available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Calretinin distribution in the octopus brain: An immunohistochemical and *in situ* hybridization histochemical analysis

*Giovanna G. Altobelli, Vincenzo Cimini**

Department of Biomorphological and Functional Sciences, "Federico II" University of Naples, Via Pansini 5, 80131 Napoli, Italy

ARTICLE INFO

Article history:
Accepted 13 November 2006
Available online 26 December 2006

Keywords:
Calcium-binding protein
Calretinin
Brain
Cephalopod
Immunocytochemistry
In situ hybridization
Octopus
Western blot
Evolution
Neuropil
Cortex

ABSTRACT

The distribution of calretinin containing neurons examined by *in situ* hybridization mapping was compared with that obtained by immunocytochemistry in the brain of octopus. Results revealed a close correspondence between the two types of investigations. Western blot analysis disclosed a 29 kDa protein immunostained with anti-calretinin antibody. Calretinin containing neurons were localized mainly in the cortex of octopus lobes, including the vertical, frontal, basal, buccal, palliovisceral, pedal and branchial, with variations of staining intensity and density of immunoreactive cells. The amacrine cells surrounding calretinin containing neuronal bodies of the cortex were also labeled unlike the glial cells. The close correspondence of blotting analysis, immunocytochemistry and *in situ* hybridization indicates with no doubt that calretinin, like other calcium-binding proteins previously studied, is also present in the nervous system of cephalopods. Furthermore, although recent findings localize calretinin also in endocrine glands, the presence of this calcium-binding protein in the brain of octopus indicates that calretinin appeared early in the phylogeny as a neuronal protein already in invertebrates.

1. Introduction

The octopus nervous system is made by a central and a peripheral part. The central nervous system includes brain and optic lobes, while the peripheral nervous system includes the body and tentacles. The central body of the nervous system is encapsulated by cartilage. The central nervous system is composed of about fifty lobes and lobules. Nerve centers may be divided in five groups with interesting functional similarity to that of vertebrates: (1) inferior motor centers (tentacle ganglia, stellate ganglia); (2) intermediate motor centers (subesophageal ganglia); (3) superior motor centers (basal supraesophageal centers); (4) reception and memory analyzers (optic lobes, maybe brachial and posterior buccal lobes); (5) association centers (inferior and superior frontal, vertical and subfrontal lobes). Structurally, a cortex of neurons covers a deeper neuropil containing a number of organized tracts and pathways. For an accurate description of anatomy and physiology of octopus brain, we recommend Young (1971).

* *Corresponding author*. Fax: +39 081 7463427.
E-mail address: cimini@unina.it (V. Cimini).

Abbreviations: CR, calretinin; SpE, supraesophageal; SbE, subesophageal; V, vertical; SV, subvertical; SMF, superior median frontal; IMF, inferior median frontal; SbF, subfrontal; SB, superior buccal; DB, dorsal basal; MB, medial basal; AB, anterior basal

0006-8993/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2006.11.056

Calretinin (CR) is a calcium-binding protein which participates in a variety of functions including calcium buffering and neuronal protection (Billing-Marczak and Kuznicki, 1999; Heizmann and Hunziker, 1991; Rogers et al., 1990). It also serves as a developmental marker of retinal ganglion cells (Yan et al., 1996). It has been considered a neuronal marker until localized also in some endocrine glands (Afework and Burnstock, 1995; Bertschy et al., 1998; Cimini et al., 1997; Pohl et al., 1992; Pochet et al., 1994; Strauss et al., 1994).

Only some EF-hand proteins, such as calmodulin, have been extracted and sequenced in squid neurons (Krinks et al., 1988).

The presence of calcium-binding proteins has been investigated in the brain of a number of vertebrates, but very little in the invertebrate brains (Head, 1989). Christakos et al. (1987) were the only authors to study the immunocytochemical localization of the calcium-binding protein calbindin in cephalopods.

The distribution and localization of the neuronal calcium-binding protein calretinin have been widely studied in the nervous system of both lower and higher vertebrates (see Jacobowitz and Abbott, 1997 for mouse brain immunohistochemical mapping). The present report provides a detailed description of CR-containing lobes in the octopus brain. This is accomplished by using together with immunoblotting analysis both calretinin immunochemistry and hybridocytochemistry in order to examine the expected colocalization of the protein and its messenger RNA.

2. Results

2.1. General observations

2.1.1. Western blot

Western blotting analysis showed that calretinin antibody used in these experiments specifically recognized a CR-like protein of about 30 kDa and failed to recognize this band in the heart, where CR is absent. A tiny band corresponding to lower molecular weight proteins is present. Negative controls, with normal rabbit serum (NRS) instead of specific antibody, did not show any labeled band (Fig. 1).

2.1.2. In situ hybridization (ISH)

The CR oligonucleotide probe (Fig. 2) produced a specific pattern of labeling that, with a few exceptions, matched the distribution of calretinin immunoreactivity. Isotope-labeled structures were found to many of the brain lobes and in each lobe the cortex only was mainly labeled, not the neuropil. Incubation of sections in buffer containing labeled sense probe revealed no specific label.

2.1.3. Immunocytochemistry (ICC)

The localization of calretinin immunoreactivity was almost always cytoplasmic and in some cases also nuclear. Immunoreactive nerve fibers were also present. The use of antigen-preabsorbed anti-calretinin antibody quenched the immunohistochemical reaction (Fig. 3H).

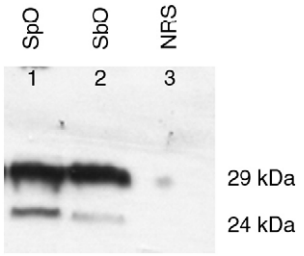

Fig. 1 – Western blot analysis. Immunoblots from octopus supraesophageal (lane 1) and subesophageal (lane 2) brain masses were stained by rabbit anti-calretinin. This antibody did not detect the protein with heart tissue's extract (not shown here). Calretinin was not detected after substitution of the primary antibody with normal rabbit serum (lane 3). The protein recognized by the antibody has a molecular weight similar to that of higher vertebrates.

A description of the octopus brain areas containing calretinin follows taking into account either immunocytochemical and *in situ* hybridization, while a summary of the semi-quantitative results is presented (Table 1).

2.2. Lobes of supraesophageal (SpE) mass

2.2.1. Vertical and superior frontal lobes

2.2.1.1. ISH. The CR oligonucleotide probe produced a specific labeling of vertical and frontal lobe cortex.

2.2.1.2. ICC. Numerous CR-immunoreactive, voluminous neurons ("large cells" according to Gray, 1970) were displaced among the amacrine cells of the vertical lobe and the neuropil CR-immunoreactive fibers (Fig. 3A). Amacrine cells of this lobe were immunolabeled as well as in the other lobes of the vertical–frontal system. Calretinin immunoreactivity appeared to be present in large neurons of the superior frontal lobe cortex (Fig. 3B).

2.2.2. Inferior frontal and buccal lobes

2.2.2.1. ISH. Either cortical regions of inferior frontal and buccal lobes appeared to be labeled by calretinin oligonucleotide probe.

2.2.2.2. ICC. The median inferior frontal lobe was characterized by numerous CR-immunoreactive neurons (Fig. 3C): their calretinin containing fibers leave the cortex toward the neuropil. The lateral inferior frontal lobes were devoid of labeling.

Within the superior buccal lobe, antibody stain appeared to identify a subpopulation of numerous, differently sized neurons, some of them confining with the neuropil (Fig. 3D), where however numerous CR-immunoreactive nerve fibers were also present. Only voluminous neurons of the posterior buccal lobe appeared to contain calretinin immunoreactivity (Fig. 3E). On the contrary, smaller neurons and neuropil were only slightly positive.

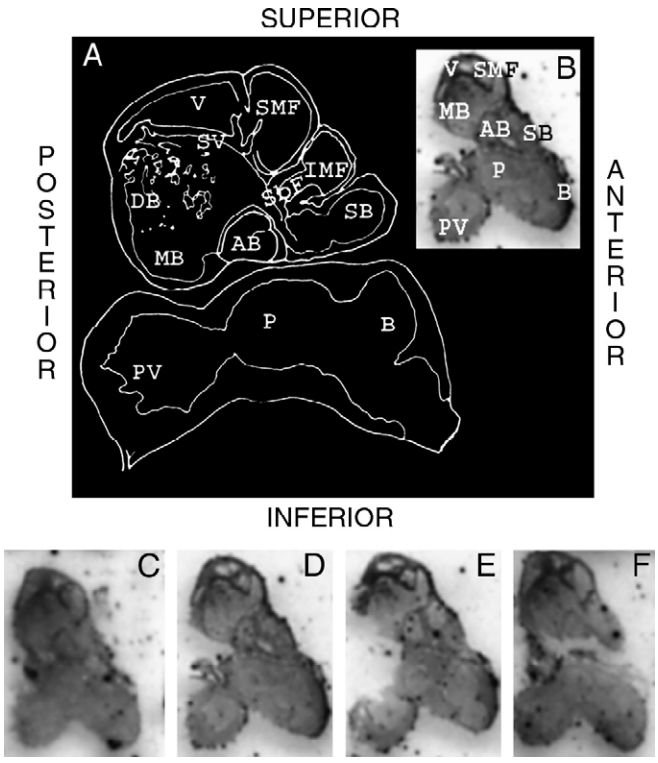

Fig. 2 – *In situ* hybridization analysis. The analysis was performed on digitalized images obtained by the autoradiograms of films exposed to isotopic labeling of antisense CR probe. Four sagittal sections out of 56 were randomly sorted out to show CR signal throughout the brain (C–F). The drawing (modified from Saidel, 1982) illustrates labeled lobes (A). SpE lobes are labeled as follows: V = vertical; SV = subvertical; SMF = superior median frontal; IMF = inferior median frontal; SbF = subfrontal; SB = superior buccal; DB = dorsal basal; MB = median basal; AB = anterior basal. SbE lobes are labeled as follows: PV = palliovisceral; P = pedal; B = branchial. Some lobes are also illustrated on the autoradiogram aside (B).

2.2.3. *Basal lobes*

2.2.3.1. *ISH.* Throughout the basal lobes, the brain cortex showed a slight labeling with calretinin oligonucleotide probe, with the exception of dorsal basal lobe, whose labeling appeared more intense (Fig. 2).

2.2.3.2. *ICC.* CR-immunoreactive neurons were present throughout the cortical wall of dorsal, median and anterior lobes. In the anterior basal lobe, the lateral anterior and median lobules showed calretinin containing cell bodies (Fig. 3F). The neuropil of both dorsal lobes was poorly labeled, as well as the lateral basal lobe.

2.3. *Lobes of subesophageal (SbE) mass*

2.3.1. *Palliovisceral, pedal and branchial lobes*

2.3.1.1. *ISH.* *In situ* hybridization revealed moderate peripheral labeling: the entire SbE mass appeared well lined by a cortical labeling with calretinin oligonucleotide probe. Labeling was also seen around the single lobes of the SbE mass.

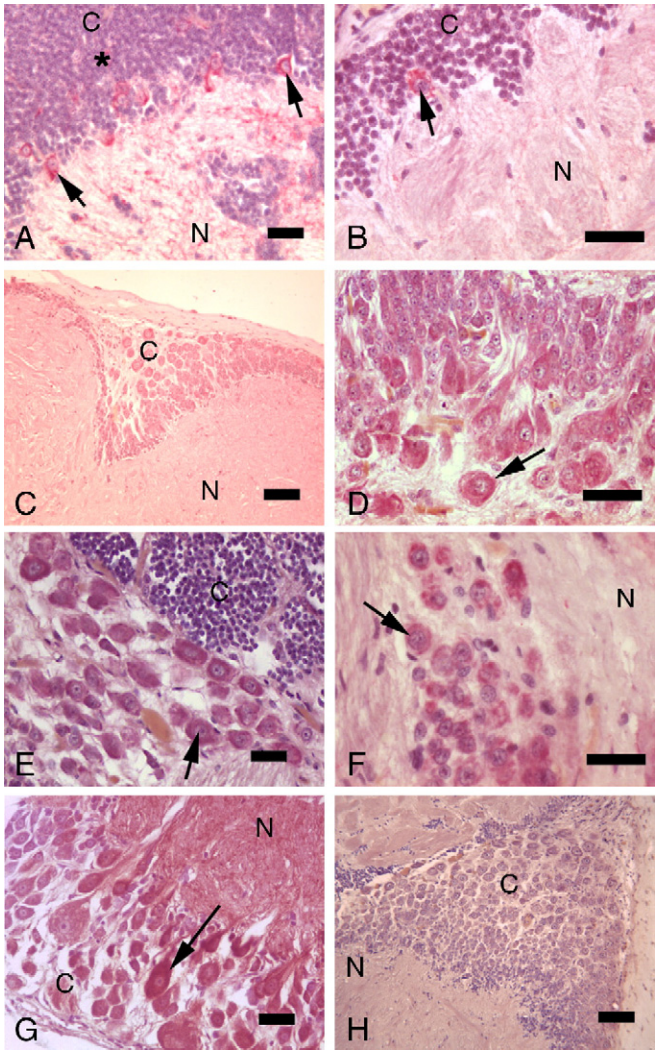

Fig. 3 – Calretinin immunostained sagittal sections of octopus brain. Brain lobes have a cortex (C) consisting of the neuronal perikarya, while the core or medulla of each lobule constitutes the neuropil (N). Two distinct classes of neuronal perikarya are found in the cortex: small cells (*) called amacrine cells, and large cells (arrows) which may lie in the inner zones of the cortex. Calretinin-immunoreactive cells were most prominent in the cortex of the vertical lobe (A), of the superior frontal lobe (B), of the inferior frontal lobe (C), of the superior buccal lobe (D), of the posterior buccal lobe (E), of the anterior basal lobe (F) and of pedal lobe (G). The distribution of calretinin immunoreactivity mostly matched the patterns of *in situ* hybridization shown in Fig. 2. A negative control obtained by incubation of pre-absorbed antiserum is shown in panel H. Magnifications: A, vertical lobe, 25×; B, superior frontal lobe, 40×; C, inferior frontal lobe, 10×; D, superior buccal lobe, 40×; e, posterior buccal lobe, 25×; F, anterior basal lobe, 40×; G, pedal lobe, 25×; H, negative control, 10×. Scale bars of 40× and 25× = 100 μm; that of 10× = 250 μm.

Table 1 – Summary of localization of CR-containing structures in the octopus brain

Brain areas	ISH—cortex	ICC—cell bodies	ICC—fibers
Vertical lobe	+	++	++
Subvertical lobe	++$^{+}$	++	+
Frontal area			
Superior median frontal lobe	++	++	+
Inferior median frontal lobe	++	+	+
Subfrontal lobe	++	±	±
Basal area			
Dorsal basal lobe	++	++$^{+}$	±
Median basal lobe	+	++	+
Anterior basal lobe	+	+	+
Buccal area			
Superior buccal lobe	+	+$^{+}$	+
Posterior buccal lobe	+	++	±
Palliovisceral lobe	±	+$^{+}$	+$^{+}$
Pedal lobe	±	+$^{+}$	+$^{+}$
Branchial lobe	+	+$^{+}$	+

Localization of CR in the brain cortex was determined by *in situ* hybridization and immunocytochemistry, while in the neuropil by immunocytochemistry only. Description of semi-quantitative analysis is as follows: ++, numerous positive cells and/or fibers or intense radiolabeling; ++$^{+}$, more than ++; +, discrete number of cells and/or fibers or moderate radiolabeling; +$^{+}$, more than+; ±, scattered positive cells and/or fibers or less intense radiolabeling.

2.3.1.2. ICC. Both palliovisceral and pedal lobe showed CR-immunoreactive cell bodies of different size, while nerve fibers of neuropil were strongly labeled (Fig. 3G). Branchial lobe as well showed cortical labeling with immunocytochemistry.

3. Discussion

The aim of the present study was to investigate the distribution of the 29 kDa calcium-binding protein calretinin in the octopus brain by means of immunochemical and *in situ* hybridization methods.

The results of blot analysis indicate that both the SpE and SbE regions of the octopus brain contain proteins that are sufficiently similar to be recognized by anti-calretinin antibody. These data strongly suggest that the calcium-binding protein stained in the immunocytochemical preparations is calretinin or a closely related protein, although definitive identification must await the cloning and sequencing of octopus calretinin. The presence of a lower molecular weight protein could be due to species differences between octopus and human calretinin, although other possibilities remain.

The examination of sagittal brain sections has shown the expression of calretinin in several areas of the octopus brain, including the vertical lobe, the superior and inferior frontal lobes, the basal lobes of the SpE mass and the palliovisceral lobe, the pedal lobe and the branchial lobe of the SbE mass. CR immunoreactivity has been observed mainly at neuronal cell body and dendritic level, often at axonal and nuclear level. Glial cells were not labeled.

The staining intensity was variable among the different examined lobes. However, either distribution of calretinin containing neurons and differential staining of the different lobes did not show any obvious feature which could account for the functional role of this calcium-binding protein. Of course the brain of higher vertebrates has been massively studied compared with that of cephalopods, but the problem of understanding the exact functional role of neuronal calretinin is still remaining. The differential staining intensity of immunostained neurons might be a useful tool to identify new subclasses of neurons in future studies.

Calretinin is present in lobes of both the SpE and SbE mass of octopus brain. That means either sensory neurons, mainly belonging to the SpE mass, or motor neurons, mainly belonging to the SbE mass, express the calcium-binding protein calretinin. Once again it is not known the meaning of why calretinin labels sensory or motor neurons. Otherwise studies with neuronal markers for specific lobes of octopus brain are still lacking: the vertical lobe, for instance, is concerned with the integrative action of the visual and chemo-tactile systems, but no one report is present throughout the literature on the basis of neuro-histochemical characterization of this lobe, except the distribution of acetylcholinesterase and catecholamines in the vertical lobe of *Octopus vulgaris* (Barlow, 1971). The systematic study of octopus cerebral structures for neuronal markers could help to better understand their function with relationship to the expression of calretinin. For example, the use of neuronal markers helped to show that calretinin is a specific marker of olfactory receptor neurons and of various neuronal populations distributed throughout the telencephalon and diencephalon of the zebrafish (Castro et al., 2006). Furthermore, the presence of NOS (nitric oxide synthase) in the octopus optic, tactile and peduncle lobes (Di Cosmo et al., 2000) has recently been reported. Some of our unpublished observations demonstrate the presence of calretinin in the optic, tactile and peduncle lobes: as NOS activity is calmodulin-modulated, it seems likely that a calcium-based system might be related to calretinin. Therefore, it might be extremely likely that this protein in the octopus brain has a novel unknown role and not simply that of a calcium buffering protein.

The variable patterns in distribution of calretinin among species can be analyzed in the context of the cephalopod brain cytoarchitecture: it would be meaningless to try comparisons between phylogenetically distant species, considering that mammalian brain is the most investigated.

On the basis of these and above considerations, we think there is no point getting deeper into the analysis of comparative aspects. Instead, the high degree of correspondence between the immunocytochemical and hybridocytochemical results, the demonstration of a calretinin-like protein by Western blot analysis and the quenching of the histochemical reactions, respectively, with the antigen-absorbed anti-CR and with the sense probe showed that the antisense calretinin probe recognized a very CR-specific mRNA. Therefore, the main topic of this report is that calretinin, a protein to date studied mainly in vertebrates, has been for the first time localized in the brain of an invertebrate.

Calretinin localization is mainly neuronal and this is related to the fundamental role of transmembrane and also

cytosolic signaling of calcium ions (Baimbridge et al., 1992), whose increase of free form triggers numerous processes such as electric activity, neurotransmitter release, fast axonal flow and memory storage. Hundreds of previous studies demonstrated its relationship with nervous system (see the reviews: Baimbridge et al., 1992; Bastianelli, 2003; Heizmann, 1993; Koutcherov et al., 2003; Kwong, 2000; Parent et al., 1996; Ren and Ruda, 1994; Rogers et al., 1990; Schwaller et al., 2002; Ulfig, 2002), although calretinin was discovered also in endocrine glands. However, this study on the nervous system of cephalopods, the lowest in the zoological scale to date studied, confirms that calretinin, from a phylogenetic point of view, represents a protein of neuronal origin. The increasing complexity of organisms linked together with the need of communication and integration among systems might have led to calretinin appearance also in the endocrine apparatus. Further studies would need to clarify whether calretinin and other calcium-binding proteins of this family are expressed in the remaining parts of cephalopod nervous system. This, together with the study of other neurochemical markers, would allow functional correlations in the octopus brain.

In conclusion, the present study has provided a partial cytotectonic map of the octopus brain based on the presence of the EF-hand calcium-binding protein calretinin that has not been done before. The cephalopod's brain is the most highly developed nervous system among the invertebrates, but it is equally important from our point of view because the perfect knowledge of calcium-binding protein role even in the octopus brain may eventually give us a system to understand the animal and human behavior. This study represents just the starting point: it is hopefully expected that patterns of calcium-binding protein distribution can be further analyzed in the context of the cephalopod brain cytoarchitecture.

4. Experimental procedures

Sexually mature octopuses (*O. vulgaris*), ranging from 500 to 1500 g body weight, were captured in the bay of Naples. The same day, animals were killed with an overdose of anesthetic (MS222, Sandoz, Germany) and brains were dissected out including supra- and subesophageal masses. Optic lobes were not considered in this study.

For ISH, freshly dissected out octopus brains were quickly frozen in liquid nitrogen-cooled isopentane and kept at −80 °C until cryostat sectioning. Specimens for immunocytochemical studies were immediately fixed by immersion for at least 12 h in Bouin's solution and then embedded in paraffin according to previous studies (Cimini, 2003).

4.1. *In situ hybridization histochemistry*

The calretinin probe was a 45-base oligodeoxyribonucleotide complementary to bases 4835–4879 of the mouse mRNA (GenBank accession no. AB037964). The probe was labeled at 3′ using [α^{35}S] dATP (>1000 Ci/mmol, Amersham Pharmacia Biotech, USA, now Amersham Biosciences), terminal deoxynucleotidyl transferase (15 units/ml) (Roche, Switzerland) and tailing buffer. The unincorporated nucleotides were separated from radiolabeled DNA using Sephadex G-50 chromatographic columns (Amersham Pharmacia Biotech).

The procedure for *in situ* hybridization was taken from several standard published protocols (Arai et al., 1994; Cicale et al., 2002; Cimini et al., 1997; Isaacs et al., 1995; Winsky et al., 1989). Briefly, 10- to 12-μm-thick sections were fixed in 1.5% formaldehyde in 0.12 M sodium phosphate buffer, quickly rinsed three times with 1× buffer and placed in 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl, pH 8.0, for 10 min. Next, the sections were dehydrated in 70%, 80%, 90% and 100% ethanol, delipidated in chloroform for 5 min, then rinsed again in 100% and 95% ethanol and air dried. Sections were hybridized with 0.4–0.6×10^6 cpm of radiolabeled oligonucleotide in buffer containing 50% formamide, 600 mM NaCl, 80 mM Tris–HCl (pH 7.5), 4 mM EDTA, 0.1% pyrophosphate, 0.2 mg/ml heparin sulfate and 10% dextran sulfate. Incubations were carried out at 37 °C in a humid chamber for 20 h. After hybridization, the sections were processed through four 15-minute washes in 2× SSC/50% formamide at 40 °C followed by two 30-minute washes with 1× SSC at 40 °C. The slides were rapidly rinsed in distilled water and then in 70% ethanol. The sections were dried and exposed to Biomax MR X-ray film (Kodak Scientific Imaging Film, USA). The ideal time of exposure was chosen to maximize the signal to noise ratio and also to avoid optical density approaching the limits of saturation. The evaluation of *in situ* hybridization experiments was done on radiograms obtained by inverting the scanned X-ray film so that the white areas corresponded to labeled structures.

4.2. *Immunocytochemistry*

Six-micrometer-thick sagittal sections were serially cut and processed for immunocytochemistry by 3 days incubation at 4 °C with 1/1500 rabbit anti-calretinin antiserum (Swant, Switzerland), which was raised against human recombinant calretinin, and then the signal was revealed by using the following standardized immunocytochemical procedure.

In order to reveal the primary antibody, the sections were incubated (30 min at room temperature) with a goat anti-rabbit IgG (1/20000, Calbiochem, Germany) diluted in 0.01 M phosphate-buffered saline (PBS), pH 7.4, containing 0.1% BSA. They were then rinsed three times (5 min each) by 0.2 M Tris buffered saline (TBS), pH 8.2. The third step was performed by a 30-minute incubation with a mouse alkaline phosphatase anti-alkaline phosphatase (APAAP) antibody (1/300, Dako, Denmark) diluted in TBS. Following the three 5-minute washes, the enzymatic reaction of alkaline phosphatase in presence of a mixture containing naphthol AS-MX-phosphate (Sigma-Aldrich, USA) as substrate and Fast red TR (Sigma-Aldrich) as chromogen colored red the immunoreactive structures. Finally, after tap water washing to stop the enzymatic reaction, sections were mounted with Ultramount (Dako) and observed with a Leitz Diaplan microscope. Photographs were taken with a Leica DC-200 digital camera.

4.3. *Negative controls*

As negative controls, tissue sections were incubated without primary antibody or with 0.1 μM recombinant CR (Swant)-absorbed anti-calretinin antibody (1/1500, Swant).

4.4. *Western blot analysis*

Tissue protein extracts (approximately 20 μg per sample) were electrophoretically separated under reduced conditions using NuPAGE 12% Bis–Tris gels (Invitrogen, USA). Recombinant CR was loaded as positive control. Low molecular weight Standard Mark12 (Invitrogen) was used as calretinin at 29 kDa, while rat heart tissue extract was used as a negative control. Proteins were then electrotransferred to 0.2 μm nitrocellulose membranes (Bio-Rad Laboratories, USA) and the immunoblots were subsequently blocked for 2 h on an orbital shaker at room temperature with PBS (pH 7.7) containing 0.1% Tween-20 (Fluka, Switzerland) and 5% non-fat dry milk. The membranes were incubated for 3 days at 4 °C with a rabbit antiserum against CR (Swant) diluted 1/1500 or a monoclonal antibody against actin (Sigma-Aldrich) diluted 1/5000, which was used as an internal control for equal loading. After rinsing the membranes with PBS/0.1% Tween-20, they were incubated in phosphatase alkaline-conjugated anti-rabbit secondary IgG (Calbiochem) diluted 1/3000 in Tris–HCl/Triton/BSA for 3 h at 37 °C. After additional rinses in Tris–HCl/Triton, membranes were stained with NBT/BCIP (Sigma-Aldrich) according to the manufacturer's instructions.

Acknowledgments

Supported by grants from Federico II's University of Naples. We are indebted to Professor A. Di Cosmo and Dr. C. Di Cristo for their initial collaboration and guidance and for access to their material. We are also grateful to Dr. Antimo D'Aniello (Stazione Zoologica di Napoli "A. Dohrn") for giving us most of the animals used in this study. We gratefully appreciated the laboratory assistance of Dr. S. Vitale during the preparation of her BSc thesis.

REFERENCES

Afework, M., Burnstock, G., 1995. Calretinin immunoreactivity in adrenal glands of developing, adult and ageing Sprague–Dawley rats. Int. J. Dev. Neurosci. 13, 515–521.

Arai, R., Jacobowitz, D.M., Deura, S., 1994. Distribution of calretinin, calbindin-D28k, and parvalbumin in the rat thalamus. Brain Res. Bull. 33, 595–614.

Baimbridge, K.G., Celio, M.R., Rogers, J.H., 1992. Calcium-binding proteins in the nervous system. Trends Neurosci. 15, 303–308.

Barlow, J.J., 1971. The distribution of acetylcholinesterase and catecholamines in the vertical lobe of *Octopus vulgaris*. Brain Res. 35, 304–307.

Bastianelli, E., 2003. Distribution of calcium-binding proteins in the cerebellum. Cerebellum 2, 242–262.

Bertschy, S., Genton, C.Y., Gotzos, V., 1998. Selective immunocytochemical localisation of calretinin in the human ovary. Histochem. Cell Biol. 109, 59–66.

Billing-Marczak, K., Kuznicki, J., 1999. Calretinin-sensor or buffer-function still unclear. Pol. J. Pharmacol. 51, 173–178.

Castro, A., Becerra, M., Manso, M.J., Anadon, R., 2006. Calretinin immunoreactivity in the brain of the zebrafish, *Danio rerio*: distribution and comparison with some neuropeptides and neurotransmitter-synthesizing enzymes: II. Midbrain, hindbrain, and rostral spinal cord. J. Comp. Neurol. 494, 792–814.

Christakos, S., Malkowitz, L., Sori, A., Sperduto, A., Feldman, S.C., 1987. Calcium binding protein in squid brain: biochemical similarity to the 28,000-Mr vitamin D-dependent calcium binding protein (calbindin-D28k). J. Neurochem. 49, 1427–1437.

Cicale, M., Ambesi-Impiombato, A., Cimini, V., Fiore, G., Muscettola, G., Abbott, L.C., de Bartolomeis, A., 2002. Decreased gene expression of calretinin and ryanodine receptor type 1 in tottering mice. Brain Res. Bull. 59, 53–58.

Cimini, V., 2003. Neuropeptides in anterior pituitary development. Int. J. Dev. Neurosci. 21, 291–301 (Review).

Cimini, V., Isaacs, K.R., Jacobowitz, D.M., 1997. Calretinin in the rat pituitary: colocalization with thyroid-stimulating hormone. Neuroendocrinology 65, 179–188.

Di Cosmo, A., Di Cristo, C., Palombo, A., d'Ischia, M., Messenger, J.B., 2000. Nitric oxide synthase (NOS) in the brain of the cephalopod *Sepia officinalis*. J. Comp. Neurol. 428, 411–427.

Gray, E.G., 1970. The fine structure of the vertical lobe of octopus brain. Philos. Trans. R. Soc. London., Ser. B 258, 379–394.

Head, J.F., 1989. Amino acid sequence of a low molecular weight, high affinity calcium-binding protein from the optic lobe of the squid *Loligo pealei*. J. Biol. Chem. 264, 7202–7209.

Heizmann, C.W., 1993. Calcium signaling in the brain. Acta Neurobiol. Exp. (Wars) 53, 15–23.

Heizmann, C.W., Hunziker, W., 1991. Intracellular calcium-binding proteins: more sites than insights. Trends Biochem. Sci. 16, 98–103.

Isaacs, K.R., Winsky, L., Strauss, K.I., Jacobowitz, D.M., 1995. Quadruple colocalization of calretinin, calcitonin gene-related peptide, vasoactive intestinal peptide, and substance P in fibers within the villi of the rat intestine. Cell Tissue Res. 280, 639–651.

Jacobowitz, D.M., Abbott, L.C., 1997. Chemoarchitectonic Atlas of the Developing Mouse Brain. CRC Press.

Koutcherov, Y., Mai, J.K., Paxinos, G., 2003. Hypothalamus of the human fetus. J. Chem. Neuroanat. 26, 253–270.

Krinks, M.H., Klee, C.B., Pant, H.C., Gainer, H., 1988. Identification and quantification of calcium-binding proteins in squid axoplasm. J. Neurosci. 8, 2172–2182.

Kwong, W.H., Chan, W.Y., Lee, K.K., Fan, M., Yew, D.T., 2000. Neurotransmitters, neuropeptides and calcium binding proteins in developing human cerebellum: a review. Histochem. J. 32, 521–534.

Parent, A., Fortin, M., Cote, P.Y., Cicchetti, F., 1996. Calcium-binding proteins in primate basal ganglia. Neurosci. Res. 25, 309–334.

Pochet, R., Van Rampelbergh, J., Bastianelli, E., Van Eldik, L.J., 1994. Calmodulin, calbindin-D28K and calretinin in rat and chicken pineal glands: immunocytochemical and immunoblotting analysis. Biochim. Biophys. Acta 1223, 318–324.

Pohl, V., Van Rampelbergh, J., Mellaert, S., Parmentier, M., Pochet, R., 1992. Calretinin in rat ovary: an in situ hybridization and immunohistochemical study. Biochim. Biophys. Acta 1160, 87–94.

Ren, K., Ruda, M.A., 1994. A comparative study of the calcium-binding proteins calbindin-D28K, calretinin, calmodulin and parvalbumin in the rat spinal cord. Brain Res. Brain Res. Rev. 19, 163–179.

Rogers, J., Khan, M., Ellis, J., 1990. Calretinin and other CaBPs in the nervous system. Adv. Exp. Med. Biol. 269, 195–203.

Saidel, W.M., 1982. Connections of the octopus optic lobe: an HRP study. J. Comp. Neurol. 206, 346–358.

Schwaller, B., Meyer, M., Schiffmann, S., 2002. 'New' functions for 'old' proteins: the role of the calcium-binding proteins calbindin D-28k, calretinin and parvalbumin, in cerebellar physiology. Studies with knockout mice. Cerebellum 1, 241–258.

Strauss, K.I., Isaacs, K.R., Ha, Q.N., Jacobowitz, D.M., 1994. Calretinin is expressed in the Leydig cells of rat testis. Biochim. Biophys. Acta 1219, 435–440.

Ulfig, N., 2002. Calcium-binding proteins in the human developing brain. Adv. Anat., Embryol. Cell Biol. 165, 1–92.
Winsky, L., Nakata, H., Martin, B.M., Jacobowitz, D.M., 1989. Isolation, partial amino acid sequence, and immunohistochemical localization of a brain-specific calcium-binding protein. Proc. Natl. Acad. Sci. U. S. A. 86, 10139–10143.
Yan, Y.H., Winarto, A., Mansjoer, I., Hendrickson, A., 1996. Parvalbumin, calbindin, and calretinin mark distinct pathways during development of monkey dorsal lateral geniculate nucleus. J. Neurobiol. 31, 189–209.
Young, J.Z., 1971. The Anatomy of the Nervous System of *Octopus vulgaris*. Oxford, Clarendon.