

# Characterization of Primary and Secondary Cultures of Astrocytes Prepared from Mouse Cerebral Cortex

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**Abstract** Astrocyte cultures were prepared from cerebral cortex of new-born and 7-day-old mice and additionally, the cultures from new-born animals were passaged as secondary cultures. The cultures were characterized by immunostaining for the astrocyte markers glutamine synthetase (GS), glial fibrillary acidic protein, and the glutamate transporters EAAT1 and EAAT2. The cultures prepared from 7-day-old animals were additionally characterized metabolically using  $^{13}\text{C}$ -labeled glucose and glutamate as well as  $^{15}\text{N}$ -labeled glutamate as substrates. All types of cultures exhibited pronounced immunostaining of the astrocyte marker proteins. The metabolic pattern of the cultures from 7-day-old animals of the labeled substrates was comparable to that seen previously in astrocyte cultures prepared from new-born mouse brain showing pronounced glycolytic and oxidative metabolism of glucose. Glutamate was metabolized both via the GS pathway and oxidatively via the tricarboxylic acid cycle as expected. Additionally, glutamate underwent pronounced transamination to aspartate and alanine and the intracellular pools of alanine and pyruvate exhibited compartmentation. Altogether the results show that cultures prepared from cerebral cortex of 7-day-old mice have metabolic and functional properties indistinguishable from those of classical astrocyte cultures

prepared from neocortex of new-born animals. This provides flexibility with regard to preparation and use of these cultures for a variety of purposes.

**Keywords** Glutamate transporters · Glial fibrillary acidic protein (GFAP) · Glutamine synthetase (GS) · Glucose and glutamate metabolism

## Introduction

Classically, cultures of astrocytes from rodent cerebral cortex are prepared from neocortex obtained from neonatal animals [1] and such cultures have been extensively characterized with regard to biochemical, pharmacological and morphological properties [2, 3]. These cultures have turned out to be extremely useful for metabolic studies related to GABAergic and glutamatergic neurotransmission [4]. Likewise astrocytes cultured from cerebellum of 7-day-old rodents have been established as an important tool to study the functional role of cerebellar astrocytes particularly related to glutamatergic neurotransmission [5, 6].

We have recently been interested in using transgenic mice in studies of glutamate metabolism. In this context it would be important to utilize cerebral cortex and cerebellum from 7-day-old animals in order to be able to carry out experiments on astrocytes cultured from these brain regions isolated from the same animal. Since cerebral cortical astrocytes prepared from 7-day-old mice have not previously been characterized properly, it was decided to carry out experiments designed to provide information about the basic biochemical properties of such astrocytes. Additionally, it would be advantageous to be able to subculture the astrocytes by passaging and hence, such secondary cultures were also characterized. Immunocytochemistry was used to

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monitor expression of the astrocytic markers glial fibrillary acidic protein (GFAP), glutamine synthetase (GS) and the glutamate transporters EAAT1 and EAAT2 [7–10] and the metabolic properties were assessed using [ $U$ - $^{13}C$ ]glucose, [ $U$ - $^{13}C$ ]glutamate and [ $^{15}N$ ]glutamate in combination with GC–MS technology to monitor amino acid, glycolytic and TCA cycle metabolism [11, 12]. Functional characterization of glutamate transport capacity was assessed using the non-metabolizable glutamate analog [ $^3H$ ]D-aspartate [13].

## Experimental Procedures

### Materials

Antibodies against GFAP-Cy3 (monoclonal Anti-Glial Fibrillary Acidic protein; Sigma C9205-.2ML) 1:400 and GS (anti-glutamine synthetase; rabbit; Sigma C5781-.2ML) were from Sigma Chem. Comp., St. Louis, MO, USA and antibodies against the glutamate transporters EAAT1 (GLAST) and EAAT2 (GLT-1) were generously donated by Prof. N.C. Danbolt, the University of Oslo (Anti-GLAST (Anti-A522, antibody#314, rabbit 8D0161) 0.3  $\mu$ g/ml and anti-GLT-1 (Anti B-12, antibody#360, rabbit 26970) 0.1  $\mu$ g/ml). Secondary antibodies, Alexa 488 (goat anti rabbit; Molecular probes) 1:400 and Alexa 633 (goat anti Mouse; Molecular probes) 1:400 were from Life Technologies (Carlsbad, CA, USA).  $^{13}C$ - and  $^{15}N$ -labeled compounds were from Cambridge Isotopes Laboratories Inc. (Woburn, MA, USA) and Isotec, a subsidiary of Sigma Chem. Comp. (St. Louis, MO, USA), respectively. [ $^3H$ ]D-Aspartate (474 MBq/mol) was from Amersham Biosciences (Hørsholm, Denmark) and the GC–MS and HPLC chemicals and chromatography columns from Phenomenex (Torrance, CA, USA) and Varian (Palo Alto, CA, USA), respectively. All other chemicals were of the purest grade available from regular commercial sources.

### Cell Cultures

Astrocyte cultures from neocortex of new-born or 7-day-old mice were prepared using the protocol for preparation of astrocytes from newborn mice [14]. Briefly, cerebral cortex was dissected from brains of new-born or 7-day-old mice (NMRI, Taconic, Ry, Denmark) and the tissue was mechanically dissociated and passed through a 80  $\mu$ m nylon sieve into DMEM tissue culture medium containing 6 mM glucose and 2.0 mM glutamine supplemented with 20% fetal bovine serum (FBS) (Biological Industries, Israel) to obtain a single cell suspension as described by Hertz et al. [14, 15]. The cell suspension was subsequently seeded in 25  $cm^2$  tissue culture flasks (NUNC, Roskilde, Denmark). The medium was changed twice a week for

3 weeks with gradual reductions in the FBS content (2nd week 15%; 3rd week 10%). During the last week of culture, 0.25 mM dibutyryl cyclic adenosine 3',5'-monophosphate (dBcAMP) was added to induce stellation of the cells [14, 15]. Cultures were maintained at 37°C in a mixture of atmospheric air and CO<sub>2</sub> (95/5%) in a humidified incubator.

In addition to preparing primary astrocyte cultures from cerebral cortex of 7-day-old mice, we have propagated the established primary astrocytes from neocortex of new-born mice up to passage 3. All of the cells meant for subculturing were seeded in 25  $cm^2$  cell culture flasks and grown for 7 days in DMEM supplemented with 20% FBS, with a single change of the medium on day 4. On days 8 and 11 the medium was changed to DMEM containing 15% FBS. Additionally, on day 11, the cells were trypsinized and subcultured. The cells destined to be characterized as passage 2 (P2) subculture were transferred into 24 well plates on day 11. Cells from two 25  $cm^2$  flasks were pooled together and used to seed one 24-well plate. From day 15 the cells were cultured for 1 week in DMEM supplemented with 10% FBS and 0.25 mM dBcAMP with media change on day 18. On day 22–23 the cells were used for experiments.

The astrocytes destined to be characterized as passage 3 (P3) subculture were split on day 11 in 1:2 ratio into new 25  $cm^2$  tissue culture flasks. On day 15 the medium was changed to DMEM supplemented with 10% FBS. On day 18, the astrocytes were subcultured into 24-well plates as described above. From day 22, the cells were cultured for 1 week in DMEM supplemented with 10% FBS and 0.25 mM dBcAMP, with a single change of medium on day 25. On day 29–30 the cells were ready for experiments.

### Immunocytochemistry

For immunocytochemical staining, the cells were seeded on poly-D-lysine coated coverslips. The culturing procedure and media were as described above. Cells were fixed in 4% paraformaldehyde at 0°C for 15 min and washed in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 7.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.9 CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, pH 7.4) solution containing 0.05% saponin. All incubations were carried out in PBS containing 0.05% saponin to permeabilize the cell membrane. The slides were boiled in 10 mM citrate buffer for 20 min to unmask the antigen epitopes whereafter they were incubated in blocking buffer containing 5% goat serum for 1.5 h. Subsequently, the slides were incubated with the above mentioned primary antibodies and incubated overnight in PBS supplemented with 1.5% goat serum at 5°C. Following a brief wash with PBS the slides were incubated with secondary antibody for 2 h at room temperature in PBS supplemented with 1.5% goat serum. The slides were mounted in Dako Faramount

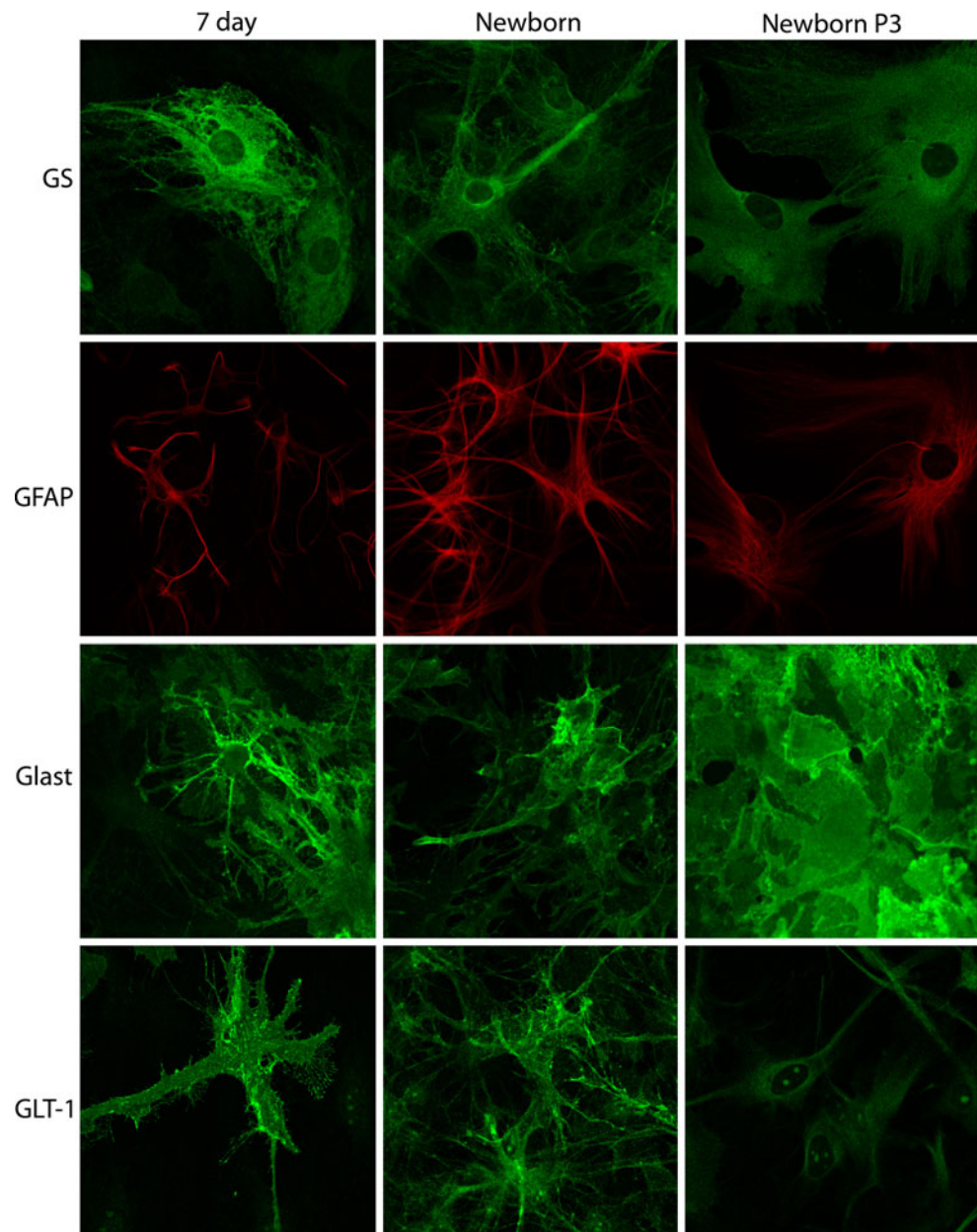
(S3025, Dako, Glostrup, Denmark) and sealed with nail polish and stored at 5°C. Imaging was performed on a Leica SP2 inverted confocal microscope using Argon and HeNe lasers to excite the alexa fluorophores. For imaging a 63 × 1.2 NA water objective was used. All images were obtained with zoom factor 2 creating images with a physical dimension of 119 × 119 μm at a resolution of 1,024 × 1,024 pixels.

#### Uptake of [<sup>3</sup>H]D-Aspartate

The kinetic characterization of glutamate transport in the cultured astrocytes using the non-metabolizable glutamate

analog D-aspartate was performed essentially as detailed by Drejer et al. [13]. Briefly, cultures were incubated for 3 min at 37°C in medium containing different concentrations of D-aspartate (range 0–500 μM) and subsequently for a further 3 min in the same medium with addition of trace amounts of [<sup>3</sup>H]D-aspartate. Incubations were finished by a rapid wash using ice-cold medium and the cellular content of radioactivity determined in extracts of the cells. The protein contents in the extracts were determined according to Lowry et al. [16] using bovine serum albumin as standard. The kinetic constants were calculated using weighted regression analysis [17]. Unspecific uptake was determined by incubation at 0°C.

**Fig. 1** Immunocytochemical staining of cortical astrocytes from 7-day-old mice (*left* column), new-born mice (*middle* column), and from new-born mice passage P3 (*right* column). Cells were cultured and immunostaining performed as detailed in “[Experimental Procedures](#)” using antibodies directed against GS (*top* row), GFAP (*2nd* row), EAAT1 (*3rd* row), and EAAT2 (*bottom* row). Image dimensions are 119 × 119 μm



## Incubation of Astrocytes with $^{15}\text{N}$ and $^{13}\text{C}$ Labeled Substrates

After 3 weeks in culture, the astrocytes cultured from neocortex of 7-day-old mice were used for metabolic labeling experiments. The astrocyte cultures were incubated for 4 h in a serum free DMEM containing 6 mM  $[\text{U-}^{13}\text{C}]$ glucose or 2 h in 100  $\mu\text{M}$   $[\text{U-}^{13}\text{C}]$ glutamate + 2.5 mM glucose or 3 h in 100  $\mu\text{M}$   $^{15}\text{N}$ glutamate + 2.5 mM glucose. Cells incubated with glutamate were spiked with additional 100  $\mu\text{M}$  of the labeled compound after 1 h. Subsequently, the cells were extracted in 70% ethanol and centrifuged (20,000g, 20 min, 4°C) to separate the soluble extract from the insoluble components. The pellets were employed for total protein determination using the Bradford protein assay [18]. Cell extracts and incubation media were lyophilized and reconstituted in water for biochemical analyses. Quantification and separation of amino acids in the cell extracts was performed by reversed-phase HPLC on an Agilent Eclipse AAA column (4.6  $\times$  150 mm, particle size 5  $\mu\text{m}$ ) employing pre-column, o-phthalaldehyde derivatization and fluorescence detection (excitation 350 nm; detection 450 nm).

The  $^{13}\text{C}$ - or  $^{15}\text{N}$ -labeling patterns in relevant metabolites from the incubation were analyzed using GC–MS. The cell extracts and reconstituted incubation media were derivatized with *N*-methyl-*N*-(tert-butyldimethylsilyl)trifluoroacetamide in the presence of 15% DMF (modified after Mawhinney et al. [19]). The samples were analyzed in a Shimadzu GC-2010 gas chromatograph linked to a Shimadzu GC–MS-Q2010plus mass spectrometer and compared to a standard sample containing the analytes of interest. All  $^{13}\text{C}$ -labeling data were corrected for natural abundance of  $^{13}\text{C}$  by subtracting the mass distribution of a standard containing the relevant metabolites. The average percent of  $^{13}\text{C}$ -labeled carbon atoms, i.e., the percent molecular carbon labeling (MCL) in Asp, Ala, Glu and Gln was calculated as initially described by Bak et al. [12].

## Results

### Immunocytochemistry

Primary and passaged cultures of astrocytes prepared from cerebral cortex of new-born or 7-day-old mice were immunostained for the classical glial markers, glial fibrillary acidic protein (GFAP), glutamine synthetase (GS) and the glutamate transporters EAAT1 and EAAT2. Figure 1 shows such staining for the astrocytes cultured from 7-day-old animals as well as analogous photomicrographs obtained from astrocytes prepared from new-born mice and the passaged cultures P3. It is seen that all sets of cultures

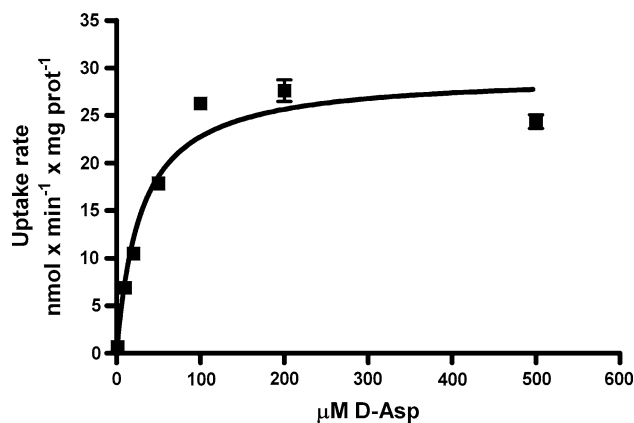
were stained for these markers, GFAP and GS being located in the cytosol and EAAT1 being associated primarily with the plasma membrane. The expression of EAAT2 was found to be different from that of EAAT1 as the EAAT2 immunostaining was more pronounced in the cytoplasm than in the plasma membrane and it was similar to that of GS. In some cultures prepared from cerebral cortex of either new-born or 7-day-old mice staining for GS and GFAP was compared to nuclear staining. Results were almost identical for the two sets of cultures. Almost all cells were stained for GS while more than 75% of the cells were stained for GFAP (results not shown).

### Uptake of D-Aspartate

Figure 2 shows the basic kinetics of  $[\text{}^3\text{H}]$ D-aspartate uptake into astrocytes cultured from cerebral cortex from 7-day-old mice. It conforms to classical Michaelis–Menten kinetics with a  $K_m$  value of  $29 \pm 9 \mu\text{M}$  and a  $V_{\text{max}}$  of  $30 \pm \text{nmol} \times \text{min}^{-1} \times \text{mg}^{-1}$  protein. Analogous results were obtained for the passaged cultures (P2 and P3) of astrocytes (results not shown) and uptake of L-glutamate in the primary cultures was found to exhibit kinetic constants similar to those of  $[\text{}^3\text{H}]$ D-aspartate uptake (results not shown).

### Amino Acid Content and Metabolism

The cellular contents ( $\text{nmol} \times \text{mg}^{-1}$  protein) of a number of amino acids as well as glutathion (GSH) in the primary cultures of astrocytes derived from cerebral cortex of 7-day-old mice are presented in Table 1. Except for aspartate which was present at a low level, the amino acids were found at levels exceeding  $10 \text{ nmol} \times \text{mg}^{-1}$  with glutamine and serine being among the most abundant.



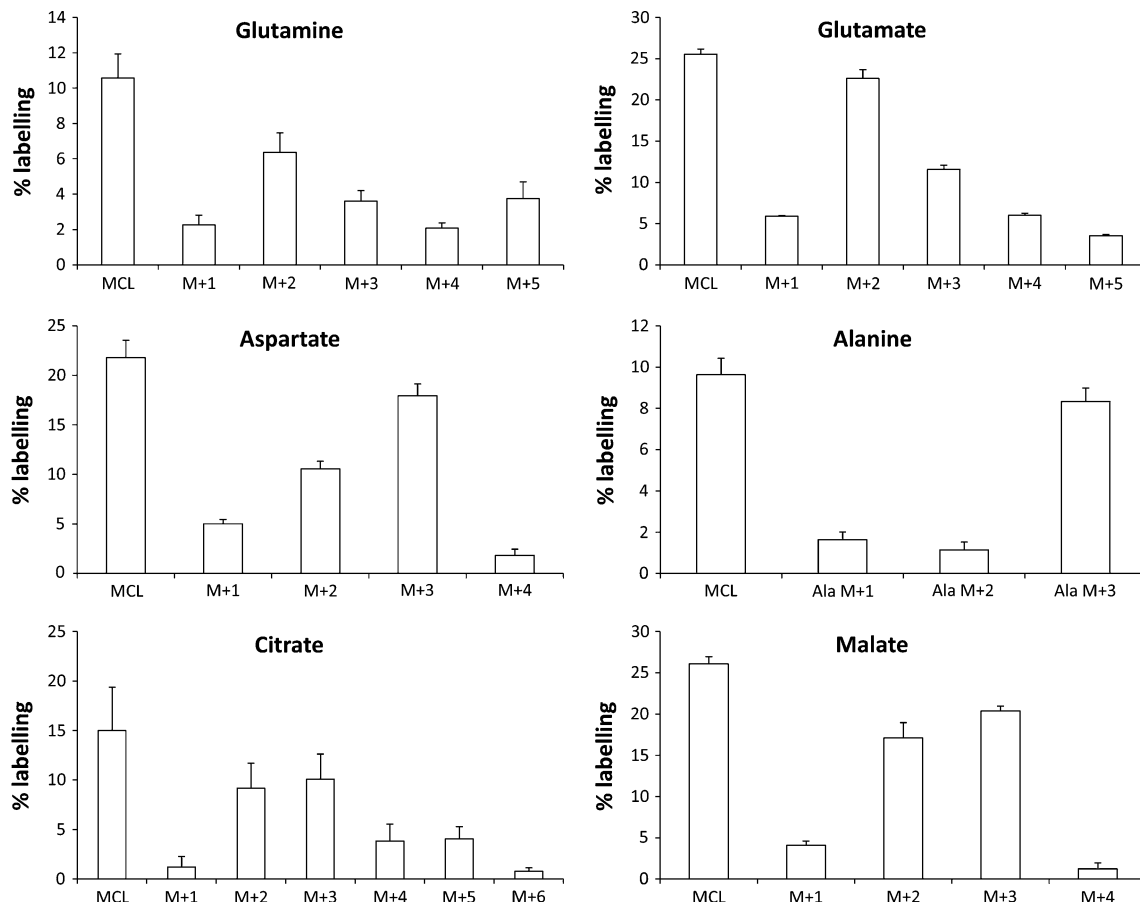
**Fig. 2** Kinetic study of  $[\text{}^3\text{H}]$ D-aspartate uptake in primary cultures of astrocytes prepared from cerebral cortex from 7-day-old mice performed as detailed in “Experimental Procedures”. The uptake followed Michaelis–Menten kinetics with a  $K_m$  of  $29 \pm 0.9 \mu\text{M}$  and a  $V_{\text{max}}$  of  $29 \pm 3.2 \text{ nmol} \times \text{min}^{-1} \times \text{mg protein}^{-1}$

**Table 1** Content of amino acids and GSH (nmol/mg protein) in astrocytes after incubation in medium containing 6 mM glucose

Amino acid/GSH	Contents (nmol/mg protein)
Gln	24.2 ± 0.8
Glu	18.8 ± 2.3
Asp	4.8 ± 0.3
Ala	13.0 ± 0.8
Ser	33.5 ± 2.0
Val	25.7 ± 4.2
Ile	27.7 ± 5.2
Leu	17.3 ± 3.5
GSH	52.8 ± 0.5

Astrocytes were cultured from neocortex of 7-day-old mice and incubated in culture medium with 6 mM glucose for 4 h and the amino acid and GSH contents were determined by HPLC analysis of cell extracts as detailed in “Experimental Procedures”. Results are averages ± SEM of four cultures

The metabolic status of the cultured astrocytes was assessed using the <sup>13</sup>C-labeled substrates glucose and glutamate as well as glutamate labeled with <sup>15</sup>N. Figure 3 shows the labeling in the intracellular pool of the amino acids glutamine, glutamate, aspartate and alanine as well as that in the TCA cycle constituents citrate and malate after incubation in medium containing 6 mM [U-<sup>13</sup>C]glucose. It is seen that glutamate and aspartate were labeled to a large extent followed by that in glutamine and alanine. The TCA cycle intermediates citrate and malate were also labeled to a considerable extent. Labeling in lactate which could only be found in the medium (Fig. 4) was considerable and essentially restricted to uniform labeling (M + 3) indicating that lactate was produced almost exclusively by glycolytic activity. It should be noted that labeling in glutamine in the medium (Fig. 4) was slightly but significantly (*P* < 0.05; Student’s *t* test) higher than that in the

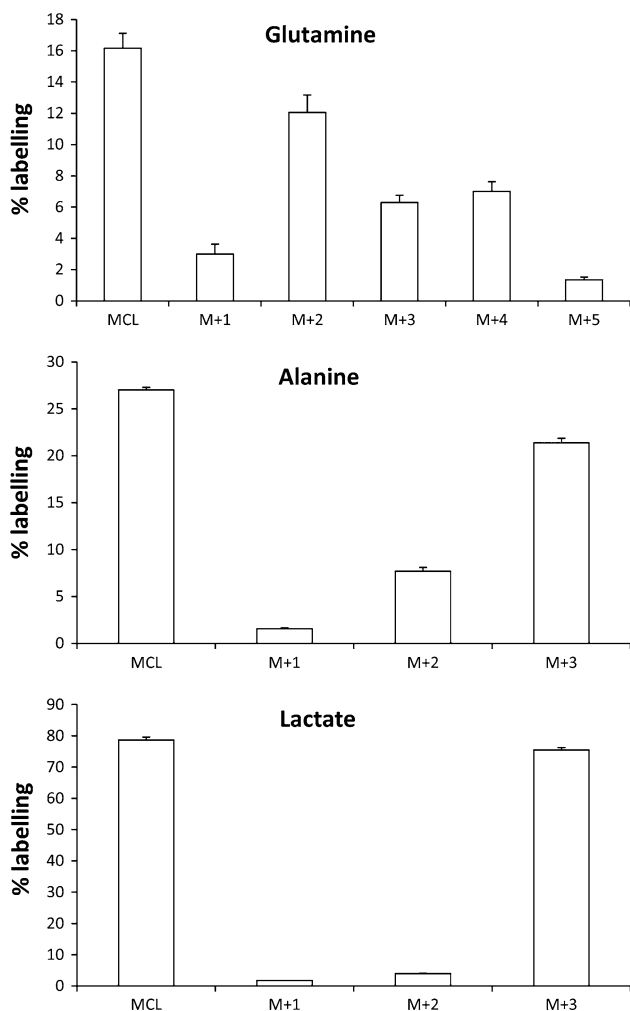


**Fig. 3** Isotopic labeling of intracellular amino acids and the TCA cycle intermediates citrate and malate determined by GC–MS analysis in cortical astrocytes from 7-day-old mice following a 4 h incubation period in the presence of 6 mM [U-<sup>13</sup>C] glucose as detailed in

“Experimental Procedures”. Values are given as MCL (%) as well as distribution (%) of label as previously described [12]. Results are averages ± SEM of four cultures

cells (Fig. 3) while that in alanine in the medium (Fig. 4) was much higher ( $P < 0.0001$ ; Student's  $t$  test) than the corresponding labeling in the intracellular pool (Fig. 3).

Using  $[U-^{13}C]$ glutamate as substrate it was demonstrated that the cells metabolized this amino acid both to glutamine and to aspartate and alanine (Fig. 5). Metabolism to alanine was paralleled with production of labeled lactate which could only be found in the medium (Fig. 6). Interestingly, after incubation with  $[U-^{13}C]$ glutamate the intracellular and extracellular pools of alanine exhibited comparable labeling (Figs. 5, 6). Moreover, the  $M + 1$  isotopomer of extracellular alanine was much more pronounced than the corresponding isotopomer of lactate, i.e., the  $M + 1$  (Fig. 6). Interestingly, the  $M + 3$  isotopomer of alanine was less pronounced than the corresponding lactate isotopomer (Fig. 6).

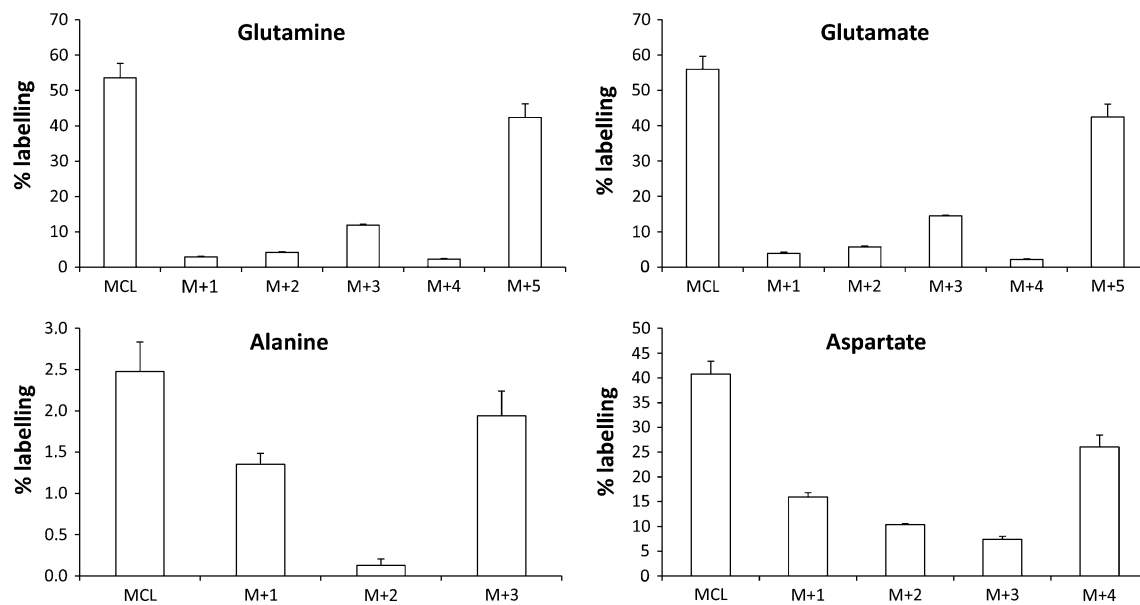


**Fig. 4** Isotopic labeling of extracellular amino acids and lactate determined by GC–MS analysis in cortical astrocytes from 7-day-old mice following a 4 h incubation period in the presence of 6 mM  $[U-^{13}C]$  glucose as detailed in “Experimental Procedures”. Values are given as MCL (%) as well as distribution (%) of label as previously described [12]. Results are averages  $\pm$  SEM of four cultures

Glutamate metabolism was additionally investigated using  $[^{15}N]$ glutamate as the substrate. It was found that glutamine was labeled to the same extent in one of the nitrogen atoms ( $M + 1$ ) and in both nitrogens ( $M + 2$ ) and that the amino acids aspartate and alanine exhibited pronounced labeling (Fig. 7). Labeled aspartate could only be found intracellularly while labeled alanine was present both intra- and extracellularly (Figs. 7 and 8). As was found using labeled glucose as precursor, alanine was much more labeled ( $P < 0.0001$ ; Student's  $t$  test) in the medium compared to the cellular pool (Figs. 7, 8), again suggesting compartmentation of the intracellular alanine pool.

## Discussion

Results from numerous studies over the past several decades have clearly placed the astrocytes in a key position with regard to maintenance of glutamatergic neurotransmission [20]. Not only are they in control of the availability of the main precursor for neuronal synthesis of transmitter glutamate, glutamine due to the exclusive astrocytic localization of the two key enzymes pyruvate carboxylase [21] and glutamine synthetase [8, 22] but they also are mandatory for termination of glutamatergic transmission by removal of glutamate from the synaptic cleft via highly efficient glutamate transporters expressed almost exclusively in the membrane of astrocytic processes [10]. In keeping with this, the astrocytes cultured from cerebral cortex of 7-day-old mice exhibited pronounced staining for GS, EAAT1 and EAAT2. This also reflects the characteristics of astrocyte cultures prepared from newborn mice which exhibit a high expression level of these proteins [9, 23, 24]. It should, however, be noted that cultured astrocytes under standard culturing conditions mainly if not exclusively express EAAT1 and not EAAT2 [24]. Expression of EAAT2 appears to require an inducing signal such as exposure to neuron-conditioned medium or dibutyl-cAMP [24–27]. In the present study the immunostaining of EAAT2 differed from that of EAAT1 since EAAT2 was less associated with the plasma membrane compared with EAAT1. This may be a consequence of the lack of factors regulating the trafficking of the transporter between the cytoplasm and the membrane [28, 29]. The expression pattern of EAAT2 relative to that of EAAT1 in the present cultures is very similar to that reported for astrocytes cultured from brains of 1–2 days old C57BL mice [29]. The finding that the immunostaining of GFAP and GS was essentially identical in the primary and passaged cultures of astrocytes is a good indication that the passaged cultures have maintained their basic astrocyte characteristics [7, 8]. This is further underlined by the



**Fig. 5** Isotopic labeling of intracellular amino acids determined by GC–MS analysis in cortical astrocytes from 7-day-old mice following a 2 h incubation in the presence of 100  $\mu$ M [ $U$ - $^{13}C$ ]glutamate and 2.5 mM glucose as detailed in “[Experimental Procedures](#)”. Values

are given as MCL (%) as well as distribution (%) of label as previously described [12]. Results are averages  $\pm$  SEM of eight cultures

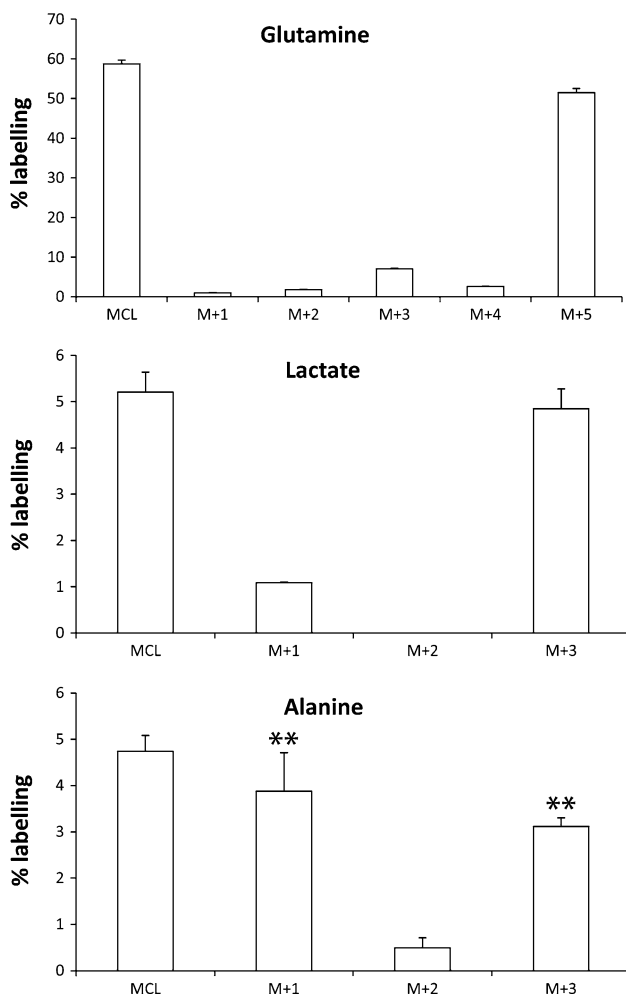
observation that uptake of [ $^3H$ ]D-aspartate, reflecting the expression of glutamate transporters [13], was similar in the primary and secondary cultures. Moreover, the kinetic characteristics of the uptake of [ $^3H$ ]D-aspartate were similar to previously published values for primary cultures of astrocytes from cerebral cortex of new-born mice [13, 30].

As astrocytes play an important role in brain metabolism and particularly in that involving glutamate and glutamine [4, 20, 31] it was important to assess the basic metabolic characteristics of the astrocytes cultured from cerebral cortex of 7-day-old mice. The contents of amino acids in these cells are similar to previously published values for astrocytes cultured from neonatal brain [32–34] albeit variations between studies exist. However, the present results indicate that these astrocytes are comparable to astrocytes derived from brains of newborn mice. That this may indeed be the case is strengthened by the findings concerning the metabolism of isotopically labeled glucose and glutamate.

Incubation of the cells with [ $U$ - $^{13}C$ ]glucose led to considerable labeling in the amino acids glutamate, glutamine and alanine similar to previously published results obtained in astrocytes cultured from cerebral cortex of newborn mice [35–37] or from cerebellum of 7-day-old mice [38]. The observation that the extracellular pools of glutamine and in particular alanine had a higher labeling than the corresponding intracellular pools may reflect compartmentation of the amino acids, a phenomenon observed in other preparations of cultured astrocytes as well [38]. It may be noted that compartmentation of amino acid

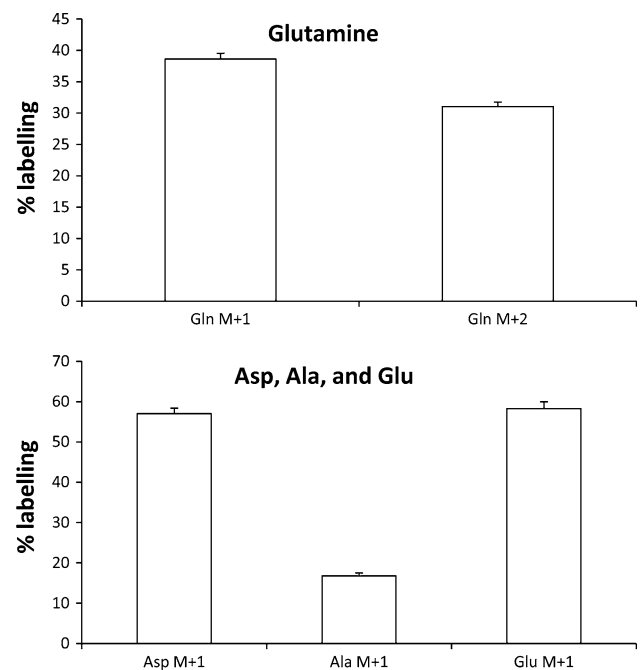
metabolism in the brain was observed several decades ago and that work from Abel Lajtha and others at that time was instrumental for the understanding this complex metabolic pattern in the brain [39, 40]. The difference in the amount of the M + 3 isotopomers of alanine and lactate in the medium constitutes another clear indication of compartmentation of the intracellular pyruvate pool from which these metabolites are derived. This result is in line with that reported for cerebellar astrocytes prepared from cerebella of 7-day-old mice [6]. The high labeling (MCL) in lactate is compatible with the pronounced glycolysis characteristic of cultured astrocytes [37, 41, 42] and this is reflected in the preferential production of uniformly (M + 3) labeled lactate. The presence of mono- and double-labeled lactate (M + 1 and M + 2) does, however, show that lactate is produced via metabolism of the glucose carbon skeleton in the TCA cycle as also seen in other studies of cultured astrocytes [36, 37, 43]. The finding of considerable labeling in malate and citrate obviously also shows that glucose is oxidatively metabolized in the TCA cycle in perfect agreement with the presence of all necessary enzymes in astrocytes acutely isolated from the brain [44].

The findings regarding metabolism of [ $U$ - $^{13}C$ ]glutamate with pronounced label in both glutamine and aspartate show that the present astrocyte preparation exhibits both oxidative metabolism of glutamate and non-oxidative conversion to glutamine as observed also for astrocytes cultured from neonatal brain tissue [45]. In this context, the extent of labeling in lactate and alanine was smaller than expected from a previous study by Sonnewald et al. [45] but it should

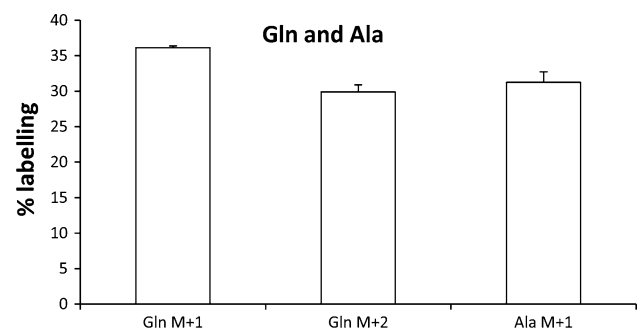


**Fig. 6** Isotopic labeling of extracellular amino acids and lactate determined by GC–MS analysis in cortical astrocytes from 7-day-old mice following a 2 h incubation period in the presence of 100  $\mu\text{M}$  [ $^{13}\text{C}$ ]glutamate and 2.5 mM glucose as detailed in “Experimental Procedures”. Values are given as MCL (%) as well as distribution (%) of label as previously described [12]. Results are averages  $\pm$  SEM of eight cultures. The asterisks indicate a statistically significant difference from the percent of M + 1 and M + 3 lactate ( $P < 0.01$ ; Student’s *t* test)

be considered that a lower concentration of glutamate was used in the present study compared to that of Sonnewald et al. [45]. This could affect the oxidative metabolism of glutamate and hence, lactate formation as shown by McKenna et al. [46] and Sonnewald et al. [33]. It may be pointed out that when glutamate was the substrate the compartmentation of the alanine pool was less pronounced than when using glucose as the labeled substrate since the difference between the total alanine labeling (MCL) in the cellular and extracellular pools was only marginal. However, comparing the cellular and the extracellular amounts of the M + 1 isotopomer of alanine, this isotopomer was more pronounced in the medium. Therefore, the alanine generated from oxidative metabolism of glutamate must be



**Fig. 7** Isotopic labeling of intracellular amino acids determined by GC–MS analysis in cortical astrocytes from 7-day-old mice following a 3 h incubation period in the presence of 100  $\mu\text{M}$  [ $^{15}\text{N}$ ]glutamate and 2.5 mM glucose as detailed in “Experimental Procedures”. Values are given as distribution (%) of label as previously described [12]. Results are averages  $\pm$  SEM of five cultures



**Fig. 8** Isotopic labeling of extracellular amino acids determined by GC–MS analysis in cortical astrocytes from 7-day-old mice following a 3 h incubation period in the presence of 100  $\mu\text{M}$  [ $^{15}\text{N}$ ]glutamate and 2.5 mM glucose as detailed in “Experimental Procedures”. Values are given as distribution (%) of label as previously described [12]. Results are averages  $\pm$  SEM of five cultures

compartmentalized. In keeping with this, lactate M + 3 in the medium was higher than alanine M + 3.

Using [ $^{15}\text{N}$ ]glutamate it could be demonstrated that glutamate undergoes both transamination and oxidative deamination as extensive labeling was found in glutamine M + 2 as well as in alanine and aspartate. This again is compatible with previous results using astrocytes from neonatal cerebral cortex and a low glutamate concentration [33]. The large proportion of M + 2 glutamine compared to the M + 1 isotopomer shows that the labeled ammonia



generated in the oxidative deamination of glutamate is trapped in glutamine formed in the GS catalyzed reaction. It should be noted that the labeling pattern in glutamine was found to be almost the same intra- and extracellularly. That this was not found to be the case for alanine where the extracellular pool exhibited a higher labeling than the intracellular pool again shows the existence of more than one pool of alanine. The lower labeling of intracellular alanine compared to aspartate which had the same labeling as glutamate may be a result of a much higher activity of aspartate aminotransferase than of alanine aminotransferase [47, 48].

In summary, the results obtained in the astrocytes prepared from cerebral cortex of 7-day-old mice strongly suggest that important astrocyte markers and metabolic characteristics are expressed and these cultures can be considered equivalent to those classically prepared from neonatal tissue. Also the passaged astrocytes exhibited basic astrocytic properties indicating that such cells may serve as alternative models for astrocytes.

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## References

- Booher J, Sensenbrenner M (1972) Growth and cultivation of dissociated neurons and glial cells from embryonic chick, rat and human brain in flask cultures. *Neurobiology* 2:97–105
- Schousboe A (1980) Primary cultures of astrocytes from mammalian brain as a tool in neurochemical research. *Cell Mol Biol Incl Cyto Enzymol* 26:505–513
- Hertz L, Juurlink BHJ, Szuchet S (1985) Cell cultures. In: Lajtha A (ed) *Handbook of neurochemistry*, vol 8, 2nd edn. Plenum Publishing Corporation, New York, pp 603–653
- Waagepetersen HS, Sonnewald U, Schousboe A (2009) Energy and amino acid neurotransmitter metabolism in astrocytes. In: Parpura V, Haydon PG (eds) *Astrocytes in (patho)physiology of the nervous system*. Springer, Boston, pp 177–199
- Westergaard N, Fosmark H, Schousboe A (1991) Metabolism and release of glutamate in cerebellar granule cells cocultured with astrocytes from cerebellum or cerebral cortex. *J Neurochem* 56:59–66
- Waagepetersen HS, Sonnewald U, Larsson OM, Schousboe A (2000) A possible role of alanine for ammonia transfer between astrocytes and glutamatergic neurons. *J Neurochem* 75:471–479
- Eng LF, Vanderhaeghen JJ, Bignami A, Gerstl B (1971) An acidic protein isolated from fibrous astrocytes. *Brain Res* 28:351–354
- Martinez-Hernandez A, Bell KP, Norenberg MD (1977) Glutamine synthetase: glial localization in brain. *Science* 195:1356–1358
- Gegelashvili G, Schousboe A (1997) High affinity glutamate transporters: regulation of expression and activity. *Mol Pharmacol* 52:6–15
- Danbolt NC (2001) Glutamate uptake. *Prog Neurobiol* 65:1–105
- Bak LK, Sickmann HM, Schousboe A, Waagepetersen HS (2005) Activity of the lactate-alanine shuttle is independent of glutamate-glutamine cycle activity in cerebellar neuronal-astrocytic cultures. *J Neurosci Res* 79:88–96
- Bak LK, Schousboe A, Sonnewald U, Waagepetersen HS (2006) Glucose is necessary to maintain neurotransmitter homeostasis during synaptic activity in cultured glutamatergic neurons. *J Cereb Blood Flow Metab* 26:1285–1297
- Drejer J, Larsson OM, Schousboe A (1983) Characterization of uptake and release processes for D- and L-aspartate in primary cultures of astrocytes and cerebellar granule cells. *Neurochem Res* 8:231–243
- Hertz L, Juurlink BHJ, Hertz E, Fosmark H, Schousboe A (1989) Preparation of primary cultures of mouse (rat) astrocytes. In: Shahar A, de Vellis J, Vernadakis A, Haber B (eds) *A dissection and tissue culture manual of the nervous system*. Alan R Liss, Inc., New York, pp 105–108
- Hertz L, Juurlink BHJ, Fosmark H, Schousboe A (1982) Astrocytes in primary cultures. In: Pfeiffer SE (ed) *Neuroscience approached through cell cultures*. CRC Press, Boca Raton, pp 175–186
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275
- Wilkinson GN (1961) Statistical estimations in enzyme kinetics. *Biochem J* 80:324–332
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Mawhinney TP, Robinett RS, Atalay A, Madson MA (1986) Analysis of amino acids as their tert-butyldimethylsilyl derivatives by gas-liquid chromatography and mass spectrometry. *J Chromatogr* 358:231–242
- Hertz L, Zielke HR (2004) Astrocytic control of glutamatergic activity: astrocytes as stars of the show. *Trends Neurosci* 27:735–743
- Yu AC, Drejer J, Hertz L, Schousboe A (1983) Pyruvate carboxylase activity in primary cultures of astrocytes and neurons. *J Neurochem* 41:1484–1487
- Norenberg MD, Martinez-Hernandez A (1979) Fine structural localization of glutamine synthetase in astrocytes of rat brain. *Brain Res* 161:303–310
- Juurlink BH, Schousboe A, Jørgensen OS, Hertz L (1981) Induction by hydrocortisone of glutamine synthetase in mouse primary astrocyte cultures. *J Neurochem* 36:136–142
- Gegelashvili G, Danbolt NC, Schousboe A (1997) Neuronal soluble factors differentially regulate the expression of the GLT1 and GLAST glutamate transporters in cultured astroglia. *J Neurochem* 69:2612–2615
- Gegelashvili G, Dehnes Y, Danbolt NC, Schousboe A (2000) The high-affinity glutamate transporters GLT1, GLAST, and EAAT4 are regulated via different signalling mechanisms. *Neurochem Int* 37:163–170
- Swanson RA, Liu J, Miller JW, Rothstein JD, Farrell K, Stein BA, Longuemare MC (1997) Neuronal regulation of glutamate transporter subtype expression in astrocytes. *J Neurosci* 17:932–940
- Schlag BD, Vondrasek JR, Munir M, Kalandadze A, Zeleniaia OA, Rothstein JD, Robinson MB (1998) Regulation of the glial

- Na<sup>+</sup>-dependent glutamate transporters by cyclic AMP analogs and neurons. *Mol Pharmacol* 53:355–369
28. Robinson MB (2002) Regulated trafficking of neurotransmitter transporters: common notes but different melodies. *J Neurochem* 80:1–11
  29. O'Shea RD, Lau CL, Farso MC, Diwakarla S, Zagami CJ, Svendsen BB, Feeney SJ, Callaway JK, Jones NM, Pow DV, Danbolt NC, Jarrott B, Beart PM (2006) Effects of lipopolysaccharide on glial phenotype and activity of glutamate transporters: evidence for delayed up-regulation and redistribution of GLT-1. *Neurochem Int* 48:604–610
  30. Hertz L, Schousboe A, Boechler N, Mukerji S, Fedoroff S (1978) Kinetic characteristics of the glutamate uptake into normal astrocytes in cultures. *Neurochem Res* 3:1–14
  31. Hertz L, Peng L, Dienel GA (2007) Energy metabolism in astrocytes: high rate of oxidative metabolism and spatiotemporal dependence on glycolysis/glycogenolysis. *J Cereb Blood Flow Metab* 27:219–249
  32. Hassel B, Westergaard N, Schousboe A, Fonnum F (1995) Metabolic differences between primary cultures of astrocytes and neurons from cerebellum and cerebral cortex. Effects of fluorocitrate. *Neurochem Res* 20:413–420
  33. Sonnewald U, Westergaard N, Schousboe A (1997) Glutamate transport and metabolism in astrocytes. *Glia* 21:56–63
  34. Westergaard N, Drejer J, Schousboe A, Sonnewald U (1996) Evaluation of the importance of transamination versus deamination in astrocytic metabolism of [U-<sup>13</sup>C]glutamate. *Glia* 17:160–168
  35. Sonnewald U, Westergaard N, Krane J, Unsgard G, Petersen SB, Schousboe A (1991) First direct demonstration of preferential release of citrate from astrocytes using [<sup>13</sup>C]NMR spectroscopy of cultured neurons and astrocytes. *Neurosci Lett* 128:235–239
  36. Sonnewald U, Westergaard N, Hassel B, Muller TB, Unsgard G, Fonnum F, Hertz L, Schousboe A, Petersen SB (1993) NMR spectroscopic studies of <sup>13</sup>C acetate and <sup>13</sup>C glucose metabolism in neocortical astrocytes: evidence for mitochondrial heterogeneity. *Dev Neurosci* 15:351–358
  37. Leo GC, Driscoll BF, Shank RP, Kaufman E (1993) Analysis of [1-<sup>13</sup>C]D-glucose metabolism in cultured astrocytes and neurons using nuclear magnetic resonance spectroscopy. *Dev Neurosci* 15:282–288
  38. Waagepetersen HS, Sonnewald U, Larsson OM, Schousboe A (2001) Multiple compartments with different metabolic characteristics are involved in biosynthesis of intracellular and released glutamine and citrate in astrocytes. *Glia* 35:246–252
  39. Lajtha A, Berl S, Waelsch H (1959) Amino acid and protein metabolism of the brain-IV. The metabolism of glutamic acid. *J Neurochem* 3:322–332
  40. Berl S, Lajtha A, Waelsch H (1961) Amino acid and protein metabolism-VI. Cerebral compartments of glutamic acid metabolism. *J Neurochem* 7:186–197
  41. Walz W, Mukerji S (1988) Lactate release from cultured astrocytes and neurons: a comparison. *Glia* 1:366–370
  42. Schousboe A, Westergaard N, Waagepetersen HS, Larsson OM, Bakken IJ, Sonnewald U (1997) Trafficking between glia and neurons of TCA cycle intermediates and related metabolites. *Glia* 21:99–105
  43. Hassel B, Sonnewald U, Unsgård G, Fonnum F (1994) NMR spectroscopy of cultured astrocytes: effects of glutamine and the gliotoxin fluorocitrate. *J Neurochem* 62:2187–2194
  44. Lovatt D, Sonnewald U, Waagepetersen HS, Schousboe A, He W, Lin JH, Han X, Takano T, Wang S, Sim FJ, Goldman SA, Nedergaard M (2007) The transcriptome and metabolic gene signature of protoplasmic astrocytes in the adult murine cortex. *J Neurosci* 27:12255–12266
  45. Sonnewald U, Westergaard N, Petersen SB, Unsgård G, Schousboe A (1993) Metabolism of [U-<sup>13</sup>C]glutamate in astrocytes studied by <sup>13</sup>C NMR spectroscopy: Incorporation of more label into lactate than into glutamine demonstrates the importance of the TCA cycle. *J Neurochem* 61:1179–1182
  46. McKenna MC, Sonnewald U, Huang X, Stevenson J, Zielke HR (1996) Exogenous glutamate concentration regulates the metabolic fate of glutamate in astrocytes. *J Neurochem* 66:386–393
  47. Schousboe A, Svenneby G, Hertz L (1977) Uptake and metabolism of glutamate in astrocytes cultured from dissociated mouse brain hemispheres. *J Neurochem* 29:999–1005
  48. Westergaard N, Varming T, Peng L, Sonnewald U, Hertz L, Schousboe A (1993) Uptake, release, and metabolism of alanine in neurons and astrocytes in primary cultures. *J Neurosci Res* 35:540–545