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# Glutamate/glutamine metabolism coupling between astrocytes and glioma cells: Neuroprotection and inhibition of glioma growth

Pei-Sen Yao [a], De-Zhi Kang [a,b,*], Ru-Ying Lin [c], Bing Ye [d], Wei Wang [c], Zu-Cheng Ye [c,e,*]

[a] The First Clinical Medical College of Fujian Medical University, Fuzhou, China
[b] Department of Neurosurgery, The First Affiliated Hospital of Fujian Medical University, Fuzhou, China
[c] Center for Neuroscience Research, School of Basic Medical Sciences, Fujian Medical University, Fuzhou, China
[d] Department of Neurology, Fujian Institute of Geriatrics, Union Hospital of Fujian, Fuzhou, China
[e] Department of Neurology, University of Washington School of Medicine, Seattle, WA, USA

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ABSTRACT

Glioma glutamate release has been shown to promote the growth of glioma cells and induce neuronal injuries from epilepsy to neuronal death. However, potential counteractions from normal astrocytes against glioma glutamate release have not been fully evaluated. In this study, we investigated the glutamate/glutamine cycling between glioma cells and astrocytes and their impact on neuronal function. Co-cultures of glioma cells with astrocytes (CGA) in direct contact were established under different mix ratio of astrocyte/glioma. Culture medium conditioned in these CGAs were sampled for HPLC measurement, for neuronal ratiometric calcium imaging, and for neuronal survival assay. We found: (1) High levels of glutaminase expression in glioma cells, but not in astrocytes, glutaminase enables glioma cells to release large amount of glutamate in the presence of glutamine. (2) Glutamate levels in CGAs were directly determined by the astrocyte/glioma ratios. Indicating a balance between glioma glutamate release and astrocyte glutamate uptake. (3) Culture media from CGAs of higher glioma/astrocyte ratios induced stronger neuronal $Ca^{2+}$ response and more severe neuronal death. (4) Co-culturing with astrocytes significantly reduced the growth rate of glioma cells. These results indicate that normal astrocytes in the brain play pivotal roles in glioma growth inhibition and in reducing neuronal injuries from glioma glutamate release. However, as tumor growth, the protective role of astrocytes gradually succumb to glioma cells.



## 1. Introduction

Glioblastomas are the most common and aggressive brain tumors. Malignant gliomas have been shown to release high amount of glutamate [1], which can promote the growth of gliomas and mediate neuronal cell death [2–4]. In normal brain, glutamate is released from neurons and predominantly taken up by astrocytes, form a glutamate/glutamine metabolism coupling between astrocytes and neurons [5–8]. Similarly, we speculate that glioma glutamate release and astrocyte glutamate uptake can also form a glutamate/glutamine metabolism coupling, with glioma cells replacing neurons. In early-stage glioma, glioma cells are surrounded by a large number of astrocytes, glutamate released from glioma cells can be near completely eliminated by astrocytic glutamate uptake. This can prevent neuronal death and reduce the stimulatory role of glutamate on glioma growth, leading to inhibition of glioma expansion. Previous studies demonstrated that inhibition of glioma glutamate release or blockade of glutamate receptors could limit tumor growth [9,10]. However, ammonia is produced when glutamine is deamidated to glutamate by glutaminase, which could induce astrocyte swelling and dysfunction of glutamate uptake via excessive oxidative stress [5,11,12]. And ammonia-induced dysfunction of astrocytic glutamate uptake may contribute to glioma-induced neuronal damage in advanced glioma. In addition, increased glutamate concentration in gliomas has been shown to be associated with a higher risk of gliomas-associated seizures [13,14].

*Abbreviations:* CGA, co-culture of glioma cells with astrocytes; HPLC, high performance liquid chromatography; GFAP, glial fibrillary acidic protein; GDM, glutamate depleted medium; TFB-TBOA, (2S,3S)-3-[3-[4-(trifluoromethyl)benzoylamino]benzyloxy]aspartate; OPA, o-phthalaldehyde; ACSF, artificial cerebrospinal fluid; HBSS, HEPES-buffered saline solution.

* Corresponding authors. Address: 88 Jiaotong Road, Fuzhou, Fujian 350004, China. Fax: +86 591 83569369 (D.-Z. Kang), +86 591 22862045 (Z.-C. Ye).
*E-mail addresses:* kdzy99988@163.com (D.-Z. Kang), zcye@uw.edu (Z.-C. Ye).

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Currently, little is known about the setting of extracellular glutamate concentration by the balance between glioma glutamate release and astrocyte glutamate uptake. We established a co-culture experimental model to evaluate the following hypotheses: (1) Glioma cells express abundant glutaminase, while astrocytes not. (2) Glioma cells release a large amount of glutamate depending on extracellular glutamine. (3) At early stage of glioma, glioma cells were outnumbered by surrounding astrocytes and extracellular glutamate could be clamped at low level to reduce neuronal injury and proliferation of glioma cells. However, at an advanced stage of glioma, glutamate uptake will be compromised by glioma cells: (1) Accompanying glutamate release, glioma cells released large amounts of ammonia may damage astrocytic glutamate uptake. (2) Furthermore, excessive extracellular glutamate can induce neuronal injury.

## 2. Materials and methods

### 2.1. Culture of glioma cells, primary astrocytes and neurons

Glioma cell lines (STTG1, U251, T98G) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen), at 37 °C in humidified atmosphere (95% air/5% $CO_2$).

Primary cultures of mouse cortical astrocytes were prepared from neonatal C57BL/6 pups as previously described [15]. Briefly, cortices were separated, minced and incubated in papain for 15–20 min. After digestion, dissociated cells were suspended in culture media consisting of DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Gibico). Cells were typically utilized after 2–3 weeks in culture. Greater than 90% of the cultured cells were glial fibrillary acidic protein (GFAP)-positive and cultures were essentially free of neurons. Culture medium was changed every 4–5 days.

Primary cultures of mouse cortical neurons were prepared similarly to astrocytes, except the digested tissues were triturated by pipettes in glutamate depleted medium (GDM) [1] and plated at a density of $1 \times 10^5$ cells/ml into 24-well plates or 6-well plates previously coated with poly-lysine, 10 M 1-β-D-arabinofuranosylcytosine was added to kill off proliferating cells.

### 2.2. Co-Culture

Glioma cells were co-cultured in direct contact with astrocytes in 96-well plates and 6-well plates. $0.5–1 \times 10^4$ glioma cells/well (U251, T98G, and sttg1) were seeded in 96-well plates, and $1–2 \times 10^5$ glioma cells/well in 6-well plates. All the glioma cells were then mixed with astrocytes at different ratios. In order to eliminate the influence of glutamate contained in serum, cells were cultured in serum-free DMEM medium containing 4 mM glutamine. After incubation for 18 h, cultured medium were sampled for HPLC measurement, or for testing effects on cultured neurons.

To evaluate whether or not astrocytes co-cultured with glioma cells remove glioma-released glutamate, TFB-TBOA, a high affinity inhibitor of glutamate transporters, was used for inhibiting astrocytic glutamate uptake.

To assess effects of ammonia on astrocytic glutamate uptake, astrocytes were seeded on 96-well plates with DMEM supplemented with 10% FBS overnight, and subsequently medium was replaced by ACSF (115.75 mM NaCl, 3 mM KCl, 1.25 mM $NaH_2PO_4$, 23 mM $NaHCO_3$, 10 mM Glucose, 2 mM $MgSO_4 \cdot 7H_2O$, 2 mM $CaCl_2$) supplemented with 100 μM glutamate and $NH_4Cl$. After a 5 h incubation, extracellular media were collected for HPLC measurement.

### 2.3. Measurement of extracellular glutamate levels

Glutamate concentration was measured by a breeze HPLC system (Waters, Milford, MA) as previously described [15]. Briefly, samples (10–20 μL) were collected and derivatized with o-phthalaldehyde (OPA), followed by separation through a C18 reverse-phase adsorbosphere OPA-HR column (Alltech, Deerfield, IL) at 37 °C. Mobile phase A consisted of 25 mM sodium acetate, 0.4% 1,4-dioxane and 4.3% 2-isopropanol with pH adjusted to 5.90 by acetic acid. Mobile phase B was a mixture of methanol (97%), 1,4-dioxane (1.5%) and 2-isopropanol (1.5%). Fluorescent signals were excited at 338 nm and detected at 450 nm by a 474 scanning fluorescent detector (Waters).

### 2.4. Proliferation of glioma cells and astrocytes in co-culture models

To assess the effect of co-culturing with astrocytes on the proliferation of glioma cells, glioma cells were co-cultured in direct contact with astrocytes in 96-well plates in serum-free medium (to eliminate the influence of glutamate contained in serum), and control groups were sister astrocytes and glioma cells grown in separate wells. After 4 days, cultured cells were dissolved with 0.05 M NaOH and subsequently neutralized with HCl. The protein concentration of the lysate was measured with the BCA protein assay kit (Pierce).

### 2.5. Ratiometric $[Ca^{2+}]_i$ measurements

To evaluate neuronal $Ca^{2+}$ influx in CGA-conditioned medium, CGA-conditioned medium was applied to cultured neurons, and recordings were obtained and analyzed as previously described [1]. Briefly, neurons were loaded with ratiometric $Ca2^+$ dye Fura-2-acetoxymethylester (10 μM; Invitrogen) in cultured medium for 45 min. Subsequently, neurons were rinsed with HBSS (126.25 mM NaCl, 2.0 mM $CaCl_2$, 3.0 mM KCl, 1.25 mM $NaH_2PO_4$, 2.0 mM $MgSO_4$, 10 mM glucose, and 25 mM HEPES, pH 7.40) and placed in a series 20 microperfusion chamber (Harvard Apparatus). To reduce the impact of non glutamate factors on inducing basal $Ca^{2+}$ response in neurons, CGA-conditioned medium was diluted 1:4 with HBSS, and used to perfuse cultured neurons at a rate of 2.0 ml/min. Neurons were excited at 340 and 380 nm, digitized images were collected at 0.1 Hz for calculating 340:380 ratio by Metafluor program (Molecular Devices).

### 2.6. Effects of CGA-conditioned medium on neuronal survival

To assess the effects of CGA-conditioned medium on neuronal survival, CGA-conditioned medium was applied to cultured neurons for 48 h, and neuronal survival was evaluated by cell counting on a Leica microscope.

### 2.7. Immunofluorescence

Cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After three washes in PBS, cells were blocked with PBS + 0.1% Triton X-100 + 5% normal goat serum (Invitrogen) for 90 min. Cells were then incubated with rabbit anti-glutaminase (abcam, ab156876, 1:500) overnight followed by rinses and incubation with fluorescent conjugated secondary antibodies. Monoclonal antibody for GFAP (Cy3 conjugated, sigma, C9205, 1:10,000) was used for co-staining with rabbit anti-glutaminase by incubation together with its compatible secondary antibody. All coverslips were then rinsed, counterstained with 1 μM DAPI (Invitrogen), and mounted onto glass slides with Prolong Gold Antifade (Invitrogen). Immunofluorescence images were collected with a CoolSNAP HQ2 camera (Photometric, Tucson, AZ)



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