

# MCT4-Mediated Expression of EAAT1 is Involved in the Resistance to Hypoxia Injury in Astrocyte–Neuron co-Cultures

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**Abstract** Hypoxic stressors contribute to neuronal death in many brain diseases. Astrocyte processes surround most neurons and are therefore anatomically well-positioned to shield them from hypoxic injury. Excitatory amino acid transporters (EAATs), represent the sole mechanism of active reuptake of glutamate into the astrocytes and neurons and are essential to dampen neuronal excitation following glutamate release at synapses. Glutamate clearance impairment from any factors is bound to result in an increase in hypoxic neuronal injury. The brain energy metabolism under hypoxic conditions depends on monocarboxylate transporters (MCTs) that are expressed by neurons and glia. Previous co-immunoprecipitation experiments revealed that MCT4 directly modulate EAAT1 in astrocytes. The reduction in both surface proteins may act synergistically to induce neuronal hyperexcitability and

excitotoxicity. Therefore we hypothesized that astrocytes would respond to hypoxic conditions by enhancing their expression of MCT4 and EAAT1, which, in turn, would enable them to better support neurons to survive lethal hypoxia injury. An oxygen deprivation (OD) protocol was used in primary cultures of neurons, astrocytes, and astrocytes–neurons derived from rat hippocampus, with or without MCT4-targeted short hairpin RNA (shRNA) transfection. Cell survival, expression of MCT4, EAAT1, glial fibrillary acidic protein and neuronal nuclear antigen were evaluated. OD resulted in significant cell death in neuronal cultures and up-regulation of MCT4, EAAT1 expression respectively in primary cell cultures, but no injury in neuron–astrocyte co-cultures and astrocyte cultures. However, neuronal cell death in co-cultures was increased exposure to shRNA-MCT4 prior to OD. These findings demonstrate that the MCT4-mediated expression of EAAT1 is involved in the resistance to hypoxia injury in astrocyte–neuron co-cultures.

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## Introduction

Brain function involves much more than signaling between neurons. Neuroglia constitute more than half the cells in the adult vertebrate brain [1]. Almost 95 % of the vascular surface of the blood–brain barrier is ensheathed by perivascular astrocytes, and the opposite pole of each astrocyte contacts approximately 30,000 synapses [2]. Actually, astrocytes serve many essential physiological functions in the central nervous system (CNS) on which neurons

depend for their survival and peak functional performance. These include inducing the formation and maintenance of the blood–brain barrier, clearing extracellular potassium that accumulates with neuronal activity and collecting and metabolizing excitatory amino acids [3]. The latter is crucial to survival of neurons under various physiological or pathological conditions. Astrocytes are the largest glycogen repository in the brain. Glycogen stores supply astrocytes with the energy required to maintain transmembrane ion gradients, such as the sodium gradient that drives glutamate uptake necessary to regulate glutamatergic neurotransmission and prevent glutamate-mediated neuronal excitotoxicity [4].

A subset of these astroglial functions are induced in adult astrocytes in response to hypoxic or chemical preconditioning [5–7]. During hypoxia/ischemia (H/I), the transport of lactate and other monocarboxylates across the glial and neuronal membranes is facilitated by a family of monocarboxylate transporters (MCTs) [8]. MCTs belong to the SLC16 gene family comprising 14 members, of which MCT1–4 are proton symporters that mediate the transmembrane transport of lactate, pyruvate and ketone bodies [9]. MCT1 is expressed mainly in endothelial cells of microvessels, ependymocytes, astrocytes and oligodendrocytes but not in neurons, MCT2 is expressed primarily in neurons, whereas MCT4 is expressed almost exclusively in astrocytes [10]. Recent studies have shown that MCT4 is involved in the pathophysiology of temporal lobe epilepsy (TLE) [11], and upregulation of MCT4 induced by hypoxia preconditioning may help decrease the susceptibility to drug-induced epilepsy in rat [12].

Glutamate is the primary excitatory neurotransmitter in the CNS. Glutamate transporters, referred to as excitatory amino acid transporters (EAATs), represent the sole mechanism of active reuptake of glutamate into the astrocytes and neurons and are essential to dampen neuronal excitation following glutamate release at synapses. There are five known members of the EAAT family, each having a unique tissue distribution. EAAT1 and EAAT2 are expressed mainly in astrocytes [13], EAAT3 and EAAT4 are expressed primarily in neurons [14], whereas EAAT5 is only present in the retina [15]. Failure of astroglial glutamate uptake leads to hyperexcitability, seizures, and excitotoxicity. Glutamate clearance impairment from any factors is bound to result in an increase in hypoxic neuronal injury [16]. In recent years, some studies have found that MCT4 expression was associated with downregulation of the astrocyte-specific excitatory EAAT1 in primary culture rat cortical astrocytes and clinical epilepsy sample sections, and co-immunoprecipitation experiments revealed a protein–protein interaction between MCT4 and EAAT1 [11]. It is possible that EAAT1 is a substrate for MCT4, implying MCT4 may directly modulate EAAT1 in astrocytes. The

reduction in both surface proteins may act synergistically to induce neuronal hyperexcitability and excitotoxicity.

Therefore we were interested in establishing whether astrocytes would respond to hypoxic conditions by enhancing their expression of MCT4 and EAAT1, which, in turn, would enable them to better support neurons to survive lethal hypoxia injury. To test this hypothesis, primary neuronal, astrocyte cultures, and astrocyte–neuron co-cultures derived from rat hippocampus, with or without MCT4-targeted short hairpin RNA (shRNA) transfection, were subjected to oxygen deprivation (OD), then cell survival, expression of MCT4, EAAT1, glial fibrillary acidic protein (GFAP) and neuronal nuclear antigen (NeuN) were evaluated.

## Materials and Methods

Reagents were obtained from Gibco Life Technologies Corporation (Gibco, Grand Island, NY, USA) except where noted otherwise.

### Rat Primary Astrocyte–Neuron co-Cultures

All procedures were approved by the Animal Care and Use Committee of Lanzhou University (Lanzhou, China) and followed the National Guidelines for Animal Experimentation. Embryonic hippocampuses were obtained from 18-day Sprague–Dawley rat embryos ( $n = 7$ ) from the Laboratory Animal Center of Lanzhou University, using a modification of a previously-described method [17]. Embryos were removed under deep isoflurane anesthesia. Hippocampuses were collected and mechanically dissociated in 2 mg/mL papain (Worthington Biochemical, Lakewood, NJ, USA) and 50  $\mu$ g/mL DNase I (Sigma-Aldrich, St. Louis, MO, USA) in sterile Hank's Balanced Salt Solution (HBSS) at 37 °C for 20 min. The tissues were then washed three times in sterile HBSS to inactivate the papain and switched to 5 % fetal bovine serum (FBS) in Neurobasal-A growth medium supplemented with 0.5 mM L-glutamine, 0.5 mM GlutaMax, 0.01 % antibiotic–antimycotic and 0.02 % SM1 (StemCell Technologies, Vancouver, BC, Canada). The tissue mixture was then triturated three times using a 5-mL pipette followed by a Pasteur pipette, and filtered through an 80- $\mu$ m pore size nylon mesh (BD Biosciences, Franklin Lakes, NJ, USA). The cell mixture was then centrifuged at 200g for 3 min and resuspended in fresh Neurobasal-A medium. The cells were plated into 0.1 % polyethyleneimine (in 25 mM borate buffer, pH 8.3)-coated culture flasks or glass coverslips (25 mm diameter, Degkassen, Warner Instruments, Hamden, CT, USA) in a 6-well uncoated plate at around 100,000–200,000 cells/cm<sup>2</sup>. Cells were maintained in

Neurobasal-A growth medium (containing 25 mM D-glucose) without FBS at 37 °C in a 5 % CO<sub>2</sub>/95 % air (balanced nitrogen and 85 % humidity) incubator (i.e., normoxic conditions). The culture medium was replaced completely 4–6 h after plating and then changed by half every 2 days until the experiment finished. On the 4th day after plating, 1.5 mM leucine–leucine methyl ester was added to the medium to deplete microglia in the astrocyte–neuron co-cultures. The cells were used for experiments 2 weeks after plating.

#### Rat Primary Neuronal Cultures

Pure neuronal cultures were derived from the same embryonic hippocampal cell suspensions and maintained in the same culture medium as the co-cultures above. Astrocyte growth was inhibited by adding cytosine arabinoside (10 μM) 72 h after plating and decreasing the serum content in the medium. The cells were used for experiments 2 weeks after plating. The culture medium was changed by half every 2 days until the experiment finished.

#### Rat Primary Astrocyte Cultures

Hippocampal astrocytes were obtained from postnatal day 1 Sprague–Dawley rats (n = 10) as for co-cultures, except that hippocampal cells were plated and maintained in Dulbecco's Modified Eagle's Medium/F-12 (DMEM/F12, 1:1; Thermo Fisher Scientific, Waltham, MA, USA) (containing 17.5 mM D-glucose) supplemented with 10 % FBS and a mixture of 100 U/mL penicillin and 100 μg/mL streptomycin. The culture medium was replaced completely 1 h after plating to eliminate fibroblasts, and then changed by half every 3–4 days until the experiment finished. On the 4th day after plating, 1.5 mM leucine–leucine methyl ester was added to the medium to deplete microglia. Cells were detached with trypsin and replated at least twice before use. The cells were used for experiments 3 weeks after plating.

#### Immunocytochemistry in Cultured Cells

The purities of the cell cultures were confirmed by immunocytochemistry, as described previously, with modifications [18]. Cell cultures plated on coverslips were fixed with 4 % (w/v) paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 30 min at room temperature and blocked with PBS supplemented with 1 % bovine serum albumin and 0.3 % Triton X-100 for 1 h at room temperature. Cells were incubated with rabbit polyclonal anti-NeuN (Catalog #: ab104225, 1:1,000; Abcam, Cambridge, MA, USA) and (or) mouse monoclonal anti-GFAP (Catalog #: 3670, 1:500; Cell Signaling Technology, Inc.,

Danvers, MA, USA) antibodies overnight at 4 °C on an orbital shaker. The following day, cells were washed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated anti-rabbit (both 1:100; CoWin Biotech, Co. Ltd., Beijing, China) secondary antibodies for 2 h at room temperature. After washing with PBS for 15 min, cells were treated with the nuclear counterstain 4',6-diamidino-2-phenylindole (DAPI) for 10 min, washed, and mounted on glass slides. Images were acquired using a fluorescence microscope (Nikon Eclipse Ti-S, Tokyo, Japan) using FITC and TRITC filter sets (excitation/emission, 480/535 and 535/610 nm, respectively). Images were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

#### OD Model and Cell Treatment

Three kinds of primary cultures were subjected to hypoxia injury by OD which was produced in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA). Oxygen levels were monitored continuously with a gas analyzer (Coy Laboratory Products Inc.). On the day of OD treatment, cells were placed in the anaerobic chamber flushed with 1 % O<sub>2</sub>/5 % CO<sub>2</sub>/95 % N<sub>2</sub> at 37 °C for 24 h, then the cells were used for subsequent experiments under normoxic conditions.

#### Transfection of Astrocytes with a shRNA Targeting MCT4

Primary astrocyte–neuron co-cultured cells with MCT4-targeted shRNA (shRNA-MCT4) or a scrambled sequence control for off-target effects (shRNA-NC) were generated using a previously published method with modification [19]. All lentiviral short hairpin RNA (shRNA) particles were purchased from Open Biosystems (Thermo Scientific, Huntsville, AL). The shRNA sequences for MCT4 are 5-CCCACGUCUACAUGUACGU-3 (forward) and 5-ACGUACAUGUAGACGUGGG-3 (reverse). Briefly, the culture medium was replaced by normal culture medium without antibiotics, then  $2 \times 10^4$  primary cells were cultured in 24 well plates and transduced with lentiviral particles or the same amount of non-target shRNA control transduction particles following the manufacturer's protocol (multiplicity of infection = 2 viral transducing units/cell, transfection time = 72 h). The transduced clones were selected in puromycin-containing cell culture medium (0.5 mg/mL) (Invitrogen Australia Pty Ltd, Melbourne, VIC, Australia), propagated and finally transferred to the 25 cm<sup>2</sup> cell culture flasks and maintained in medium containing 1.0 mg/mL puromycin for the following experiments.

## Immunofluorescence Analysis to Determine Cell Survival and Apoptosis

The survival of cells on coverslips was assessed using a live/dead cell viability assay kit (Molecular Probes, Invitrogen, Singapore). Ethidium homodimer-1 cannot penetrate live cells but stains the DNA in dead cells red when exposed to 528 nm light. In contrast, calcein-AM penetrates live cells, where an esterase cleaves the molecule, which then fluoresces green when exposed to 494 nm light. The assay was performed on the cultures 1, 2, 3, 4, 5, 6, or 7 days after OD. The reagents were diluted, applied to coverslips, incubated for 15–30 min and then examined under an epifluorescence microscope (Nikon Eclipse), using a blue excitation filter (488/515 nm) and a green excitation filter (514/550 nm). Ten fields on each coverslip were photographed using the filter sets for each fluorescent label. The percentages of live (green) and dead (red) cells were calculated, with the total cell count represented by the sum of the live and dead cells.

Cells on the coverslips were then fixed with 4 % paraformaldehyde, rinsed and treated with the nuclear counterstain DAPI for apoptotic analysis on the 7th day after OD, the cells without OD as controls. DAPI-stained cells (10 per coverslip) were captured, and apoptotic cells were identified by their bright, condensed or multi-fragmented nuclei.

## Western Blotting

The cellular protein of primary astrocyte–neuron co-cultured cells was extracted as described previously with some modifications [20]. Concentrations were determined using the bicinchoninic acid method (Pierce, Rockford, IL, USA). Samples were resolved by 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis, then transferred to immobilon polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), blocked with 5 % (w/v) non-fat milk in Tris-buffered saline with 0.1 % Tween buffer (10 mM Tris–HCl, pH 7.5; 150 mM NaCl; 1 % Tween 20) for 2 h at room temperature. Then the membranes were incubated overnight with gentle agitation at 4 °C with primary antibodies against MCT4 (Catalog #: PAB21410, 1:500; Abnova Corporation, Taipei City, Taiwan), EAAT1 (Catalog #: 4166, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), GFAP (Catalog #: 3670, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), NeuN (Catalog #: ab104225, 1:5,000; Abcam, Cambridge, MA, USA) and  $\alpha$ -tubulin (Catalog #: 2144, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), which served as a loading control. Signals were detected using anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:5,000; Abcam,

Cambridge, MA, USA) and an enhanced chemiluminescence kit (Millipore). Signal intensity was analyzed using Image J software. All experiments were performed at least three times.

## Statistical Analysis

SPSS v.16.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. All values are presented as mean  $\pm$  standard deviation (SD) and differences were determined using two-tailed Student's *t* tests or one-way ANOVA with Bonferroni or Tamhane's post hoc tests. *P* values < 0.05 were considered statistically significant.

## Results

### Composition of Primary Cell Cultures

The co-cultures were prepared from 18-day embryonic hippocampuses and maintained in Neurobasal-A growth medium with 5 % FBS. This medium is widely used for neuronal cultures. After 2 weeks in vitro, the cultures comprised >50 % neurons and >45 % astrocytes, as determined by NeuN and GFAP immunofluorescence double labeling (Table 1, Fig. 1a). Neuronal-enriched cultures were also made from embryonic hippocampal cell suspensions with the addition of cytosine arabinoside to inhibit astrocyte growth. These cultures were identified as >80 % neurons by NeuN and DAPI immunofluorescence double labeling after 2 weeks of growth (Table 1, Fig. 1b). Hippocampal astrocyte cultures were obtained from post-natal-day 1 rats, and different culture media and methods yielded a population of >95 % astrocytes, as determined by GFAP and DAPI immunofluorescence double labeling (Table 1, Fig. 1c).

### Effect of OD on Cell Survival

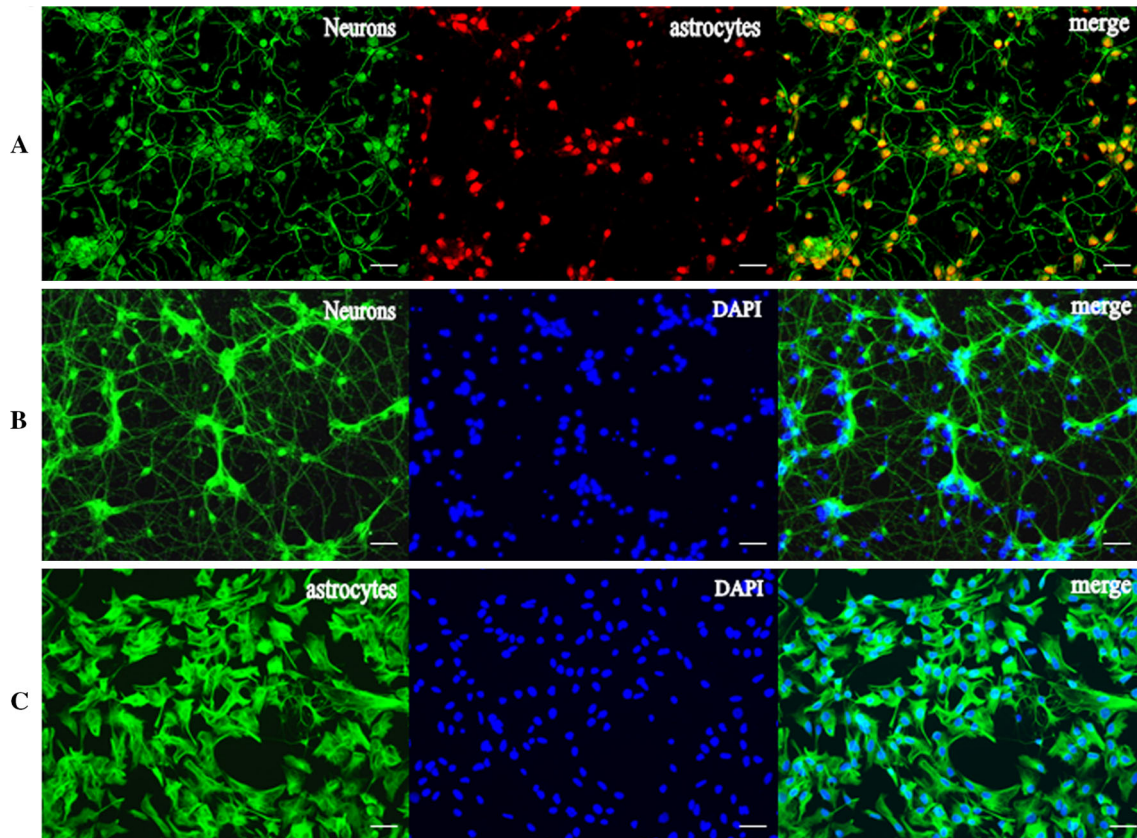
We identified the effect of OD on survival of different primary cell cultures at various time-points using a live/dead cell viability assay kit. The cell death curves constructed from independent experiments per day revealed an increased cell death percent in neuronal cultures, but no significant changes in co-cultures and astrocyte cultures. The average cell death percent for the different primary cell cultures are shown in Fig. 2. In contrast, OD resulted in significant higher average cell death percent in neuronal cultures than co-cultures and astrocyte cultures (*P* < 0.05). Apoptosis was determined by nuclear staining with DAPI on the 7th day after OD, and



**Table 1** Cell culture composition

Culture type <sup>a</sup>	N	Neurons % of total cells	Astrocytes % of total cells	Non-neurons % of total cells	Non-astrocytes % of total cells
Astrocyte–neuron Co-cultures	3	52.1 ± 1.2	45.9 ± 0.7	–	–
Neuronal cultures	3	82.6 ± 1.5	–	14.5 ± 0.4	–
Astrocyte cultures	3	–	96.2 ± 1.1	–	2.1 ± 0.5

<sup>a</sup> Cells from indicated culture type were counted following NeuN, GFAP and DAPI immunofluorescence double labeling to identify neurons, astrocytes and cell nucleus, respectively. Values are the mean ± SD

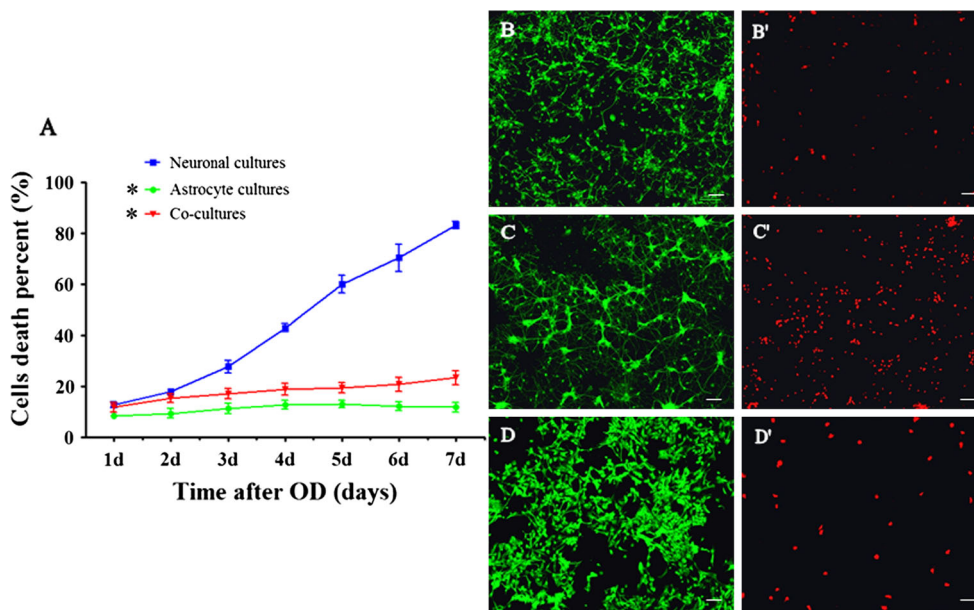


**Fig. 1** Cell culture compositions were determined by immunofluorescence double labeling. **a** Astrocyte–neuron co-culture; **b** neuronal culture; **c** astrocyte culture. Scale bar = 50 μm

the result indicated that  $64.5 \pm 5.3$  % of neurons in neuronal cultures were apoptotic, compared with  $6.2 \pm 1.1$  % in control cells ( $P < 0.05$ ) (Fig. 3). In contrast, the percentages of apoptotic cells in co-cultures and astrocyte cultures were unaffected by OD. Collectively, these data suggest that interactions between astrocytes and neurons in the co-culture system may be an important factor limiting neuronal death caused by hypoxic stressors. The main pathway of hypoxia-induced neuron death was apoptosis.

#### Effect of MCT4 Knockdown on Survival of Astrocyte–Neuron co-Cultures Under OD

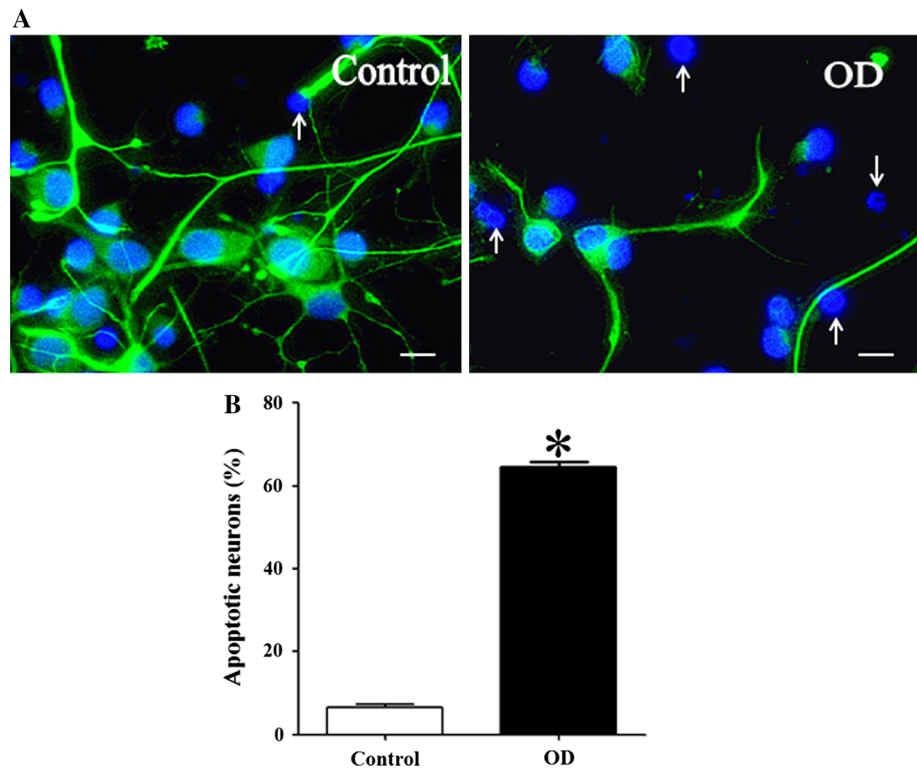
Based on the above results, OD resulted in significant cell death in cultured neurons, but not in astrocyte–neuron co-cultures. Therefore astrocyte–neuron co-cultures were transfected with a lentivirus vector containing a MCT4-targeted shRNA (shRNA-MCT4) or a scrambled control shRNA (shRNA-NC) for 72 h before exposure to OD, and the effects of MCT4 knockdown on cell survival were



**Fig. 2** Effect of OD on survival of different primary cell cultures. **a** The cell death curves constructed from independent experiments per day. Note that OD resulted in significant higher average cell death percent in neuronal cultures than co-cultures and astrocyte cultures. \* $P < 0.05$  versus neuronal cultures (ANOVA with post hoc Tamhane correction;  $n = 10$  per group). Culture coverslips were stained with

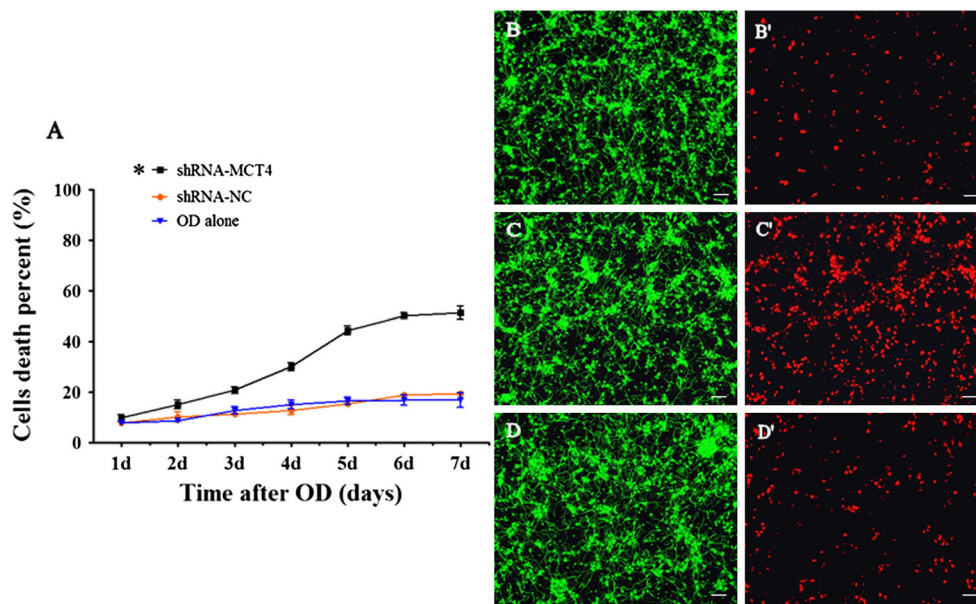
Molecular Probes live/dead assay reagents and viewed under a fluorescence microscope. **b**, **c**, and **d** Astrocyte–neuron co-culture, neuron culture and astrocyte culture, respectively, with intracellular calcein fluorescence signifying viable cells. **b'**, **c'** and **d'** Ethidium homodimer fluorescence in the same fields as **b**, **c**, and **d**, demonstrating injured and non-viable cells. Scale bar = 100  $\mu\text{m}$

**Fig. 3** Apoptotic analysis of neuronal cultures determined by immunofluorescence double labeling with specific markers of NeuN and DAPI. **a** Staining with DAPI in neuronal cultures to label nuclei blue allowed the determination of apoptotic cells, which exhibit condensed or fragmented nuclei. White arrows indicate apoptotic neurons. Scale bar = 25  $\mu\text{m}$ . **b** Percentage of apoptotic neurons was determined on the 7th day after OD in neuronal cultures. One-way ANOVA followed by Bonferroni post hoc tests indicated a significant difference between control and OD neurons. \* $P < 0.05$  versus control neurons ( $n = 10$  per group) (Color figure online)



identified by using live/dead cell viability assay kit. Cell cultures subjected to OD alone were used as controls. The cell death curves constructed from independent

experiments per day revealed an increased cell death percent in co-culture cells transfected with shRNA-MCT4 (Fig. 4). The cell death percent was  $53.2 \pm 4.9\%$  at 7th



**Fig. 4** Effect of MCT4 knockdown on survival of astrocyte–neuron co-cultures under OD. **a** The cell death curves constructed from independent experiments per day revealed an increased cell death percent in shRNA-MCT4-infected co-culture cells, still no significant changes in shRNA-NC-infected cells and OD alone co-culture cells. \* $P < 0.05$  versus OD alone cells (ANOVA with post hoc Tamhane correction;  $n = 10$  per group). Culture coverslips were stained with

Molecular Probes live/dead assay reagents and viewed under a fluorescence microscope. **b**, **c**, and **d** shRNA-MCT4, shRNA-NC and OD alone, respectively, with intracellular calcein fluorescence signifying viable cells. **b'**, **c'** and **d'**: Ethidium homodimer fluorescence in the same fields as **b**, **c** and **d**, demonstrating injured and non-viable cells. Scale bar = 100  $\mu\text{m}$

day after OD compared with OD alone co-culture cells ( $P < 0.05$ ), whereas shRNA-NC transfection had no effect ( $P > 0.05$ ). So MCT4 knockdown resulted in a significant increase in average cell death percent in astrocyte–neuron co-cultures.

#### The Evaluation of MCT4, EAAT1, GFAP and NeuN in Primary Astrocyte–Neuron co-Cultures by Western Blotting

We evaluated the expression levels of MCT4 and EAAT1 in primary astrocyte–neuron co-cultures 24 h later after transfection alone or OD alone by Western blotting, with normal cultured cells as controls. MCT4 and EAAT1 proteins were respectively visible as 43 and 58-kDa band in the immunoblot (Fig. 5a). The results of relative quantitative analysis are shown as Fig. 5b. Compared with the control cells, MCT4 and EAAT1 expression levels were respectively 68.1 and 64.5 % higher in the OD alone cells ( $P < 0.05$ ), but declined respectively by 93.5 and 89.1 % in the shRNA-MCT4 alone cells ( $P < 0.05$ ), whereas shRNA-NC transfection had no effect ( $P > 0.05$ ). A positive correlation between MCT4 and EAAT was observed in the entire astrocyte–neuron co-culture system. These data reveal that MCT4 expression was associated with expression change of the astrocyte-specific excitatory EAAT1 in primary astrocyte–neuron co-cultures. MCT4 and EAAT1

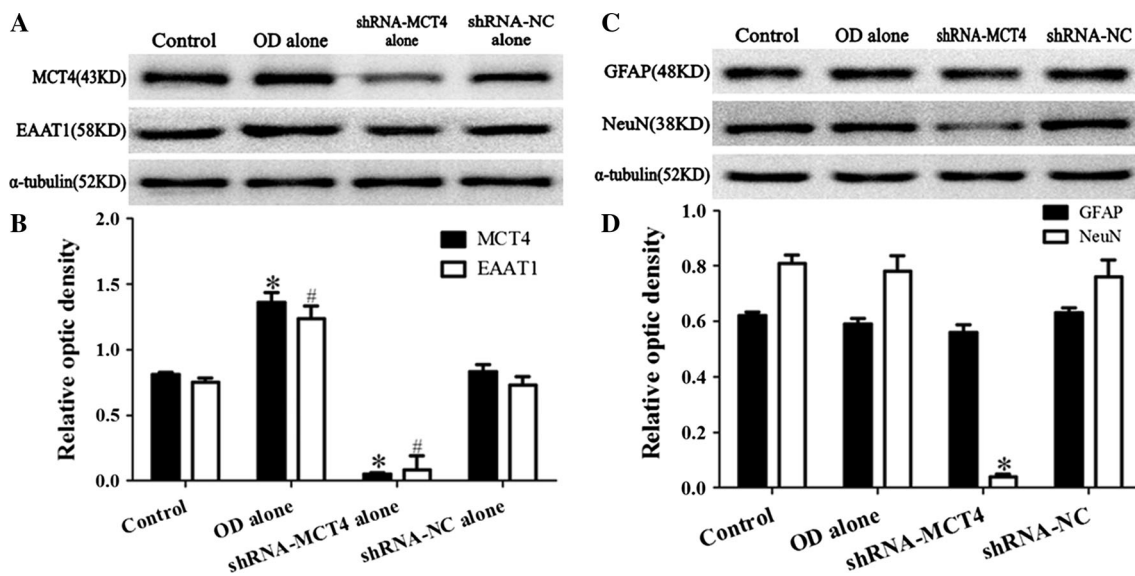
expression levels can be up-regulated by hypoxic stimulation, but significantly inhibited by RNAi-mediated suppression of MCT4.

We also evaluated the expression levels of GFAP and NeuN on the 7th day after OD in four groups of primary astrocyte–neuron co-cultures: control group (always normal cultured), OD alone group, shRNA-MCT4 group, and shRNA-NC group. GFAP and NeuN proteins were respectively visible as 48 and 38-kDa band in the immunoblot (Fig. 5c). The results of relative quantitative analysis are shown as Fig. 5d. Compared with the control group, the remaining three groups showed no comparable change in GFAP expression level ( $P > 0.05$ ), only NeuN expression level in shRNA-MCT4 group significantly declined by 95.1 % ( $P < 0.05$ ). These data suggest that the survival of astrocyte–neuron co-cultures was unaffected by 24 h OD treatment and neuronal cell death was increased by RNAi-mediated suppression of MCT4 prior to OD in astrocyte–neuron co-cultures.

#### Discussion

Recent studies have examined the role of astrocytes in ischemic injury [21]. Interactions between astrocytes and neurons have been proposed as an important factor promoting neuronal survival during ischemia or other





**Fig. 5** Western blot analysis of MCT4, EAAT1, GFAP and NeuN in primary astrocyte–neuron co-cultures. **a** Representative Western blot showing MCT4 and EAAT1 expression levels corresponding to different protocols, with  $\alpha$ -tubulin as a loading control. **b** Results of relative quantitative analysis. Compared with the control cells, MCT4 and EAAT1 expression levels were increased in the OD alone cells, but markedly decreased in the shRNA-MCT4 alone cells. \* $P < 0.05$  versus control cells for MCT4 quantitative analysis (ANOVA with post hoc Tamhane correction;  $n = 3$  independent experiment/per group). # $P < 0.05$  versus control cells for EAAT1 quantitative

analysis (ANOVA with post hoc Bonferroni correction;  $n = 3$  independent experiment/per group). **c** Representative Western blot showing GFAP and NeuN expression levels corresponding to different protocols, with  $\alpha$ -tubulin as a loading control. **d** Results of relative quantitative analysis. Compared with the control group, only NeuN expression level in shRNA-MCT4 group significantly declined. \* $P < 0.05$  versus control cells for NeuN quantitative analysis (ANOVA with post hoc Tamhane correction;  $n = 3$  independent experiment/per group)

pathological states, by limiting neuronal damage and death from excitotoxins [22], reactive oxygen species [23], and other stressors. However, evidence for the specific mechanisms involved in these astrocyte–neuron interactions is lacking. Hypoxia regulates the expression of a subset of astrocyte-specific transporters, including glucose and glutamate transporters, as well as MCTs [8, 24], which are involved in interactions between astrocytes and neurons.

Comparisons between cultured primary astrocytes and neurons in isolation, as well as in combination, have been used to investigate astrocyte–neuron interactions in several studies. The present co-culture system contained both neurons and astrocytes in similar proportions to those recently calculated to exist in vivo [25] and reported by others in vitro [26]. Compared with neuronal- or astrocyte-enriched cultures used by many researchers, the presence of astrocytes as well as neurons in mixed culture more closely mimics the in vivo environment. Astrocytes have been shown to be important for neuronal survival in vivo [27]. Several studies have shown that astrocytes protect cultured neurons against serum deprivation [28], copper-induced toxicity [29] and glutathione depletion [30]. A co-culture technique was used to show that the addition of astrocytes or astrocyte-conditioned medium during the recovery period protected neurons from oxygen and

glucose deprivation (OGD)-induced cell death [31]. Knockout mice lacking GFAP and vimentin genes, which are expressed by astrocytes, are more severely affected by ischemia [32], while increasing astrocyte resistance by targeted overexpression of Hsp72 or SOD2 decreased neuronal vulnerability to ischemia [33]. In the present study, we identified the effect of OD on cell survival using live/dead cell assays. The reported durations of OD applied in studies of ischemia and hypoxia injury range from 15 min to 48 h [26, 34]; we exposed cells to OD for 24 h, as used in most studies. The selective vulnerability of the hippocampus is considered important in primary cell OD models derived from the CNS, because animal models of stroke have reported selective delayed death in the CA1 of hippocampus, and CA1 neurons in particular appear to be more vulnerable to hypoxia injury [35]. Compared with the other two culture preparations, neuronal cultures appeared to be more sensitive to OD, which was consistent with the results of a previous research that found greater sensitivity of neurons to hypoxia in co-culture systems [36]. All these lines of evidence suggest that astrocytes in astrocyte–neuron co-cultures may protect neurons from mimetic ischemia and hypoxia injury by activating endogenous protective mechanisms, while the lack of astrocytes contributes to enhanced neuronal vulnerability. In addition,



immunofluorescence double labeling with specific markers for neurons and nuclei also revealed that OD induced apoptosis in neurons, but not astrocytes. Compared with neurons in isolation, astrocytes in isolation or neurons and astrocytes in combination appeared to be resistant to OD. However, these findings do not mean that astrocytes are unaffected by shorter durations of OD. The survival of astrocytes under hypoxic conditions did not depend totally on MCTs. Astrocytes, but not neurons, synthesize and store glycogen [37], because of the absence of glycogen synthase in the latter [38]. Astrocytes are thus the largest glycogen repository in the brain. Glycogen can be rapidly converted to pyruvate/lactate and metabolized in the tricarboxylic acid cycle or used for the biosynthesis of glutamate [39]. So we speculate that aggravation of cell injury should be observed in primary astrocyte cultures in response to further extended anoxia time.

Chemical factors that play a role in this protective mechanism interfere with the action of proteins involved in the cell-damage response, or act as intracellular signal transducers [40–42]. Many of these factors may induce protective effects, further complicating the elucidation of the underlying mechanisms [43]. The regulation mechanism of lactate transmembrane transport by MCTs between astrocytes and neurons postulates the key role during brain energy metabolism crises under hypoxic conditions. Some previous researches have already focused on the roles of MCT1 [44] and MCT2 [45] in epilepsy in vivo. However, it is not known the relationship between neuronal hyperexcitability and expression change of MCT4. Previous researches have shown that the loss of MCT4 on astrocytes may contribute to the energy impairment and seizure susceptibility in TLE. An energy deficiency in astrocytes is likely to impair the uptake of extracellular glutamate [46], which can cause seizures and neuronal death in animals [47]. Moreover, increased extracellular glutamate concentrations are present in the epileptogenic hippocampal formation in patients with TLE [48]. Glutamate is normally taken up by astrocyte glutamate transporters and rapidly converted to glutamine. EAAT1 is expressed by astrocytes [48]. Tanaka et al. [49] demonstrated that reduced expression of glutamate transporters can cause seizures in animal models. In present study, we demonstrate that MCT4 knockdown in primary astrocyte–neuron co-cultures led to reduced EAAT1 expression in astrocytes, and resulted in further disturbance of apoptosis in neurons, suggesting that neuronal death may be due to deleterious effects of hyperexcitability via allowing accumulation of extracellular glutamate in primary co-cultures.

These results taken together indicate that neuronal independent cultures were sensitive to hypoxia injury, but resistant to OD in co-culture with astrocytes. However, hypoxic tolerance in primary astrocyte–neuron co-cultures

was almost completely abolished by RNAi-mediated suppression of MCT4 prior to OD. The variation tendency of neuronal survival under different oxygen contents condition followed the same trend as the EAAT1 expression levels which were mediated by MCT4 in primary co-cultures, to some extent. So we believe that various factors contributed to hypoxic neuronal injury, of which energy crisis and excitotoxicity are two most important lethal stresses. More recently, the conceptual role of lactate metabolism and function in the normal brain have undergone major changes, shifting from developmental fuel and glycolytic waste product to include its use as a supplemental fuel and signaling molecule [50]. So MCT4-mediated suppression of EAAT1 may be due to energy failure. Further studies will investigate the concrete mechanism of MCT4-mediated EAAT1, as well as the functional synergy between these factors in hypoxia–ischemia brain injury. The results of these studies will help in the development of effective treatment strategies for patients with brain diseases characterized by hypoxia and ischemia.

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## References

1. Pelvig DP, Pakkenberg H, Stark AK et al (2008) Neocortical glial cell numbers in human brains. *Neurobiol Aging* 29:1754–1762
2. Bushong EA, Martone ME, Jones YZ, Ellisman MH (2002) Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. *J Neurosci* 22:183–192
3. Sofroniew MV, Vinters HV (2010) Astrocytes: biology and pathology. *Acta Neuropathol* 119:7–35
4. Dienel GA, Cruz NF (2004) Nutrition during brain activation: does cell-to-cell lactate shuttling contribute significantly to sweet and sour food for thought? *Neurochem Int* 45:321–351
5. Kawahara K, Kosugi T, Tanaka M, Nakajima T, Yamada T (2005) Reversed operation of glutamate transporter GLT-1 is crucial to the development of preconditioning-induced ischemic tolerance of neurons in neuron/astrocyte co-cultures. *Glia* 49:349–359
6. Zhang M, Li WB, Geng JX, Li QJ, Sun XC, Xian XH, Qi J, Li SQ (2007) The upregulation of glial glutamate transporter-1 participates in the induction of brain ischemic tolerance in rats. *J Cereb Blood Flow Metab* 27:1352–1368
7. Weller ML, Stone IM, Goss A, Rau T, Rova C, Poulsen DJ (2008) Selective overexpression of excitatory amino acid transporter 2 (EAAT2) in astrocytes enhances neuroprotection from moderate but not severe hypoxia–ischemia. *Neuroscience* 155:1204–1211
8. Simpson IA, Carruthers A, Vannucci SJ (2007) Supply and demand in cerebral energy metabolism: the role of nutrient transporters. *J Cereb Blood Flow Metab* 27:1766–1791
9. Halestrap AP, Meredith D (2004) The SLC16 gene family—from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. *Pflug Arch* 447:619–628
10. Pellerin L, Bergersen LH, Halestrap AP, Pierre K (2005) Cellular and subcellular distribution of monocarboxylate transporters in

- cultured brain cells and in the adult brain. *J Neurosci Res* 79:55–64
11. Liu B, Niu L, Shen MZ, Gao L, Wang C, Li J, Song LJ, Tao Y, Meng Q, Yang QL, Gao GD, Zhang H (2014) Decreased astroglial monocarboxylate transporter 4 expression in temporal lobe epilepsy. *Mol Neurobiol* 50:327–338
  12. Gao C, Wang C, Liu B, Wu H, Yang Q, Jin J, Li H, Dong S, Gao G, Zhang H (2014) Intermittent hypoxia preconditioning-induced epileptic tolerance by upregulation of monocarboxylate transporter 4 expression in rat hippocampal astrocytes. *Neurochem Res* 39:2160–2169
  13. Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC (1995) Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. *J Neurosci* 15:1835–1853
  14. Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW (1994) Localization of neuronal and glial glutamate transporters. *Neuron* 13:713–725
  15. Arriza JL, Eliasof S, Kavanaugh MP, Amara SG (1997) Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. *Proc Natl Acad Sci USA* 94:4155–4160
  16. Jackman NA, Uliasz TF, Hewett JA, Hewett SJ (2010) Regulation of system x(c)(-)-activity and expression in astrocytes by interleukin-1 $\beta$ : implications for hypoxic neuronal injury. *Glia* 58:1806–1815
  17. Liu B, Hong JS (2003) Primary rat mesencephalic neuron-glia, neuron-enriched, microglia-enriched, and astroglia-enriched cultures. *Methods Mol Med* 79:387–395
  18. McLendon RE, Bigner DD (1994) Immunohistochemistry of the glial fibrillary acidic protein: basic and applied considerations. *Brain Pathol* 4:221–228
  19. Qin XF, An DS, Chen IS, Baltimore D (2003) Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc Natl Acad Sci USA* 100:183–188
  20. Gao CJ, Niu L, Ren PC, Wang W, Zhu C, Li YQ, Chai W, Sun XD (2012) Hypoxic preconditioning attenuates global cerebral ischemic injury following asphyxial cardiac arrest through regulation of delta opioid receptor system. *Neuroscience* 202:352–362
  21. Nedergaard M, Dirnagl U (2005) Role of glial cells in cerebral ischemia. *Glia* 50:281–286
  22. Rosenberg PA, Aizenman E (1989) Hundred-fold increase in neuronal vulnerability to glutamate toxicity in astrocyte-poor cultures of rat cerebral cortex. *Neurosci Lett* 103:162–168
  23. Dringen R, Gutterer JM, Hirrlinger J (2000) Glutathione metabolism in brain metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. *Eur J Biochem* 267:4912–4916
  24. Voutsinos-Porche B, Bonvento G, Tanaka K, Steiner P, Welker E, Chatton JY, Magistretti PJ, Pellerin L (2003) Glial glutamate transporters mediate a functional metabolic crosstalk between neurons and astrocytes in the mouse developing cortex. *Neuron* 37:275–286
  25. Azevedo FA, Carvalho LR, Grinberg LT, Farfel JM, Ferretti RE, Leite RE, Jacob Filho W, Lent R, Herculano-Houzel S (2009) Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. *J Comp Neurol* 513:532–541
  26. Vibulsreth S, Hefti F, Ginsberg MD, Dietrich WD, Busto R (1987) Astrocytes protect cultured neurons from degeneration induced by anoxia. *Brain Res* 422:303–311
  27. Cui W, Allen ND, Skynner M, Gusterson B, Clark AJ (2001) Inducible ablation of astrocytes shows that these cells are required for neuronal survival in the adult brain. *Glia* 34:272–282
  28. Dhandapani KM, Hadman M, De Sevilla L, Wade MF, Mahesh VB, Brann DW (2003) Astrocyte protection of neurons: role of transforming growth factor-beta signaling via a c-Jun-AP-1 protective pathway. *J Biol Chem* 278:43329–43339
  29. Wang XF, Cynader MS (2001) Pyruvate released by astrocytes protects neurons from copper-catalyzed cysteine neurotoxicity. *J Neurosci* 21:3322–3331
  30. Drukarch B, Schepens E, Stoof JC, Langeveld CH, Van Muijswinkel FL (1998) Astrocyte-enhanced neuronal survival is mediated by scavenging of extracellular reactive oxygen species. *Free Radic Biol Med* 25:217–220
  31. Griffin S, Clark JB, Canevari L (2005) Astrocyte–neurone communication following oxygen glucose deprivation. *J Neurochem* 95:1015–1022
  32. Li L, Lundkvist A, Andersson D, Wilhelmsson U, Nagai N, Pardo AC, Nodin C, Ståhlberg A, Aprico K, Larsson K, Yabe T, Moons L, Fotheringham A, Davies I, Carmeliet P, Schwartz JP, Pekna M, Kubista M, Blomstrand F, Maragakis N, Nilsson M, Pekny M (2008) Protective role of reactive astrocytes in brain ischemia. *J Cereb Blood Flow Metab* 28:468–481
  33. Xu L, Emery JF, Ouyang YB, Voloboueva LA, Giffard RG (2010) Astrocyte targeted overexpression of Hsp72 or SOD2 reduces neuronal vulnerability to forebrain ischemia. *Glia* 58:1042–1049
  34. Kusumoto M, Dux E, Hossmann KA (1997) Effect of trophic factors on delayed neuronal death induced by in vitro ischemia in cultivated hippocampal and cortical neurons. *Metab Brain Dis* 12:113–120
  35. Kirino T (1982) Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Res* 239:57–69
  36. Vangeison G, Carr D, Federoff HJ, Rempe DA (2008) The good, the bad, and the cell type-specific roles of hypoxia inducible factor-1 alpha in neurons and astrocytes. *J Neurosci* 28:1988–1993
  37. Vilchez D, Ros S, Cifuentes D, Pujadas L, Vallès J, García-Fojeda B, CriadoGarcía O, Fernández-Sánchez E, Medraño-Fernández I, Domínguez J, García-Rocha M, Soriano E, Rodríguez de Córdoba S, Guinovart JJ (2007) Mechanism suppressing myoclonus epilepsy. *Nat Neurosci* 10:1407–1413
  38. Pellegri G, Rossier C, Magistretti PJ, Martin JL (1996) Cloning, localization and induction of mouse brain glycogen synthase. *Brain Res Mol Brain Res* 38:191–199
  39. Hamprecht B, Verleysdonk S, Wiesinger H (2005) Enzymes of carbohydrate and energy metabolism. In: Kettenmann H, Ransom BR (eds) *Neuroglia*, 2nd edn. Oxford University Press, New York, pp 202–215
  40. Zemke D, Smith JL, Reeves MJ, Majid A (2004) Ischemia and ischemic tolerance in the brain: an overview. *Neurotoxicology* 25:895–904
  41. Dirnagl U, Meisel A (2008) Endogenous neuroprotection: mitochondria as gateways to cerebral preconditioning? *Neuropharmacology* 55:334–344
  42. Schaller B, Graf R (2002) Cerebral ischemic preconditioning. An experimental phenomenon or a clinically important entity of stroke prevention? *J Neurol* 249:1503–1511
  43. Dirnagl U, Simon RP, Hallenbeck JM (2003) Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci* 26:248–254
  44. Lauritzen F, de Lanerolle NC, Lee TS, Spencer DD, Kim JH, Bergersen LH, Eid T (2011) Monocarboxylate transporter 1 is deficient on microvessels in the human epileptogenic hippocampus. *Neurobiol Dis* 41:577–584
  45. Lauritzen F, Heuser K, de Lanerolle NC, Lee TS, Spencer DD, Kim JH, Gjedde A, Eid T, Bergersen LH (2012) Redistribution of monocarboxylate transporter 2 on the surface of astrocytes in the human epileptogenic hippocampus. *Glia* 60:1172–1181

46. Danbolt NC (2001) Glutamate uptake. *Prog Neurobiol* 65:1–105
47. Olney JW, Collins RC, Sloviter RS (1986) Excitotoxic mechanisms of epileptic brain damage. *Adv Neurol* 44:857–877
48. Cavus I, Kasoff WS, Cassaday MP, Jacob R, Gueorguieva R, Sherwin RS, Krystal JH, Spencer DD, Abi-Saab WM (2005) Extracellular metabolites in the cortex and hippocampus of epileptic patients. *Ann Neurol* 57:226–235
49. Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Kikuchi T, Okuyama S, Kawashima N, Hori S, Takimoto M, Wada K (1997) Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science* 276:1699–1702
50. Dienel GA, Cruz NF (2008) Imaging brain activation: simple pictures of complex biology. *Ann NY Acad Sci* 1147:139–170