Selective blockade of astrocytic glutamate transporter GLT-1 with dihydrokainate prevents neuronal death during ouabain treatment of astrocyte/neuron co-cultures.

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Abstract

Glutamate (Glu) is a major excitatory neurotransmitter of the mammalian central nervous system and under normal conditions plays an important role in information processing in the brain. Therefore, extracellular Glu is subject to strong homeostasis. Astrocytes in the brain have been considered to be mainly responsible for the clearance of extracellular Glu. In this study, using mixed neuron/astrocyte cultures, we investigated whether astrocytic Glu transporter GLT-1 is crucial to the survival of neurons under various conditions. Treatment of the mixed cultures with a low concentration of Glu did not produce significant death of neurons. However, co-treatment with dihydrokainate (DHK), a specific blocker of GLT-1, resulted in significant neuronal death that was suppressed by an antagonist of N-methyl-D-aspartate (NMDA) receptors. These results suggested that asrocytic GLT-1 participated in the clearance of extracellular Glu and protected neurons from NMDA receptor-mediated toxicity. When the cultures were treated with ouabain -- an inhibitor of Na^+/K^+-ATPase -- a low concentration of Glu resulted in massive neuronal death that was also suppressed by co-treatment with an antagonist of NMDA receptors. In this case, however, co-treatment with DHK significantly protected neurons from death, suggesting that GLT-1 was responsible for the death of neurons. The present study provides evidence suggesting that astrocytes use their Glu transporter GLT-1 to protect neurons from Glu toxicity, but ironically, also use GLT-1 to kill neurons through Glu toxicity depending on their status.

Introduction

Extracellular L-glutamate (Glu) in the mammalian brain is subject to homeostasis because an elevated concentration results in the excessive activation of Glu receptors, thereby resulting in neuronal death (Choi, 1988). Astrocytes seem to be the cell type primarily responsible for the clearance of extracellular Glu in the brain (Rothstein *et al*., 1996), while the astrocytic Na^+-dependent Glu transporter GLT-1 is quantitatively the dominant form (Rao *et al*., 2001b; Tanaka *et al*., 1997). The disruption of astrocytic Glu transporters such as GLT-1 may contribute to neuronal damage in stroke, trauma, Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington's disease (Hazell and Butterworth, 1999; Li *et al.*, 1999; Rao *et al*., 2001b; Rothstein *et al*., 1994). Thus, the function of astrocytic Glu transporters seems to be crucial to the survival of neurons in the brain. However, under certain conditions such as brain anoxia, the role of astrocytic Glu transporters is reversed; that is, Glu is released from astrocytes to the extracellular space (Szatkowski *et al*., 1990), triggering the death of neurons, thus giving rise to mental and physical handicaps (Takahashi *et al*., 1997). Recently however, Rossi *et al*. (2000) have reported that the reversed uptake of Glu by neuronal Glu transporters is crucial in the death of neurons during ischemia. Therefore, it remains controversial as to what mechanisms are critical for the ischemia-induced rise of Glu and neuronal death.

Here we report experimental evidence obtained using mixed neuron/astrocyte cultures, suggesting that astrocytes utilize their Glu transporter GLT-1 for the protection of neurons from Glu neurotoxicity and, ironically, for the killing of neurons through Glu neurotoxicity, depending on their status.

Materials and methods

Cell culture.

Neurons/astrocytes were prepared from 16 to 18 day old embryonic rat cortices and grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) which was supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 10 % Ham's F12, and 0.24 % penicillin/streptomycin (culture medium). Cells were plated at a uniform density of 3.0×10^5 cells/cm^2 onto poly-L-lysine (100 μg/ml)-coated plastic dishes and maintained in a 5 % CO_2 incubator at 37 ℃. The cultures were fed a filtered (0.22 μm; Millipore, Bedford, TX) conditioned medium (CM) twice a week. To obtain the CM, cells from the 16-18 day old embryonic rat cortices were plated onto poly-L-lysine-coated 6 well dishes and cultured for more than 2 weeks. The cultures were then fed a cooled culture medium and incubated for an additional day. The culture medium was then filtered and used as a CM. After 13-15 days, the neurons in these cultures sit on the top of a confluent monolayer of astrocytes. The experiments were performed using these cultures.

Astrocyte-enriched cultures were prepared from cortical astrocytes of postnatal 1-3 day old rat pups. Cortical hemispheres were removed, cleaned, and dissociated using papain (Roche Diagnostics GmbH, Mannheim, Germany) and mechanical trituration. Cells were placed on poly-L-lysine-coated glass coverslips and maintained in the culture medium. The medium was changed 48 hours later and twice a week thereafter. The experiments described here were performed on astrocytes maintained for 13-15 days in culture.

Immunocytochemistry.

Astrocytes and neurons were identified by immunostaining with antibodies against glial fibrillary acidic protein (GFAP; Sigma, St Louis, MO) and microtubule-associated protein 2 (MAP-2; Sigma), respectively. The astrocytic glutamate transporters GLT-1 and GLAST were detected by immunostaining with anti-GLT-1 and anti-GLAST polyclonal antibodies (Chemicon, Temecula, CA), respectively. For the labeling of MAP-2, GFAP, GLAST, and GLT-1, the cortical cells were fixed with 4 % paraformaldehyde for 5 minutes at 4 ℃, followed by 95 % methanol in PBS for 10 minutes at –20 ℃. The cells were then incubated with a primary antibody over a 24 hour period using a dilution of 1:1000 for MAP-2, 1:400 for GFAP, 1:400 for GLT-1, and 1:5000 for GLAST. After being washed with phosphate-buffered saline (PBS), the cells were incubated with a secondary antibody containing 1.0 % goat serum for 30 minutes. For labeling, a 1:500 dilution of biotinylated goat antibody against mice IgG (Vector Laboratories, Burlingame, CA) was used. Bound antibodies were detected by the avidin-biotin-peroxidase complex (ABC) method, using a commercial ABC kit (Vector Laboratories). Observation of peroxidase activity was made possible by incubation with 0.1 % 3,3'-diaminobenzidine tetrahydrochloride (DAB) in a 50 mM Tris-HCl buffer (pH 7.4) supplemented with 0.02 % H_2O_2. The cells were dehydrated in 70 – 100 % ethanol, cleared in xylene, and mounted on glass coverslips in Permount (Fisher Scientific, Fair Lawn, NJ) for light microscopic observation.

Excitotoxicity protocol.

Cultures were treated with glutamate (Glu) at various concentrations for 30 minutes, and the survival rate of neurons was analyzed 24 hours after exchanging the Glu-containing test medium

with the Glu-free conditioned medium. An Mg^{2+}-free Earle's Balanced Salt Solution (EBSS) was used as the test medium. When dihydrokainate (DHK) was used to investigate whether Glu-toxicity waz enhanced by blocking GLT-1, treatment of cultures with DHK (100 μ M) was started 30 minutes before the glutamate (10 μ M) treatment and was continued throughout the treatment. When ouabain was used to block Na^+/K^+-ATPase, cultures were co-treated with ouabain (50 μ M) and Glu (10 μ M) for 15 minutes. To investigate whether DHK treatment would affect the neurotoxicity caused by inhibiting the activity of the Na^+/K^+-ATPase, treatment of cultures with DHK (200 μ M) began 15 minutes prior to and was continued throughout the ouabain (50 μ M) treatment. When DL-threo- β -benzyloxyaspartate (TBOA) -- a competitive blocker of GLAST and GLT-1 (Diamond 2001; Shimamoto *et al*., 1998) -- was used, the concentration of TBOA was set at 50 μ M. The experimental protocols were the same as those in DHK.

Survival rate of neurons.

Neuronal death was analyzed following observation of the nuclear morphology by using the fluorescent DNA-binding dyes, Hoechst 33342 (H33342) and propidium iodide (PI). Cells were incubated with these dyes for 15 minutes at 37℃. Individual nuclei were observed using fluorescent microscopy (Olympus, IX70) and subsequently analyzed. PI was used to identify nonviable cells. More specifically, an average of 450-500 neurons from random fields were analyzed in each experiment. The survival rate of neurons –- meaning the percentage of viable neurons remaining -- was determined by placing images of nuclear staining on phase-contrast images, and calculated the number as (viable neurons/total neurons before drug treatment) $\times$

100 %, since some neurons came off the dishes at the time of inspection. At least 4 independent experiments (n $\geqq$4) were conducted and analyzed.

Chemicals.

Dihydrokainate (DHK), 1 β , 3 β , 5 β , 11 α , 14,19-hexahydroxycard-20(22)-enolide 3-(6-deoxy- α -L-mannopyranoside (ouabain), bisbenzimide (Hoechst 33342), DL-2-amino-5-phosphonopentanoic acid (AP5), and PI were obtained from Sigma. TBOA was purchased from Tocris (Avonmouth Bristol, UK). All other compounds were obtained from Wako Chemical (Tokyo, Japan).

Statistics.

Data are presented as the mean$\pm$S.D. Statistical comparison of the control group with treated groups was carried out using an unpaired t-test. Differences with a value of P$<$0.05 were considered significant.

Results

We analyzed the functional role of the astrocytic glutamate (Glu) transporter GLT-1 in mixed cultures of neurons and astrocytes (Fig. 1A1 & A2) from fetal rat brain (embryonic day 17). During this process, we first tried to clarify the dose-dependent neurotoxicity of Glu in the cultures. Exposure to a relatively low concentration of Glu (5 & 10 μM) did not result in significant neuronal death in our mixed cultures (Fig. 1C & E). However, a high concentration of Glu induced significant neuronal death (Fig. 1 D & E).

The Glu toxicity observed in this study seemed rather mild compared to that found in pure neuronal cultures reported previously (Cheung *et al*., 1998). Therefore, there is a possibility that astrocytic Glu transporters play some neuro-protective role in this situation. Next, we confirmed whether or not the astrocytes in our cultures expressed the astrocytic Glu transporter GLT-1 that is crucial to Glu neurotoxicity. A previous study has revealed that astrocytes co-cultured with neurons change from a polygonal to a process-bearing morphology that is more characteristic of astrocytes in situ (Swanson *et al*., 1997). The expression of both GLT-1 and GLAST increases with such morphological changes (Perego *et al*., 2000). Flat polygonal astrocytes preferentially express GLAST, while the process-bearing cells express GLT-1 (Perego *et al*., 2000). These results suggest that neurons are directly involved in regulating the expression of astrocytic Glu transporters (Gegelachvilli *et al*., 1997; Perego *et al*., 2000; Schlag *et al*., 1998). Most of the GFAP-positive astrocytes in our cultures showed a process-bearing, but not a flat polygonal morphology (Fig. 1A2). GLT-1 was actually expressed on such process-bearing astrocytes in the mixed neuron-astrocyte cultures (Fig. 2A). Although GLT-1 is believed to be present only on

astrocytes in the brain (Lehre *et al*., 1995), there are reports that GLT-1 is also present on neurons under certain conditions (Martin *et al*., 1997; Mennerick *et al*., 1998). However, we observed hardly any GLT-1-positive neurons in our mixed cultures, despite careful inspection of the immunocytochemical data. We then analyzed the change in neurotoxicity caused by the exposure to Glu while blocking GLT-1 with dihydrokainate (DHK) -- a selective non-transportable inhibitor of GLT-1 (Levy *et al*., 1998; Rao *et al*., 2001b; Robinson, 1998).

A previously ineffective concentration of Glu (10 μ M) caused significant neuronal death when astrocytic GLT-1 was blocked with DHK (Fig. 2B &D). However, cell death was significantly reduced by co-treatment with DL-2-amino-5-phosphonopentanoic acid (AP5), a specific inhibitor of N-methyl-D-aspartate (NMDA) type Glu receptors (Fig. 2C &D). Cultures treated with only DHK (100 μ M) for 30 minutes did not result in the significant death of neurons (Fig. 2D). Almost all the astrocytes, however, seemed to remain intact under these experimental conditions. These results suggest that the astrocytic Glu transporter GLT-1 actively participated in the clearance of extracellular Glu and protected neurons from NMDA receptor-mediated excitotoxic stress. Thus, the functional operation of GLT-1 seems crucial to the survival of neurons.

The sodium-dependent astrocytic Glu transporter GLT-1 transports one Glu anion coupled to the co-transport of three Na^+ and one H^+, as well as to the countertransport of one K^+ (Levy *et al*., 1998). GLT-1 uses steep ionic gradients across the membrane to accumulate a high intracellular concentration of Glu in astrocytes. The ionic gradients are mainly maintained by Na^+/K^+-ATPase, which excludes Na^+ in exchange for extracellular K^+, in turn energizing other secondary ion transporters (e.g., Na^+-Ca^{2+} exchanger). We then tried to block GLT-1 by inhibiting Na^+/K^+-ATPase with ouabain -- a specific inhibitor of such ATPases -- instead of by using DHK.

Inhibition of Na^+/K^+-ATPase with ouabain resulted in a gradual increase of intracellular Na^+ in astrocytes (Rose *et al*., 1998). Thus, the treatment of cultures with this drug is expected to disrupt the Na^+ gradient across membranes and effectively block the Na^+-dependent glutamate transporter GLT-1, an effect which is similar to DHK, although the mechanism of inhibition is different.

The treatment using a low concentration of Glu (10 μM) for only 15 minutes resulted in massive neuronal death when the cultures were co-treated with 50 μM ouabain (Fig. 3A & D). The survival rate of neurons was markedly reduced on co-treatment with ouabain compared with DHK (Fig. 2D). The reason for this difference in the survival rate is unclear, although one possibility is that the ouabain treatment depressed the Na^+/K^+-ATPase of neurons as well as astrocytes. Inhibition of the Na^+/K^+-ATPase of neurons may result in the disruption of neuronal Na^+-dependent Glu transporters, possibly contributing to neuronal death. In fact, a recent study has demonstrated that the dysfunction of neuronal Glu transporters is involved in the ischemia-induced rise in Glu and neuronal death (Rossi *et al*., 2000). Another possibility is that the ouabain treatment reverses the role of the astrocytic Na^+-dependent Glu transporter GLT-1. The reversed uptake of Glu also contributes to the marked rise in extracellular Glu and neuronal death (Szatkowski *et al*., 1990; 1994). We then tested these possibilities.

Co-treatment of cultures with DHK, which caused neuronal death in Figure 2, significantly protected neurons from death when the activity of Na^+/K^+-ATPase was inhibited with ouabain (Fig. 3B & D). This result seems to exclude the possible involvement of dysfunctional neuronal Glu transporters in massive neuronal death caused by the inhibition of the activity of neuronal Na^+/K^+-ATPase, since the neurons in our cultures did not express GLT-1 and because DHK has

been known to specifically block GLT-1. Therefore, this result may favor the second possibility that the role of astrocytic Glu transporter GLT-1 was reversed by inhibiting astrocytic Na^+/K^+-ATPase with ouabain; that is, astrocytes took part in the killing of neurons during this experimental condition. This idea logically leads to the assumption that the neuronal death caused by ouabain treatment was due to Glu toxicity resulting from the rise in extracellular Glu released from astrocytes. We then tested this possibility. Neuronal death induced by ouabain treatment was markedly depressed by co-treatment with AP5, a blocker of NMDA-type Glu receptors (Fig. 3C & D). Thus, this result supported our assumption that the neuronal death induced by ouabain treatment was in fact due to Glu-induced excitotoxicity.

To further confirm the involvement of astrocytic Glu transporters in the killing of neurons when the activity of Na^+/K^+-ATPase was inhibited, we treated the mixed neuron/astrocyte cultures with DL-threo-β-benzyloxyaspartate (TBOA) -- a broader non-transportable blocker of both GLT-1 and GLAST (Diamond 2001; Shimamoto *et al*. 1998). Co-treatment with a low concentration of both Glu (10 μM) and ouabain (50 μM) for 15 minutes resulted in massive neuronal death (Fig. 4B &D). Co-treatment of cultures with TBOA (50 μM) significantly protected neurons from death when the activity of Na^+/K^+-ATPase was inhibited with ouabain (Fig. 4C &D). The survival rate of neurons in the TBOA treatment was not statistically different from that in the DHK treatment (Fig. 4D). An immunocytochemical study using anti-GLAST antibodies showed that there were GLAST-positive astrocytes in the mixed neuron/astrocyte cultures (Fig. 4E). However, the immunoreactivity seemed to be weak and the rate of GLAST-positive astrocytes was low in the cultures tested (n=3 cultures).

Although ouabain treatment produced massive death of neurons, almost all of the astrocytes

seemed to remain intact under these experimental conditions. We then tried to clarify whether or not the experimental conditions toxic to neurons observed in this study were also toxic to astrocytes in astrocyte-enriched cultures (Fig. 5A). Co-treatment of astrocytes with Glu and DHK, as well as with Glu and ouabain, did not induce significant cell death among astrocytes (Fig. 5B-E). These results suggested that the experimental conditions toxic to neurons were not toxic to astrocytes.

To confirm that the astrocytic Glu transporter GLT-1 is crucial for both the survival and the death of neurons, we produced mixed neuron-astrocyte cultures with little GLT-1 expression. Previous studies have revealed that astrocytic GLT-1 expression is regulated by small neuron-derived peptides (Brown, 2000; Figiel and Engele, 2000). Therefore, neuronal density was reduced to the extent that astrocytic GLT-1 expression could not be clearly detected (Fig. 6E & F). In these cultures, treatment with 10 μ M Glu, which was not toxic to neurons in the cultures with astrocytic GLT-1 (Fig. 1 & 2), resulted in slight but significant neuronal death (Fig. 6B & D). However, co-treatment of these cultures with ouabain did not further facilitate glutamate excitotoxicity (Fig. 6C & D). In contrast, ouabain treatment resulted in massive neuronal death in the cultures with astrocytic GLT-1 (Fig. 3A & D).

Discussion

The present study has provided evidence that the astrocytic Glu transporter GLT-1 is closely involved in protecting neurons from NMDA-mediated neurotoxicity, as well as in killing neurons through NMDA-mediated neurotoxicity. Treatment of cultures with DHK, a non-transportable inhibitor of astrocytic GLT-1, resulted in the significant death of neurons. However, the same DHK treatment resulted in the significant protection of neurons when the activity of astrocytic Na^+/K^+-ATPase was inhibited with ouabain.

In the brain, at least 40 % of the energy released by respiration is required by Na^+/K^+-ATPase in order to maintain the ionic gradients of sodium and potassium across cell membranes (Astrup *et al*., 1981; Hansen, 1985). Therefore, the sodium pump in the brain requires an enormous expenditure of energy, indicating that the activity of Na^+/K^+-ATPase is markedly suppressed during ischemia due to a decreased availability of glucose and oxygen (Lees, 1991). In the present study, ouabain treatment might have reduced the activity of Na^+/K^+-ATPase in both neurons and astrocytes. However, the reduction in the Na^+/K^+-ATPase activity of neurons itself did not appear to be crucial for neuronal death. Rather, the suppression of astrocytic Na^+/K^+-ATPase proved crucial for neuronal death, provided that astrocytes expressed GLT-1.

Previous studies have shown that DHK directly activates ionotropic excitatory amino acid (EAA) receptors including the NMDA receptor of neurons (Maki *et al*., 1994; Wang *et al*., 1998). These results do raise the possibility that DHK-induced effects on the survival of neurons observed in this study could be caused by such a direct action of DHK on the NMDA receptor -- not by the inhibition of astrocytic GLT-1. However, this is unlikely to be the case, since co-treatment of cultures with DHK and ouabain resulted in the significant protection of neurons

from cell death (Fig. 3). In addition, treatment with AP5, an antagonist of the NMDA receptor, suppressed the ouabain-induced death of neurons (Fig. 3). Provided that the direct action of DHK on the NMDA receptor was involved in the protection of neurons from ouabain-induced neurotoxicity, AP5 treatment could result in the exacerbation of neuronal injury -- not in the neuroprotection observed in the present study.

In the neuron/glia mixed cultures used in this study, GLT-1-positive neurons could hardly be identified, despite careful immunocytochemical inspection. However, a recent study has demonstrated the presence of GLT-1 variants in cultured neurons as well as in neurons of the rat brain (Chen *et al*., 2002). They have postulated that such GLT-1 variants in neurons uptake glutamate at pre-synaptic terminals, and are responsible for the preservation of input specificity at excitatory synapses. However, the functional significance of GLT-1 expressed by neurons in the pathophysiology of excitotoxicity in the brain remains unclear at present.

GLAST-positive astrocytes were identified in the mixed neuron/astrocyte cultures (Fig. 4E). However, the immunoreactivity seemed weak and the rate of GLAST-positive astrocytes was low in the cultures. Treatment of the cultures with TBOA, a blocker of both GLT-1 and GLAST (Diamond 2001; Shimamoto *et al*. 1998), produced similar results to the DHK treatment. Treatment of cultures with 50 μ M TBOA resulted in the significant death of neurons in the presence of a low concentration of Glu (10 μ M) (data not shown). TBOA treatment at the same concentration of 50 μ M, however, significantly protected neurons from death when the cultures were co-treated with 50 μ M ouabain and 10 μ M Glu (Fig. 4D). Dose-dependency of TBOA-induced protective effects on the survival rate of neurons were investigated (data not shown). The near-maximum protection was obtained when the concentration of TBOA was

approximately 50 μ M. The TBOA-induced protective effect on neurons was almost comparable to that induced by DHK (Fig. 4D). Thus, blocking of the activity of GLAST by TBOA was probably not the primary contributor to the protection of neurons under the experimental condition in our cultures.

Previous studies have demonstrated that brain ischemia caused a reduced expression of astrocytic GLT-1 in the post-ischemic phase (Pines *et al*., 1992; Torp *et al*., 1995). Moreover, this reduction is considered to be a contributing factor to the delayed neuronal death following brain ischemia (Rao *et al*., 2000; 2001a). Astrocytic GLT-1 expression is also down-regulated after traumatic brain injury in the rat (Rao *et al*., 1998; Samuelsson *et al*., 2000). A persistently reduced GLT-1 expression by astrocytes is in fact toxic to neurons because of the reduced capability for the clearance of extracellular Glu by astrocytes. However, another interpretation of the ischemia-induced post-ischemic reduction of astrocytic GLT-1 would be that neurons participate in the down-regulation of the expression during post-ischemic periods in order to protect neurons themselves from Glu-induced excitotoxicity. A recent finding which seems to support the idea of neuronal self-defense in response to ischemia is that preconditioning with cortical-spreading depression results in a transient reduction in the expression of astrocytic GLT-1 (Douen *et al*., 2000). Neurons could respond to a brief ischemic attack by down-regulating astrocytic GLT-1 expression to temporarily protect themselves from the Glu-induced toxicity caused by any subsequent severe ischemic insult, despite the fact that the reduced level of astrocytic GLT-1 is itself dangerous. This possibility is now being investigated in a rat model of brain ischemic preconditioning.

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Figure legends

Figure 1

Glutamate-induced neurotoxicity in mixed cortical neuron/astrocyte cultures:

Immunocytochemical analysis using anti-MAP-2 (A1) and anti-GFAP (A2) antibodies indicates that the cultures were mixed. Photomicrographs B, C, and D show glutamate (Glu)-induced neuronal death in the sham-treated, 10 μ M Glu-, and 50 μ M Glu-treated cultures, respectively. Photomicrographs C1 and D1 show the cultures before treatment, whereas C2 and D2 illustrate their state 24 hours after Glu treatment for 30 minutes. Cell nuclei were stained with bisbenzimide (Hoechst 33342) and propidium iodide (PI) (B3, C3, and D3). Red nuclei in D3 indicate dead PI-positive neurons. Treatment of mixed cultures with Glu resulted in dose-dependent neuronal death (E). Note that low concentrations of Glu (5 μ M and 10 μ M) did not induce a significant death of neurons in these cultures. Scale bars indicate 250 μ m. Data are expressed as the mean+SD (n>4). * $p<0.05$.

Figure 2

Neuroprotective role of the astrocytic glutamate transporter GLT-1 against Glu-toxicity in mixed cultures:

Immunostaining of cultures with an anti-GLT-1 antibody shows the existence of GLT-1-positive astrocytes in the mixed neuron/astrocyte cultures (A). GLT-1-positive neurons were not detected. Most GLT-1-positive astrocytes were observed under aggregates of neurons. Photomicrographs B1 and C1 show the cultures prior to Glu-treatment. Figures B2 and C2 illustrate the cultures 24

hours after 30 minute co-treatments with Glu (10 μ M) and DHK (100 μ M); and with Glu (10 μ M), DHK (100 μ M), and AP5 (100 μ M), respectively. Although treatment of cultures with only Glu (10 μ M) did not induce significant neuronal death (D), co-treatment with DHK (100 μ M) did (B3 & D). DHK-induced neuronal death was markedly suppressed by AP5, suggesting that NMDA receptor-mediated mechanisms were involved in such neurotoxicity. Treatment of cultures with only DHK for 30 minutes did not result in the significant death of neurons (D). Scale bars show 250 μ m. Data are expressed as the mean+SD (n>4). * $p<0.05$.

Figure 3

The astrocytic glutamate transporter GLT-1 responsible for the killing of neurons in mixed neuron/astrocyte cultures:

Photomicrographs A1, B1 and C1 indicate the mixed cultures before treatment. Figures A2, B2 and C2 show the cultures 24 hours following 15 minute co-treatments with Glu (10 μ M) and ouabain (50 μ M); with Glu (10 μ M), ouabain (50 μ M) and DHK (200 μ M); and with Glu (10 μ M), ouabain (50 μ M) and AP5 (100 μ M), respectively. Co-treatment of mixed cultures with ouabain resulted in massive neuronal death (A3 & D), whereas DHK co-treatment markedly suppressed the ouabain-induced neuronal toxicity (B3 & D). Ouabain-induced neuronal death was also suppressed by co-treatment with AP5 (C3 & D), suggesting that NMDA receptor-mediated mechanisms were involved in the neurotoxicity. The scale bar is 200 μ m. Data are expressed as the mean+SD (n>4). * $p<0.05$.

Figure 4

Astrocytic glutamate transporters and neuronal death in mixed neuron/astrocyte cultures:

Photomicrographs A1, B1 and C1 indicate the mixed cultures before treatment. Figure A2 shows the sham-treated control cultures, whereas B2 and C2 indicate the cultures 24 hours after 15 minute co-treatments with Glu (10 μ M) and ouabain (50 μ M); and with Glu (10 μ M), ouabain (50 μ M) and TBOA (50 μ M), respectively. Co-treatment of mixed cultures with ouabain resulted in massive neuronal death (B3 & D), whereas TBOA co-treatment markedly suppressed the ouabain-induced neuronal toxicity (C3 & D). Ouabain-induced neuronal death was also suppressed by co-treatment with 200 μ M DHK (D). Immunocytochemical analysis of cultures using an anti-GLAST antibody revealed that GLAST-positive astrocytes could be identified in the mixed neuron/astrocyte cultures. Scale bars are 250 μ m. Data are expressed as the mean+SD (n=4). * $p<0.05$.

Figure 5

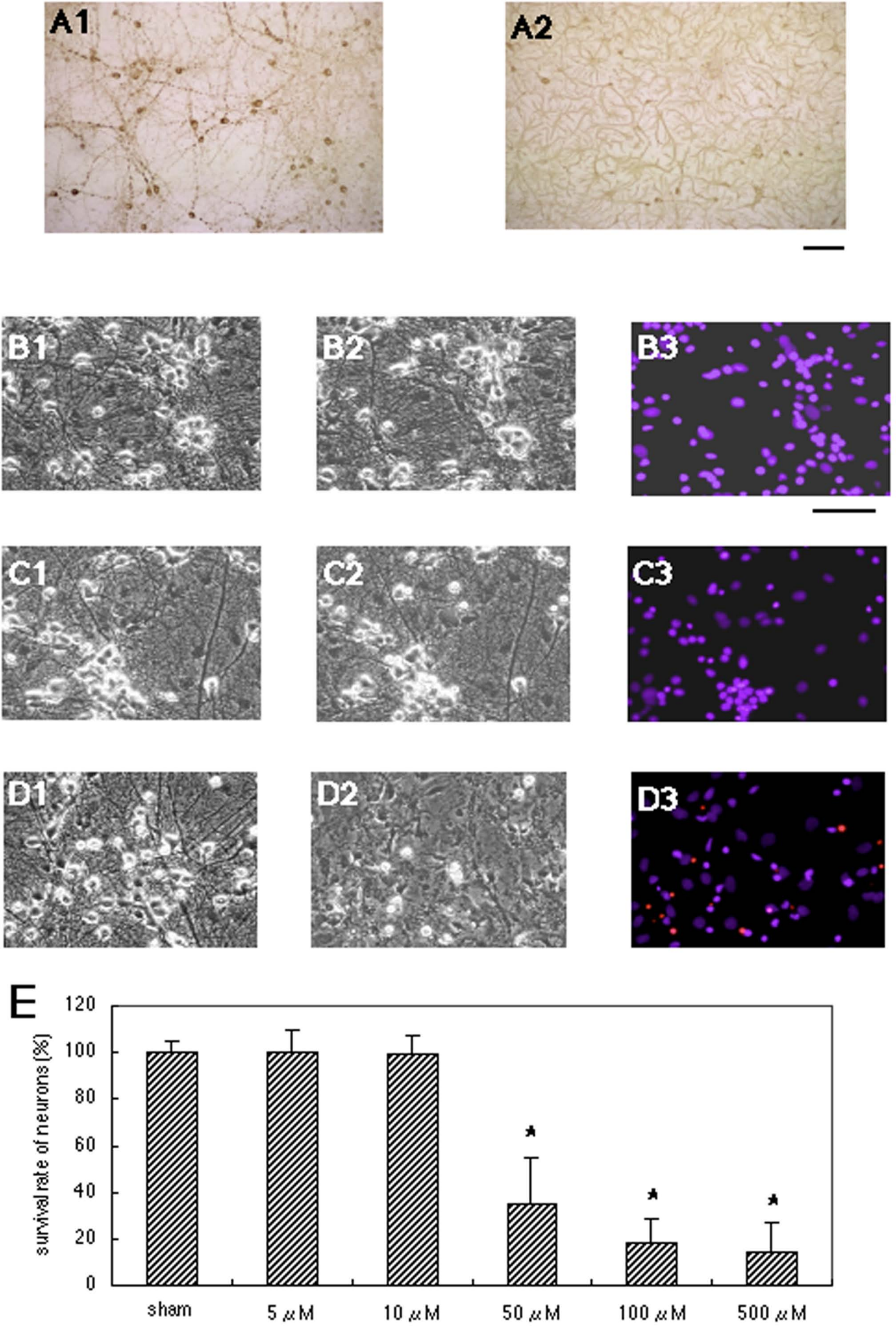
Astrocytes survived neurotoxic treatment in astrocyte-enriched cultures:

Immunocytochemical analysis of cultures using anti-MAP-2 (A1) and anti-GFAP (A2) antibodies revealed that MAP-2-positive neurons could not be clearly identified in these cultures. Photomicrograph B1 shows the sham-treated control cultures, whereas photomicrographs C1 and D1 indicate the state of the cultures 24 hours after 30 minute treatments with Glu (10 μ M) and DHK (100 μ M); and with Glu (10 μ M) and ouabain (50 μ M), respectively. Co-treatment of astrocyte-enriched cultures with Glu and either DHK or ouabain did not induce significant death of astrocytes (E). Scale bars show 250 μ m. Data are expressed as the mean+SD (n=4).

Figure 6

Inhibition of the activity of Na^+/K^+-ATPase did not induce neuronal death in mixed neuron/astrocyte cultures with little astrocytic GLT-1:

Photomicrographs A1, B1 and C1 show the mixed cultures before treatment. Figures A2, B2 and C2 indicate the cultures 24 hours after sham treatment, treatment with Glu (10 μ M), and co-treatment with Glu (10 μ M) and ouabain (50 μ M), respectively. In these cultures, treatment with only Glu (10 μ M) resulted in the slight but significant death of neurons (B3 &D). This was probably because of a deficiency of the astrocytic glutamate transporter GLT-1. However, co-treatment with ouabain did not further facilitate glutamate excitotoxicity (C3 & D). Immunocytochemical analysis of these cultures revealed that astrocytes did not have detectable levels of GLT-1 (E), although the morphology of astrocytes (F) was not so different from that of the cells with GLT-1 in Figure 2A. Scale bars show 250 μ m. Data are expressed as the mean+SD (n=4). * $p<0.05$.


A1
A2
B1
B2
B3
C1
C2
C3
D1
D2
D3
E
survival rate of neurons (%)
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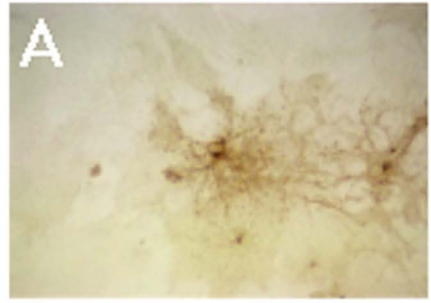
100
80
60
40
20
0
sham
5 μM
10 μM
50 μM
100 μM
500 μM
*
*
*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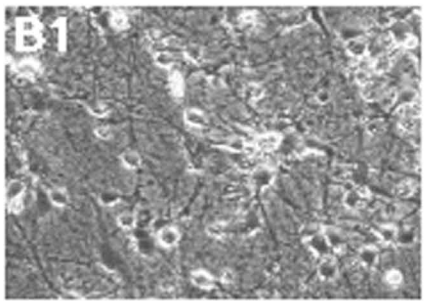
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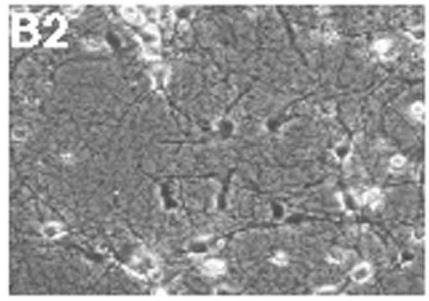
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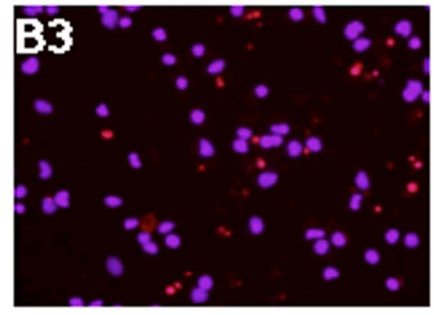
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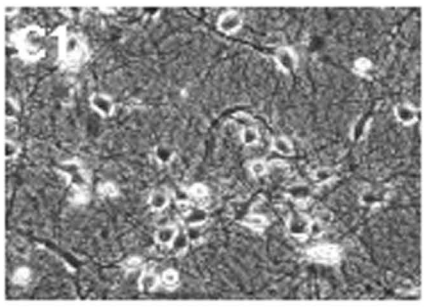
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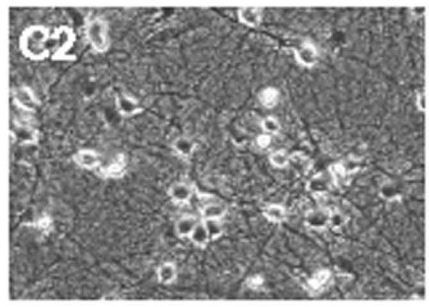
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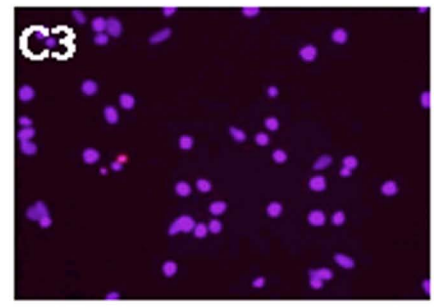
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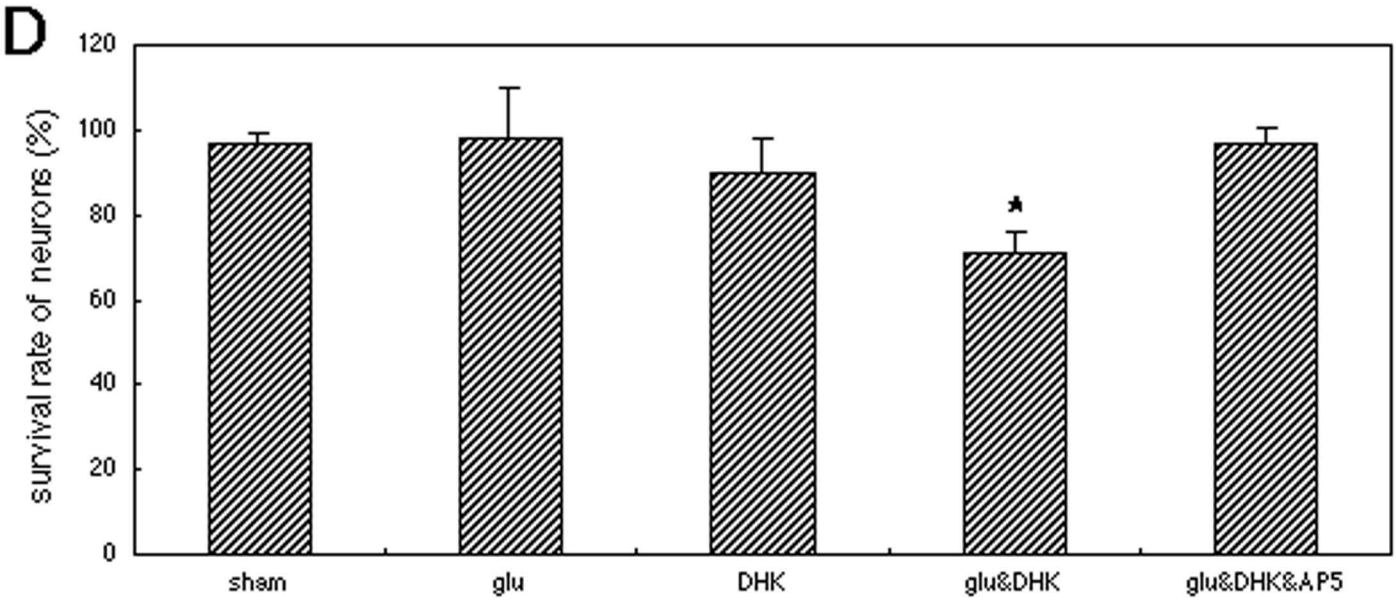
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survival rate of neurons (%)
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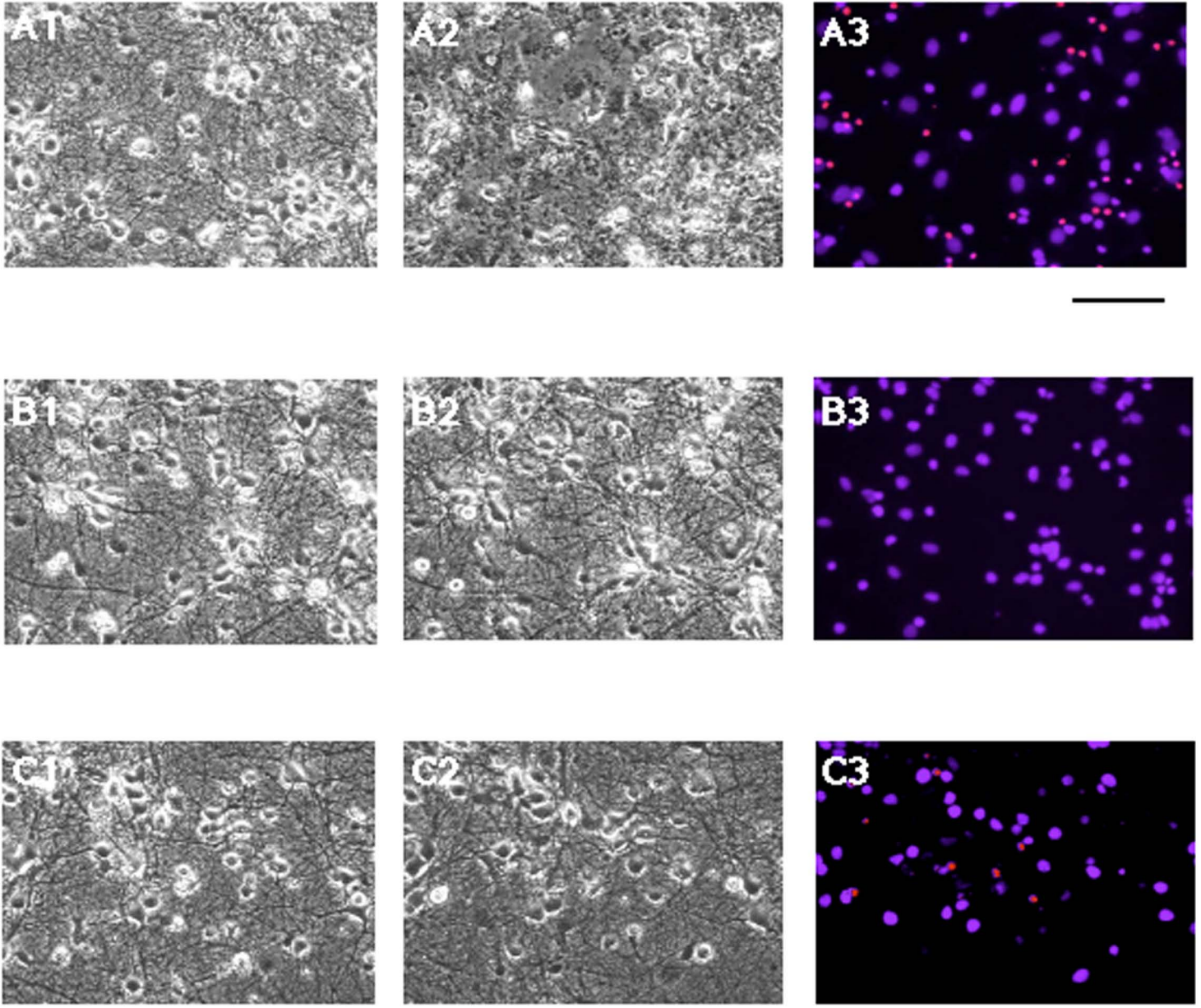
100
80
60
40
20
0
*
sham
glu
DHK
glu&DHK
glu&DHK&AP5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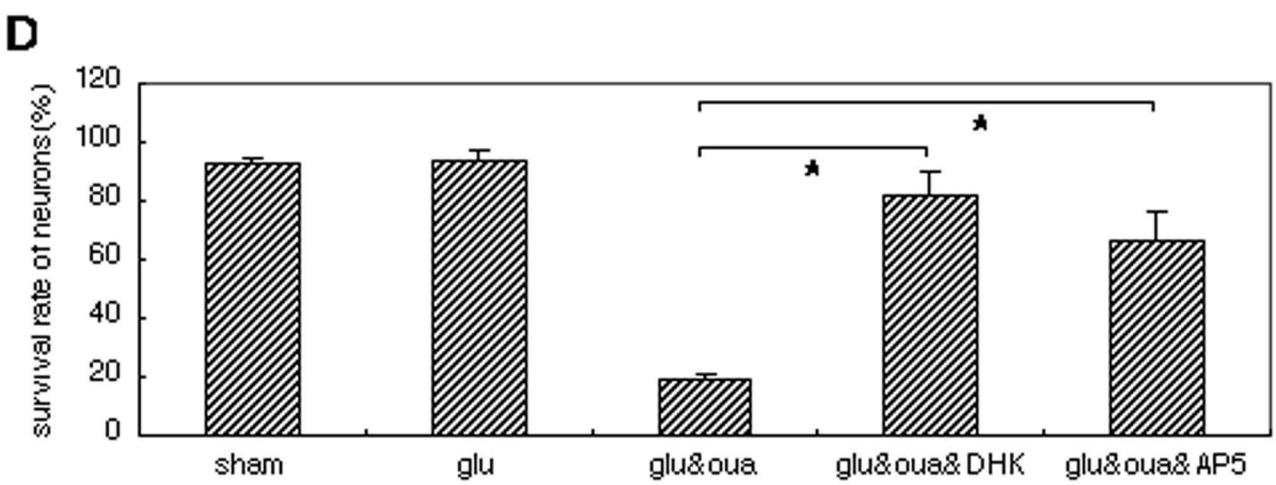
A2
A3
B1
B2
B3
C1
C2
C3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survival rate of neurons(%)
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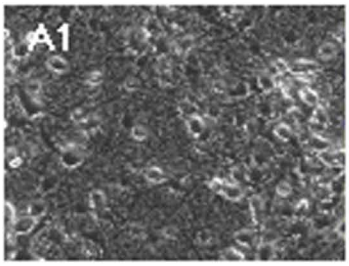
100
80
60
40
20
0
sham
glu
glu&oua
glu&oua& DHK
glu&oua& AP5
*
*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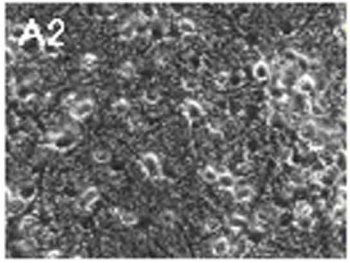
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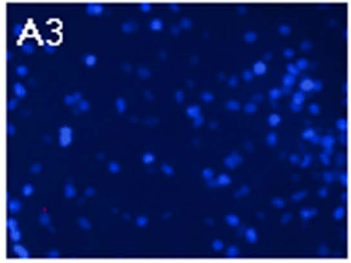
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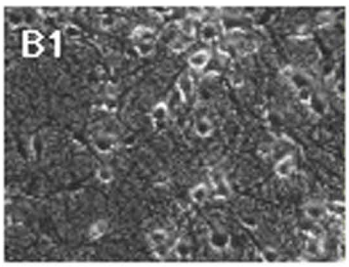
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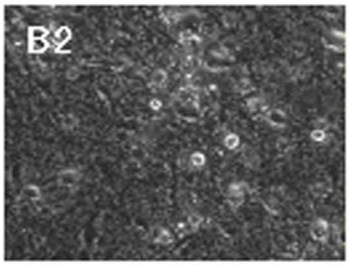
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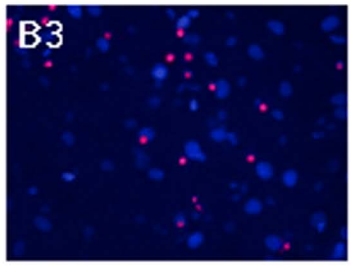
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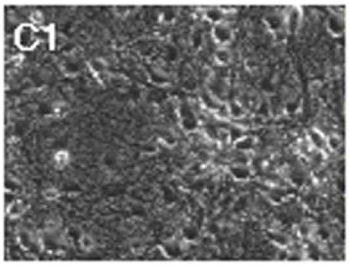
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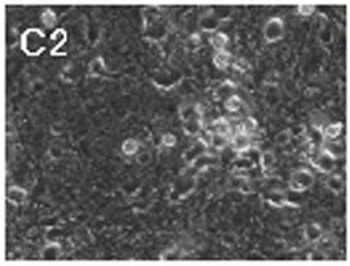
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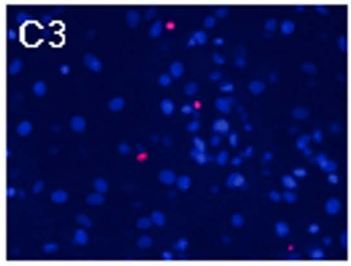
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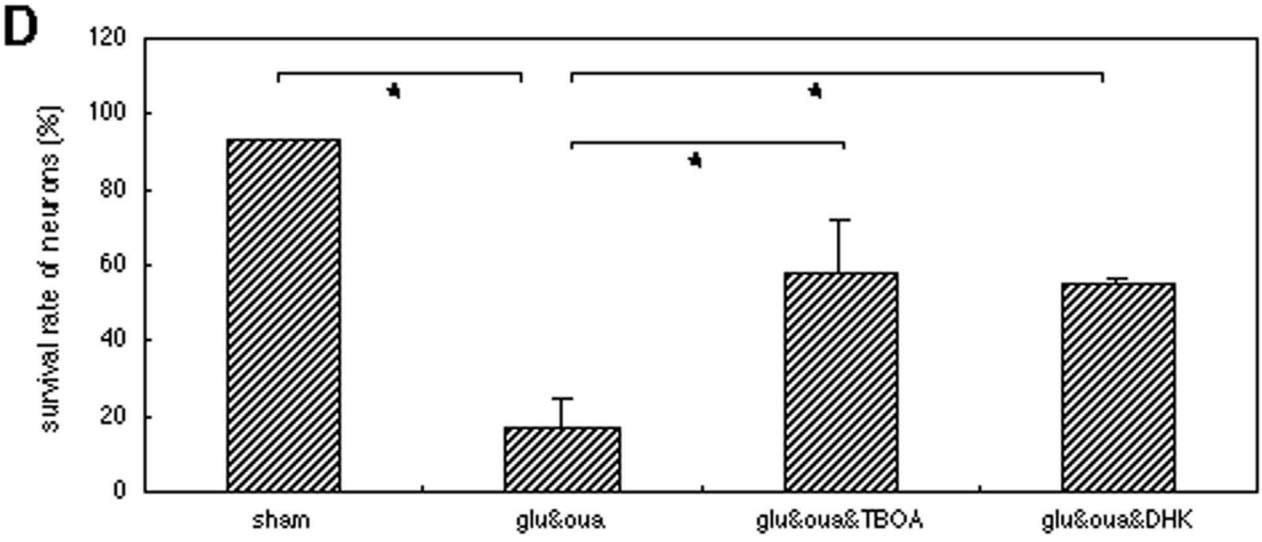
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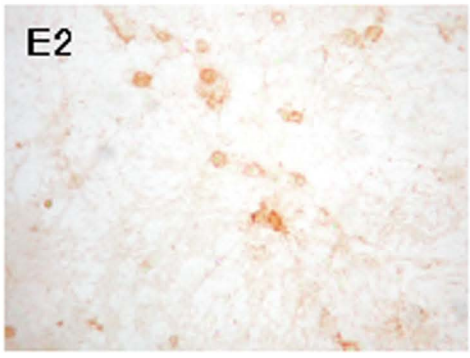
survival rate of neurons (%)
120
100
80
60
40
20
0
*
*
*
sham
glu&oua
glu&oua&TBOA
glu&oua&DHK


E1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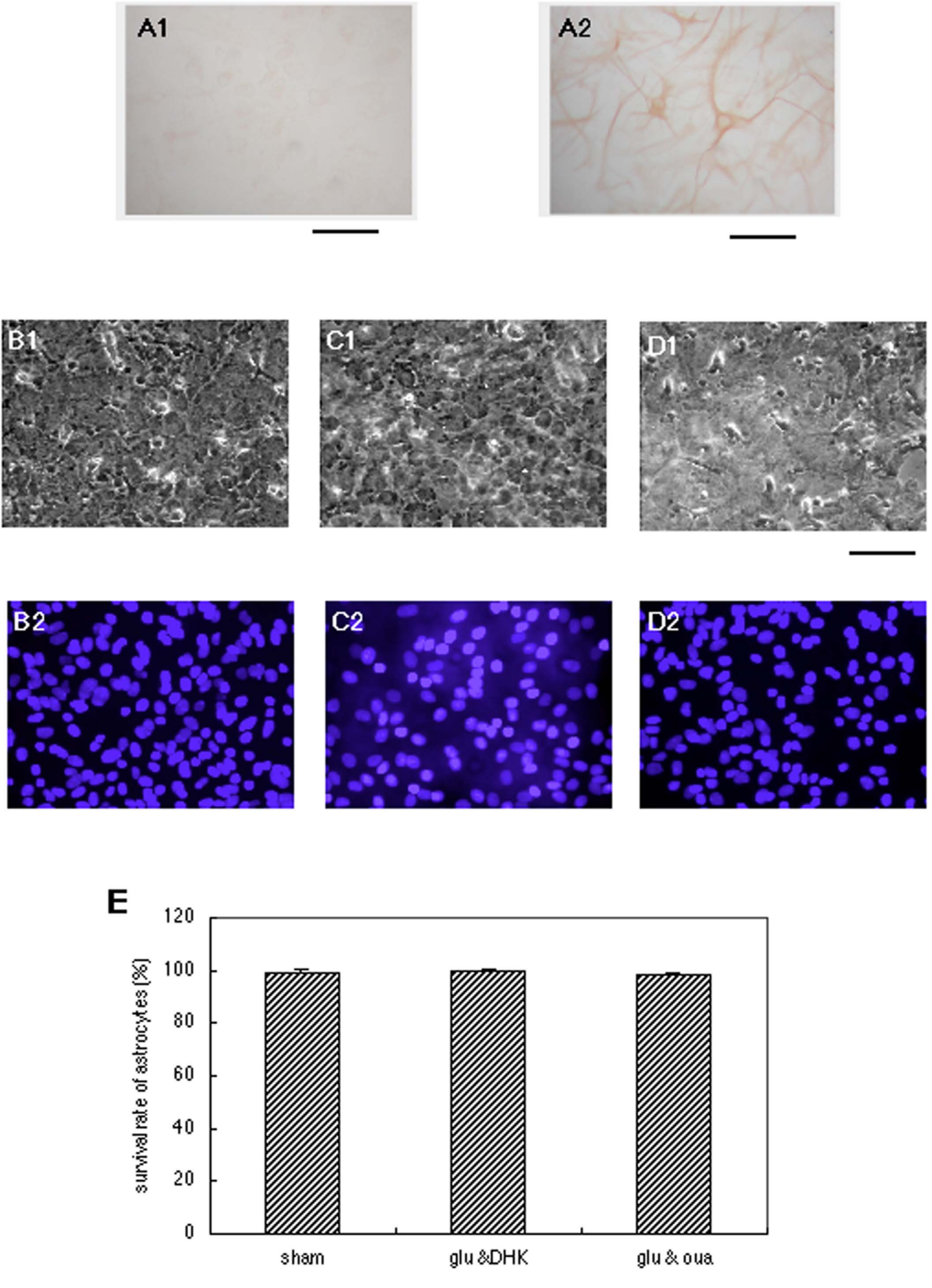
A1
A2
B1
C1
D1
B2
C2
D2
E
survival rate of astrocytes (%)
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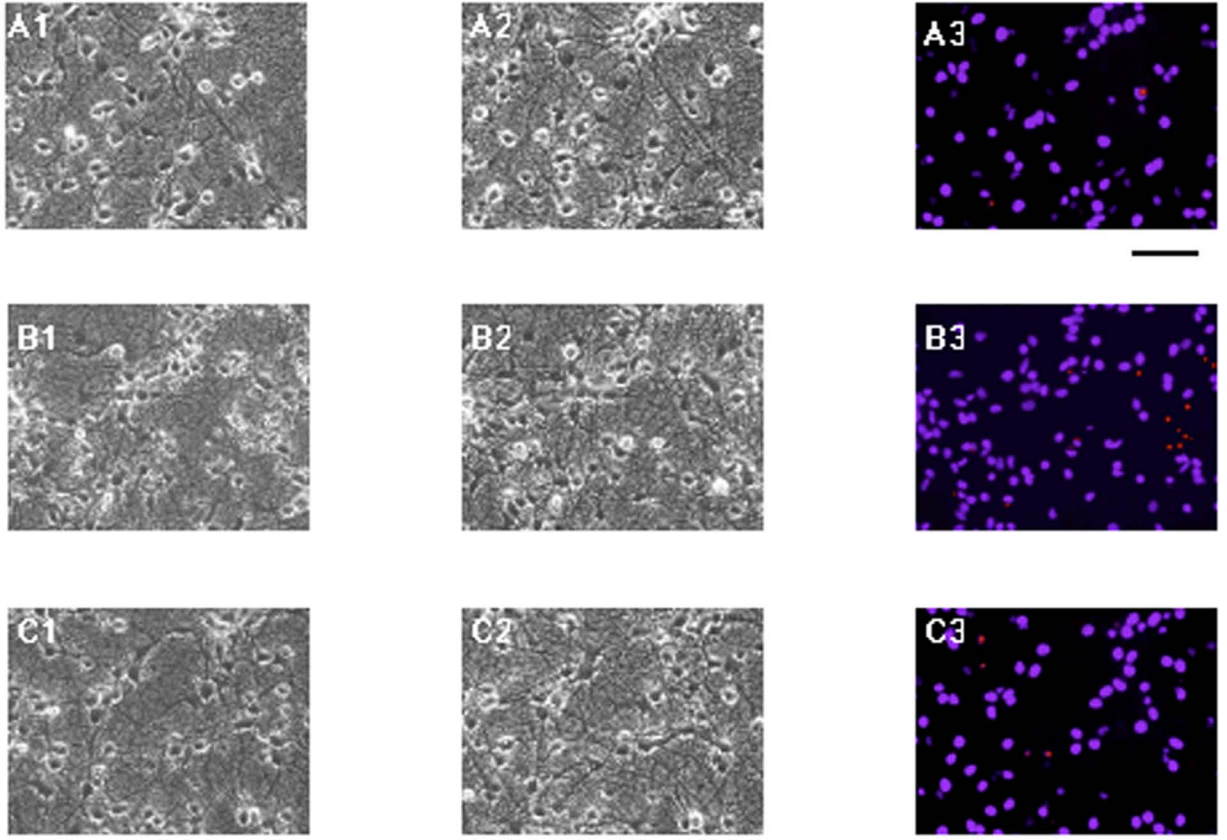
100
80
60
40
20
0
sham
glu &DHK
glu & oua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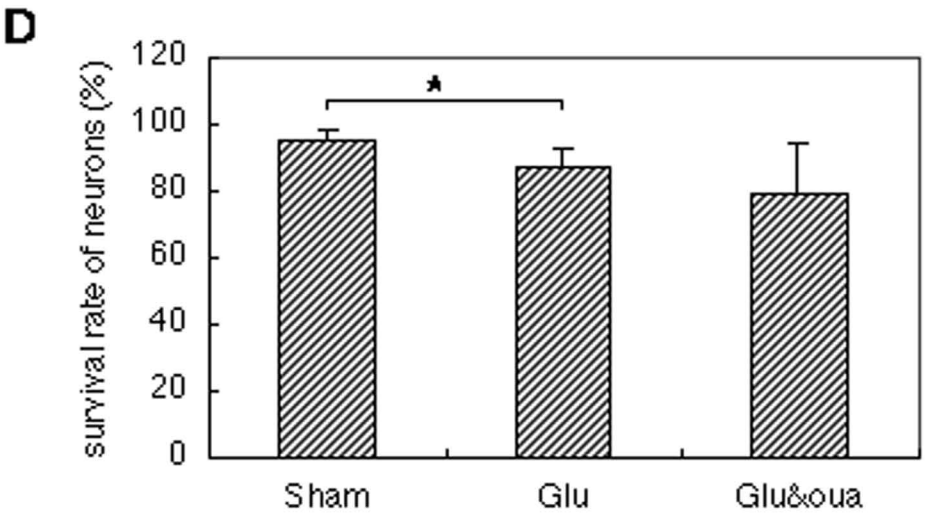
A2
A3
B1
B2
B3
C1
C2
C3


D
survival rate of neurons (%)
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100
80
60
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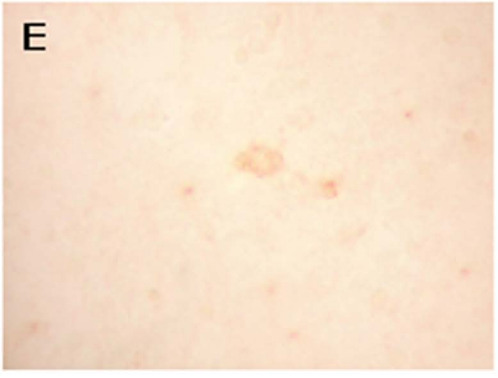
20
0
*
Sham
Glu
Glu&oua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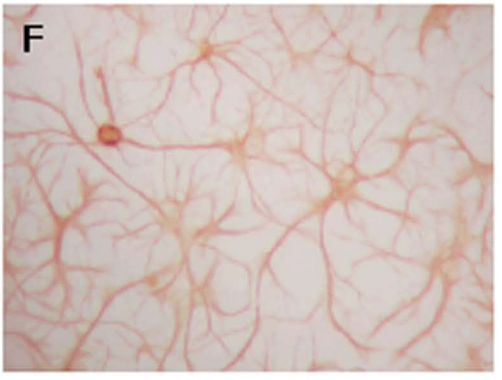
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