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Cellular distribution of chromogranin A in excitatory, inhibitory, aminergic and peptidergic neurons of the rodent central nervous system

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ABSTRACT

Immunoreactivity for both processed and unprocessed forms of chromogranin A (CGA) was examined, using an antibody recognizing the WE14 epitope, among terminal fields and cell bodies of anatomically defined GABAergic, glutamatergic, cholinergic, catecholaminergic, and peptidergic cell groups in the rodent central nervous system. CGA is ubiquitous within neuronal cell bodies, with no obvious anatomical or chemically-coded subdivision of the nervous system in which CGA is not expressed in most neurons. CGA expression is essentially absent from catecholaminergic terminal fields in the CNS, suggesting a relative paucity of large dense-core vesicles in CNS compared to peripheral catecholaminergic neurons. Extensive synaptic co-localization with classical transmitter markers is not observed even in areas such as amygdala, where CGA fibers are numerous, suggesting preferential segregation of CGA to peptidergic terminals in CNS. Localization of CGA in dendrites in some areas of CNS may indicate its involvement in regulation of dendritic release mechanisms. Finally, the ubiquitous presence of CGA in neuronal cell somata, especially pronounced in GABAergic neurons, suggests a second non-secretory vesicle-associated function for CGA in CNS. We propose that CGA may function in the CNS as a prohormone and granulogenic factor in some terminal fields, but also possesses as-yet unknown unique cellular functions within neuronal somata and dendrites.

1. Introduction

CGA has multiple functions in neurons and neuroendocrine cells. CGA forms complexes with ATP and catecholamines believed to function in catecholamine storage in secretory granules [1–6]. CGA is the precursor for the bioactive peptides pancreastatin [7,8], the vasostatins [9], and catestatin [10–12]. CGA was demonstrated to be capable of inducing large dense-core vesicles when overexpressed in non-neuroendocrine cells, and to be involved in regulated sorting, secretion, and granulogenesis in neuroendocrine cells [13–15]. Although several granins may be involved in the generation of secretory vesicles in vivo [16], CGA-deficient mice lack physiologically appropriate diurnal regulation of catecholamine secretion from the adrenal medulla, demonstrating the importance of chromogranin A for generation of large dense-core vesicles (LDCVs) within the regulated secretory pathway in peripheral neuroendocrine cells [11,16].

Although much is known about CGA localization [17–24], function in LDCV granulogenesis [13–15], and prohormone function [7,12,25] in endocrine, neuroendocrine, and peripheral neuronal systems, its role in the CNS is less clear. The distribution of CGA was first described by Winkler et al. in the sheep and rodent brain [26,27] as not restricted to a specific type of neuronal cell or neural circuit. In situ hybridization histochemical methodology revealed the widespread presence of CGA and its mRNA in brain [28,29], leading to the demonstration that virtually all CNS neuronal cell groups throughout the brain and spinal cord expressed CGA, and the concept of CGA as a pan-neuronally expressed protein in the rat central and peripheral nervous systems [30]. Subsequently, Woulfe et al. confirmed the pan-neuronal expression of CGA mRNA in rat brain, and pointed out that differential distribution of CGA immunoreactivity is highly dependent on the antibody used to visualize the CGA protein and its processed peptides [31].

The role of CGA in sequestration of biogenic amines in LDCVs within the CNS, and indeed the subcellular localization of CGA within different types of chemically defined neuronal systems in the brain, has been relatively neglected given its importance peripherally. CGA's role as a prohormone has also not been highly investigated in the CNS. This is of particular interest given recent evidence for the dominant role of cathepsin L in prohormone processing in the CNS [32] and in PC12 cells [33], rather than the classical prohormone convertase 1 that processes CGA in endocrine cells [34]. Despite its ubiquity in the CNS, chromogranin A levels are significantly lower than in peripheral

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endocrine tissues [18,27], and exist at a higher ratio of the proteoglycanated form of the protein [27]. Indeed other roles for CGA in CNS besides either prohormone or granulogenic ones have been proposed, including its function as a molecular chaperone for misfolded superoxide dismutase, important in prevention of spinal motor neuron degeneration as occurs in amyotrophic lateral sclerosis [35].

It was our intention in the present investigation, to employ an antibody directed towards the WE14 epitope of CGA, which recognizes this epitope in both processed peptide and intact CGA [36,37], to investigate the following questions about CGA chemical neuroanatomy in the brain. First, is CGA present in both fibers and cell bodies throughout the CNS? Second, is CGA in nerve terminals associated primarily with any of the classical neurotransmitter systems or with neuropeptide projections? Third, is CGA subcellular distribution in known neurotransmitter systems consistent with a role besides that of an LDCV-associated protein/peptide? These questions were examined initially in mouse brain, so that the chromogranin A-deficient brain could be used as a control for absolute specificity of CGA epitope staining using the WE14 antibody.

2. Materials and methods

2.1. Animals

Adult male mice of the C57Bl/6 strain and CGA knock-out mice (knock-out allele of Mahapatra et al., 2005; back-crossed onto C57Bl/6 strain, N6–12) and adult male Wistar rats were terminally anesthetized, perfused transcardially with Bouin Hollande fixative or immersion-fixed, and the brain and other tissues removed. All tissues were postfixed in Bouin Hollande for up to 3 h. Bouin Hollande fixative contained 4% (w/v) picric acid, 2.5% (w/v) cupric acetate, 3.7% (v/v) formaldehyde, and 1% (v/v) glacial acetic acid. Following fixation, the tissues were extensively washed in 70% isopropanol, dehydrated, cleared with xylene, and embedded in paraffin. Seven micron thick sections were cut on a Leica microtome, mounted on gelatin-coated slides and stored until immunocytochemical staining was performed.

2.2. Single and double immunohistochemistry

Immunohistochemistry (IHC) was carried out on deparaffinized sections using an antigen retrieval technique and visualized enzymatically with 3,3-diaminobenzidine (DAB; Sigma, Deisenhofen, Germany), enhanced by addition of ammonium nickel sulfate (Fluka, Buchs, Switzerland), or visualized by immunofluorescence, as previously described [38]. Tyramide signal amplification (TSA) was used to increase sensitivity for both single immunohistochemistry and for double immunofluorescence according to the manufacturer's instructions (catalog no. SAT700; PerkinElmer Life and Analytical Sciences, Boston, MA).

2.3. Primary antibodies

CGA immunoreactivity was visualized with polyclonal rabbit WE14 antibody [30]. Cholinergic phenotypes were detected by our polyclonal rabbit (80259) and goat (1624) antibodies [39,40]. Monoaminergic traits were visualized with a rabbit polyclonal antibody (W1–2) that specifically detected mouse vesicular monoamine transporter 2 (VMAT2) and a sheep polyclonal antibody against tyrosine hydroxylase (TH) from Chemicon [38,41]. In addition, a polyclonal guinea-pig anti-VMAT2 antibody (Chemicon) was used that, however, did not recognize mouse VMAT2 but reacted with rat VMAT2. This antibody was used for double immunofluorescence analysis of CGA and VMAT2 in rat brain. Pituitary adenylate cyclase-activating polypeptide (PACAP) was stained with the rabbit anti-PACAP-38 (Progen, Heidelberg) [42]. Neuropeptide Y (NPY) was visualized with polyclonal sheep anti-NPY (Auspep, Parkville, Australia) [41]. Glutamatergic synapses were stained with polyclonal guinea-pig antibodies against the vesicular glutamate transporter isoforms VGluT1 and VGluT2 [43]. For the visualization of GABAergic synapses we used a rabbit polyclonal antibody against the vesicular inhibitory amino acid transporter (VIAAT) (W1–4) also referred to as the vesicular GABA transporter (VGAT) [44].

2.4. Single immunohistochemistry

Sections were deparaffinized in xylene and rehydrated through a graded series of isopropanol. Endogenous peroxidase activity was blocked by incubation in methanol/0.3% H_2O_2 for 30 min and antigen retrieval achieved by incubation in 10 mM sodium citrate buffer (pH 6.0) at 92–95 °C for 15 min. Non-specific binding sites were blocked with 5% bovine serum albumin (BSA) in 50 mM phosphate buffered saline (PBS, pH 7.45) for 30 min, followed by an avidin–biotin blocking step (Avidin–Biotin Blocking Kit, Boehringer, Ingelheim, Germany) for 40 min. Primary antibodies were applied in 1% BSA/PBS and incubated at 16 °C overnight followed by 2 h at 37 °C. After several washes in distilled water followed by rinsing in PBS, the sections were incubated for 45 min at 37 °C with species-specific biotinylated secondary antibodies (Dianova, Hamburg, Germany), diluted 1:200 in 1% BSA/PBS, washed again several times and incubated for 30 min with avidin–biotin–peroxidase complex reagents (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). Immunoreactions were visualized by an 8 min incubation with 3,3-diaminobenzidine (DAB, Sigma Aldrich, Deisenhofen, Germany), enhanced by the addition of 0.08% ammonium nickel sulfate (Fluka, Buchs, Switzerland), which resulted in a dark blue staining. After three 5 min washes in distilled water, the sections were dehydrated through a graded series of isopropanol, cleared in xylene and finally mounted under coverslips. Digital bright-field pictures were taken with an Olympus AX70 microscope (Olympus Optical, Hamburg, Germany), equipped with a SPOT RT Slider Camera and SPOT Image Analyses software (Version 3.4; Diagnostic Instruments Inc., Seoul, Korea).

2.5. Double immunofluorescence

After deparaffinization and blocking procedures, appropriate combinations of two primary antibodies raised in different donor species were co-applied in 1% BSA/PBS and incubated overnight at 4 °C, followed by 2 h at 37 °C. After extensive washing in distilled water followed by PBS, immunoreactions for the first primary antibody were visualized with species-specific secondary antibodies labeled with Alexa Fluor 647 (MoBiTec, Göttingen, Germany), diluted 1:200 in 1% BSA/PBS. The second primary antibody was visualized by a two-step procedure including species-specific biotinylated secondary antisera (Dianova), diluted 1:200 in 1% BSA/PBS followed by streptavidin conjugated with Alexa Fluor 488, diluted 1:100 in 1% BSA/PBS. Incubation times were 45 min with biotinylated secondary antibodies, followed by a 2 h incubation with a mixture of fluorochrome-conjugated secondary antibody and streptavidin. Immunofluorescent signals were documented as digitized false color images (8-bit tiff format) with an Olympus BX50WI confocal laser scanning microscope (Olympus Optical, Hamburg, Germany) and Olympus Fluoview 2.1 software.

3. Results

3.1. Specificity and ubiquity of WE14 staining

Chromogranin A (i.e. WE14) immunoreactivity is widespread but exhibits varied levels of expression throughout the mouse central nervous system (Fig. 1). Knock-out of both chromogranin alleles has been back-crossed onto C57Bl/6 mice, a strain in which genetic,