Calretinin Immunoreactivity in the Cerebral Cortex of the Lizard Psammodromus algirus: A Light and Electron Microscopic Study

JOSÉ C. DÁVILA, JESÚS PADIAL, MANUEL J. ANDREU, M. ÁNGELES REAL, AND SALVADOR GUIRADO*
Departamento de Biología Celular, Facultad de Ciencias, Universidad de Málaga, 20971 Málaga, Spain

ABSTRACT
The present study describes the distribution and structural features of calretinin-immunoreactive neurons and fiber plexuses in the cerebral cortex of a lacertid lizard, at the light and electron microscopic levels, and also examines the colocalization of calretinin with parvalbumin and gamma-aminobutyric acid (GABA) in certain cortical regions. Calretinin-immunoreactive neurons are present throughout the cerebral cortex of Psammodromus and can be classified according to morphological and neurochemical criteria. Neurons in the medial cortex are small, spine-free and lack parvalbumin, whereas in the lateral cortex, calretinin-immunoreactive neurons display sparsely spiny dendrites and also lack parvalbumin. The dorsomedial and dorsal cortices contain most of the calretinin cortical neurons, which were located almost exclusively in the deep plexiform layer. These neurons are large, with an extensive spine-free dendritic tree. Most of the calretinin-immunoreactive neurons of dorsomedial and dorsal cortices are GABAergic and contain parvalbumin. Calretinin-immunoreactive fibers form two main afferent systems in the cortical areas. One probably intrinsic inhibitory system, arising from the calretinin and parvalbumin GABAergic neurons in the dorsomedial and dorsal cortices, makes symmetrical synapses on the soma and proximal dendrites of neurons located in the cell layers of the same cortical areas. The other system is formed by extremely thin axons running within the superficial plexiform layers of the medial, dorsomedial and dorsal cortices. These axons make asymmetrical synapses on dendrites or dendritic spines. We suggest that this system, probably extrinsic excitatory, arises from neurons located in the basal forebrain.

The cytoarchitectonic of the cerebral cortex of Psammodromus are typical of other lizards: the entire cerebral cortex is tri-laminar (a cell layer sandwiched between two plexiform layers, superficial and deep, respectively) and consists of four regions, medial, dorsomedial, dorsal and lateral (Guirado et al., 1984). From an evolutionary viewpoint, evidence from cytoarchitectonic, connectional or developmental studies support the comparison of the medial regions of the reptilian cerebral cortex (medial, dorsomedial and part of dorsal cortices) with parts of the mammalian hippocampal formation (Ramón y Cajal, 1917; Curwen, 1937; Northcutt, 1967, 1978; Ebbesson and Veneida, 1969; Lacey, 1978; Lohman and van Woerden-Verkley, 1978; López-García et al., 1992), whereas the reptilian lateral cortex has been considered homologous to the piriform cortex (Hoogland and Vermeulen-VanderZee, 1988). In this context, the use of cellular markers allows the comparison of neurons located in presumptive homologous cerebral regions (and recruited in specific neural circuits) of phylogenetically distinct species.

Grant sponsor: Spanish DGICYT; Grant number: PB93-1001.
*Correspondence to: Dr. S. Guirado, Departamento Biología Celular, Facultad de Ciencias, Campus de Teatinos, 20971 Málaga, Spain. E-mail: guirado@ccuma.sci.uma.es
Received 28 June 1996; Revised 17 January 1997; Accepted 22 January 1997

© 1997 WILEY-LISS, INC.
Calcium-binding proteins are widely accepted as cellular markers for distinct populations of neurons in the central nervous system. In mammals, the three best documented calcium-binding proteins, parvalbumin, calretinin and calbindin-D28K (members of the EF-hand family of calcium-binding proteins), are expressed by segregated gamma-aminobutyric acid (GABA)ergic cell populations both in the hippocampus (Nitsch et al., 1990; Braak et al., 1993; Gulyás et al., 1991; Seress et al., 1991, 1993; Acdsy et al., 1993; Nitsch and Léránth, 1993; Pitkänen and Amaral, 1993; Ribak et al., 1993; Deller et al., 1994) and the isocortex (Hendry and Jones, 1991; Rogers and Réseibois, 1992; Condé et al., 1994; Kubota and Kawaguchi, 1994).

Previous studies in Psammomimus demonstrated that neurons containing parvalbumin constitute a subpopulation of GABAergic neurons different from those containing neuropeptides (Dávila et al., 1993) or NADPH-diaphorase activity, the histochemical marker for nitric oxide synthase (Dávila et al., 1995). Data on the three calcium-binding proteins in the cerebral cortex of reptiles are only available for the lizard Podarcis (Martínez-Guijarro and Freund, 1992). In this species, however, the three calcium-binding proteins are not segregated as in mammals but colocalized in the same population of GABAergic neurons. We do not know whether or not this differential feature regarding the expression of calcium-binding proteins is a common feature in other lizards.

In lizards, cortical fibers containing calcium-binding proteins appear to originate from intrinsic GABAergic neurons which make inhibitory synapses on principal cells (Martínez-Guijarro et al., 1991, 1993; Dávila et al., 1993). In the mammalian hippocampus, however, two sources for calretinin fibers have been reported (Nitsch and Léránth, 1993), one provided by intrinsic calretinin-containing GABAergic interneurons, and the other by supramammillary non-GABAergic neurons projecting to the hippocampus (Nitsch and Léránth, 1993; Maglóczky et al., 1994). A comparable statement for the putative intrinsic and extrinsic origin of hippocampal calretinin in reptiles is lacking. Moreover, there are no data available about the ultrastructural features and synaptic connections of calretinin-containing neurons and axon terminals in reptiles.

The present study describes the calretinin-immunoreactive neurons and fiber plexuses in the cerebral cortex of a lacertid lizard at the light and electron microscopic levels. In addition, we performed colocalization studies of calretinin with parvalbumin and GABA in the lacertilian doro-medial and dorsal cortices, the two cortical regions in which we demonstrated a conspicuous population of inter-neurons expressing these other cellular markers.

**MATERIALS AND METHODS**

Adult lizards of the species Psammomimus algirus were used with permission of the Andalusian authorities. Throughout the experimental work, animals were treated following the European Union guidelines on treatment of experimental animals. Lizards were killed under deep anesthesia with urethane and perfused transcardially with phosphate-buffered saline 0.1 M, pH 7.4 (PBS) followed by 4% paraformaldehyde, 0.1% glutaraldehyde and 0.2% picric acid in PBS for 30 minutes at room temperature. The brains were removed from the skulls, and stored overnight in the same fixative (without glutaraldehyde) at 4°C, then they were embedded in 4% agar, and cut into 50-µm-thick sections on a vibratome. The sections were washed extensively in PBS before the immunocytochemical staining by the peroxidase-antiperoxidase method.

**Light microscopy immunocytochemistry**

Free-floating sections were first incubated in 2% normal goat serum and 0.3% Triton X-100 in PBS for 60–90 minutes at room temperature to block nonspecific binding of the antibodies (the same solution was employed as a solvent for the primary and link antibodies), and then transferred to the primary antibody diluted 1:2,000 for 18 hours. The antisera against calretinin (SWant, Switzerland) was produced in rabbits by immunization with recombinant human calretinin. The antibody reacts specifically with calretinin in tissue originating from human, monkey, rat and mouse. This antiserum does not cross-react with calbindin D-28K or other known calcium-binding proteins, as determined by its distribution in the brain, as well as by immunoblots (Schwaller et al., 1993). After three washes in PBS for 45 minutes, the sections were incubated in goat anti-rabbit IgG diluted 1:35 for 1 hour, washed again in PBS for 45 minutes, and incubated in peroxidase-antiperoxidase diluted 1:100 for 1 hour. All steps were done at room temperature with agitation. The immunolabeling was revealed with 0.05% diaminobenzidine (DAB) and 0.03% hydrogen peroxide (H2O2) in PBS. After a thorough wash in PBS, the sections were mounted on gelatinized slides, air-dried, dehydrated in ethanol, cleared in xylene and coverslipped with Eukitt.

**Electron microscopy immunocytochemistry**

The method was essentially identical to that of light microscopy except that Triton X-100 was eliminated from all solutions. After the DAB reaction, the 50-µm sections were washed in PBS, treated with 1% OsO4 in PBS for 1 hour, dehydrated in acetone, and flat-embedded between aluminum sheets in Araldite. To increase contrast, the sections were treated with 1% uranyl acetate in 70% acetone during dehydration. After a light microscopic examination, selected flat-embedded sections were glued onto prepolymerized resin blocks and cut at 70–80 nm on a Reichert Ultracut E ultramicrotome. Ultrathin sections were collected on copper grids and examined with a Philips CM 100 electron microscope.

**Colocalization studies**

For the colocalization studies, we used postembedding immunocytochemistry on semithin sections as has been described elsewhere (Dávila et al., 1993). Briefly, blocks from two brains fixed with 4% paraformaldehyde, 0.1% glutaraldehyde and 0.2% picric acid in PBS, were embedded in Araldite and sectioned at 1 µm. Series of three adjacent 1-µm sections obtained at different rostrocaudal levels of the cerebral cortex were alternatively immunostained for calretinin (rabbit anti-calretinin, SWant; diluted 1:1,000), parvalbumin (rabbit anti-parvalbumin, SWant; diluted 1:250) and GABA (rabbit anti-GABA, Sigma (St. Louis, MO); diluted 1:5,000), using the same incubation sequence described above in the "Light microscopy immunocytochemistry" section. Before incubation in the primary antibody, resin was etched from the sections with sodium methoxide, according to Mayor et al. (1961). Fifty-five series of consecutive sections taken from all rostrocaudal levels of the telencephalon (a total of 165
Immunoreactive neurons for the different markers were counted directly on the photomicrographs, and neurons not unequivocally classified as positive or negative for either of the substances were excluded from the colocalization study.

**Controls.** As a control of the immunohistochemical methods used in the present study, in every immunostaining protocol (either in pre- or postembedding), control sections were processed as indicated but incubation with the corresponding primary antisera was replaced by incubation with normal rabbit serum (1:500). No immunostaining could be detected under these conditions except for a faint background on the surface of the sections.

In addition, as a control of the specificity of the antiseraum against calretinin under the experimental conditions used in this work, we incubated control sections in the primary antibody preadsorbed with the recombinant protein (1 µg/1 ml of the diluted antibody). As a result, specific immunostaining was completely eliminated.

**RESULTS**

**Distribution and morphology of the calretinin-immunoreactive neurons**

**Light microscopy.** Calretinin-immunoreactive neurons were present in every subdivision of the cerebral cortex of Psammodromus but their number, distribution and morphology showed significant variations from one region to another (Fig. 1).

In the medial cortex, small calretinin-positive neurons were preferentially located at the outermost one-third of the superficial plexiform layer (Figs. 1, 2A). These neurons had a rounded cell body from which a few, thin and lightly immunostained dendritic processes arose. Although it was difficult to distinguish the trajectory of the immunoreactive processes, cells appeared mostly stellate. Calretinin-positive neurons were more abundant at rostral levels of the medial cortex, whereas at mid- or caudal telencephalic levels they were almost lacking (Fig. 1). In addition, a few immunoreactive neurons were found in the deep plexiform layer of the medial cortex.

Calretinin-positive neurons in both the dorsomedial cortex and in the medial part of the dorsal cortex appeared differently from those in the medial cortex. They were almost exclusively located within the deep plexiform layer of the respective cortical region (Figs. 1, 2B, C). A large cell body, oval or piriform in shape and intensely stained, characterized these cells. Dendrites were long, thick and nonspiny; they emitted a small number of branches and sometimes displayed varicosities. Most immunoreactive dendrites ran apically toward the pial surface crossing the respective cell layer (Fig. 2C). Dendrites acquired a wavy appearance while traveling through the superficial plexiform layer. Other immunopositive dendrites extended exclusively within the deep plexiform layer. Some calretinin-positive neurons, whose cell bodies were found just above the part of ependyma corresponding to the dorsomedial cortex, displayed a horizontal bipolar morphology: one dendrite ran laterally, whereas the other ran medially to reach the medial cortex, where it curved and ascended toward the cortical surface.

The number of calretinin-positive neurons in the medial part of the dorsal cortex increased at caudal telencephalic levels, whereas the number of positive neurons in the dorsomedial cortex did not increase from midtelencephalic levels onward (Fig. 1). Calretinin-immunoreactive neurons were also found in the deep plexiform layers of the intermediate and lateral parts of the dorsal cortex. They were smaller and more slightly stained than neurons in the medial part of the dorsal cortex.

Immunopositive neurons in the lateral cortex were found throughout the entire thickness of the cortex but they were preferentially located at the ventral part of the region (Fig. 1). These neurons displayed mostly bipolar or multipolar morphologies, although pyramidal-shaped neurons were also observed. Frequently, dendrites were oriented toward the cortical surface (Fig. 2D). As a special feature of these calretinin cells in the lateral cortex, a number of thin and long spines covered their immunoreactive dendrites (Fig. 2E).

**Electron microscopy.** Ultrastructural features of the calretinin-immunoreactive cell bodies and dendrites were similar to that of parvalbumin neurons described in Psammodromus and Podarcis (Martínez-Guijarro et al., 1991, 1993; Dávila et al., 1993). The nucleus appeared immunoreactive; it was eccentrically located in most cell bodies and displayed some invaginations (Fig. 3A). The perinuclear cytoplasm was abundant and filled with many cytoplasmic organelles, especially mitochondria and endoplasmic reticulum (Fig. 3A). Few synaptic contacts were seen on the cell body and most were asymmetric.

Thick dendritic profiles appeared homogeneously filled with the dark reaction product. Many long mitochondria and microtubules were present within immunopositive dendrites. In contrast to the cell body, dendrites of calretinin neurons were covered by numerous synaptic contacts, most of them of the asymmetric type (Fig. 3B).

**Distribution and ultrastructure of calretinin-immunoreactive axons and terminals**

Many axons and terminal-like immunoreactive structures were present throughout the cerebral cortex of Psammodromus and two axonal plexuses were especially prominent: one in the superficial plexiform layers and the other around neurons within the cell layers. Less frequently, very thin, varicose axons running in the superficial plexiform layer of the medial cortex penetrated the externalmost parts of the cell layer and surrounded the cell bodies of neurons located there. In addition, some thin, varicose immunopositive axons were observed throughout the deep plexiform layers of all cortical regions, although they did not form prominent plexuses.

**Superficial plexus.** The superficial plexus occupied the middle one-third of the superficial plexiform layer of the medial cortex (Fig. 4A). This plexus consisted of many extremely fine immunoreactive fibers which gave it a homogeneous aspect in vibratome sections. It extended along the vertical (medial) and horizontal (dorsal) aspects of the medial cortex parallel to the cell layer and following the same curvatures (Fig. 4A). The plexus displayed the same form and thickness throughout the antero- and caudal extension of the medial cortex. In the dorsomedial cortex it occupied a position just below the pial surface and it seemed to disappear. However, immunopositive fibers were seen again in the outermost portions of the superficial plexiform layer of the dorsal cortex and the plexus...
Fig. 1. a–h: Camera lucida drawings of eight transverse sections through the telencephalon of Psammodromus algirus, rostrocaudally arranged (a, rostral; h, caudal), showing the cortical distribution of immunoreactive neurons (only cortical immunoreactive cells have been plotted). Each drawing represents a single 50-µm vibratome section and each dot represents an immunoreactive neuronal cell body. Cell layers of different cortical regions are delimited by dotted lines, as well as the boundary between the dorsal ventricular ridge (DVR) and the subjacent striatum. MC, DMC, DC, and LC, medial, dorsomedial, dorsal, and lateral cortices, respectively. NS, nucleus sphericus; S, septum; St, striatum.
Fig. 2. **A:** Photomicrograph of the interhemispheric region of the telencephalon at a rostral level, showing bilaterally the medial cortex (MC). Several immunoreactive small neurons (arrows) are located in the most external region of the superficial plexiform layer (spl). Note the dense plexus of immunoreactive fibers (stars). Nomarski optics. **B:** Detail of the dorsal cortex (DC). A pyramidal-shaped neuron (arrow) in the deep plexiform layer (dpl) emits an apical dendrite that crosses toward the cortical surface. cl, spl: cell layer and superficial plexiform layer, respectively. Nomarski optics. **C:** Photographic reconstruction of part of the cerebral cortex, including medial (MC), dorsomedial (DMC), and dorsal (DC) cortices. Some immunoreactive dendrites (arrowheads) are directed toward the MC, whereas most dendrites (large arrows) cross the DMC toward the cortical surface. Note the wavy appearance of dendrites (small arrows). Nomarski optics. **D,E:** Immunoreactive neurons in the lateral cortex (LC). (The marked neuron of D is enlarged in E.) These neurons display thin dendrites with a few, long spines (arrows, in E). Scale bars = 25 µm in A, 50 µm in B–D, 20 µm in E.
gradually thickened until it reached the dorsalmost part of the lateral cortex where it ended (Fig. 4B).

Synaptic contacts between calretinin axons and either small profiles of presumably thin dendrites or dendritic spines were observed in the superficial plexiform layer (Fig. 4C–E). Frequently, axonal profiles parallel to the pial surface made synapses en passant on dendritic spines (Fig. 4C), and sometimes up to four post synaptic thickening profiles were observed on the same spine (Fig. 4D). Synapses made by calretinin boutons were always asymmetric, and synaptic vesicles were mostly round or oval, clear and small.

Cell layer plexus. On the other hand, many varicose immunoreactive axons were observed around the cell bodies located in the cell layers of dorsal and dorsomedial cortices, forming the second axonal plexus referred to above (Fig. 5A). These axons were thicker and with larger varicosities than that of the superficial plexus. Their distribution within the cell layers was not homogeneous. In the dorsomedial cortex, immunopositive varicosities appeared preferentially around the cell bodies located in the lateralmost part of the cortex, in contrast to the medial part that was less innervated (Fig. 5A). In the dorsal cortex, differences were more pronounced. The medialmost region of the cortex was filled with thick, varicose axons (Fig. 5A), whereas the density of calretinin fibers was lesser in the intermediate and lateral zones of the cortex. This differential distribution of calretinin fibers and terminals matches the cortical zones where calretinin neurons were found in the deep plexiform layers. The medial region of the dorsal cortex is enlarged at caudal telencephalic levels, where many immunopositive terminals were observed throughout the entire thickness of the dorsal cortex.

Electron microscopy revealed immunoreactive terminals in the cell layers of dorsomedial and dorsal cortices distributed around the cell bodies and proximal apical dendrites of the principal cells (Fig. 5B–D). Immunoreactive boutons were usually large, with several mitochondrial profiles filling the bouton (Fig. 5C,D). Calretinin-immunoreactive terminals established axosomatic and axodendritic symmetrical synapses (Fig. 5E,F). Sometimes a single bouton synapsed on the cell bodies of two adjacent neurons (Fig. 5C,E). Synaptic vesicles within immunopositive terminals were small, mostly round and clear, and no dense-core vesicles were seen.

**Colocalization of calretinin with parvalbumin and GABA**

The colocalization analysis was made only in the dorsomedial and dorsal cortices because calretinin-positive neu-
Fig. 4. **A**: Panoramic view of the medial (MC) and dorsomedial (DMC) cortices taken from a frontal section of a midtelencephalic level. A prominent fiber plexus (arrows) travels in the middle one-third of the superficial plexiform layer (spl) of the MC, parallel to the cell layer (cl, delimited by the two dashed lines). This plexus becomes more superficial and thinner in DMC. **B**: View of the dorsal (DC) and lateral (LC) cortices. The fiber plexus (arrows) occupies the external zone of the superficial plexiform layer (spl) in the DC and spreads out in the cell layer (cl) of LC. **C**: Electron micrograph of an immunoreactive axon (arrows) located in the superficial plexiform layer. Axons are oriented parallel to the surface and establish synaptic contacts en passant on small dendritic profiles or spines (s). **D**: Detail of the squared area in C. Four asymmetrical synaptic junctions (arrowheads) on the same dendritic spine (s) are observed. **E**: Two asymmetrical synapses (arrowheads) on different dendritic profiles are made by the same axon. Scale bars = 100 μm in A, B, 0.5 μm in C, 0.2 μm in D, 0.25 μm in E.
Fig. 5. A: Photomontage of part of the cerebral cortex in which the medial (MC), dorsomedial (DMC) and dorsal (DC) cortices can be observed. Cell layers (cl) in DMC and DC are densely innervated by terminal-like immunoreactive structures. Note the virtual absence of terminals in both the mediallymost region of the cell layer of the DMC (arrows) and the MC (the boundary between both regions is marked by a dashed line). Nomarski optics. B: Detail of the cell layer (cl) of the dorsomedial cortex. Immunoreactive terminals surround immunonegative neuronal cell bodies. spl, dpl: superficial and deep plexiform layers. Semithin section (1 µm) and Nomarski optics. C: Electron micrograph of the cell layer of the dorsomedial cortex. Two negative neuronal cell bodies (n) and several immunoreactive profiles (arrows), some of them contacting the cell bodies, can be observed. D: Photomicrograph of a pyramidal neuron (p) in the cell layer of the dorsomedial cortex. A thick apical dendrite (ad) arising from the cell body is contacted by an immunoreactive bouton (squared area). E: Detail of the squared area in C. An immunoreactive bouton makes two symmetrical synapses (arrows) on adjacent cell bodies. F: Detail of the squared area in D. The immunoreactive bouton makes a symmetrical synapsis (arrows). In front of this junction, an asymmetrical synapsis on the same dendrite (arrowheads) can be observed. Scale bars = 50 µm in A, 20 µm in B, 5 µm in C, 2 µm in D, 0.4 µm in E, 0.5 µm in F.
rons displayed morphological features very similar to a population of GABA/parvalbumin cells previously described in the same regions of Psammodromus (Dávila et al., 1993). This study showed no parvalbumin-immunoreactive cells in the medial nor in the lateral cortices. Furthermore, calretinin neurons in the lateral cortex displayed such a distinctive feature when compared with other cortical immunopositive neurons (presence of long spines) that it appears obvious that they form a distinct population.

Colocalization studies showed that the calretinin population in the cerebral cortex of Psammodromus varied in regards to the colocalization of calretinin immunoreactivity with parvalbumin or GABA (Fig. 6). Results for different cortical regions are shown in Table 1. A remarkable finding of this study is that most of the calretinin neurons were GABAergic (CR/GABA = 76.2%) and that colocalization between the two calcium-binding proteins was also very high (CR/PV = 68.8%), whereas neurons with calretinin alone represent a small percentage (9.1%). Results were somewhat different for the two cortical regions: colocalization of calretinin and GABA was higher in the dorsal cortex, whereas the highest percentage of colocalization between calretinin and parvalbumin was found in the dorsomedial cortex.

**DISCUSSION**

The two major findings of the present study are: (1) calretinin neurons in the cerebral cortex of Psammodromus are heterogeneous both morphologically and neurochemically; and (2) calretinin-positive axons form two main afferent systems, probably one intrinsic inhibitory and the other extrinsic excitatory, in the cerebral cortex of Psammodromus.

**Calretinin-immunoreactive neurons in the lizard cortex**

Calretinin-positive neurons in the cerebral cortex of Psammodromus fall into two main morphological types: spine-free and sparsely spiny neurons. The latter are characteristic of the lateral cortex. A further morphological subdivision of the calretinin spine-free cells correlates with a segregated distribution of these cells. Those located in the medial cortex are small neurons with dendritic trees restricted to the superficial plexiform layer in close vicinity to the afferent (superficial) calretinin fibers. On the other hand, the calretinin spine-free cells in the deep plexiform layers of the dorsomedial and dorsal cortices are larger and their extensive dendritic trees cross all layers of the cerebral cortex. These large calretinin spine-free neurons are mostly GABAergic and may be responsible for the calretinin fibers and terminals around the principal cells in the dorsomedial and dorsal cortices. Thus, three distinct morphological calretinin-immunoreactive populations are found in the cerebral cortex of Psammodromus: one in the medial cortex, another in the dorsomedial and dorsal cortices, and the last in the lateral cortex.

In addition, calretinin-positive neurons in the cerebral cortex of Psammodromus are heterogeneous in terms of colocalization properties. Those in the medial and lateral cortices do not express parvalbumin (because this calcium-binding protein is absent from these regions in Psammodromus; Dávila et al., 1993), whereas calretinin neurons in the dorsomedial and dorsal cortices coexpress parvalbumin (more than 68%) and are mostly GABAergic (76.2%).

A number of differences exists between the calretinin-immunoreactive cells found in the cerebral cortex of Podarcis and Psammodromus. In Podarcis, no calretinin-positive cells are found in the medial or lateral cortices (Martínez-Guijarro and Freund, 1992). However, a popula-
The findings in reactivity of antisera to calbindin and calretinin explain distinct populations of cells within the lateral cortex of tree vertically oriented toward the pial surface. In addition, pyramidal-shaped neurons, with their apical dendritic spines, were sparsely spiny, mostly multipolar or bipolar, whereas in the lateral cortex of Podarcis, calbindin neurons are mostly bipolar cells with horizontally arranged spine-free dendrites, whereas in the lateral cortex of Psammomimus, calretinin-positive cells were sparsely spiny, mostly multipolar or pyramidal-shaped neurons, with their apical dendritic tree vertically oriented toward the pial surface. In addition, the distribution of immunoreactive cells was also different. Calbindin neurons in Podarcis are found preferentially in the dorsal part of the lateral cortex, whereas calretinin neurons in Psammomimus were mostly located in the ventral part of the cortex. Thus, they represent distinct populations of cells within the lateral cortex of these two species. Therefore, it is unlikely that cross-reactivity of antisera to calbindin and calretinin explain the findings in Psammomimus and Podarcis.

In Podarcis, calretinin-immunoreactive neurons are only found in the dorsomedial and dorsal cortices. These neurons morphologically resemble those described in the corresponding regions of Psammomimus. In Podarcis, however, calretinin, parvalbumin and calbindin coexist in the same population of GABAergic neurons in the dorsomedial and dorsal cortices, i.e., colocalization was 100% (Martínez-Guijarro and Freund, 1992).

Since calbindin-immunoreactive neurons appear as a neuronal population different from the parvalbumin- and calretinin-containing ones in the cerebral cortex of Psammomimus (unpublished data), it is clear that interspecific differences exist between lizards as regards the distribution and colocalization of calcium-binding proteins in the cerebral cortex. Thus, subtle functional differences may be inferred from these colocalization studies. Nevertheless, coexpression of calcium-binding proteins in GABAergic neurons seems to be a common feature in lacertilian cerebral cortex.

**Colocalization of calcium binding proteins: Comparison with mammals**

The coexpression of different calcium-binding proteins in the lizard cortex contrasts with mammalian cortical areas where calretinin, calbindin, and parvalbumin are markers of separate populations of GABAergic neurons: colocalization studies showed that calretinin hippocampal neurons were completely devoid of other calcium-binding proteins, parvalbumin and calbindin (Seress et al., 1993). Similarly, in neocortex, parvalbumin, somatostatin and calretinin are present in essentially different subsets of GABAergic nonpyramidal neurons (Hendry and Jones, 1991; Rogers and Réisbois, 1992; Condé et al., 1994; Kubota and Kawaguchi, 1994).

Interestingly, a neuron in monkey primary visual cortex can express different combinations of calcium-binding proteins at different stages of its development; i.e., calretinin, parvalbumin and calbindin are transiently colocalized in the same cells at different embryonic stages (Yan et al., 1995). Presently available data on the colocalization of these proteins in reptiles are insufficient to determine whether this feature in embryonic stages in mammals resembles the adult state of nonmammalian vertebrates.

**Calretinin-containing fibers in the cortex**

Two plexuses of calretinin-containing fibers with a different topography, ultrastructural features, and putative origin have been described in this study. The most probable origin of the calretinin axons and terminals around the cell bodies in the cell layers of the dorsomedial and dorsal cortices are the calretinin-positive neurons found in the deep plexiform layers of the same regions. The distribution of this putative intrinsic plexus matches the cortical areas where the number of calretinin neurons was the highest. Since most calretinin neurons in these locations contain GABA, and calretinin terminals make symmetric contacts on both the cell bodies and proximal dendrites of principal cells, we suggest that this plexus arises from calretinin GABAergic interneurons. However, calretinin neurons in the dorsomedial and dorsal cortices form part of a GABAergic subset of neurons that coexpress parvalbumin but not neuropeptides and which may be recruited in feed-forward inhibitory pathways (Martínez-Guijarro and Freund, 1992; Dávila et al., 1993). In this case, the pathway presumably influenced by the feed-forward inhibition is the zinc-positive axonal projection originating from the principal neurons of the medial cortex (López-García and Martínez-Guijarro, 1988; Bernabeu et al., 1994).

An intrinsic inhibitory calretinin-containing system has been suggested in mammals based on the observation that most calretinin-immunoreactive neurons contain GABA (Miettinen et al., 1992; Nitsch and Léranth, 1993) and that axon terminals of calretinin-positive neurons form symmetric synaptic contacts mainly with dendrites and less frequently with somata of principal cells (Seress et al., 1993).

We suggest that the superficial plexus of immunoreactive fibers represents a putative excitatory extrinsic system based on the asymmetric character of the synaptic contacts made by these fibers and their distribution. As mentioned above, the plexus occupies the intermediate region of the superficial plexiform layer of medial cortex, and the most superficial zones of the same layer in the dorsomedial and dorsal cortices. This distribution of calretinin-containing fibers matches two extracortical afferent projections described in other lizards. First, the lacertilian dorsolateral thalamic nucleus projects to the cortex (Lohman and van Woerden-Verkley, 1978; Bruce and Butler, 1984) and the cortical topography of this projection in Podarcis (Martínez-García and Lorente, 1990) corresponds with the distribution of calretinin fibers. Second, in the lizard Gallotia, cholinergic fibers in the cerebral cortex (most probably coming from cholinergic neurons in the

<table>
<thead>
<tr>
<th>TABLE 1. Number (n) and Percentage (%) of Different Combinations of Calretinin Neurons in the Dorsomedial (DMC) and Dorsal (DC) Cortices</th>
<th>DMC n (%)</th>
<th>DC n (%)</th>
<th>DMC + DC n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR</td>
<td>16(16.5)</td>
<td>5(3.7)</td>
<td>21(9.1)</td>
</tr>
<tr>
<td>CR/GABA</td>
<td>6(6.2)</td>
<td>45(33.6)</td>
<td>51(22.1)</td>
</tr>
<tr>
<td>CR/PSV</td>
<td>30(30.9)</td>
<td>34(14.7)</td>
<td>64(27.7)</td>
</tr>
<tr>
<td>CR/GABA/PSV</td>
<td>45(46.4)</td>
<td>80(59.7)</td>
<td>125(54.1)</td>
</tr>
<tr>
<td>Total</td>
<td>97</td>
<td>134</td>
<td>231</td>
</tr>
</tbody>
</table>

1CR, calretinin; GABA, gamma-aminobutyric acid; PV, parvalbumin.
basal telencephalon) are distributed in a similar way (Medina et al., 1993). It must be noted that calretinin neurons are present in the dorsal thalamus as well as in the basal telencephalon of Psammomorus (results not shown).

Two facts argue against the putative thalamic origin of the superficial calretinin plexus. First, calretinin neurons in the dorsolateral anterior thalamic nucleus are very few, in contrast to other dorsal thalamic nuclei; and second and more significant, the course of thalamocortical fibers through the telencephalon does not coincide with the distribution of calretinin fibers.

The other putative source of calretinin fibers are neurons in the basal forebrain. A continuum of calretinin neurons along the medial telencephalic wall and the nucleus of the diagonal band of Broca is found in the mammalian hippocampus, at similar locations where cholinergic neurons have been described in other lizards (Hoogland and Vermeulen-Vander Zee, 1990; Medina et al., 1993). A study on the ultrastructural features of cholinergic fibers in the reptilian cerebral cortex has not been carried out, but in the mammalian hippocampus cholinergic varicosities establish either asymmetric or symmetric contacts, although most of the synaptic junctions on dendritic spines are asymmetric (Frotscher and Léránt, 1985). Therefore, the location of calretinin neurons in the basal forebrain, the distribution of fibers in the cerebral cortex, and the asymmetric contacts made by these calretinin fibers suggest that the calretinin plexus in the superficial plexiform layers of cortical regions most likely contains acetylcholine.

An extrinsic calretinin-containing afferent system has also been described in the mammalian hippocampus. Experimental tracing studies revealed the cells of origin of the immunoreactive axon terminals in the inner molecular layer of the dentate gyrus and in the pyramidal layer of CA2 in the monkey hippocampal formation, which is in the supramammillary nucleus (Nitsch and Léránt, 1993). These supramammillary projecting neurons contain both calretinin and substance P but lack GABA as an inhibitory transmitter (Nitsch and Léránt, 1993). In the rat, most, if not all, postsynaptic targets of the supramammillary projection are principal cells dendritic spines and somata both in the dentate gyrus and in the CA2-3a areas (Maglóczky et al., 1994).

In summary, calretinin is present in fibers that most probably use acetylcholine to produce a band of excitation which is restricted to a specific portion (different for distinct cortical regions) of the apical dendritic tree of principal neurons, and also is present in GABAergic terminals that may provide a powerful inhibition of principal cells in some regions of the lizard hippocampus.

ACKNOWLEDGMENTS

The authors are grateful to E. Ortega for technical assistance.

LITERATURE CITED


