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Expression of the γ-Aminobutyraldehyde Dehydrogenase in the Bovine Brain by Immunohistochemistry and *in Situ* Hybridization Methods

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γ-Aminobutyrate (GABA), a four carbon amino acid, was discovered in brain tissue in 1950. GABA is an inhibitory neurotransmitter in the mammalian CNS. GABA is produced mainly through enzymatic decarboxylation of glutamic acid by glutamic acid decarboxylase (GAD), and synthesized by the degradation of putrescine (1,4-diaminobutane), one of the polyamines. It provides γ-aminobutyraldehyde (γ-ABAL), substrate for the synthesis of GABA by γ-aminobutyraldehyde dehydrogenase (γ-ABALDH). We purified γ-ABALDH, another synthetic enzyme for GABA, from the bovine brain and prepared an antibody for γ-ABALDH from rabbits. We also attempted the NH_2-terminal sequencing of γ-ABALDH and were successful in producing the oligonucleotide probes based on the N-terminal sequence determined. 36 mer-oligonucleotides complementary to the mRNA were synthesized, and radiolabelled at the 5′end with ^{32}P. Then an *in situ* hybridization technique was applied to detect γ-aminobutyraldehyde dehydrogenase mRNA in the bovine brain tissue. Cells containing mRNA of γ-ABALDH were found in many regions of bovine brain tissue, the results being similar to those from immunostaining.

Putrescine (1,4-diaminobutane), one of the polyamines, is degraded into γ-aminobutyric acid (GABA) via the intermediate γ-aminobutyraldehyde (γ-ABAL) (Pegg and McCann, 1982; Tabor and Tabor, 1984). This is one of the pathways for GABA synthesis (Grillo, 1985). GABA is known to be the major inhibitory neurotransmitter in the mammalian central nervous system. The degradation pathway of putrescine has been detected in rat brain and liver (Jänne *et al.*, 1978; Abe *et al.*, 1990), mouse brain (Mach *et al.*, 1982), and some other tissues (Grillo, 1985). The physiological importance of the conversion of putrescine into GABA is, however, not yet well-understood. But this pathway suggests that GABA and polyamine metabolisms are closely associated (Fig. 1) (Seiler *et al.*, 1979; Seiler, 1980). γ-Aminobutyraldehyde dehydrogenase catalyses the dehydrogenation of ABAL to GABA specifically in the bovine brain (Lee and Cho, 1992). Aldehyde dehydrogenase catalyses the irreversible oxidation of a broad range of aldehydes to the corresponding acids in an NAD^+-dependent reaction (MacKerell and Pietruszko, 1987). Thus, the substrate specificity of this enzyme is broad. γ-Aminobutyraldehyde dehydrogenase has been known to be one of the isozymes of the aldehyde dehydrogenase in the human liver (Kurys *et al.*, 1989). In order to study the importance of the pathway that synthesizes GABA from putrescine, we studied and first reported the results of purification and characterization of γ-aminobutyraldehyde dehydrogenase from bovine brain (Lee and Cho, 1992).

γ-Aminobutyrate (GABA) is produced mainly through enzymatic decarboxylation of glutamic acid by glutamic acid decarboxylase (GAD) (Wu, 1973; Roberts, 1979), but as mentioned previously it is also synthesized by the degradation of putrescine (Seiler and Al-Therib, 1974; Ambroziak and Pietruszko, 1991; Caron *et al.*, 1988). Recently, antibodies against GABA itself have been produced (Storm-Mathisen *et al.*, 1983; Hodgson *et al.*, 1985). Antibodies to GABA could be used clearly to reveal the GABAnergic axon terminals and GABA-containing neurons. GABA-containing neurons could be observed in certain brain areas such as the neocortex, thalamus, hippocampus and cerebellar cortex (Ottersen and Strom-Mathisen 1984; Somogyi *et al.*, 1985). Localization of GABA and GAD in brain is well known, but that of γ-ABALDH, another synthetic enzyme for GABA, has been not yet reported.

In the present paper we first describe the distribution of γ-ABALDH in the bovine brain using the immunohistochemical method and *in situ* hybridization.

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The abbreviations used are: γ-ABAL, γ-aminobutyraldehyde; γ-ABALDH, γ-aminobutyraldehyde dehydrogenase; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase.

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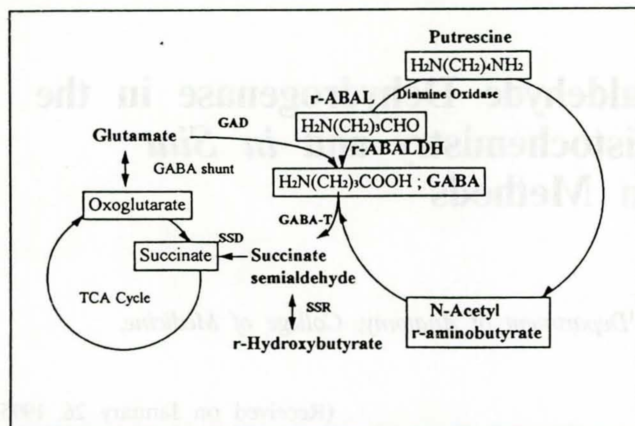

Figure 1. Scheme of putrescine catabolism via γ-aminobutyric acid. GAD, glutamate decarboxylase; γ-ABALDH, γ-aminobutyraldehyde dehydrigenase; GABA-T, GABA transaminase; SSD, succinic semialdehyde dehydrogenase; SSR, succinic semialdehyde reductase.

To our knowledge this is the first such documentation for a bovine brain. These studies are also a necessary prerequisite for further physiological and biochemical studies of the enzymes responsible for formation of neurotransmitters.

Materials and Methods

Purification and enzyme assay of the γ-aminobutyraldehyde dehydrogenase

About 400 g of bovine brain tissue kept frozen at −70 ℃ was homogenized in 4 volume. of 50 mM K^+-phosphate buffer, pH 7.0, containing 1 mM 2-mercaptoethanol. The homogenate was centrifuged at 10, 000 × *g* for 50 min, and a clear supernatant was obtained. Aldehyde dehydrogenase with low Km for γ-aminobutyraldehyde from bovine brain was purified by using CM-trisacryl, DEAE-Sephacel, and 5′-AMP-Sepharose affinity chromatography (Lee and Cho, 1992). A homogeneous enzyme was subjected to additional chromatography - fast protein liquid chromatography (FPLC) on a Mono-Q column (Phamacia) - in order to separate the major and the minor components. Fractions were pooled according to activity and then concentrated. During purification, enzyme activity was assayed in a solution containing a 0.1 M K^+-phosphate buffer (pH 8.0), 5 mM NAD^+, 0.5 mM dithiothreitol (DTT), and 2 mM γ-ABAL. All assays were performed spectrophotometrically at 340 nm (Jakoby and Fredericks, 1959). Protein concentration was determined as described by Lowry *et al.* (1951) with bovine serum albumin as a standard, or spectrophotometrically at 280 nm.

Preparation of antibodies to γ-aminobutyraldehyde dehydrogenase

Anti-γ-ABALDH antiserum was prepared from a

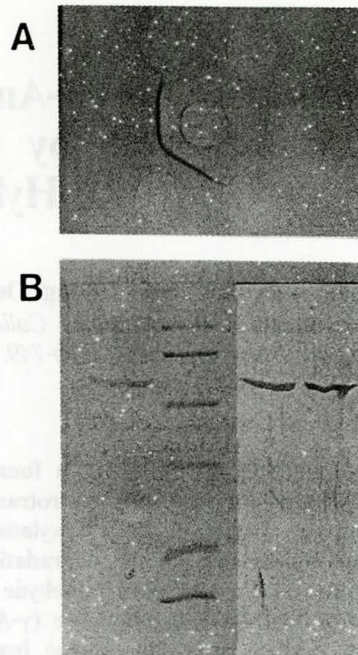

Figure 2. A: Ouchterlony double immunodiffusion. Center well, purified γ-aminobutyraldehyde dehydrogenase; 1, anti γ-aminobutyraldehyde dehydrogenase (20 μl); 2, 1/2 dilution of 1; 3, 1/10 dilution of 1; 4, 1/100 dilution of 1; 5, 1/200 dilution of 1; 6, 1/1000 dilution of 1. B: Western blot immunostaining. Lane 1, Purified γ-aminobutyraldehyde dehydrogenase; lane 2, marker proteins; lanes 1, 2 stained by coomassie brilliant blue R-250; lanes 3, 4, immunostaining by rabbit anti-bovine γ-aminobutyraldehyde dehydrogenase.

rabbit immunized subcutaneously with γ-ABALDH. Serum samples were tested by immunodiffusion. Purified enzyme was applied to the central hole of the agarose plate, and the antibodies diluted progressively were applied to the surrounding holes. The precipitates of the antigen-antibody reaction were identified (Fig. 2A). Antisera was purified using a protein A column (Harlow and Lane, 1988).

Western blotting

Proteins were separated on SDS-PAGE and transferred to nitrocellulose (NC) membranes (Towbin *et al.*, 1979). After transfer, nitrocellulose membranes were washed in TBST solution (10 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, 0.5% Tween 20) thoroughly. They were incubated in the blocking solution (1% BSA in TBST) for 30 min. Primary antibodies were added to the blocking solution, and incubated for 30 min. After being washed, membranes were incubated in the solution containing secondary antibodies, and stained with a color development solution. The color development solution was composed of 100 mM Tris-HCl buffer (containig 100 mM NaCl and 5 mM Mg_2Cl) 10 ml, nitro blue tetrazolium (NBT) solution (50 mg/ml) 66 μl, and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution (50 mg/ml) 33 μl.

Staining of the membranes was stopped at the point where they had a desirable color density, whereupon they were washed (Johnson *et al.*, 1984). Western blotting of the enzyme purified from bovine brain confirmed that the antibodies formed in rabbits were against the purified enzyme (Harlow and Lane, 1988).

Antigenicity measurement of the γ-aminobutyraldehyde dehydrogenase antibody using the ELISA test

Purified γ-aminobutyraldehyde dehydrogenase · 1 mg/ml was diluted in the coating buffer (0.05 M carbonate buffer, pH 9.6), applied to the 96 plate tissue well and incubated in a 37 ℃ oven for 2 h. Antibodies 50 μg/ml were diluted with 0.01 M PBS (pH 7.4) appropriately, diluted primary antibodies 100 μl were applied to the tissue well and incubated in a 37 ℃ oven for 1 h. After antibodies were washed, the tissue well was incubated with secondary antibodies 25 μg/ml in a 37 ℃ oven for 1 h, and with PAP solution for 1 h. Ortho-phenylene diamine (OPD) 25 g was added to 50 ml phosphate-citrate buffer (pH 5.0), and H_2O_2 was added just before use. After the reaction progressed in a dark room for 30 min, it was stopped with H_2SO_4 (Kuno and Kihara, 1967). Then, we estimated the antigenicity of each portion of bovine brain using a Dynatech MR-300 ELISA reader.

Tissue preparation for light microscope observation and in situ *hybridization*

Isolated bovine brains were immersed immediately in a fixation solution (4% paraformaldehyde solution, 0.1 M phosphate buffered, pH 7.4), and reserved for 20 h at 4 ℃. In order to prevent them from being exposed to RNAse, all solutions were made with distilled DEPC-water during tissue preparation procedure for light microscopic observation and *in situ* hybridization . Tissues were trimmed refering to the atlas of Lauder (1982). After being dehydrated and cleared, they were embedded in paraplast. Tissues were sectioned in 4 μm thickness, spread on DEPC-water within warmer, and placed on a slide treated with gelatin. The gelatin solution was also made distilled DEPC-water.

Immunoperoxidase staining

Tissues were stained by immunoperoxidase staining methods (Sternberger, 1986). Before staining, tissues were pretreated in 3% hydrogen peroxide solution for 10 min and in 10% normal goat serum (NGS, Gibco Lab., New York) for 1 h at room temperature, and washed with 0.1 M PBS (pH 7.4, containing 0.1% Triton X-100). They were incubated in PBS solution containing antibovine rabbit γ-aminobutyraldehyde dehydrogenase antibody for 72 h at 4 ℃, and washed. They also were incubated in a solution containing goat antirabbit IgG (secondary antibody) for 2 h, and in PAP (peroxidase-antiperoxidase) solution for 1 h at room temperature. They were stained by 0.05% diaminobenzidine (DAB, in 0.05 M tris-HCl, pH 7.6, containing 0.01% hydrogen peroxide) and washed with distilled water. Tissue slides were made according to general tissue preparation procedures. All incubation steps were performed in a humidified chamber.

Synthesis of the oligonucleotide probe

Oligonucleotide probes were supplied by the KIST and synthesized on an automatic DNA synthesizer (Applied Biosystems 380, Dupont Coder 300). Oligonucleotide probes were made based on the N-terminal sequence (Fig. 5). 36 mer oligonucleotides complementary to the mRNA were synthesized (Lathe, 1990).

Direct phosphorylation of the 5′-hydroxyl groups was performed by a standard method (Sambrook *et al.*, 1989) in which γ-ATP (Amersham, 3000 μCi/mmole) and T4 polynucleotide kinase (New England Biochemicals) were used. Following labelling at 37 ℃ for 2 h, the reaction mixture was applied to a Sephadex G-50 (Pharmacia, superfine) in order to remove unincorporated [^{32}P]dATPs.

In situ *hybridization*

Tissue slides were deparaffinized and hydrated through gradual concentrations of ethanols. Slides were washed with 2× SSC solution which had been set at 60 ℃ the previous day for 10 mins and repeated 3 times. They were preincubated in hybridization buffer (1× Denhart's solution, 2× SSC, 146 μg/ml denatured salmon sperm DNA, 0.2 mg/ml yeast tRNA) for 2 h at room temperature. ^{32}P-labeled γ-aminobutyraldehyde dehydrogenase oligonucleotide probes 10^4 cpm/μl were added to the slides, and incubated in a humidified chamber for 1 or 2 days at 40 ℃. After incubation, they were washed with 2× SSC solution which had been set at 60 ℃ previous day 3 times for 30 min, with 0.5× SSC solution three times for 30 min, and with 0.1× SSC solution three times for 30 min. Slides were dehydrated through a graded series of ethanols and air-dried at room temperature (Sambrook *et al.*, 1989).

Autoradiography

After washing and dehydration, slides were equipped with a standard X-ray film cassette. For a higher-resolution image, they were exposed in a Hyperfilm β-max film (Amersham) for 2 days at room temperature, developed in a Kodak D-19 developer and fixed in a Kodak fixer. Then the slides were dipped in a Kodak NTB_2 emulsion (diluted to 1 : 1 with water), exposed for 7-10 days at 4 ℃, developed and fixed (Brady and Finlan, 1990).

Slides were counter-stained with hematoxylin-eosin solution.

Results and Discussion

Characterization of antibodies to γ-aminobutyraldehyde dehydrogenase

The anti-γ-ABALDH antiserum was first obtained

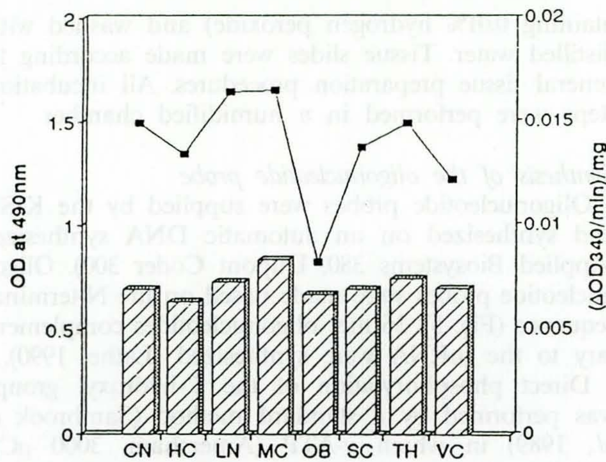

Figure 3. Comparison of the specific activity and antigenicity against anti γ-aminobutyraldehyde dehydrogenase in the regions of the bovine brain. CN, caudate nucleus; HC, hippocampus formation; LN, lentiform nucleus; MC, motor cortex; OB, olfactory bulb; SC, primary sensory cortex; TH, thalamus; VC, visual cortex. ■—■, specific activity; ▨, antigenicity against anti-γ-aminobutyraldehyde dehydrogenase.

from rabbits immunized subcutaneously with γ-ABALDH. From the immunodiffusion results, the precipitates of antigen-antibody reactions were identified (Fig. 2A). Western blotting of the enzyme purified from bovine brain confirmed the antibodies formed in the rabbits to be against the enzyme (Fig. 2B).

We obtained the motor cortex, primary sensory cortex, visual cortex, olfactory bulb, hippocampus, caudate nucleus, lentiform nucleus, and thalamus from the bovine brain, homogenized these tissues, and measured the activity of the enzyme and its antigenicity using ELISA (Fig. 3). The highest relative absorbance was obtained from the motor cortex, whereas the lowest value of 0.63 was recorded for the hippocampal formation. Concentration of the γ-aminobutyraldehyde dehydrogenase was distributed relatively evenly in the investigated tissues. The cross-reactivity of the antiserum against γ-aminobutyraldehyde dehydrogenase was investigated using Ouchterlony double diffusion. The antiserum reacted to form complexes with the enzymes contained in the rat liver, the bovine liver and rat brain. It also formed partially homologous complexes with the enzymes in the tissue of the adrenal gland, kidney, and spleen of the rat.

These results, therefore, indicate that the tissues of bovine brain, bovine liver, rat brain and rat liver have equal antigenicities, while those of rat adrenal gland, kidney, and spleen have partially equal antigenicities with the bovine brain tissue.

Localization and regulation of the γ-aminobutyraldehyde dehydrogenase

The distribution pattern of the γ-ABALDH in bovine CNS was determined by immunoperoxidase staining. The tissue slices of bovine motor cortex, primary sensory cortex, visual cortex, olfactory bulb, hippocampus, caudate and lentiform nuclei, and diencephalon with reference to Lauder (1982) were stained, and the immunoreactive cells were found to be present in all tissues. The immunoreactivity of these cells, which were usually found to be neurons, was not confined to only within the nucleus, but also was found to be present outside of the nucleus.

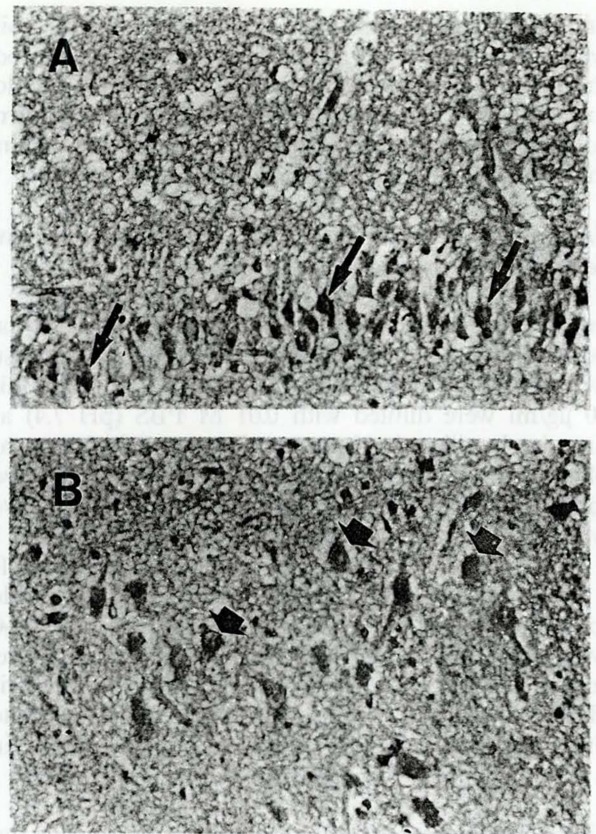

Figure 4. γ-Aminobutyraldehyde dehydrogenase-immunoreactive cells in bovine brain. A, olfactory bulb; B, thalamus; Immunoperoxidase stain, × 400.

In immunohistochemical staining, anti-γ-ABALDH immunoreactive neurons were found in many regions of bovine brain tissues such as the precentral gyrus (motor cortex), postcentral gyrus (primary sensory cortex), occipital cortex (visual cortex), olfactory bulb, hippocampus, thalamus, caudate nucleus, and lentiform nucleus. Anti-γ-ABALDH immunoreactive cells were mostly small- and medium-sized neurons (Fig. 4), generally known as the interneurons and projection neurons (Ottersen and Strom-Mathisen 1984; Somogyi *et al.*, 1985). These results coincided with the previous reports that most inhibitory neurons are interneurons (Larsson *et al.*, 1984). Since GABA is synthesized mostly by the action of glutamate decarboxylase (GAD), the localization of GABA was investigated by the anti-GAD antibody (Wu *et al.*, 1973; Oetel *et al.*, 1983; Larsson *et al.*, 1984). Recently, an antibody preparation method for small molecules has been developed,

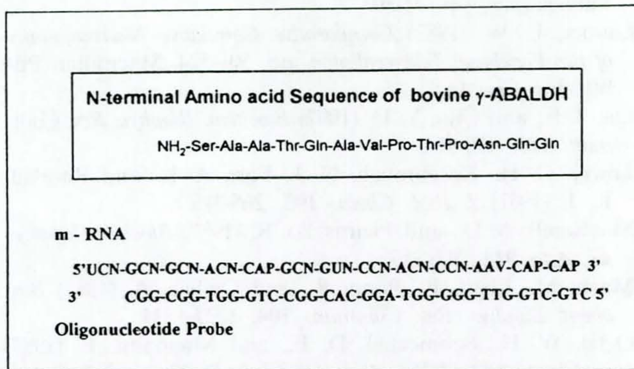

Figure 5. The N-terminal sequences of γ-aminobutyraldehyde dehydrogenase and prepared oligonucleotide sequence (Farres *et al.*, 1989; Lee and Cho, 1992).

and we are now able to identify GABA itself in the tissue using GABA antibodies (Hempel *et al.*, 1984; Otterson and Strorm-Mathisen, 1984; Hodgson *et al.*, 1985; Hougaard *et al.*, 1987). From the immunostaining results, γ-ABALDH was found to also exist in GABAnergic neurons, leading to another synthetic pathway. But it is not clear whether the γ-ABALDH-immunoreactive cells are the cells with GAD-immunoreactivity. It will be possible to identify such things with double-immunohistochemical staining methods for glutamate decarboxylase and γ-aminobutyraldehyde dehydrogenase, and this we have saved for the next project. GABAnergic neurons are modulated by other GABAnergic neurons (Wu *et al.*, 1973). We think that another synthetic pathway of GABA from putrescine is also important for the synthesis of GABA, and that the studies on the distribution of γ-aminobutyraldehyde dehydrogenase are helpful in understanding the regulation of GABA metabolism.

The activity of γ-aminobutyraldehyde dehydrogenase was inhibited by high concentrations of GABA. In a group of rats treated with glutamate decarboxylase inhibitors at 2 and 4 days after birth, γ-aminobutyraldehyde dehydrogenase appeared to be induced by glutamate decarboxylase inhibitors.

Putting all these results together, it was determined that GABA synthesis is regulated elaborately, and that the two synthetic pathways have effects on each other.

Cellular localization of γ-aminobutyraldehyde dehydrogenase transcripts

In situ hybridization technique is used for tracing the regional and cellular sites of gene expression within the tissue (Gee *et al.*, 1983). It allows the specific cell types expressing a given gene to be delineated from the other cells. We attempted the NH_2-terminal sequencing of γ-ABALDH, produced the oligonucleotide probes based on the N-terminal sequence, and investigated celluar localization of γ-aminobutyraldehyde dehydrogenase transcripts. We have described our methods for NH_2-terminal sequencing in a previous

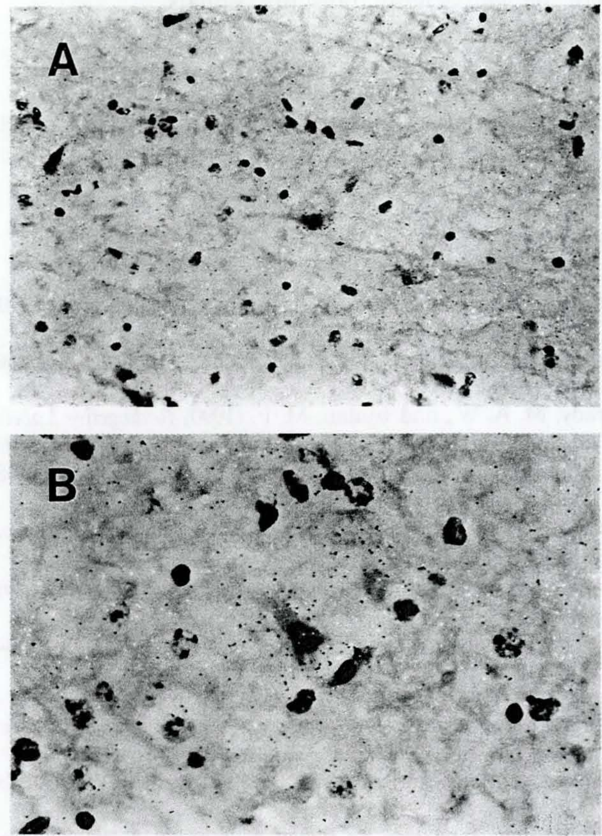

Figure 6. Micrography of γ-aminobutyraldehyde dehydrogenase mRNA hybridized neurons. Autoradiographic grains are restricted to γ-aminobutyraldehyde dehydrogenase mRNA hybridized neurons. Hematoxylin & eosin counterstained. × 400.

paper (Lee and Cho, 1992). The NH_2-terminal amino acid sequence of γ-aminobutyraldehyde dehydrogenase was identified first and was determined to be NH_2-S-A-A-T-Q-A-V-P-T-P-N-Q-Q (Lee and Cho, 1992). From this data, 36 mer oligonucleotides complementary to the mRNA were synthesized (Fig. 5), and radiolabelled at the 5′end with ^{32}P. An *in situ* hybridization technique was applied to detect γ-aminobutyraldehyde dehydrogenase mRNA in the bovine brain tissue. The probes were applied to paraffin sections of the bovine brain, and were visualized by autoradiography. Autoradiographic signals representing hybrids of the probe were localized distinctly over the loci occupied by cells containing γ-aminobutyraldehyde dehydrogenase. Cells containing mRNA of the γ-aminobutyraldehyde dehydrogenase were found in many regions of bovine brain tissues and were mainly small- and medium-sized neurons, similar to the results of immunostaining (Fig. 6).

To ensure the specificity of γ-aminobutyraldehyde dehydrogenase transcript signal, slides were incubated without oligonucleotide probes during *in situ* hybridization. No silver grain was observed in any of the slides, indicating the specificity of γ-aminobutyralde-

hyde dehydrogenase transcript signals.

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References

Abe, T., Takada, K., Ohkawa, K., and Matsuda, M. (1990) *Biochem. J.* **269**, 25-29.

Ambroziak, W., and Pietruszko, R. (1991) *J. Biol. Chem.* **266**, 13011-13018.

Brady, M. A. W., and Finlan, M. F. (1990) *Radioactive Labels: Autoradiography and Choice of Emulsions for in Situ Hybridization* (Polak, J. M., and McGee, J. O., eds) pp. 31-58, Oxford Science Publications.

Caron, P. C., Cote, L. J., and Kremzner, L. T. (1988) *Biochem. J.* **251**, 559-562.

Farres, J., Guan, K. L., and Weiner, H. (1989) *Eur. J. Biochem.* **180**, 67-74.

Gee, C. E., Chen, C. L., Robert, J. L., Thompson, R., and Watson, S. J. (1983) *Nature* **306**, 374-376.

Grillo, M. A. (1985) *Int. J. Biochem.* **17**, 943-948.

Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Habor Laboratory, pp. 511-552.

Hempel, J., Bahr-Lindstrom, H., and Jornvall, H. (1984) *Eur. J. Biochem.* **141**, 21-35.

Hodgson, A. J., Penke, B., Erdei, A., Chubb, I. W., and Somogyi, P. (1985) *J. Histochem. Cytochem.* **33**, 229-239.

Hougaard, D. M., Bolund, L., Fujiwara, K., and Larsson, L. I. (1987) *Eur. J. Cell. Biol.* **44**, 151-155.

Jakoby, W. B., and Fredericks, J. (1959) *J. Biol. Chem.* **234**, 2145-2149.

Jänne, J., Pösö, H., and Raina, A. (1978) *Biochim. Biophys. Acta* **473**, 241-293.

Johnson, D. A., Gautsch, J. W., Sportsman, J. R., and Elder, J. H. (1984) *Gene Anal. Tech.* **1**, 3.

Kuno, H., and Kihara, H. K. (1967) *Nature* **215**, 974.

Kurys, G., Ambroziak, W., and Pietruszko, R. (1989) *J. Biol. Chem.* **264**, 4715-4721.

Larsson, L. I., Morch-Jorgensen, L., and Hougaard, D. M. (1984) *Histochemistry* **76**, 159-174.

Lathe, R. (1990) in *Oligonucleotide Probes for in Situ Hybridization* (Polak, J. M., and McGee, J. O., eds) Oxford Science Publications, pp. 71-80.

Lauder, E. W. (1982) *Comparative Correlative Neuroanatomy of the Vertebrate Telencephalon*, pp. 501-524, Macmillan Publishing Co., Inc.

Lee, J. E., and Cho, Y. D. (1992) *Biochem. Biophys. Res. Commun.* **189**, 450-454.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275.

MacKerell, A. D., and Pietruszko, R. (1987) *Biochim. Biophysic. Acta* **911**, 306-317.

Mach, M., Ebert, P., Popp, R., and Ogilvie, A. (1982) *Biochem. Biophys. Res. Commun.* **104**, 1327-1334.

Oetal, W. H., Schmechel, D. E., and Mugnaini, E. (1983) in *Glutamic Acid Decarboxylase (gad): Purification, Antiserum Production, Immunocytochemistry* (Barker, J. L., and Mckelvy, J. F., eds) pp. 63-110, John Wiley & Sons, New York.

Otterson, O. P., and Strom-Mathisen, J. (1984) *J. Comp. Neurol.* **229**, 374-392.

Pegg, A. E., and McCann, P. P. (1982) *Am. J. Physiol.* **243**, 212-221.

Roberts, E. (1979) in *New directions in GABA Research I: Immunocytochemical Studies of GABA Neurons* (Krogsgaard-Larsen, P., Scheel-Kruger, J., and Kofod, H., eds) pp. 28-45, Munksgaard, Copenhagen.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed, Cold Spring Harber Laboratory Press, Cold Spring Haber, NY.

Seiler, N. (1980) *Physiol. Chem. Physics.* **12**, 411-427.

Seiler, N., and Al-Therib, M. J. (1974) *Biochem. J.* **144**, 29-35.

Seiler, N., Bink, G., and Grove, J. (1979) *Neurochem. Res.* **4**, 425-435.

Somogyi, P., Hodgson, A. J., Chubb, I. W., Penke, B., and Erdei, A. (1985) *J. Histochem. Cytochem.* **33**, 240-248.

Sternberger, L. A. (1986) *Immunocytochemistry*, 3nd Ed, New York, John Wiley.

Storm-Mathisen, J., Leknes, A. K., Bore, A. T., Vaaland, J. L., Edminson, P., Haug, F-M. S., and Ottersen, O. P. (1983) *Nature* **301**, 517-520.

Tabor, C. W., and Tabor, H. (1984) *Annu. Rev. Biochem.* **53**, 749-790.

Towbin, H. T., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.

Wu, J-Y., Matsuda, T., and Roberts, E. (1973) *J. Biol. Chem.* **248**, 3029-3034.