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Presence of mRNA for glutamic acid decarboxylase in both excitatory and inhibitory neurons

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ABSTRACT **Neurons in very low density hippocampal cultures that are physiologically identified as either GABAergic inhibitory or glutamatergic excitatory all contain mRNA for the γ-aminobutyric acid (GABA) synthetic enzyme, glutamic acid decarboxylase (GAD), as detected by single cell mRNA amplification and PCR. However, consistent with the physiology, immunocytochemistry revealed that only a subset of the neurons stain for either GAD protein or GABA. A similar fraction hybridize with RNA probes for GAD65 and GAD67. Hippocampal CA1 pyramidal neurons in slice preparations, which are traditionally thought to be excitatory, also contain mRNA for GAD65 and GAD67. Hippocampal neurons in culture did not contain mRNA for two other neurotransmitter synthesizing enzymes, tyrosine hydroxylase, and choline acetyl transferase. These data suggest that in some neurons, presumably the excitatory neurons, GAD mRNA is selectively regulated at the level of translation. We propose that neurotransmitter phenotype may be posttranscriptionally regulated and neurons may exhibit transient phenotypic plasticity in response to environmental influences.**

Most inhibitory neurons of the mammalian forebrain use γ-aminobutyric acid (GABA) as a neurotransmitter. GABA is released presynaptically in response to action potentials and diffuses across the synaptic cleft where it binds to postsynaptic receptors directly coupled to ion channels permeable to anions. Activation of these receptor-linked ion channels hyperpolarizes, or inhibits, postsynaptic neurons.

GABA-utilizing neurons require the enzyme glutamic acid decarboxylase (GAD) to convert glutamate into GABA for subsequent release. Immunohistochemical detection of GABA or GAD is commonly used to identify GABA-synthesizing inhibitory neurons (1–4). In addition, *in situ* hybridization for GAD mRNA has also been employed for phenotypic identification, based on the assumption that cells that contain the mRNA for a given protein will also synthesize significant quantities of the active form of that protein (5–8). Therefore, the presence of mRNA encoding for GAD has been taken as evidence that a cell is GABAergic.

During the course of preliminary experiments in which the mRNA content of single hippocampal (HC) neurons maintained in culture was examined, it was found that all of the examined neurons (n = 75) contained significant amounts of mRNA encoding GAD65, a low molecular weight isoform of GAD (9, 10). However, previous experiments had demonstrated that many of the HC neurons maintained under our culture conditions are in fact excitatory and release an amino acid neurotransmitter that activates postsynaptic glutamate receptors (11, 12). Excitatory neurons do not use GABA as a neurotransmitter, and thus would presumably not require mRNA encoding the enzyme that converts glutamate to GABA. Therefore, the ubiquitous presence of mRNA for GAD65 in unidentified neurons was an unexpected finding, since it is assumed that neurons transcribe mRNA only for proteins subsequently produced.

The present experiments were designed to directly determine, via single-cell antisense RNA (aRNA) amplification, if positively identified glutamatergic, excitatory neurons contained significant amounts of mRNA for GAD. The most definitive method for identifying neurons as excitatory or inhibitory, and for identifying neurotransmitter phenotype, requires stimulation of a single neuron while simultaneously recording from another neuron which is monosynaptically connected to the first. This can readily be performed in dissociated culture preparations (11, 13). It is therefore possible to identify the phenotype of a recorded neuron and then aspirate the contents of the cell into the same recording pipette for aRNA amplification and PCR analysis (14–16). Using these combined techniques, it was found that all positively identified excitatory neurons examined in this culture preparation have significant levels of mRNA encoding for GAD65. Therefore, GAD65, in excitatory neurons, appears to be regulated at the level of translation, providing a rapid mechanism for excitatory neurons to regulate neurotransmitter phenotype.

MATERIALS AND METHODS

Tissue Culture. HC cultures were prepared as described (11, 13, 17). Cells were plated at 100,000 viable cells/ml (very low density; VLD) or 400,000 (high density). The media for VLD cultures was partially replaced with one containing elevated potassium concentration (20 mM) to enhance survival (18). Cultures were fed either three times a week by replacing 0.5 ml of media with fresh media (high density) or once a week with one or two drops of the high potassium medium (VLD).

Electrophysiological Techniques. The whole cell (WC) patch clamp technique was used to simultaneously record from monosynaptically connected pairs of HC neurons maintained in culture for 14–21 days (13, 19). The bath solution [Hepes-buffered saline (HBS)] contained 140 mM NaCl, 3 mM KCl, 10 mM Hepes-NaOH buffer, 10 mM glucose, and 4 mM $CaCl_2$. The internal pipette solution contained 135 mM potassium gluconate, 5 mM KCl, 10 mM Hepes, 1 mM EGTA, 1 mM $CaCl_2$, and 10 mM glucose. Inclusion of dNTPs and reverse transcriptase in the recording pipettes allowed amplification of cellular mRNA to be initiated during the recording (14–16).

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Abbreviations: GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; HC, hippocampal; aRNA, antisense RNA; VDL, very low density; WC, whole cell; PSC, postsynaptic current; IPSC, inhibitory PSC; EPSC, excitatory PSC; TH, tyrosine hydroxylase; CAT, choline acetyltransferase; NMDA, *N*-methyl-D-aspartate; ISH, *in situ* hybridization.

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Postsynaptic currents (PSCs) were considered monosynaptic when they followed a presynaptic action potential with a short (<4.0 msec), constant latency and exhibited no failures following an action potential. Identification of a postsynaptic current as either an inhibitory PSC (IPSC) or an excitatory PSC (EPSC) was based on both reversal potential and waveform (11).

Immunocytochemistry for GABA. HC cultures (14–21 days old) were washed in phosphate-buffer saline (PBS; 0.1 M), fixed in a 4% paraformaldehyde/PBS solution, washed, and then incubated in 0.02% Triton X-100. The cultures were then incubated in a 3% H_2O_2 solution to block staining of endogenous peroxidases. Following incubation in normal goat serum, the rabbit anti-GABA antiserum (Sigma) at a dilution of 1:10,000 in PBS was applied overnight at 4°C. The cultures were then incubated in biotinylated goat antirabbit-IgG secondary antibody (Vector Laboratories) for 1–2 hr, and specific staining was visualized by the ABC–peroxidase technique (Vector Laboratories) (20). As a control for nonspecific staining, the primary antibody was omitted for several coverslips.

Immunocytochemistry for GAD. High- and low-density cultures were washed and fixed as above, although the Triton X-100 step was omitted. Following incubation in horse serum as a blocking step, the GAD-6 monoclonal antibody was applied at a dilution of 1:10 overnight at 4°C (Developmental Studies Hybridoma Bank, Johns Hopkins University). The cultures were then incubated in biotinylated horse antimouse-IgG secondary antibody and visualized by the ABC–peroxidase method as above. Percentage of positively stained cells were determined by counting all cells within a ×20 microscope field across the diameter of the coverslip at least two times.

Amplification of Single Cell mRNA and PCR. After WC patch-clamp studies, cytoplasmic material of single inhibitory or excitatory cells were aspirated into the electrode which contained intracellular solution, as defined above and 0.5 unit/μl avian myeloblastosis virus–reverse transcriptase (20 units/μl), dNTPs, and oligo(dT)24-T7 (14). The electrode solution was then placed into an Eppendorf tube with an additional 20 μl of reaction mix containing 1 unit/μl of reverse transcriptase, dNTPs, and Hepes buffer at pH 8.3 with 120 mM KCl. The first strand cDNA was synthesized for 1 hr at 37°C; the second strand cDNA synthesis was performed at 14°C for 5 hr followed by S1 nuclease treatment and blunt ending. After 4 hr of drop dialysis, one tenth of the sample was used as template for aRNA amplification. The amplification reaction, using 1000 units of T7 RNA polymerase, was incubated for 4 hr at 37°C. Second round amplification was required for expression profiling. The first round aRNA was reverse transcribed into a single-stranded cDNA using random primers to synthesize a cDNA copy of the aRNA. Double-stranded cDNA was made by using the synthetic oligo(dT)-T7 sequence to prime second strand cDNA synthesis as previously described. This double-stranded cDNA was used for 2nd round amplification with radiolabeled CTP and was also used as a template for PCR analysis of GAD mRNA. Prehybridization and hybridization of slot blots containing GAD65 and GAD67 clones were carried out at 42°C for 36 hr with 50% formamide, 6× standard saline citrate (SSC), 50 μg of salmon sperm DNA per ml, 5× Denhardt's reagents, and 0.5% SDS. After hybridization, blots were washed at 42°C for 1 hr with 2× SSC and 0.1% SDS, and then 1 hr with 0.1× SSC and 0.5% SDS. Subsequently they were placed on x-ray film.

aRNA RT–PCR. The sequences of the PCR primers used for GAD65 amplification were 5′-CCT TTC CTG GTG AGT GCC ACA GCT GGA ACC-3′ (nucleotides 1059–1088) and 5′-TTT GAG AGG CGG CTC ATT CTC TCT TCA TTG-3′ (nucleotides 1628–1657). The size of the predicted PCR product was 599 bp. The sequences of the PCR primers used for GAD67 were 5′-TTT GGA TAT CAT TGG TTT AGC TGG CGA AT-3′ (nucleotides 762–790) and 5′-TTT TTG CCT CTA AAT CAG CTG GAA TTA TCT-3′ (nucleotides 1133–1162). The predicted size of PCR product was 400 bp. The sequences of the PCR primers used for tyrosine hydroxylase (TH) were 5′-CAA GAT CAA ACC TAC CAG CC-3′ (nucleotides 1637–1656) and 5′-TTG AGA AGC AGT GTT GGG AG-3′ (nucleotides 1133–1162). The predicted size of PCR product was 361 bp. The sequences of the PCR primers used for choline acetyltransferase (CAT) were 5′-ACT GAG TAC ACA GTC ATG GC-3′ (nucleotides 1702–1721) and 5′-TAG GGT CTG ACT TCT GTA GG-3′ (nucleotides 2168–2149). The predicted size of PCR product was 466 bp.

PCR for GAD was performed for 40 cycles under the following conditions: 93°C for 30 sec, 60°C for 30 sec, and 73°C for 30 sec. One twentieth of double-stranded cDNA after one round of aRNA amplification was used as a template, 300 ng of each GAD67 primer, and 1 μg of each GAD65 primer were used in each PCR. One third of each PCR was electrophoresed on a 1.3% agarose gel, and the products were detected by ethidium bromide staining. To confirm the identity of these PCR bands, the bands were blotted to nitrocellulose and probed with [γ-^{32}P]ATP-labeled internal primers GAD65 (5′-GGG CCA ACT CTG TGA CAT GGA ATC CCC ACA-3′, nucleotides 1231–1260) and GAD67 (5′-AGA AGT GAA AAC AAA AGG CAT GGC GGC TGT-3′, nucleotides 987-1016). PCR for TH and CAT were performed for 35 cycles, with striatal cDNA used as a positive control, under the following conditions: 1 time at 95°C for 5 min; 35 times at 93°C for 30 sec; 46°C for 30 sec; 72°C for 30 sec; and 1 time at 72°C for 10 min.

***In Situ* Hybridization for GAD65 and GAD67 mRNA.** RNA probes for rat GAD65 and GAD67 were synthesized by *in vitro* transcription by using a cDNA template containing the entire coding region of each probe (10). Transcripts were labeled with digoxigenin-11-UTP using the Genius kit and protocols (Boehringer Mannheim). High- and low-density HC cultures were washed in HBS, fixed in 4% paraformaldehyde/PBS, and permeabilized in 0.02% Triton X-100/PBS. The cells were then acetylated with 0.25% acetic anhydride in 0.1M triethanolamine, rinsed, and dehydrated in ascending concentrations of ethanol. Coverslips were incubated overnight at 55°C in a hybridization solution consisting of 50% formamide, 5× SSC (1× SSC = 150 mM NaCl/60 mM Na citrate, pH 7.0), 8% dextran sulfate, 1× Denhardt's solution, 5 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 500 μg of tRNA per ml, and 2.5 ng of RNA probe per μl. Following hybridization, cultures were washed in 4× SSC and incubated in 20 μg of ribonuclease A per ml at 37°C for 30 min. Stringency washes were performed with decreasing concentrations of SSC, ending with an incubation at 60°C in 0.1× SSC for 30 min. Following incubation for 1 hr at room temperature in a blocking buffer consisting of 0.1 M Tris·HCl (pH 7.5), 0.1 M NaCl, 2 mM $MgCl_2$, and 3% bovine serum albumin, cultures were incubated overnight at 4°C with anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim) diluted 1:500 in the above buffer. Signal was detected using the chromogenic substrates Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (both from Boehringer Mannheim) diluted according to manufacturer's instructions in a buffer consisting of 0.1 M Tris·HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$, 0.1% Tween-20 and 1 mM levamisole. After the colored precipitate had formed (≈2-3 hr), coverslips were refixed in 4% paraformaldehyde/PBS (to halt the color reaction) and mounted with 80% glycerol/0.1M Tris·HCl (pH 7.4).

RESULTS

Experiments were performed in primary dissociated VLD cultures of fetal hippocampus. Low density cultures were

chosen so that isolated, monosynaptically connected pairs of neurons (Fig. 1) could be studied with WC patch clamp techniques before subsequent single-cell amplification was performed (16, 21). Individual neurons were definitively identified as either excitatory or inhibitory based on the properties of PSCs evoked following a presynaptic action potential (Fig. 1) (13). We have extensively characterized evoked PSCs between neurons in this system, and have found that IPSCs are due exclusively to the release of GABA, the activation of postsynaptic $GABA_A$ receptors, and the opening of chloride channels (11). Similarly, EPSCs in these cultures have been shown to be due exclusively to the activation of postsynaptic glutamate receptors, both the NMDA and non-NMDA subtypes (11), coupled to the opening of cation channels. Distinction between EPSCs and IPSCs is readily accomplished by determining the equilibrium potential of the evoked PSC with gluconate as the main internal anion; IPSCs reverse around −60 mV, EPSCs reverse near 0 mV. In addition, the PSC waveforms are distinct. EPSCs have a dual component due to the activation of both non-NMDA and NMDA receptors.

Ten neurons were unequivocally identified as inhibitory and 6 as excitatory. The conversion of mRNA into amplified aRNA was initiated during the WC recording, and completed after each recording (16, 21). By analysis of the aRNA population of each of the 16 neurons identified as excitatory or inhibitory, it was possible to develop a "profile" of identifiable mRNAs whose relative relationship to one another in a given neuron was quantifiable.

Using the aRNA amplification technique, mRNA for GAD65 was found in *all* 16 neurons examined; the 10 inhibitory and the 6 excitatory neurons (Fig. 2*A* and Table 1). Since aRNA amplification is able to amplify mRNAs in a linear manner to approximately the same extent (22), the autoradiographic intensity of the hybidization bands in slot blots reflects the relative abundance of the original mRNAs. In each of the analyzed neurons, GAD65 mRNA was present at a relatively high level compared with other mRNAs, such as that for brain-derived neurotrophic factor. GAD67 mRNA was not detected above background using the slot blot technique (Table 1).

As a second method for independently showing the presence of the GAD mRNA in the aRNA population, PCR was performed on the aRNA population. The aRNA was reverse transcribed into cDNA that served as the template for the PCR studies. In this manner, multiple PCR reactions can be performed on the aRNA from a single cell; indeed, 1/200 of the amplification products of a single cell was used in each of these reactions. PCR confirmed the identity of the GAD65 mRNA (PCR band, 599 bases) in 14/16 cells; it was equivocal in the two remaining neurons (one inhibitory and one excitatory neuron), possibly because the amplified product was too short for the primers used, which would not interfere with the ability

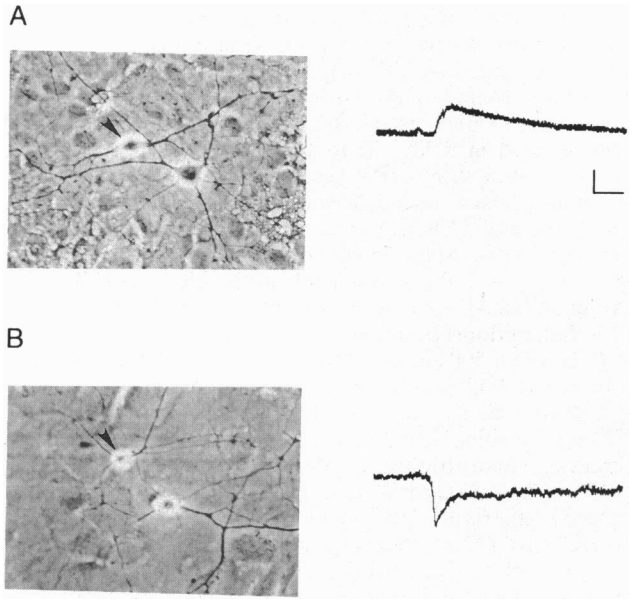

FIG. 1. Identification of inhibitory and excitatory neurons in VLD HC cultures. (*A*) Two isolated HC neurons that were simultaneously recorded using the WC patch clamp technique. An action potential in the presynaptic neuron (arrowhead) evoked an outward current at a holding potential of −50 mV, indicating that it was an IPSC mediated by the presynaptic release of GABA. The trace is an average of four evoked IPSCs. (*B*) Pair of neurons whose presynaptic cell (arrowhead) was identified as an excitatory cell; the evoked postsynaptic current (average of four evoked EPSCs) was inward at hyperpolarized potentials and had a characteristic biphasic waveform. This type of current has been found to be due to the activation of two types of colocalized postsynaptic glutamate receptors, non-*N*-methyl-D-aspartate (NMDA) (α-amino-3-hydroxy-5-methyl-4-isoxazolepropidnic acid) and NMDA. Both the excitatory and inhibitory neurons from this figure were found to contain relatively large amounts of mRNA encoding for GAD65. (Calibration = 200 pA and 10 msec.)

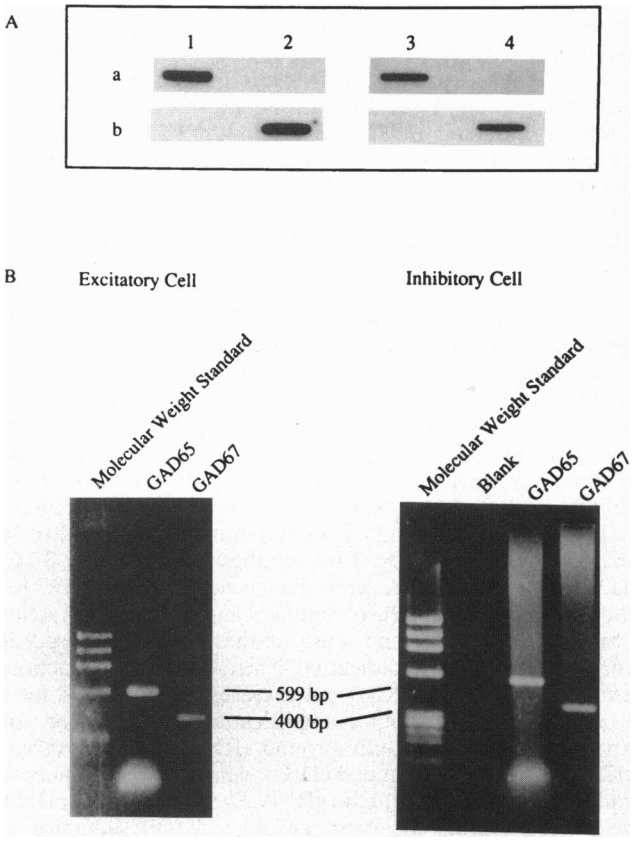

FIG. 2. Presence of GAD mRNAs in excitatory and inhibitory neurons. aRNA amplification was used to examine the mRNA population in individual cells. (*A*) Slot blots containing equal amounts of cDNAs were probed with ^{32}P-labeled second round aRNA. Representative expression profiles for an excitatory cell (*Left*) and an inhibitory cell (*Right*) are illustrated. *a1* and *a3*, GAD65; *a2* and *a4*, GAD67 (which was not detectable with blot hybidization at this level of sensitivity); *b1* and *b3*, pBS (Bluescript plasmid, negative control); *b2* and *b4*, brain-derived neurotrophic factor (positive control). (*B*) PCR products for GAD mRNAs in a single excitatory cell and an inhibitory cell analyzed on a 1.3% agarose gel. Lanes for the excitatory cell: 1, molecular weight standard (ϕX174 RF DNA/*Hae*III); 2, PCR product for GAD65; 3, PCR product for GAD67. Lanes for the inhibitory cell: 1, molecular weight standard; 2, blank well; 3, PCR product for GAD65; 4, PCR product for GAD67. Note that PCR detects both forms of GAD mRNA and is nonquantitative, whereas the quantitative slot blots only detected relatively large amounts of mRNA for GAD65.

Table 1. Comparative results of different single cell mRNA assays performed on identified neurons

	Inhibitory HC neuron (n = 10)	Excitatory HC neuron (n = 6)	CA1 pyramidal cell (n = 5)
GAD65 by aRNA hybridization	9	6	5
GAD65 by PCR from aRNA	10	5	5
GAD67 by aRNA hybridization	0*	0*	0*
GAD67 by PCR from aRNA	10	6	5
TH by PCR from aRNA	0	0	ND
CAT by PCR from aRNA	0	0	ND

ND, not done.
*None detected at this level of sensitivity.

of the aRNA to hybridize to a GAD65 cDNA (Fig. 2*B*). In addition, PCR of the aRNA detected GAD67 (PCR band, 400 bases) in all 16 neurons (Table 1). Thus, using PCR, we were able to confirm the results obtained with the aRNA amplification, that all the neurons contained GAD mRNA, either for one or both isoforms. As positive controls for the PCR amplification step, plasmid cDNAs of GAD65 and GAD67 were used, while at the same time incubations of reagents without template were used as negative controls to eliminate the possibility of reagent contamination.

As a further control for PCR specificity, oligonucleotides internal to the PCR primers for each of the GAD sequences were synthesized and end labeled with [γ-^{32}[P]ATP. PCR bands were transferred to nitrocellulose and hybridized with these internal oligonucleotide probes. Hybridization was only detected in the positive control lanes and not detected in the negative control lanes. This ruled out the possibility of low level contamination.

To determine if the finding of mRNA for GAD was relatively specific for this enzyme we examined the amplified aRNA for the presence of both TH and CAT by PCR. Neither of these mRNAs was found in any of the HC neurons (Table 1), although simultaneous PCR of striatal aRNA revealed the presence of aRNA for both TH and CAT. This indicates that the presence of mRNA for neurotransmitter synthesizing enzymes was not a universal property of our cultured HC neurons, and that the presence of mRNA encoding for GAD was selective.

We next examined both low-density and high-density cultures to determine whether all the neurons expressed either GAD protein or GABA. Our previous work on identifying GABA utilizing neurons in both high-density neocortical (23) and HC (24) cultures indicated that ≈33-50% of the neurons were GABAergic. When our low-density cultures were stained for GABA (20), positive staining was noted in ≈35-58% of the neurons in different cultures (Fig. 3*A* and Table 2). In high-density cultures from the same dissections, a range of 34–41% of the neurons stained positively for GABA. When low-density cultures were stained for GAD with the antibody, GAD-6, which recognizes GAD65, 31–46% of neurons were positive (Fig. 3*B*); high-density cultures had 42–58% positive (Table 2). Despite the fact that 100% of the neurons that we analyzed contained significant amounts of GAD mRNA, it was clear that many of the neurons in these cultures were not producing detectable amounts of GAD protein or GABA.

To determine whether our observations about the expression of GAD mRNA in both excitatory and inhibitory neurons was unique to cultured neurons, we analyzed the aRNA of individual CA1 pyramidal neurons from HC slices (25). As with the cultured neurons, GAD65 mRNA was observed in all the CA1 pyramidal neurons examined with slot blot hybridization (Fig. 4) while GAD67 mRNA was generally not detected with this technique. However, GAD67 mRNA was detected in 5 of 5 CA1 pyramidal neurons with PCR from the amplified aRNA. The specific HC neurons analyzed were not identified in these experiments as excitatory with physiological techniques, but it is generally believed that CA1 pyramidal cells are predominantly excitatory. Moreover, immunohistochemical staining for GAD (2) and GABA has shown CA1 pyramidal cells to be mostly negative. Thus, neurons in the central nervous system that are thought to be excitatory and that do not stain for either GAD or GABA, do, in fact, contain mRNA for this synthetic enzyme.

Our findings of GAD mRNA in CA1 pyramidal neurons in slices raised an important issue. Prior studies had failed to demonstrate GAD mRNA by *in situ* hybridization (ISH) in these neurons (2, 8, 26–29). We therefore performed ISH on the cultured HC neurons, using both antisense and sense full-length probes of GAD65 and GAD67 mRNA, to determine if the GAD mRNA could be detected by this methodology. Antisense probes for GAD65 labeled ≈31% and 18% of neurons in the high and low density cultures, respectively (Fig. 5) and Table 2); in both cases smaller fractions than labeled by immunohistochemistry for GAD65 (46% high and 30% low density) and GABA (61% high and 29% low density) in sister

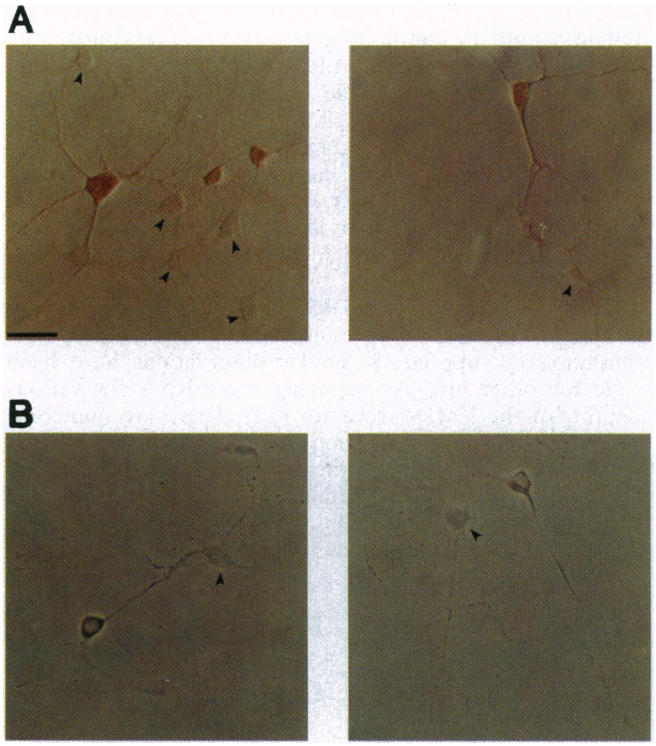

FIG. 3. Immunohistochemical staining for GABA and GAD showing both positive and negative cells. (*A Left*) HC neurons in high density culture stained for GABA-immunoreactivity. Arrowheads in this and all panels indicate unstained neurons. (*Right*) Similar stain for GABA-immunoreactivity in VLD cultures from the same culture preparation. (*B*) *Left* and *Right* demonstrate HC neurons from VLD cultures stained with monoclonal antibody, GAD-6. Note positive neurons and positive synaptic boutons contacting soma and processes of negative neurons. (Bar = 30 μm.)

Table 2. Immunohistochemistry for GABA and GAD and *in situ* hybridization for GAD65

Culture types	Immunohistochemistry for GABA	Immunohistochemistry for GAD	*In situ* hybridization for GAD65
High density	34–50% positive in 15 cultures	42–58% positive in 6 cultures	31% (23–40%) positive in 4 cultures
VLD	35–58% positive in 10 cultures	31–46% positive in 12 cultures	18% (14–27%) positive in 2 cultures

cultures from the same dissection. Antisense probes for GAD67 labeled approximately the same fraction of cells, and when both probes were used, only ≈30% of neurons in the high density cultures were labeled (i.e., labeling by the two probes was *not* additive). Positive neurons showed labeling most heavily in the soma, but proximal and even some distal processes also demonstrated labeling. No labeling was noted with sense probes. Thus, ISH does not detect mRNA for GAD in all the neurons in which it is demonstrated with the aRNA amplification technique, either in the cultures or in tissue sections. ISH also does not demonstrate mRNA for GAD in all the cells which make GAD protein and GABA.

DISCUSSION

Our results indicate that neurons which are unequivocally identified as excitatory by electrophysiological methods contain mRNA for the enzyme that synthesizes the inhibitory neurotransmitter, GABA. The relative amounts of GAD65 mRNA were not noticeably different for the excitatory or inhibitory neurons. Our results also indicate that CA1 pyramidal neurons in the HC slice preparation, which are thought to be excitatory, also contain GAD65 and GAD67 mRNA. Thus, it does not appear that this observation is either an artifact of culture or a property of immature or developmentally arrested neurons. The negative control PCR experiments make it unlikely that our results are the result of an inadvertent contamination. In addition, some mRNAs are consistently absent in the neurons (e.g., mRNA for TH, CAT, and glial fibrillary acidic protein) while other mRNAs are found at varying levels within a single cell, suggesting that our technique is not simply amplifying genomic DNA. Finally, in an independent set of experiments, another group in one of our laboratories has made a similar observation of GAD mRNA in cerebellar granule cells grown in culture, neurons thought to be excitatory (L. Nowak and J. Eberwine, personal communication).

The inability to detect GAD65 protein in all neurons where GAD65 mRNA is clearly present suggests that the mRNA is translationally suppressed. Similar observations have been made for other mRNAs including the mRNA for various subunits of the NMDA receptor (30). There are numerous mechanisms by which translation of mRNA into protein can be regulated. An attractive hypothesis for GAD expression is that there is an RNA binding protein that interacts with the mRNA to either stimulate or inhibit translation. This hypothesis suggests that during development when a cell is determined to become excitatory, an RNA binding protein either turns on and binds to GAD mRNA to inhibit translation or an RNA binding protein that would normally stimulate GAD translation ceases to be expressed so that translation is inhibited. Regulation of the expression of such RNA binding proteins would facilitate the possible intermittent expression of GAD protein in excitatory cells. While there is no evidence for the existence of such RNA binding proteins in our experiments, these hypotheses are testable.

The results from the aRNA amplification procedure are somewhat different from those of ISH from the same cultures. With the former technique, GAD65 mRNA is found in all the neurons assayed, whereas with the latter, only a subset of neurons is labeled. Although we did not perform double labeling for GAD and ISH, it is presumed that the inhibitory neurons that are positive for GAD and GABA would be the ones also labeling with ISH. A similar discrepancy is found in CA1 pyramidal neurons in the intact hippocampus. The aRNA amplification demonstrates the presence of relatively large amounts of GAD mRNA, but ISH fails to detect GAD mRNA in these cells (26–29). It is not likely that this is simply a quantitative issue, as with the aRNA amplification procedure, GAD65 mRNA appears to be present in amounts that are comparable to other abundant mRNAs in these neurons. One possible explanation for these differences in sensitivity of two techniques is that a common mechanism, such as RNA binding proteins attached to the mRNA, would prevent translation of the GAD65 mRNA and block ISH. Precedence exists for such a mechanism in other systems (31). This raises the important question as to whether ISH only detects mRNAs in particular functional states.

The relative abundance of GAD65 mRNA in neurons that normally do not utilize GABA as a neurotransmitter suggests that GAD may have a functional role in such neurons. In many cells, including neurons, GABA may participate as an intermediate in energy metabolism as part of the "GABA shunt." It is possible that the GAD mRNA is translated intermittently to supply enzyme to metabolize excess glutamate to GABA when the GABA shunt is overactive.

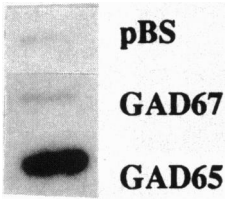

FIG. 4. Presence of GAD65 mRNA in CA1 pyramidal neurons from HC slices. Slot blots for GAD65, GAD67, and plasmid control from amplified aRNA from a single CA1 pyramidal neuron. Note that GAD65 mRNA is abundant but that GAD67 mRNA is not detectable at this level of sensitivity by this technique. However, PCR for GAD67 mRNA from the amplified aRNA did demonstrate the presence of this form in the CA1 pyramidal neurons.

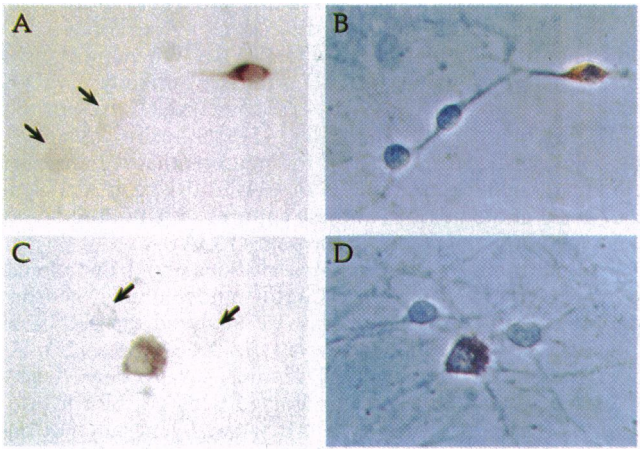

FIG. 5. Bright field (*A* and *C*) and phase contrast (*B* and *D*) photomicrographs of ISH for GAD65 mRNA in low density (*Upper*) and high density (*Lower*) HC cultures. Note that in *A* and *C* one heavily labeled neuron is present and two negative neurons (arrows) are noted.

In addition to its role as a neurotransmitter, GABA is known to act as a trophic substance during development (32–34). It is possible that neurons maintain the capacity to synthesize and secrete GABA, perhaps only in localized portions of the neuron, as a mechanism for maintaining some forms of synaptic connections or for maintaining or altering dendritic architecture. The recent observation of abundant mRNAs with differential distribution in dendrites (15) could provide a mechanism for such localized molecular signaling. Alternatively, the capacity to rapidly synthesize GABA without requiring transcription of the synthetic enzyme, could be part of the neuron's defense against injury. GABA may reduce excitotoxic damage and could be "neuroprotective" under other circumstances as well.

Finally, one has to consider the possibility that neurons intermittently change their neurotransmitter phenotype in response to physiological stimuli—a "standby neurotransmitter" hypothesis. It is well known that neurons can change their phenotypes during development and in response to denervation or injury (35, 36), but this has been attributed to transcriptional events. Neurons may contain more than one neurotransmitter, often a neuropeptide and an amine, but possibly even two neurotransmitters such as GABA and dopamine (37). Our data suggest that a rapid, and possibly transient, phenotypic regulation based on posttranscriptional controls may in fact occur. This could occur throughout the neuron or be localized to particular neurites or axon branches. Such alterations in phenotype could be a component of normal circadian rhythms or result from cyclic hormonal changes. Standby neurotransmitter functioning could be part of transient neuronal plasticity in response to specific activation of local pathways. It could also be a mechanism for transiently dampening excitability in a localized region of the central nervous system after prolonged and intense activation (e.g., after epileptic seizures or in response to excitotoxic damage). Consistent with this latter hypothesis is a recent observation of a possible change in phenotype of HC granule cells in response to recurrent seizures (38). Mossy fibers that arise from dentate gyrus granule cells and synapse on CA3 pyramidal cell dendrites are excitatory (39). The somata of the dentate gyrus granule cells have not been demonstrated to stain for either GABA or GAD65 and appear not to hybridize with GAD probes. It has been controversial as to whether the mossy fiber terminals contain small amounts of GABA, in addition to their excitatory neurotransmitter (40–42). However, new immunohistochemical procedures have demonstrated that the granule cells do contain GAD67 and GABA constituitively, and both of these are dramatically increased in only these cells within the hippocampus, after seizure activity (38).

The data presented in this paper demonstrate that excitatory neurons in HC cultures contain GAD mRNA, but do not express detectable GAD protein or GABA and do not utilize GABA during synaptic activity. Nor do the neurons in culture express mRNA for other neurotransmitter synthesizing enzymes. Further investigations into possible translational control of GAD mRNA and the factors that activate or inhibit GAD expression at the cellular level are required to determine the functional consequence of the presence of GAD mRNA in many excitatory neurons. The implications of this finding for our understanding of translational control of mRNA in neurons and the functional role of translational control in central nervous system development and plasticity, as well as the regulation of neuronal phenotype, are currently being addressed.

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