Potentiation of Excito-toxicity by Glutamate Uptake Inhibitor Rather than Glutamine Synthetase Inhibitor

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ABSTRACT—The neuroprotective functions of glia cells in the presence of excessive amounts of extracellular glutamate (Glu) were examined using glia-rich and glia-poor cultured cerebellar granule cells that contained the same number of neurons. In order to focus on the metabolic enzyme glutamine synthetase (GS) and the uptake system in glia cells, selective inhibitors such as L-methionine sulfoximine (MSO) and 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) were used as pharmacological tools. The increased amount of lactate dehydrogenase (LDH) leakage induced by 50 μM Glu and SITS was equivalent to that of 1 mM Glu. However, the simultaneous treatment with 50 μM Glu and 5 μM MSO did not increase the LDH leakage. The larger quantities of extracellular Glu were sustained in both glia-rich and glia-poor cultures. After the administration of Glu and MSO, however, the larger quantities of Glu were not sustained. Taking these results into consideration, the Glu uptake system in glia cells seems to be more important than the Glu metabolic enzyme system in the regulation of neuronal protection from Glu toxicity.

Keywords: Glial cell, Cerebellar granule cell (cultured), Glutamate, L-Methionine sulfoximine, 4-Acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid

It is well documented that extracellular glutamate (Glu) levels rise greatly during ischemia or anoxia (1, 2). Extracellular potassium concentration also rises during such pathological conditions. This increased potassium level promotes the release of Glu and is regulated by a positive feedback system that serves to increase the extracellular Glu concentration (3). Furthermore, the cause of the extracellular Glu increase may be related to the alteration of uptake systems and the metabolic fate of Glu in neuronal and/or glial cells.

Recent reports have proposed that the glial-neuronal interaction might be very important to the maintenance of brain functions, and that glial cells, especially astrocytes, play a role in preventing neuronal disorders resulting from Glu toxicity (4). For instance, glial cells possess two important mechanisms for maintaining the extracellular Glu level. One is an uptake system located on the plasma membrane of neurons and glial cells (3). The other is the metabolic enzyme glutamate synthetase (GS), located in glial cells, that converts Glu to glutamine (Gln) by coupling Glu with ammonium cation (5, 6).

Although recent studies have made considerable progress in elucidating the protective effects of NMDA or Ca antagonists against excessive Glu neuronal toxicity, most of these studies focused on post-synaptic neuronal events (7, 8). Also, most of the in vitro studies using a tissue culture employed neuronal cultures to study the protective functions.

To evaluate the underlying mechanisms of the metabolic enzymes and uptake system in glial cells under Glu toxicity, we believe it is important to examine the function of glia cells using a co-culture system of neurons and glial cells. We have employed cultured rat cerebellar granule cells in which the ratio of glial cells may be altered without changing the number of neuronal cells as shown in our previously reported paper (9). Culturing cells in this fashion may facilitate the investigation of the two protective functions of glial cells, Glu uptake by glial cells and Glu metabolism by GS.

In this report, we investigated which functions of glial cells are more protective against the toxicity induced by excessive amounts of extracellular Glu. This was achieved mainly by quantitative analyses of intracellular and extracellular amino acid levels and by paying close attention to lactate dehydrogenase (LDH) leakage. To achieve this objective, we used two pharmacological tools: 1) a gluta-

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mine synthetase inhibitor, methionine sulfoximine (MSO; Sigma Chem. Co., St. Louis, MO, USA) and 2) a glutamate re-uptake inhibitor, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS; ICN Biochem. Inc., Cleveland, OH, USA).

MATERIALS AND METHODS

Cell culture

Ten cerebellums of 8-day-old Wistar rats were prepared by modifying the procedure of Gallo et al. (10). After tripsinization, isolated cells were suspended in Basal Eagle Medium solution (Kyokuto Pharm. Ind. Co., Ltd., Tokyo) containing 0.165% W/V KCl (Wako Pure Chem. Ind. Co., Tokyo), 0.029% W/V glutamine (Sigma Chemical Co.), 10% V/V FBS (Bockneck, Toronto, Canada) and 90 μg/ml gentamicin (Sigma Chemical Co.) at a concentration of 1.25×10^6 cells/ml. At approximately 16 hr after seeding, half of the prepared dishes were treated with 10 μM cytosine arabinoside (AraC) (Sigma Chemical Co.) to prevent astrocytic proliferation. Hereafter, the cultures treated with and without AraC will be referred to as the glia-poor culture and glia-rich culture, respectively. It should be noted that our previous study detected no significant difference in the number of neurons between glia-poor and glia-rich cultures, but that the percentages of glial cells were approximately 17% and 32% in 7 DIV, respectively (9)

Assessment of neuronal toxicity by LDH acitivity assay

According to our recent evidence (11), the extent of neuronal damage under each experimental condition was estimated after a 6-hr exposure to the chemicals at day 6 of the in vitro culture and by means of determining LDH activity in the culture medium: extracellular LDH activity. The supernatants of the remaining medium and cells (frozen twice) were assayed as the total LDH activity defined as the sum of intracellular and extracellular LDH activity. The ratio of LDH leakage was calculated as a percentage of the extracellular activity to the total activity (international units: IU) of each aliquot as determined by the Wróblewski-La Due method using COBAS/FARAII (Roche, Basel, Switzerland).

Measurement of intracellular and extracellular amino acids amounts

To determine the function of the Glu uptake systems, intracellular and extracellular Glu and Gln contents were quantified at the end of each experiment. Glu- and Gln-containing aliquots were measured as described previously (12) by an HPLC-ECD system (Jasco, Tokyo). The obtained concentrations of Glu and Gln are shown as pmol/30 μl.

Measurement of glutamine synthetase activity

The effect of the experimental condition on the GS activity of glia, including the K_m and V_{max}, was evaluated by the modified Sher and Hu method (13). One unit of Gln synthetase was defined as the amount of enzyme that produced 1 μmol glutamyl-γ-hydroxamate/hr at 37℃.

Statistical analyses

All values are expressed as means$\pm$S.D. values. The total number of experiments is indicated in the figure or table. Statistical significance of the result was evaluated by the *F-t* test. A probability value of 5% or less was considered indicative of a significant effect.

RESULTS

Difference between glia-rich and glia-poor culture in toxicity induced by excessive Glu with or without MSO or SITS

Glu induced neuronal toxicity as it appeared in glia-poor and glia-rich cultures was compared using the LDH leakage method (Table 1). When incubating with Glu alone at a concentration of 1 mM for 6 hr, the LDH leakage increased about 70% above the control values of either culture. Incubating at a concentration of 50 μM Glu, however, did not significantly increase the LDH leakage in either culture media. The contrast between glia-rich and glia-poor cultures became more pronounced when 100 μM Glu was applied. The LDH leakage of the glia-poor culture was significantly higher than that of the glia-rich culture, and the treatment of 1 mM Glu significantly increased the LDH leakage in both culture media. These results suggest that more than 100 μM Glu causes severe neuronal damage, although less than 50 μM Glu does not induce any neuronal damage.

After incubation with the GS inhibitor, MSO (5 μM) for 6 hr, the increase of LDH leakage in glia-poor and glia-rich cultures was not significant. It was also observed that the simultaneous incubations for 6 hr with 5 μM MSO and 50 μM Glu in both cultures did not cause any significant increases in LDH leakage (Table 1).

After incubation for 6 hr with the Glu uptake inhibitor SITS, the neuronal damage in cultured glia-poor and glia-rich cultures was examined. A simultaneous incubation with 500 μM SITS and 50 μM Glu showed a significant increase of LDH leakage, although the treatment of 500 μM SITS alone showed no significant increase in either culture. A comparison between glia-poor and glia-rich cultures incubated with 500 μM SITS revealed the same increased percentage of LDH leakage (Table 1).

Table 1. Toxicological effect of Glu, MSO and/or SITS on glia-rich and glia-poor culture

Group	Glia-poor culture	% Control	Glia-rich culture	% Control
Control	20.4± 7.8 (24)	100	17.4± 6.4 (26)	100
50 μM Glu	23.2± 9.0 (22)	114	18.4± 8.5 (22)	106
100 μM Glu	27.3± 4.3 (5)*	134	20.4± 5.2 (5)	117
1 mM Glu	35.2± 3.4 (9)**	173	30.2± 5.0 (9)**#	174
5 μM MSO	22.4± 3.8 (5)	110	18.4± 2.6 (5)	106
5 μM MSO + 50 μM Glu	23.1± 5.8 (5)	113	21.5± 5.3 (5)	124
500 μM SITS	23.9± 1.3 (4)	117	22.4± 2.0 (4)	129
500 μM SITS + 50 μM Glu	32.8±10.2 (6)**	161	29.9± 7.3 (6)**	172

Each value represents the mean and S.D. of LDH leakage {(Extracellular LDH activity/Total LDH activity) × 100} in percent. The figure in parentheses represents the number of experiments. Significantly different from the control group, *: $P<0.05$, **: $P<0.01$ (*F-t* test). Significantly different from the glia-poor culture, #: $P<0.05$ (*F-t* test).

Effects of MSO on GS activity in glia-poor and glia-rich cultures

The time-dependent effects of MSO on glutamine synthetase activity are shown in Table 2. The initial GS activity (μmol/hr/dish) in the glia-poor culture was 0.56±0.02 and 0.94±0.04 in the glia-rich culture. The initial GS activity in the glia-rich culture was significantly higher than that in the glia-poor culture. After a 30-min incubation with 5 μM MSO, the GS activity of the glia-rich culture and the glia-poor culture were reduced by approximately equivalent values.

The K_m of GS in the glia-rich culture (4.01×10^{-1} M) was slightly higher than that in the glia-poor culture (2.74×10^{-1} M). The V_{max} of GS (M/hr) in the glia-rich culture (1.71×10^{-3}) was comparable to that in the glia-poor culture (1.48×10^{-3}).

Table 2. Effects of MSO on glutamine synthetase activity

Group		Time		
		0 min	5 min	30 min
Glia-poor culture	control	0.56±0.02	0.55±0.01	0.60±0.03
	5 μM MSO	0.54±0.03	0.52±0.02	0.34±0.01**
Glia-rich culture	control	0.94±0.04##	1.00±0.01##	1.12±0.08##
	5 μM MSO	0.94±0.04##	0.92±0.02##	0.35±0.05**

GS activities are shown in the production of 1 μmol glutamyl-γ-hydroxamate/hr. Each value represents the mean and S.D. of 4 experiments. Significantly different from the corresponding control group. **: $P<0.01$ (*F-t* test). Significantly different from the corresponding glia-poor culture group. ##: $P<0.01$ (*F-t* test).

Table 3. Effect of simultaneous incubation of MSO and Glu on the intracellular Glu and Gln contents

Group		50 μM Glu	5 μM MSO + 50 μM Glu	% of change
Glu content	Glia-poor culture	716.9±174.2 (10)	767.6±22.3 (3)	107.0
	Glia-rich culture	1049.7±172.8 (9)	1314.7±33.6 (3)	125.2
Gln content	Glia-poor culture	114.9± 36.4 (8)	87.9±22.4 (3)	76.5
	Glia-rich culture	261.1±136.8 (10)	205.8±19.3 (3)	78.8

Each value represents the mean and S.D. of the Glu or Gln content at 30 min after Glu incubation in pmol/30 μl. The culture was incubated with 5 μM MSO simultaneously with 50 μM Glu for 30 min. No significant difference was detected by the *F-t* test.

Effects of simultaneous incubation with MSO on the intracellular amino acids levels induced by excessive Glu treatment

The effect on the intracellular Glu and Gln levels in 30-min simultaneous incubations with 5 μM MSO and 50 μM Glu, which followed the pre-incubation of MSO for 30 min, is shown in Table 3. The intracellular Glu levels in the simultaneous incubation groups increased in both cultures with respect to the 50 μM Glu incubation groups, although the increase was more obvious in the glia-rich culture than the glia-poor culture.

The reduction of the Gln levels in the simultaneous incu-

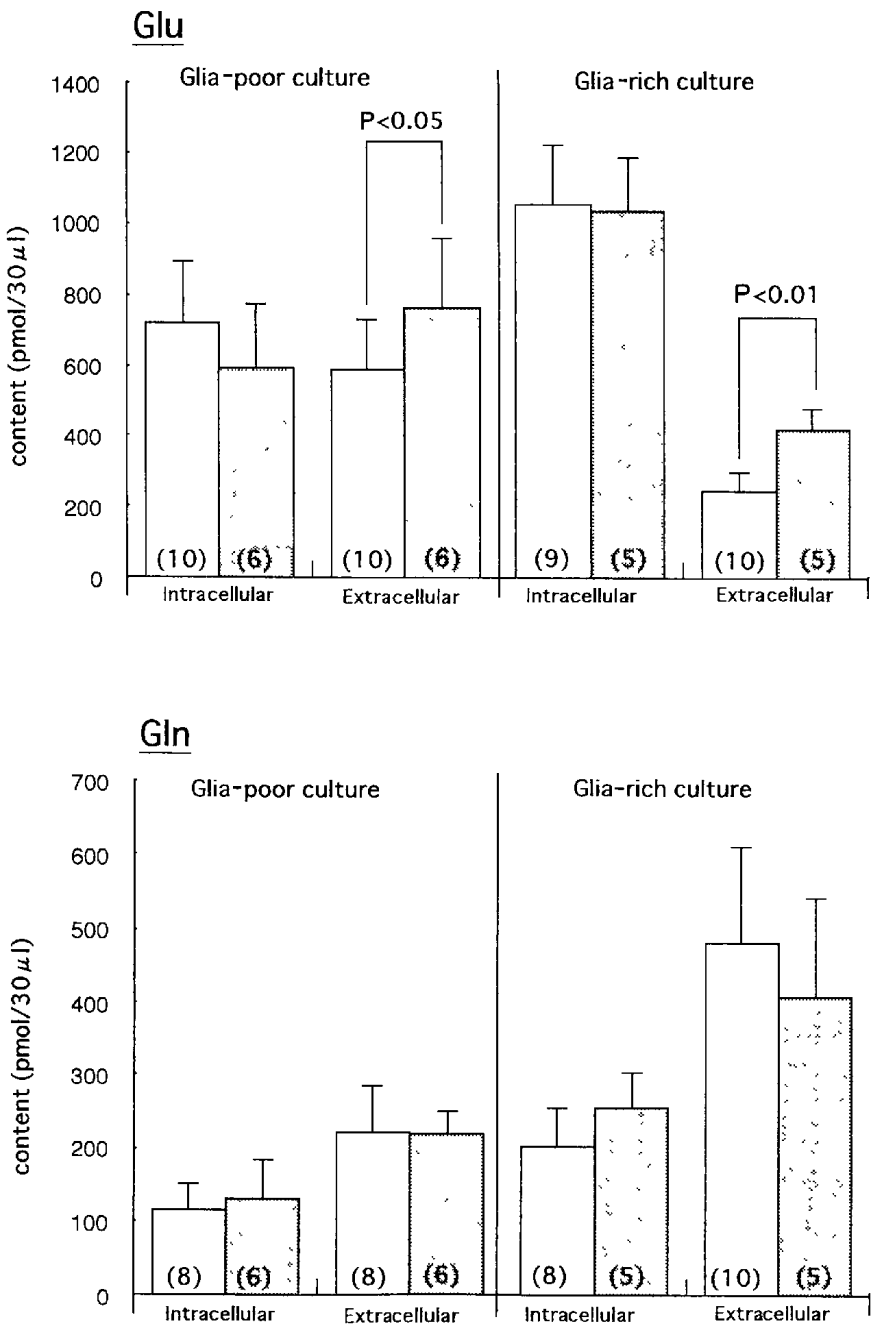

Fig. 1. Intracellular and extracellular Glu and Gln contents after simultaneous incubation with 50 μM Glu and 500 μM SITS for 30 min. Intracellular and extracellular Glu and Gln contents are shown in pmol/30 μl, and reported as the mean and S.D. of the results from the number of experiments shown in parentheses. Symbols in the figure are as follows: Open column: 50 μM Glu incubation, Dotted column: simultaneous incubation with 50 μM Glu and 500 μM SITS.

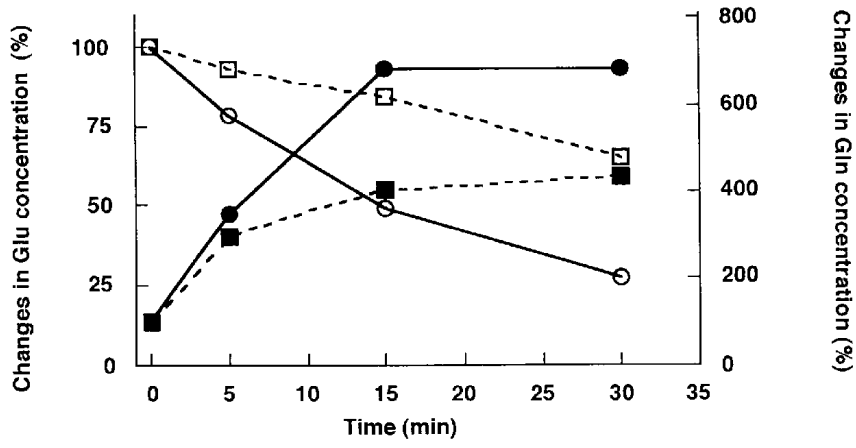

Fig. 2. Changes in extracellular concentration of Glu and Gln induced by 50 μM Glu. Each plot is shown as the percentage value against the initial concentration that is taken as 100%. Each plot was calculated from the mean value of 3–4 experiments. Symbols in the figure are as follows: ○, Glu/Glia-rich culture; ●, Gln/Glia-rich culture; □, Glu/Glia-poor culture; ■, Gln/Glia-poor culture.

bation groups was observed in both cultures as compared with the 50 μM Glu incubation groups. In contrast, the reduction of Gln levels in the glia-poor culture and the glia-rich culture show little variance.

Effects of the simultaneous incubation of SITS on the intracellular and extracellular amino acids levels induced by excessive Glu treatment

Figure 1 shows the effects of simultaneous incubation with Glu and SITS on extracellular and intracellular Glu levels. At 30 min after the simultaneous treatment with Glu and SITS, the intracellular levels of Glu in both cultures did not change significantly compared to those treated solely with 50 μM Glu. On the other hand, the extracellular levels of Glu in both cultures showed significant increases ($P<0.05$) compared to the incubation with 50 μM Glu alone.

In the case of changes in Gln levels, slight increasing and decreasing tendencies were observed in the intracellular and extracellular levels, respectively.

Table 4. Contents of intracellular Glu levels at 0 and 30 min after Glu treatment

Group	0 min	30 min	Increasing %
Glia-poor culture	346.0±143.7	331.0± 55.7	−4.3
Glia-rich culture	665.1± 14.1	1131.8±172.2*	70.2

Each value represents the mean and S.D. of 4 experiments and is shown in pmol/30 μl of K-H buffer solution. Significantly different from 0 min, *: $P<0.05$ (*F-t* test).

Difference between glia-rich and glia-poor culture extracellular amino acids and intracellular Glu level induced by excessive Glu

Figure 2 shows the percentage of change for 30 min in extracellular levels of Glu and Gln in both glia-poor and glia-rich cultures induced by 50 μM Glu treatment.

A decreased level of Glu and an increased level of Gln were observed in both cultures, although these changes were more obvious in the glia-rich culture than in the glia-poor culture. The 30-min value of Glu in the glia-poor and glia-rich cultures was 65% and 27% of the initial concentrations, respectively, and the values of Gln was 430% and 690% of the initial concentrations, respectively.

The intracellular Glu levels at 0 and after 30-min Glu incubation are shown in Table 4. In the glia-poor culture, the intracellular Glu levels did not change significantly after 30-min 50 μM Glu incubation. In the glia-rich culture, however, a significant increase in intracellular Glu level was observed at 30 min after treatment. In comparison, after the treatment with 50 μM Glu, the decreased levels of extracellular Glu and the changes detected in intracellular Glu were less in the glia-poor culture.

DISCUSSION

Recent studies have revealed that the glial cell plays an important role in maintaining the homeostasis of the central nervous system (4, 14, 15). One main purpose of this study is to estimate which function of glia cells, i.e., Glu uptake system or GS activity, is more protective against the toxicity induced by the excessive amounts of extracellular Glu. The other purpose is whether to determine if cultured glia-poor and glia-rich cerebellar granule cells are useful for examining glial cell functions.

For the main purpose of this study, we employed two pharmacological tools to resolve this issue: one is the GS inhibitor MSO and the other is the Glu uptake inhibitor SITS.

In the Glu toxicity induced by more than 100 μM Glu, the glia-poor culture showed more severe neuronal damage than that seen in the glia-rich culture (Table 1). However, simultaneous incubations with 50 μM Glu and 500 μM SITS showed the same significant potentiation of Glu toxicity in both glia-rich and glia-poor cultures, and these potentiations were of the same magnitude as seen in 1 mM Glu treatment. On the other hand, simultaneous incubation with 50 μM Glu and 5 μM MSO revealed no significant potentiation in either culture (Table 1).

Many studies have demonstrated the important roles of GS in Glu metabolism (3, 14, 16, 17). Under low energy conditions in the brain, it has been demonstrated that GS activity in rats was increased following ischemia (18) and chronic hypoxia (13) by the increase of extracellular Glu levels. Furthermore, the inhibition of GS by MSO has resulted in increases of Glu uptake and decreases of Glu release (19, 20).

We also examined the effects of a dose of 5 μM MSO on the GS activity and intracellular Glu and Gln levels. The assay of GS activity showed that inhibitions of the same magnitude were observed 30 min after MSO treatment in both cultures, although the initial activity of the glia-rich culture was approximately twofold that of the glia-poor culture (Table 2). In addition, these differences parallel the deviation of glia cell numbers in each culture. By the treatment of MSO, the Glu level increased and the Gln levels decreased, respectively (Table 3). These results suggest that 5 μM MSO inhibits GS activity. However, contrary to these pharmacological actions of MSO seen in the previously reported results, we could not detect any potentiated toxicity of 5 μM MSO in this study.

Kollegger et al. using slices of rat corpus striatum demonstrated that 500 μM MSO caused slight direct toxic effects and potentiated both kainate and NMDA toxicities (21). In our study, the single administration of 500 μM MSO did cause severe neuronal damage (data are not shown), but the dose of 5 μM MSO did not induce any damage in the cerebellar granule cell culture.

From these results, it might be inferred that the protective effects of glial cells do not have a strong relationship to changes in GS activity, although GS plays an important role in maintaining the normal homeostasis as described in the previous reports (14, 15).

Regarding SITS, we examined the Glu and Gln intracellular and extracellular levels. The most important effect of SITS was the significant increase in the extracellular level of Glu even at 30 min after its introduction (Fig. 1). This result suggests that the toxic potentiation of 50 μM Glu by SITS might be due to these Glu accumulations, since SITS is known to be a selective glial uptake inhibitor of Glu in cultured rat astorocytes (22). Futhermore, the combined treatment of 50 μM Glu and SITS did not change the intracellular Glu levels when compared to those observed in the treatment with 50 μM Glu alone (Fig. 1). This discrepancy might be explained by the following three reasons: 1) The larger amounts of Glu uptake to glial cells might be faster than its inhibition by SITS (23). 2) Variation in the amount of Glu that is reuptaken into the neuronal cells. (23). 3) The maximum amount of Glu uptake by either glial cells or neuronal cells might be restricted (or controlled). Also, increasing and decreasing tendencies in the intracellular and extracellular Gln levels were detected, although no significant change in intracellular Glu levels was measured (Fig. 1). These changes can be explained by the relationship between Glu uptake and GS activity (19, 20). This data supports the claim that the neuro-protective functions of glia cells are at least partially dependent on the Glu uptake system rather than GS activity.

For the other purpose of this study, we measured the intracellular and extracellular Glu and Gln levels, because rapid changes in Glu and Gln levels were observed at even 30 min incubation as described above. As shown in Fig. 2 and Table 4, obvious differences were observed between the glia-rich and glia-poor cultures. This data suggests that the glia-rich culture has greater abilities to uptake Glu and to release Gln than the glia-poor culture. The significant difference of LDH leakage in the glia-rich and glia-poor cultures induced by 1 mM Glu (Table 1) might be related to this observation.

On the other hand, the kinetic parameters showed little differences between cultures, and the affinity and metabolic velocity of GS were reasonably high. From these results, it might be suggested that Glu is attacked by the enzyme GS to form Gln smoothly in both cultures.

Moreover, the percentages of intracellular Glu levels in the simultaneous incubation of 50 μM Glu and 5 μM MSO against 50 μM Glu incubation were 107% in the glia-poor culture and 125% in the glia-rich culture. Taking into consideration that the same magnitudes of MSO inhibitions were observed in both cultures, the above results elucidate the difference in the ability of glia-rich and glia-poor cultures to maintain storable amounts of Glu.

These findings suggest that not only the number of glial cells but also the functions of the Glu uptake system are the main differences between glia-poor and glia-rich cultures. The detailed functions of glial cells, especially the Glu uptake system remain obscure, but recent studies have characterized the glutamate transporter on the molecular level (24). As a further study, the molecular

function of the glutamate transporter in combination with our culture systems consisting of diverse glial cell numbers may be useful for answering this question, although it has to be considered that the efflux of Glu from glia cells may be induced by the brain energy failure conditions (25).

In conclusion, our present data suggest that the primary neuroprotective effect of glial cells is dependent on the uptake system rather than GS activity, and the capacity for storage of Glu in glial cells might be an important factor in regulating the brain functions under low energy brain conditions such as ischemia or anoxia.

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