GLUTAMATE DECARBOXYLASE IMMUNOREACTIVITY AND γ -[^3H] AMINOBUTYRIC ACID ACCUMULATION WITHIN THE SAME NEURONS IN DISSOCIATED CELL CULTURES OF CEREBRAL CORTEX¹

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Abstract

In order to evaluate the reliability of high affinity [^3H]GABA accumulation as a marker for GABAergic neurons, murine cerebral cortical neurons were studied in dissociated cell culture. Cultures which had been incubated in [^3H]GABA were stained immunohistochemically for the GABA-synthesizing enzyme, glutamate decarboxylase, fixed with paraformaldehyde, and subsequently processed for radioautography. In mature cultures, there was an 84 to 94% correlation between the presence of the enzyme and [^3H]GABA uptake within the same cortical neurons. These data provide direct evidence that those neurons which synthesize GABA are the same neurons which are labeled by high affinity [^3H]GABA uptake.

γ -Aminobutyric acid (GABA) is generally considered to be a major inhibitory neurotransmitter in the mammalian central nervous system (Roberts et al., 1979). GABAergic neurons have been identified morphologically by the immunohistochemical localization of the endogenous specific marker, glutamate decarboxylase (GAD; EC 4.1.1.15) (Ribak, 1978; Roberts, 1979), the biosynthetic enzyme for GABA. Since high affinity GABA uptake has been demonstrated in cortical slices, homogenates, and GAD-containing particle fractions (Iversen and Neal, 1968; Neal and Iversen, 1969), radioautographic methods have been employed to visualize specific [^3H]GABA-accumulating neurons and nerve endings in cortical preparations (Bloom and Iversen, 1971; Chronwall and Wolff, 1978; Hökfelt and Ljungdahl, 1972; Iversen and Bloom, 1972).

Further, it appears likely that GABA functions as a

neurotransmitter in neuronal cell culture systems. GABA content, uptake, and release (Farb et al., 1979; Hauser et al., 1980; Neale et al., 1980; Snodgrass et al., 1980; White et al., 1980) as well as GAD activity (Hauser et al., 1980; Snodgrass et al., 1980) and immunoreactivity (Barker et al., 1981) have been demonstrated in neuronal cultures. The postsynaptic actions of GABA on cultured cortical (Dichter, 1980) and spinal cord (Barker et al., 1981) neurons have been well documented.

Although GAD immunoreactivity and high affinity [^3H]GABA uptake have been demonstrated previously in identified neuronal cell types (Brandon et al., 1979), the present study directly compares GAD localization and [^3H]GABA uptake in neurons in dissociated cell cultures of mammalian cerebral cortex. The data show that GAD-immunoreactive label and [^3H]GABA radioautographic label coexist in the same neurons indicating that those cortical neurons which synthesize GABA are the neurons which accumulate GABA by a high affinity transport mechanism.

Materials and Methods

Neuronal cell cultures were prepared by the trypsinization of fetal mouse cerebral cortex (Swaiman et al., 1982) or spinal cord (Ransom et al., 1977) as described previously. For radioautographic localization of high affinity [^3H]GABA uptake, 1- to 5-week-old cultures were washed three times in Eagle's Minimal Essential Medium (MEM; Gibco) and incubated for 10 to 30 min at 37°C in

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0.1 μ M [3 H]GABA (New England Nuclear; specific activity, 28.5 to 40 Ci/mmol) (Farb et al., 1979). Following incubation, cultures were washed rapidly three times with MEM at 25°C and fixed for 1 hr at 25°C in either 2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer, pH 7.3, or in freshly depolymerized 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. Cultures were coated with undiluted Kodak NTB2 nuclear track emulsion at 42°C, dried, stored at 4°C for 3 to 12 days, and developed in Kodak Dektol (1:1) at 16°C for 3 min.

Although glutaraldehyde is the fixative of choice for [3 H]GABA retention (Bloom and Iversen, 1971; Davis and Himwich, 1971; Peters and Ashley, 1967), as little as 0.1% glutaraldehyde abolished specific GAD immunoreactivity in these cultures. Experiments were performed to determine whether paraformaldehyde would "fix" accumulated [3 H]GABA and whether subsequent processing steps required for immunohistochemistry, particularly the rinse in Triton X-100, might result in a significant further loss of [3 H]GABA from neurons. The amount of radioactivity accumulated by cultures of spinal cord (SC) was determined after incubation in 0.1 μ M [3 H]GABA for 30 min at 37°C, followed by three or four rapid rinses in MEM. Cells were dissolved in 1.5 ml of 0.2 N NaOH, a 500- μ l aliquot of which was neutralized with 100 μ l of 1 N HCl and counted with 10 ml of Hydrofluor (National Diagnostics). Sister cultures were fixed with 2.5% glutaraldehyde or 4% paraformaldehyde, and the fixed cultures were rinsed, treated with Tris-HCl containing 0.25% Triton X-100, rinsed, and processed for radioautography. [3 H]GABA lost from cells during fixation and further processing was determined by counting a 500- μ l portion of each processing solution with 10 ml of Hydrofluor.

For combined GAD immunohistochemistry and [3 H]GABA radioautography, cultures were fixed following [3 H]GABA incubation in 4% paraformaldehyde. The cultures were washed with Tris-buffered saline and GAD immunoreactivity was localized with the unlabeled antibody enzyme method (Sternberger, 1979) using a sheep GAD antiserum S3 (second bleed) (Oertel et al., 1981). After development of the chromogen, 3,3'-diaminobenzidine tetrahydrochloride (DAB), areas of the cultures were photographed and neurons were scored for GAD immunoreactivity. Neuronal somata displaying perinuclear brown reaction product were considered GAD positive. The cultures then were coated with emulsion and were developed as described above after exposure for 3 days. The previously photographed areas were relocated and the neurons were scored a second time for high affinity [3 H]GABA uptake. Neuronal somata showing a silver grain density at least 3 times that over background neuronal densities (see "Results") were considered [3 H]GABA positive.

Results

The cerebral cortical cell cultures contained networks of neurons which grew and differentiated on top of flattened fibroblast-like cells. Cortical neurons in culture have been characterized both morphologically (Swaiman et al., 1982; Yavin and Yavin, 1974) and electrophysiologically (Dichter, 1978). [3 H]GABA accumulation was vis-

ualized by light microscopic radioautography in approximately 10 to 14% of the neurons, with silver grains overlying both somata and neurites (Fig. 1A). Labeled somata were bipolar or polymorphous, ranging in size from 10 $\times$ 12 to 15 $\times$ 20 μ m. The size, shape, and percentage of labeled neurons in these cultures were in

Figure 1. Radiolabeling and immunohistochemistry of 22-day-old dissociated cerebral cortical cell cultures. **A**, Light microscope radioautogram following incubation with 0.1 μ M [3 H]GABA for 30 min at 37°C. The culture was fixed in 2.5% glutaraldehyde, coated with NTB2, and exposed for 11 days. Phase contrast optics were used. **Scale bar**, 20 μ m. **B**, GAD immunoreactivity surrounding the nucleus of a neuron in a 22-day-old culture of cerebral cortex. Immunoreactive varicose fibers are abundant and appear to contact (at arrows) the GAD-positive neuron. The culture was fixed with 4% paraformaldehyde and was incubated in GAD antiserum S3 (1:2000) for 12 hr at 22°C, in rabbit anti-sheep immunoglobulin (1:100) for 30 min, in goat peroxidase-antiperoxidase complexes (1:100) for 30 min, and in 0.05% 3,3'-diaminobenzidine tetrahydrochloride in 0.01% hydrogen peroxide for 20 min. **Scale bar**, 20 μ m. **C**, area of a culture treated with preimmune serum S3 (1:1800). **Scale bar**, 20 μ m.

agreement with studies of [^3H]GABA accumulation by neurons of the intact postnatal rat visual cortex (Chronwall and Wolff, 1980).

The pattern of [^3H]GABA uptake was not altered significantly by preincubation with 1 mM β -alanine, a competitive blocker of non-neuronal [^3H]GABA uptake (Schon and Kelly, 1975), although labeling over the flattened background cells was somewhat reduced. Radioautography following incubation with 0.1 μM β -[^3H]alanine resulted in a uniform distribution of silver grains over neurons and most background cells. Higher grain densities were seen occasionally over groups of flattened non-neuronal cells.

GAD immunoreactivity was found in approximately 12% of neuronal somata. The reaction product was distinctly perinuclear. Some varicose fibers were also intensely immunoreactive and impinged upon the somata and neurites of both unlabeled and GAD-positive cells (Fig. 1*B*). Pretreatment of cultures for 12 hr with 10^{-8} M colchicine (Ribak et al., 1978) did not enhance substantially either the proportion of stained neurons or the intensity of somatic GAD immunostaining. No specific staining was seen in untreated or colchicine-treated cultures incubated with preimmune serum (Fig. 1*C*).

As a control for chemography, cultures stained for GAD, but not incubated with [^3H]GABA, were processed for radioautography. There was no silver grain density above emulsion background over either GAD-positive or GAD-negative neuronal somata (Fig. 2*A*). In addition, radioautograms were prepared from cultures incubated in [^3H]GABA and processed for immunohistochemistry, substituting preimmune serum for anti-GAD antiserum. In these cultures, there was no neuronal immunostaining, whereas approximately 12% of the neurons showed radioautographic labeling (Fig. 2*B*). While apparent debris and some fibroblast-like cells did demonstrate nonspecific reaction product, there were no silver grains associated with these stained elements.

In experiments in which cultures were incubated in [^3H]GABA and then processed as for immunohistochemistry, the greatest loss of accumulated [^3H]GABA occurred during fixation (25% lost in glutaraldehyde; 78% in paraformaldehyde). With either aldehyde, counts in the detergent rinse together with five subsequent Tris-buffered saline rinses over 18 hr accounted for less than 10% of accumulated [^3H]GABA. From the totals of counts in all processing solutions, it was determined that approximately 50% of accumulated [^3H]GABA was lost from cultures fixed with glutaraldehyde and approximately 93% from cultures fixed in paraformaldehyde.

In spite of the fact that substantial [^3H]GABA was lost

TABLE I
Number of [^3H]GABA-accumulating neurons as a percentage of total neurons^a

Culture	Exposure (days)	Fixative	
		Glutaraldehyde	Paraformaldehyde
SC	12	10.9	10.3
SC	13	9.9	7.8
Cortex	3	10.8	11.9

^a Both total and [^3H]GABA-labeled neurons were counted in 150 fields/culture. More than 9000 SC and 1000 cortical neurons were counted.

from cells following fixation in paraformaldehyde, radioautograms prepared from such cultures demonstrated labeling over selected neuronal somata. Radioautograms prepared from 3-week-old SC or cortical cultures incubated with [^3H]GABA, and fixed with either glutaraldehyde or paraformaldehyde and exposed for 3 days are shown in Figure 2, *C* to *F*. Differences in intensity of label were consistent with data obtained by scintillation spectroscopy. A higher grain density was seen over [^3H]GABA-accumulating neurons after glutaraldehyde fixation, and silver grains were disposed over both neuronal somata and processes.

In paraformaldehyde-fixed cultures, the grain density, although reduced relative to glutaraldehyde, was sufficient to label a discrete population of neuronal somata. Radioautographic labeling of neuronal processes was not observed after this brief exposure interval. Counts of silver grains per soma showed a "background" neuronal grain density of 0 to 5 grains/soma. However, some neuronal somata showed grain densities 3 and most frequently greater than 6 times higher than that over neighboring neurons. "Specific" labeling thus was taken as a grain density at least 3 times higher than that over the majority of neuronal somata in the same area of the culture. In experiments from three dissections, [^3H]GABA-accumulating neurons constituted roughly equivalent proportions of the total neuronal population, regardless of the fixative used (Table I).

Examples of neurons in cultures incubated in [^3H]GABA, fixed, stained for GAD, and processed radioautographically, are represented in Figure 3. Of the 44 neuronal somata shown in two fields, 11 show GAD immunoreactivity (Fig. 3, *A* and *C*) and 10 of these same neurons are labeled with silver grains (Fig. 3, *B* and *D*) as evidence of [^3H]GABA accumulation.

Cell counts from these experiments are presented in Table II. Of the 4923 neurons examined in six cultures from two separate experiments, 593 neurons were labeled

Figure 2. Radioautograms prepared from neuronal cultures. Photomicrographs *A*₁ to *D*₁ are focused on neuronal somata and *A*₂ to *D*₂ are focused on the layer of emulsion overlying the same neurons. All arrows indicate neurons specifically labeled by [^3H]GABA accumulation. *A*, Cerebral cortical cell cultures stained for GAD, without preincubation in [^3H]GABA, and coated with emulsion. No silver grains are seen over the two GAD-positive neurons indicating that there is no chemographic interaction between the horseradish peroxidase/DAB reaction product and the emulsion. *B*, Cultures incubated for [^3H]GABA uptake and processed for immunohistochemistry with preimmune serum substituted for anti-GAD antiserum. *C* and *D*, Cerebral cortical (*C*) and spinal cord (*D*) cell cultures incubated with [^3H]GABA and fixed with 4% paraformaldehyde. Although silver grain density is lower than that observed after glutaraldehyde fixation (see below), specifically labeled neurons can be demonstrated. *E* and *F*, Cortical (*E*) and SC (*F*) cultures incubated with [^3H]GABA and fixed with 2.5% glutaraldehyde. Radioautographic exposure for all cultures was 3 days. Magnification $\times 500$. Bar, 25 μm .

Figure 3. Combined GAD immunohistochemistry (A and C) and high affinity [^3H]GABA uptake radioautography (B and D) shown on two fields from a 22-day-old dissociated cerebral cortical cell culture fixed with paraformaldehyde. Neurons in which GAD is localized also show accumulation of [^3H]GABA, with the exception of the neuron marked with an *asterisk* (in B), which appeared GAD positive and [^3H]GABA negative. Radioautographic silver grains (3 days exposure) are photographed at a focal plane above the neuronal somata. Arrows in A mark nonspecifically stained debris, seen also in control cultures treated with preimmune serum (see Fig. 1C). Scale bar, 20 μm .

TABLE II
Counts of neurons in dissociated cerebral cortical cell cultures^a

	Experiment			
	A		B	
Days in culture	14	22	28	36
Labeled neurons ^b	98	108	162	163
GAD-[^3H]GABA neurons ^c	75	87	152	152
Total neurons counted ^d	838	1140	1328	1316
% GAD-[^3H]GABA neurons	77	81	94	93
% Labeled neurons	88	84		

^a Counts were made of neurons in dissociated cerebral cortical cell cultures ($N = 6$) that were exposed to [^3H]GABA, fixed in paraformaldehyde, stained for GAD immunoreactivity, and processed for radioautography.

^b Labeled neurons, those neurons labeled by immunohistochemistry and/or radioautography.

^c GAD-[^3H]GABA neurons, those neurons showing the coincidence of both labels.

^d Total neurons counted, labeled plus nonlabeled neurons.

by either GAD immunohistochemistry or [^3H]GABA radioautography or both. The coincidence of GAD and [^3H]GABA labels was seen in 519 neurons. Thus, GAD immunoreactivity and [^3H]GABA accumulation were

correlated in 88% of those neurons classified as GABAergic by one or the other of these morphologic criteria.

In optimal preparations (experiment A; 22 days in culture) in which somatic GAD staining was distinct, 310 neurons were [^3H]GABA positive and 304 (98%) of these showed GAD immunoreactivity. GAD-positive neurons numbered 319, and 304 (95%) of these accumulated [^3H]GABA. These results indicate that [^3H]GABA-accumulating neurons in dissociated cell cultures of mammalian cerebral cortex are those neurons that synthesize GABA.

Discussion

Radioautography of cultures after high affinity [^3H]GABA uptake (Farb et al., 1979), particularly in conjunction with the appropriate pharmacological manipulations (White et al., 1980), has provided evidence that [^3H]GABA uptake labels a selected population of neurons. This study provides direct evidence that the GABA-accumulating neurons in cortical cell cultures are those same neurons which synthesize GABA.

The finding that paraformaldehyde does not fix GABA as efficiently as glutaraldehyde was not unexpected (Cuénod and Streit, 1979; Peters and Ashley, 1967; Price et al., 1978; Streit et al., 1978). However, since paraform-

aldehyde fixation of neuronal cell cultures resulted in the retention of sufficient [³H]GABA within neurons to allow the identification of a [³H]GABA-accumulating subpopulation, this fixative was used for combined immunohistochemistry and radioautography. Radioautographic exposure for 3 days yielded low grain densities which did not obscure the intracellular GAD reaction product. Silver grain densities after high affinity [³H]GABA uptake were higher over somata, whereas GAD staining was more intense in neurite varicosities. The short exposure times used in these experiments biased against the simultaneous detection of the two labels within neurites. Data indicating the apparent existence of high affinity GABA transport sites in the membrane of the neuronal soma as well as in neurites (Bordon et al., 1981) are consistent with patterns of somatic labeling seen in this and other studies (Farb et al., 1979; Hauser et al., 1980; White et al., 1980).

The coincidence of GAD and [³H]GABA labels was lowest (79%) in the 14-day-old cultures from experiment A. In young cultures from experiment B, neurons were well labeled by [³H]GABA uptake in cultures examined 8 and 14 days after plating, although GAD immunoreactivity could not be detected on day 8 and the GAD-containing neurons were poorly stained on day 14 (data not presented). After 28 and 36 days, neurons in cultures from this same experiment showed distinct GAD staining. These results appear to reflect the early development of a high affinity [³H]GABA transport system and a later development of GAD activity in cerebral cortical neurons both *in vivo* (Coyle and Enna, 1976) and in culture (Hauser et al., 1980; Snodgrass et al., 1980).

The results of this study indicate that [³H]GABA-accumulating neurons in dissociated cell cultures of mammalian cerebral cortex are the same neurons which synthesize GABA. The radioautography of [³H]GABA-accumulating neurons is particularly useful in cell culture systems where specific labeling can be correlated with electrophysiologic observations and intracellular tracing techniques. Radioautographic labeling of [³H]GABA uptake neurons with paraformaldehyde fixation, while less intense than with glutaraldehyde fixation, may be used in conjunction with immunohistochemistry to define the coexistence of GABA with neuropeptides, structural proteins, or enzymes in individual neurons.

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