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Neocortical neuron morphology in Afrotheria: comparing the rock hyrax with the African elephant

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The mammalian neocortex contains a great variety of neuronal types. In particular, recent studies have shown substantial morphological diversity among spiny projecting neurons in species that diverged close to the base of the mammalian radiation (e.g., monotremes, afrotherians, and xenarthrans). Here, we used a Golgi technique to examine different neuronal morphologies in an afrotherian species, the rock hyrax (*Procavia capensis*), and provide a comparison with the related African elephant (*Loxodonta africana*). Results showed that spiny neurons in the rock hyrax neocortex exhibit less morphological variation than in elephants, displaying a higher frequency of relatively "typical" pyramidal neurons. A quantitative comparison of rock hyrax pyramidal neuron morphology between frontal and visual areas, moreover, revealed greater spine density of neurons in frontal cortex, but no differences in other morphological aspects. Regional variations in pyramidal structure have also been observed in the African elephant, as well as a number of primate species.

Keywords: rock hyrax; elephant; Afrotheria; golgi; dendrite; evolution

Introduction

The mammalian neocortex is characterized by a diverse array of neurons, varying in shape, biochemistry, and patterns of connectivity.[1–6] Examination of cortical microstructure has revealed that distinct neuronal types also exhibit considerable evolutionary diversity, with species- and order-specific distributions and morphologies.[7,8] As such, comparing species of different lineages may help identify ancestral and derived features of the neocortex. To this end, a number of studies have analyzed the cyto- and chemoarchitecture of the neocortex of species that diverged close to the base of the mammalian radiation, such as the monotreme short-beaked echidna (*Tachyglossus aculeatus)*,[9,10] as well as the marsupials Tammar wallaby (*Macropus eugenii)*,[11–13] common brush-tailed possum (*Trichosurus vulpecula*),[14] fat-tailed dunnart (*Sminthopsis crassicaudata*), and quokka (*Setonix brachyurus*).[15]

Among living eutherian (placental) mammals, four major phylogenetic groups have been identified by molecular genetic studies, including Euarchontoglires (e.g., rodents, primates, scandentians), Laurasiatheria (e.g., carnivores, chiropterans,[a] cetartiodactyls), Xenarthra (e.g., armadillos, anteaters, and sloths), and Afrotheria (i.e., elephants, hyraxes, manatees and dugongs, golden moles, tenrecs, and elephant shrews).[17] Compared to Euarchontoglires and Laurasiatheria, however, there is less information available on the neocortical architecture of xenarthrans and afrotherians, thus making it difficult to reconstruct how

[a]See Dell *et al.*[16] for a discussion of the monophyletic or diphyletic origins of chiropterans.

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neocortical circuitry has changed in placental mammal evolution.

To address this limitation, a recent immunohistochemical analysis of the neocortex of several xenarthrans and afrotherians has provided new data on neuron types and distributions in these species.[18] Taken together, these studies highlight substantial similarities across all mammals in the morphology of nonprojection inhibitory interneurons (however, see De Felipe *et al.*[2] for interspecies differences in double-bouquet cells). A remarkable degree of variation, however, has been observed among pyramidal cells, which exhibited a greater number of "atypical" features (e.g., inverted somata, widely bifurcating apical dendrites) in monotremes, marsupials, xenarthrans, and afrotherians compared to rodents and primates.[10,11,18]

To elucidate the evolution of mammalian neuromorphology, we examined the neocortex of an afrotherian species, the rock hyrax. As a member of the order Hyracoidea, family Procaviidae, the rock hyrax is a medium-sized (∼4 kg), herbivorous animal that inhabits sub-Saharan Africa and the Middle East. Among mammals, the Afrotheria is a group of particular interest not only for its basal phylogenetic position—it diverged from other mammals about 100 million years ago[17]—but also for its striking diversity. Indeed, the Afrotheria is comprised of species with diverse ecological niches, behavioral adaptations, and brain sizes, which range from the ∼1.5 g brain of elephant shrews[19] to the ∼5 kg brain of elephants.[20] Afrotherians, moreover, remain relatively underrepresented in the comparative neuroanatomical literature,[21] with only a handful of studies published on the cortical organization of the elephant shrew (*Elephantulus edwardii),*[22] the tenrec (*Echinops telfairi*),[23] the manatee (*Trichechus manatus*),[24] and the African elephant (*Loxodonta africana*).[25,26] Previous work on the neocortex of the hyrax includes the mapping of the somatosensory area,[27] volumetric measurements of gray and white matter,[28] and immunohistochemical analyses;[18] however, no study has provided a quantitative analysis of cortical neuromorphology.

In the present study, we used Golgi staining to describe and quantify a variety of neuron types in the rock hyrax neocortex. In so doing, we furnish a direct comparison with another afrotherian species, the African elephant, for which a detailed neuromorphological study was recently completed.[26] In the examination of the elephant neocortex, a variety of neuron types were identified, revealing remarkable morphological heterogeneity, especially among spiny neurons.[26] In addition, quantitative analyses that compared the morphology of pyramidal neurons within superficial cortical layers found significant differences between frontal and occipital regions.[26] Previous studies measuring regional variation in pyramidal cells of humans and other primates[29–31] have revealed greater neuromorphological complexity in anterior compared to posterior regions, suggesting increased computational demands within regions of the frontal lobe. Consistent with these findings, the elephant exhibited more complex dendritic arbors and spine complement in frontal compared with occipital neurons.[26] Here, we offer a similar morphometric assessment of pyramidal neurons in the rock hyrax by quantifying and comparing a sample of neurons across frontal and occipital regions. Furthermore, we present a description of variation in neuronal morphology in these neocortical areas.

Material and methods

Specimens and tissue preparation

The brains of two adult rock hyraxes, one female (age: one year, five months) and one male (age: 11 months, 22 days), were obtained from the Cleveland Metroparks Zoo after the animals had died for reasons unrelated to the current study. Tissue samples were removed within 14 hours after death and immersion fixed in 10% buffered formalin for seven days. They were then transferred to a phosphate buffer saline (PBS) solution with 1% azide and stored at 4° C until staining. Both brains appeared normal upon routine pathology examination.

Two small tissue blocks (1–2 cm) from frontal and occipital areas were removed from the right hemisphere of each individual. By reference to the pattern of staining against Nissl substance, myelin, and parvalbumin from the left hemisphere of these same specimens,[18] the occipital region blocks were removed from an area that was in the location of primary visual cortex, and the frontal blocks were taken from a dorsolateral region anterior to the primary motor cortex. The frontal cortex was dysgranular, and the occipital was granular. In keeping with previous studies,[26,30,31] the tissue was processed by a modified rapid Golgi technique[33] and sectioned at 120 μm with a vibratome.

Neuron selection and morphological quantification

Ten supragranular pyramidal neurons per region per brain were selected for the quantitative analysis of regional differences. Criteria for selection required that neurons be fully impregnated, relatively isolated and unobscured, the soma centered within the 120 μm–thick section, and the dendritic systems as complete as possible.[30,31] To ensure relative homogeneity among sampled neurons, the soma depth of each neuron from the pial surface was recorded, and comparable average depths were maintained across regions.

In addition to the 20 supragranular pyramidal neurons, two inverted pyramidal neurons, nine other spiny, and four aspiny nonpyramidal neurons were traced. Because these neurons were often not fully impregnated, only a qualitative description of their morphology is provided. Our qualitative description of variation in neuromorphology was based on examination of many other neurons that were not traced.

All neurons were manually traced using a 40x dry objective on a Zeiss Axioplan 2 photomicroscope (Ludl Electronics, Hawthorne, NY, USA), Heidenhain *z*-axis encoder (Heidenhain, Schaumburg, IL, USA), an Optronics MicroFire color videocamera (Optronics, Goleta, CA, USA), and a Dell PC workstation running Neurolucida software (MBF Bioscience, Williston, VT, USA). Tracing involved drawing the contour of the soma, following all dendrites along their entire length, and marking all visible spines. Tracing was not continued in adjacent sections, and those dendrites that were either broken or not fully impregnated were coded as incomplete terminations.

Neuronal morphology was quantified according to six measures adapted from Jacobs *et al.*[26] These included (1) cell soma area (μm^2); (2) total dendritic length (TDL, μm)—the sum of the individual lengths of all dendritic segments; (3) dendritic segment count (DSC)—the number of all dendritic segments; (4) mean segment length (MSL, μm); (5) dendritic spine number (DSN)—the number of all spines marked on the dendritic arbor; and (6) dendritic spine density (DSD)—the ratio of spines per unit (1 μm) of dendritic length. For each of these measures, values for the basilar and apical dendrites were separately computed. As most apical dendrites were incomplete, they were only described qualitatively; however, basilar arbors were examined quantitatively. In addition to these measures, a Sholl analysis[33] was performed, which assessed neuronal morphological complexity as the number of intersections made by each dendritic tree with a series of concentric virtual spheres at increasing increments of 20 μm.

All tracings were obtained by two researchers (S.B., C.D.S.), who were normed with another rater (A.L.B.) and checked by the primary investigator (C.C.S.). To ensure accuracy, intrarater reliability was assessed by tracing the same dendritic branch 10 times. Coefficients of variation calculated for S.B. (cell soma area = 5.1%, TDL = 1.8%, and DSN = 2.6%) and C.D.S. (cell soma area = 9.16%, TDL = 1.93%, DSN = 5.72%) showed little variation, and a split-plot design revealed no significant differences between the first and the second half of the tracings ($P > 0.05$). As assessed by the coefficient of intraclass correlation, interrater reliability was high in all three measures of interest: cell soma area = 0.903, TDL = 0.977, DSN = 0.906 (A.L.B-S.B), and cell soma = 0.579, TDL = 0.995, and DSN = 0.993 (S.B-C.D.S).

Results

Supragranular pyramidal neurons

Supragranular pyramidal neurons usually appeared well impregnated. Neurons selected for tracing came from layer III, at a similar soma depth across regions (frontal = 736.16 ± 251.18 μm, occipital = 808.30 ± 269.46 μm). A Mann–Whitney

Table 1. Mean and standard deviation of soma area, TDL, DSN, DSC, MSL, and DSD for basilar dendrites of supragranular pyramidal neurons in the frontal and occipital region

	Frontal $N = 20$	Occipital $N = 20$
Soma area (μm^2)	261.92 ± 73.04	243.27 ± 71.95
TDL (μm)	1721.77 ± 688.99	1700.80 ± 709.62
DSC	28.45 ± 9.48	29.5 ± 11.15
MSL (μm)	55.57 ± 11.03	59.72 ± 8.84
DSN	518.90 ± 333.79	387.85 ± 302.37
DSD (number per μm)	0.28 ± 0.10	0.21 ± 0.11

U-test showed no significant differences in the soma depth between regions ($U = 152$, $P > 0.05$).

Morphology. Supragranular pyramidal neurons typically exhibited spiny basilar dendrites extending radially from the cell body and a single apical dendrite traveling toward the pial surface. Quantitative data are provided in Table 1. Visual inspection of 15 relatively complete apical dendrites revealed one thick dendrite that ascended perpendicularly to the pial surface while forming thinner, but spiny, oblique branches in most cases. In some instances, however, apical dendrites originated from the soma as a single shaft, split after approximately 30 μm into two thick branches that either traveled obliquely toward the pial surface, forming a V-shaped pattern, or, more frequently, ascended in parallel.

Regional differences. We analyzed regional differences in pyramidal neuron morphology using one-way ANOVAs with MSL and DSD as the dependent variables and region as the independent variable. These analyses showed that DSD ($F_{1,38} = 5.167$, $P = 0.029$) was significantly greater in the frontal region. No significant differences were found in MSL ($F_{1,38} = 0.252$, $P > 0.05$), which was similar across regions. Because the other variables of interest, TDL, DSN, DSC, and cell body area were not normally distributed, we used Mann–Whitney *U*-tests to examine regional differences. Results revealed no significant differences in either cell body

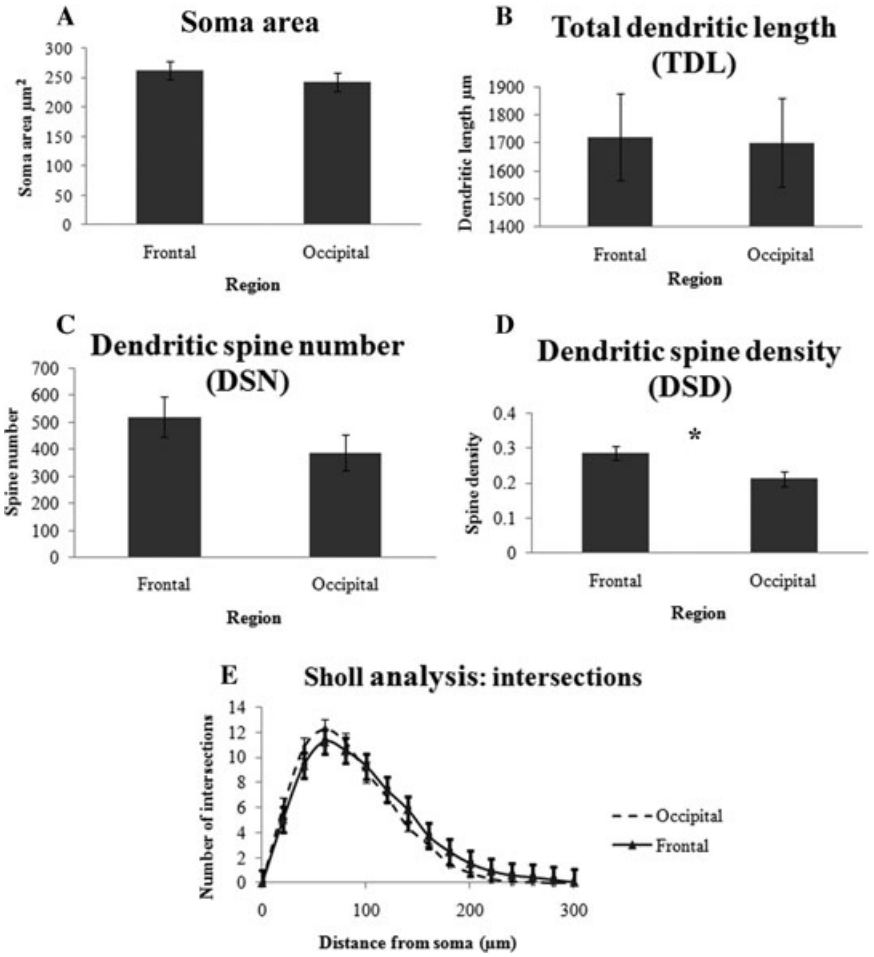

Figure 1. **A–D illustrate regional differences in the soma area, TDL, DSN, and DSD for basilar dendritic arbors of supragranular pyramidal neurons. E shows different Sholl profiles for frontal and occipital supragranular pyramidal neurons, calculated as the total number of intersections on each dendritic system per 20 μm of dendritic length.**

area ($U = 155.00$, $P > 0.05$), TDL ($U = 199.00$, $P > 0.05$) or DSC ($U = 193.00$, $P > 0.05$). Greater DSN was found in neurons in the frontal region, but it fell short of conventional significance levels ($U = 136.500$, $P = 0.086$, Fig. 1).

In a Sholl analysis, similar profiles of basilar dendritic branching characterized frontal and occipital neurons in both the average of the total number of intersections (frontal $= 4.59 \pm 4.09$, occipital $= 5.07 \pm 4.53$) and the distance from the soma, which peaked at 60 μm in both regions. In the frontal region, however, the number of intersections tended to decrease less steeply as the distance from the soma increased, possibly indicating a relatively larger receptive field (Fig. 1).

Other neuronal morphologies

In addition to the supragranular pyramidal neurons, a variety of other spiny and aspiny neuron types were identified, including inverted pyramidal, fork-shaped, bitufted, bipolar, and multipolar cells (Figs. 2 and 3). For these neuronal types, a qualitative description of morphology and laminar distribution is provided here.

Inverted pyramidal neurons. Two inverted pyramidal neurons were traced in the infragranular layers of the occipital region. These neurons exhibited a spiny basilar dendritic skirt that ascended toward the pial surface, and a thick apical dendrite with several side branches that descended toward the white matter.

Fork neurons. One fork neuron was traced in layer III of the frontal region. Fork neurons have been previously described in the human insular cortex.[34] They have a single large tapered basal dendrite, with two thick apical dendrites that bifurcate close to the soma and project toward layer I in a narrow Y-like fashion. However, only the cell body and proximal apical dendritic segments were fully impregnated in this neuron.

Bitufted neurons. Three bitufted neurons (spiny: $n = 2$; aspiny: $n = 1$) were traced in layer III and V of the occipital cortex. Both spiny and aspiny types showed similar vertical morphologies, with two dendritic processes emerging from each pole of an elongated soma. A possible variant of these neurons showed an elongated soma, from which a spiny basilar skirt developed vertically in a horse tail–like fashion, giving the neuron an

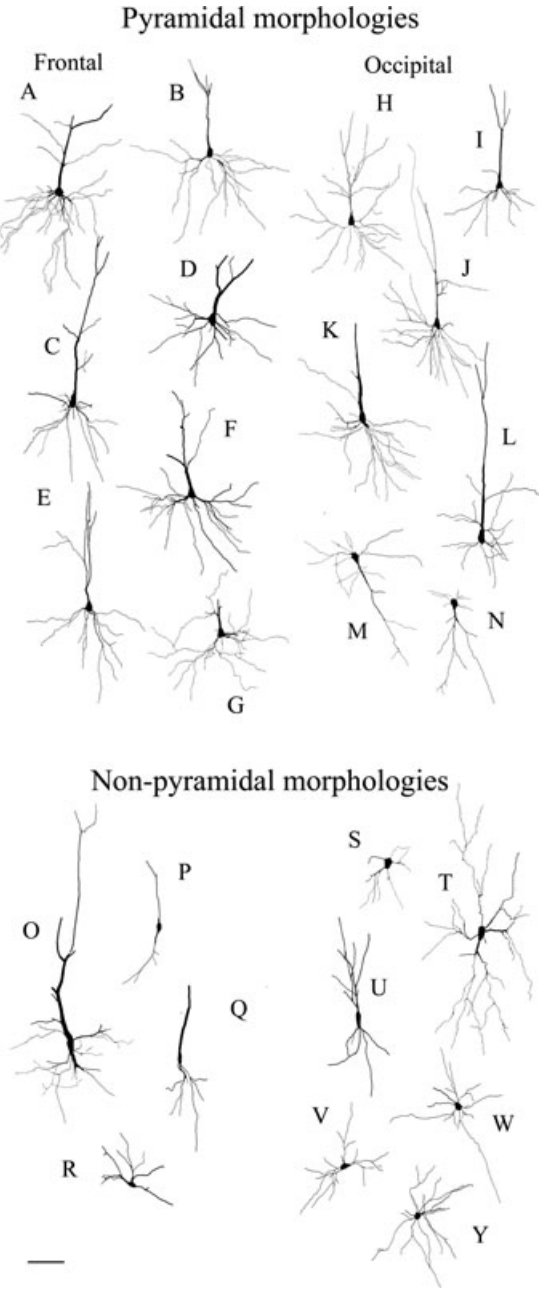

Figure 2. Tracings of pyramidal and nonpyramidal morphologies in the rock hyrax neocortex. Among pyramidal morphologies, A–G illustrate frontal pyramidal neurons, H–L illustrate occipital pyramidal neurons, and M and N illustrate inverted pyramidal neurons. Among nonpyramidal morphologies, O, R–T, and V–Y are multipolar cells; P and Q are bipolar cells; and U and T are bitufted cells. O, P, R, and S are aspiny or sparsely spined. Q, T, U, W, and Y are spiny. Pial surface is toward the top of the figure. Scale bar = 100 μm.

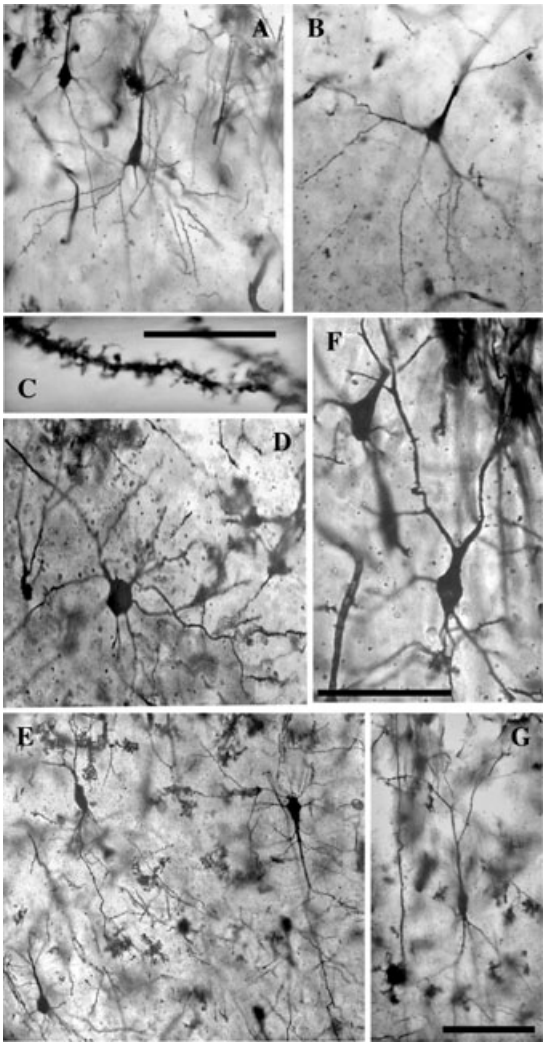

Figure 3. **Photomicrographs of neuronal types in the rock hyrax neocortex. A–B are supragranular pyramidal neurons; C provides a higher magnification image of a spiny dendrite in a pyramidal neuron; D is a spiny multipolar cell; E are inverted pyramidal cells; and F–G are bitufted cells. Pial surface is toward the top of the image. Scale bars: A–B, D–E, G = 50 μm; C, F = 25 μm.**

elliptical orientation. In contrast to bitufted neurons, these cells possessed either a single or a bifurcating apical dendrite that ascended toward the pial surface.

Bipolar neurons. Two bipolar neurons were traced in layer III of both frontal and occipital cortex. These cells exhibited a fusiform soma, with two main processes departing vertically from each pole. Typically, the descending process possessed a few, sparsely spined collaterals.

Nonpyramidal multipolar neurons. Nine nonpyramidal multipolar neurons (spiny: $n = 8$; aspiny: $n = 1$) were traced, mainly located in the infragranular layers of both frontal and occipital cortex. Their somata were either elliptic, round, or quadrangular. In all cases, dendrites emerged from different sides of the soma and spread in all directions, forming a spherical dendritic arbor. Similar morphologies characterized both the spiny and aspiny types.

Discussion

The present study provides a morphological characterization of different neuronal types in the rock hyrax neocortex, with a focus on regional differences in supragranular pyramidal neurons between frontal and occipital areas. By adopting the same methodology used in a recent Golgi impregnation study of the African elephant neocortex,[26] our analyses provide the opportunity to compare neuronal morphology between two closely related afrotherian species that differ significantly in brain size, body size, ecological niche, reproductive strategies, and general behavior.

Neuronal morphologies

Cell types in the neocortex include spiny and smooth or sparsely spiny neurons. In primates, the predominant (70–90%) type of spiny neuron is the pyramidal cell,[5] which is typically defined by a triangular-shaped cell body, a wide "skirt" of basilar dendrites, one axon descending into the underlying white matter, and one single apical dendrite ascending vertically toward the pial surface. However, as more comparative studies accumulate, it has become apparent that such a structure constitutes one end of a broad scope of morphologies, spanning from "typical" pyramidal to stellate neurons.[5,18]

An illustration of such a continuum has been provided in a recent examination of neuromorphology in the African elephant neocortex. In the elephant, there is a remarkable heterogeneity of spiny neurons[27] (Figs. 4 and 5), from those appearing more pyramid-like (e.g., magnopyramidal, multiapical, and fork neurons), to those radically differing from the "typical" pyramidal shape (e.g., horizontal and inverted pyramidal neurons; "crab-like" and flattened pyramidal neurons). In the rock hyrax, a spectrum of spiny neuron morphologies was also

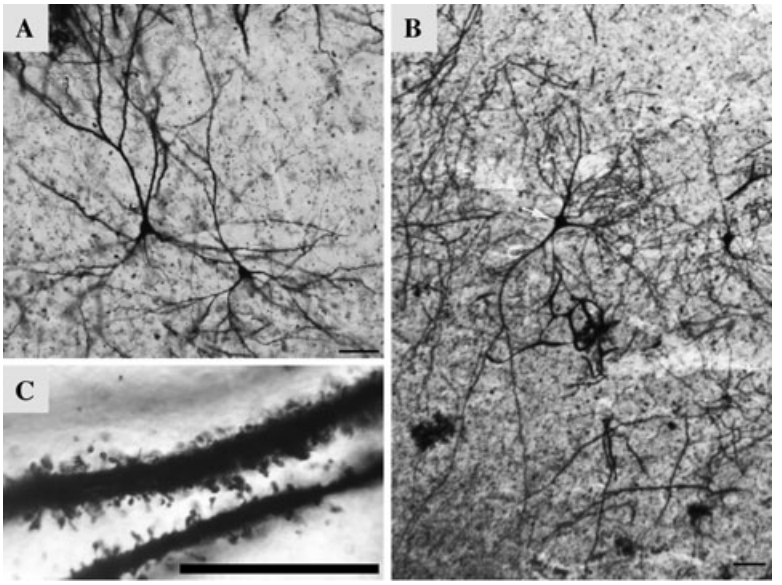

Figure 4. **Photomicrographs of two supragranular pyramidal neurons from the African elephant frontal cortex (A), a layer III/V inverted pyramidal neuron (arrowhead) from frontal cortex (B), and a high magnification view of a spine-rich apical dendrite from occipital cortex (C). Pial surface is toward the top of the image. For A, scale bar = 100 μm. For B, scale bar = 200 μm. For C, scale bar = 50 μm.**

observed; however, fewer separate neuronal types were identified, including only pyramidal, forked shaped, inverted pyramidal, and bitufted neurons.

Of the pyramidal-like neurons, some presented "atypical" features (e.g., bifurcating apical dendrites, multiapical and inverted soma) that have been similarly observed in other Atlantogenata (i.e., the clade formed by Afrotheria and Xenarthra), such as elephant shrews, anteaters, and sloths,[18] as well as a monotreme, the short-beaked echidna.[10] The rock hyrax, nevertheless, was characterized by a predominance of "typical" pyramidal cells. Indeed, one main respect in which the rock hyrax differed from the elephant was the high frequency of pyramidal cells with "canonical" apical dendrites that ascended vertically toward the pial surface. In contrast, in the elephant, most spiny neurons had apical dendrites that bifurcated at or near the soma, resulting in two obliquely ascending secondary branches that joined with others to form V-shaped apical bundles (Fig. 5).

While being consistent with descriptions in the horse and the cow,[35] as well as the two-toed sloth and anteater,[18] the widely bifurcating structure observed in the elephant represents a striking departure from the vertically orientated apical dendrites that typify the rodent and primate neocortex.[36] Together with previous observations, this suggests that the notion of a neocortical architecture common to all mammals, defined by pyramidal-shaped projection neurons with apical dendrites bundled together at the core of minicolumns,[36] may not capture the actual variation that is present among different mammalian lineages. In fact, the observation of a greater frequency of "atypical" features in taxa close to the origin of the mammalian radiation has led to the suggestion that, while variation in projection neuron morphology characterizes most mammalian species, strong selection for vertically oriented apical dendrites might have begun to emerge with the evolution of Boreoeutheria (i.e., Euarchontoglires and Laurasiatheria).[18]

As shown by a recent reevaluation of the concept of the cortical column as elementary unit of the neocortex, however, the functional and evolutionary significance of a vertical neocortical organization remains unclear.[37] Thus, while it is interesting that the afrotherian rock hyrax exhibits high frequency of "typical" pyramidal features, further studies on species such as shrews, flying lemurs, lagomorphs, and megachiropterans will be needed to determine whether these features universally characterize Boreoeutheria as a phylogenetic group.

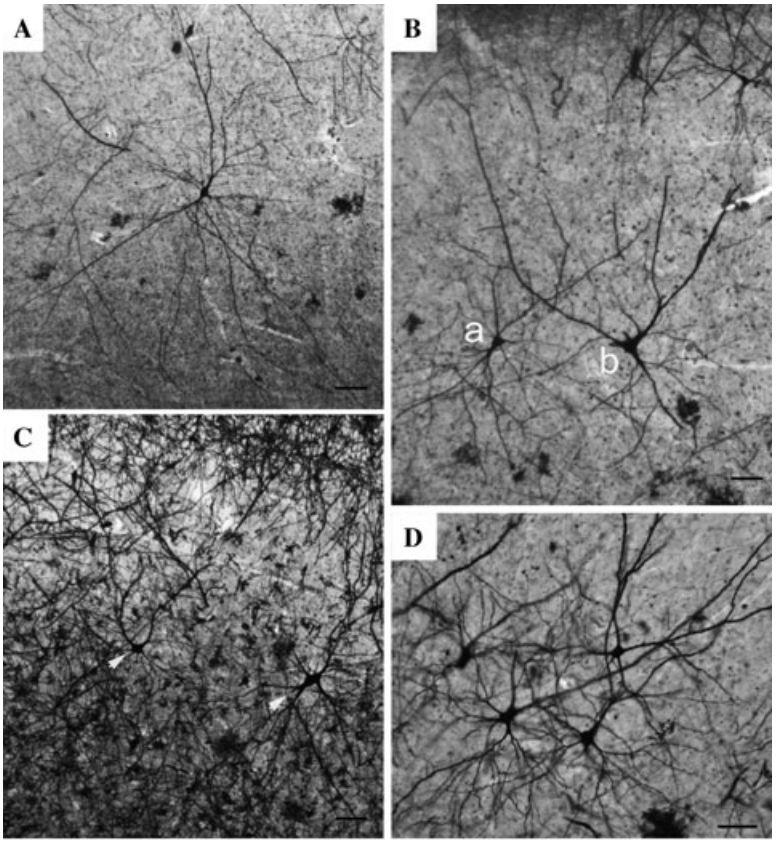

Figure 5. **Photomicrograph of neuronal morphologies in the African elephant. In A, a magnopyramidal-taproot or matriarch neuron, a large layer V neuron with a long descending taproot dendrite. An inverted pyramidal neuron (a) and a large, multiapical pyramidal neuron (b) from frontal cortex are represented in B. Two multiapical pyramidal neurons (arrowheads) from occipital cortex can be seen in C. In D, several supragranular pyramidal neurons from the frontal cortex are present, each with widely bifurcating apical dendrites projecting toward the pial surface. For A, B, C, scale bar = 200 μm. For D, scale bar = 100 μm.**

Greater similarity with other eutherian mammals was observed in the morphology of aspiny and sparsely spiny neurons, including those with multipolar, bitufted, and bipolar shapes. In terms of morphology and laminar distribution, these neurons resembled those described in monotremes,[10] humans, carnivores, artiodactyls, lagomorphs,[38] rodents,[39] dolphins,[40] and elephants.[26] Because aspiny cortical neurons generally correspond to inhibitory GABAergic subtypes, this suggests that there may be relatively more evolutionary conservation in regard to the morphology of cell types that comprise the intrinsic microcircuitry of the mammalian cerebral cortex[41] (however, see Ref. 5). Considerable phylogenetic diversity in the density and biochemical phenotype of these inhibitory interneurons, however, is apparent.[3,7,8,18]

Regional differences in supragranular pyramidal morphology

A comparison of regional differences in the morphology of supragranular pyramidal neurons in the rock hyrax revealed spinier dendrites in frontal compared to occipital cortex. This result is consistent with previous findings in the elephant[26] as well as several primate species,[5,29] including humans.[6] In contrast with these species, however, the rock hyrax showed no differences in the extent and complexity of dendritic arbors.

Spine density and dendritic branching patterns are essential in determining the receptive field and integrative capacity of the neuron.[42] In primates, regional variation in neuronal morphology is thought to reflect area-specific functional specializations.[29,30] Specifically, neurons with greater

dendritic complexity and higher spine density might subserve the synthesis of a more diverse array of inputs.[29] Thus, the higher spine density found in the frontal cortex of the rock hyrax provides indirect evidence that this region is involved in functions that entail greater computational demand. However, given the scarcity of data on the physiology and connectivity of the rock hyrax cerebral cortex, it is uncertain whether the functional significance of regional differences in spine density is comparable to that of primates. Moreover, our failure to find significant differences in other important aspects of the cell morphology indicates more similarity in neuronal structure across regions than has previously been reported in primates. The relatively small sample size used this study, however, may have prevented the observation of greater morphological variation. As such, our interpretation of structural and functional regional differences remains tentative. The present study contributes, however, to the growing body of research delineating neuromorphological variation in the neocortex of afrotherian species, and adds to our understanding of the evolution of neuronal diversity in mammals.

Acknowledgments

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Conflicts of interest

The authors declare no conflicts of interest.

Supporting Information

Additional supporting information may be found in the online version of this article:

Supplementary Figure 1. Low magnification photomicrograph of Golgi-impregnated neurons in the elephant occipital cortex. Note the wide lateral spread of the bifurcating apical dendrites in the large layer V neurons (arrowheads). Pial surface is toward the top of the image. Scale bar = 200 μm.

Supplementary Figure 2. Low magnification photomicrograph of Golgi-impregnated neurons in elephant frontal cortex illustrating several neuronal types: (A) supragranular pyramidal neurons with widely bifurcating apical branches, (BB) horizontal pyramidal neurons with sectioned apical dendrites, and (C) several inverted pyramidal neurons. Pial surface is toward the top of the image. Scale bar = 200 μm.

Supplementary Figure 3. Low magnification photomicrograph of Golgi-impregnated neurons in elephant frontal cortex illustrating several neuronal types: (A) a supragranular pyramidal neuron, (B) a magnopyramidal-taproot or matriarch neuron, and (C) a magnopyramidal neuron with a sectioned apical dendrite. Pial surface is toward the top of the image. Scale bar = 200 μm.

Supplementary Figure 4. Photomicrograph of a multipolar, Golgi-impregnated aspiny interneuron in elephant frontal cortex. Pial surface is toward the top of the image. Axon is highlighted by the arrowhead. Scale bar = 100 μm.

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