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Original Article

Chromogranin A In Neurons of the Rat Cerebellum and Spinal Cord: Quantification and Sites of Expression[1]

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Chromogranin A (CGA) is an abundant protein of dense-cored secretory vesicles in endocrine and neuronal cells. The present study, for the first time, compares CGA of neurons of the central nervous system with the CGA of adrenal origin. By S1 nucleus protection assay, we found that the 3′ part of the CGA mRNA between exons 5–8 of the cerebellum and the spinal cord of the rat is homologous to that of the adrenal. In situ hybridization histochemistry revealed that CGA mRNA in the cerebellar cortex is present in cell bodies of Purkinje cells and in neurons of the deep cerebellar nuclei. The perikarya of these cells also exhibit CGA-like immunoreactivity. CGA mRNA and CGA-like immunoreactivity are also present in the motoneurons of the ventral, lateral, and dorsal horns of the rat spinal cord. The amounts of CGA, as determined by radioimmunoassay in cerebellum and spinal cord, were about one tenth of the amounts detected in the adrenal, adenohypophysis, or the olfactory bulb. The sites of CGA expression suggest that CGA may be involved in signal transduction in the motor system. (*J Histochem Cytochem 40:993–999, 1992*)

KEY WORDS: Chromogranin A: Cerebellum; Spinal cord; Immunocytochemistry; Isotopic and non-isotopic in situ hybridization histochemistry; cDNA probe; cRNA probe.

Introduction

Chromogranin A (CGA) is a member of the chromogranin/secretogranin protein family. This acidic protein was found first in chromaffin vesicles of the adrenal medullary cells, where it is stored and co-released with catecholamines (1,3). Various studies have indicated that CGA is not restricted to the adrenal medulla but is also found in a variety of peptidergic endocrine cells (cf. 8,9,17,24,31,42).

CGA binds calcium (4,27,29,30). There is evidence that the CGA–calcium complex participates in the intracellular storage of catecholamines and polypeptide hormones (12,13,39,41). CGA itself, or proteolytic fragments of CGA, has been shown to inhibit secretion by various endocrine cells such as adrenal chromaffin cells, pancreatic B-cells, and parathyroid cells (7,10,14,32), suggesting that this protein plays an important function in the regulation of hormone release by endocrine glands.

In contrast to the endocrine system, little information is available on the synthesis and the function of CGA in the central nervous system. Its occurrence and distribution have first been determined with immunological techniques in ovine and bovine neural tissues (16,19,24,25,33). CGA was found to be widely distributed in brain and spinal cord without a clear-cut correlation with known transmitter systems (cf. 5,33) and, compared with endocrine tissues, appears to be of low abundance in the central nervous system (19,25,38). One exception is the olfactory system, which contains CGA in amounts comparable to those found in the adrenal medulla or the adenohypophysis (22). In the olfactory system, we have found that CGA is present mainly in the centrally projecting axon terminals of the secondary neurons of the olfactory system.

Secretory proteins such as CGA, which are synthesized in the perikarya of neurons, are rapidly transported to their axons. Therefore, their detection by immunocytochemistry is often very difficult. The recent elucidation of the complete adrenal cDNA coding sequences of CGA of different species (cf. 17) allows the identification of CGA mRNA in perikarya of neurons by in situ hybridization histochemistry. This method complements immu-

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nocytochemistry. Information about the sequences of CGA mRNA from different tissues can be obtained by S1 nuclease protection assays. The 2 KB-long CGA mRNA of rat and mouse is composed of eight exons. Some CGA domains, deduced from the primary structure, are correlated to specific exons (40).

Here we report the gene expression of CGA at protein and mRNA levels in cerebellum and spinal cord of the rat, using both radioactive and non-radioactive cRNA probes, S1 nuclease protection assays, immunocytochemistry, and radioimmunoassay.

Materials and Methods

Experimental Procedures

Animals. Adult male rats (Sprague–Dawley) were purchased from Charles River (Sulzfeld, FRG) and housed in our vivarium under standard conditions. Animals were sacrificed by decapitation and tissues were immediately removed and further processed as described below.

Immunocytochemistry and Radioimmunoassay. Brains and segments of thoracic spinal cords (approximately 1 cm long) of male rats were immersed in Bouin's fixative for 12 hr. Subsequently, they were embedded in paraffin and serial sections were cut (5 μm) and mounted on gelatin-coated glass slides. For immunocytochemistry, sections of the cerebellum and spinal cord were selected. A rabbit antiserum directed against the C-terminal peptide (16-mer) of bovine CGA was used. Bovine CGA differs only slightly in sequence from rat CGA, and therefore this antiserum also recognizes rat CGA. The specificity of this antiserum (kindly provided by D.T. O'Connor, San Diego, CA) and its use for immunocytochemistry and immunoblotting of neural and endocrine tissue from rat have been described previously (2,22). To detect CGA immunoreactivity, we used the peroxidase–anti-peroxidase (PAP) method. In brief, paraffin-embedded sections were deparaffinized, permeabilized with 0.5% Triton X-100 in Tris-buffered saline (TBS; 0.05 M Tris and 0.15 M NaCl, pH 7.6), and were then incubated in TBS containing 10% methanol and 0.03% H_2O_2 to block endogenous peroxidase activity. Subsequently, they were pre-incubated with 2% normal swine serum (NSS) in TBS, followed by incubation with CGA antiserum diluted in 2% NSS in TBS. Different dilution steps of this antibody were tested and a dilution of 1:1000 was used in all experiments reported here. Incubation with a second swine anti-rabbit antiserum and incubation with a rabbit–PAP complex followed. Diaminobenzidine was used as a chromogen to visualize the immunoreaction. Controls consisted of incubation with 2% NSS in TBS instead of the specific antibody and of incubation with normal serum (1:1000). Sections were photographed using a Zeiss (Oberkochen, FRG) Axioplan microscope.

For extraction of CGA, small pieces of tissue (about 20–200 mg) were rapidly frozen. After addition of 0.1 M HCl (300–1000 μl) the tissue was sonicated until it was completely homogenized. After lyophilization and appropriate dilution, CGA was quantified by radioimmunoassay as described previously (26,34,37). The antisera to CGA were raised in rabbits to a synthetic peptide 17-mer corresponding to the amino terminus of bovine/human CGA. After an overnight incubation of antibody and radiolabeled N-terminal CGA peptide (10,000 cpm) in assay buffer at 4°C, an excess titer of second antibody (goat anti-rabbit gamma globulin; Scantibodies, Lakeside, CA) was added. After vortexing, polyethylene glycol was added to precipitate the immune complexes (leaving the free antigen in solution). The solution was centrifuged at 1700 × g for 20 min, the supernatant aspirated, and the radioactivity of the pellet was subjected to gamma counting for ^{125}I (Searle Mark IV). Immunoreactive CGA was determined by parallel displacement of CGA[1–16]–^{125}I-[tyr_{17}] (tracer) and unknown sample from the antibody in the radioimmunoassay. The assay had intra- and interassay coefficients of variation of 4.8% and 8.4%, respectively.

cDNA and cRNA probes. For cRNA and cDNA probe synthesis we used a rat CGA cDNA (28) (kindly provided by R. Parmer and D.T. O'Connor, San Diego, CA) as previously described (22). A clone referred to as rCGA M13(−) was used to generate cRNA and cDNA anti-sense strands of the CGA mRNA, originating within Exon 8 and extending towards the 5′ end.

For S1 analysis a 950 nucleotide (nt) fragment yielded as calculated by the sequence map (28) was used. This cDNA probe contains 644 nt of the translated (Exons 5–8) and 192 nt of the untranslated 3′ end from the rat CGA sequence and 114 nt of vector sequences and extends from Exon 6 to Exon 8 (40).

Synthesis of ^{35}S-labeled cRNA (a) (spec. act. 5×10^8 cpm/μg) for in situ hybridization was carried out as described by Lahr et al. (22). The T3 promoter was used to synthesize the cRNA probe within Exon 8 280 nucleotides in length.

Synthesis of Dig-11-UTP-labeled cRNA (b) for non-isotopic in situ hybridization was carried out according to the protocol of Boehringer (Mannheim, FRG) using T3-RNA polymerase, ATP, GTP, CTP, 1 mM each and 0.65 mM UTP and 0.35 mM Dig-11-UTP. As template we used 1 μg rCGA M13(−) linearized with PvuII. The resulting digoxigenin-labeled cRNA probe originates with Exon 8 and contains 280 nucleotides.

Controls for the specificity of hybridization signals consisted of treating tissues with 100 μg/ml RNAse A (Boehringer) in 10 mM Tris-HCl, 15 mM NaCl (pH 7.0) for 1 hr at 37°C before hybridization.

S1 Nuclease Protection Assay

Total RNA was isolated by a modified guanidinium thiocyanate–CsCl method (18) from male rat adrenals, cerebelli, and spinal cords. Twenty μg total RNA (determined photometrically) were hybridized with an excess of ^{32}P-labeled cDNA probe (5×10^4 cpm; spec. act. 1×10^8 cpm/μg) in 75% formamide, 400 mM NaCl, 1 mM EDTA, and 20 mM Tris-HCl (pH 7.4), for 16 hr at 58°C. Hybridization was terminated by digestion with 680 U S1 nuclease (AGS; Heidelberg, FRG) for 2 hr at 37°C. After phenol extraction and ethanol precipitation, samples were separated electrophoretically on 0.3-mm thick 5% polyacrylamide gels (8.3 M urea). Gels were dried and exposed to X-ray film at −70°C, using intensifying screens.

In Situ Hybridization Histochemistry

For isotopic in situ hybridization of the rat spinal cord, (a) tissues were immediately frozen and stored in liquid nitrogen. Cryostat sections (10 μm; Reichert-Jung, Nussloch, FRG) were mounted on 3-aminopropyltriethoxysilan (Sigma; Munich, FRG)-coated slides. Sections were stored until needed at −80°C. They were fixed in 4% paraformaldehyde/PBS for 30 min at room temperature, rinsed twice in PBS, and dehydrated by an ascending series of graded alcohol.

For non-isotopic in situ hybridization and isotopic in situ hybridization of cerebellum (b) tissues were immersed in Bouin's fixative for 12 hr, embedded in paraffin, and serial sections were cut (5 μm) and mounted on 3-aminopropyltriethoxysilan-coated glass slides. Sections were deparaffinized by runs through xylene, ethanol, chloroform, ethanol, and were then air-dried.

The sections (a) and (b) were pre-hybridized at 50°C for 3 hr with 1 ml hybridization solution, as described by Lahr et al. (22). Subsequently, they were hybridized overnight at 50°C with either (a) 5 ng labeled cRNA probe (spec. act. 7×10^8 cpm/μg) or with 200 ng Dig-labeled cRNA (b) in 150 μl hybridization solution in a humidified chamber. Controls consisted of sections pre-treated with 100 μg/ml RNAse A at 37°C for 60 min before hybridization, or of sense RNA. After hybridization, sections with isotopic cRNA probes were washed and further treated as described. Slides were rinsed in ascending alcohol solutions, air-dried, and dipped in Ilford K2

emulsion diluted 1:1 with H_2O. They were exposed for 6 weeks at 4°C and developed with Kodak D19 at 16°C for 4 min. The sections were counterstained with hemalum (Mayer) and eosin.

After hybridization, sections with non-isotopic cRNA probes (b) were rinsed twice in 2 × SSC (0.3 M sodium chloride, 30 mM sodium citrate, pH 7.0), 50% formamide for 20 min at 50°C and once in 2 × SSC at room temperature. To reduce background, sections were treated for 30 min at 37°C in 2 × SSC and 100 µg/ml RNAse A. They were washed twice at room temperature in 2 × SSC and once with 2 × SSC, 50% formamide at 50°C for 5 min. Sections were incubated overnight in 2 × SSC, 0.084% saponin, 2% normal sheep serum at room temperature. Sections were washed twice for 10 min in Buffer 1 containing 0.1 M Tris-HCl, 0.15 NaCl (pH 7.5) and incubated for 5 hr at room temperature in a humidified chamber with anti-digoxigenin-AP, Fab fragments (1:500) (Boehringer) in 1% NSS, 0.084% saponin in Buffer 1. Sections were washed for 10 min at room temperature in Buffer 1 and for 10 min in Buffer 3 containing 0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$ (pH 9.5). Sections were incubated overnight in a dark, humidified chamber in 0.41 mM 4-nitrobluetetrazoliumchloride (NBT), 0.38 mM BCIP (5-bromo-4-chloro-3-indolyl-phosphate) in Buffer 3 (NBT and BCIP from Boehringer). Sections were washed in Buffer 4 containing 10 mM Tris-HCl, 1 mM EDTA (pH 8.0), and then covered with a coverslip using glycerine–gelatin.

Results

S1 nuclease protection analysis revealed a completely protected band of approximately 836 nucleotides. The size of the protected band (836 nt) was calculated from the rat sequence map of CGA (28). This result indicates the presence of mRNA co-linear to the whole probe covering one third of the chromogranin A mRNA at the 3′ terminal portion in cerebellum, spinal cord, and adrenal. In contrast to the high amount of CGA-specific mRNA in adrenals, the level of CGA-specific mRNA in cerebellum and in spinal cord was lower (Figure 1).

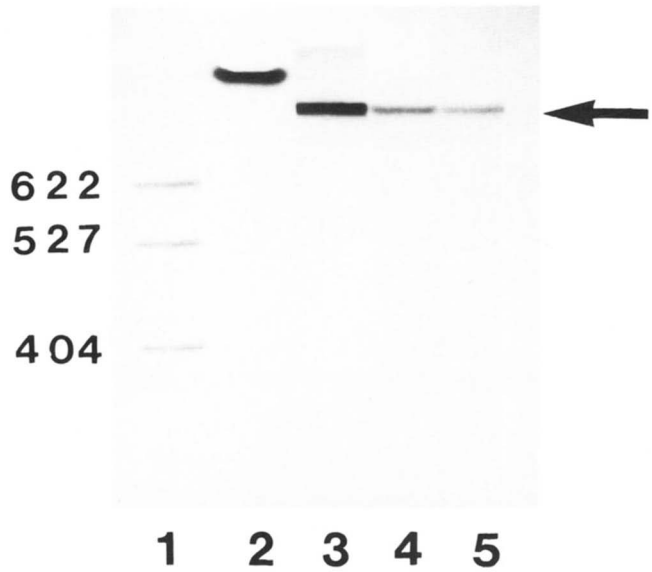

Figure 1. S1 nuclease protection analysis of extracted mRNA of rat tissues. A single-stranded anti-sense DNA probe derived from rCGA M13(−) clones, as described by Lahr et al. (22), was used for hybridization. In Lanes 3–5 the ^{32}P-labeled cDNA served as hybridization probe for 20 µg total RNA each. Lane 3: adrenal medulla; Lane 4: cerebellum; Lane 5: spinal cord. As a size marker, HpaII digested pBR322 was applied to Lane 1. The sizes are indicated on the left side. The upper band of approximately 950 nt, as calculated by the sequence published (28), in Lane 2 represents undigested probe containing flanking Bluescribe M13(−) vector sequences, while the protected band of approximately 836 nt fragment (arrow), as calculated by the sequence published (28), indicates rCGA mRNA in the tissues analyzed. Duration of film exposure was 36 hr.

By in situ hybridization histochemistry with isotopic and non-isotopic labeled anti-sense cRNA probe, we found that CGA-specific mRNA within the cerebellum is mainly confined to Purkinje cells and neurons of the deep cerebellar nuclei (Figures 2A and 2B). Weak labeling was also seen over the granular layer of the cerebellar cortex. In the thoracic spinal cord, cell bodies of neurons (Laminae I–X) were positive for CGA mRNA. Figure 3A shows heavily labeled large (alpha-) and small (gamma-) motoneurons in Lamina IX of the ventral horn. Cell bodies in Laminae III–V of the dorsal horn, heterogeneous in size, were also positive for CGA mRNA (Figure 3B).

Within the cerebellum, neurons of the cerebellar nuclei and of Purkinje cells showed CGA-like immunoreactivity. Faint immunoreaction which could not be localized to cells was seen over the entire cerebellar cortex (Figure 4A). In the thoracic spinal cord, CGA-like immunoreactivity was observed in cell bodies of the neurons of all laminae (Figure 4C). Controls did not show any CGA-like immunoreactivity in the Purkinje cells (Figure 4B) and in the cell bodies of the neurons of all laminae of the thoracic spinal cord (not shown).

As determined by radioimmunoassay, the amounts of CGA in acid extracts of the spinal cord and the cerebellum are low and are comparable to the values observed in brainstem, forebrain, and pancreas (Table 1). By contrast, the levels of CGA (as detected by radioimmunoassay) in the olfactory bulb were found to be on the same order of magnitude as observed in the pituitary or the adrenals, confirming an earlier report (22).

Discussion

The present study analyzes for the first time the distribution of CGA mRNA and of CGA-like immunoreactivity in the cerebellum and the spinal cord of the rat. The data presented here indicate a high degree of homology within a stretch of approximately 800 nucleotides around the 3′ coding and non-coding region covering Exons 6–8 between peripheral (adrenal) CGA and that of the central nervous system. CGA-like immunoreactivity is mainly localized in Purkinje cells of the cerebellar cortex, in neurons of the deep cerebellar nuclei, and in the neurons of the ventral, lateral, and dorsal horns of the rat spinal cord. As in other brain areas, CGA levels in cerebellum and spinal cord are low in contrast to the olfactory system, which contains CGA in amounts similar to those found in the adrenal gland and the adenohypophysis.

A secretory protein such as CGA is rapidly transported away from the site of transcription to the endings, and therefore may easily escape immunochemical detection. This may explain the weak immunoreactivity of the perikarya of Purkinje cells, as well as the faint staining in the entire cerebellar cortex, which could reflect axonal/dendritic transport of CGA. In the present study we combined determination of both CGA mRNA and the protein to detect sites of CGA production. In addition to Purkinje cells, which

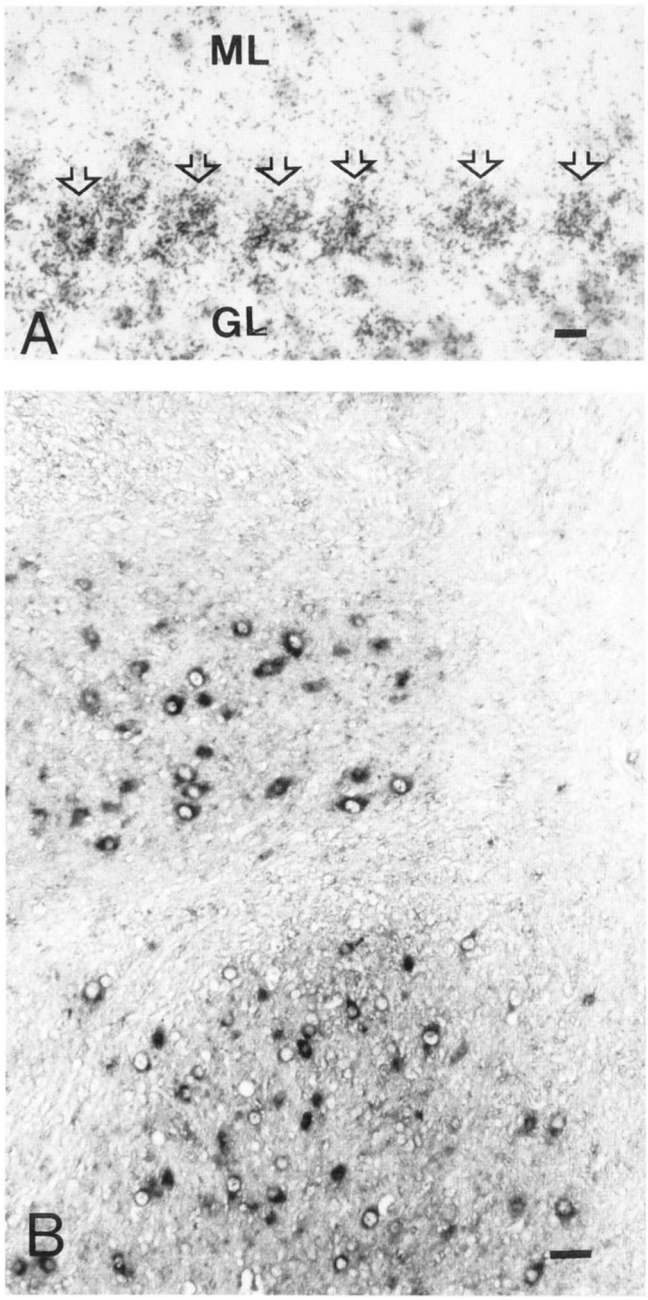

Figure 2. Detection of CGA mRNA in situ hybridization of cerebellar tissue sections using (**A**) radioactive and (**B**) Dig-11-UTP-labeled cRNA probe. (**A**) Purkinje cells (open arrows) and the heterogeneous group of cells in the granular layer contain rCGA-specific cRNA. ML, molecular layer; GL, granular layer. (**B**) Perikarya of the deep cerebellar nuclei are stained with Dig-11-UTP-labeled cRNA probe. Bars: **A** = 10 μm; **B** = 40 μm.

could be distinguished by their localization and size, additional cells of the granular layer expressed CGA mRNA. Since the cells of this layer are Golgi cells and glial cells, the faint CGA-like immunoreactivity in the entire cerebellar cortex could be due to other CGA-producing cell types as well as to positive fibers. It should be mentioned at this point (see also below) that neurons of the dorsal, lateral, and ventral horns of the thoracic spinal cord also

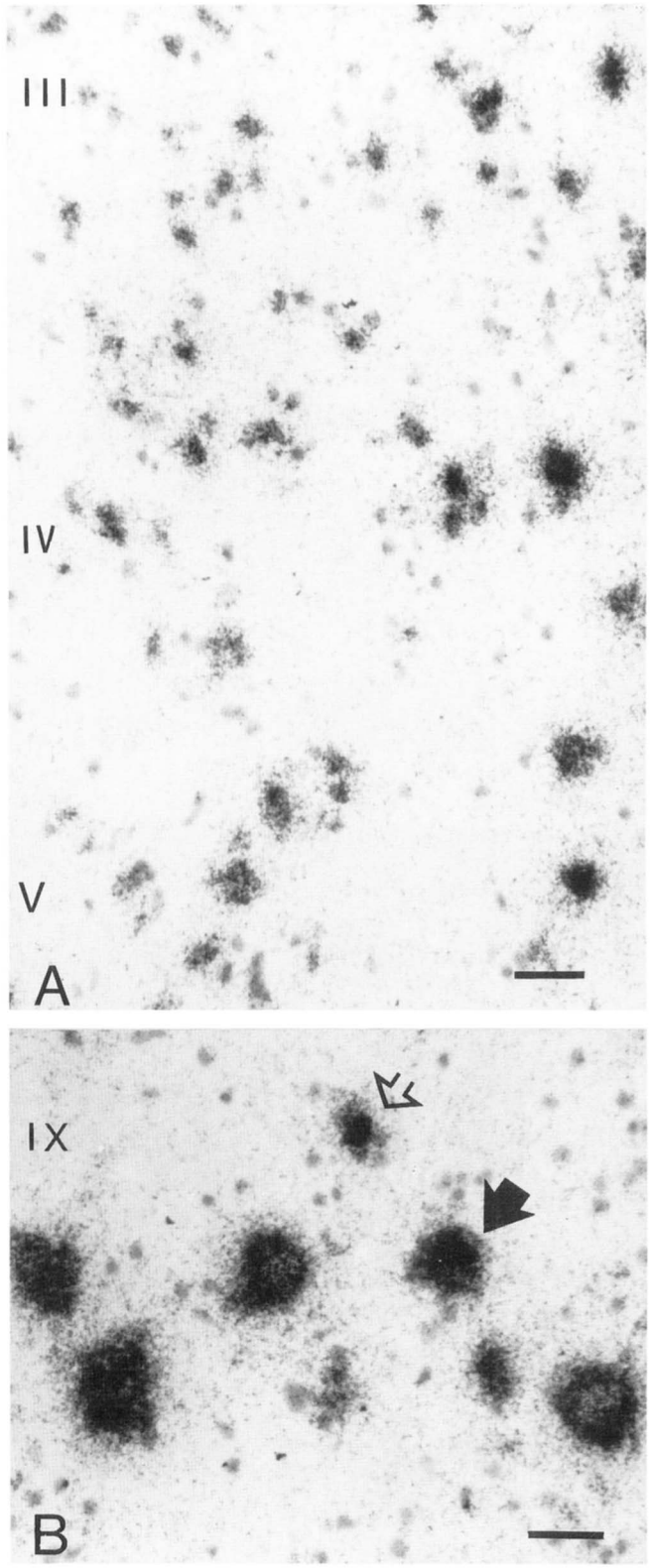

Figure 3. Detection of CGA mRNA by situ hybridization in sections of the thoracic spinal cord using radioactive cRNA probe. (**A**) Cell bodies in Laminae III–V of the neurons in the dorsal horn and (**B**) perikarya in Lamina IX of the motoneurons (alpha, arrow; gamma, open arrow) in the ventral horn are strongly labeled. Bars = 10 μm.

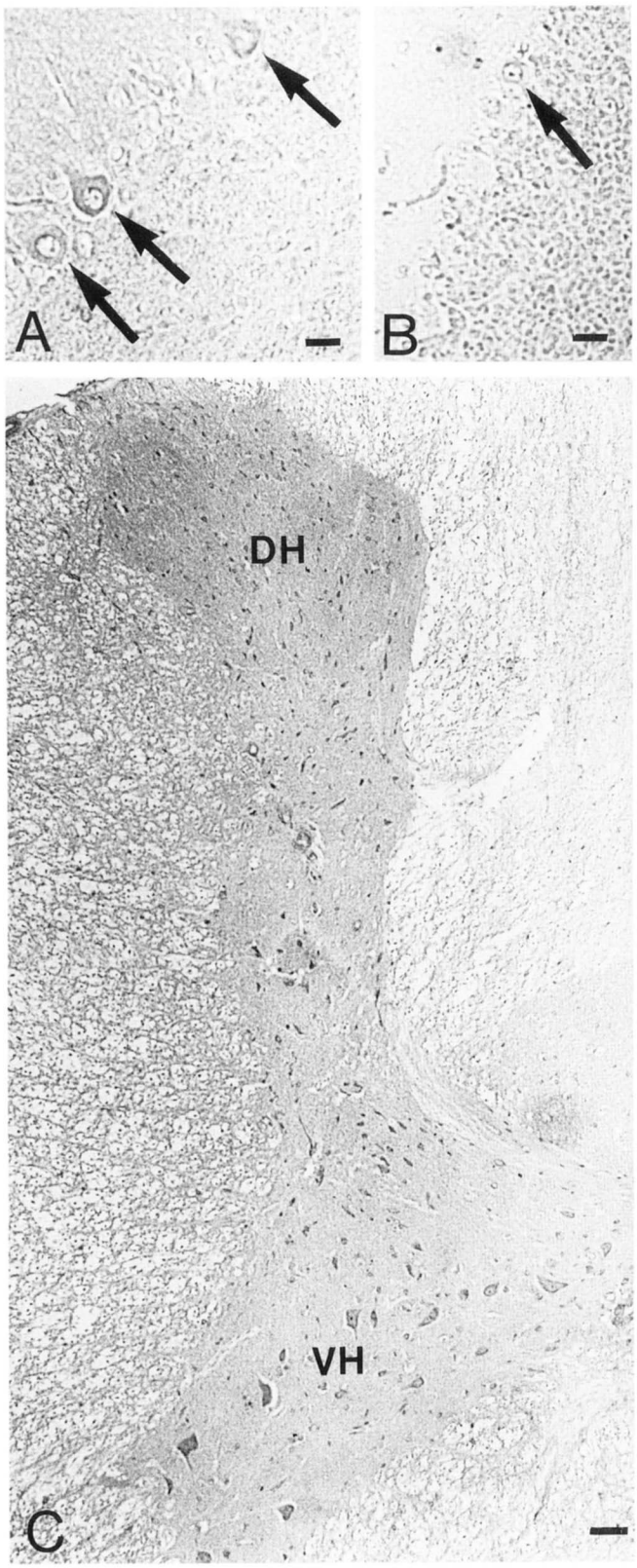

Figure 4. Immunocytochemical detection of chromogranin A in the cerebellum and in the thoracic spinal cord. (**A**) The immunoreaction is confined mainly to the Purkinje cells (arrows). (**B**) Note absence of CGA immunoreactivity of Purkinje cells (arrow) in a control section (omission of CGA antiserum). (**C**) CGA immunoreactivity is confined to the neurons of the ventral horn (VH) and in the lateral as well as the dorsal horn (DH) in the thoracic spinal cord. Bars: **A,B** = 20 μm; **C** = 40 μm.

Table 1. *Levels of immunoreactive chromogranin A in rat endocrine and neural tissues*[a]

	Chromogranin A		
Tissue	ng/mg protein	Relative amount	*n*
Adrenal gland	422.2 ± 33.8	1.00	10
Adenohypophysis	360.8 ± 95.9	0.85	4
Olfactory bulb	359.2 ± 29.6	0.85	6
Brainstem	94.2 ± 8.4	0.22	6
Forebrain	55.4 ± 13.8	0.13	6
Cerebellum	32.1 ± 4.6	0.08	5
Spinal cord	23.8 ± 3.8	0.06	6
Pancreas	48.0 ± 11.8	0.11	6
Liver	7.6 ± 1.1	0.02	10

[a] Amounts of CGA (mean ± SD; *n*, number of tissues from different male animals analyzed).

expressed CGA. Therefore, afferent sensory neurons, which project back to the cerebellum and reach the granular layer (cf. 36), might have contributed to the diffuse CGA immunoreactivity in this layer.

Large species differences exist in the central parts of the CGA molecule, although the C- and N-terminals are very similar (cf. 17). Because of this, immunocytochemical investigations of CGA must be interpreted with caution when the topology of the epitope in the protein recognized by the antiserum, is unknown. One example is the recent observation that a monoclonal antibody to an undefined domain of human CGA exclusively stained Bergmann glia of the rat cerebellum, whereas rabbit antibodies to bovine or porcine CGA do not (23). In this study we used an antiserum raised against the 16 C-terminal (Exon 8) amino acids of CGA, which differ only slightly in sequence in rat, cow, and human. With this antiserum we did not find the pattern typical for Bergmann glia. Furthermore, our data, which show that Purkinje cells and perikarya of the deep cerebellar nuclei in the rat contain CGA-like immunoreactivity, are further supported by in situ hybridization histochemistry using cRNA probes complementary to the C-terminal portion of the rat CGA mRNA. In earlier studies carried out in the sheep brain with an antiserum against bovine CGA, it was observed that CGA occurs in Purkinje cells, in Golgi cells of the granular layer, and in neurons of the deep cerebellar nuclei (33). Our findings in the rat on distribution of CGA-like immunoreactivity and CGA mRNA using in situ hybridization techniques support and extend these data.

Our results show the presence of CGA-like immunoreactivity and its mRNA in deep cerebellar nuclei. Interestingly, there is evidence for the presence of another member of the chromogranin/secretogranin protein family, i.e., chromogranin B, in the rat perikarya of deep cerebellar nuclei, since its mRNA was described there (11). On the other hand, these perikarya were not immunoreactive for secretogranin II (chromogranin C), although they were surrounded by a loose network of immunoreactive fibers (6), which

may at least in part present endings originating from immunoreactive cerebellar Purkinje cells. Therefore, it can be concluded from the data presented here and the work of other groups that in the rat cerebellum all known members of the chromogranin/secretogranin protein family are detectable in cerebellar Purkinje cells, whereas the neurons of the deep cerebellar nuclei contain chromogranin A and B but not secretogranin II (chromogranin C). On the other hand, the human cerebellum was negative for CGA but the Purkinje cells stained strongly for chromogranin B (31).

It is well known that the neurons of the cerebellar nuclei project to the motoneurons present in the ventral horn of the spinal cord (cf. 36), where we found CGA and its mRNA. The present study shows that in the spinal cord, CGA-like immunoreactivity was also present in the cells of the lateral and dorsal horn. Moreover, these cells of the spinal cord possess specific mRNA for CGA. Therefore, our data support and extend immunocytochemical results in the spinal cord of the sheep (33) as well as in cholinergic nerve endings of the rat (5,35).

As mentioned above, projections of neurons of the dorsal horn reach the cerebellum and might be responsible for the diffuse CGA immunoreactivity in the granular layer. The fact that neurons of all layers of the spinal cord express CGA may indicate that CGA is involved in both efferent and afferent signal transduction. One could therefore speculate that CGA may be important for motor control in general.

Clearly, the function of CGA in neurons has not been examined. However, hormone release by endocrine cells is modulated by CGA or its fragments (cf. 7,10,14,15,32). In addition, CGA within the endocrine cells is involved in sorting of hormones to the regulatory pathway and in the control of intracellular calcium (20,21, 29,30). Since the basic mechanisms underlying secretion of hormones and release of neurotransmitters are very similar, CGA may fulfill similar roles in neurons.

Acknowledgments

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