

Morphomolecular Neuronal Phenotypes in the Neocortex Reflect Phylogenetic Relationships Among Certain Mammalian Orders

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ABSTRACT

The cytoarchitecture of the cerebral cortex in mammals has been traditionally investigated using Nissl, Golgi, or myelin stains and there are few comparative studies on the relationships between neuronal morphology and neurochemical specialization. Most available studies on neuronal subtypes identified by their molecular and morphologic characteristics have been performed in species commonly used in laboratory research such as the rat, mouse, cat, and macaque monkey, as well as in autopsic human brain specimens. A number of cellular markers, such as neurotransmitters, structural proteins, and calcium-buffering proteins, display a highly specific distribution in distinct classes of neocortical neurons in a large number of mammalian species. In this article, we present an overview of the morphologic characteristics and distribution of three calcium-binding proteins, parvalbumin, calbindin, and calretinin, and of a component of the neuronal cytoskeleton, nonphosphorylated neurofilament protein in the neocortex of various species, representative of the major subdivisions of mammals. The distribution of these neurochemical markers defined several species- and order-specific patterns that permit assessment of the degree to which neuronal morphomolecular specialization, as well as the regional and laminar distribution of distinct cell types in the neocortex, represents derived or ancestral features. In spite of the remarkable diversity in morphologic and cellular organization that occurred during mammalian neocortical evolution, such patterns identified several associations among taxa that closely match their phylogenetic relationships. © 2005 Wiley-Liss, Inc.

Key words: artiodactyls; brain evolution; cerebral cortex; cetaceans; chemoarchitecture; interneurons; primates; pyramidal cells

In general terms, neurons in the mammalian neocortex can be divided into two generic classes, pyramidal excitatory cells and inhibitory interneurons. Each class includes many subtypes that can be identified by their size, shape, dendritic and axonal morphology, and connectivity. About a dozen morphologically different subtypes of inhibitory interneurons have been described and there exist an unknown number of pyramidal neuron subpopulations. These neuronal subtypes are known to exhibit a differential distribution among cortical layers and regions, and some of them are also differentially represented among species (for review, see Hof et al., 1999). In addition, neurons can be further classified based on their content of various proteins that can serve as neurochemical markers of identifiable subpopulations. Such neurochemical patterns have also been used to define cytoarchitectural bor-

ders or transitions among cortical domains in several mammalian species. The most commonly used markers

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are dephosphorylated epitopes of the neurofilament protein triplet (NFP) and the calcium-binding proteins parvalbumin (PV), calbindin (CB), and calretinin (CR) (Hof et al., 1999, 2000).

In primates, NFP predominates in a subset of large pyramidal neurons that have an extensive dendritic arborization, a well-defined laminar distribution, and form highly specific long corticocortical projections in macaque monkeys (Campbell et al., 1989, 1991; Hof and Morrison, 1995; Hof et al., 1995b; Nimchinsky et al., 1996; Preuss et al., 1997, 1999; Sherwood et al., 2003a). In the macaque monkey visual cortex, the distribution and density of NFP-containing neurons define quantitatively the regional boundaries of more than 20 visual cortical areas (Hof and Morrison, 1995). Similarly, regionally specific distribution patterns of NFP-immunoreactive pyramidal neurons have been reported in the orbitofrontal, cingulate, retrosplenial, and inferior frontal cortex in macaques, great apes, and humans (Hof and Nimchinsky, 1992; Carmichael and Price, 1994; Hof et al., 1995a; Nimchinsky et al., 1995, 1997; Vogt et al., 2001, 2005; Sherwood et al., 2003a). NFP-immunoreactive pyramidal neurons have also been reported in the neocortex of mouse, rat, hamster, cat, and dog, where they exhibit clear regional and species differences in their distribution and densities (Hof et al., 1996; van der Gucht et al., 2001, 2004, 2005; Kirkcaldie et al., 2002; Boire et al., 2005), and in bottlenose dolphins, where they may identify a major subpopulation of output neurons throughout the neocortex (Hof et al., 1992).

Data from rat and primate neocortex show that PV-, CB-, and CR-immunoreactive neurons represent morphologically nonoverlapping subtypes of GABAergic interneurons (Hendry et al., 1989; Andressen et al., 1993; Condé et al., 1994; DeFelipe, 1997; Gonchar and Burkhalter, 1997; Glezer et al., 1998; Morrison et al., 1998; Hof et al., 1999). CB- and CR- expressing interneurons share many morphologic similarities and are mainly bitufted, bipolar, and double bouquet neurons, as well as a few pyramidal neurons, with minimal overlap among these subpopulations in the rodent and primate neocortex (Rogers, 1992; DeFelipe, 1997; Morrison et al., 1998). PV-immunoreactive neurons are mainly observed in layers II to V and are principally basket and chandelier cells (Blümcke et al., 1990; Van Brederode et al., 1990; Hof and Nimchinsky, 1992; Condé et al., 1994; Nimchinsky et al., 1997). PV has been reported to occur in certain pyramidal neurons in primates as well (Preuss and Kaas, 1996; Sherwood et al., 2004). CB immunoreactivity is found in subpopulations of pyramidal and nonpyramidal cells (DeFelipe et al., 1989; Hof and Morrison, 1991; DeFelipe and Jones, 1992; Hayes and Lewis, 1992; Hof and Nimchinsky, 1992; Condé et al., 1994). Most CB-immunoreactive interneurons are double bouquet cells located in layers II and III (DeFelipe et al., 1989). CB is also present in Martinotti cells in layers V and VI, in some neurogliaform neurons, and Cajal-Retzius cells in layer I (Derer and Derer, 1990). Finally, a population of weakly labeled CB-immunoreactive pyramidal neurons has been reported in layer III in monkeys and human, with clear rostrocaudal density gradients among neocortical areas (Hof and Morrison, 1991; Hayes and Lewis, 1992; Kondo et al., 1994). Most CR-immunoreactive neurons have bitufted, vertically oriented dendrites and a vertically oriented axon, defining a narrow radial domain (Jacobowitz and Winsky, 1991; Hof and Nimchin-

sky, 1992; Résibois and Rogers, 1992; Condé et al., 1994; Meskenaite, 1997). They are densest in layers II and III and represent the bipolar and double bouquet subtypes. CR-immunoreactive Cajal-Retzius cells are also seen in layer I, and isolated pyramidal neurons containing CR have been reported in several mammalian species (Nimchinsky et al., 1997; Hof et al., 1999). These three calcium-binding proteins are also observed in neurons in a variety of mammalian species, particularly in domesticated carnivores and in cetaceans (Demeulemeester et al., 1991; Glezer et al., 1992, 1993, 1998; Hof et al., 1996, 1999, 2000).

In this article, we describe several aspects of neurochemical specialization in large-brained primates, ungulates, and cetaceans. We compare these features to observations from about 40 species illustrating more than 10 mammalian orders (Hof et al., 1999). We report specific chemoarchitectural characteristics in the context of regional anatomy and cortical microcircuitry, and, based on descriptions of large brained species such as cetaceans, large artiodactyls, and hominoid primates, we discuss how these cellular phenotypes could be used to assess taxonomic affinities among species.

MATERIALS AND METHODS

Specimens were obtained from laboratory animals used in the context of unrelated studies sacrificed for scientific purposes, from animals suffering from a terminal illness and euthanized in zoological facilities for humane reasons, or from animals that died naturally. All euthanasia protocols were reviewed and approved by the relevant institutional animal care and use committees. We had access to over 50 representative species of 12 mammalian orders, including 2 prototherians, 5 marsupials, 1 xenarthran, 1 insectivore, 3 chiropterans, 1 scandentia, 20 primates, 4 rodents, 7 carnivores, 9 artiodactyls, 1 perissodactyl, 12 cetaceans, 1 sirenian, and 1 proboscoid [see Hof et al. (1999) for a detailed summary; the elephant is discussed separately in this issue by Akeem et al. (2005)]. The prototherians, marsupials, the hedgehog, chiropterans, the tree shrew, rodents, the dogs, cats, macaque monkeys, ceboids, and two bottlenose dolphins and one pilot whale were perfused transcardially after injection of a lethal dose of anesthetic with 4% paraformaldehyde [the cetaceans were terminally ill and could be perfused through the descending aorta; in this case, the fixative was a mixture of 2.5% glutaraldehyde and 4% paraformaldehyde (Glezer et al., 1993, 1998; Hof et al., 1999)]. All of the other specimens were obtained after the animal had died of natural causes or was sacrificed for humane reasons and fixed by immersion for several weeks in neutral formalin (cetaceans, sea lion, artiodactyls, great apes, manatee, elephant) or 2–3 weeks in 4% paraformaldehyde [humans (Hof et al., 1995a; 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., 1996). 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, 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 hylobatids and great apes, where incomplete specimens were recovered, and in

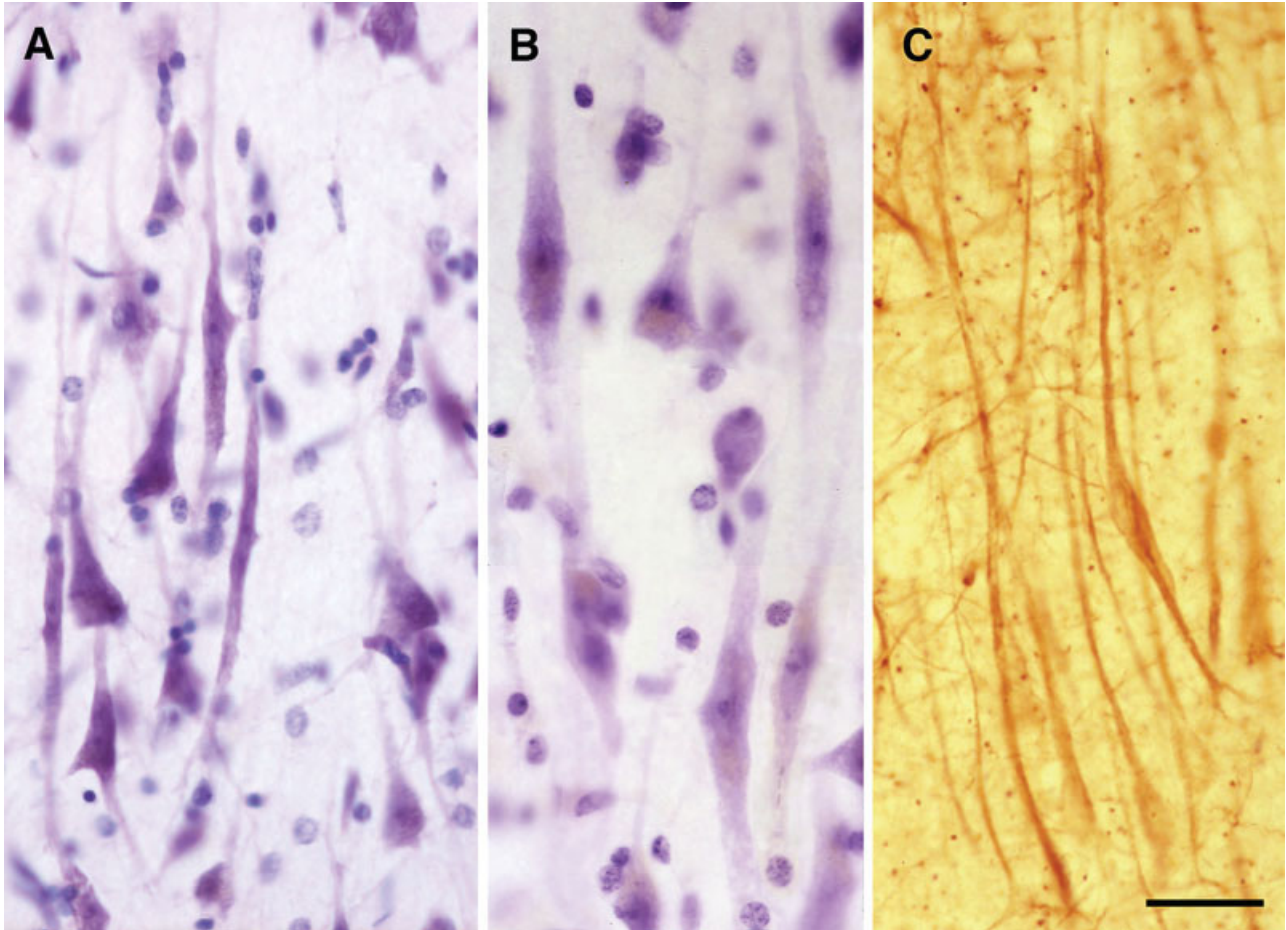


Fig. 1. Examples of spindle neurons in layer Vb of the anterior cingulate cortex in hominids. These particular neurons are numerous in human (A) and are characterized by their vertical fusiform morphology and large somatic size. They are abundant in the bonobo (B) and, as in

human, exhibit a certain degree of clustering. A and B show cresyl violet-stained preparations. Spindle cells are enriched in NFP (C; human). Scale bar (in C) = 60 μ m (A and C); 40 μ m (B).

humans, where only one hemisphere was collected for immunohistochemistry.

All specimens were cut in 40–60 μ m thick sections on a sliding microtome for large samples or on a cryostat. Sections were mounted every 500–1,000 μ m, depending on the species, onto chrom-alum subbed slides and processed for Nissl staining (Fig. 1) and immunohistochemistry. The remaining sections were cryoprotected and stored in serial order at -20°C . For immunohistochemistry, the 40 μ m thick free-floating sections were incubated for 48 hr at 4°C with monoclonal antibodies against dephosphorylated epitopes of the heavy and mid molecular weight subunits of NFP (SMI-32; 1:1,500 dilution; Sternberger Monoclonals, Lutherville, MD), PV, or CB, or with a polyclonal antibody against CR (Swant, Bellinzona, Switzerland), 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 species-relevant antimouse or antirabbit secondary antibodies (1:200–1:500 dilution) and 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% H_2O_2 for 20 min to quench endogenous peroxidase reaction, and then by microwaving them in citrate buffer pH 6.5 for 3 min at maximum intensity using a commercial microwave oven. After cooling, sections were rinsed in PBS and processed for immunohistochemistry. Although consistent immunohistochemical labeling was generally obtained from these specimens, it should be kept in mind that differences in fixation protocols, duration of fixation, storage conditions, or postmortem delay, which were unavoidable, may have affected the staining patterns in some cases. In view of the size of certain brains, the entire cortex could not be exhaustively sampled. Rather, regions were targeted based on existing knowledge of homologies with monkeys, cats, and rodents, so that a consistent number of areas could be analyzed in most specimens. These included at least

the primary and secondary visual cortex, the auditory cortex, and the dorsolateral frontal cortex.

RESULTS

Emergence of a Unique Cellular Specialization in Hominids

The evolution of the neocortex in primates has long been recognized to be the result of great expansion of cortical areas, with a roughly sevenfold increase in cortical volume between strepsirrhines and humans after correcting for body size. In spite of gross morphology and morphometric differences, pyramidal neurons and nonpyramidal interneuron populations have remained remarkably constant across primate species. In fact, the regional distribution of these cell classes has permitted the definition of anatomical boundaries between cortical domains not only in primates but in other mammalian orders as well. A number of studies have pointed to the usefulness of NFP and calcium-binding protein as markers of regional chemoarchitectural features in the primate visual and auditory cortex (Hof and Morrison, 1995; Preuss and Coleman, 2002), as well as in high-order association cortices that have a less distinct cytoarchitecture than primary sensory or motor fields (Carmichael and Price, 1994; Nimchinsky et al., 1997; Vogt et al., 2001, 2005; Sherwood et al., 2003a, 2003b, 2004). In addition, some cortical domains are characterized by the presence of specialized neurons, such as Meynert and Betz cells in the primary visual and primary motor cortices in primates, that exhibit distinct morphology and distribution patterns related to specific projections and functions (Sherwood et al., 2003b, 2004; Rivara et al., 2003). Such specializations are also observed in other species. For example, the primary motor cortex of large carnivores exhibits gigantic NFP-immunoreactive neurons as well as very large multipolar CR-expressing interneurons in layers III and V that are not encountered in other species. Large Meynert-like neurons enriched in NFP have also been reported in the primary visual cortex of the cat (van der Gucht et al., 2001, 2005).

The cingulate cortex and insula of hominids are distinguished by a remarkable cellular specialization, the spindle cells, that are characterized by a vertical, fusiform morphology, very large size, and high levels of NFP immunoreactivity (Fig. 1) (Nimchinsky et al., 1995, 1999). They are prevalent in a restricted sector of the anterior cingulate cortex [areas 25, 24a, and 24b (Vogt et al., 1995)] and are also numerous in the anteroventral agranular insular cortex. These neurons are found exclusively in hominids and have not been reported in any other mammalian species investigated thus far (including other primate species) (Nimchinsky et al., 1999; Hof et al., 2000). In the human and in the bonobo (*Pan paniscus*) anterior cingulate cortex (Fig. 1A and B), spindle cells occur most often in small clusters and are found exclusively in layer Vb. The common chimpanzee (*Pan troglodytes*) has abundant spindle cells, but clusters are not as conspicuous. In the gorilla (*Gorilla gorilla*), the distribution is similar to that in the common chimpanzee, but there are far fewer spindle cells. In the orangutan (*Pongo pygmaeus*), spindle cells are rare, and they are notably absent in lesser apes (e.g., the white-handed gibbon, *Hylobates lar*). As a percentage of pyramidal cell numbers in layer V of area 24, spindles cells account for < 1% in orangutan, ~ 2% in gorilla, ~ 4% in common chimpanzee, ~ 5% in bonobo,

and ~ 6% in human, demonstrating their rarity. Stereologic volume estimates indicate that spindle cells are larger than neighboring layer V pyramidal cells and considerably larger than the small fusiform neurons seen in layer VI. They are larger in chimpanzees and human than in gorilla and orangutan, in contrast to pyramidal cells and small fusiform neurons for which no size difference occurs among these species. The volume of spindle cells is strongly correlated with encephalization, which is not the case for the other neuron types (Nimchinsky et al., 1999). These observations suggest the occurrence of functional modifications during the last 15–20 million years in cortical regions that play major roles in the regulation of autonomic function, cognition, self-awareness, emotionality, and vocalization. These spindle cells are a particular type of projection neuron, as they send an axon in the subcortical white matter, although their exact domain(s) of projection cannot be ascertained in the species in which they are present. They may, however, provide well-defined projections, similar to Meynert cells or Betz cells, and their exclusive presence in humans and their closest relatives suggests that the anterior cingulate and insular cortices have been subjected to strong adaptive pressure along the hominid lineage in recent evolution. This view is also supported by the fact that areas 24 and 25 of the anterior cingulate cortex of hominids also contain a population of CR-expressing small pyramidal neurons in layer Va that, much like the spindle cells, are not observed in other species (Hof et al., 2001).

Cetaceans and Artiodactyls Share Several Cytoarchitectural and Neurochemical Features

Extensive paleontologic and molecular evidence indicates that extant cetaceans (whales, dolphins, and porpoises) had a terrestrial origin and are closely related to artiodactyls; particularly among them, hippopotamuses (Thewissen, 1998). In fact, recent genetic data indicate a sister-group relationship between cetaceans and hippopotamuses, placing cetaceans and artiodactyls within a superorder Cetungulata (Milinkovitch, 1995; Buntjer et al., 1997; Gatesy, 1998; Milinkovitch et al., 1998; Nomura et al., 1998). Reports of the cyto- and chemoarchitecture of Odontoceti, particularly of visual and auditory regions, and analysis of neocortical neurons in a few large artiodactyls have indeed revealed commonalities in cortical organization across these species (Morgane et al., 1988, 1990; Glezer et al., 1992, 1993, 1998; Hof et al., 1992; 1999).

The cetacean neocortex is characterized by a general absence of granularity, a thicker and far more cellular layer I 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. This pattern is observed throughout the neocortex with few variations among regions (Morgane et al., 1988), although the local complexity of cellular architecture in the dolphin neocortex is increasingly recognized (see Hof et al., 2005; Manger et al., 1998). Comparable cytoarchitectural patterns have been described in the neocortex of large artiodactyls (Hof et al., 1999). The distribution and morphology of NFP-immunoreactive neurons are comparable in cetaceans and artiodactyls, but differ considerably from that in primates, carnivores (Fig. 2A–C), and rodents. In cetaceans and artiodactyls, NFP is expressed in very large pyramidal neurons located in the deep portion of layer III

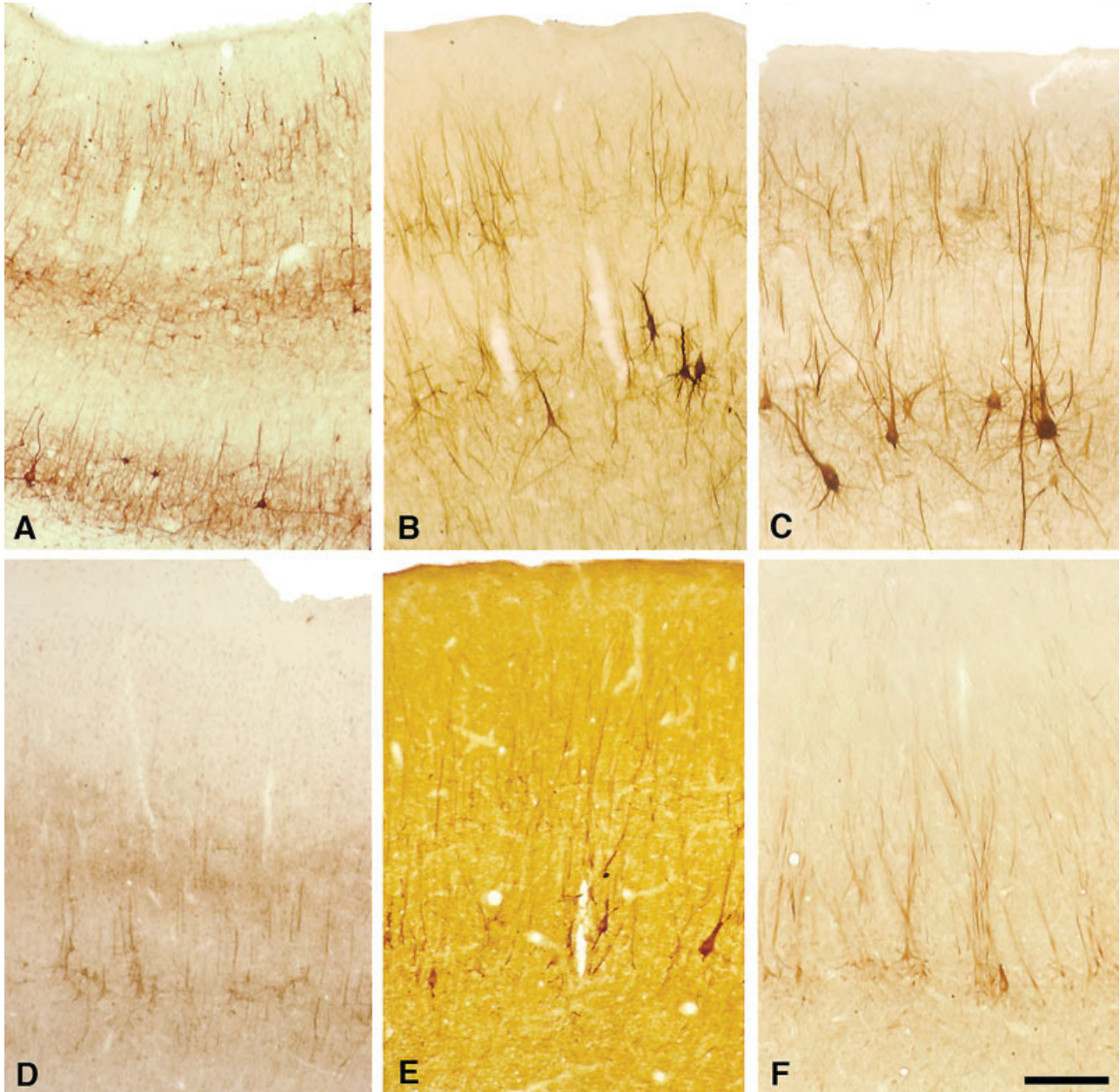


Fig. 2. Examples of cytoarchitecture and cellular typology using an antibody against NFP. **A:** Organization of the primary visual cortex in a long-tailed macaque monkey (*Macaca fascicularis*). NFP-immunoreactive neurons are predominant in layers III, IVB, and V. **B** and **C:** Distribution of NFP-immunoreactive neurons in the primary visual cortex of two carnivores, the dog (*Canis familiaris*; B) and the California sea lion (*Zalophus californianus*; C). Note the prominent labeling of very large cells in layer V and smaller cells in layer III, whereas layer IV in the middle is devoid of labeled neurons.

The large size and extensive dendrites of NFP-immunoreactive neurons are obvious. **D:** NFP-immunoreactive cells in the visual area of the camel (*Camelus dromedarius*). The labeled neurons are relatively small and predominate in the deep layers. **E** and **F:** NFP immunolabeling in the visual cortex of the pigmy sperm whale (*Kogia breviceps*; E) and of the beluga whale (*Delphinapterus leucas*; F). Note the prominent clusters of large immunoreactive neurons in layer IIIc/V only. Cortical layers are shown by Roman numerals. Scale bar (on F) = 300 μm .

and in upper layer V (Hof et al., 1992). The later neurons present as clusters of 3–6 neurons regularly spaced throughout the cortical mantle and intensely labeled with prominent apical dendrites extending well into layer I (Fig. 2D–F), with no major regional variability in their densities, whereas in primates and carnivores, large pyramidal NFP-immunoreactive pyramidal cells are present

in layers III and V–VI with clear regional patterns of distribution.

Cetaceans and artiodactyls also share generally comparable staining patterns for the three calcium-binding proteins in the neocortex (Glezer et al., 1992, 1993, 1998; Hof et al., 1999), which generally set them apart from other mammals. Figure 3 shows several examples of calcium-

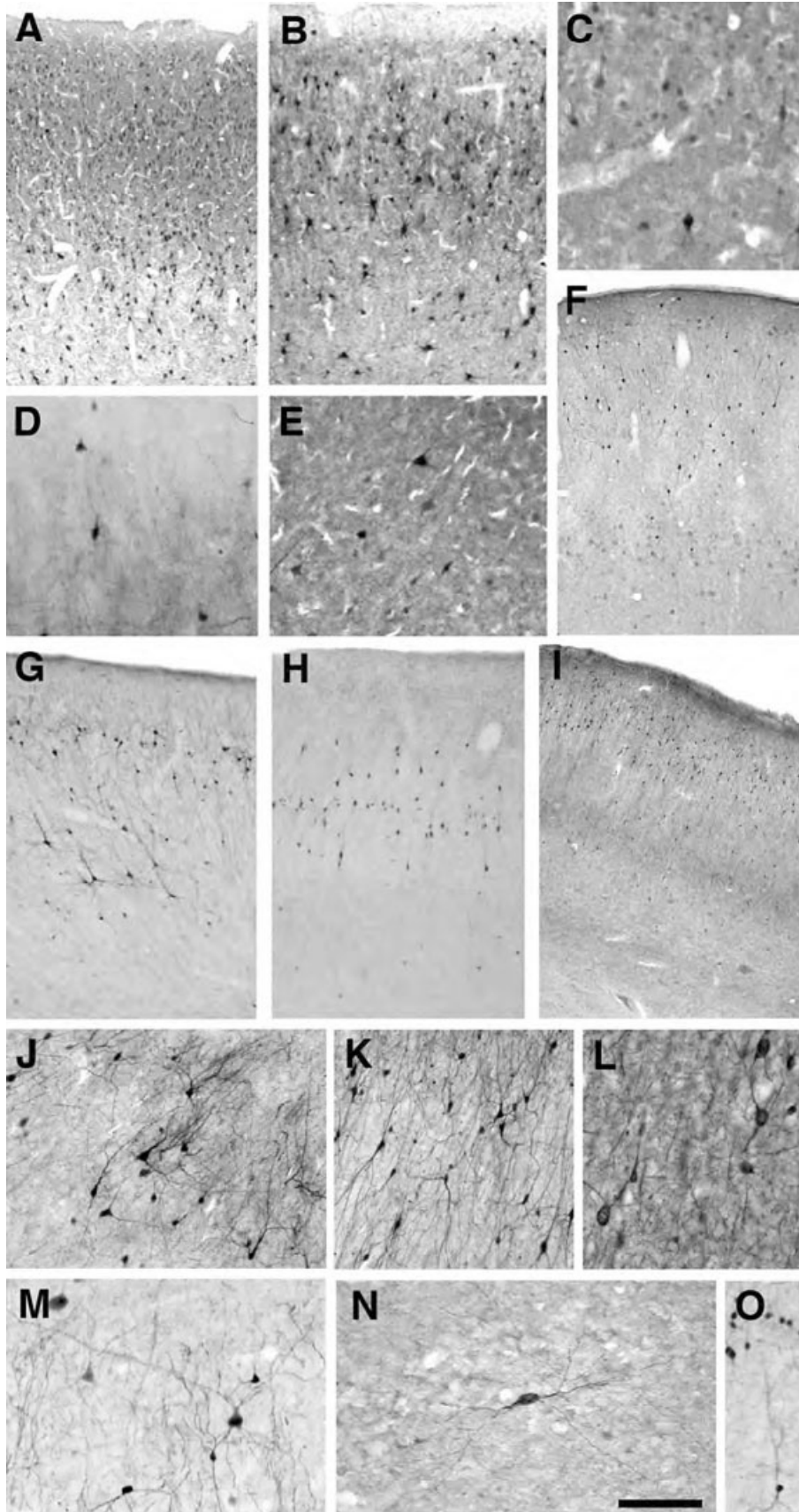


Figure 3.

binding protein distribution in a monotreme and a marsupial (Fig. 3A–C), a xenarthran (Fig. 3D and E), rodents (Fig. 3F and O), and carnivores (Fig. 3G and I–M) that can be used as a comparison to the specializations seen in a dolphin (Fig. 3H) and a giraffe (Fig. 3N). In cetaceans, PV is present in sparsely distributed large stellate neurons located in layers IIIc/V, and a comparable paucity of labeled neurons is found in artiodactyls. A few small pyramidal neurons in layer III also exhibit PV immunoreactivity in dolphins. In cetaceans and artiodactyls, CB and CR are far more numerous and occur in large fusiform, bipolar or multipolar neurons in layers I, II, and superficial III. CB-containing neurons are much less numerous and less intensely stained than CR-immunoreactive neurons. The CR-containing neurons located in layer I have a morphology quite comparable to that of the bipolar/bifurcated CB- or CR-expressing neurons typically seen in layer II of other mammals such as rats, carnivores, and primates (Ballesteros Yáñez et al., 2005), whereas the CR-containing neurons in layers II and III are much larger and more variable in shape than in other species, with a predominance of multipolar and fusiform types (Fig. 3). These neurons have long dendrites that extend into layers I and III (Fig. 3H). Very large CR-immunoreactive neurons are also encountered in layers V and VI, especially in the neocortex of large artiodactyls, such as the giraffe (*Giraffa camelopardalis*), llama (*Lama glama*), and camel (*Camelus dromedarius*), whereas they are less numerous in the pig and in smaller ruminants (Fig. 3N). 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. Interestingly, a population of NFP-immunoreactive neurons in layer III of neocortex in anthropoid primates also expresses CB (Kondo et al., 1994). The distribution and

typology of calcium-binding proteins in artiodactyls and cetaceans show some commonalities with those in insectivorous bats and hedgehogs but differ from those in rodents and primates (Glezer et al., 1993, 1998; Hof et al., 1999), suggesting that these traits may distinguish among some members of Laurasiatheria and Euarchontoglires (Murphy et al., 2001a, 2001b). Large multipolar PV-containing neurons are observed in the deep layers of the neocortex of hedgehogs as in dolphins, unlike the patterns in rodents, carnivores, and primates, where PV-expressing cells are more numerous in layers III and IV. As in hedgehog, a basal member of the Laurasiatheria, CB and CR are the dominating calcium-binding proteins in cetaceans and artiodactyls. In the cetacean primary visual and auditory cortices, CB- and CR-immunoreactive neurons represent about 40% of the total number of neurons, whereas PV is present only in about 5% of the neurons (Glezer et al., 1993), and these values are probably lower in large artiodactyls. In cetaceans, the proportion of calcium-binding protein-containing neurons is about twice as high as in primates and rodents (Glezer et al., 1993). This may represent a shared trait in whale and ungulate brains. That the cetacean and artiodactyl neocortex is dominated by CB and CR may also constitute an ancestral trait, observed as well in some insectivorous bats and hedgehogs, making it ancestral for just Laurasiatheria, but not for all mammals as insectivores are no longer recognized as a valid monophyletic clade (Murphy et al., 2001a, 2001b); in contrast to PV, these two proteins predominate in phylogenetically older neural systems mammals (Glezer et al., 1993, 1998; Jones, 1998).

DISCUSSION

Relationships of Neurochemical Phenotype and Phylogenetic Affinities

The variable patterns in distribution of NFP and the three calcium-binding proteins among species can be analyzed in the context of the general cytoarchitecture of the mammalian neocortex. In fact, several sets of cortical organizational patterns emerge from our data and the available literature. Generally, species showing a high degree of morphologic differentiation of neocortical areas and a variable development of layer IV and substantial variation in neuronal size and packing densities across the cortical plate are characterized by a balanced representation of the three calcium-binding proteins and morphological diversity of NFP-immunoreactive pyramidal neurons across cortical regions. In contrast, species characterized by a greater cytoarchitectural monotony throughout the cortical mantle, a poorly defined or lack of layer IV in most regions, and the presence of very large pyramidal cells in all neocortical areas display a predominance of CB- and CR-containing populations in comparison to PV-immunoreactive neurons and rather uniform NFP-containing pyramidal cell morphology. The first type occurs in primates, rodents, carnivores, and, to some extent, megachiropterans, as well as in tree shrews and lagomorphs, whereas the second type is present in cetaceans and ungulates. This fits nicely with a Laurasiatheria vs. Euarchontoglires split (Murphy et al., 2001a, 2001b); allowing for some convergent evolution in the Carnivora, the distribution and typology of calcium-binding proteins in this order show some commonalities to those seen in rodents and primates (Fig. 3) (Glezer et al., 1993, 1998; Hof et al.,

Fig. 3. Immunoreactivity patterns of calcium-binding proteins in the neocortex of various mammals. **A** and **B**: CB (A) and PV (B) immunoreactivity in the primary somatosensory cortex of the echidna (*Tachyglonus aculeatus*). There is a large population of small neurons that express CB, whereas PV is contained in a heterogeneous collection of neurons, some very large and multipolar. This differs radically from other members of the Australian fauna (Hof et al., 1999), such as the koala (*Phascolarctos cinereus*); **C**: that displays a rather sparse population of CB-containing cells. **D** and **E**: The giant anteater (*Myrmecophaga tridactyla*) is also characterized by a sparse population of relatively large CB-immunoreactive multipolar neurons (D) and PV-expressing neurons (E). These patterns are fully distinct from that in rodents (**F**: CR immunoreactivity in the somatosensory cortex of the chinchilla, *Chinchilla laniger*), carnivores (CR immunoreactivity in a dog motor cortex displaying bipolar neurons as well as pyramidal-like neurons; **G**), and a cetacean visual cortex (the bottlenose dolphin, *Tursiops truncatus*; **H**). Layers I and II are particularly enriched in the cetacean (layer II is located about one-third down from the top of the photomicrograph). **I–L** show details of the chemoarchitecture of the frontal cortex of a Siberian tiger (*Panthera tigris*). CR-immunoreactive neurons exhibit a typical distribution predominating in the superficial layers (I and K), and large multipolar CB-immunoreactive (J) and PV-immunoreactive (L) neurons are encountered in deep layer III. Large multipolar CR-containing neurons are found in layer III of a dog (*Canis familiaris*) primary motor area (**M**). Note the large size of this neuron and compare it to the CR-immunoreactive giant neuron located in layer VI of a giraffe (*Giraffa camelopardalis*; **N**), and to a typical bipolar neuron in layer III of the mouse visual cortex (*Mus musculus*; **O**). Scale bar (in N) = 300 μ m (A and B); 400 μ m (F–I); 50 μ m (C–E and J–O).

1999; Ballesteros Yáñez et al., 2005). In addition, hedgehogs and microchiropterans display a combination of both character complexes, with the second type being generally predominant (Hof et al., 1999). These observations in insectivores need to be confirmed in other species of this paraphyletic group such as representative Afrosoricids and Macroscelids. As a rule, while general patterns are shared by many species, frequently certain patterns are unique to a single taxon or occur at the superfamily level. Thus, the general classes of calcium-binding protein-containing interneurons characterized in the rat, macaque monkey, and human are consistently observed among rodents, lagomorphs, primates, carnivores, as well as to some degree in megachiropterans and tree shrews (Hof et al., 1999; Ballesteros Yáñez et al., 2005). Yet megachiropterans are characterized by the unique absence of neocortical expression of CR in interneurons (while thalamic neurons do express it). However, in prototherians, marsupials, xenarthran, cetaceans, and ungulates, PV, CB, and CR are encountered in diverse types of pyramidal cells, as well as in classes of interneurons unique to these species, with much less consistency than in other mammals (Fig. 3) (Hof et al., 1999). In some cases, these features may even be interpreted as examples of homoplasy, with certain characters occurring in nonrelated groups of mammals, such as the paucity of PV expression observed in the neocortex of cetungulates and marsupials, or the fact that CB and CR predominate in cetaceans, microchiropterans, hedgehogs, and artiodactyls, not only in the neocortex, but also in subcortical systems (Glezer et al., 1993, 1998; Hof et al., 1999). The functional repercussions of these specializations remain, however, unknown.

It is interesting that in canids, felids, and pinnipeds, very large CR-immunoreactive, multipolar neurons occur in layer III of motor cortices (Fig. 3). These neurons are morphologically comparable to the very large CR-containing neurons found in layers III, V, and VI throughout the neocortex of large artiodactyls and cetaceans (Hof et al., 1996, 1999). In carnivores, these neurons are seen only in agranular cortices and the cortex of large ungulates and cetaceans shows a poorly differentiated layer IV only in certain regions (Morgane et al., 1990; Glezer et al., 1993, 1998; Hof et al., 1999). The presence of these particular CR-immunoreactive cells in agranular cortex deserves further investigation and may point to possible phylogenetic affinities between carnivores, ungulates *sensu lato* (i.e., "Fereungulata") (see Johnson et al., 1994), carnivores being a sister taxon to the clade containing perissodactyls and cetartiodactyls (Murphy et al., 2001a, 2001b). In this context, the dog neocortex displays several differences in neocortical neurochemical organization compared to anthropoids in spite of many similarities in cortical organization and connectivity between carnivores and primates (Hof et al., 1996, 1999). Canids have a high degree of cellular specialization in primary motor and sensory cortices, contrasting with fairly homogeneous patterns in association cortices. Generally, the distribution patterns and typology of PV- and CB-immunoreactive interneurons in dogs and cats are comparable reflecting their close phylogenetic proximity (Demeulemeester et al., 1991; Hof et al., 1996). Large PV- and CB-containing pyramidal cells are present only in primary motor and visual regions, and high numbers of large PV-immunoreactive basket cells and multipolar CR-containing interneurons occur only in primary motor, somatosensory, and visual areas (Hof et

al., 1996). It is not known whether the large PV- and CB-containing pyramidal cells in these species represent subclasses of NFP-enriched neurons and double-labeling studies would be required to answer this question in carnivores and in other taxa. It should be noted in this context that at least some CB-expressing pyramidal cells in primates also display NFP immunoreactivity (Hayes and Lewis, 1992). That the association cortex in the dog appears less differentiated neurochemically than presumably homologous areas in primates supports the concept that cortical regions such as inferior temporal and prefrontal cortex achieved a particular degree of elaboration in primates compared to other mammals (Preuss, 1995). Whether this argument can in fact be generalized awaits further analyses in additional canid and felid species in comparison to primates as well as representative taxa of other orders. Further limitations of our study is that our sample is limited to representative species of Boreoeutheria, permitting assessment of affinities and character specificity only among Laurasiatheria and Euarchontoglires to the exclusion of Afrotheria, for which no detailed regional studies of such patterns are currently available. As such, some of these neurochemical features may relate to event in mammalian brain evolution dating back to about 80–90 million years ago at the time Laurasiatheria (i.e., cetungulates, carnivores, pangolins, bats, hedgehogs, and moles) diverged from Euarchontoglires (i.e., rodents, lagomorphs, primates, tree shrews, and colugos). It is also worth noting that the Australian echidna presents an NFP-expressing cells in layer V in its neocortex (Hassiotis et al., 1994), whereas calcium-binding proteins in this species show a fairly variable morphology and distribution compared to other mammals, including marsupials (Fig. 3) (Hof et al., 1999). Although it would be important to analyze NFP distribution species directly related to echidna such as the platypus and in representative marsupials, the presence of layer V NFP-rich cells may be interpreted has a conservative trait among mammals.

Implications for Cortical Function

Calcium-binding protein-containing interneurons are known to influence the activity of pyramidal neurons in a manner specific to each cell class (Condé et al., 1994; DeFelipe, 1997). Parvalbumin-immunoreactive basket and chandelier cells provide an innervation of the perikaryon and axon initial segment, respectively, whereas CR- and CB-containing bipolar and double bouquet cells target mostly the apical dendritic arbors of pyramidal neurons, and as comparable types of interneurons have been described in many species (Condé et al., 1994; DeFelipe, 1997; Hof et al., 1999; Ballesteros Yáñez et al., 2005), the role of calcium-binding protein in cortical integration is likely to be similar to a large degree at least in rodents, carnivores, and archontans. Yet certain differences exist at the level of particular neuronal subclasses, as recently revealed in a study of CB-expressing interneurons in primates compared to rodents, lagomorphs, carnivores, and artiodactyls (Ballesteros Yáñez et al., 2005). These authors reported that in the nonprimate species they investigated, the vertical bundles of axons of CB-immunoreactive double bouquet cells are not observed except in the visual cortex of carnivores. Even there these axonal "horse tails" are far less prevalent than in the primate neocortex. These data indicate that although CB-expressing cell types comparable to the typical primate

double bouquet cell occur in these species, their axonal projections are likely to be organized very differently and may not contribute to the columnar connectivity of the neocortex in the same manner as is observed in primates (Ballesteros Yáñez et al., 2005). Whether this sort of difference in axonal organization of interneurons can be extended to typical PV- and CR-immunoreactive neurons remains to be demonstrated. In any case, the degree to which such functional relationships can be extended to all mammalian orders is difficult to define owing to such differences within a given cell type across species. The relative rarity of PV-immunoreactive neurons in cetaceans and ungulates could be interpreted as an ancestral retention. It also occurs in echolocating bats and in hedgehogs, which have been claimed to have retained many plesiomorphic features (Glezer et al., 1988). This suggests that the inhibitory microcircuitry of the cetacean and ungulate neocortex may be characterized by primitive features involving different cellular interactions compared to other mammalian lineages. In fact, the neocortex of cetaceans and large ungulates appears to contain an inordinate number of cortical columns as revealed by clusters of large NFP-containing layer V pyramidal cells in layer IIIc/V (Glezer et al., 1988; Morgane et al., 1988; Hof et al., 1992). Cortical integration in cetaceans may take place mostly in the highly cellular, comparatively thick layer I that contains approximately 70% of the neocortical synapses in these species (Glezer and Morgane, 1990). This is consistent with the observation that the majority of CB- and CR-containing interneurons are located in layers I and II in cetaceans, the less abundant PV-immunoreactive cells being in the vicinity of layers IIIc/V and VI pyramidal cells. The distribution of PV-immunoreactive neurons indicates that these cells may represent basket cells and that the axons of CB- and CR-immunoreactive interneurons may be located where most of the inputs to the neocortex terminate around the apical dendrites of the deep-layer pyramidal neurons (Glezer et al., 1988; Morgane et al., 1988). In spite of lack of ultrastructural evidence at the synaptic level, it is possible that in cetaceans and large ungulates, CB-, some CR-, and PV-immunoreactive neurons play a very similar role in neocortical microcircuits as in primates and rodents. It is therefore likely that functionally, interspecies differences in neocortical organization notwithstanding, calcium-binding proteins identify classes of interneurons that subservise similar tasks across mammals.

Although the precise function of NFP is not understood, it has a restricted distribution among certain subsets of corticocortical circuits in primates (Hof et al., 1995b, 1996) and is involved in neurodegenerative lesions in subsets of pyramidal neurons particularly vulnerable in dementing disorders in humans (Bussière et al., 2003; Hof and Morrison, 2004). This fact is particularly important considering that NFP-enriched spindle neurons of the hominid cingulate and fronto-insular cortex have been shown to be severely affected in the degenerative process of Alzheimer's disease, suggesting that the neuronal susceptibility that occurs in the human brain in the course of age-related dementing illnesses appeared only recently during primate evolution (Nimchinsky et al., 1995, 1997, 1999; Rapoport, 1999). We have proposed that NFP confers unique neurochemical and morphologic properties to select neuronal subpopulations subserving a range of highly specialized functions in the neocortical connectivity (Hof et al.,

1995a, 1995b; Nimchinsky et al., 1996; Bussière et al., 2003; Hof and Morrison, 2004). NFP may thus be present, to some degree, in functionally homologous subsets of cortical output neurons in cetaceans and artiodactyls. The similarities in neurochemical specialization of the cetacean and artiodactyl neocortex parallel the paleontological and molecular evidence that these species share a relatively recent common ancestor, and that much like primates, the evolution of the species with the largest brains (the delphinids) is a recent event (Marino et al., 2005). Also, it is intriguing that in both cetacean and artiodactyls, compared to other mammals, the calves are born with precocious physical maturity, a crucial factor for survival in the aquatic milieu and, in the case of terrestrial herbivores, to escape predators. It is possible that pedomorphosis occurs in these species. In the case of cetaceans, possible pedomorphic features include the retention in adults of the pontine, mesencephalic and cephalic flexures that are visible only in embryos in other mammals, and the very large size of the brain at birth (Glezer et al., 1998; Hof et al., 1999). Furthermore, that the neocortex of cetaceans and ungulates is dominated by CB- and CR-containing interneurons may also represent a pedomorphic feature because these calcium-binding proteins appear first during development and in rodent, carnivores, and primates and are preferentially distributed in phylogenetically older neural systems (Glezer et al., 1993, 1998; Jones, 1998; Hof et al., 1999).

Although there are major gaps in our knowledge of the evolutionary history of neocortical organization in mammals and of the chemical organization of the cerebral cortex in most species, these observations together indicate that brain organization and neurochemical cellular specialization reflect evolutionary relationships among many mammalian species.

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