Gradients in cytoarchitectural landscapes of the isocortex: diprotodont marsupials in comparison to eutherian mammals

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Running title: Neuron numbers across the marsupial isocortex

Associate Editor: Kathleen Rockland

11 Figures, 3 tables

Key words: cortex, evolution, layer, marsupials, RRID:SCR_002526, RRID:SCR_001905

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Grant sponsor: The James S. McDonnell Foundation; Grant number: 22002078.

Abstract

Although it has been claimed that marsupials possess a lower density of isocortical neurons compared with other mammals, little is known about cross-cortical variation in neuron distributions in this diverse taxonomic group. We quantified upper layer (layers II-IV) and lower layer (layers V-VI) neuron numbers per unit of cortical surface area in three diprotodont marsupial species (two macropodiformes, the red kangaroo and the parma wallaby, and a vombatiform, the koala) and compared these results to eutherian mammals (e.g., xenarthrans, rodents, primates). In contrast to the notion that the marsupial isocortex contains a low density of neurons, we found that neuron numbers per unit of cortical surface area in several marsupial species overlap with those found in eutherian mammals. Furthermore, neuron numbers vary systematically across the isocortex of the marsupial mammals examined. Neuron numbers under a unit of cortical surface area are low towards the frontal cortex and high towards the caudo-medial (occipital) pole. Upper layer neurons (i.e., layers II-IV) account for most of the variation in neuron numbers across the isocortex. The variation in neuron numbers across the rostral to the caudal pole resembles primates. These findings suggest that diprotodont marsupials and eutherian mammals share a similar cortical architecture despite their distant evolutionary divergence.

Introduction

The isocortex of many mammals can be subdivided into six layers. Neurons across the depth of the isocortex can be further distinguished by their patterns of connectivity with neurons located in supragranular and granular layers (layers II-IV) predominantly connecting to other cortical structures whereas many lower layer neurons (layers V-VI) project to subcortical structures (Gilbert and Kelly, 1975; Barbas, 1986; Nudo and Masterton, 1985; Hof et al., 1995). During development, newly born neurons migrate from the proliferative zones to the cortical plate. Neurons born earlier in development migrate to assume locations within lower layers and neurons born later in development are positioned within upper layers (Rakic, 1974, 2002; Sanderson and Weller, 1990; Bayer and Altman, 1991). Consequently, neurons across the depth of the cortex and across the cortical surface are organized according to their birth order and connectivity patterns (Gaspard and Vanderhaeghen, 2011).

Several recent quantitative comparisons have been made to examine the evolution of upper and lower layer neuron numbers across mammals. This approach is useful in identifying evolutionary changes in distributions of neurons projecting cortically from those projecting subcortically. It is also useful in relating cross-cortical variation in neuron numbers with the developmental mechanisms that generate them (Finlay and Uchiyama, 2015; Cheung et al., 2010; Charvet et al., 2016b). Recent studies have shown that primates possess disproportionately more upper layer neurons than many other studied mammals, which suggests that primates possess increased numbers of corticocortically projecting neurons (O'Kusky and Colonnier, 1982; Charvet et al., 2015, 2016a,b).

There is extensive variation in neuron numbers across the isocortex of primates (Beaulieu, 1993; Beaulieu and Colonnier, 1989; Collins et al., 2010; Charvet al., 2015; Srinivasan et al., 2015). In primates, neuron numbers vary systematically across the isocortex with more neurons per unit of cortical surface area found towards the posterior pole (i.e., primary visual cortex) than towards the anterior pole (Collins et al., 2010; Cahalane et al., 2012; Collins et al., 2016; Turner et al., 2016). Most of this variation is

accounted for by upper layer neurons (Charvet et al., 2015). In other species examined to date, such as rodents, carnivores and manatees, the variation in neuron numbers per unit of cortical surface area is not as extensive as it is in primates (Beaulieu, 1993; Beaulieu and Colonnier, 1989; Charvet et al., 2015, 2016a,b). Yet, we still know very little about cross-cortical variation in neuron numbers in mammals other than rodents, primates, and a few other species.

Marsupials represent an interesting infraclass of mammals to investigate variation in cortical neuron distributions. Marsupials comprise a highly diverse taxonomic group in that they exhibit a wide range of adaptations (Karlen and Krubitzer, 2007) and they diverged from other mammals more than 125-140 million years ago. Consequently, they are distantly related to eutherian mammals (Bininda-Emonds et al., 2007; Luo et al., 2011). It has been claimed that marsupial mammals possess fewer isocortical neurons and few upper layer neurons per unit of cortical surface area compared to eutherian mammals. This notion primarily derives from a handful of studies that almost exclusively focused on the gray short-tailed opossum (e.g., *Monodelphis domestica,* Haug, 1987; Cheung et al., 2010; Seelke et al., 2013, 2014). As such, how neuron densities vary across this diverse taxonomic group remains poorly understood.

In the present study, we quantified neuron numbers per unit of cortical surface area in upper (layers II-IV) and lower layers (layers V-VI) systematically across the rostral to caudal and medial to lateral axes of the isocortex of several diprotodont marsupial species, including red kangaroo (*Macropus rufus*), parma wallaby (*Macropus parma*), and koala (*Phascolarctos cinereus*). We combined layers II, III, and IV as "upper layers" because layer IV can be rather difficult to define reliably across the isocortex of marsupials. We compared these values to those observed in some eutherian mammals (i.e., mouse, sloths, anteaters, chimpanzees). These eutherian species were chosen to span a wide range of orders (i.e., xenarthrans, rodents, primates) and brain weights, some of which overlap with those of the studied marsupial mammals. We sought to identify to what extent marsupials, as a group, possess lower isocortical neuronal densities compared with eutherian mammals as has been previously claimed. We also sought to compare cross-cortical variation in marsupials with that of eutherian mammals.

We used the optical disector method to quantify neuron densities systematically across the rostro-caudal and medial to lateral axes of the isocortex of kangaroo, wallaby, and koala. Our analysis departs from the traditional approach of investigating neuron numbers in select cortical areas (O'Kusky and Colonnier, 1989; Naumann et al., 2012; Young et al., 2013). Data from electrophysiological recordings of primary cortical areas are sparse or non-existent in these taxa, rendering parcellation and comparative analyses of cortical areas difficult. Rather, we adopted a more systematic cross-cortical approach in which we focus on variation in neuron numbers per mm^2 of cortical surface area across the isocortex and relate the variation in neuron numbers to the developmental mechanisms that serve to produce them. In the marsupials we examined, we found that upper and lower layer neurons per mm^2 of cortical surface area fall within the range of those observed in eutherian mammals. We also found that neuron numbers per unit of cortical surface area are higher in the caudo-medial pole of the isocortex than in many other regions. Most of the variation in neuron numbers per mm^2 of cortical surface is accounted for by superficially located neurons (layers II-IV neurons) as has been observed in primates (Charvet et al., 2015, 2016b).

Materials and Methods

Specimens

The brains used for the present study were collected opportunistically at necropsy, mostly from animals that had lived in zoos (Tables 1, 2). The brains of one red kangaroo, one wallaby, and one koala were used to assess cross-cortical variation in neuron numbers under a unit of cortical surface area (Figs. 1-3). Additional brains were collected to assess cross-cortical variation in neuron numbers between eutherian and marsupial mammals. Table 1 and 2 list information about the specimens used in the current study. These brains were immersion-fixed in 4% paraformaldehyde and stored in phosphate-buffered saline (PBS) with 0.1% sodium azide at 4° C. The postmortem interval prior to brain fixation did not exceed 14 hours for any specimen. The parma wallaby was 1 year and 3 months old and the red kangaroo was 1 year and 5 months old. The age of the koala

is unknown. The kangaroo brain weighed approximately 46 g, whereas the parma wallaby and koala brain weighed 19 and 12 g, respectively. Brain weights vary by a factor of more than two in the present study.

Tissue processing

Brains were immersed in a graded series of sucrose solutions for cryoprotection and sectioned coronally on a freezing sliding microtome at a thickness of 40-50 µm (Table 1). The brain of the koala was embedded in gelatin prior to sectioning. Serial sections were Nissl-stained with a solution of 0.5% cresyl violet. The sections were subsequently dehydrated and coverslipped.

We quantified neuron numbers under a unit of cortical surface area from these coronally sectioned Nissl-stained sections. We defined neurons as having a pale cytoplasmic soma and a dense nucleus in comparison with to glial cells, which are smaller and circular (Fig. 4). We applied this same definition across the cortex and across species. Endothelial cells can be distinguished from neuronal or glial cells by their elongate shape in contrast to neurons and glial cells, which are either circular or pyramidal. These definitions follow that of previous studies that utilized Nissl-stained section to quantify neuron numbers (Sherwood et al., 2006; Charvet et al., 2015, 2016a,b).

Neuronal quantification and statistics

We quantified neuron numbers under a unit of cortical surface area by systematically sampling sites across the rostral to caudal and medial to lateral axes of the cortex (Figs. 1, 3). To do this, we measured the cortical surface area of coronally sectioned brains and subdivided the cortical surface area into percentiles. The cortical surface length includes a number of cortical regions (e.g., motor cortex, primary visual cortex, cingulate cortex, insula; Ashwell et al., 2005). At every 20th percentile, we sampled sites orthogonal to the cortical surface area. An additional sampling site was applied between the medial and lateral surfaces of the cortex to better visualize the pattern of variation in neurons per unit of cortical surface area across the isocortex (Fig.

3). In total, we selected 5 sampling sites per coronal section and used 6 or 7 equidistantly spaced sections. Thus, for each specimen, 30 to 35 sites were sampled across the isocortex with a total of 688 to 1403 neurons counted depending on the specimen (Table 1). We applied counting frames (40 x 40 μm) at regular intervals (100 μm apart) along the vertical tangent perpendicular to the cortical surface (Fig. 3). Counting frames spanned the boundary between layers I and II to the boundary between layer VI and the white matter. If layer IV was clearly present, the boundary between layer IV and V was defined as an abrupt change in cell size and density. If layer IV was not clearly observed, the presence of large cells in layer V was used to define the boundary between upper and lower layers. If the counting frame lay intermediate between two layers, it was included within a given layer depending on where the counting frame preferentially laid. If the region that was selected lay within an area of high curvature, we sampled an immediately adjacent region with less curvature.

In this study we focused on variation in layers II-IV versus layers V-VI. We did not specifically examine variation in layer IV across the isocortex for several reasons. The distinction between layer III and IV was not unambiguously evident across cortical regions in the species we examined. Moreover, cytoarchitecture alone may not be sufficient to define layer IV neurons (García-Cabezas and Barbas 2014; Yamawaki et al. 2014; Barbas and García-Cabezas 2015). For these reasons, we combine layers II-IV to layers V-VI in our analyses.

To reconstruct neuron numbers per unit of cortical surface area, we used the following equation:

Total neuron numbers under a unit of cortical surface area = total neurons counted x 1/hsf x 1/asf,

where hsf is the height of the counting frame/tissue thickness and asf the area between counting frame/area of the counting frame (Williams and Rakic, 1988; Williams et al., 2003). Because tissue shrinkage may vary across the cortex, we estimated the tissue thickness throughout the depth of the cortex for each sampled site empirically during counting (Carlo and Stevens, 2011). Quantification of neurons under a unit of cortical

surface area were performed with a computerized stereology system consisting of a Zeiss Axioplan 2 photomicroscope equipped with a Ludl XY motorized stage, Heidenhain z-axis encoder, an Optronics MicroFire color videocamera, a Dell PC workstation, and StereoInvestigator software version 10 (MBF Bioscience, Williston, VT, USA; RRID:SCR_002526).

A linear model was used to test whether neuron numbers per mm^2 of cortical surface area vary significantly across the axes of the cortex. More specifically, we tested whether the rostral to caudal and medial to lateral axes as well as the interaction between the rostral to caudal and medial to lateral axes of the cortex account for significant variance in neuron numbers. Statistical analyses were performed with the R programming language (RRID:SCR_001905) with the function (lm).

Cortical curvature

Estimates of neuron numbers under a unit of cortical surface area may be biased by the curvature of the cortex. To address this potential limitation, we used MRI scans of kangaroo, koala, and red-neck wallaby brains. Structural MRI scans were available from the koala brain (1.5 T, SE sequence, TR = 12.54, TE = 12.53, flip angle = 90°; Brucker scanner) and kangaroo brain (3 T, GR sequence, TR = 13.37, TE = 6.47, flip angle = 8°; Philips scanner) that were used in the present study. A Structural MRI of a red-neck wallaby was obtained from the website www.braincatalogue.org. In our inspection of the MRI scans of marsupial brains (e.g., kangaroos, wallabies, koalas), we noted that rostral and caudal regions of the cortex exhibit high curvature in all examined species (Figs. 1, 3). The rostro-lateral regions of the cortex in kangaroos and wallabies likewise exhibit high curvature although this was less evident in the lissencephalic koala brain (Figs. 1-3). Therefore, estimates of neuron numbers under a unit of cortical surface area are prone to being overestimated in very rostral and rostro-lateral regions of the cortex (Fig. 3).

To address this potential limitation, we avoided regions with high curvature where possible. This was achieved by comparing our Nissl-stained sections with equivalent locations in the structural MRIs. Because the aim of the present study was to examine variation in neuron numbers across the isocortex, we selected some regions in which the

depth of the cortex was not fully orthogonal to the surface of the cortex. To investigate the extent to which the curvature might have overestimated neuron numbers across the rostral to caudal axis, we compared the estimates of the thickness (depth) of the cortex from Nissl-stained sections to those obtained from the structural MRI. We used the structural MRIs to measure the cortical depth in the plane in which the brain was cut histologically. We also measured the cortical depth in the plane orthogonal to the cortical surface. The correction factor consists of the ratio between the two measurements. We used the corrected cortical thickness estimates when reconstructing neuron numbers per mm^2 of cortical surface area. Correction for curvature was generally highest towards the rostral pole.

A three-dimensional model of the koala brain shown in Figure 1 was obtained from the structural MRI scan using automatic segmentation tools in ITK-Snap v. 3.4.0 (Yushkevich et al., 2006). This process consists of thresholding the scans, placing some spherical seeds, and letting these seeds evolve to fill the thresholded region (Yushkevich et al., 2006), which in this case corresponded to the complete brain. The 3D model was subsequently smoothed in MeshLab (Cignoni and Ranzuglia, 2005). Sampling points were identified on 3D surface models using IDAV Landmark Editor (Wiley et al., 2005), and later represented together with brain models in *Mathematica* v. 10 (Wolfram Research). Only sampled sites from the lateral cortex are shown.

Marsupials in comparison to eutherian mammals

To investigate how upper and lower layer neuron densities of diprodont marsupials compare to those observed in eutherian mammals, we quantified neuron numbers per unit of surface area within the caudo-medial pole, corresponding to the likely position of primary visual cortex, in one mouse (wild-type), one two-toed sloth, one lesser anteater, one chimpanzee, as well as three additional marsupials (a swamp wallaby, a parma wallaby, a koala). We selected this region because cortical curvature is minimal in this location. Table 2 lists tissue thickness, brain weights, age, and sex of the examined species. We also include data from previous studies that report upper and lower layer neuron numbers under 1 mm^2 of cortical surface area for the rat (*Rattus*

norvegicus, Beaulieu, 1993), the cat (*Felis catus*, Beaulieu and Colonnier, 1989), as well as the macaque (*Macaca mulatta*, O'Kusky and Colonnier, 1982). Brain weights for the rat, cat, and a macaque were taken from Charvet et al. (2016b).

Although the selected regions may not be fully homologous across species, this analysis suffices to determine whether the range of upper and lower layer neuron numbers per unit of cortical surface area overlap with those observed in marsupial mammals. We selected regions with a well-defined layer IV when possible to identify the location of the presumptive primary visual cortex as had been done previously for some of the selected species (Sherwood et al., 2009). We followed the same protocol to quantify neuron numbers as that described above with a few exceptions.

The mouse brain was sectioned at 30 µm. We therefore applied an optical disector height of 6 rather than 8 µm due to tissue shrinkage. The cortical thickness is very small in mice (less than 1 mm in the caudo-medial pole) compared to the examined marsupial mammals, which on average ranged between 1.9 to 2.6 mm, as sampled across the cortex (Table 1). We therefore sampled sites across the cortical depth more densely. We sampled counting frames at 50 µm intervals in the mouse, sloth, and anteater rather than 100 µm as was done for the marsupial mammals. This yielded an average of 14 counting frames through the cortical depth in mice (e.g., 8 for upper layers and 6 for lower layers), which is less than those obtained for marsupial mammals (e.g., average 25 counting frames through the cortical thickness of the kangaroo).

To identify whether the distance between counting frames was of sufficient resolution to capture the number of neurons in upper and lower layers, we quantified neuron numbers exhaustively in continuous steps across the whole cortical thickness in the mouse primary visual cortex and compared those estimates to similar regions sampled with counting frames. We applied counting frames of 40 µm in width orthogonal to the cortical surface and quantified neuron numbers across the entire depth of the cortex (layers II-VI). Such an analysis shows that the two approaches yield very similar values. Neuron numbers (layers II-VI neurons) sampled throughout the 'cortical column' ($\bar{x}$ = 122,654; SEM = 8,271; n = 4) yielded very similar values to those sampled with counting frames ($\bar{x}$ = 129,987; SEM = 11,044; n = 4). Upper layer neuron numbers sampled throughout the cortical depth ($\bar{x}$ = 60,105; SEM = 7,102; n = 4) yielded similar values to

those sampled with counting frames ($\bar{x}$ = 75,502; SEM = 4,464, n = 4). Finally, lower layer neuron numbers sampled throughout the cortical column ($\bar{x}$ = 62,549; SEM = 7,225; n = 4) yielded similar values to those obtained with counting frames ($\bar{x}$ = 54,485; SEM = 7,224 n = 4). Neuron number per mm^2 of cortical surface area reported in the present study are comparable, although higher, to those in previous studies, which report approximately 90,000 to 100,000 neurons under 1 mm^2 of cortical surface area in the mouse primary visual cortex (Herculano-Houzel et al., 2013; Srinivasan et al., 2015).

Results

Neuron numbers per unit of cortical surface area

We systematically sampled neurons under a unit of cortical surface area across the rostro-caudal and medial to lateral axes of three marsupial species (Figs. 1, 3). Layers II-VI neurons per mm^2 of cortical surface area across the isocortex averaged $73x10^3$ in the koala, $144x10^3$ in the parma wallaby, and $91x10^3$ neurons in the red kangaroo. Cortical thickness does not significantly co-vary with neuron numbers under $1mm^2$ of cortical surface area in the koala (adj R^2= 0.01; F=1.23; p=0.27), but cortical thickness significantly co-varies with neuron numbers under $1mm^2$ in the wallaby (adjR^2= 0.56; F= 38.08; p=1.157e-06), as well as in the kangaroo (adj R2= 0.51; F=35.82; p=1.009e-06; Fig. 5).

We examined the range of variation between sampled sites with the average 5 highest and average 5 lowest values in neuron numbers in each of the examined specimens. Layers II-VI neurons per mm^2 of cortical surface area ranged between $41x10^3$ to $112x10^3$ in the koala, between $87x10^3$ to $233x10^3$ in the parma wallaby, and between $45x10^3$ to $145x10^3$ in the kangaroo. That is, neuron numbers per mm^2 of cortical surface vary by a factor of 2.7, 2.7, and 3.2 across the koala, parma wallaby, and kangaroo cortex, respectively. There is extensive variation in neuron numbers per unit of cortical surface area across the isocortex (Table 3).

To appreciate the spatial variation in neurons per mm^2 of cortical surface area, we plotted total neuron numbers, upper layer neuron numbers, lower layer neuron numbers

per a unit of cortical surface area as well as the percentage of upper layer neuron numbers relative to total neurons under 1 mm^2 of cortical surface area across the rostral to caudal and medial to lateral axes as variables in the kangaroo (Fig. 6), parma wallaby (Fig. 7), and koala (Fig. 8). Neurons per mm^2 of cortical surface area are generally highest towards the caudo-medial pole of the isocortex, which corresponds to the presumptive primary visual cortex as assessed with Nissl-stained sections (Fig. 6-8). We did, however, find a few exceptions to these overall trends. Elevated neuron numbers per mm^2 of cortical surface area were also observed in the rostral-lateral cortex in the kangaroo and the koala (Fig. 7, 8). The rostral to caudal and medial to lateral axes account for a larger percentage of the variation in the kangaroo (49%) and parma wallaby (56%) than in the koala (30%; Table 3) with the greatest increase in neuron numbers per unit of cortical surface area located towards the caudo-medial pole.

Upper and lower layer neuron numbers per unit of cortical surface area

Similar to what we observed for total layer II-VI neurons, upper layer (layers II-IV) neuron numbers are generally greater towards the occipito-medial pole of the lateral cortex and values are generally low towards lateral and rostral regions of the cortex (Figs. 5-7). We also observed increased upper layer neurons in a few rostro-lateral regions in the kangaroo and koala. We examined the range of variation in upper layer neuron number per unit of cortical surface area by comparing the sampled sites with the five highest values and five lowest values for upper layer neurons. Upper layer neurons per mm^2 of cortical surface area ranged between $18x10^3$ to $63x10^3$ in the koala, $43x10^3$ to $170x10^3$ in the parma wallaby, and $20x10^3$ to $102x10^3$ in the kangaroo. The rostral to caudal and medial to lateral axes account for a significant amount of variation in upper layers neuron numbers in the kangaroo (51%) and parma wallaby (56%) but less in koala (29%; Table 3). The variation in upper layers neuron numbers per mm^2 of cortical surface area mirrors the variation in layer II-VI neurons: the highest number of upper layer neurons per mm^2 of cortical surface area are found in the caudo-medial pole (Fig. 6-8).

Neuron numbers in layers V-VI do not exhibit the pattern of variation observed for total layers II-IV neurons (Figs. 5C, 6C, 7C). Whereas the rostral to caudal and

medial to lateral axes account for a large percentage of variation in layers II-IV neurons, there is little variation in lower layers neuron densities across the cortex (Table 3). The rostral to caudal and medial to lateral axes account for 15-33% of the variation in lower layer neuron numbers per unit of cortical surface area, which is not statistically significant in the kangaroo and koala (Table 3).

Marsupial mammals in comparison to eutherian mammals

In order to compare neuron numbers per unit of cortical surface area between marsupials and eutherian mammals, we quantified neuron numbers per unit of cortical surface area within the caudo-medial pole in select eutherian (i.e., lesser anteater, two-toed sloth, mouse, chimpanzee) and marsupial species (i.e., swamp wallaby, parma wallabies, koalas, kangaroo; Fig. 9). We observed that Macropodiformes (wallabies, kangaroo) possess a well-defined layer IV, as defined cytoarchitecturally, but that anteaters, sloth, and koalas do not possess a well-defined layers IV. The layer IV of marsupial mammals is similar to that observed in primates (Fig. 9). We compare upper and lower layer neuron numbers within the presumptive visual cortex across marsupial and eutherian mammals.

Neuron numbers, including upper and lower layer neurons per mm^2 of cortical surface area in marsupial mammals fall well within the range of several eutherian mammal species (e.g., anteater, sloths, rat, cat; Fig. 10). We found that some species such as sloths and the lesser anteater, actually exhibit fewer neuron numbers per mm^2 of cortical surface area than those observed in marsupial species (Fig. 10A). Neurons per mm^2 of cortical surface area averaged 69,000 in the sloth and 47,000 in the lesser anteater and these values are well within the range of cross-cortical variation in neuron numbers observed for the examined marsupial mammals. Neuron numbers per mm^2 of cortical surface area average 122,286 per mm^2 of cortical surface area across the studied marsupial species but only 88,884 per mm^2 of cortical surface area across the studied non-primate mammals. As is evident in Figure 9A, neuron numbers per unit of cortical surface area of marsupial species overlap with those of eutherian mammals (Fig. 10A).

Upper and lower layer neuron numbers of marsupials likewise fall well within the range of the examined eutherian mammals (Fig. 10B). For instance, upper layer neurons average 37,000 in the sloth whereas they average 83,000 neurons per mm^2 of cortical surface area in the swamp wallaby. The number of upper layer neurons in most of the marsupial species examined is greater than those of the examined eutherian mammals (Fig. 10B). Upper layer neurons per mm^2 of cortical surface area average 85,385 in marsupial mammals but only 53,292 in non-primate mammals. Upper layer neurons of marsupial mammals fall between those of eutherian non-primate mammals and those of primates. Taken together, these findings demonstrate that the examined marsupial species do not possess fewer neurons compared to eutherian mammals.

Discussion

We found considerable variation in neuron numbers per unit of cortical surface area across the rostro-caudal and medial to lateral axes of the cortex in kangaroo, wallaby, and koala with more neurons located towards the caudo-medial pole of the isocortex. Most of the variation in neuron numbers per mm^2 of cortical surface area is accounted for by layer II-IV neurons. These findings have implications for understanding the evolution of cortical neuron numbers, patterns of connectivity and the developmental mechanisms generating evolutionary changes in neuron numbers across the cortex.

Comparative analyses of neurons per unit of cortical surface area

Previous studies that have almost exclusively focused on the gray short-tailed opossum (*Monodelphis domestica*, order Didelphimorphia) concluded that the isocortex of marsupials contains relatively few neurons under a unit of surface area (Haug, 1987; Cheung et al., 2010; Seelke et al., 2014). For instance, Haug 1987 found that the short-tailed opossum contains less than 40,000 neurons per mm^2 of cortical surface area. In this study, we broadened the comparative analysis to consider cross-cortical variation in neuron numbers in a wider range of marsupial mammals. We found that the number of neurons per mm^2 of cortical surface area in marsupials belonging to the order

Diprotodontia fell well within the range of those reported for eutherian mammals (Beaulieu, 1993; Beaulieu and Colonnier, 1989; Collins et al., 2011; Cahalane et al., 2012; Herculano-Houzel et al., 2013; Charvet et al., 2016a). It is important to keep in mind that the species available to the present study represent only one order of marsupials, the diprotodonts, and likely do not fully capture the whole range of diversity across marsupial mammals. Therefore, whether isocortical neuron numbers per unit of cortical surface area increased within the lineage leading to kangaroos, wallabies, and koalas independently within marsupials or whether the low density of cortical neurons reported in *Monodelphis* (Seelke et al., 2013, 2014) actually represent the derived condition remains to be investigated in a broader sample of marsupials.

Cross-cortical variation in upper and lower layer neurons

We found that the variation in overall neuron numbers per unit of cortical surface area is mostly accounted for by upper layer neurons. This is similar to what is observed in primates where increases in neuron numbers per unit of cortical surface area towards the posterior pole are likewise mostly accounted for by upper layer neurons (Charvet et al., 2015). Whether eutherian mammals other than primates also possess these features is still largely unknown. The expansion of upper layer neuron numbers is accompanied by a well-defined layer IV, which is evident in the macropodiformes (e.g., wallabies, kangaroos) and in primates (Figs. 3, 8). A distinct layer IV, defined cytoarchitecturally, however is not shared among all mammals. It is entirely absent in some species such as cetaceans and hippopotamids (Hof et al., 2005; Butti et al., 2014) and is uncertain in anteaters and sloths (Fig. 9). Thus, some marsupial species resemble primates in that neuron numbers and upper layer neuron numbers steadily increase towards the posterior pole of the isocortex. Some marsupial species also resemble primates in possessing a well-defined layer IV in the presumptive primary visual cortex. These observations suggest that the primary visual cortex of macropodiformes and primates may have independently evolved similar cytoarchitectural traits.

Comparative analyses of variation in neuron numbers across the isocortex

In primates, as in the diprotodont marsupial mammals examined in our study, neuron numbers under a unit of cortical surface area are higher towards the posterior or caudal regions of the isocortex (Collins et al., 2010; Charvet et al., 2015). Notably, however, there are differences in the pattern of cross-cortical variation in neuron densities between primates and marsupial mammals. Increases in neuron numbers per unit of cortical surface area occur extensively across the posterior pole in primates (Collins et al., 2010; Cahalane et al., 2012; Charvet et al., 2015). This is in contrast to what we observed in kangaroos, wallabies, and koalas, where neuron densities are high in a more restricted location towards the caudo-medial pole of the isocortex but are clearly lower in the occipital lateral cortex. The primary visual cortex of marsupial mammals (as in many placental mammals) is located within the occipito-medial pole (Haight et al., 1980; Tyler et al., 1998; Kahn et al., 2000; Seelke et al., 2014; Karlen and Krubitzer, 2007). This is in contrast to primates where the primary visual cortex extends over the caudal pole and along the calcarine sulcus. In other words, evolutionary changes in the location of the primary visual cortex coincide with evolutionary changes in the spatial variation in neuron numbers per unit of cortical surface area.

The evolutionary changes in the location of peak neuron numbers under a unit of cortical surface area observed between primates and marsupial mammals are mirrored in differences in the pattern of neurogenesis duration (Rakic, 1974, 2002; Sanderson and Weller, 1990; Bayer and Altman, 1991). In primates, neurogenesis is prolonged in the occipital cortex (i.e., the primary visual cortex) and extends across the occipital pole (Rakic, 2002). This is in contrast to what has been reported for non-primate species (e.g., rat, brush-tailed possum; Sanderson and Weller, 1990, Bayer and Altman. 1991) for which the duration of neurogenesis is most protracted in the medial regions of the occipital pole (Fig. 11). Variation in neuron numbers per unit of cortical surface area coincides with variation in neurogenesis duration in a number of these taxa. One exception is the South American short-tailed opossum where protracted neurogenesis towards the caudo-medial pole of the cortex is not associated with greater neuron numbers per unit of cortical surface area in adulthood (Seelke et al., 2014). Despite this exception, we hypothesize that the rostral to caudal gradient in neurogenesis timing

during development is an important source of evolutionary changes in neuron numbers across the cortex and is evident in the cortical cytoarchitecture of the adult brain.

In the kangaroo and in the koala, we found that some sampled sites within the rostro-lateral cortex also possess elevated neuron densities. We hypothesize that this region lies within the primary somatosensory cortex. Elevated neuron densities have been described in the primary somatosensory cortex of the opossum (Seelke et al., 2014) as well as in several eutherian mammals (e.g., primates, mice; Collins et al., 2010; Cahalane et al., 2012; Herculano-Houzel et al., 2013). The increase in neuron density observed in the mid-rostral cortex may therefore correspond to the primary somatosensory cortex. However, electrophysiological recordings have not been carried out in these less well-studied species, making it difficult to conclusively identify whether regions with elevated neuron density correspond to the primary somatosensory cortex.

The elevated neuron numbers under a unit of cortical surface area observed in the rostral cortex are not directly in line with the predictions generated from the gradients of neurogenesis duration reported for non-primate mammals (Rakic, 1974, 2002; Bayer and Altman, 1991; Sanderson and Weller, 1990). Other factors such as differential cell death across the cortex may sculpt variation in neuron numbers (Finlay and Slattery, 1983).

In conclusion, our data disprove the notion that marsupials possess fewer upper and lower layer neuron numbers per unit of cortical surface area in comparison to eutherian mammals. Our data show that there is cross-cortical variation in neuron numbers per mm^2 of cortical surface area with more neurons located towards the caudo-medial pole of the cortex. Most of this variation is accounted for by upper layer neurons rather than lower layer neurons. As such, macropodiformes possess a number of characteristic features of isocortical organization that resemble primates.

Conflicts of interest:

The authors declare no conflicts of interest

Data accessibility

Data on upper layer lower layer neuron numbers of all species studied in the present manuscript are available on the Dryad website

Authors contributions to the work:

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: CJC, FMK; Acquisition of data: CJC, CDS, YDK, MAR; Analysis and interpretation of data: CJC, AGR; Drafting of the manuscript: CJC; Statistical analysis: CJC; Obtained funding: CCS, PRH; Critical revision of the manuscript for important intellectual content: FMK, PRH, CCS. Administrative, technical, and material support: MAR, AHL, PRH, AGR. All authors critically read and contributed to the writing of the manuscript.

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Figure legends

Figure 1. Dorsal (A) and lateral view (B) of the three-dimensional model of the koala brain. Regions that were selected for neuron number counts are shown with points superimposed on the model. Regions selected through the medial cortex are not shown. Only regions selected for the lateral cortex are shown here. Scale bar=1cm.

Figure 2. Images of whole brains of marsupial species used in the present study and the phylogeny of these species in relation to monotremes and eutherian mammals. Within marsupials, the gray short-tailed opossum is basal to wallabies, kangaroos, and koalas. The phylogeny follows that of Bininda-Emonds et al. (2007). Scale bar = 1 cm.

Figure 3. (A) Structural MRI of a red kangaroo brain, from which 3 coronal sections (B-D) are shown. Filled arrowheads point to the locations sampled for neuron counts. Letters E-I in panel C show the locations of higher power photomontages in panels E-I. Neurons per unit of cortical surface area are relatively few in the medial cortex, (E) numerous in medial regions of the lateral cortex (F-G) and few in lateral regions of the cortex (H-I). Notably, a well-defined layer IV is evident within the caudo-medial pole of the isocortex of the kangaroo. We estimated neuron numbers under the cortical surface by applying equidistant counting frames across the depth of the cortex as shown in F. Scale bar = 1 mm (C), and 0.5 mm (C-G).

Figure 4. Counting frames at high resolution were made across the depth of the isocortex. Neurons were distinguished from non-neuronal cells by the presence of a dense nucleus, and a less cell dense cytoplasm. This definition was applied systematically

across the cortex and across species. Representative regions through the caudo-medial pole of the kangaroo isocortex (A) and the isocortex of the lesser anteater (B) are shown. Black arrowheads point to what we defined as a neuron. White arrowheads point to what we defined as a non-neuronal cell. Only cells in focus are highlighted with arrows. Scale bar = 10 µm.

Figure 5. Cortical thickness co-varies with neuron numbers under 1mm^2 of cortical surface area in the parma wallaby, as well as in the kangaroo, but not in the koala. Cortical thickness is in µm.

Figure 6. In the kangaroo, neurons under 1 mm^2 of cortical surface area vary across the cortex. Layers II-VI neuron numbers (A) as well as upper layer neurons (B) steadily increase from the rostral to caudal pole of the cortex in the kangaroo. In contrast, lower layer neurons (D) area are relatively invariant across the isocortex. The percentage of upper layer neurons under a unit of cortical surface area are plotted across the isocortex and occupy 23% to 78% of layers II-VI per unit of cortical surface area. Across the medial to lateral axes (ML), values represent the distance of the samples sites from the medial/lateral cortex boundary to the sampled site along the cortical surface. Samples were selected within the lateral cortex and medial cortex. Distances selected within the medial cortex were multiplied by -1.

Figure 7. In the wallaby, layers II-VI neuron numbers per mm^2 of cortical surface (A) area and upper layer neurons (B) increase from the rostral to the caudal pole of the isocortex. In contrast, lower layer neuron numbers (C) vary relatively little across the isocortex in the wallaby. (D) The percentage of upper layer neurons under a unit of cortical surface area occupy 36% to 87% of layers II-VI neurons under a unit of cortical surface area. The x-y coordinates are as in Figure 6.

Figure 8. In the koala, layer II-VI neuron numbers (A), and upper layer neurons (B) slightly increase slightly towards the caudal pole of the isocortex. Lower layer neurons

are relatively invariant across the isocortex. (D) The percentage of upper layer neuron numbers range between 27% to 73%. The x-y coordinates are as in Figure 6.

Figure 9 Nissl-stains of coronal sections through the presumptive primary visual cortex of the eutherian and marsupial mammals. Upper layer neurons occupy a large fraction of neurons under a unit of cortical surface area in the chimpanzee (A), the kangaroo (B), the parma wallaby (C), and the swamp wallaby (D). In contrast, upper layer neurons occupy a smaller fraction of neurons under a unit of cortical surface area in the koala (E), the anteater (F), the sloth (G), as well as the mouse (H). Scale bar: 500 μm.

Figure 10. (A) Neuron numbers under $1mm^2$ of cortical surface area are plotted against brain weight in marsupial species as well as eutherian mammals. Neurons number per unit of cortical surface area in marsupial species fall intermediate between those of primates and those of other eutherian mammals. (B) Upper layer neuron numbers under $1mm^2$ of cortical surface area of marsupial mammals fall within the range of eutherian mammals.

Figure 11. Previous studies that examined neurogenesis timing across the isocortex in a brush-tailed possum and a rat show that terminal neurogenesis is protracted in the caudo-medial pole of the isocortex in both of these species. The protracted duration of neurogenesis in the caudo-medial pole of the cortex coincides with increased number of neurons per unit of cortical surface area in the caudo-medial pole in marsupial mammals. The spatiotemporal pattern of neurogenesis timing observed in the brush-tailed possum coincides with variation in the number of layer II-VI neurons across the cortex in the kangaroo. Data on neurogenesis timing for the brush-tailed possum and rat are from Sanderson and Weller (1990), and Bayer and Altman (1991), respectively.

	Red kangaroo	Parma wallaby	Koala
Species name	*Macropus rufus*	*Macropus parma*	*Phascolarctos cinereus*
Sex	Female	Female	Unknown
Brain weight	46.0 g	18.8 g	12.4 g
Tissue thickness	40 µm	40 µm	50 µm
Optical disector height	8 µm	8 µm	8 µm
Average correction factor	94.1%	91.9%	94.5%
Number of sampling locations	35	30	30
Number of neurons counted	1212	1344	688
Average height of a ‘cortical column in mm (SD)	2.64 (0.42)	2.28 (0.42)	1.87 (0.27)

Table 1. Information about specimens used in the present analyses.

Species name	Common name	Sex	Age	Brain weight	Tissue thickness	Optical disector height	Samples	Neurons counted
Choloepus didactylus	Toe-toed Sloth	F	2 y	36 g	40 µm	8 µm	6	205
Macropus parma	Parma wallaby	F	1 y 3 m	18.8 g	40 µm	8 µm	6	348
Macropus parma	Parma wallaby	F	2y 2 m	17.4 g	40 µm	8 µm	6	277
Macropus rufus	Red kangaroo	F	1 y 5 m	46.0 g	40 µm	8 µm	6	251
Mus musculus	Mouse	M	?	0.6 g	30 µm	6 µm	4	257
Pan troglodytes	Chimp-anzee	F	41 y	327.8 g	40 µm	8 µm	6	255
Phascolarctos cinereus	Koala	?	?	?	50 µm	8 µm	5	115
Phascolarctos cinereus	Koala	?	?	?	40 µm	8 µm	2	70
Tamandua tetradactyla	Lesser Anteater	F	>6 y	30 g	40 µm	8 µm	6	194
Wallabia bicolor	Swamp wallaby	M	2 y, 1 m	32.7 g	40 µm	8 µm	6	208

Table 2. Information about specimens selected for cross-species comparisons.

Kangaroo	Variance explained	F statistics	Intercept value, (t-test)	RC slope value, (t-test)	ML slope value, (t-test)	RC*ML (t-test)
Total neurons	48.75	9.83*	51,219.58 (4.571)*	3,035.88 (4.597)*	597.89 (0.848)	-75.44 (-1.924)*
Upper layer neurons	50.78	10.66*	18,321.49 (2.051)*	2,694.18 (5.118)*	624.90 (1.112)	-64.76 (-2.073)*
Lower layer neurons	14.78	1.793	32,898.09 (6.134)*	341.70 (1.081)	-27.01 (-0.080)	-10.68 (-0.569)
Parma wallaby						
Total neurons	55.87	10.97*	52,359.60 (2.241)*	16545.90 (4.901)*	1082.90 (0.807)	-436.0 (-2.277)*
Upper layer neurons	55.32	10.73*	10,883.00 (0.570)	14,543.80 (5.271)*	1,621.80 (1.480)	-408.7 (0.0148)*
Lower layer neurons	33.18	4.304*	30,792.70 (2.326)*	4,372.40 (2.286)*	-172.00 (-0.226)	-113.8 (-1.049)*
Koala						
Total neurons	29.85	3.688	58,006.00 (3.519)*	1,652.1 (1.379)	3,035.4 (1.277)	-358.1 (-2.083)*
Upper layer neurons	28.92	3.527*	24,621.2 (2.432)*	1,361.0 (1.850)	2,817.3 (1.930)	-272.2 (-2.578)*
Lower layer neurons	18.31	1.942	33,384.80 (3.306)*	291.10 (0.397)	218.12 (0.150)	-85.93 (-0.816)

Table 3. Summary statistics of neuron numbers per mm^2 of cortical surface area with rostral to caudal (RC) and medial to lateral axes (ML) as variables, as well as the interaction between these two variables. *, $p < 0.05$

Figure 1

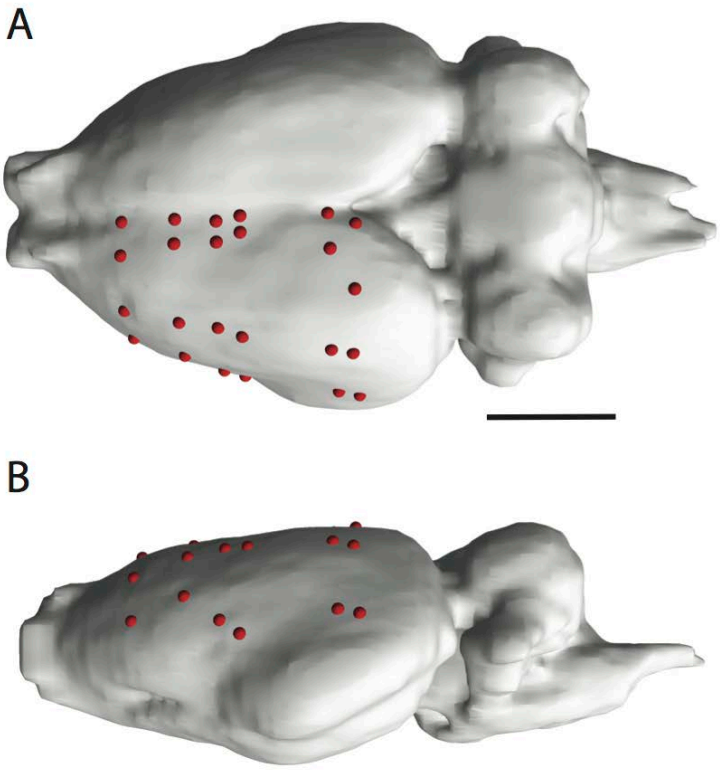


Figure 2

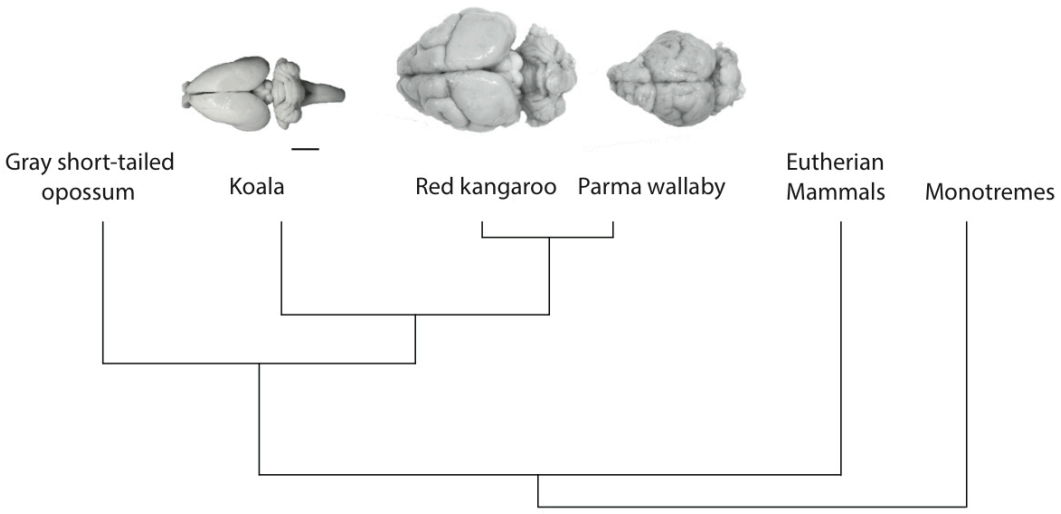

Gray short-tailed opossum
Koala
Red kangaroo
Parma wallaby
Eutherian Mammals
Monotremes

Figure 3

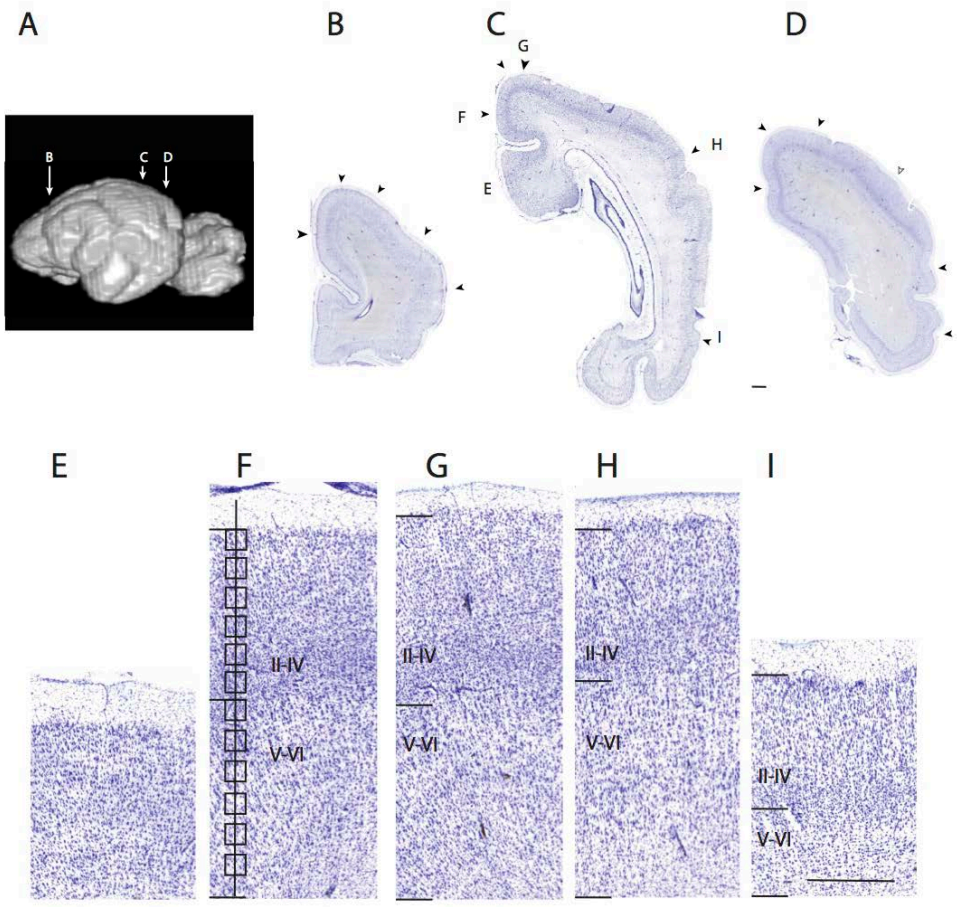


Figure 4

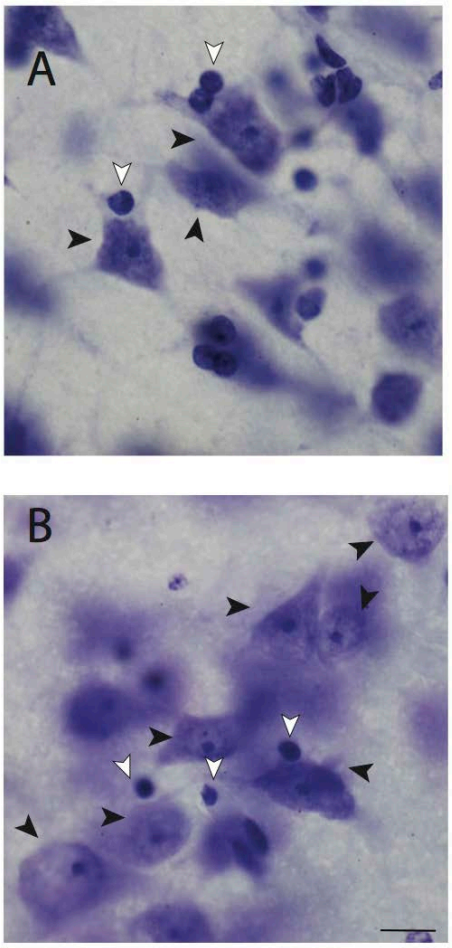
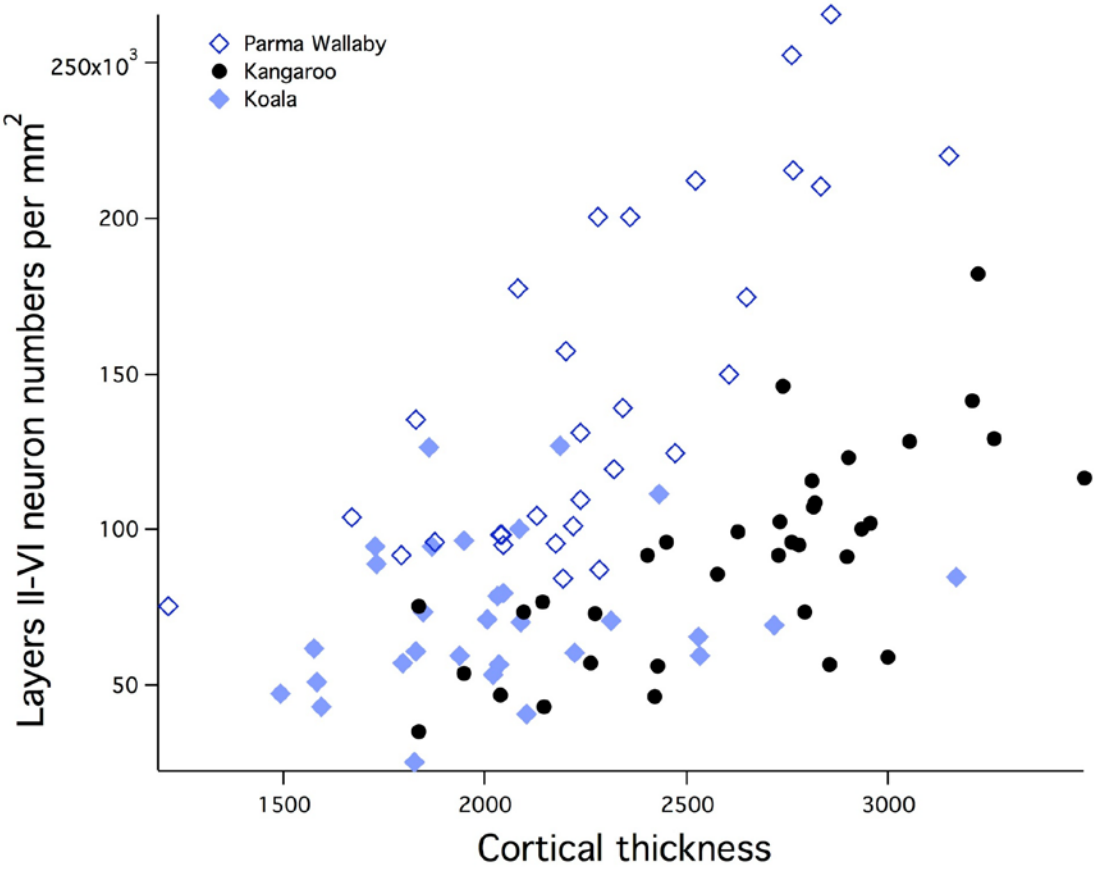


Figure 5


Parma Wallaby
Kangaroo
Koala
250x10^3
200
150
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50
Layers II-VI neuron numbers per mm^2
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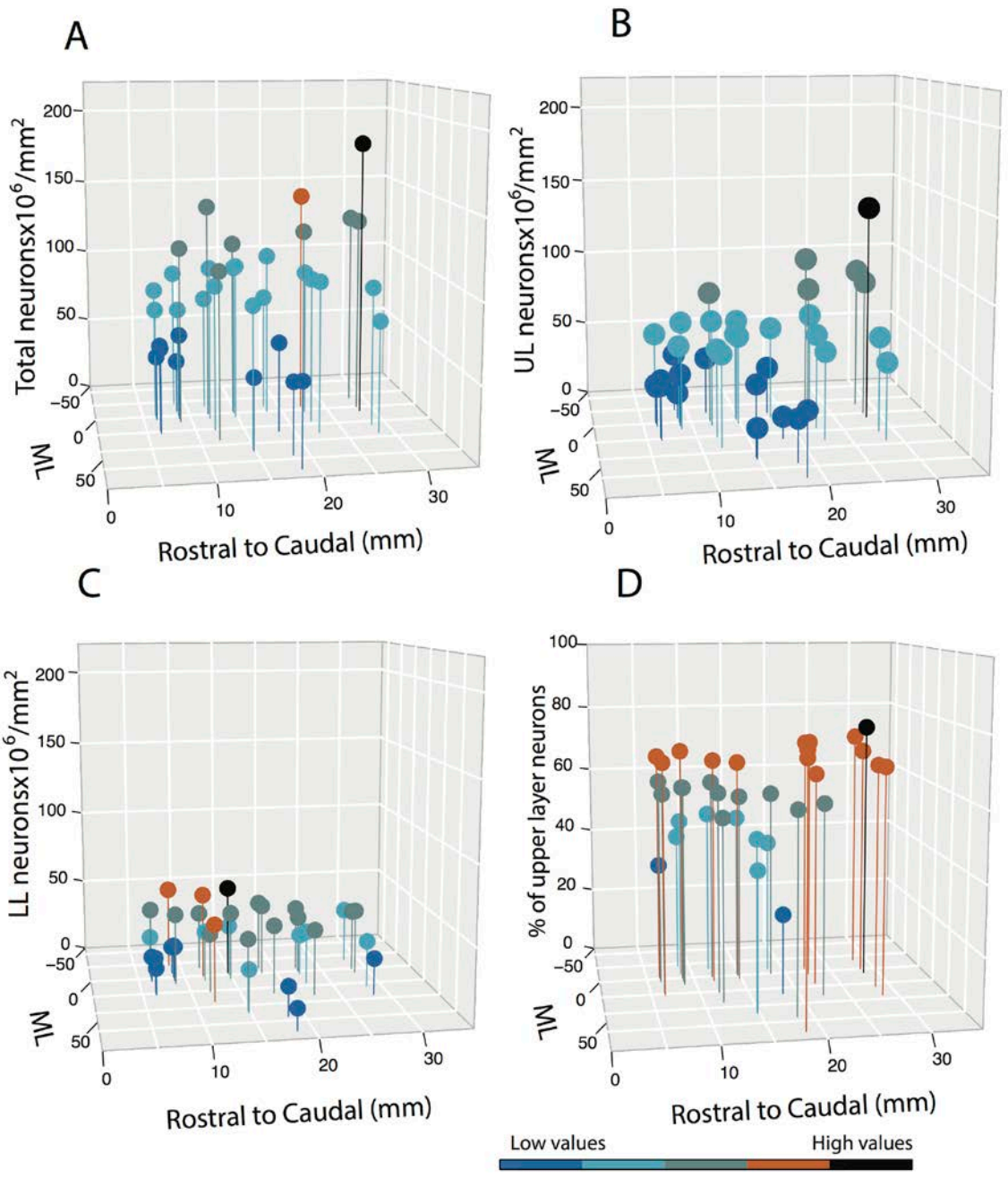
2000
2500
3000
Cortical thickness

Figure 6


A
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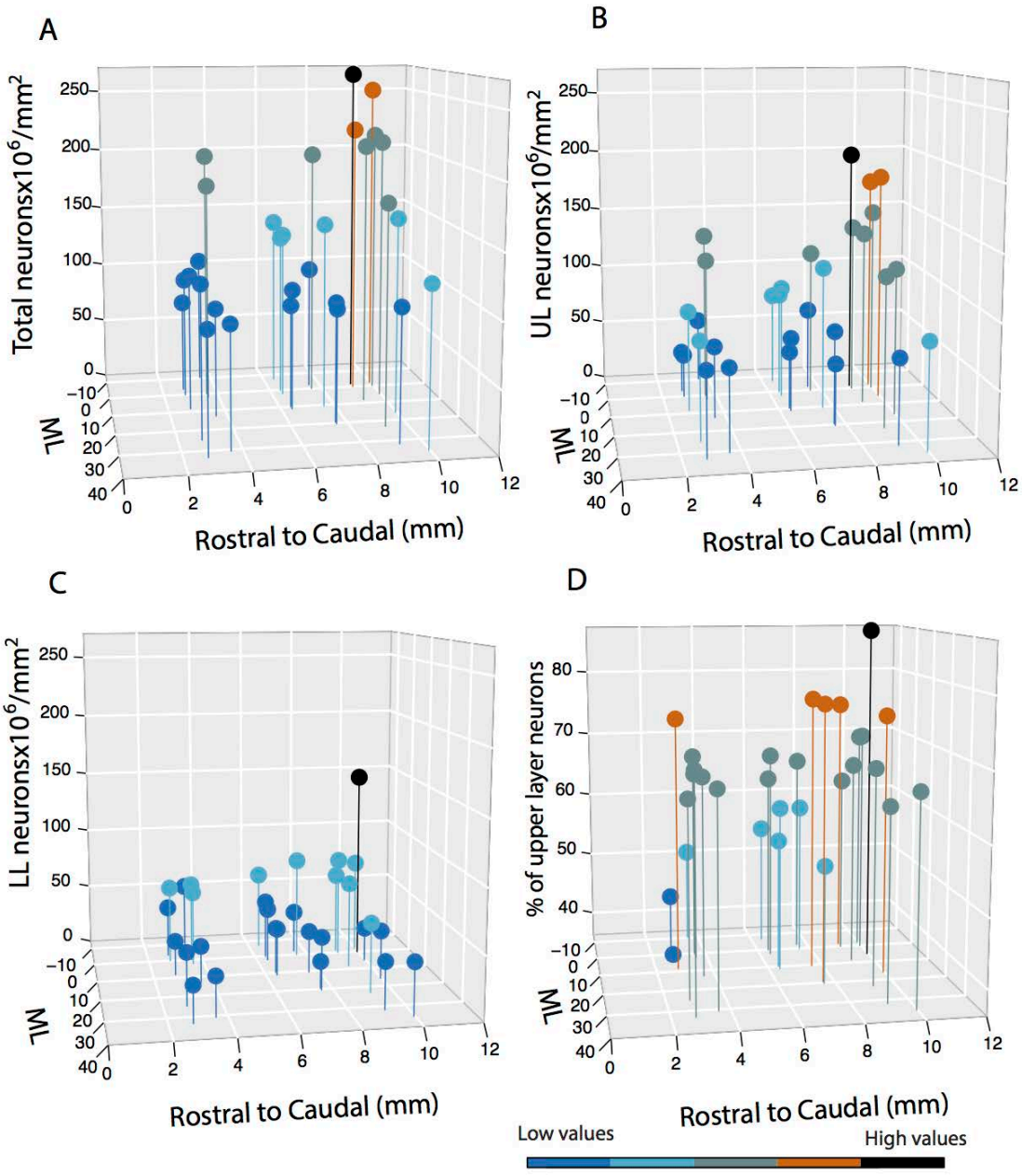
C
D
Total neuronsx10^6/mm^2
UL neuronsx10^6/mm^2
LL neuronsx10^6/mm^2
% of upper layer neurons
Rostral to Caudal (mm)
ML
Low values
High values

Figure 7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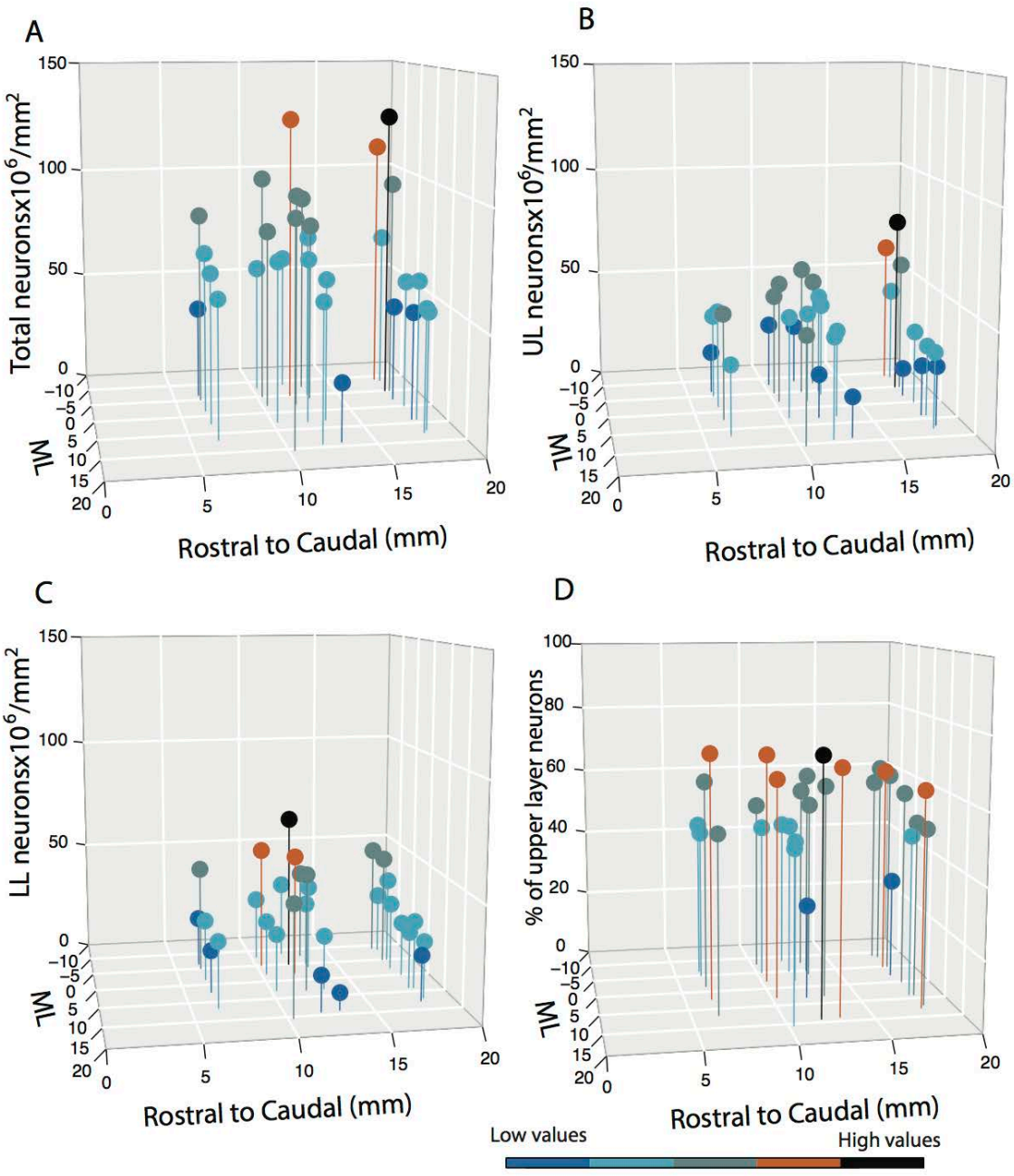
Total neuronsx10^6/mm^2
UL neuronsx10^6/mm^2
LL neuronsx10^6/mm^2
% of upper layer neurons
ML
Rostral to Caudal (mm)
Low values
High values

Figure 8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Total neuronsx10^6/mm^2
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15
20
Rostral to Caudal (mm)
D
% of upper layer neurons
100
80
60
40
20
0
−10
−5
0
5
10
15
20
ML
0
5
10
15
20
Rostral to Caudal (mm)
Low values
High values

Figure 9

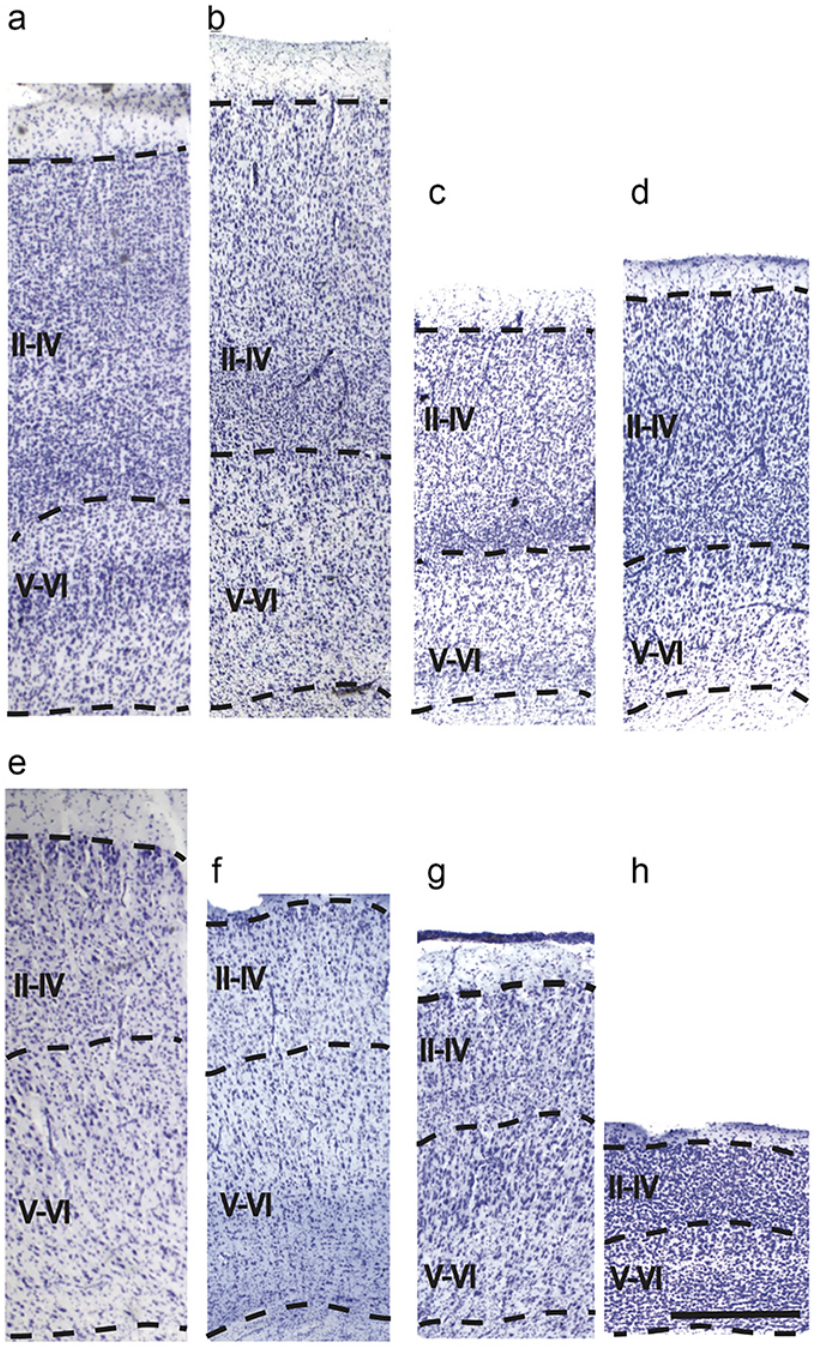

a
b
c
d
II-IV
V-VI
II-IV
V-VI
II-IV
V-VI
II-IV
V-VI
e
f
g
h
II-IV
V-VI
II-IV
V-VI
II-IV
V-VI
II-IV
V-VI

Figure 10

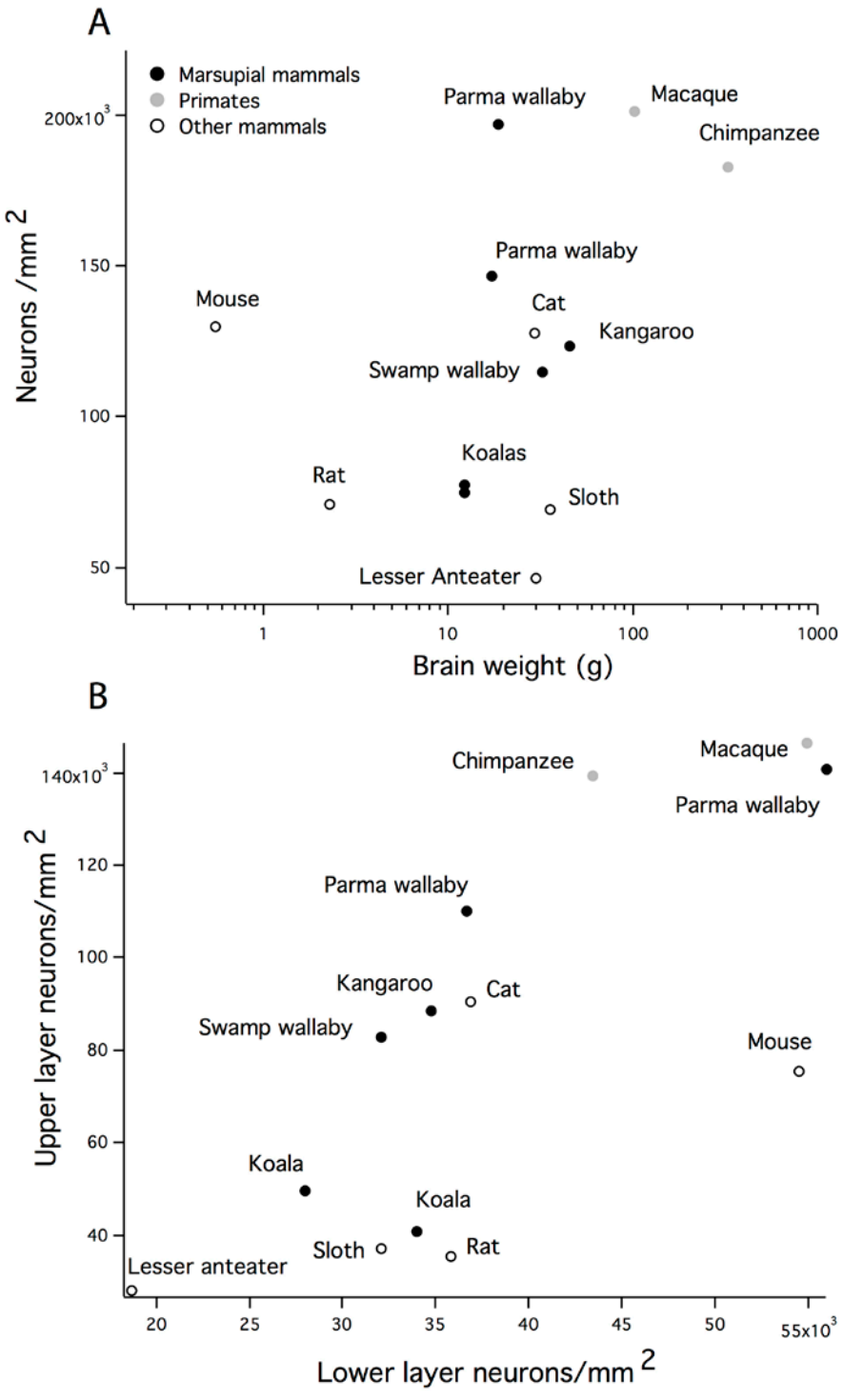
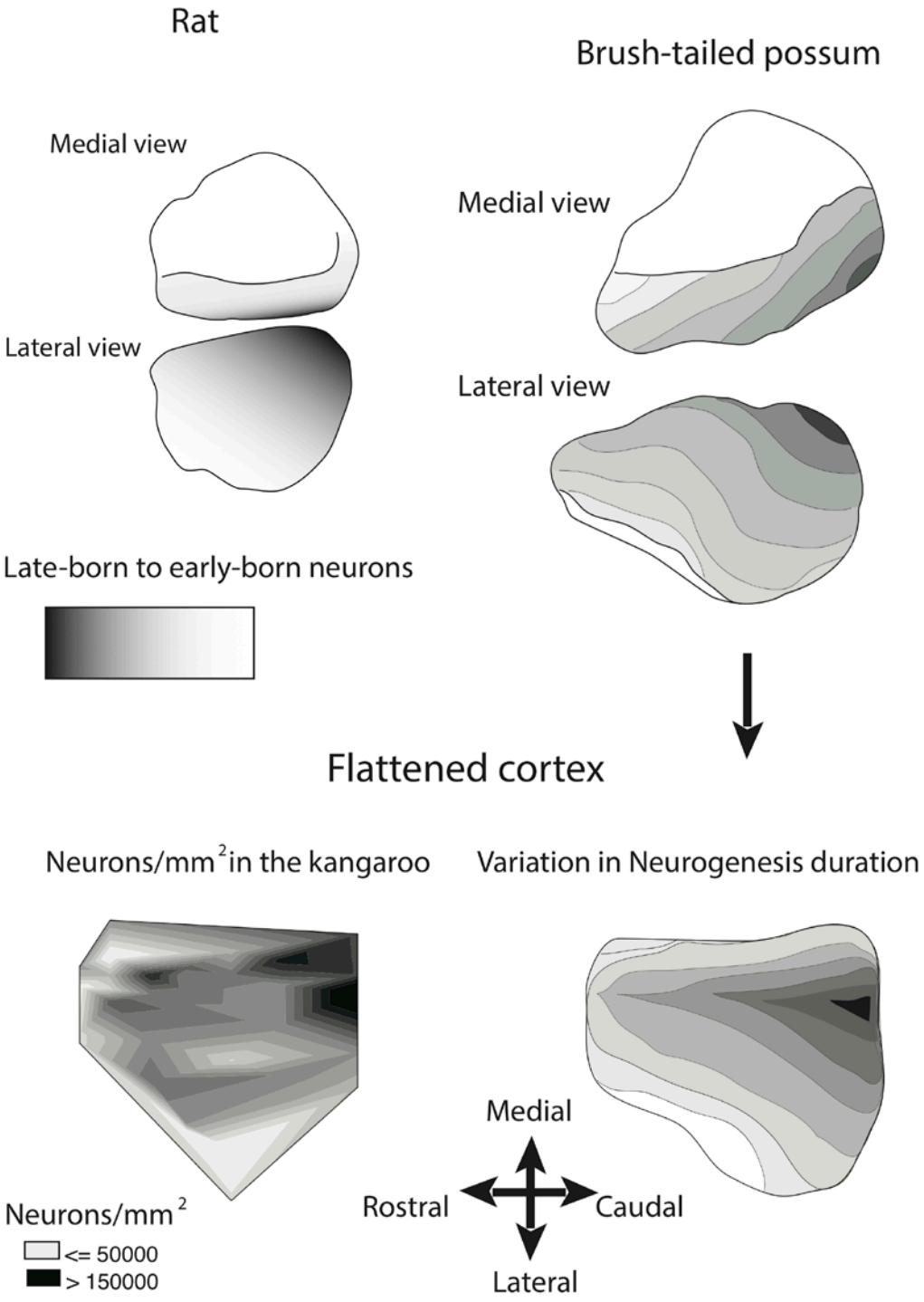

A
Marsupial mammals
Primates
Other mammals
Parma wallaby
Macaque
Chimpanzee
Parma wallaby
Mouse
Cat
Kangaroo
Swamp wallaby
Koalas
Rat
Sloth
Lesser Anteater
Neurons /mm 2
200x10 3
150
100
50
1
10
100
1000
Brain weight (g)
B
Chimpanzee
Macaque
Parma wallaby
Parma wallaby
Kangaroo
Cat
Swamp wallaby
Mouse
Koala
Koala
Sloth
Rat
Lesser anteater
Upper layer neurons/mm 2
140x10 3
120
100
80
60
40
20
25
30
35
40
45
50
55x10 3
Lower layer neurons/mm 2

Figure 11


Rat
Brush-tailed possum
Medial view
Lateral view
Medial view
Lateral view
Late-born to early-born neurons
Flattened cortex
Neurons/mm 2 in the kangaroo
Variation in Neurogenesis duration
Neurons/mm 2
<= 50000
> 150000
Medial
Rostral
Caudal
Lateral