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Immunohistochemical and in situ hybridization studies of choline acetyltransferase in large motor neurons of the human spinal cord

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Summary. The localization of choline acetyltransferase (ChAT) protein and mRNA was investigated in large motor neurons of the lumbar spinal cord of 10 autopsied individuals without neurological diseases, by immunohistochemistry and in situ hybridization. In the immunohistochemistry using 20 serial tissue sections with a total thickness of 80 pm, about 58–85% (average 67%) of the large motor neurons (30 pm and more in somal minimal diameter) in the ventral horn were stained with the anti-human ChAT antibody. In the positive neurons, most immunoreactive products were observed focally in the perikarya. Occasionally, the perikarya of some neurons were stained diffusely. In situ hybridization with a single 4 pm-thick tissue section showed that almost all large motor neurons had positive signals (93~100%, average 98%), which were distributed diffusely in the perikarya. The positivity rate in the in situ hybridization was higher than that in the immunohistochemistry for all 10 cases. These results indicate that ChAT mRNA is transcribed in almost all large motor neurons in the ventral horn of the human spinal cord, but ChAT protein cannot always be detected in the cytoplasm by immunohistochemistry.

Key words: Choline acetyltransferase, Spinal cord, Motor neuron, Human

Introduction

Choline acetyltransferase (ChAT, acetyl CoA: choline *O*-acetyltransferase, EC 2.3.1.6), the enzyme responsible for the biosynthesis of acetylcholine, is presently the most specific indicator for monitoring the functional state of cholinergic neurons in the central and peripheral nervous systems. Motor neurons in the spinal cord are one of the representative cholinergic neurons. Previous immunohistochemical studies on experimental animals, such as rat (Houser et al., 1983; Barber et al., 1984; Borges and Iversen, 1986) and cat (Woolf, 1991) have shown that not only perikarya but also the cell processes of almost all motor neurons in the spinal ventral horn are diffusely stained with anti-ChAT antibodies. In situ hybridization studies of rats have revealed that positive grains are observed evenly in the perikarya of most motor neurons of the ventral horns (Ibáñez et al., 1991a; Butcher et al., 1992; Lauterborn et al., 1993; Mori et al., 1993). However, our previous study on the human spinal cord demonstrated that most of the immunoreactive products for ChAT are observed focally in the perikarya of large motor neurons, and that several of the cell bodies of motor neurons lack immunoreactive products for ChAT (Oda et al., 1995). Our morphological observations are quite consistent with the results of a biochemical study using human materials (Kato and Murashima, 1985).

In the present study, we performed immunohistochemistry and in situ hybridization (ISH) on the human spinal cord to investigate the locational relationship between ChAT protein and mRNA in the perikarya of the large motor neurons.

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Materials and methods

Tissue specimens

Lumbar enlargement segments of the spinal cords were obtained at autopsy from 10 adult individuals with neither a clinical history nor pathological findings suggestive of neurological or psychiatric diseases (Table 1). The spinal cord was sliced at approximately 5-mm thickness, fixed in freshly prepared 4% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.4) and embedded in paraffin. For conventional light microscopic examination, deparaffinized tissue sections were stained with hematoxylin-eosin, Bodian or Klüver-Barrera.

Immunohistochemistry

Twenty serial tissue sections (4 μm/section) of the spinal cord of each subject were obtained for investigation of the whole cell body of neurons. The tissue sections were deparaffinized and treated with 1% H_2O_2 for 10 min to quench the endogenous peroxidase activity. The sections were then incubated overnight at 4 °C with a 1:1,000 dilution of the polyclonal rabbit antibody against a ChAT fusion protein developed in this Department by recombinant technology (Oda et al., 1995). Specificity of the antibody was verified by enzyme immunoprecipitation assay (Oda et al., 1995) and immunoblot analyses (Oda et al., 1995; Kasashima et al., 1999). The slides were subsequently incubated for 30 min at room temperature with biotinylated goat anti-rabbit IgG antibody (diluted 1:200; Dako, Glostrup, Denmark), followed by incubation with avidin and biotinylated horseradish peroxidase complex (Dako). Antibody binding was visualized with 3,3'-diamino-benzidine tetrahydrochloride and sections were counterstained with hematoxylin. Immunohistochemical comparison was done between sections pretreated with or without antigen retrieval procedures using Target Unmasking Fluid (Kreatech Diagnostics, Amsterdam, Netherlands), but there was no significant difference in the results of ChAT immunostaining between the two groups. Thus, serial tissue sections examined in the present study were not subjected to the antigen retrieval procedures. Sections incubated with non-immune rabbit IgG instead of the primary antibody served as negative reaction controls. The immunohistochemical evaluation was performed on photographs of the series of tissue sections with a final magnification of x100. We took photographs of all immunohistochemical tissue sections in serial order (20 tissue sections/case) and counted the number of large multipolar neurons (30 μm and more in somal minimal diameter) whose whole cell bodies were included in the total series sections in order to avoid multiple counting of neurons. ChAT-positive large neurons were also counted. Neurons with a minimum diameter greater than 30 μm are generally accepted to be α-motor neurons (Tomlinson et al., 1973; Tsukagoshi et al., 1979). Individual α-motor neurons were recognizable to count in the series photographs (x100), judging from the location and shape of the neurons and the surrounding structures such as blood vessels and globules.

In situ hybridization

A single tissue section (4 μm in thickness) was examined from each of the 10 subjects. The in situ hybridization was performed as previously described (Oda et al., 1992, 1996). Briefly, the human ChAT cDNA fragment spanning nucleotide positions 685 to 2455 (Oda et al., 1992) was subcloned into the plasmid Bluescript (Stratagene, La Jolla, CA, USA) between the *Apa I* and the *Xba I* sites. After linearization with *Sca I*, the plasmid was transcribed by T3 RNA polymerase using [α-^{35}S] UTP (DuPont/NEN, Wilmington, DE, USA) to produce an antisense RNA probe. A sense RNA probe was transcribed by T7 RNA polymerase after linearization with *Xba I*. Both probes were truncated to a length of approximately 100 nucleotides by alkaline treatment (Cox et al., 1984). The sections from paraformaldehyde-fixed, paraffin-embedded tissues were mounted on poly-L-lysine-coated slides. The deparaffinized sections were treated for 5 min at 37 ºC with proteinase K (100 μg/ml in phosphate-buffered saline) and incubated for 20 h at 50 ºC with $2x10^5$ cpm/μl of the ^{35}S-labeled antisense probe in a solution containing 50% deionized formamide, 0.3M NaCl, 20 mM Tris-HCl (pH 8.0), 2.5 mM EDTA, 1 mg/ml tRNA, 1x Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin) and 10% dextran sulfate (Pardue, 1985). After washing for 2 h at 50 ºC in a solution of 50% formamide and 1x SSC (0.15M NaCl and 0.015M sodium citrate, pH 7.0) and drying, the sections were processed for auto-radiography. The sections were counterstained with hematoxylin-eosin. Sections that were similarly treated but incubated with the sense RNA probe served as negative control. The number of large motor neurons was counted with bright-field photographs with a final magnification of x100, and the number of the neurons containing positive in situ hybridization signals was also counted with the corresponding dark-field photographs. Neurons in which grains more densely accumulated than the background and whose cell contours were clearly recognized under the dark-field microscopy were judged as 'neurons with positive signals.'

Results

Immunohistochemistry

The number of large neurons whose whole cell bodies were included in the total serial sections varied from case to case (28~73, average 49). About 58~85% (average 67%) of the neurons were stained with the anti-human ChAT antibody (Table 1). In the positive neurons, most immunoreactive products were observed focally in the perikarya (Fig. 1). In the same tissue sections, neurons whose perikarya were stained diffusely were occasionally seen (Fig. 2). Some motor neurons contained no detectable immunoreactive products, although the Nissl substance was well preserved (Fig. 1). Areas of perikarya with accumulated lipofusucin were negatively stained. Control experiments in which the primary antibody was replaced by a non-immune rabbit IgG did not result in specific staining of large motor neurons.

In situ hybridization

The number of large motor neurons included in the ventral horns of a single tissue section was between 29

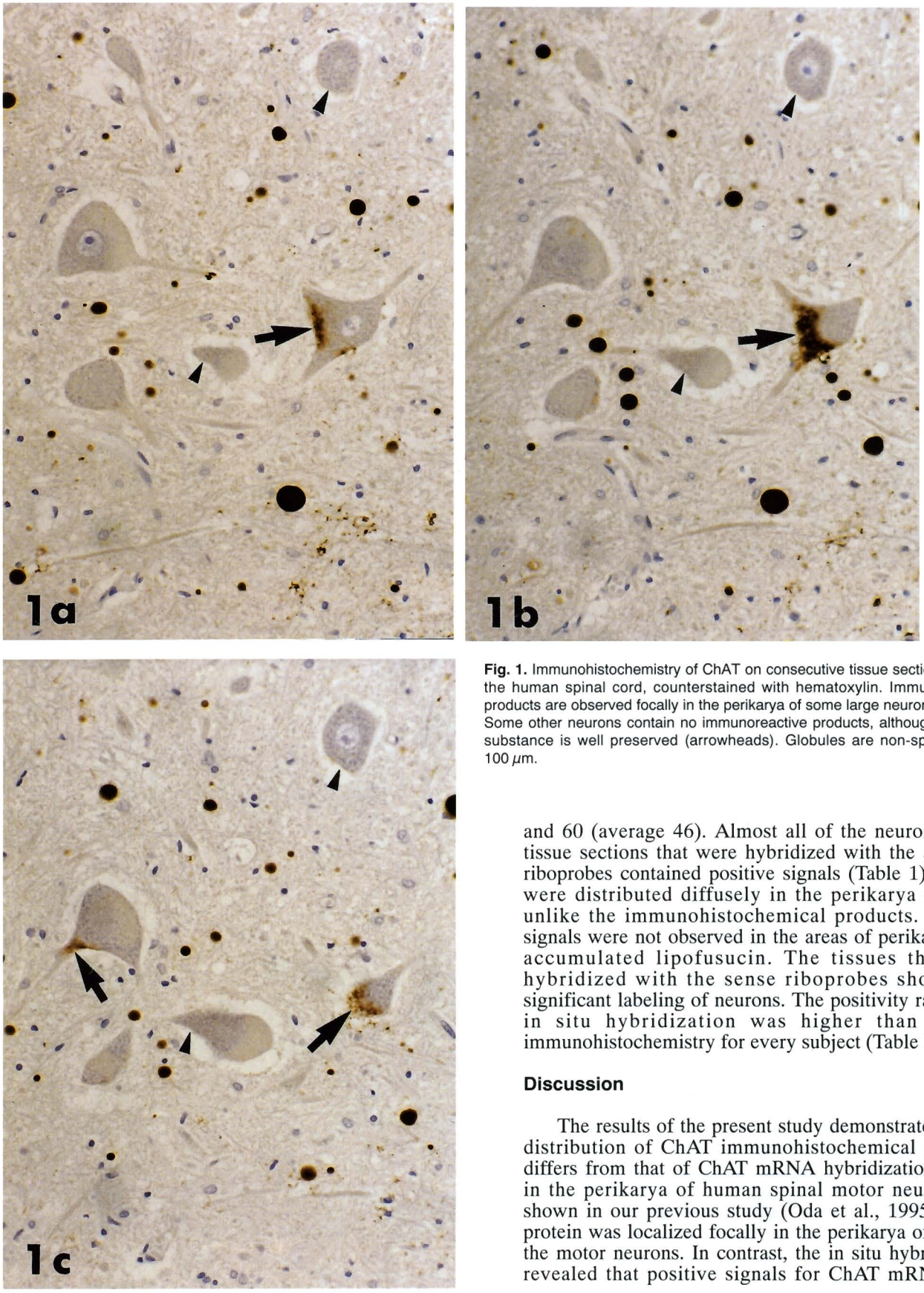


Fig. 1. Immunohistochemistry of ChAT on consecutive tissue sections **(a-c)** of the human spinal cord, counterstained with hematoxylin. Immunoreactive products are observed focally in the perikarya of some large neurons (arrows). Some other neurons contain no immunoreactive products, although the Nissl substance is well preserved (arrowheads). Globules are non-specific. Bar: 100 μm.

and 60 (average 46). Almost all of the neurons in the tissue sections that were hybridized with the antisense riboprobes contained positive signals (Table 1). Signals were distributed diffusely in the perikarya (Fig. 3), unlike the immunohistochemical products. Positive signals were not observed in the areas of perikarya with accumulated lipofusucin. The tissues that were hybridized with the sense riboprobes showed no significant labeling of neurons. The positivity rate in the in situ hybridization was higher than that in immunohistochemistry for every subject (Table 1).

Discussion

The results of the present study demonstrate that the distribution of ChAT immunohistochemical products differs from that of ChAT mRNA hybridization signals in the perikarya of human spinal motor neurons. As shown in our previous study (Oda et al., 1995), ChAT protein was localized focally in the perikarya of most of the motor neurons. In contrast, the in situ hybridization revealed that positive signals for ChAT mRNA were

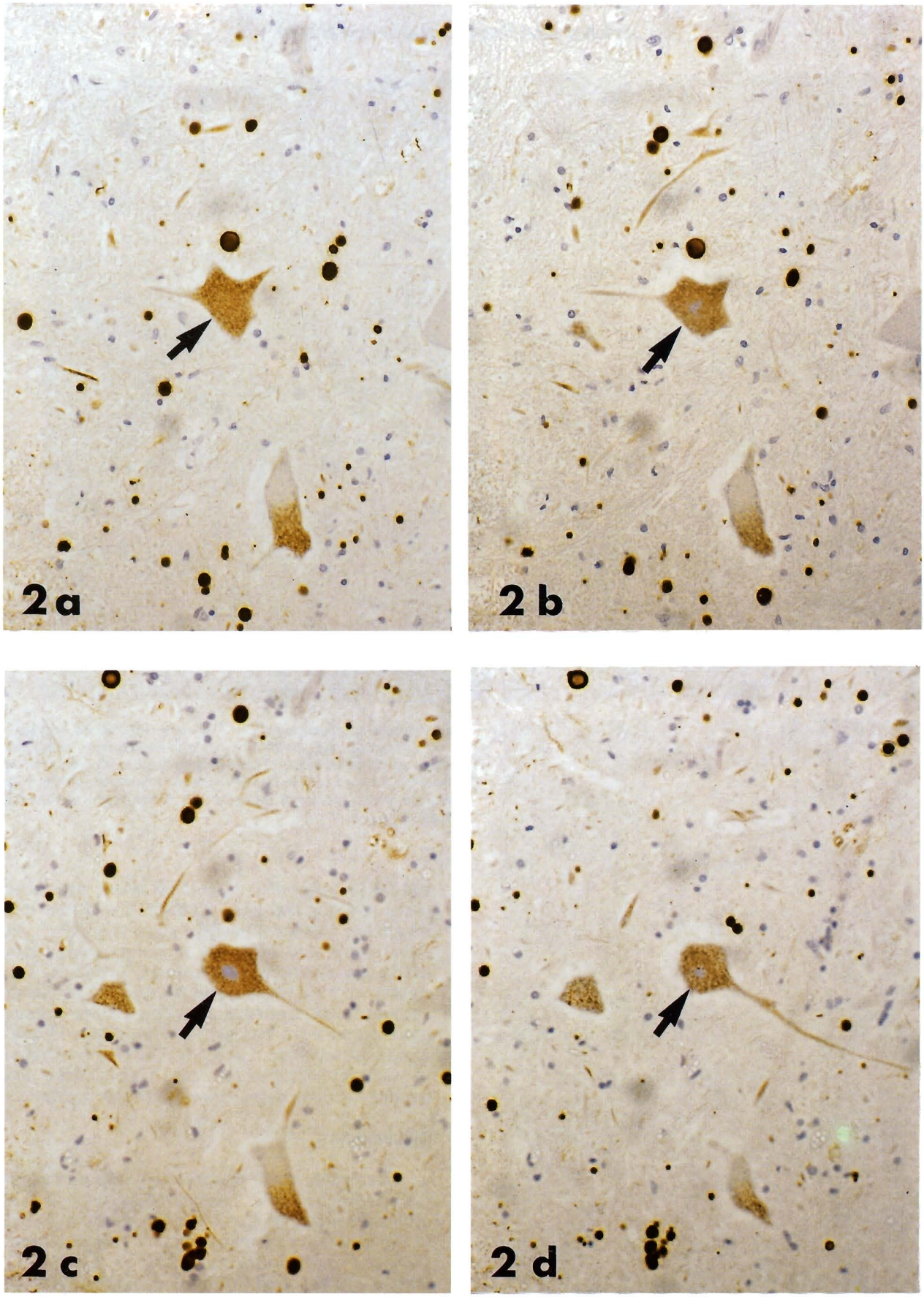
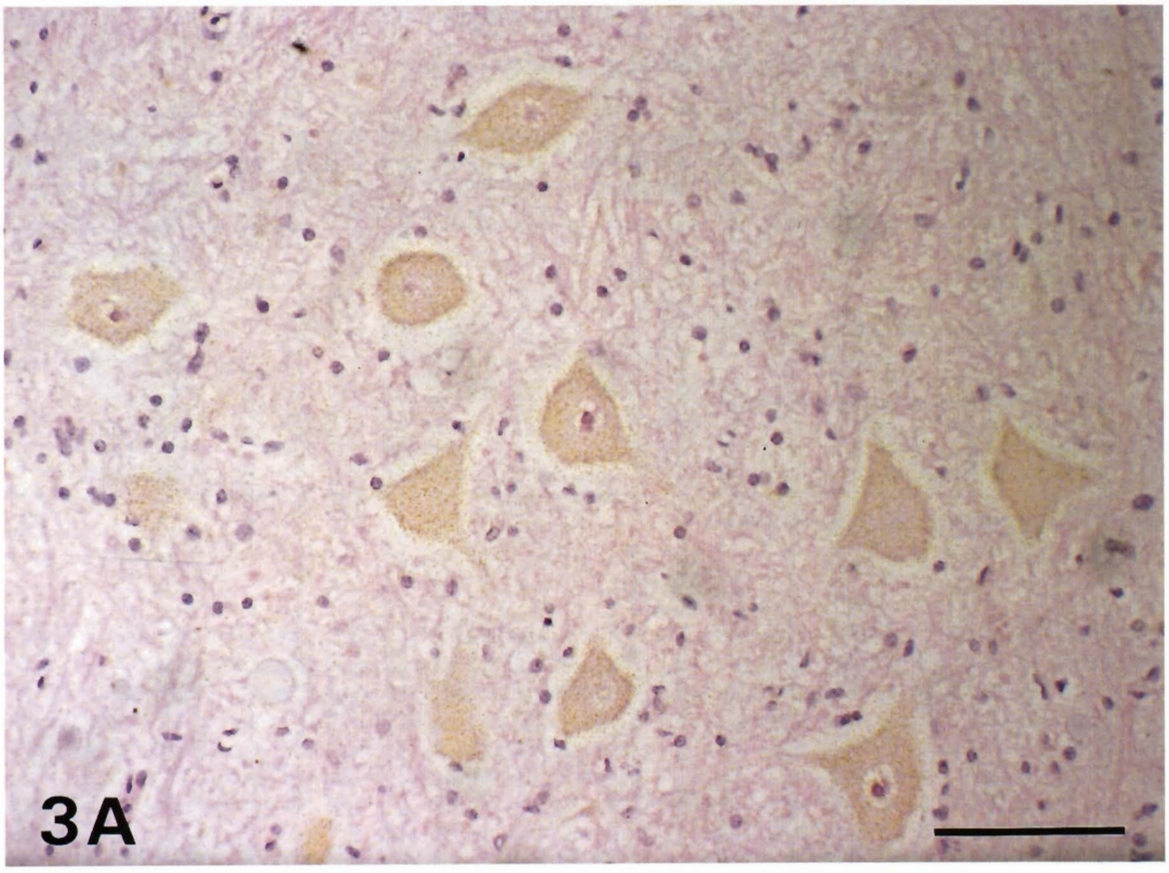


Fig. 2. Immunohistochemistry of ChAT on consecutive tissue sections **(a-d)** of the human spinal cord, counterstained with hematoxylin. The perikaryon of a large neuron is stained diffusely with the antibody (arrows). Globules are non-specific. Bar: 100 μm.



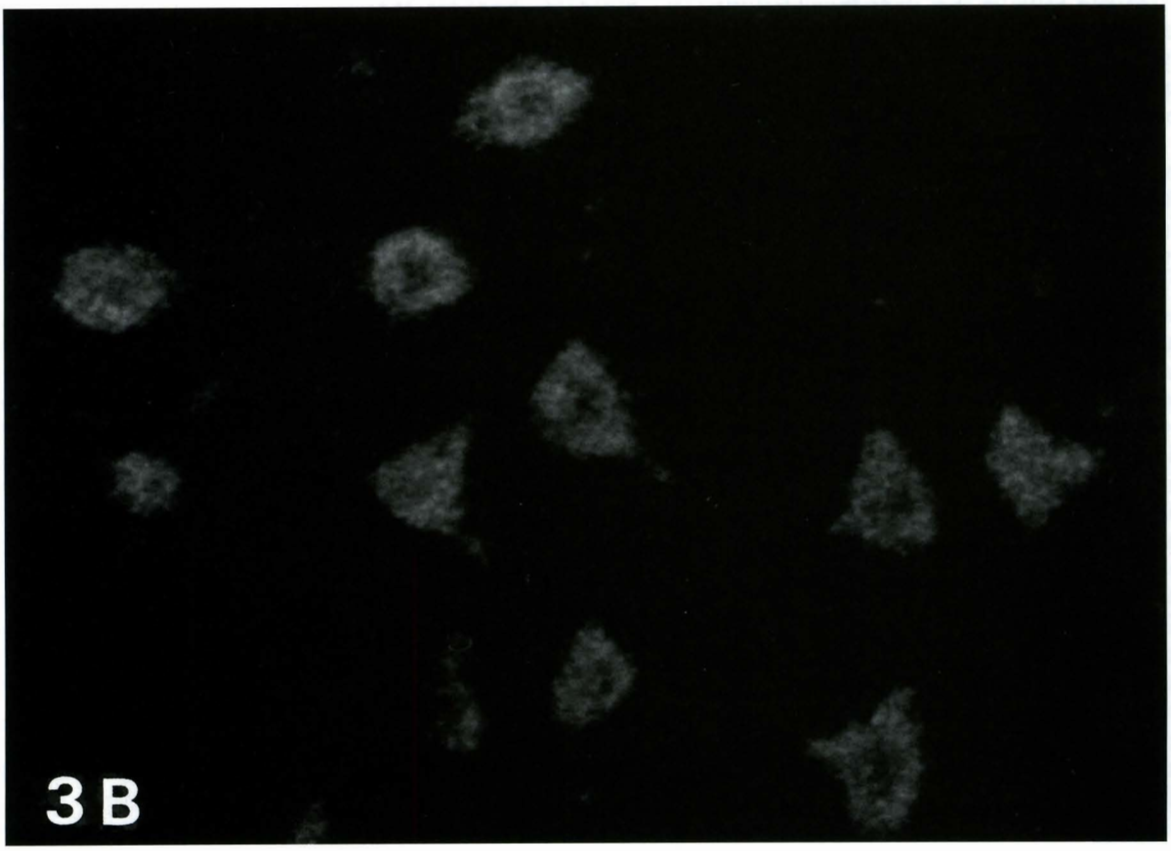


Fig. 3. In situ hybridization of ChAT. **A** Bright-field micrograph of a section with ^{35}S-labeled antisense RNA probe, counterstained with hematoxylin-eosin. **B.** Dark-field micrograph of the section shown in A. The hybridization signals are evenly distributed in the perikarya of all large neurons. Bar: 100 μm.

Table 1. Clinicopathological findings and results of immunohistochemical and in situ hybridization studies.

CASE	AGE (years)	SEX	PATHOLOGICAL DIAGNOSIS	POSTMORTEM PERIOD BEFORE FIXATION (hours)	IMMUNOHISTOCHEMISTRY Number of positive large neurons/Total number of large neurons	IN SITU HYBRIDIZATION Number of large neurons with positive signals/Total number of large neurons
1	49	M	Liver cirrhosis	8	25/36 (69)	34/35 (97)
2	68	F	Gastric carcinoma	4	44/71 (62)	36/37 (97)
3	63	M	Liver cirrhosis	5.5	33/47 (70)	58/58 (100)
4	60	F	Combined valvular disease	6	25/42 (60)	29/29 (100)
5	77	M	Liver cirrhosis	14.5	40/61 (66)	60/60 (100)
6	66	M	Esophageal carcinoma	5.5	17/28 (61)	41/41 (100)
7	70	F	Hemorrhagic infarction of the small intestine	5	17/28 (61)	32/33 (97)
8	67	M	Colon carcinoma	5.5	43/53 (81)	57/59 (97)
9	69	F	Gastric carcinoma	3	45/53 (85)	40/43 (93)
10	56	F	Flauminant hepatitis	3.5	42/73 (58)	62/62 (100)

Numbers in parentheses indicate positivity rates (%). F: female; M: male.

distributed diffusely in the perikarya. Similar differences between the distribution of ChAT mRNA and that of ChAT protein have been reported in rat and human testes (Ibáñez et al., 1991b) and in the syncytiotrophoblast of the human chorion (Oda et al., 1996). In the testis, ChAT mRNA is distributed evenly over spermatocytes and spermatids, whereas the protein is restricted to the postacrosomal region of the spermatozoa head and to the proximal region of the tail. In the human chorion, ChAT mRNA hybridization signals are evenly distributed throughout the trophoblastic layers, but the protein is detected mainly in the cytotrophoblast and very rarely in the syncytiotrophoblast.

It is not yet known why there is such a discrepancy in distribution between the in situ hybridization signals and the immunohistochemical products. Several reasons may explain the findings in the present study: (1) the sensitivity of in situ hybridization with a radio-labeled riboprobe may be higher than that of immunohisto-chemistry; (2) perhaps not all molecules of ChAT mRNA are translated simultaneously, or ChAT mRNA may be intermittently translated, and following the synthesis, ChAT protein may be redistributed in the perikarya; (3) some molecules of ChAT mRNA may be abortive and not utilized for translation; (4) ChAT mRNA may diffuse in the cytoplasm more rapidly than the protein during tissue preparation does; (5) ChAT mRNA may be more stable than the protein; and (6) postmortem changes such as chromatolysis may lead to changes in the intracellular distribution of protein, but not mRNA. The perikarya of some motor neurons were stained diffusely and others were stained focally in the same tissue sections. Despite the preservation of the Nissl substance, some neurons were devoid of immunoreactive products for ChAT. These immuno-histochemical findings make the fourth and sixth possibilities listed above less likely. ChAT is an enzyme with a long half-life; approximately 16-20 days in rats (Fonnum et al., 1973) and 4 days in cats (Tandon et al., 1996). In addition, ChAT protein can be detected immunohistochemically in human brain tissue after 24 h of postmortem delay (Brandel et al., 1990; Selden et al., 1994). Selden et al. (1994) showed that the ChAT-immunoreactivity of intrinsic ChAT neurons in the human neostriatum is not affected by postmortem intervals (between 2 h and 38 h). We obtained similar results; the cytoplasm of most ChAT neurons in the human neostriatum was stained diffusely (data not shown), whereas that of most of the spinal motor neurons was stained focally. Thus, the fifth possibility is also not likely.

Almost all of the motor neurons were labeled by in situ hybridization, whereas on average 67% of them were immunohistochemically positive. It is intriguing that the average positivity rate in the immunohisto-chemical study with serial tissue sections is similar to that in a biochemical study on ChAT activity in human spinal motor neurons using a highly sensitive microassay system (approximately 75%) (Kato and Murashima, 1985) which indicated that not all motor neurons in the spinal cord contain detectable ChAT protein in the perikarya. Kato and Murashima (1985) also demonstrated that the enzyme is unevenly distributed in the cytoplasm of single neurons. These biochemical results are very consistent with our morphological observations. Therefore, the discrepancies in the localization and positivity rates between ChAT protein and mRNA in the human spinal cord cannot be completely explained by the different sensitivity between immunohistochemistry and in situ hybridization, and/or by tissue preparation artifacts. Perhaps not all molecules of the mRNA are utilized simultaneously for ChAT synthesis; the synthesis of ChAT protein may take place partially under the posttranscriptional regulation. In fact, it has become clear that the synthesis of some proteins is controlled not only at the transcriptional level but also at the translational level (Nur et al., 1995; Parry et al., 1995; Bate et al., 1996; Comer et al., 1996). Nur et al.

(1995) examined the effect of starvation and refeeding on glycogen metabolism and showed that starvation and refeeding alter the total amount of glycogen synthase (GS) in the rat liver with no significant changes in GS mRNA, and they also found that the amount of GS mRNA associated with polyribosomes was 90% lower in the starved compared to the fed rats. Their results suggest that the efficiency of GS mRNA translation regulates GS expression in the fed-to-starved transition. In an in vitro study, Parry et al. (1995) demonstrated that COS cells transfected with the expression vector containing spermidine/spermine N'-acetyltransferase (SSAT) cDNA produce a large amount of SSAT mRNA but express very little activity of SSAT. When N^1N^{12}-bis(ethyl)spermine (BESM), one of the inducers of SSAT was added, the cells expressed high activity without any increase in the mRNA. In addition, in BESM-treated cells, a significant proportion of the SSAT mRNA was moved from the monoribosome fraction into the small-polysome fraction of the gradient. Based on these observations, Parry et al. (1995) proposed the hypothesis that a protein interacts with the mRNA to inhibit the binding of SSAT mRNA to ribosomes, and BESM acts to relieve the inhibition. However, Sadler et al. (1996) demonstrated that epidermal growth factor receptor mRNA is present in parathyroid tumors, but the corresponding protein cannot be detected by immunohistochemistry; they suggested that there is a failure of mRNA translation. Therefore, the possibility that some molecules of ChAT mRNA detected by in situ hybridization are abortive and not utilized for translation cannot be denied. Further controlled experiments are necessary to explain the discrepancies in the localization and positivity rates between ChAT protein and mRNA in the motor neurons of the human spinal cord.

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