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Uptake and Metabolism of Malate in Neurons and Astrocytes in Primary Cultures

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Uptake and oxidative metabolism of [^{14}C]malate as well as its incorporation into aspartate, glutamate, glutamine, and GABA were studied in cultured cerebral cortical neurons (GABAergic), cerebellar granule neurons (glutamatergic), and cerebral cortical astrocytes. All cell types exhibited high affinity uptake of malate (K_m 10–85 μM) with slightly higher V_{max} values in neurons (0.1–0.2 nmol × min^{-1} × mg^{-1}) than in astrocytes (0.06 nmol × min^{-1} × mg^{-1}). Malate was oxidatively metabolized in all three cell types with nominal rates of $^{14}CO_2$ production of 2–15 pmol × min^{-1} × mg^{-1}. The oxidation of malate was only slightly inhibited by 5 mM aminooxyacetic acid (AOAA). In granule cell preparations [^{14}C]malate was incorporated into aspartate and glutamate and, to a *much* less extent, into glutamine. This incorporation was blocked by 5 mM AOAA. Astrocytes exhibited slightly higher incorporation rates into aspartate and glutamate, but in these cells glutamine was labelled to a considerable extent. AOAA (5 mM) inhibited the incorporation by 60–70%. In cultures of cerebral cortical neurons, very low levels of radioactivity derived from [^{14}C]malate were found in aspartate and glutamate, and GABA was not labelled at all. Glutamine had the same specific activity as glutamate, indicating that the low rates of incorporation of radioactivity into amino acids in this preparation is likely to exclusively represent metabolism of malate in the small population of astrocytes (5% of total cell number), contaminating the neuronal cultures. The findings suggest that exogenous malate to a quantitatively limited extent may serve as a precursor for transmitter glutamate in glutamatergic neurons. Astrocytes are able to metabolize malate to glutamate and related amino acids, but oxidize little malate to CO_2.

Key words: malate, aspartate, glutamate, metabolism, neurons, astrocytes

INTRODUCTION

During neurotransmission, GABA and glutamate are released from neurons and are, to a considerable extent, accumulated into astrocytes (for reviews see Hertz and Schousboe, 1986; 1988; Schousboe et al., 1988; Hertz et al., 1992). The resulting drain from neurons of glutamate and GABA, which are derived from tricarboxylic acid (TCA) cycle intermediates, must be compensated for by neuronal synthesis or accumulation of TCA intermediates or other precursors for glutamate and GABA, such as glutamine. Net synthesis of TCA cycle constituents in brain occurs mainly by carboxylation of pyruvate to oxaloacetate (Berl and Clarke, 1969; Patel, 1974). However, pyruvate carboxylation takes place only in astrocytes and not in neurons (Yu et al., 1983; Shank et al., 1985; Sonnewald et al., 1991; Kaufman and Driscoll, 1992). Accordingly, transfer of TCA cycle intermediates, e.g., malate and α-ketoglutarate (Shank and Campbell, 1981, 1984; Shank and Aprison, 1988) and/or of glutamine (Van den Berg and Garfinkel, 1971; Hamberger et al., 1978; Besson et al., 1981; Ward et al., 1983) from astrocytes to neurons must play a major role in the maintenance of the neuronal pools of TCA cycle constituents and neurotransmitter amino acids. The "classical" concept is that glutamate and GABA after reaccumulation into astrocytes are metabolized to glutamine and that glutamine (which has no transmitter activity) is returned to neurons in amounts similar to those originally diverted into astrocytes [the "GABA-glutamate-glutamine cycle" (Van Den Berg and Garfinkel, 1971; Benjamin and Quastel, 1975)]. However, there is little doubt that such a cycle, at least

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in the case of glutamate, does not operate in a stoichiometrical manner and that additional transmitter precursors may be needed (Schousboe et al., 1979; Hertz et al., 1980; Hertz and Shousboe, 1986, 1988; Shank et al., 1989; Yudkoff et al., 1989). TCA cycle constituents synthesized in astrocytes and subsequently transferred to neurons could serve this function.

The present study is an investigation of the role of the TCA cycle constituent malate as a percursor for glutamate and GABA in cerebral cortical neurons, a GABAergic preparation (Dichter, 1978; Yu et al., 1984a; Kuriyama and Ohkuma, 1987; Drejer et al., 1987), and in cerebellar granule cells, a glutamatergic preparation (Gallo et al., 1982; Drejer et al., 1982, 1985; Schousboe et al., 1985). Since neurons compete with adjacent astrocytes for malate and since neuronal cultures show a small contamination (≈5% of total cell number) with astrocytes, the uptake and subsequent metabolic fate of malate were also studied in cultured astrocytes. Malate may be converted, via α-ketoglutarate, to glutamate either by a transamination, catalyzed by aspartate aminotransferase (EC 2.6.1.1; AAT) or by a reductive amination, catalyzed by glutamate dehydrogenase (EC 1.4.1.3; GLDH). In order to delineate the relative contribution by each of these processes aminooxyacetic acid (AOAA), an AAT inhibitor (Meijer and Van Dam, 1974), was included in some of the experiments.

MATERIALS AND METHODS

Materials

All chemicals for medium production as well as malate were from Sigma Chemical Company (St. Louis, MO); [U-^{14}C]malate from Dupont (Mississauga, Ontario); and serum from selected horses.

Culturing Methods

Monotypic primary cultures of cerebral cortical neurons were prepared from the cerebral hemispheres of 15-day-old Swiss mouse embryos as described by E. Hertz et al. (1989a). The neopallium was cut into 1 mm cubes, dissociated by trypsinization, filtered through a Nitex® mesh (pore size 80 μm) and seeded into polylysine-coated Falcon plastic tissue culture dishes. After a 15 min incubation at 37°C, unattached (non-neuronal) cells were removed with the medium, which was replaced with fresh medium containing horse serum. The cultures were then incubated at 37°C in a 5% CO_2/95% atmospheric air atmosphere. After 3 days, the cultures were exposed to cytosine arabinoside (40 μM) for 24 hr to curtail astrocytic growth. Thereafter, the cultures were "refed" with fresh medium and used without further medium change at the age of 14 days. Such neurons show several characteristics of mature GABAergic neurons, and the astrocytic contamination is slight (probably ≈5%), as also indicated by a very low glutamine synthetase activity (Hertz and Schousboe, 1987).

Granule cells were obtained in an analogous manner from cerebella of 7-day-old mice as detailed by Schousboe et al. (1989). In order to enhance differentiation and purity of the cells, 24.5 mM KCl, 7 μM para-aminobenzoic acid, 100 mU/l insulin, and 30 μM cytosine arabinoside were included in the medium. The cells were used for experiments after 9–11 days in culture. Such cultures resemble mature granule cells in vivo morphologically and functionally (Hertz and Schousboe, 1987).

Astrocytes were prepared as described by Hertz et al. (1989b) and Juurlink and Hertz (1992). The neopallium of newborn Swiss mice was dissected out and, after removal of the meninges, the tissue was cut and dissociated by vortexing. The resulting cell suspension was filtered twice through sterile Nitex® nylon mesh sieves (80 followed by 10 μm pore size). The cell suspension was introduced into uncoated plastic Falcon tissue culture dishes and incubated at 37°C in a 5% CO_2/95% air atmosphere. After 2 weeks, the cultures reached confluency, and were subsequently grown for at least 1 week in the presence of 0.25 mM dibutyryl cyclic AMP, which induced a morphological differentiation with pronounced extension of cell processes (Hertz, 1990; Meier et al., 1991).

The use of brain tissue from animals of different ages for the preparation of each of the three cell types is dictated by the different ontogenetic development of these cell types. In all three cell cultures, the cells are well-differentiated at the time when they are used (Juurlink and Hertz, 1992; Schousboe et al., 1985).

Malate Uptake

Each culture was washed in tissue culture medium and incubated at 37°C in a medium containing malate concentrations between 5 and 250 μM and 0.17 μCi/ml L-[U-^{14}C]malate. After incubation, the cultures were washed 3 times with ice-cold medium, and 1 ml 1N NaOH was added overnight to dissolve the tissue. Radioactivity was determined in digested cells and in media, and uptake rates were calculated from the specific radioactivity of [^{14}C]malate in the media and expressed per mg protein, determined by the conventional Lowry technique (Lowry et al., 1951).

Malate Metabolism

For determination of pool sizes and specific radioactivities of amino acids, each culture was incubated at 37°C for 7.5–60 min in a normal tissue culture medium containing 10 μM [U-^{14}C]L-malate [0.5–3.5 μCi/ml depending on the cell type (highest for cortical neurons and

lowest for granule cells); however, in order to make the results for the 3 cell types comparable, *all* specific activities presented in Figs. 3–5 have been *recalculated to express the labelling which would have occurred if the medium had contained 1.0 μCi/ml*]. After the incubation, the cultures were rinsed 5 times with ice-cold phosphate buffered saline, scraped off the culture dishes in 1 ml of 70% (v/v) ethanol, left in this solution at room temperature for at least 30 min, and centrifuged. Protein in the pellets was determined as described above. The contents of aspartate, glutamate, GABA, and glutamine in the supernatants were determined by HPLC after pre-column derivatization with o-phthaldialdehyde essentially as described by Lindroth and Mopper (1979), and Yu et al. (1984b), employing a Waters HPLC system (Waters Associates, Toronto, Ontario) equipped with an M420 fluorescence detector and a Resolve 5 μ Bondapak C18 column. The mobile phase was 0.1 M potassium acetate (pH 5.7) and methanol. Initially, the methanol concentration was increased from 25% to 70% (v/v) in a single linear step (flow rate 0.8 ml/min during 15 min), then it was maintained at 70% (v/v) for 10 min and finally reversed (over 5 min) to equilibrate the column with 25% (v/v) methanol. Effluents corresponding to aspartate, glutamate, GABA and glutamine were collected for subsequent determination of radioactivities.

For determination of $^{14}CO_2$ production rates from [U-^{14}C]L-malate (0.1–0.3 μCi/ml), individual culture dishes without lids were placed in an air-tight chamber (Yu et al., 1982) or culture flasks were treated as described by Lopes-Cardozo et al. (1986). At the beginning of the experimental period, the radioisotope, diluted in medium, was added to the cultures by injection. At the end of the incubation, hyamine hydroxide or NaOH was injected into an enclosed beaker to trap CO_2 after acidification of the culturing medium with perchloric acid. After quantitative trapping of CO_2, the radioactivity was determined. This value, together with the specific radioactivity of malate in the media and the protein content in the cultures were used to calculate CO_2 production per mg protein.

RESULTS

Malate Uptake

In both neurons and astrocytes equilibration of malate uptake was slow and not complete during the experimental period (60 min). The uptakes were rectilinear for the first 3–5 min (Fig. 1). For this reason the concentration dependence of the uptake was studied using a 3 min uptake period. As can be seen from the Hanes plots in Figure 2, both types of neurons, and astrocytes, expressed high affinity uptake. The V_{max} values for the uptake were highest in cortical neurons (≈0.2 nmol × min^{-1} × mg^{-1}), intermediate in cerebellar granule cells (≈0.1 nmol × min^{-1} × mg^{-1}), and lowest in astrocytes (0.06 nmol × min^{-1} × mg^{-1}). The affinity was higher in astrocytes than in cortical neurons and cerebellar granule cells as indicated by Km values of 12, 28, and 85 μM, respectively. Estimated uptake rates at 10 μM malate, calculated on the basis of these kinetic constants are shown in Table I.

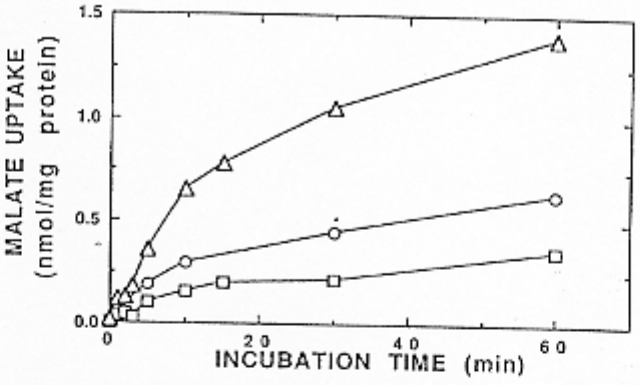

Fig. 1. Rate of uptake of malate (10 μM) into cerebellar granule cells (○), cerebral cortical neurons (△) and astrocytes (□) in primary cultures. Results are the averages of 3–5 experiments. No standard errors are indicated but they were in general less than 10% of the measured averages.

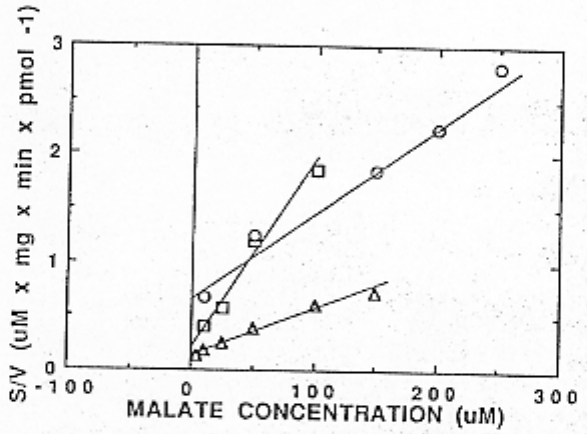

Fig. 2. Hanes plot describing uptake kinetics for malate uptake into cerebellar granule cells (□), cerebral cortical neurons (△) and astrocytes (○) in primary cultures. From the graph, V_{max} values of 120, 200, and 55 pmol × min^{-1} × mg^{-1}, respectively, can be calculated and the K_m values can be determined as 85, 28, and 12 μM, respectively. Values are averages of 3–6 experiments.

Malate Metabolism

Figures 3–5 demonstrate incorporation of radioactivity from malate into aspartate, glutamate and glutamine in the different cell types in the presence or ab-

TABLE I. Glutamate Contents, Malate Uptake Rates and Nominal Rates for Glutamate Synthesis and CO_2 Production at 10 μM [^{14}C]Malate in Cerebellar Granule Cells, Cerebral Cortical Neurons, and Astrocytes in Primary Cultures, Expressed per mg Protein*

Cell type	Glutamate content[a] (nmol × mg^{-1})	Glutamate synthesis (pmol × min^{-1})		Malate uptake (10 μM) (pmol × min^{-1})	CO_2 production (pmol × min
		Min	Max		
Cerebellar granule cells	60 ± 4	5–6	1,000	5	10
Cortical neurons	45 ± 4	0.2	—	35	15
Astrocytes	45 ± 2	16	1,600	30	2

*Metabolic rates (expressed per mg protein) are calculated based on the specific activity of malate in the incubation medium, rate of incorporation of radioactivity into glutamate (Fig. 2) or CO_2, and protein contents per culture. Since the actual specific activities in the immediate precursor pool are unknown the values are indicated as "nominal," but both minimum or maximum values have been estimated.
[a]Values for glutamate content are the average glutamate contents measured during 0–60 min of incubation with malate (since no apparent changes occurred during this period). They are the means of 19–32 experiments ± SEM.

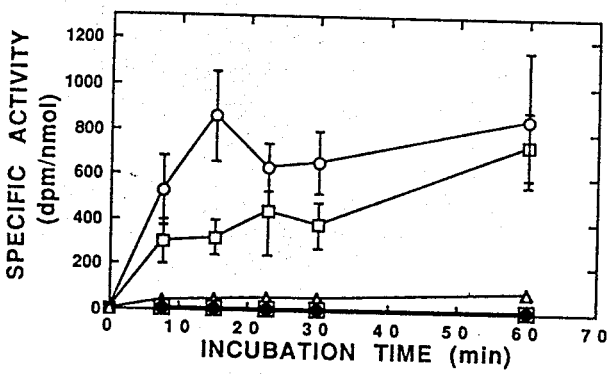

Fig. 3. Specific radioactivity of aspartate (circles), glutamate (squares) and glutamine (triangles) in the absence (open symbols) and presence (closed symbols) of 5 mM aminooxyacetic acid (AOAA) in cerebellar granule cells. Results are averages of 6–8 experiments with SEM values shown as vertical bars, if they extend beyond the symbols.

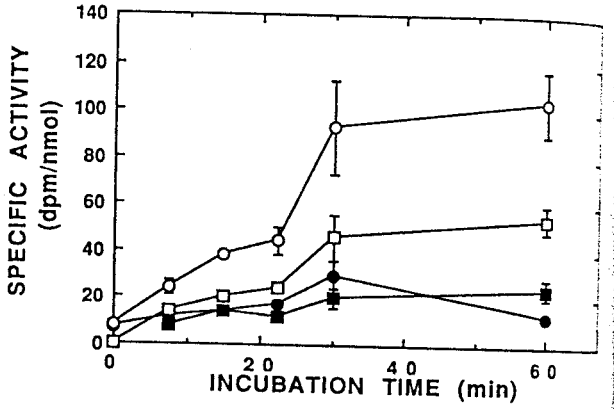

Fig. 4. Specific radioactivity of aspartate (circles), and glutamate (squares) in the absence (open symbols) and presence (closed symbols) of 5 mM aminooxyacetic acid (AOAA) in cerebral cortical neurons in primary cultures. Results are averages of 3–4 experiments with SEM values shown as vertical bars, if they extend beyond the symbols.

sence of 5 mM AOAA. In cerebellar granule cells (Fig. 3), the specific activity of aspartate reached a constant value after 15 min of incubation and the specific activity in glutamate approached the same level after 60 min. AOAA completely inhibited the labeling of both amino acids. The incorporation into glutamine was very low. In cerebral cortical neurons (Fig. 4), the specific activity of aspartate reached a much lower level (about one tenth of that in cerebellar granule cells). The specific activity of glutamate did not approach that of aspartate, even after 1 hr of incubation. AOAA inhibited labeling of these amino acids less efficiently than in cerebellar granule cells. The specific activity of glutamine (result not shown) was virtually identical to that of glutamate at all incubation periods (e.g., 58 ± 10 DPM/nmol after 60 min). The specific activity of GABA was not significantly different from zero even after 60 min of incubation (results not shown). In astrocytes, the specific activity of aspartate increased rapidly and equilibrated at around 2,000 DPM/nmol (results not shown), i.e., 10 times less than that of malate in the incubation medium (2.2×10^5 DPM/nmol). The specific activity of glutamate followed a similar course (Fig. 5) but reached a level that was only about one half of that in aspartate. The incorporation of radioactivity into glutamate (50 DPM/nmol already after 7.5 min) was much faster than in either type of neurons, and was only partly inhibited by AOAA. Labeling of glutamine was about one half of that in glutamate. The effect of AOAA was similar to that observed for incorporation of radioactivity into glutamate.

From the total rate of accumulation of radioactivity into glutamate and the specific activity of malate in the incubation medium, nominal rates of glutamate synthesis can be calculated. These rates are nominal, because it cannot be assumed that the specific activity of cellular α-ketoglutarate, which is the immediate precursor for

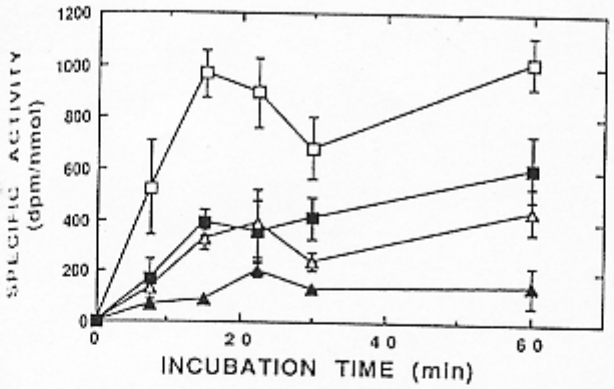

Fig. 5. Specific radioactivity of glutamate (squares) and glutamine (triangles) in the absence (open symbols) and presence (closed symbols) of 5 mM aminooxyacetic acid (AOAA) in astrocytes in primary cultures. Results are averages of 4–6 experiments with SEM values shown as vertical bars, if they extend beyond the symbols.

glutamate synthesis, is similar to that of extracellular malate. However, they are minimum values, since the *maximum* specific activity in any malate-derived pool is equal to that of malate in the incubation medium. From the pool sizes (contents) of glutamate shown in Table 1, the specific activity of malate in the incubation medium (220 DPM/pmol), and increases per min of specific activity in glutamate (DPM × nmol^{-1} × min^{-1}) of 20, 1 and 80 in, respectively, cerebellar granule cells, cortical neurons and astrocytes (estimated from the initial, relatively rectilinear parts of Figs. 3–5), the nominal rates of glutamate synthesis can be calculated. As seen from Table I, these rates are higher in cultures of cerebellar granule cells (5–6 pmol/min per mg protein) and in astrocytes (16 pmol/min per mg protein) than in cultures of cerebral cortical neurons (0.2 pmol/min per mg protein). If, on the other hand, the specific activity of aspartate, at equilibrium, is regarded as identical to that of the TCA cycle intermediates (which is probably also not the case but will provide estimates of *maximum* rates), these nominal rates could represent underestimates of actual rates of 100 and 200 times in astrocytes and granule cells, respectively. These values are also indicated in Table I.

In addition to being converted to amino acids, malate was also oxidatively degraded. Nominal rates of malate oxidation can be calculated on the basis of the specific activity in the incubation medium, and the production of $^{14}CO_2$, which was rectilinear with time during a 60-min incubation period (results not shown). The nominal rates of CO_2 production calculated in this way are shown in Table I. It can be seen that they are similar to rates of glutamate synthesis in cerebellar granule cells but much higher than the rate of glutamate synthesis in cortical neurons, and much lower than the glutamate synthesis rate in astrocytes. They were only slightly affected by AOAA (results not presented).

DISCUSSION

Due to the relatively low uptake rate for malate determined in this study it is unlikely that *exogenous* malate can be a quantitatively important precursor for glutamate in the cellular pool in any of the three cell types. Nevertheless, it is of at least theoretical interest that radioactivity from labelled malate *can* be incorporated into aspartate and glutamate in cerebellar granule cells. The finding that AOAA abolished the incorporation of radioactivity into glutamate suggests that a transamination process is responsible for glutamate formation. This is in agreement with the evidence presented by Palaiologos et al. (1988, 1989), Peng et al. (1991), and Christensen et al. (1991) that transamination processes are mandatory for biosynthesis of transmitter glutamate both in cultured neurons and in hippocampus in vivo.

The estimated rate of glutamate production from malate in astrocytes was higher than in the granule cells (Table I). The finding that glutamate synthesis is only partly (60–70%) inhibited by AOAA suggests that it occurs via both transamination and reductive amination. This observation is different from that in glutamatergic neurons (where no reductive amination occurred), probably reflecting the fact that oxidative deamination of glutamate the (GLDH catalyzed process in the opposite direction) is pronounced in astrocytes but almost absent in cerebellar granule cells (Hertz et al., 1988). Since astrocytes are capable of generating TCA cycle constituents by carboxylation of pyruvate, the low uptake rate for exogenous malate is no indication of the potential rate of glutamate synthesis from endogenously formed TCA cycle constituents. The *maximum* rate of glutamate synthesis from α-ketoglutarate in astrocytes was calculated to be ≈1.6 nmol × min^{-1} × mg^{-1}. On the assumption that one-third of the conversion from α-ketoglutarate to glutamate occurs via reductive amination, the rate of reductive amination is at most 0.5–0.6 nmol × min^{-1} × mg^{-1}. The metabolic flux in this direction thus appears to be several times lower than the rate of oxidative deamination (2–4 nmol × min^{-1} × mg^{-1}) under similar experimental conditions (Yu et al., 1982; Hertz and Schousboe, 1988). This difference between the fluxes in the two directions supports the concept that production of labelled CO_2 from glutamate in astrocytes represents a net metabolic degradation, not just a reversible exchange between glutamate and α-ketoglutarate (Yu et al., 1982; Hertz and Schousboe, 1986, 1988; Yudkoff et al., 1989; Hertz et al., 1992). The production of labelled CO_2 from [^{14}C]malate in astrocytes is in agreement with previous results by McKenna et al. (1990). Also, the rate of CO_2

production found in the present study is compatible with the value for high affinity uptake of malate that can be calculated at 10 μM from the kinetic constants reported by these authors.

In cultures of cerebral cortical neurons the glutamate formation from malate was only 1 and 3% of that in astrocytes and cerebellar granule cells, respectively (Table I). This very low rate, suggests that astrocytic contamination (≈5%) in the cultures of cerebral cortical neurons is more than high enough to quantitatively account for the observed incorporation of radioactivity into amino acids. This conclusion is further supported by the finding that no label could be found in the GABA pool of the neurons and by the rapid formation of glutamine, catalyzed by glutamine synthetase, an astrocytic enzyme (Norenberg and Martinez-Hernandez, 1979; Drejer et al., 1985; Hertz and Schousboe, 1986). Actually, the glutamine formation from glutamate may be even more pronounced than in pure cultures of astrocytes where the specific activity of glutamine did not reach that of glutamate. This difference can be explained by the enhancement of glutamine synthetase activity observed in astrocytes in contact with neurons by Linser and Moscona (1983) and Wu et al. (1988). The comparatively high uptake and oxidation rates of malate show that the lack of incorporation into amino acids is not due to a deficient uptake system in GABAergic neurons. This difference between GABAergic neurons and glutamatergic neurons with regard to utilization of malate may suggest that TCA cycle constituents are specifically involved in transmitter synthesis in glutamatergic neurons, where the quantitative demand for precursors also is much higher than in GABAergic neurons (Hertz, 1979; Hertz and Schousboe, 1986; Hertz et al., 1992). This conclusion is in agreement with the observations that labeling from malate is incorporated into the releasable pool of glutamate (Hertz and Schousboe, 1988) and that α-ketoglutarate is a precursor for transmitter glutamate in cerebellar granule cells (Peng et al., 1991) and in brain slices, but maybe not a precursor for transmitter GABA in brain slices (Kihara and Kubo, 1988). The complete inability of exogenous malate to function as a glutamate precursor in GABAergic neurons is also consistent with the observation that incorporation of ^{15}N-labelled ammonia into glutamate in these cells is not enhanced in the presence of exogenous α-ketoglutarate (Yudkoff et al., 1990), and with the concept (Hertz et al., 1992) that there must be compartmentation of TCA cycle intermediates in these cells.

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Altered C Receptor Glioma C

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Previous studies h phenotype of rat study, we compa β-adrenergic reg (P39–47) and late logical changes w derived, late pass the untreated cel broblast-like par exhibited proces ment. Untreated ulation had longe tal cells and re process outgrowt shorter generatio early passage cel agonist, isoprote in c-fos mRNA a bits RM, Yu H: Both of these in irreversibly lost l ferences in basal observed for β-A for a number of cussed in relatio ences in the abil to accumulate c Biophys Acta 67

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INTRODUCTIO

The C6 cel astrocytoma indu al., 1968). Altho as a model of th demonstrated tha