EMBRYONIC CEREBELLAR NEURONS ACCUMULATE [³H]- γ -AMINOBUTYRIC ACID: VISUALIZATION OF DEVELOPING γ -AMINOBUTYRIC ACID-UTILIZING NEURONS *IN VITRO* AND *IN VIVO*¹

MARY E. HATTEN,^{*,2} ANN M. FRANCOIS,* EUGENE NAPOLITANO,* AND SUZANNE ROFFLER-TARLOV[‡]

^{*} Department of Pharmacology, New York University School of Medicine, New York, New York 10016 and [‡] Departments of Anatomy and Neurology, Tufts University School of Medicine, Boston, Massachusetts 02111

Received August 19, 1983; Revised December 7, 1983; Accepted December 13, 1983

Abstract

γ -Aminobutyric acid (GABA) is the proposed neurotransmitter for four types of cerebellar neurons—Purkinje, Golgi, basket, and stellate neurons. With this investigation we have begun studies to establish when these neurons acquire their neurotransmitter “identification.” Autoradiographic studies of both cultured embryonic (embryonic day 13) cerebellar cells and of intact embryonic cerebellum (embryonic day 13) were conducted with tritiated GABA.

Two to 5% of the embryonic cerebellar cells accumulated [³H]GABA *in vitro*. By morphological and immunocytochemical criteria, labeled cells were large neurons with either a thick, apical process, a multipolar shape, or were bipolar with longer processes. The identification of cells which accumulated [³H]GABA as neuronal precursors was supported by the differential sensitivity to drugs that preferentially inhibit accumulation of [³H]GABA by neurons and glia.

The results of the *in vitro* experiments were confirmed and extended with *in vivo* experiments. When intact cerebellar tissue was removed at embryonic day 13, stripped of meninges and choroid plexus, exposed to low concentrations of [³H]GABA, and processed for light microscopic autoradiography, heavily labeled cells were seen in the middle of the cerebellar anlage. Labeled cells were not seen in the ventricular zone of proliferating neuroblasts lining the fourth ventricle or in the external granular layer emerging at the lateral aspect of the pial surface. The accumulation of [³H]GABA by these cells also showed the pharmacological characteristics of uptake by neurons.

This study shows that among migrating, immature forms of the larger neurons of the embryonic cerebellum, there is a select group which accumulates [³H]GABA and other classes of cells which do not. These results indicate very early acquisition of transmitter expression by cerebellar neurons, far in advance of their final positioning and establishment of synapses.

During mammalian brain development, in many brain regions, neuronal precursors proliferate in ventricular zones, migrate through the developing cortical wall, and subsequently form synaptic connections which utilize specific neurotransmitter systems. Although the mechanisms underlying cell migration in the CNS are under

study in a number of laboratories (Rakic, 1971; Rakic and Sidman, 1973; Sidman and Rakic, 1975; Trenkner and Sidman, 1978; Hatten and Liem, 1981), little is known about whether neurotransmitter systems, which are presumably required for synaptic activity to commence, develop before, during, or following neuronal migration.

In mouse cerebellum, the thirteenth day of gestation (E13) is an opportune developmental stage to address this issue. At E13, this tissue consists of proliferating young neurons in the ventricular and subventricular zones, postmitotic neurons which are migrating outward to form the intermediate zone, and immature glia (Boulder Committee, 1970; Ramon y Cajal, 1960; Miale and Sidman, 1961; del Cerro and Swartz, 1976; Levitt et al., 1981). The precursors of the Purkinje cell, having under-

¹ We thank Drs. P. Bernt, J. Blanks, L. A. Greene, C. A. Mason, and M. Shaw for their advice and criticism. Susan B. Hutchison, Lois Bricken, and Mary Repo participated in some of the experiments presented. Supported by National Institutes of Health Grants NS 19340 (S. R. T.) and NS 15429 (M. E. H.). M. E. H. is a recipient of the Irma T. Hirsch Career Scientist Award and is a Sloan Research Foundation Fellow.

² To whom correspondence should be addressed.

gone their final mitosis on embryonic days 11 to 13 (Miale and Sidman, 1961; Altman and Bayer, 1978), have started their migrations into the cerebellar anlage (Sidman and Rakic, 1975). The precursors of Golgi neurons are generated between E12 and E15 (Miale and Sidman, 1961; Altman and Bayer, 1978), and, although some of these cells are still proliferating at the ventricular surface on E13, many are postmitotic and have started to migrate through the thickening cortical wall.

The precursors of the other major neuronal cell type, the granule neuron, are proliferating and beginning to spread across the surface of the cerebellar anlage, forming the external granular layer (EGL) (Miale and Sidman, 1961; Altman, 1972). Precursors of the basket and stellate neurons are also proliferating in the EGL and are not yet distinguishable from precursors of granule neurons (Altman, 1975). At this stage, none of the major types of precursors of cerebellar neurons have either arrived at their proper cortical address or formed synaptic connections (Larramendi, 1969; West and del Cerro, 1974; Sidman and Rakic, 1975).

Four types of neurons are thought to utilize γ -aminobutyric acid (GABA) in the adult cerebellum. They have been identified by several methods, including the autoradiographic localization of [3 H]GABA captured by high affinity uptake systems. The transmitter role for GABA is more firmly established for the Purkinje cell than for any other mammalian neuron. The evidence comes from several sources: endogenous GABA and its synthetic enzyme glutamic acid decarboxylase (GAD) are located in Purkinje nerve terminals as shown by degeneration studies (Fonnum et al., 1970; Storm-Mathisen, 1972; Roffler-Tarlov et al., 1979). Purkinje cell soma, dendrites, and terminals contain GAD as shown by the immunocytochemical localization of this enzyme (McLaughlin et al., 1975; Ribac et al., 1978). In addition, GABA iontophoretically applied to Purkinje cell targets elicits an inhibitory response similar to that elicited by Purkinje cell stimulation (Obata et al., 1970).

Each of the other classes of neurons present in cerebellar cortex except the excitatory granule cell, is also a suspected GABA neuron. The evidence in the case of stellate, basket, and Golgi cells is based upon autoradiographic localization of [3 H]GABA and the immunohistochemical localization of GAD (Hokfelt and Ljungdahl, 1970, 1972; Saito et al., 1974; Kelly et al., 1975; McLaughlin et al., 1975; Ribac et al., 1978).

Cells dissociated from early postnatal mouse cerebellum and maintained in tissue culture (Lasher, 1974; Messer, 1977; Burry and Lasher, 1978a, b; Burry, 1982) also accumulate exogenously presented GABA, suggesting that [3 H]GABA uptake can be used as a marker for GABA-utilizing neurons *in vitro*. The present study uses [3 H]GABA uptake to visualize precursors of GABA-utilizing neurons in embryonic mouse cerebellum, both in dissociated cell cultures and in tissue sections taken at the thirteenth embryonic day.

Materials and Methods

Cerebellar cultures

All studies were carried out with C57BL/6J mice derived from a timed-pregnancy breeding colony at New

York University. Females were checked daily for the presence of vaginal plugs, the day of impregnation being designated E0. Whole cerebellum was removed from embryos at E13 as described (Hatten and Sidman, 1978).

Single cell suspensions of cerebellar tissue were prepared as described previously (Hatten and Sidman, 1978), and the cells were plated in glass coverslip microcultures (Hatten and Sidman, 1978) for autoradiography and immunochemical procedures or in microwells (Hatten and Sidman, 1978) for quantitative measurements of [3 H]GABA uptake. In all experiments, the culture surface was pretreated with poly-D-lysine (25 μ g/ml) (Hatten and Sidman, 1978), and the final plating density was 1.0 to 2.0×10^6 cells/ml. Throughout the time period studied, plating efficiency was 90 to 95% (Hatten, 1981). Cells were maintained at 35.5°C with 5% CO₂ and 100% humidity.

[3 H]GABA accumulation

Autoradiography of cerebellar cultures. Cultures were washed three times with unsupplemented Eagle's basal medium (BME, Gibco) (30 sec/wash, 37°C) and incubated with [3 H]GABA (5.7×10^{-7} M in BME, specific activity 34.7 Ci/mmol, New England Nuclear) for 30 min at 37°C. After three washes (BME, 30 sec/wash, 37°C), the cultures were fixed with 4% paraformaldehyde (Fisher Scientific) and 0.1% glutaraldehyde (Fisher Scientific) for 30 min at room temperature and washed three times with calcium and magnesium-free phosphate-buffered saline (CMF-PBS) (5 min/wash, 20°C). In experiments where the cells were subsequently labeled with antisera, the cultures were fixed with paraformaldehyde alone.

For autoradiography, the coverslips were removed, dehydrated through graded alcohols and xylene to remove the paraffin/Vaseline mixture which affixed them to the bottom of the culture dish, and mounted on glass slides. After air drying, the slides were rinsed with water, dipped in fresh Ilford L-4 emulsion, exposed for 2 to 5 days, and developed with D-19 developer (Kodak). Slides that were double labeled with antisera were exposed for 10 to 14 days.

To assess the cellular specificity of [3 H]GABA accumulation, cultures were pre-incubated with either β -alanine (1.0 mM in BME) or diaminobutyric acid (DABA) (1.0 mM in BME) for 15 min at 37°C prior to exposure to and during incubation with [3 H]GABA as described. Autoradiographs of more than 200 cultures were analyzed, prepared from 12 litters of embryos.

Quantitative [3 H]GABA uptake studies in vitro. Cerebellar cells (1 to 2×10^6 cells/ml) were plated in microwell cultures (10 μ l each) and maintained for 24 hr *in vitro*. Each of the microwells was rinsed three times with unsupplemented BME and incubated with [3 H]GABA (5.7×10^{-7} M) for 30 min at 37°C. To assess the cellular specificity of [3 H]GABA uptake, 24 microwells were pre-incubated with either DABA (100 μ M or 1.0 mM, 12 microwells each) or β -alanine (100 μ M or 1.0 mM, 12 microwells each) for 15 min prior to exposure to and during incubation with [3 H]GABA. Immediately after exposure to [3 H]GABA, each microwell was washed five times with unsupplemented BME and the radioactivity

was extracted from the cells by homogenization in a total of 300 μ l of 5% perchloric acid. After centrifugation at $24,000 \times g$ for 30 min, the supernatant was removed from each sample, divided into two aliquots, added to scintillation fluid, and counted with a Beckman scintillation counter. Coverslip microcultures of aliquots (30 μ l) of these cell suspensions were processed for autoradiography as described above.

Autoradiography of cerebellar tissue. Whole cerebellum was removed with a portion of the brainstem left in place to facilitate orientation. The tissue was rinsed with BME, exposed to [3 H]GABA (5.7×10^{-7} or 5.7×10^{-8} M, specific activity 34.7 Ci/mmol, New England Nuclear) at room temperature for 30 min, washed with BME, fixed in glutaraldehyde (3%), postfixed with OsO_4 (1%), and rinsed with distilled water. After dehydration in a series of alcohols, the tissue was infiltrated with Epon (Fullam) overnight. Embedding was followed with fresh Epon polymerized at 60°C for 3 days, after which the block was sectioned at 1 μ m with a JEOL ultramicrotome, and the sections were mounted on gelatin-coated glass slides (Thomas Scientific). For autoradiography, the slides were rinsed in alcoholic NaOH, washed with distilled water, and dipped in fresh Ilford L-4 emulsion. After exposure for approximately 2 months, the slides were developed with D-19 developer (Kodak).

To evaluate the cellular specificity of [3 H]GABA uptake, whole cerebella taken from mice from the same litter were pre-incubated with either DABA (1.0 mM, Sigma) or β -alanine (1.0 mM, Sigma) for 15 min prior to exposure to and during the incubation with [3 H]GABA and processing for autoradiography. Other embryos in the litter were processed for quantitative measurement of [3 H]GABA accumulation as described below. Six litters of embryos were studied.

Quantitative [3 H]GABA uptake *in vivo*. Whole cerebellum was removed by dissection, the meninges were carefully peeled off, and the tissue was washed three times with BME. [3 H]GABA was added to a final concentration of either 5.7×10^{-7} or 5.7×10^{-8} M (specific activity 34.7 Ci/mmol), and the tissue was incubated at room temperature for 30 min. After rinsing with BME, the tissue was solubilized in NaOH (1 N), transferred to scintillation fluid (Aquasol, New England Nuclear), and counted with a Beckmann counter. In duplicate experiments, either DABA (1.0 mM) or β -alanine (1.0 mM) was included in the incubation medium with [3 H]GABA after pre-incubation with the indicated drug.

Immunocytochemical identification of cell types in cerebellar cultures

Antibody. Antisera against purified glial filament protein (AbGF, 51,000 daltons), an antiserum which labels astrocytes (Hatten and Liem, 1981; Liem, 1982), were the generous gift of Dr. Ronald K. H. Liem. In tissue culture, astroglia dissociated from E13 cerebellar tissue stain with this antisera only after 3 to 4 days *in vitro* (Hatten, 1984).

Immunocytochemical procedures. The procedure followed was a slight adaptation of a method described previously (Hatten et al., 1984). In brief, after 2 to 4 days *in vitro*, cultures were exposed to [3 H]GABA as de-

scribed, fixed with paraformaldehyde (4%, 30 min, 20°C), rinsed three times with CMF-PBS, and stained with AbGF with the peroxidase-antiperoxidase method (Sternberger, 1979). In control experiments (data not shown) cultures were labeled with AbGF preabsorbed with purified glial filament protein or with preimmune serum, and autoradiography was carried out as described.

Results

The cells in the autoradiograms of cultures prepared from E13 mouse cerebellum ranged in size from 5 to 12 μ m, the vast majority being small (5 to 8 μ m) cells which were phase-bright, a characteristic feature of neurons *in vitro*. The other cells, approximately 5 to 10% of the total number of cells present, were not phase-bright, had thicker processes, and were probably glial in origin. More than 90% of the neurons present in the cultures resembled precursors of the granule neuron, having small (5 to 8 μ m) perikarya and short, fine processes (Hatten and Sidman, 1978). Twelve to 24 hr after plating, 2 to 5% of the cells in the autoradiograms were densely labeled after a short incubation with [3 H]GABA. By phase-contrast microscopy, heavily labeled cells had the characteristic phase-bright appearance of neurons (not shown). Nearly all of the cells which accumulated [3 H]GABA were larger than 8 μ m in diameter (Fig. 1, Table I). Some of these had a single, thick apical process with either branched, short arms (Fig. 1 *a* and *b*) or unbranched, longer arms (Fig. 1*c*). Other heavily labeled cells were multipolar (Fig. 1*d*). In all cases, both the cell soma and processes of labeled cells accumulated [3 H]GABA. Very few, if any, phase-bright cells larger than 8 μ m were unlabeled.

The vast majority of phase-bright, presumed neurons which did not accumulate [3 H]GABA were small cells (5 to 8 μ m) with short, fine processes which resembled immature granule cells. No heavily labeled small neurons were seen. Cells which resembled cerebellar astroglia and other non-neuronal cells in the culture were not densely labeled. In areas where the cells formed small cellular reagggregates, a higher background was seen, possibly caused by thickening of the emulsion for autoradiography.

Some of the cells in autoradiograms of the cultures were lightly labeled. Most of these were not phase-bright and were probably glial cells. In some instances, a rim of silver grains was observed around unlabeled cell bodies, possibly representing terminations of the processes of GABA-utilizing cells in the culture.

To confirm the characterization of cell types made by phase-contrast light microscopy, we combined [3 H]GABA autoradiography with immunocytochemical localization of antisera raised against the glial filament protein, a marker of astrocytes, and with antisera raised against two neuronal markers: the NILE glycoprotein and tetanus toxin (Fields et al., 1978; Hatten and Liem, 1981; Salton et al., 1983; Hatten et al., 1984). Cells which were heavily labeled with [3 H]GABA were also labeled with AbNILE or tetanus toxin, but they were not stained with AbGF (data not shown). This was easiest to visualize when the cultures were labeled with [3 H]GABA and AbGF. The AbGF staining was carried out after 4 days *in vitro*, because this was the earliest time point when

Figure 1. Accumulation of [^3H]GABA by embryonic cerebellar cells *in vitro*. Cells were dissociated from mouse cerebellum at E13 and maintained in microculture. After 22 hr *in vitro*, cultures were washed, exposed to [^3H]GABA (5×10^{-8} M), fixed, and processed for autoradiography as described in the text. Four fields (a to d) are shown. Two to 5% of the cells were labeled with [^3H]GABA. Densely labeled cells, indicated by black arrows, were large with a single, thick apical process (b), or were large with unbranched long processes (a and c), or were large and multipolar (d). Small cells ($< 8 \mu\text{m}$) never accumulated label and resembled granule neurons, by far the most numerous cell type present in the cultures. The curved arrows indicate an unlabeled cell; the black arrows indicate cells which accumulated [^3H]GABA. Phase-contrast microscopy. Magnification $\times 325$.

TABLE I
 Characteristics of E13 cerebellar cells which accumulate [^3H]GABA

Cells were dissociated from mouse cerebellum at E13, maintained *in vitro* for 12 hr, exposed to [^3H]GABA (5×10^{-8} M) in the presence or absence of specific blockers (1.0 mM), and processed for autoradiography. The total number of cells which accumulated [^3H]GABA and the number of those which had an apical or stellate shape were counted by phase-contrast light microscopy. Percentages given are the percentage of the total cell population. Very few, if any, large labelled cells or small unlabeled cells were seen among the 2507 cells counted.

	Total No. of Cells Examined	No. of Large ($>6 \mu\text{m}$) Cells Labeled with [^3H]GABA		
		Total (%)	Multipolar (%)	Apical (%)
[^3H]GABA	2507	142 (5.7)	31 (1.2)	111 (4.4)
[^3H]GABA + β -alanine (1.0 mM)	3808	167 (4.4)	52 (1.4)	115 (3.0)
[^3H]GABA + DABA (1.0 mM)	3940	0 (0)	0 (0)	0 (0)

AbGF stained embryonic astroglia (Fig. 2) (Hatten, 1984).

AbNILE-positive cells which accumulated [^3H]GABA included several cell types. The majority had cell somata which were larger than $8 \mu\text{m}$ in diameter and a large, thick apical process or a multipolar shape (Fig. 2). Small (5 to $8 \mu\text{m}$) AbNILE-positive cells in the cultures did not accumulate [^3H]GABA.

Three forms of immature astroglia were identified by staining with AbGF: cells with one or two thick, short processes; cells with a stellate shape; and cells with processes as long as $500 \mu\text{m}$. None of these types of immature astroglia accumulated [^3H]GABA. The details of the forms of immature glia in cultures of E13 cerebellar tissue are presented elsewhere (Hatten, 1984).

The identity of cells which accumulated [^3H]GABA was also evaluated by the differential sensitivity to two blockers of [^3H]GABA uptake. Total [^3H]GABA uptake in cerebellar microcultures was reduced 93% in the presence of 1 mM DABA and 66% in the presence of 100 μM DABA (Table II), a blocker of neuronal uptake in mature systems (Iversen and Kelly, 1975). When 20 autoradiograms of cultures (more than 6×10^6 cells) treated with both [^3H]GABA and DABA (1 mM) were examined, no densely labeled cells were seen (Table I, Fig. 3b). Total uptake of [^3H]GABA was less affected in the presence of β -alanine, a blocker of glial uptake (Schon and Kelly, 1975) (Table II). Accumulation of [^3H]GABA was reduced 35% by 1 mM β -alanine and was virtually unchanged in the presence of 100 μM β -alanine. The level of background was reduced in autoradiograms of cultures treated with 1 mM β -alanine, but the accumulation of [^3H]GABA by large, phase-bright cells appeared to be unaffected (Fig. 3a).

To confirm and extend the *in vitro* studies, we analyzed [^3H]GABA accumulation by cellular tissue taken at E13. When the meninges and choroid plexus were left in place, [^3H]GABA uptake was confined to the pial membrane and to the choroid plexus. When these were carefully stripped away prior to exposure to [^3H]GABA, accumulation of [^3H]GABA was seen in some cells in the middle of the developing cerebellar anlage (Fig. 4). Heavily labeled cells were not seen in the ventricular layer of

proliferating cells along the fourth ventricle, except occasionally in the most medial aspect or in the external granular layer (EGL), a layer of proliferating cells along the outer surface (Fig. 4). In the presence of DABA (1.0 mM), although some labeling was evident, no heavily labeled cells were seen (Fig. 5). In contrast, β -alanine (1.0 mM) had little effect on the pattern of [^3H]GABA labeling (not shown).

Total uptake of [^3H]GABA in whole E13 cerebellar tissue taken from littermates used to prepare the tissue sections for autoradiograms was blocked 82% by DABA (1.0 mM) and 22% by β -alanine (1.0 mM), suggesting that *in vivo* uptake of [^3H]GABA was primarily by neuronal precursors.

Discussion

At E13, three types of postmitotic, immature neurons which originate of common parentage in the ventricular zone are present in the mouse cerebellar anlage (Miale and Sidman, 1961; Altman and Bayer, 1978). These are the immature forms of two populations of inhibitory GABA-utilizing neurons—Purkinje cells and Golgi neurons and the immature excitatory deep cerebellar neurons. Our results show that as early as embryonic day 13, a select group of these immature cerebellar neurons has begun to express the specific uptake of the neurotransmitter GABA. The neuronal character of the cells which accumulated the exogenous GABA was evident by morphological and immunocytochemical criteria, and the identification was supported by pharmacological evidence which showed that the total accumulation of [^3H]GABA was nearly totally suppressed by DABA, which competes with GABA at uptake sites in mature neurons (Iversen and Kelly, 1975), whereas β -alanine, an inhibitor of GABA uptake in some glial cells (Schon and Kelly, 1975), was much less effective an antagonist of GABA uptake in these embryonic cerebellar cells. The differential sensitivity of [^3H]GABA uptake to these two drugs was evident in autoradiograms.

The most convincing evidence for neuronal uptake of [^3H]GABA in cultures of embryonic cerebellar cells was provided by a combination of [^3H]GABA autoradiogra-

Figure 2. Combination of [^3H]GABA uptake and immunocytochemical staining AbGF of microcultures of embryonic mouse cerebellar cells dissociated at E13 and maintained *in vitro* for 4 days. Cultures were rinsed three times, exposed to [^3H]GABA, fixed with paraformaldehyde, stained with AbGF, and processed for autoradiography. AbGF was visualized with the immunoperoxidase method. AbGF-positive cells are brownish-red (*curved arrow*). These immature astroglia are not heavily labeled with [^3H]GABA and have a larger cell soma, thicker and longer processes than cells labeled with silver grains. Labeled neurons (*straight arrow*) are larger than unlabeled, AbGF-negative cells and have either short, apical processes (*top*), a multipolar shape, or a bipolar shape with a longer process (*bottom*). At this developmental stage, most neurons do not associate with embryonic astroglia (Hatten and Liem, 1981; Hatten, 1984). Brightfield microscopy. Magnification $\times 470$.

phy and the immunocytochemical localization of specific markers for neurons and glia. Cells which accumulated [^3H]GABA were stained with antisera raised against the NILE glycoprotein or with tetanus toxin, both neuronal markers, but were not stained with antisera raised against glial filament protein, suggesting that they are

immature neurons. Immature astrocytes, identified by staining with AbGF, did not accumulate [^3H]GABA.

Analysis of autoradiograms stained for specific cell markers showed that [^3H]GABA uptake *in vitro* was restricted to the larger neuronal precursors. Among these, many had a single, thick apical process and resembled immature Purkinje neurons (Ramon y Cajal, 1960). Cells with the morphological characteristics of immature Purkinje cells have previously been seen with electron microscopy in cultures of E13 cerebellum (Hatten and Sidman, 1978).

The other likely candidates for the neurons labeled with [^3H]GABA are the Golgi neurons. It is unlikely that heavily labeled cells are basket or stellate interneurons, since they are not born until the first postnatal week (Fugita et al., 1966; Altman, 1975). Thus, the results reported here with cultures of early embryonic mouse cerebellar tissue contrast with studies of cells taken from tissue at the end of the first postnatal week which showed GABA uptake by basket and stellate neurons (Lasher, 1974; Messer, 1977; Burry and Lasher, 1978a, b; Burry, 1982).

The vast majority of neurons present in the cultures were not labeled with [^3H]GABA. Since unlabeled cells were generally small, AbNILE-positive, and phase-

TABLE II
Pharmacological characteristics of [^3H]GABA accumulation by E13 cerebellar cells

Each value is the mean $\pm$ Standard error of the mean for 12 different microwell cultures. Each microwell contained 1.2×10^{-5} cells dissociated from mouse cerebellum at E13, maintained *in vitro* for 24 hr, and incubated with [^3H]GABA (5.8×10^{-7} M) for 30 min at 37°C . DABA (3,4-diaminobutyric acid) and β -alanine were present throughout the incubation and during a 15-min pre-incubation period in the indicated wells.

	[^3H]GABA	Percentage of Control
	cpm/well	
Control	2389 $\pm$ 83	
01 mM DABA	814 $\pm$ 36	34
1.0 mM DABA	173 $\pm$ 9	7
0.1 mM β -alanine	2189 $\pm$ 100	92
1.0 mM β -alanine	1557 $\pm$ 88	65

Figure 3. Accumulation of [^3H]GABA in the presence of β -alanine, a blocker of glial uptake, and DABA, a blocker of neuronal uptake. After 22 hr *in vitro*, cultures were exposed to [^3H]GABA (5×10^{-8} M) in the presence of (a) β -alanine (1.0 mM) or (b) DABA (1.0 mM), fixed, and processed for autoradiography as described in the text. A large, bipolar neuron with a heavily labeled cell body (black arrow) and long process that courses among unlabeled cells is evident in a. In b, a DABA-treated culture, none of the many neurons present (curved arrow) is labeled. Phase-contrast microscopy. Magnification $\times 325$.

Figure 4. Uptake of [^3H]GABA *in vivo*. Whole cerebellum was removed at E13, exposed to [^3H]GABA (5×10^{-7} M), and processed for autoradiography as described. Sections were counterstained with cresyl violet to view unlabeled cells. *a*, At low magnification, heavily labeled cells are seen in the cerebellar anlage but not in the ventricular zone (V) along the fourth ventricle or in the external granular layer (EGL) emerging from the rhombic lip (RL) and spreading across the pial surface (P).

bright, it is likely that these are precursors of granule neurons, cells which do not utilize GABA *in vivo*, and undifferentiated basket and stellate cells.

The *in vitro* results presented are consistent with other studies of embryonic neurons *in vitro*, which showed GABA utilization very early in the life of CNS neurons (Farb et al., 1979; Lamborghini and Iles, 1982; Walker and Peacock, 1982). All of these studies suggest that the early expression of neurotransmitter utilization may well be a general feature of developing neurons. The *in vitro* experiments presented also suggest that the combination of immunocytochemical localization of specific cell markers with autoradiographic localization of neurotransmitters will be a useful approach for sorting out glial and neuronal utilization of neurotransmitter substances.

The differential labeling of E13 cerebellar tissue *in vivo* by [^3H]GABA confirms the results seen with *in vitro* preparations and suggests that it is not an abnormally "early" expression of this characteristic in culture. As was the case for dissociated embryonic cerebellar cells, by morphological criteria, [^3H]GABA uptake appeared to be restricted to a subpopulation of immature neurons. The interpretation that heavily labeled cells were neurons was supported by the pharmacological evidence presented that DABA, much more effectively than β -alanine, antagonized the accumulation of [^3H]GABA in tissue sections. Double labeling the sections with specific cell markers could not be carried out, because AbGF does not stain cells in embryonic mouse cerebellum and antisera raised against vimentin, the only available marker for embryonic astroglia, also stains embryonic neurons (Bovolenta et al., 1984).

The time of origin of the cerebellar neurons thought to use GABA suggests that at E13 an immature form of the Purkinje neuron is the most likely candidate for cells which take up [^3H]GABA *in vivo*. In the mouse, all of the precursors of this neuron are postmitotic and have started to migrate away from the ventricular zone into the cerebellar anlage at E13, making them the most numerous of the GABAergic cells present. Many of the precursors of the Golgi neuron have left the ventricular zone at E13, and these may also be represented among the labeled cells.

It is unlikely that any of the heavily labeled cells seen in the middle of the cerebellar anlage are precursors of basket or stellate neurons, because these cells are still in

Brightfield microscopy. Magnification $\times 190$. *b*, Higher magnification of the area around the asterisk in the EGL. A heavily labeled cell internal to the EGL is marked with an arrow. An unlabeled cell in the EGL is marked with a curved arrow. Brightfield microscopy. Magnification $\times 750$. *c*, Higher magnification of the area around the asterisk in the ventricular zone. Labeled cells (arrow) in the more medial aspect of the cerebellar anlage are generally smaller than those seen nearer to the EGL. A few labeled cells are seen close to the ventricular surface, but the vast majority of cells in the ventricular zone are unlabeled (curved arrow). Brightfield microscopy. Magnification $\times 750$.

Figure 5. Effect of DABA, a blocker of neuronal uptake of GABA, on [^3H]GABA uptake *in vivo*. Whole cerebellum was removed at E13, exposed to [^3H]GABA (5×10^{-7} M) in the absence (a to c) or presence (d to f) of DABA (1.0 mM), and processed for autoradiography. a, At low magnification, labeled cells are seen in the cerebellar anlage but not in the ventricular zone (V) or external granular layer (EGL). P, pial surface; RL, rhombic lip. Brightfield microscopy. Magnification $\times 190$. b, Higher magnification of the area around the asterisk in the EGL. A heavily labeled cell internal to the EGL is marked with a straight arrow. Brightfield microscopy. Magnification $\times 750$. c, Higher magnification of the area around the asterisk in the cerebellar anlage medial to the ventricular zone (V). A heavily labeled cell is marked with a straight arrow. Brightfield microscopy. Magnification $\times 750$. d, At low magnification, in the presence of DABA, very few heavily labeled cells are seen. Some labeling is evident in the portion of the cerebellar anlage medial to the rhombic lip (RL), very little of which is visible in this section. EGL, external granular layer; V, ventricular zone along the fourth ventricle; P, pial surface. Brightfield microscopy. Magnification $\times 190$. e, At higher magnification, although some labeling is seen in the area around the asterisk at right in d, it is considerably less dense than that seen in b. Brightfield microscopy. Magnification $\times 750$. f, At high magnification, silver grains are present in the area around the asterisk on the left side of d, but the amount of labeling is considerably less than that shown in c. Brightfield microscopy. Magnification $\times 750$.

the EGL at E13 (Ramon y Cajal, 1960; Fugita et al., 1966; Altman, 1975). From the shapes and location of the heavily labeled cells, it is also unlikely that they are immature forms of cerebellar glia (Sidman and Rakic, 1975).

Since heavily labeled cells were generally not seen in the proliferative zones, that along the fourth ventricular or in the emerging EGL, it is likely that the cells which accumulated [^3H]GABA are postmitotic (Miale and Sidman, 1961; Altman, 1972, 1975; Sidman and Rakic, 1975; Altman and Bayer, 1978). These results suggest that cells express some differentiated properties relating to neurotransmitter choice just after they leave the proliferative zone, much in advance of arriving at their correct position or of forming synaptic connections.

Since heavy labeling was not seen in the EGL, a layer which contains proliferating precursors of two neurons which will ultimately use GABA, the basket and stellate neurons, and one, the granule neuron, which will not utilize GABA, was not seen, it is likely that the lineages of these three classes of neurons have yet to diverge at E13. With [^3H]GABA uptake studies, it should be possible to determine the timing of this event.

These studies suggest that [^3H]GABA autoradiography can be used to visualize developing GABAergic neurons in tissue culture and allow a detailed analysis of the migration, positioning, and maturation of these neurons in developing brain.

References

- Altman, J. (1972) Postnatal development of the cerebellar cortex in the rat. I. The external germinal layer and the transitional molecular layer. *J. Comp. Neurol.* 145: 353-398.
- Altman, J. (1975) Experimental reorganization of the cerebellar cortex. VII. effects of late x-irradiation schedules that interfere with cell acquisition after stellate cells are formed. *J. Comp. Neurol.* 175: 65-76.
- Altman, J., and S. A. Bayer (1978) Prenatal development of the cerebellar system in the rat. I. Cytogenesis and histogenesis of the deep nuclei and the cortex of the cerebellum. *J. Comp. Neurol.* 179: 23-48.
- Boulder Committee (1970) Embryonic vertebrate central nervous system: Revised terminology. *Anat. Rec.* 166: 257-262.
- Bovolenta, P., R. K. H. Liem, and C. A. Mason (1984) Development of cerebellar astroglia: Transitions in form and cytoskeletal content. *Dev. Biol.* 102: 248-259.
- Burky, R. W. (1982) A unit density method of grain analysis used to identify GABAergic neurons for electron microscopic autoradiography. *J. Neurosci. Methods* 5: 369-381.
- Burky, R. W., and R. S. Lasher (1978a) Electron microscopic autoradiography of the uptake of ^3H -GABA in dispersed cell cultures of rat cerebellums. I. Morphology of GABAergic synapses. *Brain Res.* 151: 1-18.
- Burky, R. W., and R. S. Lasher (1978b) Electron microscopic autoradiography of the uptake of ^3H -GABA in dispersed cell cultures of rat cerebellums. II. Development of GABAergic synapses. *Brain Res.* 151: 19-29.
- del Cerro, M., and J. Swartz (1976) Prenatal development of Bergmann glial fibres in rodent cerebellum. *J. Neurocytol.* 5: 669-676.
- Farb, D. H., D. K. Berg, and G. D. Fischbach (1979) Uptake and release of ^3H - γ -aminobutyric acid by embryonic spinal cord neurons in dissociated cell culture. *J. Cell Biol.* 80: 651-661.
- Fields, K. L., J. P. Brookes, R. Mirsky, and L. M. B. Wendon (1978) Cell surface markers for distinguishing different types of dorsal root ganglion cells in culture. *Cell* 14: 43-51.
- Fonnum, F., J. Storm-Mathisen, and F. Walberg (1970) Glutamate decarboxylase in inhibitory neurons. A study of the enzyme in Purkinje cell axons and boutons in the cat. *Brain Res.* 20: 259-275.
- Fugita, S., M. Shimada, and T. Nakanuna (1966) ^3H -Thymidine autoradiographic studies on the cell proliferation and differentiation in the external and internal granular layers of the mouse cerebellum. *J. Comp. Neurol.* 128: 191-209.
- Hatten, M. E. (1981) Different cell assembly patterns of embryonic mouse cerebellar cells on carbohydrate-derivatized substrata. *J. Cell Biol.* 89: 54-61.
- Hatten, M. E. (1984) Embryonic cerebellar astroglia *in vitro*. *Dev. Brain Res.* 13: 309-313.
- Hatten, M. E., and R. H. K. Liem (1981) Astroglia form a template for the positioning of developing cerebellar neurons *in vitro*. *J. Cell Biol.* 90: 622-630.
- Hatten, M. E., and R. L. Sidman (1978) Cell reassociation behavior and lectin-induced agglutination of embryonic mouse cells from different brain regions. *Exp. Cell Res.* 113: 111-125.
- Hatten, M. E., R. H. K. Liem, and C. A. Mason (1984) Two forms of cerebellar astroglia interact differently with cerebellar neurons *in vitro*. *J. Cell Biol.* 98: 193-204.
- Hokfelt, T., and A. Ljungdahl (1970) Cellular localization of labeled gamma amino-butyric acid (^3H -GABA) in rat cerebellar cortex: An autoradiographic study. *Brain Res.* 22: 391-396.
- Hokfelt, T., and A. Ljungdahl (1972) Autoradiographic identification of cerebral and cerebellar cortical neurons accumulating labeled gamma amino-butyric acid (^3H -GABA). *Exp. Brain Res.* 14: 354-362.
- Iversen, L. L., and J. S. Kelly (1975) Uptake and metabolism of γ amino-butyric acid by neurones and glial cells. *Biochem. Pharmacol.* 24: 933-938.
- Kelly, J. S., F. Dick, and F. Schon (1975) The autoradiographic localization of the GABA-releasing nerve terminals in cerebellar glomeruli. *Brain Res.* 85: 255-259.
- Lamborghini, J. E., and A. Iles (1982) Development of a high affinity GABA uptake system in embryonic amphibian spinal neurons in culture. *Soc. Neurosci. Abstr.* 8: 130.
- Larramendi, L. M. H., (1969) Analysis of synaptogenesis in the cerebellum of the mouse. In *Neurobiology of Cerebellar Evolution and Development*, R. Llinas, ed., pp. 803-844, American Medical Association, Chicago.
- Lasher, R. S. (1974) The uptake of [^3H]GABA and differentiation of stellate neurons in cultures of dissociated postnatal rat cerebellum. *Brain Res.* 69: 235-254.
- Levitt, P., M. L. Cooper, and P. Rakic (1981) Coexistence of neuronal and glial precursor cells in the cerebral ventricular zone of the fetal monkey: An ultrastructural and immunoperoxidase analysis. *J. Neurosci.* 1: 27-39.
- Liem, R. H. K. (1982) Simultaneous separation and purification of neurofilament and glial filament proteins from brain. *J. Neurochem.* 38: 142-150.
- McLaughlin, B. J., J. G. Wood, K. Saito, E. Roberts, and Y. Y. Wu (1975) The fine structural localization of glutamate decarboxylase in developing axonal processes and pre-synaptic terminals of rodent cerebellum. *Brain Res.* 85: 355-371.
- Messer, A. (1977) The maintenance and identification of mouse cerebellar granule cells in monolayer culture. *Brain Res.* 130: 1-12.
- Miale, I. L., and R. L. Sidman (1961) An autoradiographic analysis of histogenesis in mouse cerebellum. *Exp. Neurol.* 4: 277-296.

- Obata, K., K. Takeda, and H. Shinozaki (1970) Further study on pharmacological properties of cerebellar-induced inhibition of Deiter's neurones. *Exp. Brain Res.* 11: 327-342.
- Rakic, P. (1971) Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electron microscopic study in *Macacus monkey*. *J. Comp. Neurol.* 141: 283-312.
- Rakic, P., and R. L. Sidman (1973) Sequence of developmental abnormalities leading to granule cell deficit in cerebellar cortex of weaver mutant mice. *J. Comp. Neurol.* 152: 103-132.
- Ramon y Cajal, S. (1960) *Studies on Vertebrate Neurogenesis*, Charles C Thomas, Springfield, IL.
- Ribac, C. E., J. E. Vaughn, and K. Saito (1978) Immunocytochemical localization of glutamine acid decarboxylase in neuronal somata following colchicine inhibition of axonal transport. *Brain Res.* 140: 315-332.
- Roffler-Tarlov, S., P. M. Beart, S. O'Gorman, and R. L. Sidman (1979) Neurochemical and morphological consequences of axon terminal degeneration in cerebellar deep nuclei of mice with inherited Purkinje cell degeneration. *Brain Res.* 168: 75-95.
- Saito, K., R. Barber, J. -Y. Wu, T. Matsuda, E. Roberts, and J. E. Vaughn (1974) Immunohistochemical localization of glutamate decarboxylase in cat cerebellum. *Proc. Natl. Acad. Sci. U. S. A.* 71: 269-273.
- Salton, S. R. J., M. L. Shelanski, and L. A. Greene (1983) Nerve growth factor-inducible large external (NILE) glycoprotein: Studies of a central and peripheral neuronal marker. *J. Neurosci.* 3: 441-454.
- Schon, F., and J. S. Kelly (1975) Uptake and metabolism of ³H β -alanine by glia: Association with the glial uptake system for GABA. *Brain Res.* 86: 243-257.
- Sidman, R. L., and P. Rakic (1975) Neuronal migration with special reference to human brain. A review. *Brain Res.* 62: 1-35.
- Sternberger, L. A. (1979) *Immunocytochemistry*, John Wiley & Sons, New York.
- Storm-Mathisen, J. (1972) Glutamate decarboxylase in the rat hippocampal region after lesions of the afferent fibre systems. *Brain Res.* 40: 215-235.
- Trenkner, E., and R. L. Sidman (1977) Histogenesis of mouse cerebellum in microwell cultures; cell reaggregation and migration, fiber and synapses formation. *J. Cell Biol.* 75: 915-940.
- Walker, C. R., and J. H. Peacock (1982) Development of GABAergic function of dissociated hippocampal cultures from fetal mice. *Dev. Brain Res.* 2: 541-555.
- West, M. J., and M. del Cerro (1974) Early formation of synapses in the molecular layer of the fetal rat cerebellum. *J. Comp. Neurol.* 165: 137-160.