Review article

Cellular distribution of the calcium-binding proteins parvalbumin, calbindin, and calretinin in the neocortex of mammals: phylogenetic and developmental patterns

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Abstract

The three calcium-binding proteins parvalbumin, calbindin, and calretinin are found in morphologically distinct classes of inhibitory interneurons as well as in some pyramidal neurons in the mammalian neocortex. Although there is a wide variability in the qualitative and quantitative characteristics of the neocortical subpopulations of calcium-binding protein-immunoreactive neurons in mammals, most of the available data show that there is a fundamental similarity among the mammalian species investigated so far, in terms of the distribution of parvalbumin, calbindin, and calretinin across the depth of the neocortex. Thus, calbindin- and calretinin-immunoreactive neurons are predominant in layers II and III, but are present across all cortical layers, whereas parvalbumin-immunoreactive neurons are more prevalent in the middle and lower cortical layers. These different neuronal populations have well defined regional and laminar distribution, neurochemical characteristics and synaptic connections, and each of these cell types displays a particular developmental sequence. Most of the available data on the development, distribution and morphological characteristics of these calcium-binding proteins are from studies in common laboratory animals such as the rat, mouse, cat, macaque monkey, as well as from postmortem analyses in humans, but there are virtually no data on other species aside of a few incidental reports. In the context of the evolution of mammalian neocortex, the distribution and morphological characteristics of calcium-binding protein-immunoreactive neurons may help defining taxon-specific patterns that may be used as reliable phylogenetic traits. It would be interesting to extend such neurochemical analyses of neuronal subpopulations to other species to assess the degree to which neurochemical specialization of particular neuronal subtypes, as well as their regional and laminar distribution in the cerebral cortex, may represent sets of derived features in any given mammalian order. This could be particularly interesting in view of the consistent differences in neurochemical typology observed in considerably divergent orders such as cetaceans and certain families of insectivores and metatherians, as well as in monotremes. The present article provides an overview of calcium-binding protein distribution across a large number of representative mammalian species and a review of their developmental patterns in the species where data are available. This analysis demonstrates that while it is likely that the developmental patterns are quite consistent across species, at least based on the limited number of species for which ontogenetic data exist, the distribution and morphology of calcium-binding protein-containing

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neurons varies substantially among mammalian orders and that certain species show highly divergent patterns compared to closely related taxa. Interestingly, primates, carnivores, rodents and tree shrews appear closely related on the basis of the observed patterns, marsupials show some affinities with that group, whereas prototherians have unique patterns. Our findings also support the relationships of cetaceans and ungulates, and demonstrates possible affinities between carnivores and ungulates, as well as the existence of common, probably primitive, traits in cetaceans and insectivores. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Calcium-binding proteins are intracellular calcium acceptors that belong to two different families: the EF-hand proteins and the annexins. The annexin family is characterized by proteins that bind calcium in the presence of phospholipid-containing membranes. The former family consists of proteins showing a general structural principle in the calcium-binding domain called the EF-hand, which is a stretch of amino acids forming a typical helix-loop-helix structure (Andressen et al., 1993). The EF-hand family of calcium-binding proteins contains about forty known calcium-regulated proteins, of which several are found in the central nervous system. The EF-hand proteins may function either as ‘triggers’, starting a cascade of reactions or as calcium ‘buffers’, decreasing the free cytoplasmic concentration of this ion (Dalgaro et al., 1984). The prototype of a ‘trigger’ protein is the ubiquitous calmodulin that activates at least twenty different enzymes. The ‘buffer’ proteins, such as parvalbumin (PV), calbindin (CB), and calretinin (CR), represent a more passive system responsible for decreasing the amplitude of calcium signals. PV was originally purified from fish muscle, and is present in high levels in the central nervous system where it is observed in a large number of neurons belonging to several functional systems (Heizmann, 1984; Celio, 1990). CB was extracted from the chick duodenum where it was thought to facilitate calcium transport across the mucosa (Wassermann and Taylor, 1966), and was later detected and mapped in the brain (Jandé et al., 1981). CR is a recently described protein specific to the nervous system, with several amino acid sequence homologies to CB (Jacobowitz and Winsky, 1991; Rogers, 1992).

Although the function of many of these proteins is not yet known (see reviews by Baimbridge et al., 1992; Andressen et al., 1993), these molecules are interesting from a neuroanatomical point of view, since they are specifically observed in well-defined subpopulations of neurons belonging to multiple functional systems in a large number of vertebrate species, including birds, reptiles, amphibians, and mammals (Rogers, 1989; Celio, 1990; Baimbridge et al., 1992; Martinez-Guijarro and Freund, 1992; Résoibois and Rogers, 1992; Glezer et al., 1993, 1998; Davila et al., 1997). They are useful to study the development of specific systems as well as their evolution, as these calcium-binding proteins exhibit preferential distribution within functionally distinct pathways (Rausell et al., 1992; Glezer et al., 1993, 1999; Hashikawa et al., 1995; Molinari et al., 1995; Jones, 1998). In the cerebral cortex, they are powerful markers for studying the complexity of the GABAergic systems, as each of these three calcium-binding proteins is mostly colocalized with GABA, in distinct subpopulations of non-pyramidal cells (Kosaka et al., 1987 Hendry et al., 1989; for review see DeFelipe, 1997).

2. Differential localization of parvalbumin-, calbindin-, and calretinin-containing neurons

The distribution of these calcium-binding proteins in the neocortex is mainly known from detailed analyses of the rat, macaque monkey, and human. This section is a brief overview of the patterns observed in these species and will serve as a general description for the comparative analysis in Section 3 of the present article.

The greatest density of PV-immunoreactive neurons is found in layers III–V, and they pertain to two large types of local circuit neurons (Blümcke et al., 1990; Kobayashi et al., 1990; Lewis and Lund, 1990; Van Brederode et al., 1990; Hof et al., 1991, 1995b; Hof and Nimchinsky, 1992; Ren et al., 1992; Condé et al., 1994; Gabbott and Bacon, 1996a,b; Gabbott et al., 1997a; Meskenaite, 1997; Nimchinsky et al., 1997; Morrison et al., 1998). PV-immunoreactive cells of the first type have large round multipolar somata with wide radiate dendritic arbors. Generally, they have a very well stained axon that originates directly from the soma through a small axonic cone. The initial part of the axon is very thin, then its diameter increases. In most cases, the strongly immunoreactive main axonal trunk is oriented in an ascending, radial fashion, but descending axons are also found. Collaterals tend to leave the axon at right angles and to extend horizontally in both mediolateral and rostrocaudal directions. The morpho-
logical features of these PV-immunoreactive neurons are similar to those of the basket cells identified in Golgi studies (Jones and Hendry, 1984). In addition, in some cases, ‘baskets’ of PV-immunoreactive terminal boutons have been reported around pyramidal cell somata (Akil and Lewis, 1992a; Gabbott and Bacon, 1996a). Mostly located in layers II and III, the PV-immunoreactive cells of the second type have a small- to medium-sized multipolar cell body, that gives rise to three or four radially oriented, beaded, primary dendrites. An axon-like process, very thin and weakly immunoreactive, is rarely seen. As these PV-immunoreactive cell bodies are associated with the typical axonal terminals of the chandelier neurons (DeFelipe et al., 1989b; Akil and Lewis, 1992b), as described in Golgi studies (Peters, 1984), they are assumed to be the somata of the chandelier neurons. In addition, patterns of PV-immunoreactive processes in the neuropil reveal a specific aggregation of thalamic afferents in layers IV and V (DeFelipe and Jones, 1991; Hashikawa et al., 1995; Hof et al., 1995b; Molinari et al., 1995).

CB immunoreactivity is found in subpopulations of pyramidal and non-pyramidal cells, but the pyramidal cells are only weakly stained (DeFelipe et al., 1989a; Kobayashi et al., 1990; Van Brederode et al., 1990; Hof and Morrison, 1991; DeFelipe and Jones, 1992; Ferrer et al., 1992a; Hayes and Lewis, 1992; Hof and Nimchinsky, 1992; Condé et al., 1994; Gabbott and Bacon, 1996a,b; Gabbott et al., 1997a; Morrison et al., 1998). Mostly located in layers II and III, the majority of CB-immunoreactive non-pyramidal neurons have a multipolar cell body with four to six relatively thin, radially oriented dendrites. As their axon is not immunoreactive it is difficult to compare these CB-immunoreactive cells with Golgi types. Nevertheless, it seems they could belong to a subpopulation of cells with double bouquet axon (DeFelipe et al., 1989a). A second group of CB-immunoreactive non-pyramidal neurons, mostly located in layers V and VI, has a large elongated or multipolar cell body, with three to six thick long dendrites, and an intensely immunoreactive axon. Their axon arises from a proximal dendrite or the cell body, and has an ascending orientation. This group of CB-immunoreactive neurons has some of the features of Martinotti cells (Fairén et al., 1984), with an ascending axon extending into layer I, where it ramifies in long tangential branches. In layers II–VI, a third group of CB-immunoreactive neurons is characterized by a small perikaryon with numerous thin dendrites that extend about 30–50 μm. Although their axon is not seen, the appearance of their dendritic tree is distinctive enough to identify these cells as the neogliaform neurons described in Golgi studies (Jones, 1984). Finally, in some studies, CB-immunoreactive cells located in superficial layer I, immediately below the pial surface, and appear as an adult version of the Cajal–Retzius cells described in the cortical plate during development (Marin-Padilla and Marin-Padilla, 1982; Derer and Derer, 1990). Their cell body is round, oval, or triangular, very close to the cortical surface. Their dendrites, thick and tortuous, with long spine-like processes, are organized in a plane tangential to the pial surface, and their axon is thin and beaded. Finally a population of small, faintly labeled CB-immunoreactive pyramidal neurons has been reported, principally in monkeys and human, in layer III of many neocortical areas (Hof and Morrison, 1991; Hayes and Lewis, 1992; Kondo et al., 1994). These neurons show a progressive increase in their density in cortical regions belonging to functional sensory systems. Thus, they are less numerous in the primary visual cortex than in cortical areas representing higher hierarchical levels in the cortical processing of the visual input (Kondo et al., 1994).

The most frequently observed CR-immunoreactive neurons have bifurcated, vertically oriented dendrites and a vertically oriented axon, with collaterals confined to a narrow radial arbor (Jacobowitz and Winsky, 1991; Lewis et al., 1991; Glezer et al., 1992; Hof and Nimchinsky, 1992; Résoibois and Rogers, 1992; Hof et al., 1993; Condé et al., 1994; Hof et al., 1995b; Gabbott and Bacon, 1996a,b; Gabbott et al., 1997b; Meskenaite, 1997; Nimchinsky et al., 1997; Morrison et al., 1998). They are located in all layers, with highest densities in layers II and III. They belong to the bipolar and double bouquet classes of interneurons, as described in Golgi studies (Somogyi and Cowey, 1984). In addition, CR-immunoreactive Cajal–Retzius cells have been described in layer I, in the same location as CB-immunoreactive Cajal–Retzius cells. Overall, the relative density of CR-immunoreactive neurons in the neocortex of primates and rodents is approximately twice that of the CB-immunoreactive and PV-immunoreactive neurons.

PV, CB, and CR, have been shown to be markers of separate populations of interneurons defined by their morphology, their laminar distribution, and their developmental pattern, nevertheless it seems that there is a certain degree of colocalization between CB and CR, and CB and PV in the adult, and the detection by single-cell RT-PCR (Cauli et al., 1997) is in agreement with immunohistochemical studies (Kubota et al., 1994; Del Rio and DeFelipe 1997a,b; Kawaguchi and Kubota, 1998). Most of the interneurons containing PV, CB, and CR appear to be GABAergic, and consequently are thought to be inhibitory. In addition, PV, CB, and CR can be colocalized with neuropeptides, cell surface markers and receptors (for review, see DeFelipe, 1997). According to data from rats and primates (Hendry et al., 1989; Andressen et al., 1993; Condé et al., 1994; DeFelipe, 1997; Morrison et al., 1998), CR-, CB-, and PV-immunoreactive neurons represent morphologically non-overlapping populations of neurons,
indicating that these proteins do not colocalize in most cases. However, CB and CR have many structural similarities (Rogers, 1987), and both are present in bitufted, bipolar, and double bouquet neurons, as well as in some pyramidal neurons. Recent data on the colocalization of CB and CR have demonstrated minimal overlap among these neuronal subpopulations in rodents and primates (Del Rio and DeFelipe, 1996; DeFelipe, 1997; Gonchar and Burkhalter, 1997; Leuba and Saini, 1996; Jacobowitz et al., 1998; Morrison et al., 1998). Overall, 4–7.1% of labeled neurons have been shown to colocalize CR and CB, yet this degree of colocalization has not been ascertained in all studies (Volgucu et al., 1997). As calcium-binding proteins are thought to regulate the intracellular calcium balance, the presence of more than one of them in a given neuron type might reflect differential metabolic needs. In addition, CB is in some neurons colocalized with somatostatin and neuropeptide Y, a few PV-immunoreactive neurons also contain corticotropin-releasing factor and cholecystokinin, whereas CR-immunoreactive neurons show a high degree of colocalization with vasoactive intestinal peptide (see DeFelipe, 1997, for details). Correlations between calcium-binding protein content and physiological properties of these cells have been investigated. Calcium-binding protein-containing interneurons show different kinds of physiological properties, but they are not specific of any one type of cell as defined by morphology and calcium-binding protein profile (Kawaguchi and Kubota, 1993, 1998; Kawaguchi, 1995; Cauli et al., 1997), indicating that each population of interneurons defined by its content in calcium-binding protein can be subdivided into discrete subpopulations displaying a large diversity in their firing properties and biochemical patterns of coexpression.

3. Comparative analysis of calcium-binding protein distribution in the mammalian neocortex

The distribution of neurons and fibers containing PV, CB, and CR has been mostly described in the brains of common laboratory animals such as rats, cats, and macaque monkeys (Hendry et al., 1989; Blümcke et al., 1990; Celio, 1990; Deemulemeester et al., 1991; Hof and Nimchinsky, 1992; Conde et al., 1994; Kondo et al., 1994; Gabbott and Bacon, 1996a,b; Gabbott et al., 1997a; DeFelipe, 1997). A few studies have reported these distribution patterns in select areas of the human cerebral cortex (Blümcke et al., 1990; Ferrer et al., 1992a; Hof et al., 1995b; Del Rio and DeFelipe, 1996, 1997a,b; Nimchinsky et al., 1997). Only scarce data are available from other mammalian species and as a result potential differences in the cellular and regional distribution of these proteins are not well documented among the major groups of mammals. However, some evidence points to the fact that substantial variation exists in the regional distribution and morphology of neocortical neurons containing PV, CB, or CR, not only among mammalian subclasses and orders, but in some cases at the interspecies level as well (Glezer et al., 1993, 1998; Hof et al., 1996a,b). In addition, it has been proposed that some of these morphological and distribution characteristics may represent consistent traits that can be interpreted in a phylogenetic context (Glezer et al., 1993, 1998; Hof et al., 1996a,b). Below, we review the major patterns of distribution of PV-, CB-, and CR-immunoreactive neurons in the mammalian neocortex, with no intention of providing a detailed regional analysis in each species or order, a task much beyond the scope of this review, but rather to present some recent data on representative taxa that altogether provide an overview of the presence and cellular typology of these proteins in a large number of mammalian orders. To date, our series includes prototherians, marsupials, representative rodents, insectivores and archontans (primates and megachiropterans), carnivores, artiodactyls, and cetaceans. Details on the orders and species, number and localization of the samples, specimen fixation and tissue processing can be found in Appendix A. Fig. 1 shows a schematic taxonomic tree of mammalian order for general guidance, and Fig. 2 shows representative Nissl-stained examples of neocortical organization in some of the species documented in this study.

3.1. Prototherians

The extant prototherians comprise the order Monotremata with two families, the Ornithorhynchids (the duck-bill platypus) and Tachyglossids (short- and long-nosed spiny anteaters or echidnas), and three living genera from Australia and New Guinea. The monotremes resemble reptiles in several anatomical features, and differ from all of the other mammals in that they lay eggs that are incubated and hatch outside of the mother’s body. Monotremes are known from the early Cretaceous and are considered to represent an ancestral stock of mammals with affinities to therapsid reptiles from the late Triassic (Griffiths, 1978; Nowak, 1991). Until recently, information on brain organization in monotremes was scant and sensory processing in the platypus and echidnas was known from only a few studies (Rowe, 1990). Recent evidence from observations of live platypuses as well as from cortical recordings in both the platypus and the echidna demonstrate the existence of well-defined somatosensory, visual, auditory and motor regions in the cerebral cortex of these animals (Krubitzer et al., 1995). Interestingly, while the general organization of the sensory cortices in the extant monotremes appears to be roughly similar, the
structure and surface anatomy of the cerebral cortex differ greatly between the semiaquatic platypus and the terrestrial, foraging echidnas. The platypus possesses a cortex with no remarkable pattern of gyrification, whereas the echidnas are characterized by an extremely convoluted cortical surface (Krubitzer et al., 1995). However, the causes and functional repercussions of such differences are still poorly understood. We had the opportunity to study a few brain specimens of *Ornithorhynchus anatinus* and *Tachyglossus aculeatus* and report below the distribution and cell typology of PV, CB, and CR in the neocortex of these two species.

In the platypus, the three proteins display a comparable range of distribution within the neocortex, with the frontal and dorsomedial regions containing fewer labeled neurons than the ventral, posterior, and lateral regions. The neuropil staining pattern matches closely the localization of immunoreactive neurons, except in the case of CB that exhibits a dense, widespread labeling of the neuropil. These distribution patterns suggest that primary sensory and motor areas contain more PV and CB immunoreactivity than other cortical regions. In particular, a dense PV innervation and large numbers of PV-containing neurons are observed in the region of cortex that was described as the primary sensory representation of the bill (Krubitzer et al., 1995). PV is present in very large multipolar neurons in layers III–VI, resembling the basket cells known from the neocortex of placentals (although we did not find clear example of basket terminals in the platypus), as well as small round interneurons in layers II and III. CB is present in a large population of small, lightly stained round interneurons in layers III and V, and in some larger multipolar neurons that predominate in the deep layers (Fig. 3A). CR is prominent only in the piriform cortex compared to other cortical regions, and predominates in small bipolar and round interneurons in layers II and III associated with an intense neuropil staining (Fig. 4A). In addition, the deep layers of the piriform cortex show a population of intensely labeled, small polymorphic CR-containing neurons that are not observed in other cortical regions.

The distribution patterns in the echidna are generally similar to those observed in the platypus, with the frontal, medial and ventral regions showing less immunoreactivity than the lateral and posterior regions of the neocortex that correspond, as in the platypus, to sensory and motor regions (Krubitzer et al., 1995).
Fig. 2. Representative examples of neocortical organization in some of the mammalian species included in this study. All materials were stained with cresyl violet (Nissl stain) to reveal the cytoarchitecture. All panels show the region of neocortex corresponding to the best of existing knowledge to the primary visual cortex. The two monotremes (the platypus, A, and the echidna, B) show a relatively thin layer I, and no clearly defined layer IV. The platypus shows large pyramidal cells in the deep layers, while the echidna has large pyramidal and polymorphic neurons in layers III–VI. (C) The marsupial quoll has a relatively thin cortex with a well defined layer II and a visible granular layer IV underneath a relatively thin layer III. Note the horizontal rows of fusiform neurons in layer VI. The grey-headed flying fox, a megachiropteran, is characterized by a distinct, large layer IV in the primary visual cortex, a feature more visible in this mammalian superfamily than in rodents or carnivores. This trait is indeed shared by other archontans such as primates and tree shrews, but not by echolocating microchiropterans and other insectivores. Layer IV is thinner but visible in between the pyramidal layers III and V of the chinchilla primary visual cortex (E). Panel (F) shows the typical laminar pattern seen in the primary visual cortex of anthropoid primates, in this case the Western Lowland gorilla. Layer IV is very large and several sublayers can be recognized. Isolated Meynert cells are well visible at the border between layers V and VI. The dog primary visual cortex contains large pyramidal cells in layers III and V and a well developed layer IV. In artiodactyls (the llama, H, and the giraffe, I), the neocortex is characterized by a rather uniform cytoarchitecture, a remarkably large layer I, the absence of or a very poorly developed layer IV, and the presence of very large pyramidal neurons forming clusters in the upper portion of layer V. Layers II and III also contain large pyramidal and polymorphic neurons. Interestingly, these characteristics are also observed in cetaceans, pointing to the relatedness of ungulates and whales. Panels (J) and (K) show two examples of the primary visual cortex in two odontocete whales (a monodontid, the beluga whale, and a small physetereid, the pigmy sperm whale). Scale bar (on F and K) = 400 μm.
However, the morphology of immunoreactive neurons in the echidna differs greatly from the neuron types in the platypus. PV is present in a unique cell type characterized by a large pyramidal-like or multipolar morphology that predominate in layers V and VI (Fig. 5A). Such PV-labeled neurons have not been observed in any other mammalian species, although typical pyramidal neurons containing PV have been reported in other mammals (Hof et al., 1996a,b; Preuss and Kaas, 1996). Slender PV-immunoreactive modified pyramidal and smaller multipolar cells that resemble basket cells are also numerous in layer III. Large fusiform and horizontal neurons are seen in layers III–VI (Fig. 5A). There is also a rostrocaudal gradient in the staining intensity of the neuropil, with the posterior cortical regions displaying more PV labeling compared to the rostral regions, except in the piriform cortex that is also strongly labeled. CB is distinguished by a comparable regional immunoreactivity pattern, with large fusiform, modified pyramids in layers V and VI and bipolar-bitufted-like neurons in layer III (Fig. 3B). As in the platypus, the echidna piriform cortex displays a dense CR innerva-
tion, and thin CR-immunoreactive fibers can be followed from the olfactory bulb into layers II and III. The neocortex contains fewer immunoreactive neurons than the piriform cortex. Small round interneurons and strongly labeled, elongated, vertically oriented neurons are also observed (Fig. 4B). CR-containing pyramidal neurons occur in layers III and V in the sensory and motor areas. Also small CR-containing neurons, not as strongly stained as in other species, exist in layers II and III that resemble the more typical CR-containing interneurons seen in other mammals. These observations indicate that these calcium-binding proteins have a substantially different regional cellular distribution in the platypus and the spiny anteater neocortex compared to other extant mammals, and that there exist considerable differences between the two monotreme families.

3.2. Metatherians

‘Metatherians’, or marsupials, include morphologically and ecologically highly diverse groups of animals, generally grouped in one order containing about 16 families living in North and South America, Australia, New Guinea and nearby islands. They probably originated from the mid-Cretaceous period in North America (Nowak, 1991). They are all characterized by a large number of morphological differences compared to placental mammals. The most conspicuous difference is the presence of an abdominal pouch, more or less developed depending on the species, in which the young completes its development after a relatively short gestational period. Marsupials are known to have sophisticated learning capabilities that make them comparable to more recent mammals, in spite of a host of differences in brain organization (Rowe, 1990). In fact, although there has been a renewed interest in brain morphology and physiology of marsupials, data of the organization of the neocortex are still lacking. Interestingly, a description of the somatosensory nuclei of the thalamus in the phalangerid Trichosurus vulpecula (brush-tailed possum) has recently shown that PV and CB labeling is generally comparable to that in primates, with PV associated with lemniscal inputs and CB with non-lemniscal inputs, although the densities of labeled neurons are generally lower in this marsupial than in primates, indicating that similarities in neurochemical organization between these orders may exist at the level of specific sensory systems (Herron et al., 1997). We had access to brain specimens from three marsupial species: two Australian dasyurids, the quoll or native cat (Dasyurus hallucatus) and the dunnart or narrow-footed marsupial mouse (Sminthopsis crassicaudata), and one Australian peramelid, the long-nosed bandicoot (Perameles nasuta). Clearly, these three species are hardly representative of marsupial variety in the context of neocortex chemoarchitecture, but they can offer a reliable description of cortical organization in small bodied, nocturnal marsupials.

All three species display comparable staining patterns in the neocortex for PV, CB and CR. The most prevalent calcium-binding protein in these marsupials appears to be CR that is present in numerous small bipolar neurons located in layer II and the upper
Fig. 4. CR immunoreactivity in the neocortex of two monotremes, the platypus (A), and the echidna (B), and a marsupial, the quoll (C). The photomicrographs show the regions corresponding to the piriform cortex in the platypus the primary somatosensory cortex in the echidna, and the primary visual cortex in the quoll. CR is dense in the superficial layers in the platypus piriform cortex, which is the only cortical region to show CR immunoreactivity in this species. The echidna has large bipolar neurons predominating in layers II–IV, and the quoll displays a pattern that resembles that in rodents and primates. Scale bar (on C) = 100 μm.

portion of layer III, in a similar fashion as in many placental mammals (Fig. 5B). These neurons are present throughout the entire cortical mantle. In addition, small pyramidal-like neurons are occasionally seen in layers V and VI in the lateral cortex. As in prototherians, the piriform cortex is characterized by a dense band of CR-immunoreactive fibers in layer I. These fibers can be followed in the olfactory nerve and many neurons in the anterior olfactory nucleus also contain CR immunoreactivity. CB is present in bipolar and bitufted neurons in the supragranular layers throughout the cortex, and in some larger multipolar in the deep layers. Interestingly, in a posterolateral region of the neocortex of the quoll, a dense population of small round CB-immunoreactive neurons is observed (Fig. 4C), that is reminiscent of the population of CB-containing neurons in layer IV of the primary visual area of primates (Fig. 3C). It is therefore possible that this region may correspond to the primary visual cortex, based on the CB staining pattern. A major difference, however, between these marsupials and other mammalian taxa is the remarkable paucity of PV-immunoreactive neurons and fibers. PV is observed only in a few small interneurons, whereas it is much more prevalent in other small bodied mammals as well as in primates and carnivores. In the quoll, PV-immunoreactive are most numerous in the region possibly corresponding to the primary visual cortex, where they are present in layers II–VI. They predominate in layers II and III, and layer IV shows a strong neuropil staining that may correlate with the presence of thalamic afferent as is the case in primates. Surprisingly, PV-containing neurons in layer II have a morphology resembling double bouquet cells that are usually labeled by CB in rodents and primates, which may represent a neuronal specialization in certain marsupials that is not found in placentals. While these differences may point to particularities of these marsupials, it should be kept in mind that these observations are limited due to the small sample from this order and that they should be confirmed in additional specimens and in particular in larger species such as wallabies, kangaroos, and wombats. Aside of the apparently low expression of PV it is worth noting that the staining patterns and neuronal densities and morphology observed for CB and CR in these small marsupials are consistent with those found in many placental species, particularly in rodents.

3.3. Placentals

Placental mammals or eutherians are an extremely diversified subclass of mammals that range in body
weight from a few grams in some insectivore species to several tons in the case of baleen whales. The brains of placentals are characterized by substantial differences in encephalization, and gyral and sulcal anatomy, even within the same order. For example, among primates certain prosimian species have a nearly lissencephalic cortex, whereas many anthropoids have a complex pattern of gyri and sulci, particularly among cercopithecids and hominoids. Similar variability exists among carnivores, and ungulates, cetaceans, and proboscideans that are all characterized by rather large, highly convoluted brains (Welker, 1990). Neocortical chemoarchitecture is known in detail in a number of rodent, carnivore and primate species that are commonly used in scientific research, but not much is available on the neuronal organization of the neocortex in other taxa. The patterns of calcium-binding protein immunoreactivity in the neocortex of common laboratory animals have been briefly reviewed above. Based on these patterns, we provide in this section comparative data on the distribution and cellular typology of PV-, CB-, and CR-immunoreactive neurons in less current placental species.

3.3.1. Insectivores and archontans

Archontans constitute a large superorder of mammals based on shared skeletal features, that has relationships with insectivores (hedgehogs, moles, tenrecs, desmans and related species), and includes micro- and megachiropterans (echolocating and non-echolocating bats), dermopterans (colugos or flying lemurs), scandentia and macroscelids (tree shrews and elephant shrews), and primates (McKenna, 1975). The exact relationships between insectivores and archontans are still a matter of debate particularly with respect to the position of scandentia and macroscelids (McKenna, 1975; Novacek, 1986), but it is generally accepted that they form two somewhat related superorders. The insectivores, which are known from the late Cretaceous to the Recent, are considered to have retained to some extent some of the primitive features of the original ‘generalized’ stock of mammals and to be living representatives of early mammals. Notwithstanding uncertainties about archontans origins, it is likely that bats evolved (as did primates and flying lemurs), from an insectivorous ancestor during the Eocene. We will summarize briefly the patterns in primates that are known from numerous studies, pointing to major differences among species or families when data are available, and describe the staining patterns in one insectivore, the European hedgehog (Erinaceus europaeus), a microchiropteran (the big brown bat, Eptesicus fuscus), two species of megachiropteran (the grey-headed and little red flying foxes, Pteropus poliocephalus and Pteropus scapulatus), and in the tree shrew (Tupaia glis).

In the hedgehog, the number of PV-immunoreactive neurons in the neocortex appears to be comparable to
Fig. 6. PV immunoreactivity in the primary visual cortex of a megabat (the grey-headed flying fox, A), in comparison to a New World monkey (the owl monkey, B) and a hominid, the common chimpanzee (C). The primates exhibit the typical laminar pattern of PV immunoreactivity. The labeling in the megabat differs in that the laminar staining is less visible that in the primates. Note also the presence of many PV-immunoreactive neurons in layer I of the flying fox, a feature not observed in other species. Scale bar (on C) 100 μm (A, B), and 200 μm (C).

Interestingly, the echolocating insectivorous bat displays a calcium-binding protein distribution quite comparable to that observed in the hedgehog, with large PV-containing multipolar neurons concentrated in layers V and VI, CB-immunoreactive large bipolar or fusiform neurons equally distributed in superficial and deep layers, and a similar population of CR-immunoreactive neurons in layers II and III (Glezer et al., 1993). These patterns are, however, remarkably different in the non-echolocating megabat P. poliocephalus. In this frugivorous bat, the morphology and distribution of PV-containing neurons is highly comparable to the patterns observed in primates, with the presence of immunoreactive basket cells in the deep layers of the neocortex, well visible basket terminals around the soma of pyramidal neurons, and numerous smaller PV-containing interneurons, with round, bipolar or ovoid shapes in layers II–VI. The neuropil displays a rich innervation by PV-labeled fibers and the primary visual cortex [as defined by Rosa et al. (1993)], and shows a distinct band of afferent fibers in the subcortical white matter that may represent thalamic inputs from the lateral geniculate nucleus, as observed in primates (Blümcke et al., 1991). Interestingly, PV is also expressed in small layer I neurons that have a bipolar or multipolar morphology and that have apparently no equivalent in other species. These PV-expressing layer I neurons are particularly numerous in the primary visual cortex (Fig. 6A). The distribution of CB in the megabat neocortex also shares similarities to that in primates. Intensely labeled bipolar and bitufted interneurons are numerous in layers II and III and small multipolar neurons are observed in layers V and VI (Fig. 7A). In addition, faintly labeled small pyramidal neurons are frequent in layer III, a feature consistently seen in the primate neocortex (Hof and Morrison, 1991; Kondo et al., 1994). However, the staining pattern for CR differs strikingly from that in primates, as well as in rodents and carnivores, in that virtually no neurons contain CR in the neocortex of Pteropus (Fig. 8A). This is unlikely
due to staining artefacts, since it was observed in all of the available Pteropus specimens. Furthermore, CR is present in neurons in many subcortical regions, and the primary visual cortex exhibits a dense CR-immunoreactive band of neuropil staining in layer IV that correlates with the presence of many CR-containing neurons in the lateral geniculate nucleus. (This is another difference from primates where the lateral geniculate nucleus projections to layer IV of primary visual cortex are dominated by PV.) It is worth noting that similar to primates and some carnivores, the flying fox entorhinal cortex contains a dense population of CR-immunoreactive pyramidal neurons forming a row in layer V, with long apical dendrites reaching layer I. It appears that megabats, which are thought to share many characteristics with primates in terms of postcranial anatomy as well as in organization of sensory systems (Pettigrew et al., 1989; Krubitzer and Calford, 1992), differ considerably with respect to CR distribution. In fact, the work of Pettigrew and colleagues on brain organization of megachiropterans led to the concept that non-echolocating megachiropterans are closely related to an ancestral stock of primates, although genetic analyses failed to support this hypothesis (Bennett et al., 1988; Pettigrew et al., 1989). The drastic difference in CR immunoreactivity between Pteropus and primates (and all other mammals) indicates that neurochemical organization of megabats differs considerably from placentals, and sets them apart from the primates. It is possible that another, unidentified calcium-binding protein takes the place of CR in the neocortex megabats, or that CR is simply not expressed in bipolar neurons in these species. Analyses of taxa related to Pteropodids as well as developmental studies will be necessary to resolve this question.

Tree shrews are close relatives of primates and until recently were considered a family within the order Primates, but have since been reclassified as their own order, Scandentia, that emerged around the same time as the common ancestors of prosimians in the Paleocene–early Eocene. The analysis of a few samples from a tree shrew revealed that overall, the types of neocortical interneurons that contain PV, CB or CR are similar in this species to those seen in primates and rodents. Their laminar distribution is also comparable, and their density appears to be intermediate between primates and rats. CB-containing pyramidal neurons were not observed in our tree shrew materials, but this needs to be examined further in additional materials.

Fig. 7. CB immunoreactivity in the primary visual cortex of a megachiropteran, the flying fox (A) and of a hominid, the chimpanzee (B). The megabat has fewer labeled neurons than the primate, and no defined laminar staining pattern. The immunoreactive cell types in the megabat are generally comparable to that in primates, with small multipolar and double bouquet neurons, as well as some lightly labeled pyramidal neurons in layer III. In primates CB-immunoreactive pyramidal neurons are much more numerous than in the megabat, particularly in association neocortical areas. Scale bar (on B) = 100 μm.
Fig. 8. Comparison of CR immunoreactivity in the primary visual cortex of a megachiropteran, the flying fox (A) and a New World monkey, the owl monkey (B). The flying fox is characterized by a complete absence of CR-immunoreactive neurons, a feature unique to this species. It does, however, contain a band of neuropil staining in layer IV. The owl monkey shows the typical primate CR staining pattern with small bipolar neurons located principally in layers II and superficial III. Scale bar (on B) = 100 μm.

The neocortical staining patterns for PV, CB and CR has been extensively described in primates (see Section 2 above). Most studies are on macaque monkeys and human (Morrison et al., 1998), but there exist some data on less commonly used primates such as New World cebids (owl monkeys, squirrel monkeys, and capuchin) and a callithricid (marmoset) that report the distribution of these proteins in the primary visual cortex (Spatz et al., 1994; Blümcke and Celio, 1992; Goodchild and Martin, 1998). There are no differences in the distribution of PV, CB and CR in the primary visual cortex of these species, regardless of diurnal or nocturnal activity patterns, or the fact many New World monkey species display polymorphism in red-green color vision (Goodchild and Martin, 1998; Fig. 6B,C, Fig. 7B, Fig. 8B, and Fig. 9). The laminar distribution, cellular typology, and neuropil labeling appears generally similar in all anthropoid primates, and preliminary data on the distribution of the three calcium-binding proteins in the visual cortex of the lesser apes (gibbons and siamang) and great apes (orangutan, gorilla, and chimpanzee) demonstrate staining patterns and neuronal morphology essentially similar to those in humans (Hof, unpublished data; Blümcke et al., 1990). Very scant immunohistochemical data are available on prosimians. The localization of visual cortical areas in tarsiers, lemuroids and loroids seems to follow the same general organization as in New World and Old World monkeys, as demonstrated by physiological mapping and distribution of cytochrome oxidase (Allman and Zucker, 1990; Preuss et al., 1993), although differences in the packing and size of cytochrome oxidase patches in the primary visual cortex of several anthropoids has been recently shown to vary in size and packing density (Farias et al., 1997). It is therefore likely that the distribution of calcium-binding protein is comparable in prosimians and anthropoids. In fact, in the visual cortex of a slow loris...
Fig. 9. Comparison of CR immunoreactivity in two different cortical areas in anthropoid primates. The primary visual cortex (in a chimpanzee, a hominid, A), is characterized by a dense population of small immunoreactive neurons in layers II–IVB, whereas layers IVC–VI contain fewer neurons. Layer V shows a band of dense CR immunolabeling in the neuropil. The prefrontal cortex (area 10) of the macaque monkey (a cercopithecid, B) shows a very dense population of CR-containing bipolar neurons in layers II and III and fewer neurons in the deep layers. Note also the presence of a few putative, horizontally oriented Cajal–Retzius cells in layer I. Comparable patterns were observed in all primate species investigated. Scale bar (on B) = 200 μm (A), and 100 μm (B).

Calcium-binding protein can also be used as reliable chemoarchitectonic markers, as significant differences in the laminar and regional distribution patterns exist in primates. For example, PV, CB and CR are differentially distributed among cortical fields in the cingulate gyrus in macaque monkeys as well as in humans (Hof and Nimchinsky, 1992; Gabbott and Bacon 1996a; Gabbott and Bacon, 1996b; Gabbott et al., 1997b; Nimchinsky et al., 1997). Similar observations have been made in the orbitofrontal cortex in macaques and human (Carmichael and Price 1994; Hof et al., 1995b). CB is also present in a population of small pyramidal cells confined to layer III in humans and macaques (Hof and Morrison, 1991; Hof and Nimchinsky, 1992; Kondo et al., 1994) and the density of these CB-immunoreactive pyramidal neurons increases progressively from primary sensory areas to association cortices (Kondo et al., 1994). While CB has been reported in pyramidal neurons in the neocortex of other species, such density gradients have not yet been observed. A particularity of anthropoid primates appears to be the presence of PV-containing pyramidal neurons in layer V of the primary motor cortex (Preuss and Kaas, 1996), and the existence of CR-containing small pyramidal neurons in layer Va of area 24 in hominid primates. CR-immunoreactive pyramidal neurons have been reported in the entorhinal cortex of several mammalian species, as well as rare ones in layer III of the human and macaque monkey neocortex, where they are small and lightly stained; however, this particular layer Va population is apparently present exclusively in hominids, possibly indicating a recent evolutionary character among primates (Nimchinsky et al., 1997, and in preparation; Hof et al., 1998). Calcium-binding proteins do not appear to be reliable to discriminate among visual cortical regions in primates, except for the very distinctive features of the primary visual cortex. The distribution of PV-, CB- and CR-immunoreactive interneurons in the visual system of macaque monkeys shows a rather homogeneous pattern, with a subtle increase in neuronal density in parietal and temporal (Nycticebus coucang), the patterns of distribution were qualitatively not different from those observed in other primates such as small ceboids (e.g., the owl monkey, Aotus lemuinus, or the squirrel monkey, Saimiri sciureus), as well as in cercopithecines and hominoids (Adams et al., 1996; Gattass et al., 1996; Hof, unpublished observations).

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3.3.2. Rodents and lagomorphs

Rodents are by far the largest mammalian order with more than 1200 species that can be grouped in three general families, the sciuromorphs (squirrel-related), myomorphs (rat-related) and hystricomorphs (porcupine-related), although such classification is uncertain. There is in fact growing evidence for very complex relationships within this order, and the position of rodents vis-à-vis related taxa such as lagomorphs (rabbits and relatives), and caviomorphs (guinea pigs and relatives), and their inclusion in a large superorder (i.e. Glires) is not fully resolved (Nowak, 1991). Rodents and lagomorphs are known from the Paleocene to the Recent. The chemoarchitecture of the neocortex has been described in great detail in various strains of laboratory mice and rats (Celio, 1990), and there exist several analyses of the functional organization of sensory systems in various rodents including squirrels, beavers, capybaras, degus, guinea pigs, chinchillas, rats and mice (Campos and Welker, 1976; Carlson and Welker, 1976; Welker and Carlson, 1976; Johnson, 1990). The distribution of calcium-binding proteins is known mostly from studies in rats and mice, and further studies of these proteins in rodents is clearly warranted in view of the biodiversity of this order. Here, we will summarize the main features seen in rats and mice (Sprague–Dawley strain, derived from Rattus norvegicus; and strain C57BL/6J derived from Mus musculus), and report recent observations in the neocortex of the Chinchilla (Chinchilla laniger). We also review available data from the neocortex of the Mongolian gerbil (Meriones unguiculatus), and of one lagomorph, the rabbit (Oryctolagus cuniculus).

In the rat (Celio, 1990; Kubota et al., 1994; Gabbott et al., 1997a), as in primates, the morphological and immunocytochemical data suggest that PV is expressed by basket and chandelier neurons, CB in double bouquet cells, as well as in Martinotti and neurogliaform cells, and that CR is found in double bouquet and bipolar neurons. In addition, CR-immunoreactive neurons in the superficial part of layer I, are considered to represent Cajal–Retzius cells (Gabbott et al., 1997a). Compared to primates, the distribution of PV- and CB-immunoreactive neurons, with a peak in lower layer III, is slightly different mostly due to the fact that layer IV is not as thick in rodents than it is in primates. Also, the rare pyramidal neurons containing calcium-binding proteins described in primates are not or very rarely observed in rats. Only PV has been studied in the cerebral cortex of the gerbil, where patterns of immunoreactivity and neuronal morphology similar to those in the rat have been demonstrated (Seto-Ohshima et al., 1990).

The chinchilla has calcium-binding protein-immunoreactive neuronal types and distribution similar to those observed in rats and mice, but differs from these species by some features that are potentially restricted to hystricomorph rodents. A remarkable characteristic of the chinchilla is the presence of CR-immunoreactive pyramidal neurons in the deep part of layer III in most of the neocortex (Fig. 10A). These neurons are particularly prevalent in the anterior cingulate cortex and anterior frontoparietal cortex. These neurons are small pyramidal cells that are lightly stained compared to the CR-containing interneurons, and are more prevalent than populations of pyramidal neurons containing CR in other species, in particular primates. Also in the same cortical layer, CB-immunoreactive neurons, similar to those seen in monkeys, are observed in lower numbers than the CR-containing pyramidal neurons. It is possible that both calcium-binding proteins are colocalized in some of the layer III neurons. Conversely, CB- and CR-immunoreactive neurons represent different morphological types and have a distinct laminar distribution in the chinchilla as they do in other species. This unexpected result points to the fact that hystricomorphs may be characterized by the expression of a specific neurochemical phenotype in certain neuronal populations, and this issue deserves further investigations in other species within this group. It is worth noting in this context that another hystricomorph rodent, the guinea pig (Cavia porcellus), also exhibits a large number of CR-immunoreactive pyramidal-like neurons in the superficial layers of the piriform cortex (Frassoni et al., 1998). Such neurons are also present in the chinchilla, but are far less prevalent in rats and mice, indicating that the hystricomorphs may be char-
characterized by neurochemical features not observed in the neocortex of myomorphs. Further studies on a larger number of rodent species are evidently needed to confirm this observation.

In the rabbit, only PV immunocytochemistry of the auditory cortex has been documented (McMullen et al., 1994; De Venecia et al., 1998). These authors describe PV-immunoreactive basket cells with their characteristic axon arbors, and another type of PV-immunoreactive neurons with cell body and dendritic arborization resembling the chandelier cells of primates, although the presence of PV-immunoreactive cartridges has not been reported. The presence of PV in specific thalamic terminals from the medial geniculate nucleus to the primary auditory cortex of the rabbit has also been reported, as well as the existence of PV-containing reciprocal connections from the primary auditory region to the medial geniculate nucleus (De Venecia et al., 1998). Unlike in primates, intensely stained neurons are present in the whole extent of layer I, and none of these cells has the morphology and the localization of the Cajal–Retzius cells of adult primates. Nevertheless, as in primates, the highest density of PV-immunoreactive neurons is observed in layer IV.

3.3.3. Carnivores

Carnivores include eight major families [dogs, cats, hyenas, bears, raccoons, weasels, and mongooses, plus the pinnipeds (seals, sea lions, and walruses, that are now considered part of the carnivores)], with well over 200 species, and evolved most likely from a common (possibly insectivorous) ancestor in the late Paleocene or early Eocene. Pinnipeds emerged later during the Oligocene–Miocene (Nowak, 1991). Among carnivores, only the domesticated dog (Canis familiaris) and the cat (Felis catus), and to some extent the raccoon (Procyon lotor) have been commonly used in brain research, and little is known on the brain of other carnivores beyond incidental observations (Fish, 1898; Alderson et al., 1960; Johnson, 1980, 1990; Welker, 1990; Rajkowska et al., 1993). We compare below the staining patterns of PV, CB and CR in the dog and cat neocortex and report some observations we had the opportunity to make in the visual and auditory cortex of the California sea lion, Zalophus californianus. The distribution of calcium-binding proteins has been recently reported in detail in the cerebral cortex of the dog (Hof et al., 1996a,b). In the dog neocortex, PV is present in a large population of morphologically diverse interneurons.
Fig. 11. PV staining patterns in the frontal cortex of two carnivores, the dog (A), and the cat (B), and in the primary visual cortex of an odontocete, the beluga whale (C). Note the major differences between these orders. The PV-immunoreactive neuron types and their laminar distribution are comparable in the two carnivores, and resemble patterns in rodents and primates. The whale is characterized by large, darkly stained PV-immunoreactive neurons in layer IIIc/V that tend to be regularly spaced on one row, and lightly stained multipolar neurons in layers III and VI. The large PV-containing neurons in layer IIIc/V are observed only in cetaceans and in artiodactyls. Scale bar on (B) = 100 μm.

with a typology generally comparable to that observed in monkey and humans, with the difference that in the latter species the vast majority of labeled neurons in layer III are to small- or intermediate-size multipolar neurons (Fig. 11A). In the dog, lightly stained, small ovoid and multipolar neurons predominate in layers II and IV, and layers III and V–VI contain larger and intensely immunoreactive neurons with bipolar, bitufted or multipolar morphology. Some of these large multipolar neurons may be basket cells, due to the presence of PV-immunoreactive basket terminals around unstained pyramidal perikarya. Also, depending on the cortical region, many small PV-immunoreactive pyramid-like neurons are observed, which do not seem to have an equivalent in the primate neocortex. CB is present in a dense population of interneurons with clear layer-specific distribution patterns. Layer II contains small round cells and darkly stained, multipolar neurons that frequently formed clusters. Layer III has typical double bouquet cells with elongated apical and basal dendrites forming dense arborizations in layers I and IV–V. Layer IV contains few labeled neurons, and layers V and VI show a few double bouquet neurons and large, intensely labeled multipolar neurons. Lightly stained CB-immunoreactive pyramidal neurons are also seen in layer V, a difference from primates where they are observed in layer III (Fig. 12A). Overall, the morphological types of CB-immunoreactive interneurons were largely comparable among dogs, macaque monkeys, and humans. CR is present in a very dense population of bipolar and double bouquet cells in layer II and the upper portion of layer III, as observed in primates and rodents (Fig. 10B). The lower part of layer III and layers IV–VI contain much fewer CR-immunoreactive neurons, mostly small bipolar and small multipolar neurons. Layer III also contains intensely labeled, very large, isolated multipolar neurons with extensive and finely beaded dendritic arborizations. Interestingly, such neurons are absent from the rodent and primate neocortex, but are found in cetaceans and artiodactyls as well, possibly indicating a shared trait between the clade containing the ungulates plus the whales and the carnivores (Figs. 10–14). A few lightly labeled pyramidal neurons are also observed in layer III, but large layer V pyramidal neurons are visible in the primary motor cortex. Similar small pyramidal neurons exist in the monkey and human neocortex, but usually in lower numbers than in the dog. As in primates, layer I displays occasional CR-immunoreactive Cajal–Retzius cells. The distribution and cellular typol-
ogy of the three calcium-binding proteins in the cat neocortex is generally comparable to that observed in the dog (Stichel et al., 1987; Demeulemeester et al., 1991; Hendry and Jones, 1991). In our materials, we observed that generally the regional density of calcium-binding protein-containing neurons is somewhat lower in cats compared to dogs, and that the large CR-immunoreactive neurons observed in the dog primary motor cortex are very rare in the cat (Fig. 11B, Fig. 14).

In comparison to macaque monkeys and humans, there is a greater inter-regional variability in the laminar distribution of PV and CB in carnivores, based on the data from cats and dogs. In primates, the regional and laminar distribution of PV- and CB-immunoreactive interneurons shows an overall larger population of labeled cells in layers II–IV than in V and VI in association cortices, and a dense population of immunoreactive neurons in layer IV of the primary visual cortex. In contrast, the carnivore neocortex demonstrates substantial differences in the laminar distribution of PV- and CB-immunoreactive neurons among areas 4, 3a–b, 17 and 18, whereas the association cortices show more similar patterns (Hof et al., 1996b). In addition, a major difference between primates and these carnivores is the intense PV immunoreactivity in the neuropil observed in monkey and human, but generally not in dogs and cats. Given that a portion of the immunoreactivity for calcium-binding proteins has been related to the distribution of specific and non-specific thalamocortical projections (Blümcke et al., 1991; Rausell et al., 1992; Hashikawa et al., 1995; Molinari et al., 1995), the apparently low neuropil staining intensity in carnivores compared to primates could reflect differences in the neurochemical coding of certain thalamic efferents. Interestingly, CR immunoreactivity is remarkably comparable across the cortical areas that have been comparatively investigated in dog, cat, macaque monkey and human, aside of the large isolated layers III and V neurons observed in dogs (Hof et al., 1994, 1996a,b).

### 3.3.4. Artiodactyls and cetaceans

Artiodactyls (even-toed ungulates) form together with the perissodactyls (odd-toed ungulates) the superorder Ungulata. Only representative species of artiodactyls will be discussed as well as data on one domestic horse (Equus caballus; a perissodactyl). Artiodactyls are divided in three major families, the suiformes (pigs, peccaries, and hippopotamuses), tyloodonts (llamas, camels, and their relatives), and rumi-
Beluga whale  

Camel

Fig. 13. Comparison of CR immunoreactivity in the primary visual cortex of an odontocete, the beluga whale (A) and an artiodactyl, the camel (B). The camel contains isolated, large multipolar neurons in layers II, III, V and VI as well as small faintly labeled pyramidal neurons in layer V. The pattern in the beluga whale is similar to that in the bottlenose dolphin (Fig. 10C) and in the pigmy sperm whale (Fig. 12A), indicating that CR has a consistent distribution among different families of cetaceans (monodontids, delphinids, and physeterids, respectively). Also note that these large CR-immunoreactive interneurons are observed only in cetaceans and artiodactyls. Less intensely labeled, but morphologically comparable neurons are observed in carnivores (see Fig. 12). Scale bar (on B) = 100 μm.

nant (chevrotains, deers and their relatives, giraffes, cattle, goats, sheep, and antelopes). Ungulates apparently emerged during the Eocene, but the ancestor of perissodactyls may be as old as the late Paleocene in America. Cetaceans are presented here together with artiodactyls in view of the many shared characteristics they bear with them. Extensive paleontological and molecular evidence have substantiated a link between whales and different groups of large ungulates (Milinkovitch, 1995; Buntjer et al., 1997), and recent genetic data indicate a close relationship between cetaceans and extant hippopotamuses (Nomura et al., 1998), permitting the grouping of these orders into a superorder, Cetungulata. Cetaceans are known from the Eocene and include two suborders, the Mysticeti (or baleen whales: rorquals, right, and grey whales) and the Odontoceti (or toothed whales: dolphins, porpoises, killer whales, narwal and beluga, beaked whales, freshwater and estuarine dolphins, and sperm whale and relatives).

A few reports of sensory systems in certain artiodactyls (Welker et al., 1976) are available, and we were able to perform recently some limited immunohistochemical studies on the cerebral cortex of the llama (Lama glama), the giraffe (Giraffa camelopardalis), the one-humped camel (Camelus dromedarius), the domestic cow (Bos taurus), the goat (Capra hircus), the sheep (Ovis aries), the sika deer (Cervus nippon), and the wild boar (Sus scrofa). Description of the central nervous system in Mysticeti have been limited to general anatomy, but more detailed anatomical and physiological studies have been performed in a variety of odontocetes (Bullock and Gurevich, 1979; Garey et al., 1985; Morgane et al., 1988, 1990; Glezer et al., 1988; Glezer and Morgane, 1990; Glezer et al., 1992, 1993, 1998; Hof et al., 1995a; Revishchin and Garey, 1996; Manger et al., 1998). We have reported in recent years several studies of the cyto- and chemoarchitecture of the dolphin brain, with an emphasis on presumptive visual and auditory regions in the bottlenose dolphin (Tursiops truncatus), the striped dolphin (Stenella coeruleoalba), the harbor porpoise (Phocoena phocoena), the long-finned pilot whale (Globicephala melas), and the beluga whale (Delphinapterus leucas; Morgane et al., 1988, 1990; Glezer and Morgane, 1990; Glezer et al., 1992, 1993, 1998; Hof et al., 1995a).
Fig. 14. Examples of large atypical neuronal types containing CR in the neocortex of cetaceans, artiodactyls and carnivores. (A) Layers I to superficial III of the anterior cingulate cortex of the pigmy sperm whale (a cetacean). Most of the CR-immunoreactive neurons are located in layers I and II, and include large bipolar and multipolar neurons. (B) A giant CR-containing multipolar neuron in layer V of the primary visual cortex of the giraffe (an artiodactyl). This cell type resembles the large CR-immunoreactive multipolar neurons that are seen in layer V of the primary motor cortex of carnivores such as the dog (arrows, C), and the cat (D). Scale bar (on D) = 200 μm (A), 40 μm (B), 150 μm (C), and 100 μm (D).
Fig. 15.
tival cytoarchitecture (Bullock and Gurevich, 1979; Garey et al., 1985; Morgane et al., 1988, 1990; Glezer et al., 1992, 1993, 1998; Hof et al., 1995a; Revishchin and Garey, 1996; Manger et al., 1998). In cetaceans, the cortex is characterized by a generalized absence of granularity, a very prominent, thick layer I that is far more cellular than in most terrestrial species, the presence of large, atypical neurons in layer II and very large pyramidal neurons at the border between layers III and V (Morgane et al., 1988). This pattern is observed throughout the neocortex with only minor variations among cortical regions (Morgane et al., 1988). Interestingly, a similar cytoarchitecture was observed in the neocortex of artiodactyls. Furthermore, the distribution and typology of calcium-binding proteins in artiodactyls and cetaceans show some resemblance to that observed in insectivorous bats and hedgehogs but differs significantly from that in rodents and primates (Glezer et al., 1993, 1998). In cetaceans and artiodactyls, CB and CR are present in large fusiform, bipolar or multiform neurons residing in layers I, II and superficial III. The CB-containing neurons are much less numerous and less intensely stained than the CR-immunoreactive neurons. The CR-containing neurons located in layer I have a morphology quite comparable to that of the bipolar neurons seen in layer II of other mammals such as rats, carnivores and primates, whereas the CR-containing neurons in layers II and III are much larger than in other species and have a more variable morphology with a predominance of multipolar and fusiform types (Fig. 10C, Figs. 13 and 14). These neurons have long dendrites that extend into layers I and III and some have the general morphology of the ‘extraverted’ neurons described by Sanides and Sanides (1974). These large CR-immunoreactive neurons are also encountered in layers V and VI, especially in the artiodactyl neocortex, although they are much less frequent than in layers II and III (Fig. 14). Among artiodactyls, the large CR-containing neurons are particularly prominent in the giraffe, llama and camel (Figs. 13 and 14), whereas they appear to be less numerous in the pig, deer, cow, goat, and sheep. A few pyramidal-like neurons in layer III are also faintly CR-immunoreactive in dolphins, and the large pyramidal neurons in layer IIIC/V contain low levels of CB. Like in insectivores, CB and CR appear to be the dominating calcium-binding proteins in cetacean and artiodactyl neocortex, whereas there is a relative paucity of PV staining in neurons and neuropil. In whales and dolphins, PV is present in a few very large stellate neurons located in layer IIIc/V (Fig. 11C, showing an example from a monodontid species, D. leucas; Glezer et al., 1993, 1998). Comparable large multipolar PV-containing neurons have also been observed in the deep layers of the neocortex of some insectivores (see above). A few small pyramidal-like neurons in layer III also exhibit a faint PV labeling in dolphins. In the cetacean primary visual and primary auditory cortices, CB- and CR-immunoreactive neurons have been shown to represent about 40% of the total number of neurons, whereas PV is present only in about 5% of the neurons (Glezer et al., 1993). However, these proportions are likely to be lower in large artiodactyls since, based on the available specimens, these species have generally fewer labeled neurons than cetaceans. It is possible that this difference is related to the preservation of our specimens and this issue clearly deserves further analysis. Nonetheless, it is important to note that in cetaceans the proportion of calcium-binding protein-containing neurons is about twice as high compared to primates and rodents (Glezer et al., 1993), which may also represent a phylogenetically relevant brain trait of whales and ungulates. The staining patterns in the horse specimen was comparable to that seen in the small artiodactyls such as the pig, goat and sheep. It is worth noting that at subcortical levels CB and CR predominate in non-lemniscal systems that are considered to be phylogenetically older, whereas PV occurs in lemniscal pathways (Glezer et al., 1993, 1998; Jones, 1998). The fact that in cetaceans (and artiodactyls), the neocortex as a whole is dominated by CB and CR could be interpreted as an ancestral trait, that is also observed in some insectivores (Glezer et al., 1993, 1998).

3.4. Calcium-binding proteins and phylogenetic patterns

The analysis of the distribution of PV, CB, and CR in the neocortex of a large series of mammals, reveals that some general patterns are shared by a large num-
Fig. 16. Schematic representation of comparable and shared neurochemical features among the major mammalian orders investigated in the present study. Comparable staining patterns are shown by dashed lines between boxes, while orders that share similar patterns are grouped in the same box. The gray scale gradient reflects the overall difference in staining patterns. Note the association of primates, rodents and carnivores, the relatedness of megabats with archontans, except in the case of CR, and the similarities of CR immunoreactivity among carnivores, cetaceans, and artiodactyls. Monotremes show unique features as do cetaceans and ungulates.

Number of species from different orders, while more subtle differences appear to be specific of a particular species or are observed at the level of a superfamilly. At the other end of the spectrum certain patterns are unique to a taxon or to an entire group of species (Figs. 15 and 16). Overall, the general types of calcium-binding protein-containing interneurons that have been extensively characterized in the rat, macaque monkey and human, appears to be consistently observed in rodents, primates, carnivores, as well as to some degree in megachiropterans and tree shrews (Fig. 15). However, certain mammals such as prototherians, cetaceans and ungulates show unique neuronal types, and PV, CB, and CR are variably encountered in diverse types of pyramidal cells. The two prototherian species examined differ substantially from each other, and from all other mammals, and show neuron types observed in no other species. To some extent, small marsupials show comparable neuronal types, particularly in the case of CR and CB, but differ from other mammals, except whales and ungulates, by the very low numbers of PV-immunoreactive neurons. This fact may be interpreted as an example of parallel evolution, the same trait (i.e. paucity of PV expression) occurring in non-related group of mammals. Pteropodids are comparable to primates and rodents with respect to CB and PV, but their neocortex lacks CR-immunoreactive neurons. Cetaceans, microchiropterans, hedgehogs, and artiodactyls are all dominated by CB and CR, not only in the neocortex, but in subcortical systems as well, even though on average, ungulates display fewer labeled neurons than cetaceans (Glezer et al., 1993, 1998). Interestingly, a relative paucity of PV in the neocortex is also present in marsupials. However, the cytoarchitecture of the marsupials neocortex resembles more that of rodents and primates than that of cetaceans, ungulates, and insectivores. It is therefore possible that absence of PV in neocortical systems is a trait that evolved twice among mammals.

While similar calcium-binding protein-immunoreactive neuron types are observed in the neocortex of carnivores in comparison to primates and rodents (Figs. 15 and 16), it is interesting to note that in dogs and cats, very large non-pyramidal neurons are observed in layer III of the motor areas. These neurons are morphologi-
cally similar to the very large CR-containing neurons found in layer III throughout the neocortex of cetaceans and ungulates. Interestingly in the carnivores, these peculiar neurons occur only in an agranular cortex and the cortex of ungulates and cetaceans is notably agranular overall (it contains in certain regions an ‘incipient’ layer IV, defined as small aggregates of granular cells at the boundary between layers III and V; Glezer et al., 1993, 1998). This particular trait would be important to analyze in other families of carnivores, since it may indicate how similar these patterns are in closely related families like canids and felids, and may be one argument in favor of the debated assemblage of carnivores with ungulates and related taxa within a superorder (‘Ferungulata’; see Johnson et al., 1994).

A detailed analysis of the dog neocortex using calcium-binding proteins as cellular markers as in fact demonstrated several differences in neocortical neurochemical organization in dogs compared to macaque monkeys and humans, suggesting that some of the regional and cellular specializations observed in the dog may represent interesting phylogenetic traits regarding the relationships among these mammalian taxa, although many aspects of neuronal typology in the dog and primates indicate that the cellular (and to some extent functional) organization of the neocortex may be quite comparable (Hof et al., 1996a,b). The canid brain shows a high degree of cellular specialization in primary motor and sensory cortices, contrasting with the relatively homogeneous staining patterns in association cortices. Thus, large PV- and CB-containing pyramidal cells were observed only in areas 17 and 4, respectively, and large PV-immunoreactive putative basket cells or large multipolar CR-containing interneurons are found at high densities only in areas 4, 3a–b, 17 and 18. The observation that association cortices in the dog display generally less remarkable neurochemical features than homologous areas in primates may be related to the fact that cortical regions such as inferior temporal and prefrontal cortex achieved a unique degree of differentiation in primates compared to other mammalian orders. For instance, although there is no doubt that prefrontal cortex exists in many mammals, the dorsolateral expansion of the frontal lobe seems unique to primates (Preuss, 1995). The comparable staining patterns observed in association areas and the more diverse chemoarchitecture of primary cortices in carnivores could be tentatively interpreted as characteristic evolutionary traits of the canid (and felid) neocortex, although it is not clear whether these neurochemical features represent a less derived state of cortical organization in carnivores compared to primates, or if it represents a specific feature of carnivores. The many similarities in neuronal organization between carnivores and primates is also in agreement with the fact that sensory integration is likely to follow comparable hierarchical rules in both orders, in view of the relatively comparable distribution of corticocortical connections in the visual system of cats and monkeys (Payne, 1993; Scannell et al., 1995), although it should be kept in mind that such generalizations may not be valid in all of the species within a given order.

Calcium-binding protein-containing interneurons are known to influence differentially the activity of pyramidal neurons (for review see DeFelipe and Fariñas, 1992; Condé et al., 1994; DeFelipe, 1997). For example, PV-immunoreactive basket and chandelier cells make synaptic contact with the perikaryon and axon initial segment, respectively, whereas CR- and CB-containing bipolar and double bouquet cells target mostly the apical dendritic arbor of pyramidal neurons, and since similar morphological classes of interneurons have been described in several species (Meyer, 1983; Marin-Padilla, 1987; DeFelipe and Fariñas, 1992; Ferrer et al., 1992a,b; Glezer et al., 1993, 1998; Condé et al., 1994; Hof et al., 1995b), the role of calcium-binding protein in cortical integration is likely to be highly conserved among at least in rodents, carnivores and archontans. The degree to which these functional relationships can be extended to other orders is difficult to assess in view of the considerable differences in neocortical organization and calcium-binding protein-immunoreactive neuronal typology encountered in other mammalian groups. For example, the relative rarity of PV-immunoreactive neurons in cetaceans has been interpreted as an ancestral trait. Interestingly, it also occur in echolocating bats and hedgehogs that have been considered extant representatives of early mammalian forms (see Glezer et al., 1988).

These characters point to the fact that the inhibitory microcircuitry of the cetacean and ungulate neocortex may be characterized by more primitive features that in other mammals lineages and may be subserved by considerably different intercellular relationships. Compared to other mammals, cetaceans and to some extent, ungulates, have a very large neocortical surface and their neocortex appears to contain an inordinate number of cortical columns (Glezer et al., 1988; Morgane et al., 1988). It has been proposed that in cetaceans integration of cortical activity occurs in the highly cellular, thick layer I compared to other mammals, which contains the majority of the neocortical synapses (approximately 70%; Glezer and Morgane, 1990). Interestingly, the vast majority of CB- and CR-containing interneurons is located in layers I and II in cetaceans, while the less abundant PV-immunoreactive are located in the vicinity of presumed output neurons in layers IIIc/V and VI. The distribution of CB- and CR-immunoreactive cell types indicates that they are located in layers where most of the inputs to the neocortex terminate and around the ascending dendrites of the large layer IIIc/V pyramidal neurons, while the PV-immunoreactive neurons are in a position to represent putative basket cells. Although there is no direct evidence at the synaptic level to test this
hypothesis, it is possible that in cetaceans (and in ungulates) CB-, some CR-, and PV-immunoreactive neurons play the same role in neocortical microcircuits as in primates and rodents based on the distribution of these neurons with respect to pyramidal neurons, in spite a major differences in their morphology. It is therefore likely that on a functional standpoint, calcium-binding proteins identify classes of interneurons that have a similar role in the mammalian neocortex, suggesting that this neurochemical categorization represents an ancestral trait among mammals, although we cannot extend this argument with any certainty to prototherians.

4. Developmental patterns

Although the development of calcium-binding protein-containing neurons in the cerebral cortex has received a considerable attention in recent years, studies are still limited to human and to a restricted number of commonly used laboratory animal species (i.e. mouse, rat, macaque monkey, and cat). Here we review the temporal and spatial patterns of expression of PV, CB, and CR in the neocortex of a rodent (rat), a felid (cat), and two primates (macaque monkey and human). The Cajal–Retzius cells will be treated separately. The mechanisms thought to regulate the expression of these calcium-binding proteins are summarized, as well as their possible functional implications during neocortical development. Overall, the available data demonstrate that during corticogenesis the patterns of expression vary for each of the three calcium-binding proteins, but are rather similar among the different species. The expression of PV occurs relatively late, since it begins when the cortical lamination is nearly achieved, whereas the expression of CR and CB starts in the developing cortical plate. In addition, the first PV-immunoreactive cells appear in middle layers, whereas the first CB- and CR-immunoreactive cells appear in the deep part of the cortical plate. The developmental patterns of PV, CB and CR in the neocortex of the rat, cat and macaque monkey are summarized in Figs. 15–17.

4.1. Developmental patterns in the rat neocortex

The first PV-immunoreactive neurons neurons are seen
between P6 (Sanchez et al., 1992) and P8–P9 (Solbach and Celio, 1991; Alcántara et al., 1993) in the middle layers of the cortex, and then their number progresses toward the pia and the white matter to cover the whole extent of layers II–VIA. The adult distribution pattern is achieved by the end of the third postnatal week. Neurons expressing PV are multipolar, with axon-like processes similar to those of basket cells, but the pericellular PV-immunoreactive puncta are only observed in adult. PV-immunoreactive axonal cartridges, characteristic of chandelier cells, have not been described in rats (Sanchez et al., 1992; Alcántara et al., 1993). Regional differences in the developmental pattern of PV expression have been reported: the first PV-expressing neurons are found in the retrosplenial and cingulate cortex, the primary somatosensory cortex and visual area 18, followed by the primary visual, auditory and motor cortices (Alcántara et al., 1993). In the primary visual and the somatosensory cortical areas, layer IV shows a densely stained neuropil from the second postnatal week on, probably partially due to its invasion by PV-containing afferent fibers from the thalamus (Fig. 17; Seto-Ohshima et al., 1990; Sanchez et al., 1992; Alcántara et al., 1993; de Lecea et al., 1995).

In other rodents, such as the gerbil (Meriones unguiculatus, Seto-Ohshima et al., 1990) and in the mouse (Del Rio et al., 1995) the first PV-immunoreactive cells appear later than in the rat, at P15 and P10, respectively, in layer V of the parietal cortex. Then, immunoreactivity expands to the upper and inner cortical layers.

In contrast to PV, CB and CR are expressed very early in development (Figs. 18 and 19). A first population of neurons expressing both CB and CR have been recently described by Meyer et al. (1998). They represent the ‘cortical pioneer neurons’ and are present at E11.5 within the outer margin of the eurepithelium, at a time when the primordial plexiform layer does not yet exist. They are thought to play important developmental roles in the first stages of corticogenesis, but not in events related to the laminar ordering of neurons within the cortical plate. They probably disappear a few days after E16.

A second population of CB- and CR-immunoreactive neurons is present in the primordial plexiform layer (Enderlin et al., 1987; Liu and Graybiel, 1992; Sanchez
Fig. 19. Schematic representation of the developmental expression of CR in the rat and monkey neocortex. The development of CR has not yet been analyzed in the cat brain. The black dots represent interneurons expressing CR. The open triangles represent pyramidal cell expressing CR transiently in the rat. I–VI, cortical layers; cp, cortical plate; mz, marginal zone; sp, subplate; E, embryonic day; P, postnatal day or week (w). Compiled from Yan et al. (1995a) and Fonseca et al. (1995).

et al., 1992; Fonseca et al., 1995; Meyer et al., 1998). When the cortical plate emerges, this population of neurons becomes split into an abundant subpopulation in the marginal zone, the Cajal–Retzius cells (see below) and a few immunoreactive cells in the subplate. The immunostaining in the subplate is transient and from P12 on for CR, and from P20 on for CB, only a few immunoreactive neurons remain in layer VIB and virtually none in white matter (Sanchez et al., 1992; Fonseca et al., 1995).

The third population of CB- and CR-immunoreactive neurons appears in the cortical plate, at about E14 with its emergence. Subsequently, the temporal pattern of CB and CR differ. The cortical plate contains many fusiform CB-immunoreactive neurons but they did not seem to have a single radial orientation, which suggests that they are migrating cells (Enderlin et al., 1987; Liu and Graybiel, 1992; Sanchez et al., 1992; Alcántara et al., 1993). Around birth (Fig. 18), CB-immunoreactive neurons are distributed within the whole cortical plate. By P2 and P4 the number of CB-immunoreactive cells increases, and most cells have already acquired a mature morphology. The number of CB-immunoreactive cells reaches a peak value between P8 and P11, and decrease in number after P11. By P11 the laminar distribution of CB-immunoreactive neurons becomes evident, with a peak density in layers II–IV. The adult pattern is achieved by the end of the third postnatal week. During early stages of postnatal development neurons expressing CB are mainly multipolar, whereas at the end of the third postnatal week most neurons have a bipolar or fusiform morphology. In addition to nonpyramidal cells, a population of pyramid-like cells in layers II, III, and IV expresses low levels CB during the second postnatal week (Alcántara et al., 1993). The adult morphology of CB immunoreactivity is seen at the end of the third postnatal week.

In the cortical plate the first CR-expressing neurons are found at E14, as soon as this structure emerges, but their number remains low during embryonic stages. During the first postnatal week (Fig. 19) the number of CR-immunoreactive neuron increases, with a peak density in layers II–III and V–VIA. The adult laminar pattern of CR immunostaining is acquired between P8 and P21 (Fonseca et al., 1995) but the density of CR-immunoreactive cells reaches a peak at P10, and decreases sharply to adult level between P12 and P15 (Schierle et al., 1997). Between P0 and P21 pyramidal cells transiently immunoreactive for CR are present throughout the cortex (particularly in the motor cortex; Fonseca et al., 1995).

4.2. Developmental patterns in the cat neocortex

In the cat (Stichel et al., 1987; Hogan and Berman, 1993, 1994; Alcántara and Ferrer, 1994), the first PV-expressing neurons can be observed in the late fetal period (E54), mostly in layers V and VI, and they follow the ‘inside-out’ pattern of maturation of cortical layers. When cortical lamination is mature (P20), PV-immunoreactive cells are found in all corti-
cal layers except layer I, in a distribution similar to that seen in the adult (Fig. 17). The expression of PV in terminals of basket cells precedes that in axonal cartridges from chandelier cells, as described in the monkey (Akil and Lewis, 1992a,b). During the second and third postnatal weeks, some pyramidal cells in layer V show a transient PV immunolabeling. As in the rat, there are regional differences in the developmental PV expression in the cat neocortex. PV-immunoreactive neurons first appear in the primary somatosensory cortex, and the primary auditory and visual areas, followed by the primary motor cortex and the polysensory association areas and, finally, the association auditory areas and cortical areas related to the limbic system. In the neuropil, a prominent band of terminals probably of subcortical origin, develops until adult stages in layer IV of the primary sensory areas, but not in other regions. In this context, PV-expressing thalamocortical fibers penetrate the inner cortical mantle at the end of the third postnatal week, and their number increases until adult stages. In the white matter, PV-expressing fibers predominate in the white matter underlying the primary sensory areas. In addition, some PV-containing callosal and ipsilateral corticocortical fibers are seen after the fourth postnatal week, indicating the presence of PV in certain long projection pathways.

There is no data describing the expression of CB during early corticogenesis in cat. At E54 and until P0–P3 the most numerous population of CB-immunoreactive cells are observed in the subplate and white matter, and only a few CB-immunoreactive neurons are present in supra- and infragranular layers (Stichel et al., 1987; Hogan and Berman, 1993, 1994). Between P14 and P20 their number greatly increases, and at P20 their laminar distribution resembles the adult pattern (Fig. 18). Around birth, CB-expressing interneurons in layers II and III are chiefly bipolar or bitufted, reminiscent of the dendritic morphology of the double bouquet cells seen in adult. In addition between P0 and 4 weeks of age a very prominent transient expression of CB is observed in pyramidal-like, non-GABAergic cells in layer V (Hogan and Berman, 1993, 1994). Regional differences in the development of CB expression in the cat neocortex are striking. There are no pyramidal CB-expressing neurons in the primary visual cortex, and in contrast to the expression of PV, the developmental pattern of CB expression is more advanced in secondary cortical areas compared to primary areas throughout the entire development. As in the rat, no expression of CB in subcortical fiber bundles is observed.

The developmental pattern of CR has not yet been documented in the cat cerebral cortex.

4.3. Developmental patterns in the monkey neocortex

As in rats and cats, the development of PV expression is not synchronous in the whole cortical mantle in macaque monkey, but proceeds in a sequential fashion from primary to association areas over a protracted period of time (Anderson et al., 1995; Condé et al., 1996; Berger et al., 1997). Therefore, we will consider the temporal pattern of PV expression in one area, the primary visual cortex (V1), which is one of the first area to complete PV maturation. The first PV-immunoreactive cells appear at E132, in layers V and VI and they have a large multipolar cell body. At P2 PV-immunoreactive neurons are yet numerous in all layers, except layer I and by 22 days of age the number of PV-immunoreactive neurons in area V1 is comparable to that of adult (Fig. 17; Hendrickson et al., 1991; Condé et al., 1996; Morrison et al., 1998). The characteristic PV-immunoreactive pericellular 'baskets' outlining the soma and proximal dendrites of pyramidal cells are observed perinatally. In area V1 very few chandelier cell cartridges are PV-immunoreactive. In other cortical areas, such as visual area V2, the expression of PV in the chandelier cell cartridges visualizes the protracted development of the cortex. In this area, the first PV-immunoreactive cartridges appear at P22 in layer IV, their number increases in layer IV and superficial layers to reach a peak at about 5 months of age, and then decreases to finally disappear after 2 years of age. In prefrontal area 46, the adult distribution of PV-immunoreactive cartridges is reach between 2.8 and 5.8 years of age.

In a New World monkey (the common marmoset, Callithrix jacchus, Spatz et al., 1994), the laminar pattern of PV-immunoreactive cells in the primary visual cortex is different from that of macaque monkey: in the deep layers IV through VI of the neonate marmoset, a large number of PV-immunoreactive neurons are visible that form band-like patterns. Their number in layers IV and V is larger than in the adult. In addition, some pyramidal cells in layer V express PV transiently. The adult distribution of PV-immunoreactive neurons is comparable in the macaque monkey and the marmoset.

Only the primary visual cortex has been examined thus far for developmental expression of CB in the monkey neocortex. Although the cortical plate appears at E45, the first CB-immunoreactive cells are only observed at E90, in its deepest layers (Hendrickson et al., 1991). By E125, CB-immunoreactive cells are observed in layers II–VI, but the heaviest stained are located in layers VI, IVC and IVB. Shortly after birth CB-immunoreactive neurons are numerous, and include some immunoreactive pyramidal cells. Then the number of CB-immunoreactive neurons declines during postnatal weeks 3–6 (Fig. 18). A second wave of CB-immunoreactive pyramidal and non-pyramidal cells ap-
appears during postnatal weeks 20–36 in the supragranular layers, before declining to adult levels at 1–2 years after birth (Morrison et al., 1998). In the adult cortex only a few weakly labeled pyramidal cells are found in the primary visual cortex, although their densities are much higher in association cortices and exhibit clear regional differences (Kondo et al., 1994). Non-pyramidal neurons are mostly bitufted or multipolar, and are first seen in the infragranular layers, and appear subsequently in the supragranular layers, leaving the middle layers of the cortex relatively free of CB-containing cells in adulthood, resulting in a bilaminar pattern. The white matter contains some bipolar CB-expressing neurons. Staining of the neuropil develops in layers II, III and IVC after birth. In layer IVA, a transient honeycomb-like pattern of CB-immunoreactive neuropil is present around birth. Expression of CB in fibers in the subcortical white matter begins in the early postnatal period.

The first CR-expressing cells appear at E55 in the deep cortical plate of the monkey visual cortex (Yan et al., 1995a). Between E85 and E101 their number rapidly increases, particularly in the upper cortical plate. Most of the CR-immunoreactive neurons are bipolar with an ascending and descending process. By E130 the adult laminar distribution pattern of CR expression is achieved (Fig. 19), with CR-containing neurons predominating in layers II and III. However, CR-immunoreactive neurons reach their morphological maturity only 6 weeks after birth. In their vicinity, a band of weakly stained neurons is transiently present at the border between layers IVA and IVB. No CR-positive pyramidal cells are encountered in the monkey primary visual cortex during development.

4.4. Developmental patterns in the human neocortex

In the cortical plate of the human primary visual cortex, the first PV-immunoreactive cells are identified in layer V at gestational week 20. The development of PV-expressing neurons follows an inside-out sequence, the number of PV-immunoreactive neurons increasing in layer V and upper layer VI by gestational week 26, then in deep layer IVC (gestational week 30), and finally in layers IV–II (gestational week 34). Their peak distribution is located in two bands, the deep one occurring from layer IVC to upper layer VI, and the superficial one, in layer IVB and the upper part of layer IVC. At that time, two major neuron types can be distinguished: large cells with an elaborated dendritic tree located mainly in layer IVC, and more abundant, small cells with fewer dendrites distributed in all layers of the developing cortex. In addition to these non-pyramidal neurons, there are a few pyramidal cells in layer V and glia-like cells, particularly in neonates, that express low levels of PV (Cao et al., 1996). As in the monkey, no PV-immunoreactive chandelier neuron cartridges have been reported. Postnatally (i.e. 79 weeks), the number of PV-immunoreactive neurons still increases (Honig et al., 1996; Letinic and Kostovic, 1998).

In human, the development of CB has been documented only in the primary visual cortex, from gestational week 15 to gestational week 40 (Yan et al., 1997). By gestational week 15, the cortical plate does not contain CB-immunoreactive cells. The first faintly immunoreactive cells appear in layers V and VI by gestational week 20. By gestational week 26, bipolar and multipolar CB-immunoreactive cells are more common in layers V and VI, and in the newly differentiated layer IV. By gestational week 30, CB-immunoreactive neurons are distributed in two bands, one in layer V, the other in layer IVA. At gestational week 34, the density of CB-immunoreactive neurons increases and these neurons are also located in layers II and III. In addition, numerous lightly stained pyramidal cells are observed in layers II–IVA, and occasionally in deep layers. Finally, during infancy and childhood, the reorganization of PV and CB expression has been found to match cortical synaptogenesis (Letinic and Kostovic, 1998).

Except for Cajal–Retzius cells, the development of the expression of CR in the human cerebral cortex has not yet been documented.

4.5. The Cajal–Retzius cells

With the ‘cortical pioneer neurons’ recently described by Meyer et al. (1998), the Cajal–Retzius cells are the earliest neuronal population arising in the cortical anlage prior to the formation of the cortical plate and are located in the primordial plexiform layer (for review, see Huntley and Jones, 1990; Verney and Derer, 1995; Meyer et al., 1998). The emergence of the cortical plate splits this primordial plexiform layer into the subplate and layer I, in which the Cajal–Retzius cells are the main neuronal type. They have a very large somata, variable in shape, and located in the superficial half of layer I, close to the pia mater. Their dendrites are thick, horizontally oriented, with vertical spine-like branchlets (Marin-Padilla and Marin-Padilla, 1982), and they appear to be excitatory neurons as they contain glutamate as a neurotransmitter (Del Rio et al., 1995). Their persistence in the adult remains a controversial matter (see Marin-Padilla and Marin-Padilla, 1982, for review). The observation that the calcium-binding proteins are markers for the Cajal–Retzius cells (Huntley and Jones, 1990; Condé et al., 1994; Vogt Weisenhorn et al., 1994) permits to study their fate and to appreciate the unique characteristics of these neurons.

Complete longitudinal studies are scarce, but from the available data, it can be assumed that the development of the Cajal–Retzius cells is a characteristic of the
cerebral cortex rather than of a given species, as far as human, monkey, rat, mouse, and cat are considered. In human, the first CB- and CR-immunoreactive Cajal–Retzius cells appear at 6 weeks of gestation in the primordial plexiform layer (Meyer and González-Hernández, 1993). In the rat, Cajal–Retzius cells immunoreactive for CR and for CB are reported to occur between E12 and E14 in the primordial plexiform layer (Sanchez et al., 1992; Vogt Weisenhorn et al., 1994). Then in all species, they attain their mature morphology, they increase in number, and reach a peak before birth, followed by a decrease in their number, and a nearly complete disappearance shortly after birth (Huntley and Jones, 1990; Hendrickson et al., 1991; Berger and Goldman-Rakic, 1993; Berger and Alvarez, 1994; Del Rio et al., 1995; Yan et al., 1995b; Cao et al., 1996; Yan et al., 1997). Nevertheless, in the adult human and monkey, a few of them are detected by their immunoreactivity to CR or CB (Ferrer et al., 1992a; Condé et al., 1994; Belichenko et al., 1995), confirming previous Golgi studies (Marín-Padilla, 1990; Mrzljak et al., 1990).

Cajal–Retzius cells also express PV, but this expression has only been reported in primates (Huntley and Jones, 1990; Berger and Goldman-Rakic, 1993; Berger and Alvarez, 1994, 1996; Verney and Derer, 1995; Cao et al., 1996; Condé et al., 1996; Honig et al., 1996). In monkey, Cajal–Retzius cells express PV at the same time as deep layer cells begin to be PV-immunoreactive (Huntley and Jones, 1990; Condé et al., 1996), that is relatively late in development compared to the expression of CB and CR. This immunoreactivity is transient and disappears before birth (Huntley and Jones, 1990; Berger and Alvarez, 1994; Condé et al., 1996).

4.6. Colocalization of calcium-binding proteins during development

In contrast to the mature cortex, where PV, CB and CR are in most cases found in distinct, mutually exclusive subsets of interneurons, there is a more substantial degree of colocalization of these proteins during development. The most conspicuous and first described cell class in which calcium-binding proteins are coexpressed are the Cajal–Retzius cells in the marginal zone (Huntley and Jones, 1990; Verney and Derer, 1995; Yan et al., 1995b). In these cells, all three calcium-binding proteins are expressed at specific periods during development. CB and CR are present in Cajal–Retzius cells early during development, and PV is later expressed in the same cells. However, not all Cajal–Retzius cells coexpress all three calcium-binding proteins, revealing a chemical heterogeneity within this morphologically homogeneous cell population. PV expression is transient and disappears before birth, whereas CB and CR are expressed in the Cajal–Retzius cells that persist into adult stages (Ferrer et al., 1992a; Condé et al., 1994; Belichenko et al., 1995).

Colocalization of calcium-binding proteins can be also found in layers II–VI during the three first postnatal weeks in the rat (Alcántara et al., 1996b; Cauí et al., 1997). CB- and PV-double labeled cells are found in all cortical layers between postnatal days 9 and 12, coinciding with the onset of PV expression. This colocalization pattern reaches a peak during the second postnatal week and decreases thereafter to adult levels by the end of the third postnatal week (Alcántara et al., 1996b). In addition, coexpression of CB with CR was also frequently detected, but coexpression of PV and CR was never observed (Cauí et al., 1997). In the monkey (Yan et al., 1995b), CB and CR are never colocalized in layers II–VI, but PV and CR are colocalized from term until postnatal week 6 in layer III. Before birth nearly all PV-IR neurons in layers IV and VI also contain CB, as in rats.

A possible explanation for this transient colocalization of calcium-binding proteins during cortical development could be that cells require more than one of the three proteins to maintain calcium homeostasis in response to increases in calcium influx during particular periods of synaptogenesis, in order to protect them from calcium-mediated excitotoxicity (Yan et al., 1995b). In addition the balance of expression of the different calcium-binding proteins might lead to different calcium buffering properties.

4.7. Regulation of the onset of calcium-binding proteins

It was recently demonstrated using retroviral lineage analysis, that clonally related cell populations that are either GABAergic or glutamatergic, comprise neurons expressing different calcium-binding proteins, indicating that their expression is lineage-independent and is probably regulated by exogenous factors (Mione et al., 1994). It is possible that the presence of trophic factors plays a role in the onset of calcium-binding protein expression during development. For instance, studies in primary neuronal cultures have shown that neurotrophins NT4/5, NT3 and brain-derived neurotrophic factor (BDNF) elevate the number of neurons expressing CB (Collazo et al., 1992; Ip et al., 1993; Widner and Hefii, 1994b; Ventimiglia et al., 1995; Pappas and Parnavelas, 1997), BDNF and NT4/5 enhance CR-expression (Widner and Hefii, 1994a), and BDNF increases the numbers of PV-immunoreactive neurons (Mizuno et al., 1994). However, it remains to be determined, whether these neurotrophins have a primary effect on the expression of calcium-binding proteins or whether their expression is rather a secondary effect of the actions of neurotrophins, which induce neuronal differentiation, of which calcium-binding proteins expression is one aspect.
In the light of developmental studies it is worth mentioning that in a medulloblastoma cell line exhibiting a neuronal phenotype, CB has been shown to be induced by retinoic acid (Wang and Christakos, 1995), and in breast cancer cells its promoter has been shown to be estrogen-responsive (Gill and Christakos, 1995). This points to the possible role of other exogenous factors that are known to influence somatic development, on the expression of these calcium-binding proteins.

The late onset of PV expression, as well as its region-specific distribution patterns have fostered the belief that its production is linked to coordinated functional activity across related cortical regions. In organotypic cortical slice cultures that are deprived of all exogenous influences, PV develops comparably to the in vivo situation only if the slices are prepared after postnatal day 6. In younger cultures, PV expression does not occur, indicating that its onset depends on factors extrinsic to the cortex during this period (Vogt Weisenhorn et al., 1996). In similar preparations, CR expression is also determined by the presence of serum at early stages, although CR-containing neurons do survive in serum-deprived cultures, suggesting a neuroprotective role of CR in these conditions (Vogt Weisenhorn et al., 1996). However, the nature of such factors that can initiate PV or CR expression in these in vitro conditions has not yet been determined. In addition, a recent observation suggests that PV and CB expression can be reversibly modulated by different cortical afferents during postnatal development, implicating a role of functional circuits in their expression (Alcántara et al., 1996a).

4.8. Possible functional roles for calcium-binding proteins during development

Altogether, it appears that the distribution of each calcium-binding protein in the developing neocortex is comparable in all species thus far examined. CR and CB are the earliest calcium-binding proteins to be expressed. After the cortical layers begin to differentiate, CB expression is first present in the infragranular layers before its expression shifts to the superficial layers. CR shows a very prominent transient expression in the infragranular layers early in development and a sharp decline in neuron number in later developmental stages. In contrast to CR and CB, PV appears late during development (with the exception of its expression in the Cajal–Retzius cells), and is first expressed in layer V before invading layers VI and IV–II. The comparison of the specific spatio-temporal expression patterns of each calcium-binding protein, as well as their capability to buffer calcium, with specific calcium-dependent developmental events have led to speculations about their possible functional role during development. PV is thought to be involved in the maturation of inhibitory circuitry, CB in the production, stabilization and maintenance of cytoskeletal elements, CR in synaptogenesis, axonal elongation and dendritic remodeling, and both CB and CR in neuronal migration (Enderlin et al., 1987; Hendrickson et al., 1991; Solbach and Celio, 1991; Yan et al., 1995a; Cao et al., 1996; Condé et al., 1996; Schierle et al., 1997).

The production of calcium-binding protein deficient mice by creating animals carrying a null mutation of their genes may offer new avenues to tease out the function of these proteins during development. Yet, it has been reported that deficiency in either CB (Airaksinen et al., 1997), CR (Schurmans et al., 1997), or PV (Schwaller et al., 1997; B. Schwaller, personal communication) does not affect the development of the nervous system at the light microscopic level. Given the complex and overlapping expression patterns during development of these calcium-binding proteins, it is possible that complete or partial compensation of the missing protein by other calcium-binding proteins occurs in these knockout models.

5. Conclusion

The developmental and phylogenetic differences in distribution of the three calcium-binding proteins should be considered in the context of the general cytoarchitectonic and neuronal organization of the mammalian neocortex. On the basis of our materials and the available literature, we can tentatively suggest the existence of at least two major traits in ontogeny and phylogeny of the mammalian neocortex. The first trait is characterized by a high degree of morphological differentiation of neocortical areas and a variable degree of granularity (evidenced by the presence of layer IV), and disparities in neuronal size and packing densities across the cortical plate. As a rule, a balanced representation of the three calcium-binding proteins is associated with this trait. The second trait is characterized by cytoarchitectural homogeneity throughout the cortical plate, the absence or extreme reduction of layer IV, and the prevalence of magnocellular neuron types across all neocortical areas. In this trait, the balance of calcium-binding proteins is shifted to a predominance of CB- and CR-containing populations in comparison to PV-immunoreactive neurons. The first trait is seen in primates, rodents, carnivores, and megabats (in which CR is not observed in neocortical neurons), as well as Scandentia and lagomorphs, whereas the second trait is present in cetaceans and ungulates. Insectivores (including non-echolocating chiropterans), may contain a mixture of both traits, however, with a predominance of the features of the second trait.
Among mammals, major inter- and intrageneric differences in neocortical organization exist that in some cases are fairly conspicuous on Nissl preparations (Johnson et al., 1994). Examples of such differences are visible between the platypus and the echidna, or among different families of primates. In addition, scaling effects due to variation in brain size, or the size of a given brain region, are likely to play a major role in shaping neocortical cytoarchitecture (Haug, 1987; Finlay and Darlington, 1995). Such effects are obvious in primates and particularly in hominoids (Semendeferi et al., 1997, 1998). Even greater differences in cytoarchitecture are evident among taxa belonging to the various mammalian orders or superorders. As an example, major differences in neocortical organization are clearly visible between carnivores and primates, whereas in both orders the functional organization of neocortical areas follows a rather comparable layout with a set of recognizable, homologue connections linking the cortical fields (Scannell et al., 1995). In fact, it seems that the distribution of sensory and motor systems is determined by a general blueprint that has not been modified in any major way during mammalian evolution (Krubitzer, 1995; Scannell et al., 1995), yet the localization in the neocortex of certain sensory areas varies substantially among distant taxa. However, the cellular organization of these functional areas has varied tremendously.

Furthermore, cytoarchitectonic differences are further enhanced at the chemoarchitectonic level and neurochemical markers such as the three calcium-binding proteins used in our analyses are useful to reveal possible evolutionary traits among taxonomic groups of mammals. A particularly interesting case is that the similarities in neurochemical specialization of the cetacean and ungulate neocortex parallel the paleontological and molecular evidence that these taxa are ancestrally related. Placed in an evolutionary and developmental context it is worth noting that in both orders, compared to other mammals, the calves are born with a very early physical maturity, a crucial factor for survival in the aquatic environment and in the case of herbivores to escape the danger of predators. It is possible that in these species characteristics that were present in juvenile ancestral forms are passed to the adult stage, a phenomenon known as pedomorphosis. In the case of cetaceans, pedomorphic features may be the presence in adults of pontine, mesencephalic and cephalic flexures (seen only in embryos in other mammals), and the very large size of the brain at birth (see Glezer et al., 1998). It is possible that the general development of the neocortex in ungulates and cetaceans also evolves according to a pedomorphic model. A reflection of this may be found in the fact that the neocortex of cetaceans and ungulates is dominated by CB- and CR-containing interneurons, which are the calcium-binding proteins that appear first during development in rodent, carnivores, and primates (Glezer et al., 1998).

The cellular and neurochemical makeup of neocortex is further determined by developmental patterns. In fact, it is possible that the major differences that exist among taxa in a given order are related to the development sequence in each of these taxa. For example, genes known to determine the degree of convolution of the cerebral cortex (Kuida et al., 1996) may be inactive in the platypus but not in the echidna. However, nothing is known on the functional repercussions of such differences in brain development and at the level of specific neural population there are no available data in species other than the rat, mouse, cat, macaque monkey, and to some extent, the human. Detailed analyses of the development of chemically identified cortical systems in less common species would be highly valuable in the context of phylogenetic studies of the mammalian neocortex. The existing data on the development of calcium-binding proteins in the neocortex of mammals is limited to the most commonly used laboratory animals and the human, and overall reveal generally comparable developmental sequences and patterns. This is not surprising since primates, rodents, and carnivores are the taxa that show highly comparable and consistent distribution patterns and cell types among mammals. It would be very interesting to compare the development of PV, CB, and CR in the neocortex of taxa that are characterized by a completely different cortical cytoarchitecture, an evolutionary history divergent from that of primates and rodents, and in the case of cetaceans showing an adaptation to a highly specialized ecological niche. In this context, it is worth noting that in commonly used laboratory animals, calcium-binding proteins do not extensively colocalize with each other, and each of PV-, CB- and CR-immunoreactive neuron types show comparable colocalization patterns with other neurochemical markers (see DeFelipe, 1997). Such degree of neurochemical specialization among species is supported by similar developmental patterns of these neuronal classes. The facts that in whales there is minimal degree of colocalization between CB and CR (about 1–2%) and none with PV (Glezer et al., 1998), and that these interneurons classes appear to have a generally comparable role in the neocortical microcircuitry suggest that similar developmental sequences may occur in other species as well. Clearly, the data in the present article are far from being comprehensive as many representative species from several orders were not available for analysis. However, they indicate that markers like PV, CB and CR permit the visualization of regional specialization that goes beyond the simple cytoarchitectural description but also accounts for differences (or similarities) in neuronal typology, laminar distribution...
of neuron classes, as well as patterns of neuropil labeling that may correspond to the presence of specific projection systems. Altogether, such patterns may represent general characters that can be used reliably as cellular criteria to establish possible relationships among different groups of mammals.

Acknowledgements


Appendix A

1. Species available for study

Since part of the data reviewed in the present article have never been presented before, it is necessary to briefly review the specimens used in this study as well as fixation and tissue processing protocols. All of the specimens were obtained from animals used in other studies and were sacrificed for scientific purposes or, in some cases, for humane reasons if the animal was suffering from a terminal illness, in accordance to the relevant Institutional Animal Care and Use guidelines. We had access to 47 representative species of 11 mammalian orders including 2 prototherians, 3 marsupials, 1 insectivore, 3 chiropterans, 1 scandentia, 16 primates, 3 rodents, 3 carnivores, 8 artiodactyls, 1 perissodactyl, and 6 cetaceans (see Table 1). Additional data from the literature were available for 2 rodents, 1 lagomorph, and 1 marsupial. Notably missing taxa are representative xenarthrans (armadillos, sloths, and relatives), dermopterans (flying lemurs), proboscideans (elephants and relatives), and sirenia (manatees and relatives). The prototherians, marsupials, the hedgehog, chiropterans, the tree shrew, rodents, the dogs, cats, macaque monkeys, ceboids, and 2 bottlenose dolphins and one pilot whale were perfused transcardially with 4% paraformaldehyde [the cetaceans were perfused through the descending aorta and the fixative was a mixture of glutaraldehyde and paraformaldehyde [(Glezer et al., 1993, 1998; Hof et al., 1995a)]. All of the other specimens were obtained after the animals had died of natural causes or were sacrificed for humane reasons, and fixed by immersion for several weeks in neutral formalin (cetaceans, sea lion, artiodactyls, great apes) or approximately one week in 4% paraformaldehyde (humans, Hof et al., 1995b; Nimchinsky et al., 1997).

The perfusion protocol generally follows the one developed and previously described for macaque monkeys and dogs (Hof and Nimchinsky, 1992; Hof et al., 1996a,b). Following fixation, all specimens were transferred to phosphate buffered saline (PBS) containing 0.1% sodium azide at 4°C or were immersed in graded sucrose solutions and stored in a cryoprotectant solution at −20°C (Hof et al., 1995b). Some specimens were cryoprotected and frozen on dry ice, and stored in a −80°C freezer. In most cases the entire brain was available to the authors, except in some lesser and great apes where incomplete specimens were recovered, and in humans where only one hemisphere was collected for immunohistochemistry. In cetaceans and ungulates, samples were limited to the presumed primary visual cortex and auditory cortices [located in the midposterior portion of the entolateral gyrus and the midposterior portion of the suprasylvian gyrus, respectively (Glezer et al., 1998)], and to the anterior cingulate cortex.

2. Staining procedures and data analysis

All specimens were cut in 40 μm thick sections on a sliding microtome for large samples, or on a cryostat. Sections were mounted every 500 μm onto chrom–alum subbed slides and processed for Nissl staining (see Fig. 2), and immunohistochemistry. The remaining sections were cryoprotected and stored in serial order at −20°C. For immunohistochemistry, the 40 μm thick free-floating sections were incubated for 48 h at 4°C with monoclonal antibodies against PV or CB, or with a polyclonal antibody against CR (Swant, Bellinzona, Switzerland; Celio et al., 1988, 1990; Schwaller et al., 1993), at a dilution of 1:5,000, 1:2,000, and 1:3,000, respectively, in PBS containing 0.3% Triton X-100 and 0.5 mg/ml bovine serum albumin. The sections were then processed with the avidin–biotin method using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and 3,3’-diaminobenzidine as a chromogen. The immunoreactivity was subsequently intensified in 0.005% osmium tetroxide. In some specimens that had been kept over a long period in formalin, it was necessary to use a microwave pretreatment to visualize reliably the immunoreactivity. This was achieved by washing the sections in 3:1 v/v mixture of methanol and 3% H2O2 for 20 min, and then by microwaving them in citrate buffer pH 6.5 for 3 min at maximum intensity using a commercial microwave oven. After allowing the sections to cool, they were rinsed in PBS and processed for immunohistochemistry as described above. While
Table 1
List of available specimens

<table>
<thead>
<tr>
<th>Order and Species</th>
<th>Common name</th>
<th>N</th>
<th>Fixation</th>
<th>Notes</th>
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<td>a,h</td>
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<td>a,h</td>
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<td>a,h</td>
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<td>a</td>
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<td>a,b</td>
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<td>–</td>
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<td>a</td>
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<tr>
<td></td>
<td><strong>Globicephala melas</strong> Pilot whale</td>
<td>1</td>
<td>4% PF + Glut</td>
<td>f</td>
</tr>
<tr>
<td></td>
<td><strong>Phocoena phocoena</strong> Harbor porpoise</td>
<td>1</td>
<td>Immersion</td>
<td>f</td>
</tr>
<tr>
<td></td>
<td><strong>Delphinapterus leucas</strong> Beluga whale</td>
<td>1</td>
<td>Immersion</td>
<td>f,h</td>
</tr>
<tr>
<td></td>
<td><strong>Kogia breviceps</strong> Pigmy sperm whale</td>
<td>1</td>
<td>Immersion</td>
<td>c,h</td>
</tr>
</tbody>
</table>

* The taxonomic nomenclature follows Nowak (1991), and the number of available specimens is indicated for each species. Refer to Fig. 1 for general phylogeny of mammalian orders, and to the text for more details on the taxonomic position of the families and species. PF: paraformaldehyde; Glut, glutaraldehyde; Immersion, fixed by immersion in 10% neutral formalin. (a) The entire brain was available; (b) the quality of some of the available specimens was poor and required microwave pretreatment; (c) incomplete specimens; (d) only one hemisphere was available for immunohistochemistry; (e) data were available only from the literature; (f) the entire brain was available but only limited samples were processed and analyzed; (g) species for which developmental data on calcium-binding proteins exist in the literature; (h) neocortical cytoarchitecture shown in Fig. 2.

consistent immunohistochemical labeling was obtained overall from these materials, it should be kept in mind that differences in fixation protocols, duration of fixation, storage conditions, or postmortem delay, that were unavoidable in the present series, may have affected the staining patterns in some of these species.

All materials were analyzed on a Zeiss Axiophot photomicroscope. Photomicrographs were taken with kodachrome 64 ASA daylight slide film using 5x Fluar, or 10x and 20x Plan-Apochromat Zeiss objectives. The slides were subsequently scanned on a Nikon LS 4500 multiformat high resolution scanner, and the images were assembled and turned into black and white documents using Adobe Photoshop 5.0. Only minimal adjustments were made to the original scans to ensure optimal contrast and brightness of the resulting prints of the plates, which in no way altered the data. The plates were printed on a Fujix Pictography 3000 color printer.

### References


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