation to neurodegenerative diseases remains unresolved [6]. Although several aluminum compounds/complexes—some of which neither exist in biological system nor consumed as such—have been studied, there is paucity of data on aluminum amino acid complexes. Aluminum glycinate is a constituent of important drugs including antacids and analgesics. Previous investigations have revealed that when volunteers took drugs containing aluminum glycinate, percentage aluminum absorbed from the intestinal tracts was 0.38%, which is more than 100-fold compared to when drugs containing $Al(OH)_3$ were taken, which was only 0.003% [44,46]. Although this may not be directly related to aluminum uptake by brain cells, however, it deserves attention. Furthermore, De Voto and Yokel [22] included some amino acids in the list of some components in serum recognized to be available to bind aluminum and glycine has the highest binding capacity than any other components in the list. Food is the primary common source of aluminum, and it is well established that amino acid concentrations of the brain extracellular fluids are affected by the quantity and composition of the food ingested [14,15,19] as well as by central nervous system (CNS) illness [43,49]. Detailed information on the uptake of aluminum amino acid complex by primary cultured astrocyte is important, therefore, for understanding the mechanism of aluminum toxicity in brain. Hence, the present study was conducted to investigate the availability of aluminum solubilized by some amino acids, especially glycine, for uptake by astrocytes. Aluminum citrate was also employed for comparison. The apoptotic effect of such internalized aluminum also forms part of the subject of the present report.

Glutamate–glutamine metabolism is central in glial metabolic activity. Methionine sulfoximine (MSO) is an irreversible inhibitor of astrocytic enzyme glutamine synthetase, and it is also known to block glutathione synthesis by inhibiting γ-glutamylcysteine synthetase [60,64,65]. Differential influence of MSO application on astrocyte transport of some amino acids has been reported [3,60]. We have therefore employed MSO to determine how the perturbation of astroglial metabolism will influence the uptake of aluminum complexed with different amino acids. Moreover, the ability of glial cells for transporting amino acids has been well established. But little is known of whether amino acid transport is, in any way, influential to the uptake and/or toxicity of aluminum in complex with it. We have therefore employed glycine and glutamate transporters blockers in the uptake studies. Ouabain, a widely known inhibitor of Na^+/K^+-ATPase, was employed to investigate whether the transport of aluminum requires energy in this form.

Thus, we describe herein the differential and real uptake of aluminum complexed with different amino acids by primary cultured astrocytes and suggested the possible mechanism. We also report how metabolic perturbation can aggravate aluminum internalization as well as the apoptotic effect of aluminum in complex with amino acid on the cells, and discussed the implications of compromised astrocytes on neurodegeneration.

2. Materials and methods

2.1. Astrocyte culture

Primary astrocyte cultures were prepared from cerebral cortices of newborn ICR mice (postnatal days 5–7) by the method previously described [50], with some modifications. Briefly, dissociated neocortical cells were suspended in appropriate volume of medium and plated at about 5 ×

10^5 ml^{-1} into culture dishes containing D-MEM/F12 (Gibco cat. no. 11330) supplemented with 15% fetal bovine serum (Gibco), 1.5 mM L-glutamine, and 0.05 mg/ml gentamicin (Sigma). After 3 days, the dishes were gently tapped and the medium aspirated to remove the loosely attached oligodendrocytes, fresh medium was added to the culture, and thereafter replaced two or three times a week. The first passage was done between 7 and 10 days following the primary culture. The astrocytes were washed twice with PBS, trypsinized by 1× trypsin–EDTA (Sigma) in Hanks' balanced salt solution (Ca^{2+}- and Mg^{2+}-free), incubated at 37 °C for about 5 min, harvested, and then replated in new 9-cm dishes. Cultures were maintained at 37 °C with 95% air and 5% CO_2 throughout the incubation periods. Cells were cultured in 5-cm dishes during the second passage and used about a week later for experiments (i.e., 3–4 weeks after the start of primary culture during which time the culture had passed through two passages by trypsinization). The culture consisted almost exclusively of glial fibrillary acidic protein (GFAP)-positive cells and displayed a flat, polygonal morphology when maintained in the growth medium described above. At the start of each experiment, the medium was replaced by DMEM (Gibco cat. no. 11885) supplemented with 15% fetal bovine serum and gentamicin (0.05 mg/ml).

2.2. Aluminum uptake

Following the second passage after about 80% confluent monolayer astrocytes, the cells were stressed with aluminum amino acid complexes freshly prepared at a final concentration of 0.1 mM in culture medium except otherwise stated. In a similar study, Levesque et al. [41] employed 1 mM aluminum salts, but we could still accurately measure aluminum content of cells cultured in 5-cm dishes at the concentration employed in the present study. Cells were exposed to test compounds for periods varying from 0.5 to 24 h. Four replicates of dishes each were measured. To distinguish real uptake or internalized aluminum from mere membrane-bound, aluminum uptake by the intact cells was compared with that of lysed cells, which received similar treatments. Lysed astrocytes were prepared by exposing the cells to sterilized distilled water for 15 min at room temperature. To prevent loss of detached lysed cells, the medium or wash buffer used for it was centrifuged at $5 \times 10^4 g_{max}$ min each time [59]. Amino acid transporter blockers as well as ouabain were used to determine the possible mechanism of aluminum internalization in the forms employed here. Reviewing previous reports and evaluating the effect of a given experimental condition on cell viability, we arrived at the effective and safe doses of the blockers. The condition whereby cell viability of treated cells was similar to that of untreated cells within the period of aluminum uptake studies was selected. The doses were also varied in some aluminum uptake experiments to ascertain that the observation in higher doses was not due to effects other than transporter blockade. Aluminum uptake of the harvested intact and lysed cells was quantified by atomic absorption spectrophotometer (AAS) with Zeeman background correction equipped with a graphite furnace (Hitachi 180-80) following wet ashing with ultrapure nitric acid at 80 °C for 4 h. Protein quantification was carried out with Protein Assay Rapid Kit, comprising of BSA standard solution (Wako) according to the manufacturer's guide, and Hitachi U-1100 spectrophotometer was used to read the color formation. The aluminum content was determined following protein analysis and results were expressed as nanograms of Al per microgram of protein. In all studies, following exposure to test compounds, the harvested cells were washed twice with PBS containing 1 mM EDTA. Plastic materials were used throughout the study to avoid aluminum contamination by glassware.

2.3. Cell proliferation and viability test

Cells were exposed to test compounds at indicated concentrations for 24 or 48 h in 96-well plate and about eight replicates per sample were studied. Quick cell proliferation assay kit (BioVision Research Products, USA) was used for the quantification of cell proliferation and viability according to the manufacturer's guide. The assay is based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. The formazan dye produced by viable cells was quantified by multiwell spectrophotometer (TOSOH micro plate reader, model MPRA4*i*). The viability of cells following exposure to test compounds was expressed as percentage of the viability of control (i.e., cells not exposed to test compounds).

2.4. Morphological analysis of apoptosis

Cells were exposed to aluminum glycinate at final concentrations varying from 0.0125 to 0.1 mM for 6 h after which the medium was exchanged, and the cells were cultured for 1–10 days in normal medium before apoptotic analysis, except otherwise stated. MSO was also employed in the apoptotic study. The cover slips on which the cells were cultured were carefully removed, washed with PBS, fixed with methanol (−20 °C), stained with Hoechist 33258 dye (Sigma), and then observed under the fluorescence microscope. The ratio of apoptotic to normal nuclei in both control and treated samples was determined as the mean percentage of every 100 cells counted. The nuclear shrinkage in astrocytes was determined as changes in the nuclear perimeter (arbitrary unit) by *Scion Image Beta 4.02* for Windows downloadable from the Scion website at www.scioncorp.com. It is an image processing and analysis program for the IBM PC based on the popular NIH Image on the Macintosh platform.

2.5. Chemicals

All reagents were of the highest purity grade available. Aluminum chloride (Aldrich Chemicals), L-serine (Wako), L-glutamine, L-glutamic acid, and citric acid monohydrate (Kanto Kagaku) were used for the preparation of aluminum amino acid and citrate complexes with Milli-Q water (Millipore). All the amino acids used in the experiment were of the L form, except glycine. Working solutions were freshly prepared by equimolar mixture of aluminum chloride and the respective amino acids or citrate, with the exception of aluminum glycinate ($H_2NCH_2COOAl(OH)_2$), which was purchased in anhydrous form (Tokyo Kasei Kogyo). The quality of complexation was verified by proton NMR [11]. Ultrapure nitric acid (Kanto Kagaku) was used for wet ashing and aluminum standard solution (Wako) was used to prepare AAS standards. Sarcosine, doxepin, L-*trans*-pyrolidine-2,4-dicarboxylic acid, and dihydrokainic acid (DHK; Sigma) were used as blockers, and L-MSO and ouabain (Sigma) as inhibitors.

3. Results

3.1. Differential uptake of aluminum

The different aluminum amino acid complexes employed in the present report are glycine, serine, glutamine, and glutamate complexes. Aluminum was differentially taken up by primary cultured mouse astrocytes (Fig. 1A). The lysed astrocytes that served as control almost uniformly bind aluminum from all complexes, and no significant difference exists between test and control in all complexes at 0.5 h. There was significant uptake in all forms of the aluminum complex above their respective control following 24 h of exposure, with the exception of aluminum glutamate. The uptake of aluminum from glycine complex was significantly higher than that of glutamine, while aluminum uptake in all forms was significantly higher than that of aluminum glutamate. The uptakes of aluminum from citrate and serine complexes were compared in another experiment following exposure to test compounds at 0.5, 8, and 24 h, respectively

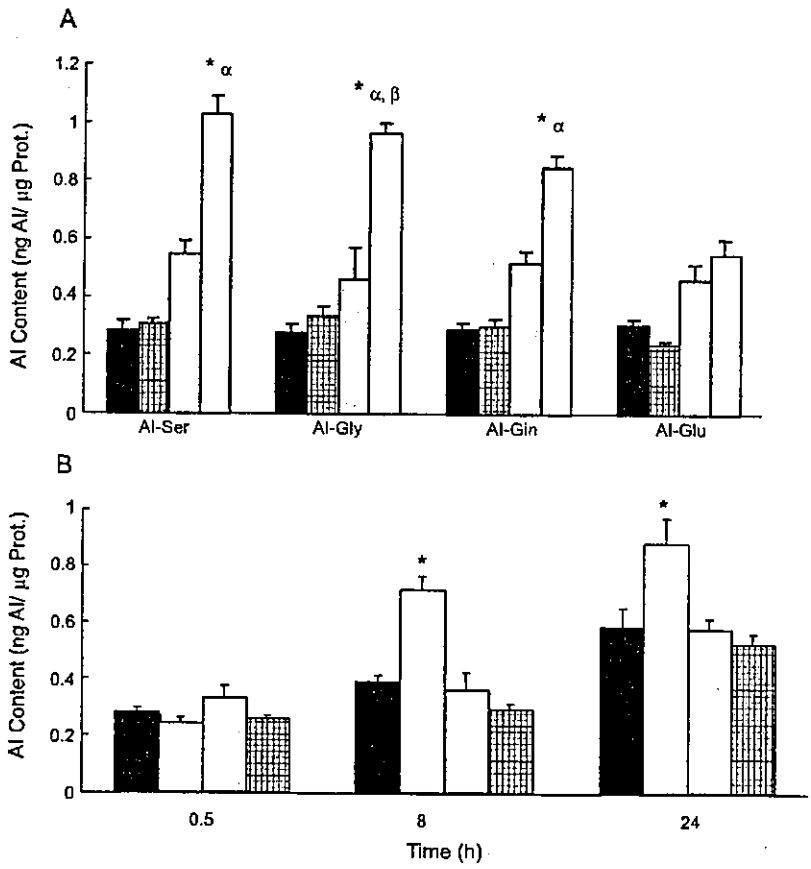

Fig. 1. Differential accumulation of aluminum by primary cultured astrocytes. Intact (test) and lysed cells (control) maintained in culture medium described under Materials and methods were exposed to (A) indicated aluminum amino acid complexes (0.1 mM) for 0.5 h (control, dark bars; test, grid bars) or 24 h (control, grey bars; test, plain bars). (B) Aluminum serine complex (control, dark bars; test, plain bars) and aluminum citrate complex (control, grey bars; test, grid bars) at a final concentration of 0.1 mM for 0.5, 8, and 24 h. Plotted values represent aluminum content shown as mean ± S.E.M. ($n = 4$) expressed as aluminum (ng) per cellular protein (µg). *Statistical difference from control condition (or Al-Cit at 8 or 24 h exposure), while α and β indicate statistical difference from 24 h of aluminum uptake from Al-Glu and Al-Gln, respectively, at $p < 0.05$ using Welch's t test.

(Fig. 1B). As early as 8 h of exposure, there was significant uptake of aluminum from serine complex; unexpectedly, no actual uptake took place in aluminum citrate complex even after 24 h of exposure.

3.2. Uptake of aluminum in the presence and absence of MSO

Due to no significant uptake of aluminum from glutamate complex after 24 h of exposure, the uptake was then studied under an impaired metabolism of astrocytes using MSO. Interestingly, the uptake of aluminum from glutamate complex was enhanced in the presence of MSO and this was significantly higher than adsorption to lysed cells and uptake by intact cells without MSO as early as 4 h following the exposure (Fig. 2A). Based on this observation, coupled with the possibility of general influence of MSO on aluminum uptake, the experiment was extended to other amino acid complexes. With the exception of glutamine complex, the uptake of aluminum in the presence of MSO was significantly enhanced in glutamate, serine, and glycine complexes (Fig. 2B). The uptake of aluminum from glutamate complex in the presence of MSO was higher than the uptake in the absence of MSO as early as 30 min following exposure, whereas aluminum uptake from other amino acid complexes in the presence of MSO was not higher than in the absence of MSO at this time. Following 4 h of exposure, on the other hand, there was much uptake in glutamate and serine complexes in the presence of MSO, whereas that of glycine complex was small. However, at 8 h of exposure in the presence of MSO, aluminum uptake from glycine complex was significantly higher than those of glutamate and serine complexes. The uptake from glutamine complex, on the other hand, was consistently suppressed.

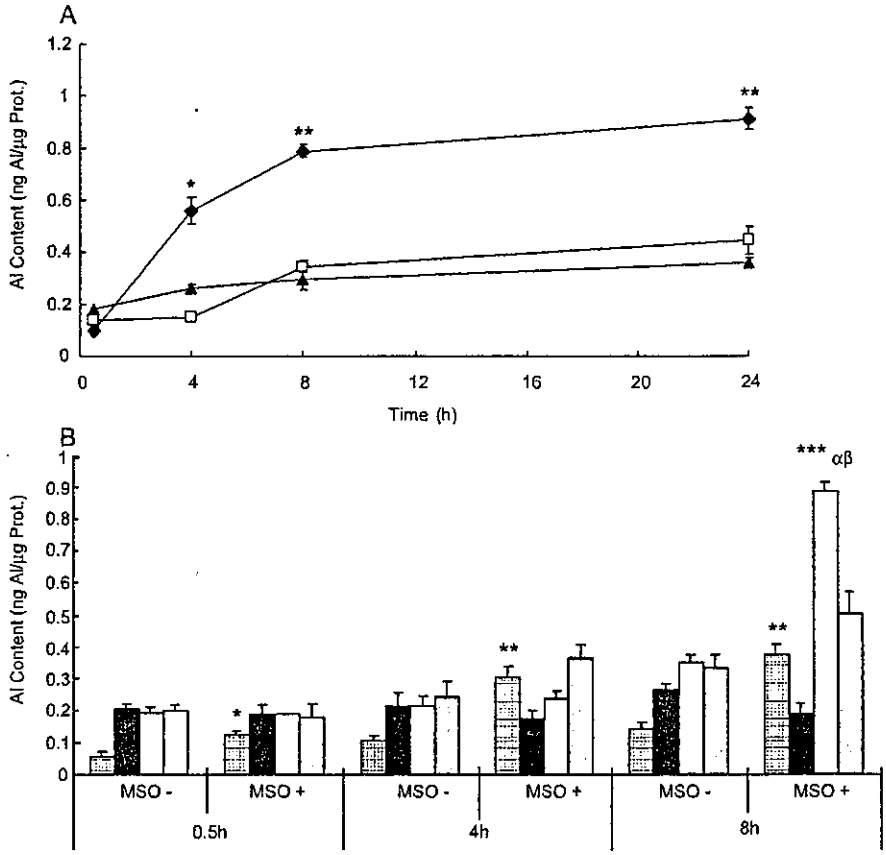

Fig. 2. Effect of MSO on uptake of aluminum. Primary cultured astrocytes maintained in culture medium described under Materials and methods were exposed to (A) aluminum glutamate complex (0.1 mM) in the presence (♦) and absence [intact cells (□); lysed cells (▲)] of MSO (1 mM) for 0.5, 4, 8, and 24 h (MSO and aluminum glutamate were applied simultaneously). (B) Aluminum–glutamate (grid bars), glutamine (dark bars), glycine (plain bars), and serine (grey bars) complexes, at a final concentration of 0.1 mM for 0.5, 4, and 8 h in the absence (−) and presence (+) of MSO (1 mM). MSO was applied 0.5 h prior to the addition of aluminum amino acid complexes. Plotted values represent aluminum content shown as mean ± S.E.M. (n = 4) expressed as aluminum (ng) per cellular protein (μg). *, **, and *** indicate statistical difference from aluminum uptake from corresponding amino acids in the absence of MSO at $p < 0.05$, 0.01, and 0.001, respectively, while α and β indicate statistical difference from 8 h of aluminum uptake from serine and glutamate complexes in the presence of MSO at $p < 0.01$ and 0.001, respectively, using Welch's t test.

3.3. Uptake of aluminum in the presence and absence of amino acid transporter blockers and ouabain

In order to determine whether aluminum in complex with amino acids shares the same transporters with the respective free amino acids, primary cultured astrocytes were exposed to aluminum glutamate and aluminum glycinate at concentrations of 0.1 and 1 mM in the presence and absence of their respective amino acid-specific and nonspecific transporter blockers. While L-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC) and sarcosine (on low aluminum dose) had no effect on uptake of aluminum complexed with the respective amino acids, doxepin and DHK consistently enhanced it (Fig. 3A). In order to determine whether the uptake of aluminum glycinate derives any energy via Na^+/K^+-ATPase and that the enhancement observed above was not due to effect other than transporter blockade, in another experiment, the effect of graded doses of specific and no specific glycine transporter blockers and ouabain on aluminum uptake from aluminum glycinate (0.1 mM) was also observed. None of the blockers inhibited the uptake of aluminum at the various doses employed, but ouabain and lower doses of doxepin and sarcosine did not significantly affect aluminum uptake (Fig. 3B).

3.4. Cell viability and proliferation

Generally, there was no significant adverse effect on the cell viability/proliferation among the cells exposed to test compounds at all concentrations employed in this study compared with control (results not shown).

3.5. Morphological analysis of apoptosis

The apoptotic effect of aluminum amino acid complex on cultured astrocytes was observed as nuclear shrinkage and chromatin condensation (Fig. 4). There was an observable effect after 3 days in normal culture medium following pulse

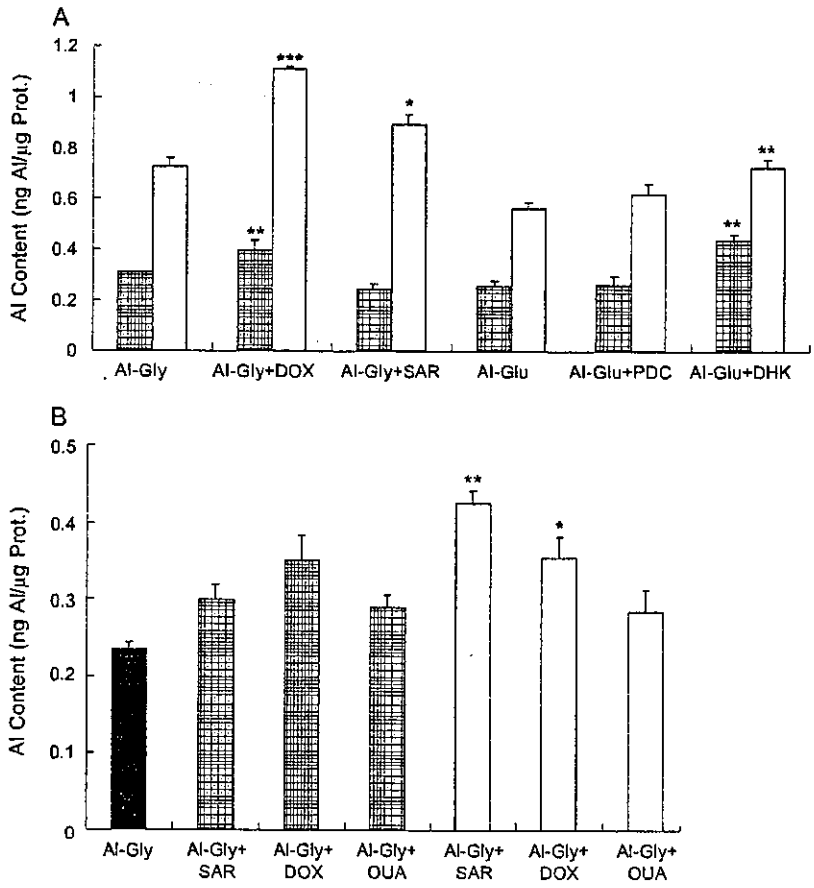

Fig. 3. Effect of some transporter blockers on uptake of aluminum. Primary cultured astrocytes maintained in culture medium described under Materials and methods were exposed to (A) aluminum glycinate (0.1 mM, grid bars; or 1 mM, plain bars) in the absence or presence of doxepin (DOX) or sarcosine (SAR), as well as aluminum glutamate (0.1 mM, grid bars; or 1 mM, plain bars) in the absence or presence of L-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC) or dihydrokainic acid (DHK) for 6 h. (B) Aluminum glycinate in the absence (dark bar) or presence of blockers/inhibitors (0.025 mM, grid bars; or 0.05 mM, plain bars) for 6 h. Doxepin (DOX), sarcosine (SAR), and ouabain (OUA) were used as blockers/inhibitors. Transporter blockers were applied 0.5 h prior to the addition of aluminum amino acid complexes. Plotted values represent aluminum content shown as mean ± S.E.M. (n = 4) expressed as aluminum (ng) per cellular protein (µg). *, **, and *** indicate statistical difference from aluminum uptake from corresponding amino acids in the absence of blocker at $p < 0.05$, 0.01, and 0.001, respectively.

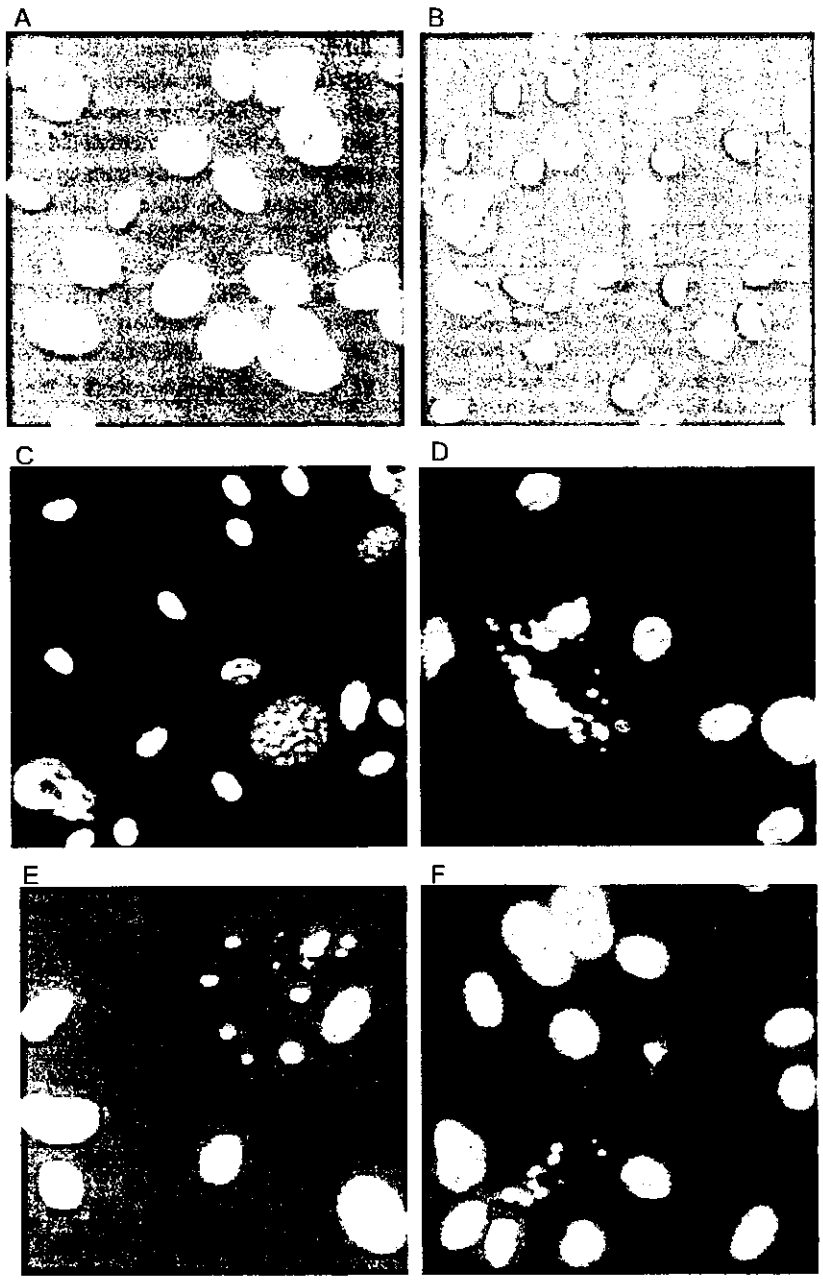

Fig. 4. Nuclear morphological changes in aluminum-treated primary cultured astrocytes. Cells maintained in culture medium described under Materials and methods were exposed to graded doses of aluminum glycinate for 6 h, the medium was exchanged with fresh medium without aluminum glycinate, and the culture was continued for a period between 24 h and 8 days before analysis. The figure shows representative Hoechst 33258 dye photomicrographs. (A) Control cells in 8-day culture. (B, E, F) Four days of culture following pulse exposure to 0.1, 0.025, and 0.1 mM Al-Gly, respectively. (C and D) Eight and 7 days of culture following pulse exposure to 0.1 and 0.0125 mM Al-Gly, respectively. Cells with homogeneously stained nuclei were considered to be viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis.

exposure to 0.1 mM aluminum glycinate, and the ratio of apoptotic to normal nuclei increased with time. Pulse exposure to lower concentrations of aluminum glycinate as low as 0.0125 mM also caused apoptosis in primary cultured astrocyte. The nuclei of astrocytes in control cultures appeared with regular shape and size with very rare smaller nuclei. On the other hand, many nuclei of aluminum-treated astrocytes appeared shrunk with irregular shapes and sizes, and hypercondensed and extensive fragmentation of their chromatin. Less than 5% of untreated cells were apoptotic, while over 25% of the treated cells became apoptotic following 8 days of culture in normal medium after pulse exposure to 0.1 mM aluminum glycinate in the presence and absence of MSO (Fig. 5). The time course changes in the mean perimeters of the shrunk nuclei in samples exposed to 0.1 mM aluminum glycinate were

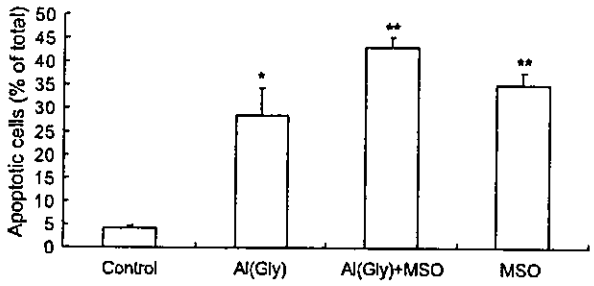

Fig. 5. Apoptotic rate in aluminum-treated primary cultured astrocytes. Cells maintained in culture medium described under Materials and methods were exposed to 0.1 mM aluminum glycinate for 6 h, the medium was exchanged with fresh medium without aluminum glycinate, and the culture was continued for 8 days before analysis. The nuclear morphology of astrocytes was studied using Hoechist 33258 dye. Both normal and apoptotic cells totaling about 100 cells were counted each time and the plotted values represent the mean apoptotic cells as percent of total cells (±S.E.M.) in at least four different counts. * and ** indicate statistical difference from control at $p < .05$ and 0.01, respectively.

also presented. The nuclei shrunk steadily until day 5, after which the shrinkage reached a plateau (Fig. 6).

4. Discussion

Aluminum absorption, tissue retention and deposition, as well as excretion depend on the properties of the Al^{3+} complexes formed with biological ligands [22,32]. Unfortunately, the form by which aluminum crosses membranes and the intracellular consequences of this form remain speculative. The present investigation revealed that aluminum, in complex with different amino acids, is differentially taken up by astrocytes. Thus, the amino acids do not only solubilize aluminum but also influence its uptake. The internalization of aluminum from the complex forms studied here was ascertained by comparing the uptake by living cells stressed with the test compounds with that of lysed astrocytes. Cellular internalization of metals, in general, and aluminum, in particular, has been determined by either of the following two methods. The first method is by mere washing of harvested cells with chelating agents such as EDTA or citrate [54,55,67], and the second is by comparing cells exposed to aluminum with cells incubated in aluminum-free medium following washing [41]. While the latter method only takes care of systematic error, the former may not and the two, nonetheless, may exaggerate the actual uptake. It has been recognized that minor metal fraction comprising of metal tightly bound onto cell surfaces may remain after repeated washing with chelators [67]. The use of lysed cells had confirmed the presence of residual aluminum following washing with chelators (EDTA) in the present study. The internalized aluminum is therefore the difference between aluminum adsorbed to the membranes (lysed cells) and the aluminum content of the intact cells. Thus, we have unequivocally demonstrated here the actual internalized aluminum. Although we are aware of the differences in cultured cells and culture conditions of the previous report on aluminum chloride [67], a close analysis, after adjusting for the exposed time and dose, revealed that aluminum is better internalized from aluminum amino acid complexes, especially with glycine and serine. Therefore, they seem to be better candidates with respect to cellular uptake.

The present results show a rapid initial binding of aluminum following 30 min of exposure. However, this seems to be mere adsorption to membranes due to lack of significant difference between test and control. Levesque et al. [41] have demonstrated a biphasic mode of aluminum accumulation earlier proposed by Shi and Haug [67]. This is characterized by an initial rapid binding (within few minutes) of aluminum to cell surfaces, followed by the slower internalization of membrane-bound aluminum. The differential internalization of aluminum observed in the present study demonstrates a diverse nature of the complexes formed with different amino acids. This is in agreement with the view of Levesque et al. [41] that incorporation and toxicity of aluminum in different compounds or different biological settings cannot be generalized. Moreover, the present observations show that uptake of aluminum depends on their chemical forms or species [22,32]. Necessary conditions for crossing biological membranes involve either a specific carrier, or a passive transfer by a small, neutral, or weakly charged molecule [20]. Variations in the above conditions as well as the extent of alteration of membrane permeability [21] among different amino acid complexes may be responsible for their differential uptake. Banks and Kastin [9] had earlier reported that aluminum salts affect the blood–brain barrier (BBB) permeability and this has been shown by other authors to

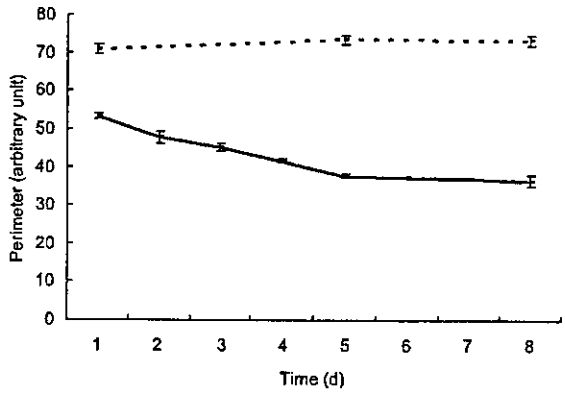

Fig. 6. Time course changes in mean perimeter of apoptotic cells and normal control. Cells maintained in culture medium described under Materials and methods were exposed to 0.1 mM aluminum glycinate for 6 h, the medium was exchanged with fresh medium without aluminum glycinate, and the culture was continued for periods varying from 24 h to 8 days before analysis. The nuclear shrinkage in astrocytes (control, broken line; treated, solid line) was determined as the mean (±S.E.M.) changes in the nuclear perimeter (arbitrary unit) of at least five representative nuclei, by *Scion Image Beta 4.02* for windows after using Hoechist 33258 dye for nuclear staining.

vary depending on the salt of aluminum [38]. The stability constant of different complexes formed may also be influential to their differential uptake.

Unexpectedly, there was no apparent uptake of aluminum from aluminum citrate complex at 24 h of exposure time compared to control. Thus, mere washing of the harvested cells with chelator without comparison with lysed cells would have given the impression that aluminum was internalized from aluminum citrate complex. It is surprising that our study revealed a reduced and no uptake of aluminum in complex with glutamate and citrate, respectively. The stability of the two complexes may influence their readiness to donate aluminum for uptake. Other experiments had also suggested a very low permeation or inhibition of aluminum transport in the form of citrate complex across liposomal membranes or neuroblastoma cells [2,66,67]. However, in contrast to our observation and other previous reports on aluminum citrate, a recent report [79] had shown the uptake of Al ^{14}C citrate in immortalized brain endothelial cells, but the method of analysis and control employed were different from ours. Aluminum uptake was indirectly quantified as level of ^{14}C in cell lysate. In the same study, however, there was no uptake of aluminum citrate in erythrocytes similar to other finding [45], which means that the rate and mechanism of aluminum uptake from citrate complex may vary between cells. It is also probable that aluminum citrate may have a preferred route or transport system not present in other cells but abundant in endothelial cells. On the alternative, citrate may be a major aluminum chelator both in extracellular and intracellular fluids in order to detoxify it. This is physiologically relevant in view of the abundance of aluminum in the form of citrate in the body fluid. Citrate has been employed as chelator to wash cultured cells following exposure and prior to aluminum analysis in aluminum uptake study [67].

MSO is a rare amino acid that occurs in nature, or as a by-product of some forms of food processing [65]. Perturbation of astrocyte metabolism with this substance has greatly enhanced the uptake of aluminum in the form of all except one of the amino acids studied. While MSO had an early effect on the uptake from glutamate and serine complexes, that of glycine was delayed; nevertheless, uptake of aluminum from glycine complex was significantly higher than that of glutamate and serine complexes in the presence of MSO following 8 h of exposure. Conversely, MSO did not enhance aluminum uptake from glutamine complex within the period of study but rather suppressed it. The reason for the observed differences is not clear. However, it has been reported that MSO increased glial glutamate and aspartate uptake via high-affinity transport site arising from the disruption of glutamate metabolism [60]. MSO had no effect on leucine transport in the same study, indicating a specific action on the glutamate/aspartate transport system. On the other hand, MSO had caused a massive efflux of glutamine from cortical astrocytes in primary culture [3] and exchange diffusion via the putative glutamine transporter on astrocytes has been implicated in the massive efflux of glutamine. Aluminum itself has been reported to have a stimulating effect on the enzyme, glutamine synthetase. Thus, it enhances the conversion of glutamate to glutamine, thereby increasing the intake of glutamate into astrocytes and efflux of glutamine out of the cell [72,80]. It is probable that the present observation may be an adaptive response to inhibition of the enzyme, which catalyzes the conversion of glutamate to glutamine. Thus, glutamine synthetase may play an important role in the transport kinetics of aluminum complexed with amino acids. The differential effects of MSO on aluminum uptake in different complexes studied here demonstrate that aluminum uptake is associated with the metabolic state of the astrocytes.

The perturbation of astrocytes in the present work with the attendant increase in aluminum uptake seems to provide an interesting model for the clarification of the cause of excessive accumulation of aluminum in AD brain. Impairment of the normal glutamate–glutamine cycle is known to be characteristic of AD [58] and MSO impairs glutamate–glutamine cycle by irreversibly inhibiting glutamine synthetase, an enzyme exclusively present in the astrocyte that is responsible for the conversion of glutamate to glutamine. In fact, the presence of MSO in the diet, as a contaminant and through inhibition of glutamine synthetase, has been hypothesized to be partially responsible for the occurrence of some neurological human diseases such as AD, Parkinson's disease, and amyotrophical lateral sclerosis [64]. The results of viability/proliferation study did not show any detrimental effect on astrocytes at doses of the test compounds employed in the present work within the time frame of uptake studies when compared with control. The present observation of the effect of aluminum amino acid complex on astrocytes viability is consistent with other findings [30,62,74].

The transport of amino acids across cell membrane is known to be carrier-mediated and the respective carriers have been isolated. Two Na^+-dependent transporters have been recognized for glutamate: GLAST (EAAT1) and GLT-1 (EAAT2) [12]. Two distinct types of glycine transporter, GlyT-1 and GlyT-2, have also been characterized [35]. In the present study, we employed DHK, a selective blocker of GLT-1 (EAAT2) [37]; PDC, a nonspecific inhibitor of glutamate transporters [52]; sarcosine, a selective blocker of GlyT1 [42]; and doxepin, a nonspecific inhibitor of glycine transporters [51]; as well as ouabain, a known inhibitor of Na^+/K^+-ATPase. However, none of these blockers inhibited aluminum uptake from aluminum glutamate and aluminum glycinate by cultured astrocytes. Thus, amino acid transporters may not be involved in transporting the aluminum in complex with the respective amino acids into astrocytes. It has been recently suggested that the fact that a substance solubilizes aluminum does not necessarily imply that its transfer into cytoplasm follows the receptor-mediated pathway of the solubilizing agent [34]. However, the enhanced

aluminum uptake observed in certain circumstances is puzzling. Although the employed doses had no effect on astrocyte viability, the fact that lower doses of doxepin and sarcosine had no significant effect shows that higher doses might have effects other than blockade of the respective amino acid transporter. Surprisingly, a dose of 0.1 mM ouabain did not have effect on aluminum uptake (results not shown) and this is consistent with a previous report [67] that dose as high as 0.25 mM had no effect. A dose of 0.1 mM had also been employed for DHK [37] to block GLT-1 (EAAT2) in primary astrocyte without any detrimental effect, but it enhanced aluminum uptake in the present study. Higher doses might have severely affected the metabolism of astrocyte as in the case of MSO. It is also probable that as an adaptive response to inhibition of the amino acid transporters, aluminum amino acid complex may utilize alternative and accelerated pathways that are responsible for the enhanced aluminum uptake. For example, passive diffusion alone is known to cause a rapid decline in the glutamate concentration in the synaptic cleft after release [40]. Even if energy is required for the transport of aluminum in the form employed, it is not likely derived via Na^+/K^+-ATPase and this is consistent with the earlier reports [67,79]. The failure of the blockers employed to inhibit aluminum uptake eliminates the possibility of amino acid transporters or Na^+/K^+-ATPase as being responsible for its uptake but seems to suggest passive diffusion. However, passive diffusion alone may not sufficiently explain the differential uptake in the absence and presence of MSO; hence, another pathway of aluminum internalization may be implicated in addition to passive diffusion.

Endocytosis via the pathway normally involved in iron uptake has been proposed to contribute to aluminum uptake by first binding to transferrin and then internalized by transferrin receptors [16,45], although this observation according to Levesque et al. [41] may be ligand-specific. Recently, a more carefully designed study did not detect any interaction between transferring receptor 1 and aluminum-saturated or mixed C-site iron-loaded/N-site aluminum-loaded transferrin under the same conditions as iron-loaded or C-site iron-loaded transferrin alone [34]. This has led to the conclusion that aluminum may not follow the receptor-mediated pathway of iron transport by transferrins. Thus, the serum Tf in the present culture system might not have significantly influenced the observed increased aluminum uptake in the presence of MSO or amino acid transporter blockers. Levesque et al. [41], in a similar study, have reported no significant difference in aluminum uptake in the presence and absence of serum in culture medium.

Apoptosis is a major form of cell death, characterized by a series of distinct morphological and biochemical alterations. It is an important process in a wide variety of different biological systems, including normal cell turnover, the immune system, embryonic development, metamorphosis and hormone-dependent atrophy, as well as chemical-induced cell death [17]. There are emerging evidences that aluminum is capable of inducing apoptosis in astrocytes, although the mechanism by which aluminum selectively induces apoptosis in cultured astrocytes remains unclear [30,74]. The present study further confirmed the apoptotic effect of aluminum. Pulse exposure to aluminum glycinate caused apoptosis in cultured astrocytes with evidence of nuclear fragmentation and chromatin condensation. Aluminum glycinate at 0.1 mM concentration caused nuclear shrinkage or chromatin condensation in more than 25% of the cells following 8 days of culture in normal medium after pulse exposure and the nuclear shrinkage reached the minimum possible in 5 days. It is probable that when a nucleus reaches a critical level of shrinkage, it fragments. The previous authors reported apoptotic effect of aluminum on cultured astrocytes at higher concentrations (0.4–1 mM) and longer exposure than the present study. The normal aluminum in serum (AIS) of healthy individual is generally accepted to be <0.4 μM, and <2 μM in asymptomatic dialyzed patients [23,68], while it was once thought that the average maximum AIS in dialysis patients is about 20 μM [16,27]. However, recent findings have reported cases of dialysis encephalopathy with mean AIS ranging between 9.4 and 29.9 ± 4.7 μM and a maximum value of 44 μM [10,23]. Here we observed apoptosis at concentrations of aluminum glycinate as low as 12.5 μM. Conversely, aluminum chloride at a concentration of 200 μM was said to have blocked apoptosis in astrocytes [30]. The differences in observation might be due to different experimental conditions such as aluminum species employed, concentration, exposure times, animal species, and methodology. Nevertheless, cell death from prolonged exposure of cultured cells to toxicants might result from causes other than apoptosis.

Apoptosis is an active process requiring energy, and it has been recently suggested that in the absence of an energy pool sufficient to execute apoptosis or maintain ionic homeostasis, cells die quickly by necrosis [28]. Paradoxically, DNA degradation is possible as a late event in necrosis [24,63] or early event not involving the participation of caspases [26]. However, nuclear shrinkage occurs relatively early in apoptosis [36,77]. According to Kalai et al. [36], a single stimulus can initiate different death signaling pathways, leading to either necrotic or apoptotic cell death depending on inhibition of either of the key events in these signaling pathways, such as caspase activation, cytochrome *c* release, or mitochondrial reactive oxygen species production. The present report demonstrates that aluminum amino acid complex is capable of committing astrocytes to death via apoptosis following short time exposure and lower concentrations than previous reports, and this is a significant finding.

The apoptotic rate observed in 0.1 mM aluminum glycinate is comparable with that of MSO alone or MSO and aluminum glycinate due to lack of significant difference. MSO has inhibitory effects on γ-glutamylcysteine synthetase and consequently block glutathione synthesis [60,64,65].

Glutathione is believed to have many functions, among which is scavenging of hydrogen peroxide and free radicals, which are important death signaling pathway. Thus, MSO could have caused apoptosis via its blockade of glutathione synthesis. However, other findings with buthionine sulfoximine, a structural analog of MSO, showed that glutathione depletion is probably not sufficient to trigger death signal and, therefore, not the end point in the death pathway but only an intermediate, eventually reversible event in the chain leading to cell lysis [29]. Further research is necessary to clarify the different mechanisms leading to apoptosis under extended and pulse aluminum exposure and the mechanism of MSO-induced apoptosis. Such clarification may give an insight into the mechanism of aluminum neurotoxicity.

The emerging view that astrocytes have an active regulatory role rather than merely supportive roles traditionally assigned to them [70,73] calls for more research on astrocytes to unravel the mystery of neurodegeneration. Astrocytes constitute nearly half of the cells in our brain, [73,76] and are known to provide essential support, forming a scaffold to hold the neurons in place, insulating the neuronal protrusions, providing nutrients, and clearing debris. Astroglia could act as reservoir of neurotrophins, which are crucial for the normal development and functioning of the brain [4,57]. In fact, according to Song et al. [70], adult astrocytes from hippocampus are also capable of regulating neurogenesis by instructing the stem cells to adopt a neuronal fate. Moreover, few synapses are formed in the absence of glial cells and the few synapses that do form are functionally immature [76]. Neuron-to-astrocyte signaling is also a key mechanism in the control of brain microcirculation [81]. Very recently, Wyss-Coray et al. [78] demonstrated a direct role for astrocytes in the degradation of Aβ and implicated deficits in astroglial clearance of Aβ in the pathogenesis of AD. If aluminum therefore targets astrocytes for its toxic action, then compromised astrocytes could spell doom for the neurons as evident in other reports. The present results, however, show that aluminum could compromise astrocytes via apoptosis.

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Uptake of aluminum amino acid complexes by cultured astrocytes

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Abstract

The form by which Al enters brain cells as well as the intracellular consequences of Al in relation to neurodegenerative diseases remains unresolved. In this report, Al was differentially taken up from Al amino acid complexes by primary culture of cortical astrocytes. Aluminum uptake from different amino acid complexes in the presence and absence of the respective amino acid transporter blockers were compared. The results indicate that none of the amino acid transporter blockers, as well as ouabain, employed in the present study apparently inhibited the uptake of Al. There is a possibility that passive diffusion, influenced by concentration gradient and exposure time, is a major mechanism involved in the Al transport in the forms employed here. The apoptotic effect of Al amino acid complex on astrocytes was also confirmed in the present study with evidence of nuclear shrinkage and chromatin condensation that occurred in more than 20% of the cells, as early as 3 days and also at concentrations as low as 0.0125 mM.

Key words: aluminum uptake, apoptosis, astrocyte, Hoechist33258 dye, glutamate and glycine transporters

Introduction

Aluminum is a ubiquitous element used extensively in contemporary life, nevertheless, evolution has not conferred essentiality or utility on it as far as is known in biological system. Although newer evidences continue to emerge in support of aluminum's participation in neurodegenerative diseases [1], the subject remains controversial. Unless the controversial role of Al as an environmental factor in the pathogenesis of neurodegenerative diseases is well resolved therefore, it will continue to be a subject of man's curiosity. Thus, it is important to clarify the metabolism of Al in brain. The forms of Al being taken up by brain cells have not been specified so far. Astrocytes have been recognized as the primary or potential target for Al toxic action [2], therefore, Al amino acid complex as possible form of Al internalization by astrocytes is the subject of the present report. The ability of cultured astrocytes to differentially internalize Al in complex with some amino acids is reported here. We also report here the possible mechanism of uptake of Al amino acid complex and the apoptotic effect of internalized Al.

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Methods

Primary culture of cortical astrocytes was prepared from 4 - 5 day old mice. The cells were grown in DMEM/F12 (or DMEM during experimentation) containing 15% fetal calf serum and 0.05 mg/ml gentamicin, incubated at 37 °C in humidified atmosphere of 5% CO_2 and were used for experiments after second passages by trypsinization. In all experiments, the cells were stressed with 0.1 mM Al amino acid complex for times ranging between 0.5 - 8h, except otherwise stated. In order to test the effects of transporter blockers, the cells were exposed to 0.1 mM each of dihydrokainic acid or sarcosine, a selective blocker of EAAT2 or GlyT1 respectively, as well as trans-pyrollidine-2,4-dicarboxylic acid or doxepin, a non-selective blocker of glutamate or glycine transporter respectively for 30 min (induction time). EAAT2 (also known as GLT-1) and GlyT1 are the dominant CNS astrocytic glutamate and glycine transporters respectively. The cells were then stressed with the respective Al amino acid complex (0.1 or 1 mM). In another experiment, the cells were exposed to 0.1 mM ouabain. Uptake of the respective Al amino acid complex in the presence and absence of transporter blockers were compared. Al content was measured by electrothermal atomic absorption spectrophotometer with Zeeman background correction, following wet digestion with ultra pure HNO_3 (Kanto Kagaku) and results expressed in ngAl/μg prot. For apoptotic study, the cells were exposed to graded doses (0.0125 – 0.1 mM) of Al(Gly) complex for 6h after which

the medium was exchanged and the cells cultured for the next 1 – 10 days in normal medium. The cover slips on which the cells were cultured were carefully removed, washed with PBS, fixed with methanol (-20 °C), stained with Hoechist33258 dye, and then observed under fluorescence microscope.

Results and Discussion

Aluminum from different complexes of amino acid was differentially internalized by cultured astrocytes (Fig. 1). Thus the amino acids do not only solubilize Al but also influence its uptake. It is expected that the amino acid transporter blockers may influence the uptake of Al in complex with the respective amino acids, however none of the transporter blockers employed in the present study apparently inhibited the uptake of Al. Unexpectedly, some of the blockers rather enhanced the uptake (Fig. 2 and 3).

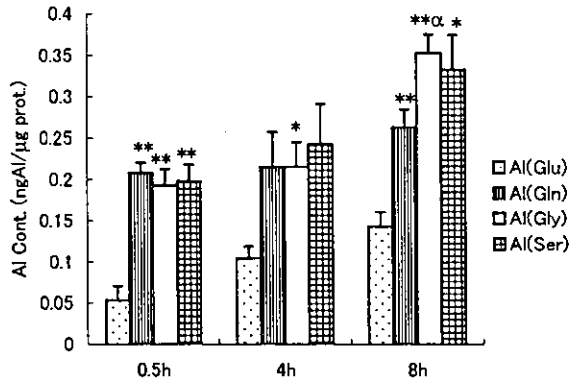

Figure 1. Cellular accumulation of Al following 0.5 - 8h exposure of primary culture of cortical astrocytes to 0.1 mM of Al(Glu), Al(Gln), Al(Gly) and Al(Ser). Plotted values represent the mean ± SE (n=4). ** and * indicate statistical difference from Al(Glu) at $p<0.01$ and 0.05 respectively, while α indicates statistical difference from Al(Gln) at $p<0.05$ using Welch's t test.

There is a possibility that other mechanisms influenced by concentration gradient are involved in the Al transport in the forms employed here, more so, when more Al was taken up at higher concentration (Fig. 2) and longer exposure time (Fig. 1 and 3). It has been recently suggested that the fact that a substance solubilizes Al does not necessarily imply that its transfer into cytoplasm follows the receptor-mediated pathway of the solubilizing agent [3]. Ouabain has also failed to block the uptake of Al in complex with glycine in the present study (Fig. 3). Thus, uptake of Al in the present forms can be said to be mainly by passive transport and this agrees with previous findings [4].

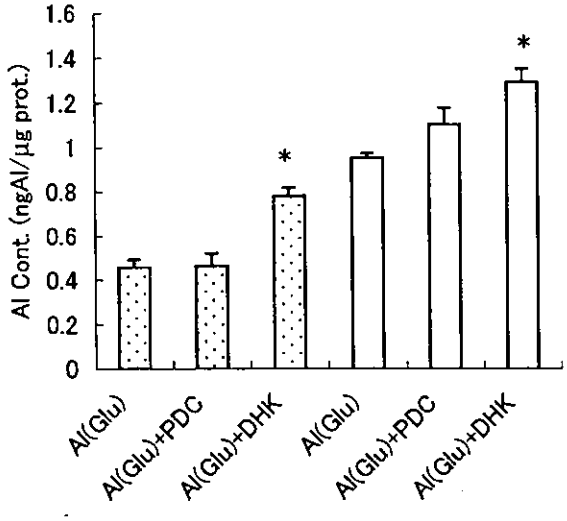

Figure 2. Cellular accumulation of Al following 6h exposure of primary culture of cortical astrocytes to 0.1 mM (dotted bar) or 1 mM (plain bar) of Al(Glu) in the presence and absence of PDC, trans-pyrollidine-2,4-dicarboxylic acid; and DHK, dihydrokainic acid. Plotted values represent the mean ± SE (n=4). * indicates statistical difference from control condition (i.e., in the absence of blockers) at $p<0.05$ using Welch's t test.

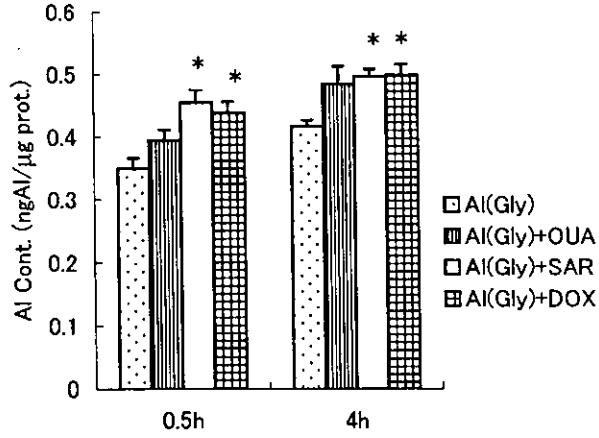

Figure 3. Cellular accumulation of Al following 0.5 or 4h exposure of primary culture of cortical astrocytes to 0.1 mM Al(Glu) in the presence and absence of OUA, ouabain; SAR, sarcosine; and DOX, doxepine. Plotted values represent the mean ± SE (n=4). * indicates statistical difference from control condition (i.e., in the absence of blockers) at $p<0.05$ using Welch's t test.

Apoptosis is a major form of cell death, characterized by a series of distinct morphological and

biochemical alterations. It is an important process in a wide variety of different biological systems, including normal cell turnover, the immune system, embryonic development, metamorphosis and hormone-dependent atrophy as well as in chemical-induced cell death. There are emerging evidences that Al is capable of inducing apoptosis in astrocytes [5]. The apoptotic effect of Al amino acid complex on cultured astrocytes is further confirmed in the present study with evidence of nuclear shrinkage and chromatin condensation that occurred in more than 20% of the cells. The significance of the present finding is that nuclear shrinkage and chromatin condensation occurred as early as three days following 6h exposure to test compounds and also at concentrations as low as 0.0125 mM. This is much lower concentration and earlier effect than those in previous reports [5]. Although the route of Al transport was yet to be clarified, Al amino acid complex is still candidate species relevant to its internalization.

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Evidence of novel neuronal functions of dysbindin, a susceptibility gene for schizophrenia

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Genetic variation in dysbindin (DTNBP1: dystrobrevin-binding protein 1) has recently been shown to be associated with schizophrenia. The dysbindin gene is located at chromosome 6p22.3, one of the most promising susceptibility loci in schizophrenia linkage studies. We attempted to replicate this association in a Japanese sample of 670 patients with schizophrenia and 588 controls. We found a nominally significant association with schizophrenia for four single nucleotide polymorphisms and stronger evidence for association in a multi-marker haplotype analysis ($P = 0.00028$). We then explored functions of dysbindin protein in primary cortical neuronal culture. Overexpression of dysbindin induced the expression of two pre-synaptic proteins, SNAP25 and synapsin I, and increased extracellular basal glutamate levels and release of glutamate evoked by high potassium. Conversely, knockdown of endogenous dysbindin protein by small interfering RNA (siRNA) resulted in the reduction of pre-synaptic protein expression and glutamate release, suggesting that dysbindin might influence exocytotic glutamate release via upregulation of the molecules in pre-synaptic machinery. The overexpression of dysbindin increased phosphorylation of Akt protein and protected cortical neurons against neuronal death due to serum deprivation and these effects were blocked by LY294002, a phosphatidylinositol 3-kinase (PI3-kinase) inhibitor. SIRNA-mediated silencing of dysbindin protein diminished Akt phosphorylation and facilitated neuronal death induced by serum deprivation, suggesting that dysbindin promotes neuronal viability through PI3-kinase-Akt signaling. Genetic variants associated with impairments of these functions of dysbindin could play an important role in the pathogenesis of schizophrenia.

INTRODUCTION

Schizophrenia is a complex genetic disorder characterized by profound disturbances of cognition, emotion and social functioning. It affects ~1% of the general population worldwide. Chromosome 6p is one of the most consistently replicated susceptibility regions in linkage studies of schizophrenia (1). A recent study implicated a gene on chromosome 6p, dysbindin (DTNBP1: dystrobrevin-binding protein 1), as a susceptibility locus in the Irish pedigrees (2). Since then, four studies have reported evidence supporting the association between genetic variants in dysbindin and schizophrenia in

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German, Chinese, Swedish and Irish populations (3–6), while one study failed to replicate positive association in an Irish case–control design (7). In the present study, we attempted to perform an independent association study in a Japanese population of schizophrenic cases and controls.

The pathophysiology of schizophrenia is still unclear; however, this disease is believed to involve genetic abnormalities in developmental processes leading to abnormal synaptic plasticity, including glutamatergic transmission (8,9). Several genes, e.g. dysbindin, neuregulin 1, G72, D-aminoacid oxidase, the regulator of G-protein signaling-4, GRM3 and PPP3CC are described as susceptibility genes for schizophrenia, and those genes may have convergent effects on glutamatergic synapses (10,11). Neuregulin affects the expression and plasticity of the *N*-methyl-D-aspartate (NMDA) receptor (12,13). D-aminoacid oxidase metabolizes D-serine, an endogenous modulator of the NMDA receptor (14), and G72 is probably an activator of D-aminoacid oxidase (15). The regulator of G-protein signaling-4 is the negative regulator of G-protein-coupled receptors, including metabotropic glutamate receptors (16). GRM3 encodes the mGlu3 receptor gene. PPP3CC, the calcineurin γ-subunit, is critical for certain types of NMDA-mediated plasticity. However, no evidence of a role in glutamatergic transmission has been imputed to dysbindin, although dysbindin is believed to play a role in synaptic plasticity and signal transduction. Although dysbindin has recently been cloned as a dystrobrevin-binding protein in mouse (17), little is known about the functions in neurons. Here, we examined neuronal functions of dysbindin and found two novel actions: (1) increased glutamate release with upregulation of pre-synaptic proteins and (2) neurotrophic effect through Akt signaling pathway.

RESULTS

Genetic association analysis

We genotyped six single nucleotide polymorphisms (SNPs) in dysbindin in 670 schizophrenic patients and 588 controls in a Japanese population. The genotype distributions of the six SNPs for the schizophrenic patients and the control subjects were in Hardy–Weinberg equilibrium (data not shown). Allele frequencies of the six SNPs among the patients and controls are shown in Table 1. A significant difference in allele frequency was observed between cases and controls for four SNPs, but not for the remaining two SNPs (Table 1). The G allele of P1635 was in excess in our cases when compared with controls ($\chi^2 = 10.3$, df = 1, $P = 0.0013$, odds ratio = 2.71, 95% CI 1.46–5.79, corrected $P = 0.0078$).

To further analyze the haplotype structure in our sample, we computed the linkage disequilibrium (LD) between the SNPs using D'. D' values ranged between 0.5 and 1.0 and indicated strong to intermediate LD between the markers. Thus, adjacent combinations of up to six markers were examined for association with schizophrenia. Global and individual *P*-values corresponding to haplotypes consisting of adjacent markers and estimated haplotype frequencies in patients and controls are shown in Table 2. All haplotype combinations were significantly associated with schizophrenia, except the P1320–P1763 haplotype. Given this result, we tested the contribution of individual haplotypes to the global result. The G–G haplotype (P1635–P1325), including the G allele of P1635, which was significantly more frequent in our cases (Table 2), was enriched in patients with schizophrenia when compared with controls (estimated frequencies: patients 3.0% versus controls 0.9%, *P*-value = 0.00028, corrected $P = 0.0042$).

Functional analysis in dysbindin-overexpressing cultured neurons

To clarify the function of dysbindin in the central nervous system, we focused on the pre-synaptic machinery in neuronal transmission, as dysbindin is primarily expressed in axonal terminals of the mouse brain (17). Pre-synaptic machinery for exocytotic transmitter release is composed of membrane proteins, cytoskeletal proteins and synaptic vesicle proteins (18). SNAP25 (25 kDa synaptosomal associated protein) and syntaxin are membrane proteins implicated in the docking, priming and fusion of the vesicles. Synapsin I is a cytoskeletal protein associated with the synaptic vesicles in the reserve pool. Synaptotagmin is a synaptic vesicle protein, which has been identified as a calcium sensor protein. Thus, we examined the expression of these synaptic associated molecules after overexpression of dysbindin with virus-mediated gene transfer system. Infected neuronal cultures were doubly stained with GFP signal and immunostaining signal by anti-MAP2 (a neuronal dendritic marker) antibody (Fig. 1A). Approximately 80% of MAP2-positive cells in either control (GFP-infected) or dysbindin-overexpressing (dysbindin- and GFP-infected) cultures were GFP-positive, indicating that the majority of neurons were infected. As shown in Figure 1B, SNAP25 and synapsin I expression tended to be upregulated in dysbindin-overexpressing cultures compared with control (49 and 57%, respectively), whereas the changes of synaptotagmin and syntaxin expression were not observed (data not shown). The levels of class III β-tubulin (TUJ1, a neuronal marker) were not altered in the three conditions (Fig. 1B). We confirmed the overexpression of dysbindin (~17-fold when compared with control) in dysbindin-infected cultures and the expression of GFP in both control and dysbindin-overexpressing cultures (Fig. 1B).

Upregulation of synapsin I and SNAP25 raised the possibility that release of neurotransmitter might be increased by the overexpression of dysbindin. Therefore, we measured the release of glutamate, which is the principle neurotransmitter in these neurons. As expected, the amount of basal glutamate from dysbindin-infected cortical cultures was significantly increased when compared with the uninfected or control cultures (Fig. 1C), indicating that dysbindin overexpression resulted in an elevation of extracellular glutamate. Furthermore, high KCl (HK^+)-evoked exocytotic release of glutamate was enhanced in dysbindin-infected cultures. These results suggest that dysbindin might be one of the regulator proteins in the excitatory neurotransmission.

We then investigated the effects of dysbindin on neuronal viability. Interestingly, it was found that the phosphorylation of Akt, a molecule in the phosphatidylinositol 3-kinase (PI3-kinase) pathway, was significantly enhanced by 67% in the dysbindin-overexpressing cultures, whereas total Akt protein levels were unchanged (Fig. 2A). As the activation of Akt is

Table 1. Allele frequencies of six dysbindin SNPs between the patients with schizophrenia and controls

Marker name	dbSNP ID	Polymorphism major/minor	Location	Minor allele frequency		*P*-value	Odds ratio (95% CI)
				Controls	Patients		
P1655	rs2619539	G/C	Int 5	0.311	0.317	0.748	1.03 (0.87–1.22)
P1635	rs3213207	A/G	Int 4	0.011	0.030	0.0013	2.71 (1.46–5.79)
P1325	rs1011313	G/A	Int 4	0.153	0.166	0.372	0.91 (0.72–1.15)
P1320	rs760761	C/T	Int 3	0.071	0.095	0.027	1.38 (1.04–1.83)
P1763	rs2619522	T/G	Int 1	0.070	0.095	0.022	1.40 (1.05–1.86)
SNPA	rs2619538	T/A	Promoter	0.024	0.040	0.025	1.69 (1.05–2.86)

Table 2. Estimated haplotype frequencies and case–control haplotype results

Markers	*P*-value		Haplotype	Haplotype frequency	
	Global	Individual		Controls	Patients
P1655–P1635	0.0026	0.0003	G–G	0.011	0.030
P1635–P1325	0.00041	0.00028	G–G	0.009	0.030
P1325–P1320	0.0074	0.013	G–T	0.069	0.096
P1320–P1763	0.06	0.02	C–T	0.929	0.904
P1763–SNPA	0.025	0.0047	G–A	0.009	0.025
P1655–P1635–P1325	0.0055	0.001	G–G–G	0.011	0.030
P1635–P1325–P1320	0.0006	0.0009	G–G–T	0.010	0.027
P1325–P1320–P1763	0.027	0.029	G–T–G	0.068	0.095
P1320–P1763–SNPA	0.05	0.0045	T–G–A	0.009	0.025
P1655–P1635–P1325–P1320	0.011	0.0038	G–G–G–T	0.011	0.027
P1635–P1325–P1320–P1763	0.0015	0.001	G–G–T–G	0.010	0.027
P1325–P1320–P1763–SNPA	0.015	0.0019	G–T–G–A	0.007	0.025
P1655–P1635–P1325–P1320–P1763	0.025	0.0028	G–G–G–T–G	0.011	0.027
P1635–P1325–P1320–P1763–SNPA	0.003	0.0016	G–G–T–G–A	0.009	0.026
P1655–P1635–P1325–P1320–P1763–SNPA	0.024	0.0012	G–G–G–T–G–A	0.010	0.026

Case–control haplotype analysis were performed using the permutation method to obtain empirical *P*-values. Global *P*-values and individual *P*-values (lowest *P*-values among the haplotypes) are indicated. Estimated frequency for the haplotype with significant association in controls and patients were shown.

regulated by phosphorylation, overexpression of dysbindin resulted in the activation of Akt. LY294002, a PI3-kinase inhibitor, completely blocked the activation of Akt by the dysbindin overexpression, with no alteration of the expression levels of Akt and TUJ1 proteins (Fig. 2A). As the PI3-kinase pathway is involved in neuronal function and survival (19), we examined the viability of cortical neurons with our virus infection system (Fig. 2B). The overexpression of dysbindin protein itself did not alter neuronal viability when compared with control. However, dysbindin overexpression significantly blocked the reduced viability of cortical cultures by serum deprivation. Additionally, LY294002 significantly inhibited the protective effects of dysbindin, suggesting that the PI3-kinase pathway was involved in the dysbindin-dependent viability promoting effects.

Knockdown analysis of endogenous dysbindin in cultured neurons

We further examined the endogenous dysbindin function in cortical cultures using small interfering RNA (siRNA) for dysbindin. Previously, we reported siRNA-dependent down-regulation of endogenous protein expression in primary cultured neurons (20). Here, we performed transfection of siRNA for dysbindin and confirmed the robust decrease (83%) of endogenous dysbindin protein (Fig. 3A). The protein expression levels of SNAP25 and synapsin I and the phosphorylation level of Akt protein was significantly suppressed after dysbindin-siRNA transfection (43, 37 and 52% of reduction, respectively), although the expression levels of TUJ1 and Akt proteins were not altered (Fig. 3A). Thus, we investigated dysbindin function on glutamate release and neuronal viability under this condition. The amount of basal and released glutamate from dysbindin-siRNA-transfected cortical cultures significantly decreased when compared with the control (scramble) cultures (Fig. 3B), indicating that endogenous dysbindin protein plays a role in the excitatory neurotransmission. The neuronal viability was not changed by dysbindin-siRNA transfection in the presence of horse serum (Fig. 3C). However, dysbindin-siRNA transfection significantly facilitated neuronal death when horse serum was deprived (Fig. 3C), suggesting that the endogenous dysbindin protein has a promoting effect on survival.

DISCUSSION

In the present study, we report a significant association between genetic variation of dysbindin and schizophrenia in a Japanese population. In previous studies, highly significant

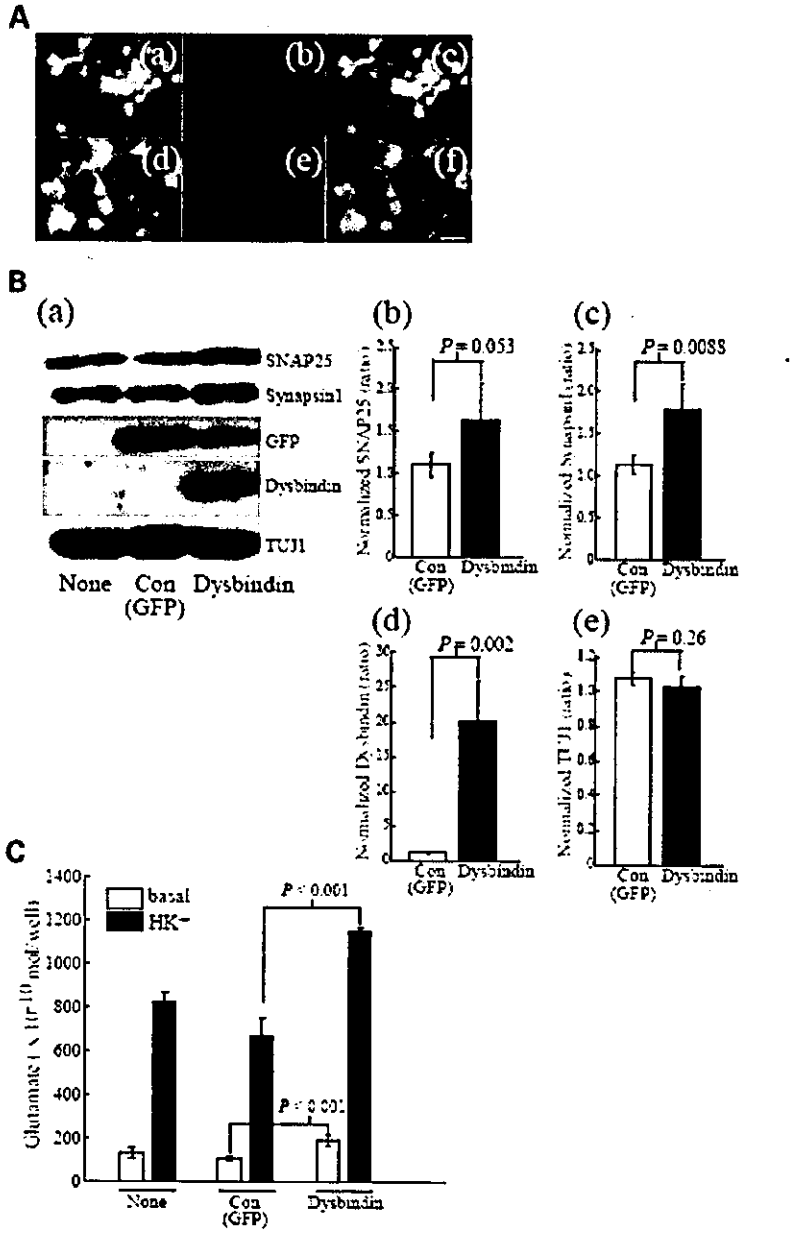

Figure 1. Dysbindin increases the expression of pre-synaptic proteins and glutamate release. (A) Double-staining of GFP and MAP2. Cortical cultures (6 days *in vitro*, DIV6) were prepared with viral infection of GFP only (a–c) or with viral infection of GFP and dysbindin (d–f) at DIV4. Images were obtained with GFP (a, d; green) and with immunostaining of anti-MAP2 antibody (b, e; red). Merged images (c, f; yellow) were also shown. (B) (a) Upregulation of pre-synaptic proteins. Cortical cultures (DIV6) were prepared without viral infection (None), with viral infection of GFP (Con) or with viral infection of GFP and dysbindin (Dysbindin) at DIV4. The cell lysates were collected at DIV6 and SNAP25, synapsin I, GFP, dysbindin and TUJ1 were detected by western blotting. The immunoblots shown are representative of four independent experiments. (b–e) Quantification of the immunoreactivity of SNAP25, Synapsin I, dysbindin and TUJ1. Data represent mean ± SD of the immunoreactivity from four independent experiments. (C) Increase of the released glutamate in dysbindin-overexpressing cortical cultures. Cortical cultures were prepared without viral infection (None), with viral infection of GFP (Con) or with viral infection of GFP and dysbindin (Dysbindin) at DIV4. Basal or HK^+ (50 mM KCl)-evoked release of glutamate was measured at DIV6 (after 48 h from infection). Data represent mean ± SD ($n = 4$).

associations were found for SNPs in introns 4–6, which is consistent with our results. The G allele of P1635, which was significantly in excess in our cases (3.0%), was also over-transmitted in Irish samples (10.2%) (2), whereas this allele was under-transmitted in German samples (17.6%) (3), suggesting that this SNP might be a marker rather than a polymorphism responsible for giving susceptibility. Notably, a high-risk haplotype in our samples was the G–G–T–G

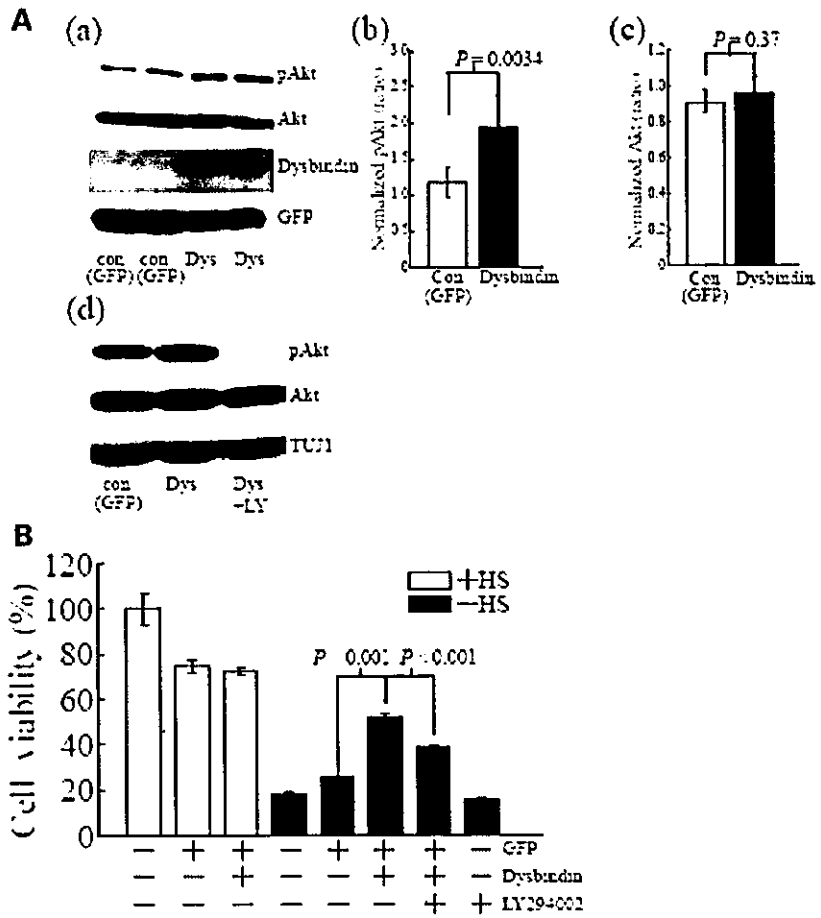

Figure 2. Dysbindin protects cortical neurons through PI3-kinase-Akt signaling. (A) (a) The activation of PI3-kinase pathway in dysbindin-overexpressing cultures. Cortical cultures after DIV4 were treated with viral infection of GFP (Con) or with viral infection of GFP and dysbindin (Dys) for 48 h. (b, c) Quantification of the immunoreactivity of pAkt and total Akt proteins. Data represent mean ± SD of the immunoreactivity from four independent experiments. (d) The inhibitory effect of LY294002 on activation of Akt. Cortical cultures at DIV4 were treated with viral infection of GFP (Con), with viral infection of GFP and dysbindin (Dys) or with viral infection of GFP and dysbindin in the presence of LY294002 (1.0 μM) (Dys + LY) for 48 h. Cortical cultures were harvested at DIV6 for western blotting for pAkt, Akt, dysbindin, GFP or TUJ1. The immunoblots shown are representative of four independent experiments. (B) Neuroprotective effects of dysbindin against serum deprivation. Cortical cultures after DIV4 were treated with viral infection of GFP (Con), with viral infection of GFP and dysbindin (Dysbindin) or with LY294002 (1.0 μM) for 48 h. Deprivation of horse serum (HS) at DIV5 24 h after viral infection is indicated as −HS. Cell viability was determined using the MTT assay at DIV6 48 h after the viral infection and/or 24 h after HS deprivation. Data represent mean ± SD ($n = 8$).

haplotype (P1635–P1325–P1320–P1763), which includes the high-risk haplotype (G–G–G–G–T–G–C–C; P1635–P1325–P1765–P1757–P1320–P1763–P1578–P1792) reported in an Irish sample (6). The frequency of our high-risk haplotype (2.7% in cases versus 1.0% in controls) is lower than that in an Irish population (6%). Novel schizophrenia risk and protective haplotypes (C–A–T, C–A–A, G–G–T; P1655–P1635–SNPA) were recently identified in Cardiff and Dublin samples (21). We also analyzed these haplotypes in our sample and obtained evidence for a significant association with a different haplotype (global P-value = 0.0086, individual P-value = 0.005; G–G–A). Furthermore, the estimated frequencies of C–A–A and G–G–T haplotypes in our sample were <0.1%, although the overall frequencies in Cardiff and Dublin were 33 and 1.4%, respectively. We failed to find a significant association for the C–A–T haplotype (overall frequency, Cardiff and Dublin versus ours, C–A–T: 18 versus 32%). These differences of the haplotype frequencies might be based on the different ethnicity. A false-positive association owing to population stratification could not be excluded in our case–control study, despite the precaution of ethnic matching of this study.

It is of interest to study how genetic variation affects dysbindin function/expression. We do not know that any of the SNPs in our haplotypes are functional. Very little is known about the potential function of specific intronic sequences with regard to protein binding, stability and splicing efficacy. A recent study showed the functional possibility of intronic SNPs on gene expression. For example, an intronic SNP affects the transcriptional efficiency of SLC22A4 *in vitro*, owing to an allelic difference in affinity to Runt-related transcription factor 1, and this SNP is associated with rheumatoid arthritis, one of