

UPTAKE AND RELEASE OF [³H]γ-AMINO BUTYRIC ACID BY EMBRYONIC SPINAL CORD NEURONS IN DISSOCIATED CELL CULTURE

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ABSTRACT

We have investigated the uptake and release of [³H]γ-aminobutyric acid (GABA) by embryonic chick spinal cord cells maintained in culture. Cells dissociated from 4- or 7-d-old embryos were studied between 1 and 3 wk after plating. At 3 °C, [³H]GABA was accumulated by a high affinity ($K_m \approx 4 \mu M$) and a low affinity ($K_m \approx 100 \mu M$) mechanism. The high affinity transport was markedly inhibited in low Na⁺ media, by ouabain, at 0°C, and by 2,4-diaminobutyric acid. Autoradiography, after incubation in 0.1 μM [³H]GABA, showed that ~50% (range = 30–70%) of the multipolar cells were labeled. These cells were neurons rather than glia; action potentials and/or synaptic potentials were recorded in cells subsequently found to be labeled. Non-neuronal, fibroblast-like cells and co-cultured myotubes were not labeled under the same conditions. The fact that not all of the neurons were labeled is consistent with the suggestion, based on studies of intact adult tissue, that high affinity transport of [³H]GABA may be unique to neurons that use GABA as a neurotransmitter. Our finding that none of fifteen physiologically identified cholinergic neurons, i.e., cells that innervated nearby myotubes, were heavily labeled after incubation in 0.1 μM [³H]GABA is significant in this regard. The newly taken up [³H]GABA was not metabolized in the short run. It was stored in a form that could be released when the neurons were depolarized in a high K⁺ (100 mM) medium. As expected for a neurotransmitter, the K⁺-evoked release was reversibly inhibited by reducing the extracellular Ca⁺⁺/Mg⁺⁺ ratio.

KEY WORDS high affinity [³H]GABA uptake · dissociated spinal cord cells · selective labeling · [³H]GABA release

Neurons dissociated from embryonic spinal cords and maintained in monolayer cell culture form excitatory and inhibitory synapses with one another (15, 16, 43). Further studies of the formation, electrophysiology, and long term regulation

of such interneuronal synapses would benefit greatly from identification of the neurotransmitters involved. Among the several candidates, γ-aminobutyric acid (GABA) is particularly attractive because a great deal is known about GABA metabolism and action on postsynaptic target cells in adult animals (see reviews: references 9, 11, 26, and 36).

There is considerable evidence that GABA

mediates presynaptic inhibition and at least some degree of postsynaptic inhibition in the adult spinal cord. GABA is present in the spinal grey matter and, in keeping with its proposed role in presynaptic inhibition, the concentration is highest in superficial laminae of the dorsal horn (33). Glutamic acid decarboxylase (GAD), the enzyme immediately responsible for GABA synthesis, has been identified immunohistochemically in synaptic boutons located on incoming afferent nerve processes and also on the cell bodies and dendrites of interneurons and motoneurons (1, 32). Afferent nerve terminals and intrinsic spinal cord nerve cell bodies are sensitive to applied GABA (3, 8, 10, 12, 30, 35, 44), and some of the synaptic interactions are blocked by the GABA antagonists picrotoxin and bicuculline (3, 7, 12, 24).

Because of the complexity of the intact central nervous system (CNS), much of the evidence cited above is qualitative and indirect. In dissociated nerve cell cultures, individual neurons and synapses can be visualized and they are accessible to microelectrodes. In principle, more precise information about postsynaptic and presynaptic inhibitory mechanisms can be obtained. However, data derived from the adult spinal cord do not insure that GABA is used as a transmitter in embryonic spinal cord cell cultures. Cells that are destined to release GABA *in vivo* might not express their unique phenotype in dissociated cell cultures. Moreover, dissociation of the embryonic CNS is a rather drastic procedure. With current techniques, cell yields range between 20 and 30% and it is possible that GABAergic neurons are selectively destroyed. We have, therefore, attempted to demonstrate the presence of GABAergic neurons in established spinal cord cell cultures.

Experiments *in vivo* suggest that neurons which take up GABA by a high affinity transport mechanism may also use GABA as a neurotransmitter (see reference 25 for review). The experiments reported here were designed to investigate the kinetics of [³H]GABA transport, its localization in spinal cord neurons, and the subsequent release of accumulated [³H]GABA into the medium. Evidence for the synthesis of GABA in spinal cord cell cultures and pharmacological evidence for inhibitory GABAergic synaptic interactions will be presented in subsequent papers (5, 14).

MATERIALS AND METHODS

Cell Cultures

Dissociated chick spinal cord cells tend to reaggregate

when plated on a naked collagen-coated substrate. They remain isolated from one another when added to established muscle cultures, perhaps because they attach more firmly. In addition, because muscle fibers are innervated *in vitro* by cholinergic neurons (15), they provide a means of distinguishing a class of neurons that presumably do not use GABA as a neurotransmitter. Therefore, most of the experiments reported in this paper were performed on spinal cord/muscle co-cultures.

Mononucleated muscle precursor cells were obtained from minced fragments of 11-d embryonic pectoral muscle by mechanical disruption (trituration). Before trituration, the fragments were incubated in 0.1% trypsin (Grand Island Biological Co. [Gibco], Grand Island, N. Y.) in a Ca⁺⁺- and Mg⁺⁺-free saline (Puck's D₁G) for 30 min at 37°C. The fragments were then centrifuged, resuspended in complete plating medium (see below), and triturated in a fire-polished Pasteur pipette. Between 1 and 2 × 10⁵ cells were added to collagen-coated (4) 35-mm plates in 1.5 ml of medium. In some experiments, each dish contained a collagen-coated (15- or 18-mm) glass coverslip (Propper Manufacturing Co., Inc., Long Island City, N. Y.; or Gold Seal Div., Clay-Adams, Inc., Parsippany, N. J., No. 1). Under our conditions, mononucleated cells fuse between the 2nd and 4th d *in vitro*. Fibroblasts were virtually eliminated by adding 10⁻⁵ M cytosine arabinoside (araC) to the medium for 48 h beginning on day 3.

Neurons were dissociated from 7-d embryonic spinal cords exactly as described above. In some experiments, neurons were obtained from 4-d cords. Meninges and attached sensory ganglia were dissected away before mincing the tissue. Between 1 and 2 × 10⁵ cells were added to established muscle cultures (usually immediately after removing the araC on day 5) or directly to fresh collagen-coated coverslips.

Transport of [³H]GABA

Carrier-free [³H]GABA (sp act = 36.7 Ci/mmol) was obtained from New England Nuclear, Boston, Mass., and further purified by electrophoresis through paper (Beckman No. 320046, Beckman Instruments, Inc., Fullerton, Calif.) at pH 1.9 for 2 h in a Beckman Durrum Cell (2).

UPTAKE: Cultures were incubated in 0.1–18.0 μM [³H]GABA in 1.0 ml of Earle's Balanced Salt Solution (BSS; see below) at 37°C and pH 7.4. At higher concentrations, the isotope was diluted with unlabeled GABA. At various times, coverslips were removed, dipped successively in four 50-ml BSS washes, and then assayed by scintillation counting. The washes were completed within 10 s. The fourth dip did not further reduce the counts per minute (cpm) compared to the third. Radioactivity was extracted by addition of 0.1 ml of 0.1 N HCl to the scintillation vials for at least 10 min before addition of 5 ml of Aquasol (New England Nuclear). The yield was not increased by prior homogenization or by extraction with 10% SDS or 10% perchloric acid. Quenching, as determined by the method of external

standards, was negligible (within 3% of values found in Aquasol alone). Counts were converted to moles of GABA after correcting for the counting efficiency of tritium (45%).

The identity of the accumulated radioactivity was determined by high voltage paper electrophoresis. After incubation in $0.1 \mu\text{M}$ [^3H]GABA, the cultures were frozen and thawed three times in $400 \mu\text{l}$ of electrophoresis buffer (pH 1.9). The cells were then scraped from the plates, spun at top speed in a Beckman Microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), and the supernate was taken to dryness under a stream of nitrogen. The residue was resuspended in $20 \mu\text{l}$ of electrophoresis buffer containing 1 mg/ml of GABA, glutamate, acetylcholine, and choline, and applied to 4-cm-wide paper strips. Electrophoresis was continued for 2 h at 5,000 V.

EFFLUX: The cells (on coverslips) were "loaded" with [^3H]GABA by incubation in $0.1 \mu\text{M}$ [^3H]GABA for 45 min at 37°C and then placed in a closed perfusion chamber (Nicholson Precision Instruments, Inc., Bethesda, Md.). The chamber, which holds 0.2 ml, was perfused at a rate of 0.15 ml/min, and samples of the effluent were collected at 1-min intervals with a Gilson fraction collector (Gilson Medical Electronics, Inc., Middleton, Wis.). The released radioactivity was shown to be [^3H]GABA by high voltage paper electrophoresis before and after enzymatic degradation in a coupled enzyme (GABAase) assay. GABAase is a mixture of GABA-transaminase and succinic semialdehyde dehydrogenase. The complete degradation of added carrier GABA was followed by measuring the absorbance caused by NADPH at 340 nm in a Cary 118 double beam spectrophotometer (Cary Instruments, Fairfield, N. J.).

Autoradiography

The cells were incubated in $0.1 \mu\text{M}$ [^3H]GABA for 45 min at 37°C , washed for 12 min with a total of 10 ml of ice-cold BSS, and then fixed in 1.6% glutaraldehyde (Taab) buffered with 0.06 M sodium phosphate (pH 7.5). Fixation was begun at 0°C and, after 10 min, was continued at room temperature for at least 1 h. Others have shown that 65–70% of the label is retained after glutaraldehyde fixation (20). The coverslips were then washed for 1 h in filtered tap water, fastened to glass slides, and dipped in Kodak NTB-3 emulsion that was warmed to 39°C and diluted 1:1 with water. The emulsion was gelled on a cold plate and then exposed in a light-tight dessicated box for 24–26 h. Some slides were exposed for five days. The emulsion was developed in Kodak D-19 (2 min at 68°C) and fixed in Kodak Rapid Fix.

Electrophysiology

Experiments were performed on the stage of an inverted, phase contrast microscope. Techniques for intracellular recording and extracellular stimulation have

been previously described (6, 15). Intracellular microelectrodes were filled with 3 M KCl. Stimulating electrodes were bumped to a tip Diam of 3–5 μm , fire polished, and filled with 1 M NaCl. While recording, the cells were perfused with a 4:1 mixture of BSS and the complete medium was maintained at 37°C and pH 7.2–7.4.

Media

Plating medium consisted of Eagle's Minimum Essential Medium (made up of Earle's BSS) supplemented with glutamine (2.4 mM), heat-inactivated horse serum (10% vol/vol), chick embryo extract (5% vol/vol), penicillin (50 U/ml), and streptomycin (50 $\mu\text{g}/\text{ml}$). After the 3rd d *in vitro*, the cells were fed every 2–3 d with an identical medium, except that the concentration of embryo extract was reduced to 2% vol/vol. Embryo extract was prepared by homogenizing 10-d-old embryos in Earle's BSS (3 ml/embryo). Horse serum was obtained from Gibco. Earle's BSS contains: 116.4 mM NaCl, 26.2 mM NaHCO_3 , 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 1.0 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 6.1 mM glucose, and 0.5 mg/ml phenol red.

RESULTS

Kinetics of [^3H]GABA Uptake

When chick spinal cord/muscle co-cultures were incubated in $0.1 \mu\text{M}$ [^3H]GABA, the cells accumulated radioactivity rapidly for the first 5–10 min and then more slowly with increasing time. Fig. 1 shows an uptake curve obtained 12 d after plating 4-d spinal cord cells on previously formed myotubes. Muscle cultures plated with the same number of myoblasts but without added spinal cord cells accumulated <3% as much label as the spinal cord/muscle co-cultures. The retained radioactivity was [^3H]GABA rather than a meta-

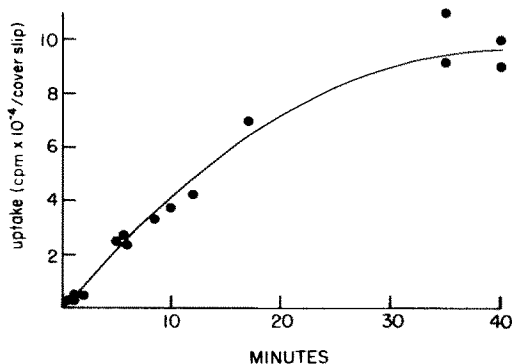


FIGURE 1 Accumulation of radioactivity by spinal cord/muscle co-cultures during incubation in $0.1 \mu\text{M}$ [^3H]GABA (sp act = 36.7 Ci/mmol) at 37°C . Each point represents one coverslip.

bolic product: 96% of the total cpm comigrated with GABA on high voltage paper electrophoresis. Moreover, neither the total uptake of radioactivity nor the percent present as [^3H]GABA was increased by prior incubation for 20 min in 10 μM amino-oxyacetic acid (AOAA), an inhibitor of GABA transaminase.

The early phase of uptake at several concentrations of [^3H]GABA is shown on an expanded time scale in Fig. 2. The initial rates increase with increasing [^3H]GABA concentration. Lineweaver-Burk plots utilizing the 1-min points and extending over a 180-fold range in [^3H]GABA concentration are shown in Fig. 3. Between 0.1 and 2.0 μM [^3H]GABA (lower graph), the points fell along a single line with an extrapolated V_{max} (y-intercept) of 14.4 pmol/min/cm 2 of culture surface and an extrapolated K_m (x-intercept) of 3.7 μM . In two other less complete experiments, K_m 's of 4.4 and 1.2 μM were obtained.

At higher concentrations (*inset* graph), the points fell below this extrapolated line. A line drawn through these points suggests a second

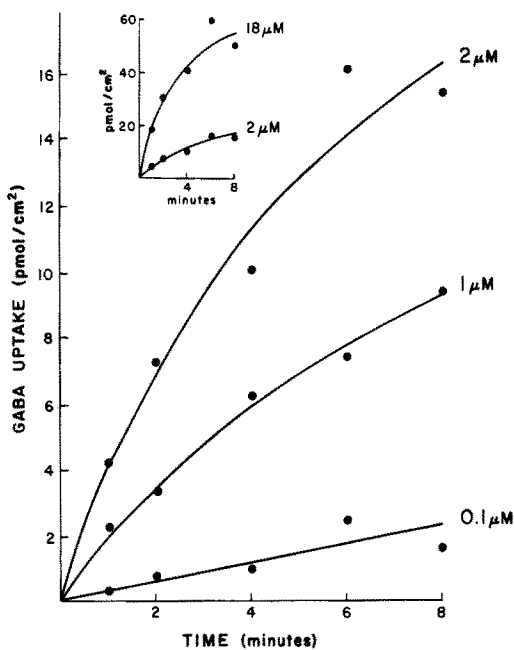


FIGURE 2 The relation between extracellular GABA concentration and GABA uptake. The specific activity of the label was reduced at the higher concentrations of GABA. In this experiment, two different-size coverslips were employed so that the data were normalized and expressed as pmoles accumulated/cm 2 of culture surface. Each point is the mean of two coverslips.

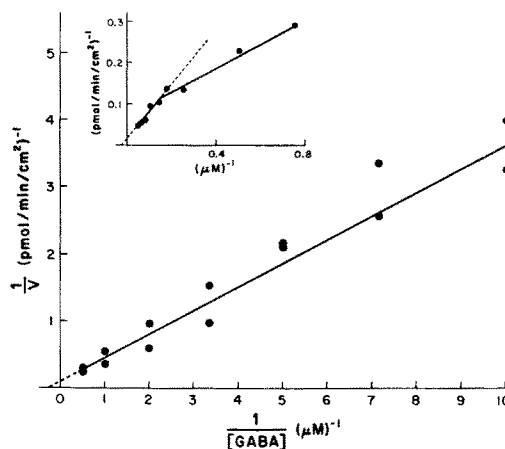


FIGURE 3 A Lineweaver-Burk plot of the initial rates of [^3H]GABA transport estimated from 1-min incubations (see Fig. 2). From 0.1–2 μM GABA, each point represents a single coverslip. Points in the *inset* (1.3–18 μM GABA) are the means of four coverslips.

TABLE I
Characteristics of High Affinity [^3H]GABA Uptake

Condition	Rate of uptake
	%
Control*	100
Muscle†	3
Low Na $^+$ (27.2 mM)§	10
0°C	16
10 $^{-5}$ M ouabain	18
1 mM 2,4-diaminobutyric acid	10
1 mM β -alanine	48

Initial (3 min) rates of [^3H]GABA uptake (0.1 μM [^3H]GABA) are expressed as a percentage of the control rate. All assays were performed 10 d after plating the spinal cord cells. Each entry represents the mean of four cultures.

* Control refers to spinal cord/muscle co-cultures seeded with 2×10^{-5} spinal cord cells and assayed at 37°C.

† Muscle cultures were plated at the same time as the control muscle but they received no neurons.

§ Choline Cl was substituted isosmotically for NaCl.

transport system with V_{max} of 63.5 pmol/min/cm 2 and K_m of 100 μM . The [^3H]GABA was purified by paper electrophoresis, so it is unlikely that the biphasic Lineweaver-Burk plot reflects the transport of a radioactive impurity. In addition, the rates of uptake measured at 0.1 and 18 μM were independent of the specific activity of [^3H]GABA (varied over a 10-fold range), and the rate of transport of [^{14}C]GABA measured at 18 μM was identical to that of [^3H]GABA.

If the high and low affinity transport mechanisms are independent, then the fraction of the total uptake caused by the high affinity system (f_H) at a given GABA concentration is determined by the kinetic constants:

$$f_H = \frac{V_H(K_L + [\text{GABA}])}{V_L(K_H + [\text{GABA}]) + V_H(K_L + [\text{GABA}])}$$

where V_H and V_L are the maximum rates of high and low affinity transport and K_H and K_L are the GABA concentrations at which transport is half maximal for the high and low affinity systems, respectively. Based on the data shown in Fig. 3, at 0.1 μM GABA, $f_H = 86\%$.

The high affinity transport was inhibited in low Na^+ medium and by 10^{-5} M ouabain (Table I). Uptake was also decreased sixfold when the tem-

perature was lowered from 37° to 0°C. Transport was not, however, acutely dependent on aerobic metabolism: brief incubations in azide (1 mM), cyanide (10 mM), or 2,4-dinitrophenol were essentially without effect. The effect of two GABA analogs, 2,4-diaminobutyric acid (DABA) and β -alanine, on GABA uptake was tested in one experiment. At 1.0 mM, DABA reduced the amount of [^3H]GABA (0.1 μM) accumulated after 45 min by 90%, whereas 1.0 mM β -alanine reduced uptake by only 50%.

Localization of Accumulated [^3H]GABA

Autoradiography of cultures exposed to 0.1 μM [^3H]GABA for 45 min showed that the label was concentrated in multipolar cells and in fine processes (Fig. 4). Grains were distributed uniformly

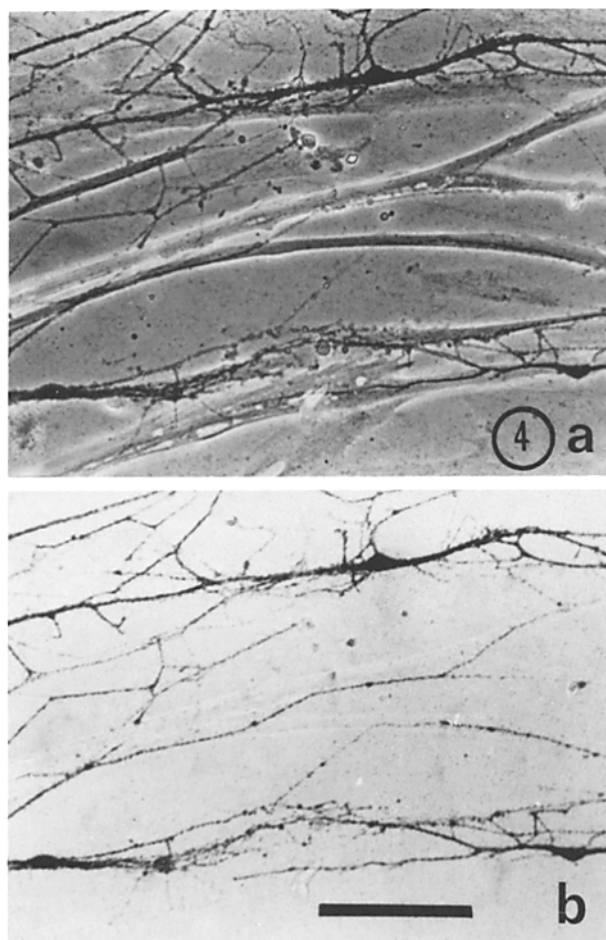


FIGURE 4 An autoradiograph of a culture exposed to 0.1 μM [^3H]GABA for 45 min: (a) phase contrast and (b) bright field. Note that multipolar cells (presumptive neurons) and fine nerve processes are heavily labeled whereas muscle fibers and fibroblast-like cells are not labeled. Exposure time, 24 h. Bar, 100 μm .

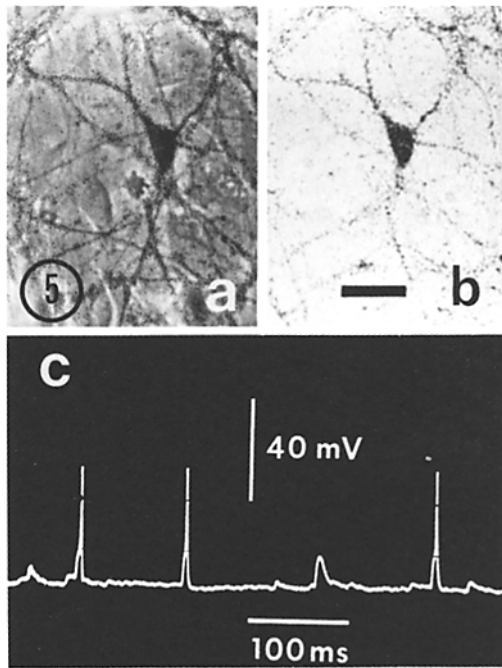


FIGURE 5 An example of a neuron that accumulated [^3H]GABA. The multipolar cell shown in Fig. 5a was identified as a neuron when spontaneous synaptic potentials and action potentials were recorded through an intracellular microelectrode (c). After exposure to [^3H]GABA and processing for autoradiography, this neuron was relocated and judged to be labeled in (a) phase contrast and (b) bright field views. Bar in Fig. 5b, 50 μm .

along the processes and over the cell bodies. Either uptake sites are present throughout the surface membrane or the newly taken-up label diffuses rapidly (within 45 min) throughout the cytoplasm before it is fixed in place.

Previous electrophysiological experiments have shown that virtually all multipolar cells with long processes in spinal cord cell cultures generate action potentials and/or receive synaptic input (16), so it is likely that most if not all of the labeled cells were neurons rather than glia. That neurons are able to concentrate [^3H]GABA was demonstrated directly in this study by recording action potentials and/or synaptic potentials in cells that were subsequently shown to be labeled (Fig. 5). As expected from the results of scintillation counting, none of the myotubes were labeled after incubation in 0.1 μM [^3H]GABA. In addition, few if any of the non-neuronal, fibroblast-like spinal cord cells were labeled.

Autoradiography was also performed on cultures seeded with cells dissociated from 16-d embryonic cerebellum and 10-d embryonic dorsal root ganglia. After incubation in 0.1 μM [^3H]GABA, some of the multipolar cells in cerebellar cultures, but none of the sensory neurons, were heavily labeled.

Not all spinal cord nerve cell bodies were labeled after incubation in 0.1 μM [^3H]GABA. Figure 6a shows two neurons within the same field of view that appear similar in size and shape: one has concentrated [^3H]GABA, the other has not. On a qualitative level, neurons were categorized as heavily labeled (grains confluent), lightly labeled (individual grains distinguishable; Fig. 6b), or unlabeled (grain density $< 3 \times$ background). Some cell bodies were crisscrossed by distinctive strings of grains (Fig. 6c) that appeared to be located within overlying neurites. These cells were considered unlabeled.

In cultures seeded with 7-d cells and assayed 1–2 wk after plating, 66% of the neurons were labeled (279 neurons counted/4 platings; range = 58–70%). A slightly lower percentage of 4-d cells (50%) were labeled (350 counted/4 platings; range = 33–67%). Aside from the fact that few of the largest neurons accumulated [^3H]GABA, there was no obvious correlation between cell shape and grain density. The percentage of neurons that were labeled did not change when the autoradiographs were exposed for 5 d instead of the usual 24 h.

The pattern of labeling was quite different after incubation in 0.1 μM [^3H]lysine. In this case, grains were distributed equally over myotubes, fibroblasts, and neurons, and none of the cells were as heavily labeled as those neurons that concentrated [^3H]GABA.

Neurons that Innervate Muscle are not Labeled by [^3H]GABA

Many of the relatively large neurons in cultures seeded with 4-d or 7-d spinal cord cells innervate nearby myotubes (15). These synaptic interactions are blocked by D-tubocurarine or α -bungarotoxin, so they are very likely mediated by acetylcholine. If high affinity transport is characteristic only of those neurons that use GABA as a neurotransmitter, then cholinergic “motoneurons” should not be labeled.

An example of a motoneuron is shown in Fig. 7a. Synaptic potentials (Fig. 7c) were detected by

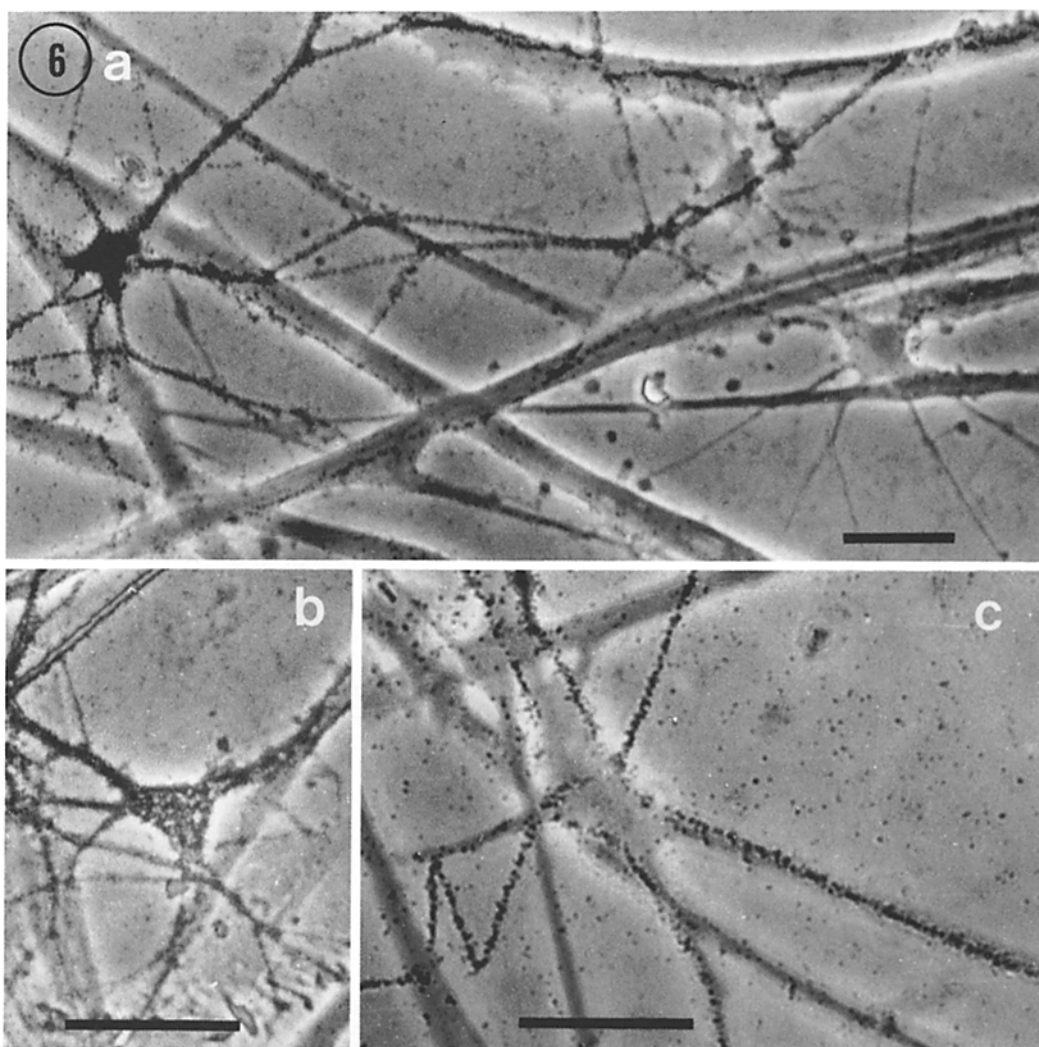


FIGURE 6 Examples of neurons in 12-d spinal cord/muscle co-cultures that were heavily labeled (*a*—left), unlabeled (*a*—right), or intermediately labeled (*b*). An unlabeled neuron traversed by strings of grains that appear to be within nerve processes that originate from other cells is shown in Fig. 6*c*. Bars, 50 μm .

an intracellular microelectrode placed in the indicated myotube while stimulating the nerve cell body through an extracellular electrode. The stimulus was extremely focal: evoked responses were lost when the stimulating electrode was moved a few microns away from the neuron. It is unlikely, therefore, that other neurons within the field of view were directly excited by the stimulating current. After the recordings were made, the culture was incubated in 0.1 μM [^3H]GABA and prepared for autoradiography. The motoneuron was not labeled (Fig. 7*b*). Another identified moto-

neuron is shown in Fig. 8. In this case, neurites overlying the neuron had accumulated [^3H]GABA but the cell body was not labeled.

In all, 15 neurons that innervated nearby myotubes were relocated after autoradiography. 14 were unlabeled; one was apparently lightly labeled. Because 30–70% of the spinal cord neurons concentrate [^3H]GABA, the lack of uptake by motoneurons cannot be because of chance.

Release of Newly Taken Up [^3H]GABA

Release of [^3H]GABA previously accumulated

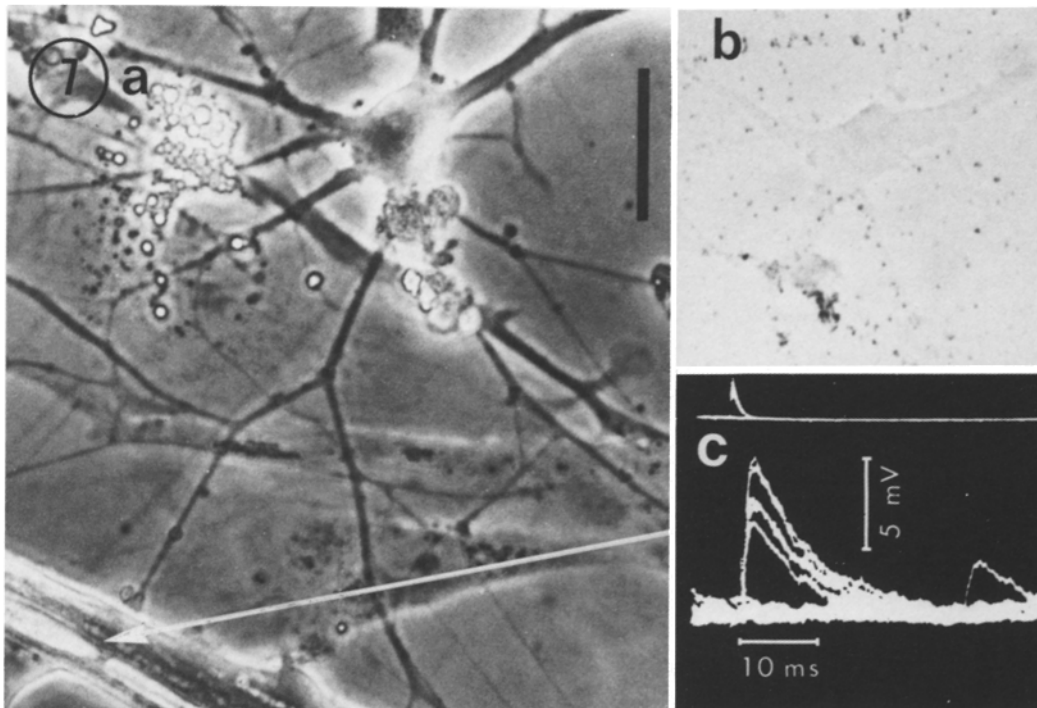


FIGURE 7 An unlabeled motoneuron. The superimposed oscilloscope traces in Fig. 7c show synaptic potentials that were evoked in the indicated myotube (a) by stimulating the nearby multipolar neuron. After the culture was incubated in $0.1 \mu\text{M}$ ^3H GABA, and processed for autoradiography, the neuron was relocated and found to be unlabeled (b). (a) Phase contrast, (b) bright field. Bar in Fig. 7a, $40 \mu\text{m}$.

by high affinity transport was evoked by perfusing the cells with a salt solution containing 100 mM K^+ . Intracellular recording showed that this concentration of K^+ depolarized the neurons to -10 mV . Fig. 9a illustrates a typical result. Immediately after the cells were loaded with ^3H GABA and transferred to the perfusion chamber (see Materials and Methods), there was a relatively high rate of isotope efflux into control salt solution ($\text{K}^+ = 5.4 \text{ mM}$, $\text{Na}^+ = 143.5 \text{ mM}$). This declined to a steady value after 10–12 min, at which time perfusion with a high K^+ salt solution ($\text{K}^+ = 100 \text{ mM}$, $\text{Na}^+ = 43.5 \text{ mM}$) resulted in a 2.5-fold increase in efflux. A second high K^+ “pulse” was approximately as effective as the first. In every case, the efflux reached a peak and began to decline during the high K^+ pulse and then remained relatively high for several minutes after return to control salt solution.

The K^+ -stimulated release depended on the $\text{Ca}^{++}/\text{Mg}^{++}$ ratio in the bathing medium. When Ca^{++} was omitted and Mg^{++} was increased to 10.5 mM , the efflux in 100 mM K^+ was decreased by

75% (Fig. 9b). The initial rapid efflux in control solution was not affected by lowering the $\text{Ca}^{++}/\text{Mg}^{++}$ ratio. Fig. 9b also illustrates that the stimulated release was not caused by the decrease in extracellular Na^+ . Substitution of choline for Na^+ was without effect.

DISCUSSION

Cultured chick spinal cord cells concentrate ^3H GABA by two mechanisms. The first is saturable with a K_m of $3.7 \mu\text{M}$ at 37°C . This K_m is within the range of values taken as characteristic of high affinity GABA transport in other systems (31). For example, slices of adult rat cerebral cortex and spinal cord take up ^3H GABA with K_m s of 22 and $26 \mu\text{M}$, respectively (21, 22). Cultured fragments of embryonic chick spinal cord exhibit a K_m of $17 \mu\text{M}$ (49). As expected from studies of amino acid transport in the nervous system (22) and in other tissues (47), the high affinity uptake of ^3H GABA by dissociated spinal cord cells depends on the transmembrane Na^+ gradient. At $0.1 \mu\text{M}$ GABA, uptake was in-

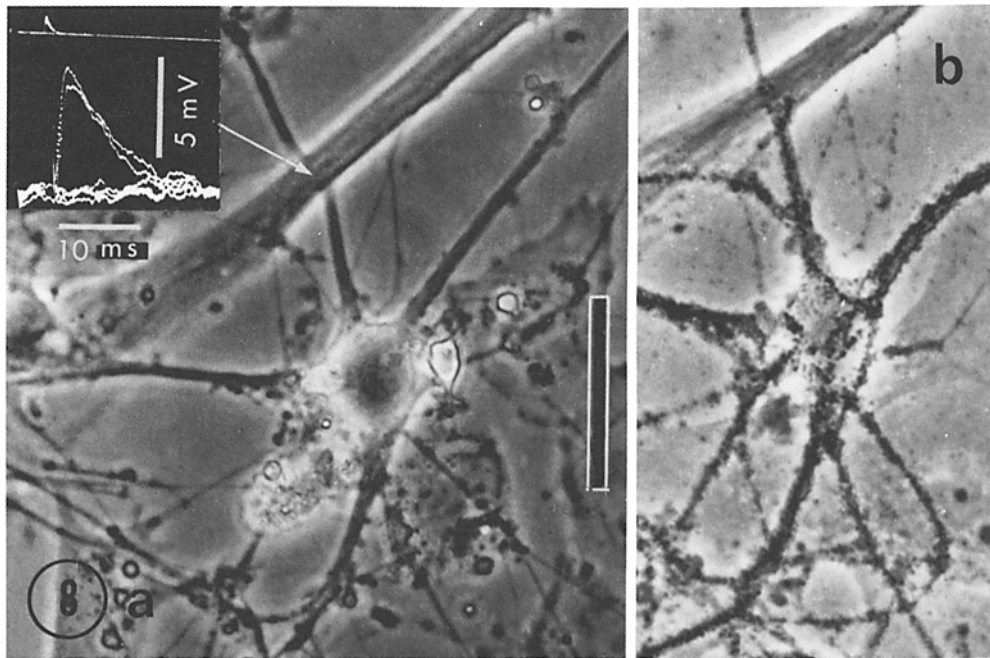


FIGURE 8 Another motoneuron that was not labeled by [^3H]GABA (see Fig. 7 legend). In this case, the unlabeled cell body is crossed by labeled nerve processes. Note the boutonlike swellings along some of the neurites. Bar in Fig. 8a, 50 μm .

hibited in low Na^+ media, by ouabain, and at low temperature. The second transport mechanism, evident at high concentrations of [^3H]GABA, is less well characterized. The kinetics are consistent with a K_m of $\sim 100 \mu\text{M}$. Although the capacity (V_{max}) of this "low affinity" system is greater than that of the high affinity system, at low concentrations of [^3H]GABA, more than 85% of the uptake is via the high affinity mechanism.

In adult CNS tissue, glia as well as neurons are labeled after exposure to low concentrations of [^3H]GABA (13, 18, 25, 28). Vertebrate sensory and sympathetic ganglia and crustacean neuromuscular junctions concentrate [^3H]GABA by a high affinity mechanism, and in these tissues only the glial elements are labeled (41, 45, 46, 50). It is important, therefore, to establish the identity of cells labeled by [^3H]GABA in culture. In our experiments most, if not all, of the labeled cells were neurons. This was shown directly by recording action potentials and/or synaptic potentials in several cells subsequently found to be labeled in autoradiographs. These were not exceptional cases. In this and in a previous study (16), >90% of adequately penetrated multipolar cells like those shown in Figs. 4, 5, and 6 were electrically

and/or chemically excitable. It is also significant in this regard that the [^3H]GABA taken up by dissociated spinal cord cells was not metabolized in the short run. Glia apparently contain high levels of GABA-transaminase, and inhibition of this enzyme with AOAA results in a several-fold increase in [^3H]GABA accumulation (25, 45, 46). The amount of radioactivity retained by the dissociated spinal cord cells was not increased by prior incubation with AOAA. Rather, the spinal cord cells stored [^3H]GABA in a form that could be released when the cells were depolarized in high K^+ media. As expected for a neurotransmitter, the K^+ -stimulated release depended on the $\text{Ca}^{++}/\text{Mg}^{++}$ ratio in the extracellular medium. It is also noteworthy that DABA, a relatively selective inhibitor of neuronal uptake (25), inhibited the transport of [^3H]GABA by spinal cord cells far more than did β -alanine, an inhibitor of glia uptake (25). Thus, while it is possible that some of the smallest labeled spinal cord cells were glia or other non-neuronal cells, most of the high affinity uptake was by neurons.

In the adult brain [^3H]GABA taken up by high affinity transport is accumulated within the endogenous GABA pool (34), and the label is probably

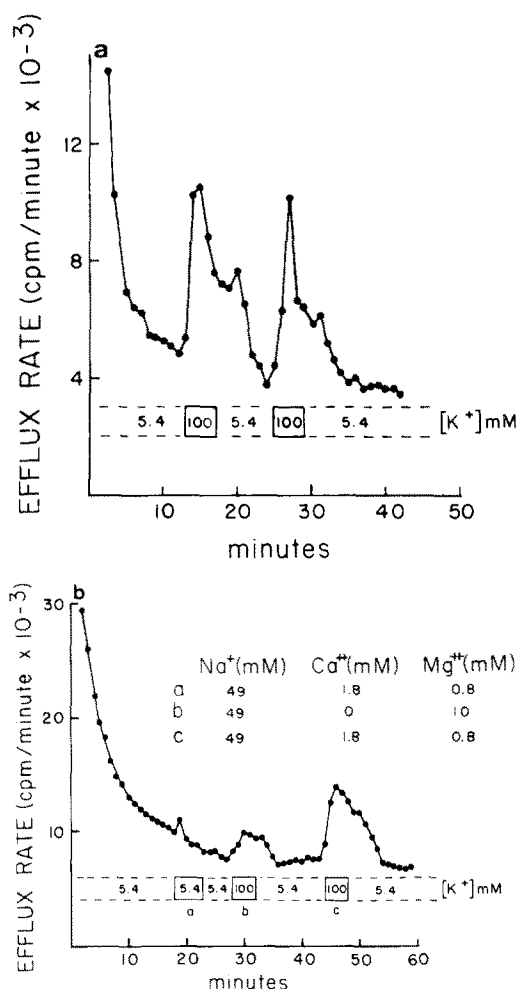


FIGURE 9 (a) Release of newly taken-up [³H]GABA in high K⁺ solutions. The spinal cord/muscle co-culture was incubated in 0.1 μM [³H]GABA for 45 min, washed, and transferred to the perfusion chamber. At the indicated times, the control salt solution (Na⁺=149 mM, K⁺=5.4 mM) was switched to the high K⁺ salt solution (Na⁺=49 mM, K⁺=100 mM). All other ions remained constant. (b) The K⁺-stimulated efflux was reduced by removing Ca⁺⁺ from the extracellular medium and raising the concentration of Mg⁺⁺ at b. The block was rapidly reversible at c. At a, the Na⁺ concentration in the perfusate was reduced and replaced with choline Cl.

concentrated in a unique subpopulation of neurons. Electron microscope autoradiography of spinal cord synaptosomes exposed to [³H]GABA and/or [³H]glycine suggests that these amino acids label different nerve terminals: 25% were labeled with [³H]GABA alone, 28% with [³H]glycine

alone, and 51% when the isotopes were added together (20). Synaptosomes that concentrate [³H]GABA can be separated from those that accumulate [³H]norepinephrine, [³H]5-hydroxytryptamine, or [³H]glycine on linear sucrose gradients (17, 21, 23, 27). Evidence for specific labeling in intact tissue by high affinity [³H]GABA transport is strongest in the cerebellum. Structures that resemble stellate and basket cells in the molecular layer and Golgi cells in the granular layer are heavily labeled (18, 19, 25, 29). These interneurons are inhibitory, and the inhibition is probably mediated by GABA. Excitatory, nonGABAergic granule cells are not labeled. Purkinje cells which exert a GABAergic inhibition on neurons in the deep cerebellar nuclei (37, 38, 39, 40, 42) are not labeled by low concentrations of [³H]GABA. However, this apparent exception may simply reflect the presence of a diffusion barrier in adult tissue, because Purkinje cells in embryonic cerebellar fragments maintained as intraocular transplants or as explant cultures are heavily labeled by exogenous [³H]GABA (28, 48).

Does GABA taken up by high affinity transport label embryonic spinal cord neurons in dissociated cell culture which use GABA as a neurotransmitter? We cannot yet answer this question with certainty, but indirect evidence suggests that this is the case. It is encouraging that many spinal cord neurons were not labeled by [³H]GABA, because GABA is certainly not the transmitter at all segmental spinal cord synapses. Our observation that ~50% of the neurons in 1-2 wk cultures accumulated [³H]GABA is consistent with the finding of Iversen and Bloom (20) that ~25% of the synaptosomes prepared from spinal cord homogenates concentrate [³H]GABA. None of the 15 physiologically identified motoneurons, i.e., neurons that innervated nearby myotubes, were heavily labeled by [³H]GABA. Motoneurons are cholinergic, and this negative result indicates that the pattern of labeling is not random. Rather, it suggests that [³H]GABA is concentrated by a distinctive class of neurons. Many cultured spinal cord neurons receive synaptic input that by pharmacological criteria are probably mediated by GABA (5). More direct evidence for the selectivity of the [³H]GABA label will require electrophysiological identification of the "driver" neurons responsible for the observed synaptic input.

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