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Alterations in Uptake and Release Rates for GABA, Glutamate, and Glutamine During Biochemical Maturation of Highly Purified Cultures of Cerebral Cortical Neurons, a GABAergic Preparation

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Abstract: This study demonstrates that virtually homogenous cultures of mouse cerebral neurons, obtained from 15-day-old embryos, differentiate at least as well as cultures which in addition contain astrocytes. This was indicated by glutamate decarboxylase activity which within 2 weeks rose from a negligible value to twice the level in the adult mouse cerebral cortex, and by a γ-aminobutyric acid (GABA) uptake rate which quadrupled during the second week in culture and reached higher values than in brain slices. Within the same period, the GABA content increased four to five times to 75 nmol/mg protein, and a potassium-induced increase in [^{14}C]GABA efflux became apparent. Although the development was faster than *in vivo*, optimum differentiation required maintenance of the cultures beyond the age of 1 week. Uptake and release rates for glutamate and glutamine underwent much less developmental alteration. At no time was there any potassium-induced release of radioactivity after exposure to [^{14}C]glutamate, and the glutamate uptake was only slightly increased during the period of GABAergic development. This indicates that exogenous glutamate is *not* an important GABA precursor. Similarly, glutamine uptake was unaltered between days 7 and 14, although a *small* potassium-induced release of radioactivity after loading with glutamine suggests a partial conversion to GABA. **Key Words:** GABA—GAD—Glutamate—Glutamine—Neurons in primary cultures. Yu A. C. H. et al. Alterations in uptake and release rates for GABA, glutamate, and glutamine during biochemical maturation of highly purified cultures of cerebral cortical neurons, a GABAergic preparation. *J. Neurochem.* **42**, 951–960 (1984).

Transport and metabolism of transmitter-related amino acids (e.g., glutamate, γ-aminobutyrate, glutamine) at the cellular level of the brain have recently attracted considerable interest, and it appears that glutamate and γ-aminobutyrate (GABA) after their neuronal release are at least partly accumulated in glial cells (e.g., Hertz, 1979; Schousboe, 1981; Schousboe and Hertz, 1983). To obtain a better understanding of such a transfer of amino acids between different cell types, or at least of the possibilities for such a transfer, in the brain *in vivo*, it is essential to possess information about actual uptake and release *rates* in all cell types. Such information seems at present to be most reliably obtained by use of cells in primary cultures (Hertz, 1979; Schousboe, 1981; Schousboe and Hertz, 1983). These cultures are generally prepared from tissue obtained from immature brains (e.g., Hertz et al., 1984).

Uptake and release of GABA, glutamate, and glutamine in well-differentiated cerebral astrocytes in primary cultures (kept in culture for a minimum of 2–3 weeks) have been quite thoroughly studied (e.g., Schousboe et al., 1977*a,b*, 1979; Hertz et al., 1978*b*; Balcar et al., 1979; Drejer et al., 1982; Ramaharobandro et al., 1982). Some information is also available about developmental changes (Hertz et al., 1978*a*; Borg et al., 1980; Yu, 1980), ensur-

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Abbreviations used: GABA, γ-Aminobutyric acid; GAD, Glutamate decarboxylase; MEM, (Eagle's) minimum essential medium.

ing that the preparations used have reached a reasonable degree of functional maturation. In the case of neurons from the cerebral hemispheres the situation is not quite as fortunate. Uptake rates have been determined for all three amino acids into 5–12-day-old cultures highly enriched in cerebral cortical neurons (Balcar et al., 1979; Hertz et al., 1980; Yu and Hertz, 1982; Drejer et al., 1982; Ramaharobandro et al., 1982; Larsson et al., 1983*a*), which seem to be mainly GABAergic (Dichter, 1980; Snodgrass et al., 1980; Yu and Hertz, 1982). Such neuronal cultures are prepared from approximately 15-day-old rat or mouse embryos (Hertz et al., 1984), and the relatively late maturation of GABA content (Agrawal et al., 1966; Cutler and Dudzinski, 1974; Coyle and Enna, 1976; Sykes and Horton, 1982), GABA transport processes (Johnston and Davies, 1974; Schousboe et al., 1976; Coyle and Enna, 1976), and glutamate decarboxylase (GAD) activity (Wu et al., 1976; Coyle and Enna, 1976; Sykes and Horton, 1982) in the mouse or rat brain *in vivo* makes it doubtful that they have reached optimal functional differentiation. This concept is supported by the fact that Borg et al. (1980) have observed a steady increase in GABA uptake rate, and Hauser and Bernasconi (1980) and Snodgrass et al. (1980) a continuous rise of GAD activity in cultured neurons over the first 2–4 weeks. However, the cultures used by these investigators contained both neurons and astrocytes. This renders the cultured cells easier to maintain over a prolonged period, but they are not suitable for uptake studies of compounds that are accumulated in both neurons and astrocytes.

In the present study, we have determined uptake and release of GABA, glutamate, and glutamine in primary cultures, highly purified in cerebral cortical neurons (prepared from 15-day-old mouse fetuses) at different developmental stages between 7 and 21 days of age in culture. This degree of longevity was achieved by seeding at high density (Hertz et al., 1984). Since nonneuronal cells, i.e., mainly astrocytes, which take up and release glutamine, glutamate, and GABA (see above), are present in untreated cultures in relatively large amounts, the use of cultures as purely neuronal as possible is essential. This was accomplished by *early* (i.e., after 3 days in culture) exposure to cytosine arabinoside, a cytotoxic agent that kills dividing (i.e., nonneuronal) cells (Dichter, 1978; Hertz et al., 1984). Using this procedure, the astrocytic contamination of the cultures was very slight until the age of about 14 days, after which some astrocytic overgrowth and neuronal degeneration started to occur. Since this technique is different from that employed by Snodgrass et al. (1980) (who did not add cytosine arabinoside until the nonneuronal cell layer was close to confluency at day 6) and by Hauser and Bernasconi (1980) (who seeded their cells on an astrocytic monolayer), we decided also to reinvestigate the development of GAD activity under the present experimental conditions.

MATERIALS AND METHODS

Cultures

Primary cultures of cortical neurons were prepared from the brain hemispheres of 15-day-old Swiss mouse embryos, in principle as described by Yavin and Yavin (1974), Sensenbrenner (1977), Dichter (1978), Hertz et al. (1980), Larsson et al. (1981), and Yu and Hertz (1982), although with some modifications. Cerebral hemispheres were removed and freed of the meninges. Subsequently, the two hemispheres were split and the olfactory bulbs, basal ganglia, and hippocampal formations were removed. This left the neopallium, i.e., the portion dorsal and lateral to the lateral ventricle, which was cut into small cubes and trypsinized for 2 min using 0.2% trypsin 1:250 (Gibco) in Puck's solution at room temperature. After inhibition of the trypsin by addition of modified (Schousboe et al., 1977*b*; Hertz et al., 1982) Eagle's minimum essential medium (MEM) containing 20% horse serum, the tissue was triturated with a pipette. The resulting cell suspension was centrifuged for 2 min at 900 × *g*. The pellet was resuspended in serum-free medium with an increased glucose concentration (30 m*M* instead of 7.5 m*M*) and filtered through a Nitex® mesh (from L. and S. H. Thompson & Co. Ltd., Montreal, P. Q.) with a pore size of 80 μm. A cell suspension corresponding to 1–1½ brain per dish was seeded in 60-mm Falcon plastic tissue culture dishes, which had been coated by exposure overnight to 12.5 μg/ml of D-polylysine in water. After 15 min of incubation at 37°C, unattached cells (mostly nonneuronal cells) were removed together with the medium, which was replaced with similar, fresh, modified MEM with 5% horse serum. The cultures were incubated at 37°C in a 95/5% (vol/vol) mixture of atmospheric air and carbon dioxide (90% humidity). After 3 days of culturing the cultures were exposed to 40 μ*M* cytosine arabinoside for 24 h to curtail astrocytic growth (Dichter, 1978). Thereafter, the cultures were refed fresh modified MEM with serum, but without the cytotoxic agent, and used for biochemical studies between the ages of 7 and 21 days. Owing to the high glucose concentration in the medium, no further change of medium was necessary; this seemed to enhance the viability of the cells.

Brain tissue

For determination of GAD activity *in vivo*, corresponding tissue samples of neopallium, i.e., mainly cortex, were dissected from the brains of animals at varying ages.

Determination of GAD (EC 4.1.1.15) activity

GAD activities in cultured cortical neurons or in intact noncultured cerebral cortex were determined by measuring the $^{14}CO_2$ production from [1-^{14}C]glutamate (Roberts and Simonsen, 1963; Wood and Peesker, 1972). A 0.4-ml aliquot of 0.11 *M* potassium phosphate buffer (pH 6.5) with 0.22 *M* L-glutamate, 0.35 m*M* pyridoxal phosphate, and approximately 0.06 μCi L-[1-^{14}C]glutamate was placed in a 25-ml Erlenmeyer flask. The flask was

en sealed with a double-septum rubber stopper from hich was suspended a plastic cup containing 0.4 ml of 0 *M* hyamine hydroxide (in methanol) and a folded filter aper. After aeration of the flask with nitrogen for 2 min, 4 ml of a homogenate of either cerebral cortex or cul-ired neurons was injected. This homogenate, containing bout 4 mg protein, had been prepared in a solution con-ining 50 m*M* potassium phosphate buffer (pH 6.5), one *M* D,L-dithiotreitol, and 0.25% Triton X-100, using a yrex® Corning 7725 tissue grinder (clearance 0.15 mm). nmediately after the introduction of the homogenate, he flask was shaken at 37°C for 10 min in a Dubnoff etabolic shaking incubator (Precision Scientific, Chi-ago, IL). After exactly 10 min, 0.1 ml of 4 *M* sulfuric cid was injected to terminate the reaction and to acidify he contents of the flask, which was then shaken at 37°C or another 30 min so that all carbon dioxide that had een formed would be liberated and trapped in the hy-mine hydroxide. Then the plastic cup with its contents as counted with 10 ml scintillation mixture (0.3% PPO, .03% POPOP, and 33% Triton X-100 in toluene) in a eckman LS-9000 liquid scintillation counter. The en-yme activity was calculated from the specific activity of he glutamate and the amount of $^{14}CO_2$ liberated per min er mg protein, determined by aid of the conventional owry technique (Lowry et al., 1951).

Amino acid release

For determination of the release of GABA, glutamate, r glutamine into media with normal or elevated potas-ium concentrations, the cultures were preloaded with 50 *M* [U-^{14}C]GABA (0.2 μCi/ml), 50 μ*M* [U-^{14}C]glutamate 0.3 μCi/ml), or 500 μ*M* [U-^{14}C]glutamine (21.5 μCi/ml) Amersham Corporation, Oakville, Ont.) for 30 min. Sub-equently, the radioactivity was "washed out" by hanges of medium (containing 5 or 55 m*M* potassium) at egular intervals (6 min) for a total of 60 min. The radio-ctivity was counted in each of the "wash out" samples, s was the remaining radioactivity in the tissue at the end f the wash-out period. The radioactivities in all the sam-les were summed to indicate the total radioactivity in he tissue at the start of the wash-out, and the release luring each wash-out period was expressed as a rate coef-icient, i.e., the release during a specific period as per-entage of radioactivity remaining in the tissue at the start f that period (Shanes and Bianchi, 1959; Arnfred and Hertz, 1971; Snodgrass et al., 1980). The rate coefficient as multiplied by the total content of the amino acid (see elow) in the tissue to provide a measurement of the ac-ual release rates in each period. This calculation em-odies the requirement that the total amino acid pool is accessible to the labeled compound and that no other amino acid pools are labeled. These conditions may not e met with *completely*, but the present amino acid con-ents are roughly comparable to the previously observed amounts of ^{14}C-labeled GABA, glutamate, and glutamine after a relatively long time (30 min) of exposure to the radioisotope (Yu and Hertz, 1982).

Amino acid content

For determination of contents of amino acids in the cultures, each individual culture was washed five times with ice-cold phosphate-buffered saline and subsequently extracted with 1 ml of 70% ethanol overnight at room temperature. The cultures were then scraped off the dish with a thin piece of plastic, and the tissue suspension was centrifuged for 3 min at 11,000 × *g* in an Eppendorf centrifuge (5412). The pellet was dissolved in 1 ml 1 *M* NaOH for protein determination (Lowry et al., 1951) and the supernatant dried with a VirTis Centrifugal Bio-Dryer and then dissolved in 0.4 *M* boric acid brought to pH 10.4 with potassium hydroxide. The determination of amino acids by HPLC comprised precolumn derivatization with *o*-phthalaldehyde as described by Lindroth and Mopper (1979), reverse-phase separation with gradient elution, and fluorescence detection. The HPLC system (Waters Associates, Toronto, Ont.) consisted of one M-45 and one 6000A pump, a model 720 system controller, a U6K sample injector, a data module M730, a model M420 fluorescence detector, and a Resolve 5μBondapak 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% in a single linear step (flow rate 0.8 ml/min during 15 min), and then it was maintained at 70% for 10 min and finally reversed (over 5 min) to equilibrate the column with 25% methanol.

The derivatization reagent was prepared by mixing 20 mg *o*-phthalaldehyde (BDH Chemicals) in 0.5 ml of methanol with 0.5 ml mercaptoethanol and adding 0.4 *M* boric acid (pH 10.4) to a final volume of 10 ml. Thirty microliters of amino acid standards (15 nmol/ml of glutamate, glutamine, and GABA) in 0.4 *M* boric acid (pH 10.4), or of samples from individual cultures, adjusted to contain comparable amounts, were added to 60 μl of this derivatization reagent. After 2 min, 10 μl of the mixture (containing 50 pmol of each amino acid in the standards) was introduced into the injector, and 1 min later (i.e., 3 min after addition of *o*-phthalaldehyde solution), the sample was injected into the column. The retention times for glutamate, glutamine, and GABA were 3.4, 8.2, and 14.3 min, respectively. The peak areas of standards and unknown samples were integrated by the data module M730 with ethanolamine as an internal standard and the content in the sample calculated and expressed per mg protein.

Amino acid uptake

Uptakes of GABA, glutamate, and glutamine were determined as previously described (Yu and Hertz, 1982). After two washings with incubation medium at 37°C, intact cultures were incubated with [U-^{14}C]GABA (generally 50 μ*M*, 0.1 μCi/ml), glutamate (generally 50 μ*M*, 0.1 μCi/ml), or glutamine (500 μ*M*, 0.1 μCi/ml) for exactly 5 min, a time short enough to ensure that the uptakes occurred at close to their initial rates (Yu and Hertz, 1982). Another advantage of the short incubation period is that it minimizes the loss of accumulated amino acid as carbon dioxide, a process which may be quite pronounced in the case of glutamine (Hertz et al., 1984). Since glutamine in the normal culture medium might to a minor extent be hydrolyzed to glutamate during storage of the medium, glutamine was not added until immediately before use of the medium. After the incubation period, the cultures were rapidly washed three times with ice-cold incubation medium without any radioisotope. Then 1 ml of 1 *M* sodium hydroxide was added and radioactivity and protein were determined. Uptake rates were calculated from the radioactivity per mg protein and the specific activity in the incubation media.

RESULTS

Culture morphology

After 7 days of culturing the cells remained dispersed as individual cells or small clumps of cells, interconnected by a dense network of processes (Fig. 1a), and the astrocytic contamination was very slight. As the cultures reached the age of 17 days, neuronal cell bodies had migrated towards each other and formed large clusters of cells, interconnected by heavy bundles of processes (Fig. 1b), and the astrocytic overgrowth was somewhat more prominent (arrows). After this age the cultures started to degenerate, with a tendency for the cell bodies to remain intact longer than the interconnecting processes. This degeneration was reflected by a decrease in the amount of protein per culture, from 1.5–1.7 mg per culture (n = 17–18) at 7–17 days of age to 1.1 mg per culture (n = 10) at day 21.

GAD activity

GAD activity, a prime GABAergic characteristic, was followed during the maturation of the cultures and compared with that in the brain cortex *in vivo* at approximately the same ages. As can be seen from Fig. 2, the activity in the brains of 15-day-old embryos, i.e., the tissue used for the preparation of the cultures, was almost negligible. At birth, and in 7-day-old cultures, the activity had increased slightly. After another week the activity in the cultures had risen sharply to almost 15 nmol/min per mg protein, whereas it remained low *in vivo*. Further maturation of the cultures to 17 days of age had little effect, and after 21 days in culture the GAD activity had declined (results not presented), probably reflecting degeneration of the cultures. This is in contrast to the *in vivo* situation, where the adult level of GAD activity [7.6 ± 0.4 (n = 5) nmol/min per mg protein] was obtained more slowly and finally reached a level which is four times higher than the activity in 7-day-old animals. It was, nevertheless, only one-half of the activity in 14–17-day-old neuronal cultures [14.2 ± 2.4 (n = 10) nmol/min per mg protein].

Content of GABA, glutamate, and glutamine

Based on the developmental alterations in GAD activity and the degeneration of the cultures at about 3 weeks of age, 17-day-old cultures were selected as a functionally differentiated preparation and 7-day-old cultures as functionally immature cells. The content of GABA increased from 15–20 nmol/mg protein in 7-day-old cultures to 75 nmol/mg in 17-day-old cultures (Table 1). During the same time period, the contents of glutamate and glutamine remained constant or declined slightly.

Release of GABA, glutamate, and glutamine

Release of GABA, glutamate, and glutamine was likewise studied in 7-day-old and 17-day-old cultures, and both during exposure to a normal medium (5.4 m*M* potassium) and during exposure to an elevated potassium concentration (55 m*M*). In the case of GABA, the rate coefficient, i.e., the percentage of the total remaining radioactivity in the tissue which was released per time period (6 min), was about the same at the two ages during exposure to the normal medium (Fig. 3, A and B). Since the GABA content increased more than fourfold from 7-day-old cultures to 17-day-old cultures (Table 1), the absolute values of the release increased from about 0.2 to about 0.9 nmol/min per mg protein (Table 2). Elevation of the potassium concentration had virtually no effect on the rate coefficient of GABA release in 7-day-old cultures (Fig. 3A), whereas there was a substantial increase in 17-day-old cultures (Fig. 3B), indicating a further enhancement in the amount of GABA actually released, to about 1.9 nmol/min per mg protein (Table 2).

The rate coefficient for glutamate release differed from that for GABA release by being slightly higher at 7 days than at 17 days and by not showing a potassium–induced release at any age (Fig. 3C and D). If anything, the release of [^{14}C]glutamate at either age decreased in the presence of the elevated potassium concentration, and this decrease was followed by an increased release immediately after the return to the normal medium. Since the glutamate content decreased between day 7 and day 17 (Table 1), the actual amount of glutamate released was lower at the greater age. In the presence of the elevated potassium concentration, it was further reduced and in 17-day-old cultures it amounted to less than one-half of the GABA release (Table 2).

The rate coefficients for glutamine release were almost identical in 7-day-old and in 17-day-old cultures, and in the older cultures there was a small but reproducible increase in the presence of excess potassium (Fig. 3, E and F). This was followed by a tendency to a slight enhancement when the cultures were again exposed to the normal medium. The rate coefficients in the medium with 5 m*M* potassium varied too much with time to calculate an exact release rate. From the glutamine content (Table 1) and the rate coefficients, the absolute amounts released from both 7-day-old and 17-day-old cultures can, however, be estimated to be about 1 nmol/min per mg protein.

Uptake of GABA, glutamate, and glutamine

Uptakes of the three amino acids were studied in functionally immature neurons (7-day-old cultures) and in presumably differentiated cells of two different ages (14- and 17-day-old cultures). The concentrations of GABA and glutamate were 50 μ*M*, i.e., a concentration well above the K_m (Yu and Hertz, 1982) and also higher than the extracellular concentrations of those amino acids in the brain

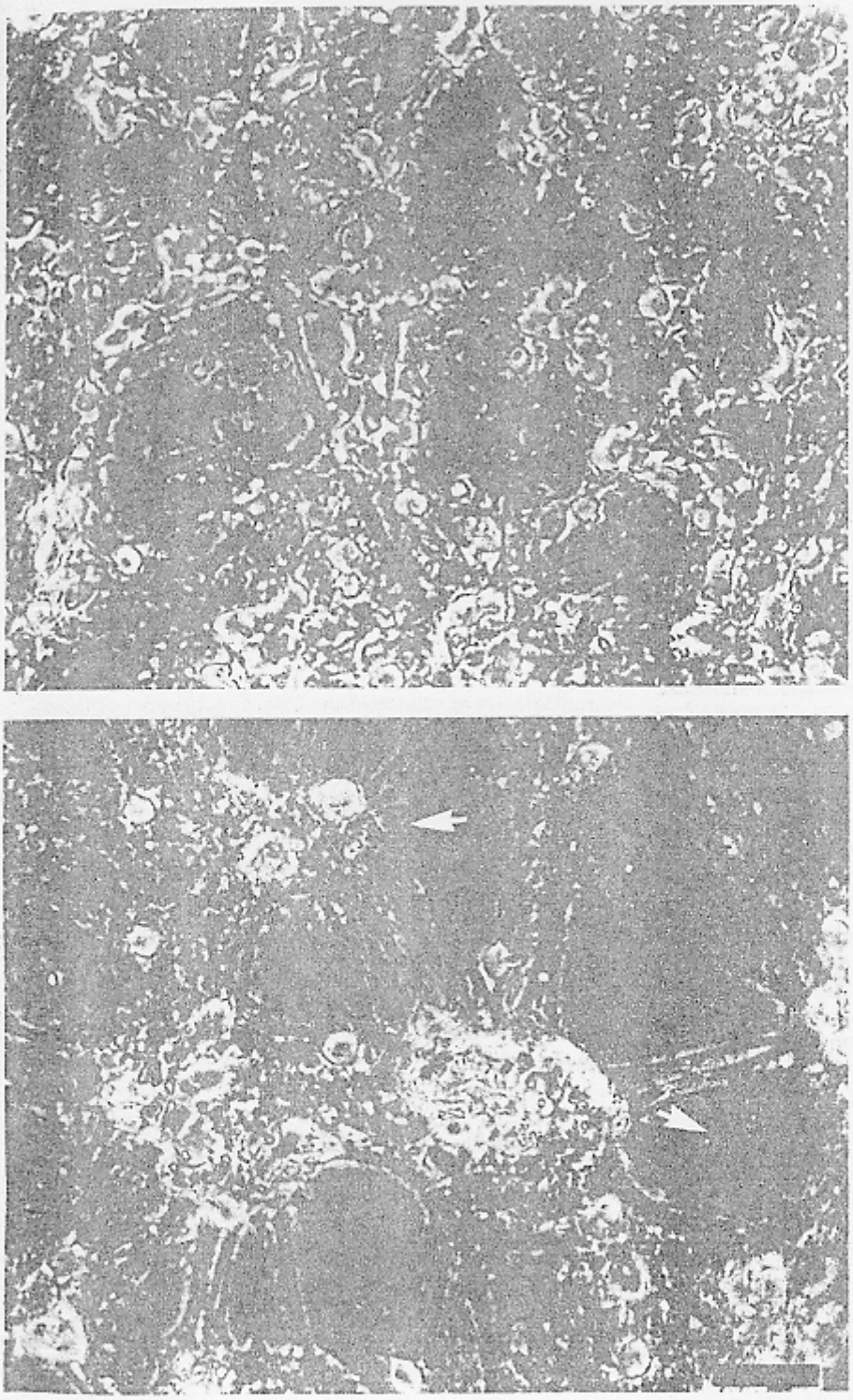

FIG. 1. Phase-contrast micrographs of 7-day-old (a, top) and 17-day-old (b, bottom) living primary culture of mouse cerebral cortical neurons. Bar equals 30 μm. Arrows indicate contaminating astrocytes in 17-day-old cultures.

(Hamberger et al., 1983). Glutamine, which is not taken up by a high-affinity uptake process, was studied at a concentration of 500 μ*M*, which is close to its concentration in cerebrospinal fluid (for references, see e.g., Hertz, 1979) and about twice the extracellular concentration in the rabbit hippocampus (Hamberger et al., 1983). Under these conditions, the uptakes of each amino acid at all ages studied fell between 2 and 10 nmol/min per mg protein (Fig. 4). The uptake of GABA increased three-

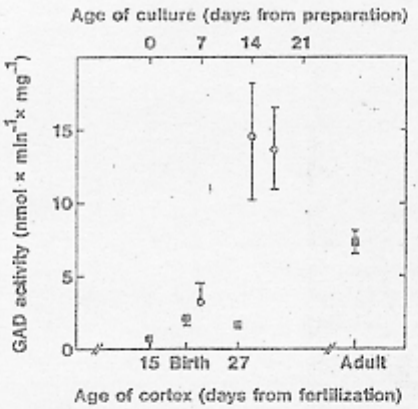

FIG. 2. Development of GAD activity in primary cultures of cerebral cortical neurons, prepared from the brains of 15-day-old mouse embryos, as a function of age in culture (O) compared with the *in vivo* activities of the enzyme (■) in the brain (neopallium) at comparable ages. Results are means of three to 10 (cultures) or two to five (brain tissue) experiments, vertical bars indicate SEM.

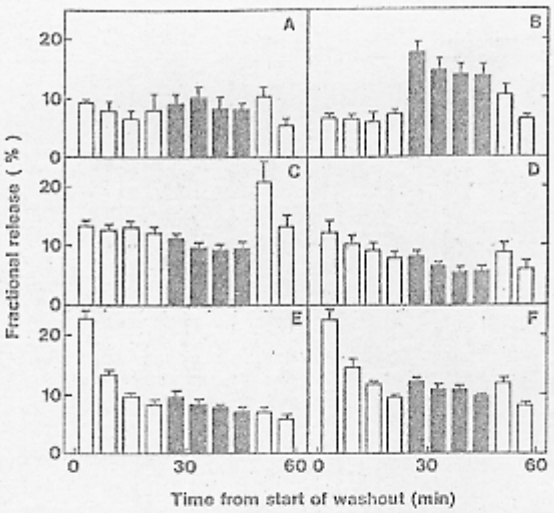

FIG. 3. Fractional release of GABA (A, B), glutamate (C, D), and glutamine (E, F) from 7-day-old (A, C, E) or 17-day-old (B, D, F) cultures of mouse cerebral cortical neurons during incubation in normal medium (open bars) or during exposure to excess (55 m*M*) potassium (filled bars). The cultures had been preloaded with radioactive GABA, glutamate, or glutamine, and the fractional release is the radioactivity released during each consecutive 6-min period as a percentage of the radioactivity remaining in the culture at the start of the period. Results are means of five or six experiments; vertical bars indicate SEM.

to fourfold at the time when the increases in GAD activity and in GABA release occurred, i.e., during the second week, and it showed no further increase at age 17 days. Glutamate and glutamine behaved oppositely; that is, the uptake rates were only slightly or not at all increased when the GABAergic development of the cultures occurred between day 7 and day 14, but they were somewhat increased between days 14 and 17.

To obtain some information about uptake rates at a lower, perhaps physiologically more relevant concentration of GABA and glutamate, a few experiments on the uptake of 5 μ*M* glutamate or GABA were performed using 17-day-old cultures. The uptakes of GABA and of glutamate were both between 0.5 and 1.0 nmol/min per mg protein (results not shown).

DISCUSSION

The approximately fourfold increases in GAD activity, GABA content, GABA uptake, and GABA release between day 7 and day 14–17 together with the development of a potassium-induced GABA release (which causes a further enhancement of the release rate) during the same time period are all indications of a maturation of GABAergic neurons. Since cultured astrocytes have practically no GAD activity, much less GABA content, and a much lower GABA uptake rate than neurons (Schousboe and Hertz, 1983), this cannot be an artifact due to the development of a small amount of astrocytes in the cultures. The increase in GAD activity, GABA content, GABA uptake, and GABA release is also in agreement with an increase in GABA transaminase activity during the same time period (Larsson et al., 1983*b*) and with electrophysiological evidence that cultured cortical neurons are mainly

TABLE 1. *Contents of GABA, glutamate, and glutamine in 7-day-old and 17-day-old primary cultures of cerebral cortical mouse neurons*

Age in culture (days)	GABA	Glutamate	Glutamine
7	16.0 ± 3.0 (3)	113.8 ± 19.8 (3)	81.5 ± 10.6 (3)
17	74.8 ± 6.5 (5)	77.6 ± 10.2 (5)	50.3 ± 9.0 (5)

Values are in nmol/mg protein ± SEM; number of experiments in parentheses.

TABLE 2. *Release of GABA and glutamate in 7-day-old and 17-day-old primary cultures of cerebral cortical neurons in the presence of normal (5 mM) or elevated (55 mM) potassium concentrations*

Age in culture (days)	GABA		Glutamate	
	5 m*M* K^+	55 m*M* K^+	5 m*M* K^+	55 m*M* K^+
7	0.22 ± 0.05	0.24 ± 0.05	2.44 ± 0.44	1.89 ± 0.34
17	0.86 ± 0.09	1.87 ± 0.20	1.27 ± 0.20	0.82 ± 0.13

These values (nmol/min per mg protein ± SEM) were calculated from the amino acid contents shown in Table 1, and the average rate coefficients during the four periods before and after the addition of excess potassium shown in Fig. 3. SEM values shown are the combined SEMs of Table 1 and Fig. 3, where the numbers of experiments are given.

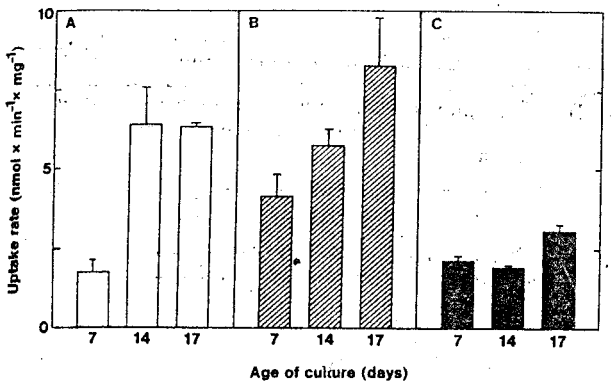

FIG. 4. Uptake rates for GABA **(A)**, glutamate **(B)**, and glutamine **(C)** into primary cultures of cerebral cortical neurons as a function of age in culture. Amino acid concentrations were 50 μ*M* **(A, B)** or 500 μ*M* **(C)**. Results are means of five to seven experiments; bars indicate SEM.

GABAergic (Dichter, 1980; Nowak et al., 1982). It shows the need to keep these cells in culture for longer than 1 week.

The maximum GAD activity (14 nmol/min per mg protein) in 14–17-day-old cultures appears slightly higher than that (10 nmol/min per mg protein) observed by Snodgrass et al. (1980) in 3–4-week-old cultures with a larger content of nonneuronal cells, and it is much higher than that (1.3 nmol/min per mg protein) reported by Hauser and Bernasconi (1980) for neurons seeded on a glioblast monolayer. In agreement with the findings of these groups of investigators, the activity increased very markedly during the second week in culture. This increase was, however, faster than that in the brain *in vivo* (Fig. 2), and it appears also to be faster than that in cultures containing more glial cells (Snodgrass et al., 1980; Hauser and Bernasconi, 1980), suggesting an enhanced rate of maturation in the absence of astrocytes. This is important in light of the inability to maintain the cultures for more than 17–21 days. The observed ontogenetic alterations in GAD activity in the mouse brain cortex *in vivo* are compatible with those described by Wu et al. (1976), Coyle and Enna (1976), and Sykes and Horton (1982), but the GAD activity in the present samples, which are enriched in cortical tissue, is two to three times that observed in whole mouse brain (Wu et al., 1976; Sykes and Horton, 1982). The further enrichment of GAD activity in 14–17-day-old cultures over that in the adult brain cortex (approximately twofold) is in reasonably good agreement with the estimates that neurons occupy about 40% of the brain cortex volume (Pope, 1978) and that a majority of cerebral cortical neurons may be GABAergic (Ribak, 1978; Dichter, 1980).

The pronounced potassium-induced GABA release in mature cultures supports the mainly GABAergic character of the cultured neurons and confirms findings by Farb et al. (1979), Hertz and Schousboe (1980), Hauser et al. (1980), Pearce et al. (1981), and Larsson et al. (1983*a*) in similar or different cultures of neurons. Since it is well known that the potassium-induced GABA release requires the presence of calcium (Farb et al., 1979; Snodgrass et al., 1980; Pearce et al., 1981; Larsson et al., 1983*a*), the calcium dependence was not studied in the present work. The absence of any potassium-induced release of GABA in 7-day-old cultures is consistent with a very slight, if any, enhancement of GABA release by excess potassium in brain slices from newborn rats compared with a pronounced effect 5 days later (Schousboe et al., 1976), and with the absence of a potassium-induced GABA release from 4-day-old neuronal cultures observed by Snodgrass et al. (1980). No effort was made to determine the exact time of onset of the potassium induced release of GABA, but Larsson et al. (1983*a*) using similar cultures, have observed that the potassium-induced release from cultures of 11–12 days of age is approximately the same as that observed in the present work with 17-day-old cultures. Glutamate differed distinctly from GABA in not being released at an enhanced rate during exposure to elevated potassium concentrations. This is not in agreement with the observations of Snodgrass et al. (1980), who reported a modest, although apparently not statistically significant, potassium-induced release of glutamate in their cultures. It is also in contrast to a potassium-induced stimulation of glutamate release from rat brain cortical slices or synaptosomes (Arnfred and Hertz, 1971; De Belleroche and Bradford, 1972). This suggests that the present culture conditions select against glutamatergic neurons, possibly because the basal ganglia containing the target cells for the cortico-striatal pathway were not included in the initial cell suspension from which the cultures were prepared, and it emphasizes, again, that the present cultures are not only highly enriched in neurons in general, but specifically in GABAergic neurons.

In spite of the enhanced GABA release in older cultures the GABA content increased markedly. The present values for GABA content (15–20 nmol/mg protein in 7-day-old cultures and 75 nmol/mg protein in 17-day-old cultures) are considerably higher than the values reported by Snodgrass et al. (1980) and by Hauser and Bernasconi (1980), probably partly reflecting dilution with astrocytes in their cultures. They are, however, in reasonably good agreement with the high GABA content (recalculated to 50–150 nmol/mg protein) demonstrated in isolated adult GABAergic neurons by Otsuka et al. (1971) and Okada (1982) and two to three times the GABA content in synaptosomes reported by Wood et al. (1979). The increase between day 7 and day 17 is compatible with the three- to sixfold increase in GABA content observed in the devel-

oping rat brain during the first postnatal weeks (Cutler and Dudzinski, 1974; Agrawal et al., 1966; Coyle and Enna, 1976). The specificity of this phenomenon to GABA is shown by the lack of corresponding increases in glutamate and glutamine content.

An increase in both GABA content and GABA release must be paralleled by an increase in synthesis and/or uptake. The observed increase in GABA uptake with age confirms observations by Borg et al. (1980) and Snodgrass et al. (1980), using less purified neuronal cultures. The uptake rate at 7 days of age is only somewhat higher than our previous observation in neurons of this age (Yu and Hertz, 1982). It is, however, three to four times higher than the uptake observed by Balcar et al. (1979) in 6-day-old rat cultures; and the uptake rate at 14–17 days of age (6–6.5 nmol/min per mg protein) is considerably higher than the value of about 1 nmol/min per mg protein observed by Larsson et al. (1981, 1983*a*) in 10–12-day-old cultures. It is also much higher than the GABA uptake rate in cerebellar neurons (Lasher, 1975) or in chick neurons (Percy et al., 1981), and far above that in neuroblastoma cells (Hutchison et al., 1974; Balcar et al., 1979). Moreover, the increase in GABA uptake rate during the second week of culturing is compatible with a corresponding increase in GABA uptake into rat brain slices or homogenates during ontogenesis (Johnston and Davies, 1974; Schousboe et al., 1976; Wong and McGeer, 1981). Assuming a protein content of 100 mg/g wet weight, it can be calculated from the data of Schousboe et al. (1976) that the GABA uptake rate into brain cortex slices is about 1.5 nmol/min per mg protein at 1 day of age postnatally (corresponding approximately to a 1-week-old culture) and 3.3 nmol/min per mg protein at 10 days of age (corresponding approximately to a 2-week-old culture). Since neurons account for 40% of the volume in brain cortex (Pope, 1978), and astrocytes (which constitute the major part of the remainder) take up GABA less intensely than neurons (Schousboe et al., 1977*a*; Yu and Hertz, 1982; Schousboe and Hertz, 1983), these results are quite compatible with the uptake rates in cultured neurons. Most of these values refer, however, to the uptake at 50 μM glutamate (Fig. 4), or they are V_{max} values. At about 5 μM GABA, which may be a physiologically more relevant concentration (Nowak et al., 1982), the uptake was less, i.e., about 1 nmol/min per mg protein. This rate of uptake is much higher than that into astrocytes (Schousboe et al., 1977*a*; Schousboe and Hertz, 1983), suggesting that most released GABA is reaccumulated into neurons. It can keep pace with the "resting" GABA release, but not with the enhanced release in the presence of an elevated potassium concentration (Table 2). *De novo* synthesis of GABA is, therefore, likely also to play a major role in the maintenance of GABA contents.

The considerable increase in GAD activity with age suggests that the *de novo* synthesis of GABA from glutamate is greater in older cultures. At least three possible sources for this glutamate exist: (1) uptake of exogenous glutamate; (2) formation of glutamate from accumulated glutamine; and (3) formation of glutamate from glucose or other compounds entering the tricarboxylic acid cycle (Shank and Campbell, 1982). The present work can probably exclude the first possibility, since glutamate uptake increased only slightly during the time period when GABA uptake and release were quadrupled, and since elevated potassium concentrations did not enhance the release of ^{14}C after labeling with [U-^{14}C]glutamate. This lack of a significant conversion of exogenous glutamate to GABA indicates also that the cells must contain more than one glutamate pool (Schousboe and Hertz, 1983; Hertz et al., 1983). Since astrocytes accumulate glutamate very avidly (McLennan, 1976; Hertz, 1979; Schousboe, 1981; Walker and Peacock, 1982; Schousboe and Hertz, 1983), it cannot, however, be excluded that part of the glutamate uptake, especially in 17-day-old cultures, which contain a larger amount of astrocytes, occurs in these cells. The second possibility, i.e., a role of glutamine as a possible GABA precursor (Reubi et al., 1978; Bradford et al., 1978; Morjaria and Voaden, 1979; McGeer and McGeer, 1979; Kemel et al., 1979) is consistent with an incorporation of radioactivity from glutamine into GABA in cultured neurons (Hertz et al., 1983) and with the present demonstration of a slight potassium-induced release of ^{14}C from glutamine (Fig. 3F). The absolute amount of this GABA formation is difficult to evaluate, but the fact that GABAergic development was not accompanied by an increased glutamine uptake suggests that relatively little glutamine is used for GABA formation. This is in keeping with the observation of substantial CO_2 production from glutamine in cerebral cortical neurons (Hertz et al., 1983). The third possibility cannot be directly evaluated from the present study, but Snodgrass et al. (1980) demonstrated GABA formation from glucose, and the possibility remains open to study in detail the metabolic fates of GABA, glutamate, glutamine, and glucose, both into metabolites stored in the cultures and into releasable compounds.

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