

The Glia/Neuron Ratio: How it Varies Uniformly Across Brain Structures and Species and What that Means for Brain Physiology and Evolution

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It is a widespread notion that the proportion of glial to neuronal cells in the brain increases with brain size, to the point that glial cells represent “about 90% of all cells in the human brain.” This notion, however, is wrong on both counts: neither does the glia/neuron ratio increase uniformly with brain size, nor do glial cells represent the majority of cells in the human brain. This review examines the origin of interest in the glia/neuron ratio; the original evidence that led to the notion that it increases with brain size; the extent to which this concept can be applied to white matter and whole brains and the recent supporting evidence that the glia/neuron ratio does not increase with brain size, but rather, and in surprisingly uniform fashion, with decreasing neuronal density due to increasing average neuronal cell size, across brain structures and species. Variations in the glia/neuron ratio are proposed to be related not to the supposed larger metabolic cost of larger neurons (given that this cost is not found to vary with neuronal density), but simply to the large variation in neuronal sizes across brain structures and species in the face of less overall variation in glial cell sizes, with interesting implications for brain physiology. The emerging evidence that the glia/neuron ratio varies uniformly across the different brain structures of mammalian species that diverged as early as 90 million years ago in evolution highlights how fundamental for brain function must be the interaction between glial cells and neurons.

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Introduction

The notion that the proportion of glial to neuronal cells in the brain increases with brain size, or with body size, to the point that glial cells represent about 90% of all cells in the human brain has recently become so common in the neuroscientific research literature as well as in textbooks that stating it no longer requires citing the original sources (see, for instance, Allen and Barres, 2009; Bear et al., 2006; Kandel et al., 2000; Nedergaard et al., 2003; Nishiyama et al., 2005). Journalistic pieces very often open with grand statements to this effect, such as “(. . .) the most numerous type of cell in the human brain—outnumbering neurons by 10 to 1” (Kast, 2001) or “Meet the forgotten 90% of your brain: glial

cells, which outnumber your neurons ten to one” (Zimmer, 2009).

Glial cells have lately ascended from a mere supporting role as brain “glue” to the status of key players in brain physiology, metabolism, development, and even neurological diseases (Barres, 2008). This new status is due to the growing recognition that, among many functions, they control synapse formation (Ullian et al., 2001), respond to neural activity, including sensory stimuli, with spatially relevant increases in intracellular $[Ca^{2+}]$ (Schummers et al., 2008), are metabolically coupled to neurons and provide them with lactate as a source of energy on demand (Lee et al., 2012; Magistretti, 2006), offer metabolic support to axons (Lee et al., 2012;

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Wender et al., 2000), regulate blood flow (Koehler et al., 2009), regulate synaptic transmission (Fields and Stevens-Graham, 2002), and modulate neuronal activity through the release of what became known as gliotransmitters (Volterra and Meldolesi, 2005). Moreover, their dysregulation can contribute to diseases such as ALS (Nagai et al., 2007) and fragile X syndrome (Jacobs and Doering, 2010), and may be involved in the mechanism of general anesthesia (Thrane et al., 2012). Beyond even this impressive array of functions, astrocytes have recently been convincingly implicated as being essential for long-term acquisition of a particular learned behavior (Suzuki et al., 2011).

With growing recognition of the importance of glial cells for normal and pathological brain function, and considering that their relative number in relation to the number of neurons is undoubtedly of functional importance, it is indeed an opportune moment to review the facts about the ratio between glial and neuronal cells in the brain. Moreover, recent findings indicate that some of the previously held notions and assumptions about this ratio in humans and other animals, how it scales with brain size, and what that implies for brain physiology and evolution, are, in a word, wrong. However, acknowledging the new data reviewed here that glial cells are often not the predominant cell type in the brain does by no means diminish the importance of these cells. Much to the contrary, the emerging evidence that the glia/neuron ratio varies uniformly across the different brain structures of mammalian species that diverged as early as 90 million years ago in evolution (Herculano-Houzel, 2011) highlights how fundamental for brain function must be the interaction between glial cells and neurons. It should be noted that this remains an active field, implying that rigid conclusions are not possible. Indeed, recent measurements of astrocyte size and complexity in human brain suggest that some exceptions to a “general” principle of glia/neuron ratio may exist.

A Historical Perspective: Larger Brains, Larger Neurons, Larger Glia/Neuron Ratios

Different animal brains—of sheep, pigeons, chicks, cats, dogs, oxen—have been the subject of investigation from the early days of neuroscience, in the hands of Thomas Willis, David Ferrier, Charles Sherrington, and Santiago Ramón y Cajal. The comparative study of what brains are made of, however, can be traced to Franz Nissl, who in 1898, through the visual inspection of human, dog, and mole brains, observed that neuronal density decreases as cortical volume increases. The decreased neuronal density, seen as a result of an increase in the “non-neuronal portion of the tissue,” he proposed, represented a higher development of “psychic functions” in humans (Nissl, 1898).

In 1954, Reinhard Friede pointed out that such an increase in the space among neurons could be explained by an increase in the volume occupied by dendrites as well as by an increase in the number of glial cells, or even by a “third substance,” a syncytial network that was supposed to connect neuronal to glial cells (Bauer, 1943). Friede, however, noticed a higher number of glial cells compared with neurons in the human cerebral cortex relative to other mammals, and decided to undertake a quantitative study of the proportion of glial cells in the cortical tissue. Thus he established, in 1954, the number of glial cells per neuron as a parameter of investigation. Friede called this parameter the glial index (the ratio between the number of glial cells, G , and the number of neurons, N , in the tissue), but it became most often referred to in the literature as the glia/neuron ratio, a term proposed by Bass et al. (1971). From the two-dimensional analysis of tissue sections cut at the same thickness, Friede observed that the glia/neuron ratio in the cerebral cortex of several species increases from frog (with an index of 0.25) to man (average index of 1.48 across cortical layers), going in ascending order of brain size through chicken (0.46), mouse (0.36), rabbit (0.43), pig (1.20), cow (1.22), and horse (1.23).

In alignment with Franz Nissl, Friede (1954) concluded that the “ascending development” (Aufwärtsentwicklung) of the cortex is associated with a relative increase in the glia/neuron ratio—with humans as the “most developed.” Such a relative increase in numbers of glial cells would be explained due to their “trophic importance,” given the suspected involvement of glial cells in brain metabolism, thus allowing the presumed “progressive development” to occur.

Until then, the human brain had been the largest to be analyzed. In 1952, Donald Tower and Allan Elliot had confirmed the observation that neuronal density decreases with increasing brain weight from mouse to man, varying from 142,500 to 8,750–10,000 N/mm^3 , through a sample of mammalian species that included rat, guinea pig, rabbit, cat, dog, monkey, and cow (Tower and Elliot, 1952). These authors described mathematically the decrease in neuronal density across species as a power function of brain mass with an exponent of -0.3 . This mathematical relationship gave rise to what in the following decades became a widespread notion that neuronal density varies continuously as a single function of brain size across the most diverse mammalian species (see, for instance, Karbowski, 2007; Prothero, 1997). Soon thereafter, Tower published a study on the cerebral cortex of the fin whale and the Indian elephant (Tower, 1954), finding neuronal densities of 6,500 to 7,100 N/mm^3 in their grey matter, values lower than the 8,750 to 10,500 he had reported earlier for humans (Tower and Elliot, 1952).

It was using the same tissue from these fin whale brains, which weighed around 7 kg, that Hawkins and Olszewski

(1957) questioned Friede's conclusion that the glia/neuron ratio is related to "ascending phylogenetic development." These authors found a glia/neuron ratio of 4.54 in the cerebral cortex of the fin whale compared with 1.78 in the human brain, of only 1.3 kg, showing that the glia/neuron ratio reflects brain size rather than "phylogenetic development."

Hawkins and Olszewski proposed that the increase in G/N ratio was related to an increase in the size of the neurons, which, with longer processes, would "require more assistance from the support tissue to cater to their metabolic needs" (Hawkins and Olszewski, 1957). Thus, it all seemed to fit: according to Tower (1952), larger brains have smaller neuronal densities, which suggests larger neurons¹; larger neurons should, intuitively, have larger metabolic needs, requiring support from more glial cells (an expectation later formalized by Attwell and Laughlin, 2001); hence, larger brains should have larger glia/neuron ratios.

White Matter and the Meaning of the Glia/Neuron Ratio in the Whole Brain

Neurons in the brain are distributed heterogeneously, and white matter tracts contain essentially no neuronal cell bodies—although they are composed of one large part of neuronal cells, the axon. One must not lose sight that the white matter is a continuation of the grey matter, and projection neurons are only whole when considered with their axons in white matter tracts.

Still, because there are no neuronal cell bodies in white matter, it is impossible to define a glia/neuron ratio in this tissue (i.e., the denominator is "0" making the glia/neuron ratio infinite). Moreover, one should bear in mind that species differ in the relative amounts of gray and white matter contained in their forebrains, the largest brain area in mammals. For example, white matter constitutes only around 12 to 14% of total forebrain volume in mice, but represents

¹Lower neuronal densities could result from several mechanisms leading to increased cortical volume. Assuming that brain tissue consists mostly of neuronal and glial cells, lower neuronal densities in larger cerebral cortices might result from an increase in the average size of the neurons (soma and/or neuropil, as Nissl proposed), in the absence of major changes in the density of glial cells in the tissue. Alternatively, a reduction in neuronal density in larger cerebral cortices could be interpreted as the result of an increase in tissue volume predominantly through the addition of glial, rather than neuronal cells, in the absence of major changes in the average size of either cell type. In both scenarios, decreased neuronal densities in larger brains would be accompanied by a relative increase in the number of glial cells per neuron. If, however, average glial cell size increased equally in larger cortices across species, then their density would also decrease, and as a consequence the number of glial cells per neuron would not increase together with cortical volume.

~55% of total forebrain volume in man (Zhang and Sejnowski, 2009). The fact that white matter progressively expands with brain size or, more correctly, with gray matter volume, and does so in a strongly non-linear fashion, makes it obvious that there is a larger amount of tissue devoid of neuronal cell bodies in human than in rodent brains. For this reason, including relatively larger volumes of white matter necessarily inflates glia/neuron ratios, and especially so in larger brains.

However, because mammalian brains share an overall layout of grey and white matter structures, it is still meaningful to define and measure a glia/neuron ratio for the entire brain and to examine how it varies across species depending on brain size and other parameters. This analysis can provide evidence of trends or shared features in a most basic property of brain tissue, the numeric distribution of glial and neuronal cells, if any such shared features exist. Similarly, it is meaningful to measure and compare glia/neuron ratios in the cerebral cortex as a whole: although neuronal cell bodies are restricted to the grey matter, possibly the largest part of the neuronal cell volume is contained in the white matter. Thus, while determining a glia/neuron ratio in the white matter alone is impossible and nonsensical, the analysis of grey matter alone, and of grey and white matter combined, offer complementary information on how glial cells are distributed around neurons. Analysis of the grey matter alone illuminates how numbers of glial cells (oligodendrocytes, astrocytes, and microglia) are distributed around neuronal cell bodies, dendrites, and resident axons; and analysis including the white matter informs on how numbers of oligodendrocytes, astrocytes, and also microglia are further added for every neuron in the attached grey matter. This is true regardless of the relative volume of the white matter compared with the grey matter, and also applies not only to the cerebral cortex, but to structures composed mostly of white matter, such as the brainstem.

An alternative to glia/neuron numeric ratio is the glia volume/neuron volume ratio. In fact, this ratio is plausibly of greater practical significance than glia/neuron cell counts, because of the physiological meaning of cell volumes towards cell function, energetic requirement and energetic capacity, for instance. When reliable glia and neuron volume data become available for different species and anatomic areas, it will be informative to reassess if systematic patterns emerge in relation to brain size or species. As described above, such a glia/neuron volume (or mass) fraction will also be meaningful for grey matter alone, for grey matter and attached white matter tracts, and for the brain as a whole. Indeed, although total neuronal cell volumes are difficult to measure (exactly because of their extension into the white matter), we were recently able to estimate how the glia/neuron mass fraction varies across brain structures and species, which provides great insight into some of most basic features of the brain (Mota and Herculano-Houzel, unpublished data).

Finally, it must be kept in mind that “glial” refers to a combination of astrocytes of various types, oligodendrocytes, and microglial cells and various precursor cells in the adult. The numbers and proportions of cell types in this heterogeneous population have yet to be determined across brain structures and species. Importantly, one must avoid equating “glia” with “astrocyte” and “glia/neuron ratio” with an “astrocyte/neuron ratio.” There is at present no systematic quantitative evidence that astrocytes are indeed the predominant glial cell type, not even in grey matter. To the contrary, astrocytes might account for only 20% of all glial cells in the grey matter of the human cerebral cortex (Pelvig et al., 2008). That said, however, much can still be learned from examining glial cells as a whole (which are the data currently available), and considering their frequency relative to neurons in structures that combine grey and white matter alike, as discussed next.

The Glia/Neuron Ratio Revisited: No Single, Uniform Variation with Brain Size

As reviewed above, the notion that the proportion of glia in the brain as a whole correlates with brain size or with an animal’s size is based on limited data from a handful of mixed mammalian species, and actually limited to the cerebral cortex alone. The correlations between brain size, neuronal density in the cerebral cortex, and the glia/neuron ratio in this structure observed in the original studies of Friede (1954), Tower (1952), Hawkins and Olzewski (1957), and later by Haug (1987) and Stolzenburg et al. (1989) are shown in Fig. 1.

These original studies predated the introduction of unbiased stereology as the standard for counting cells and estimating cell densities (Gundersen et al., 1988), and thus were mostly based on two-dimensional photomicrographs, rendering them difficult to compare directly with one another and with modern data. More recent studies have used unbiased stereology to estimate the glia/neuron ratio in different species, but most have limited their analysis to the cerebral cortex (for instance, Eriksen and Pakkenberg, 2007; Pelvig et al., 2008; but see Andersen et al., 1992, on the cerebellum). Such modern studies have confirmed a large glia/neuron ratio of 7.7 in the cerebral cortex of the minke whale (Eriksen and Pakkenberg, 2007), compared with a smaller ratio of about 1.4 in the human cerebral cortex (Pelvig et al., 2008). However, the data available so far had not been enough to grant a systematic analysis of how the glia/neuron ratio varies across species and in structures other than in the cerebral cortex, much less in the whole brain, perhaps in part because stereology can be a very time-consuming method—or, perhaps more likely, because the issue of how the glia/neuron ratio varies with brain size was thought to be settled.

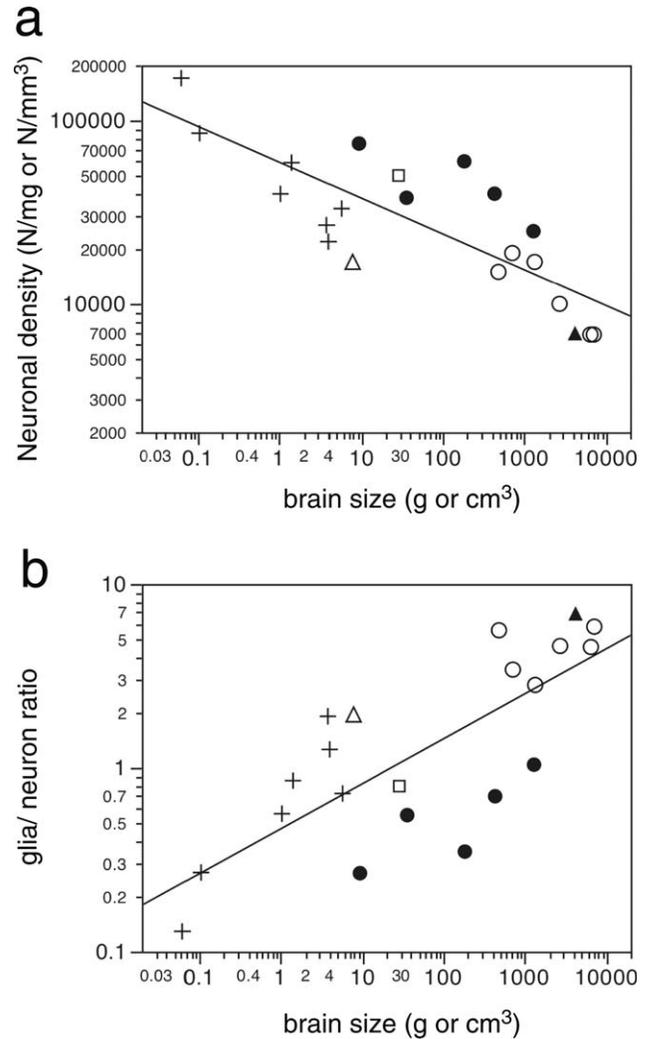


FIGURE 1: Larger brains appear to be accompanied by a decrease in neuronal density (a) and an increase in the glia/neuron ratio (b). Original dataset of Friede (1954), Tower (1952), Hawkins and Olzewski (1957), Haug (1987), and Stolzenburg et al. (1989), which gathers six primate (closed circles), six insectivore (crosses), five cetacean (open circles), one marsupial (open triangle), one carnivore (open square), and one afrotherian (closed triangle) species. Variation in neuronal density can be described as a power function of brain mass (in g or cm³) with an exponent of -0.195 ($r^2 = 0.623$, $P < 0.0001$), and variation in glia/neuron ratio, as a power function of exponent 0.245 ($r^2 = 0.558$, $P < 0.0001$).

Using a non-stereological method developed in our lab, the isotropic fractionator (Herculano-Houzel and Lent, 2005), we have determined total numbers of neuronal and non-neuronal cells (those expressing the neuronal marker NeuN or not, respectively; Mullen et al., 1992) in the cerebral cortex, cerebellum, and remaining brain areas of 29 mammalian species, distributed across the orders Rodentia, Primata and the closely related Scandentia, and Eulipotyphla (formerly known as Insectivora; Azevedo et al., 2009; Gabi et al., 2010; Herculano-Houzel et al., 2006, 2007, 2011;

Sarko et al., 2009). With this new wealth of data, it became possible to address the issue of how the glia/neuron ratio varies with brain size in a much more systematic fashion, both within and across mammalian groups. Given that the vascular volume of neuronal parenchyma is less than 3% of total volume (Lawers et al., 2008), the majority of nonneuronal cells can be presumed to be glial cells, and will be referred to as glial cells from here on. The isotropic fractionator method, which consists of transforming brain tissue into a suspension of free cell nuclei and estimating total numbers of cells from the density of nuclei in the suspension, is at least as reliable as stereology, with coefficients of variation across multiple samples of the same tissue typically below 0.10. It yields results that are comparable to those obtained with stereology (Bahney and von Bartheld, 2014; Tsai et al., 2009; and see comparison with published data in Azevedo et al., 2009) and, given the ease of application and the advantages offered by this method over stereology when estimates of total numbers of cells are sought, it is already being used independently by a number of groups (Brautigam et al., 2012; Campi et al., 2011; Duan et al., 2013; Surchev et al., 2007; Young et al., 2013).

Remarkably, we found that the overall proportion of glial cells in the brain as a whole is quite variable: from 33.3% in the eastern mole (whose brain, at 1.0 g, is more than twice the mass of the mouse brain) to 66.4% in the capybara—whose brain, at 74.7 g, is about 20 times smaller than the human brain, the largest in our sample. The human brain was found to consist roughly of 50% neuronal and 50% nonneuronal cells (Azevedo et al., 2009), relatively fewer nonneuronal cells than in the capybara brain, although more than the 35% of nonneuronal cells found in the mouse brain (Herculano-Houzel et al., 2006). Contrary to common statements in the literature, the overall proportion of glial cells in the brain is thus not a single function of brain size across species, as shown in Fig. 2. This figure shows that the proportion of glial cells increases steeply with brain size across rodent species, and less steeply across primate species; is larger in rodent brains than in primate brains of a similar size (and already larger in the capybara than in the human brain); and does not vary significantly with brain size across insectivores. Interestingly, such an order-specific relationship between glia/neuron ratios and brain size is already apparent in the original dataset (see Fig. 1b)—but, probably due to the bias of analyzing mammalian brains as scaled-up or scaled-down versions of a single plan (reviewed in Herculano-Houzel, 2011a), these clade-specific relationships had not been noticed.

The small overall proportion of glial cells in the brain, however, is skewed by the very large concentration of neurons in the cerebellum. In the cerebral cortex alone, we find the proportion of glial cells to vary between 44.3% in the

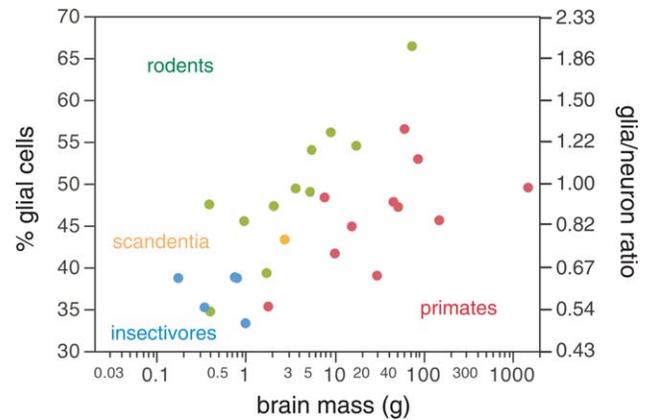


FIGURE 2: Variation in the overall glia/neuron ratio in the brain of 29 species of mammals according to brain mass. Each point represents the average proportion of glial cells (left axis) and the glia/neuron ratio (right axis) for one species, obtained by applying the isotropic fractionator separately to the cerebral cortex, cerebellum, and rest of brain of each specimen then pooling all structures together, and plotted against average brain mass for that species. Data from Herculano-Houzel et al. (2006, 2007, 2011), Azevedo et al. (2009), Sarko et al. (2009), and Gabi et al. (2010).

short-tailed shrew to 85.6% in the capybara, and in the rest of brain, between 55.4% in the mouse to 91.7% in the human. In contrast, in the cerebellum of the species analyzed, glial cells represent only from 6.3% of the cells in the baboon to 32.9% of the cells in the capybara. As found in the whole brain, the glia/neuron ratio in each of the three major brain structures does not vary as a single function of brain mass across all species. Instead, the glia/neuron ratio increases together with structure size only in the cerebral cortex, cerebellum and rest of brain of rodents, and in the cerebral cortex of insectivores, and does not vary with structure size in primates (Fig. 3).

Like the glia/neuron ratio, we find that neuronal densities in the different brain structures does not vary uniformly with brain size across all species. Neuronal density does decrease with increasing size in the cerebral cortex in rodents, primates, and insectivores; in the rodent cerebellum; and in the rodent rest of brain, but at different rates in each (Fig. 4a). In contrast, glial densities are quite similar across structures and mammalian orders, with no systematic variation with structure mass (Fig. 4b), as observed previously by other groups (Haug, 1987; Tower and Young, 1973). The source of variation in the glia/neuron ratio, therefore, must be predominantly a variation in neuronal densities.

The Glia/Neuron Ratio Revisited: Uniform Variation not with Brain Size, but Neuronal Size

Remarkably, the analysis of variation of the glia/neuron ratio with neuronal density reveals a single, uniform relationship between the two variables: the smaller the neuronal density,

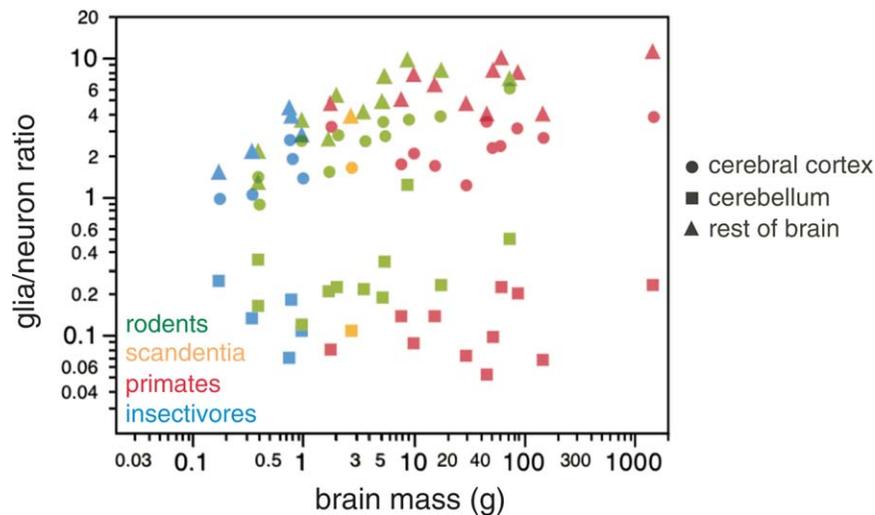


FIGURE 3: Variation in the overall glia/neuron ratio in the cerebral cortex, cerebellum, and rest of brain of 29 species of mammals according to brain mass. Each point represents the average glia/neuron ratio in the structure for one species, obtained with the isotropic fractionator (cerebral cortex, circles; cerebellum squares; rest of brain, triangles), plotted against average brain mass for that species. Data from Herculano-Houzel et al. (2006, 2007, 2011), Azevedo et al. (2009), Sarko et al. (2009), and Gabi et al. (2010).

the larger the glia/neuron ratio (Fig. 5a). This single, very strong correlation ($r^2 = 0.828$) applies not only for the whole brain, but also across brain structures, species, and mammalian orders. Thus, the lack of a universal variation in the glia/neuron ratio with brain size (Figs. 2 and 3) can be explained by the heterogeneity in variation in neuronal density with brain mass across structures and mammalian orders (Fig. 4a).

The data for the cerebral cortex presented in Fig. 5a (colored circles) include the white matter, which is relatively small in rodents but reaches 50% of cortical volume in primates. This necessarily increases the G/N ratio shown for the cerebral cortex and also decreases the estimated neuronal density compared with the grey matter alone. This might be expected to lead spuriously to a negative correlation between G/N ratio and neuronal density as larger brains gain white matter faster than grey matter, all other things being equal. However, G/N ratios found in the primate cortical grey matter alone exhibit the same pattern of a negative correlation (Fig. 5a, black circles), which is also seen in the cortical grey matter of artiodactyls and afrotherians (Kazu et al., Neves et al., 2014; unpublished data). Moreover, the recent systematic exploration of the distribution of neurons and non-neuronal cells along the human cerebral cortex allowed the analysis of the relationship between G/N and neuronal density across sites within this grey matter (Ribeiro et al., 2013). Remarkably, local G/N ratios increase with decreasing neuronal density also across sites within the grey matter of the human cerebral cortex (Fig. 6), in the same relationship that applies to other brain structures as well as species (Fig. 5a).

The finding of a similar inverse relationship between G/N ratio and neuronal densities in the cortical grey matter

alone and in the entire cerebral cortex (including the white matter) indicates that this relationship is universal to brain tissue. Although white matter contains axons rather than neuronal cell bodies, its inclusion as part of the cerebral cortex acknowledges that an important part of neuronal cells is contained therein—and is accompanied by a proportion of glial cells per neuron that follows the same relationship that applies to the grey matter alone. This is in line with the observation that the majority of glial cells in the cortical grey matter, as in the white matter, are oligodendrocytes, not astrocytes (Pelvig et al., 2008). Figure 5a shows that the inverse relationship between G/N and neuronal density is the same across brain structures that are axon rich (“rest of brain” and cerebral cortex including the white matter) or axon poor (cortical grey matter), as well as across the whole brain, and for all mammalian species examined so far. A reanalysis of the original data from the literature presented in Fig. 1 yields a similar uniform relationship, with a much better correlation between glia/neuron ratio and neuronal density ($r^2 = 0.903$) than between either variable and brain size ($r^2 = 0.558$ and $r^2 = 0.623$, respectively; Fig. 5b).

Given the apparently small variation in glial density across structures and species (Fig. 4b; but see Discussion about human glia), decreased neuronal density suggests a larger average neuronal cell size (including the soma and all dendritic and axonal arborizations). The single, uniform relationship between decreased neuronal density and increased glia/neuron ratio across brain structures and species thus suggests strongly that the glia/neuron ratio increases as a single function of increasing neuronal size—as originally proposed by Hawkins and Olszewski (1957). One must bear in mind,

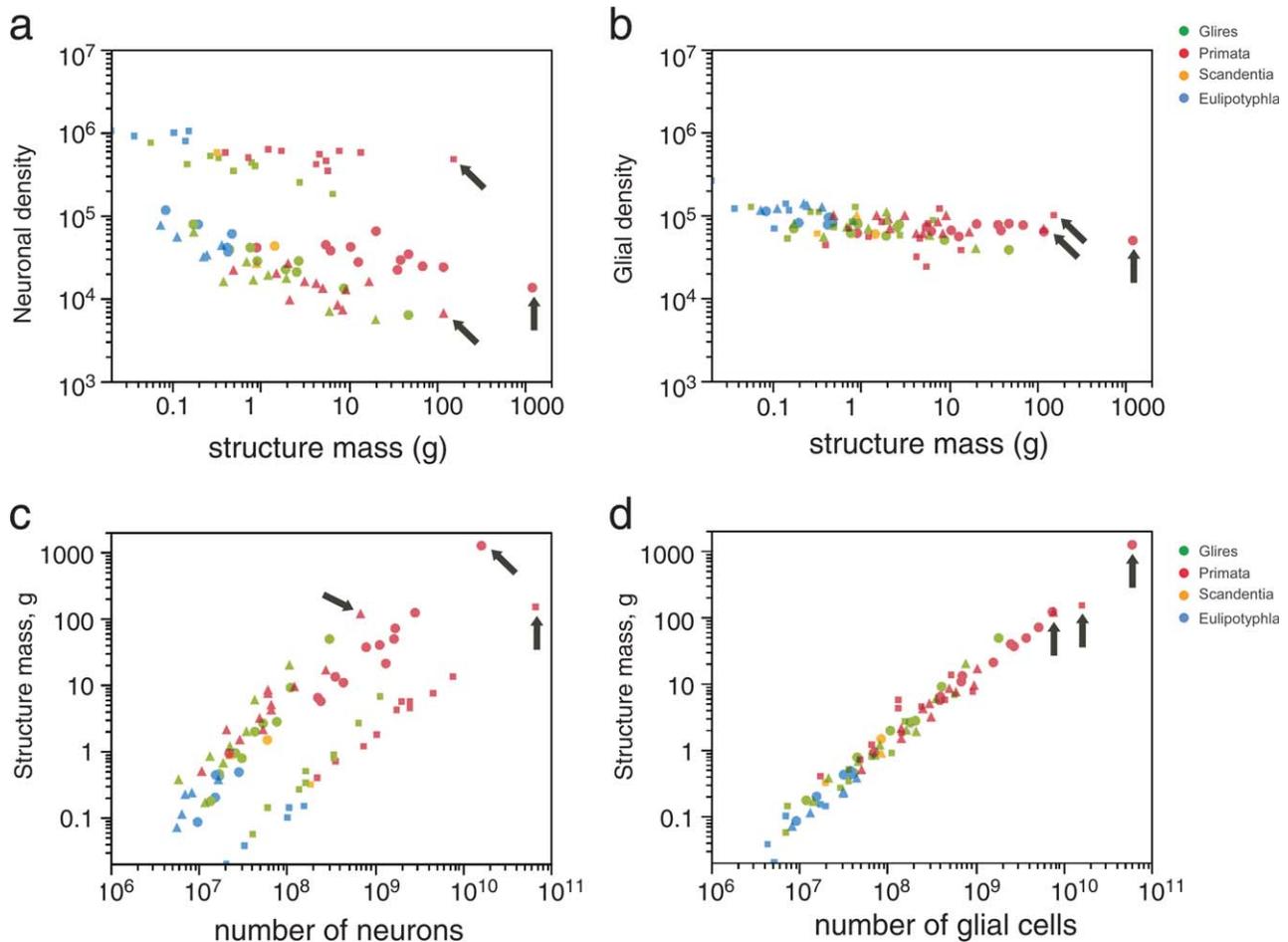


FIGURE 4: Variation in the neuronal and glial densities in the cerebral cortex, cerebellum, and rest of brain according to brain mass (a and b), and variation in the mass of brain structures according to numbers of cells (c and d). Each point represents the average neuronal density (a) or glial density (b) in the structure for one species, obtained with the isotropic fractionator, plotted against the corresponding average brain structure mass. Graphs are plotted with similar scales to show the larger variation in neuronal densities than in glial densities. (c, d) Scaling of the mass of brain structures as different functions of numbers of neurons (c) but as similar functions of numbers of glial cells (d) across structures and mammalian orders. Cerebral cortex, circles; cerebellum, squares; rest of brain, triangles. Arrows indicate human data points. Data from Herculano-Houzel et al. (2006, 2007, 2011), Azevedo et al. (2009), Sarko et al. (2009), and Gabi et al. (2010).

however, that this assertion has yet to be validated by quantitative histology.

Importantly, the glia/neuron ratio, as used in the literature, does not discriminate amongst glial cell types: it refers to the total number of microglia, astrocytes, and oligodendrocytes and their ratio to total numbers of neurons. Given the proposed metabolic cause for the increasing glia/neuron ratio in larger brains with larger neurons (Hawkins and Olszewski, 1957) and the now well established role of astrocytes in providing metabolic support for neurons (Magistretti, 2006), one might assume that astrocytes compose the majority of glial cell types and would thus contribute the most to the increasing glia/neuron ratio that accompanies larger neurons. However, a detailed study of the cellular composition of the grey matter of the human cerebral cortex showed that astrocytes are only 20% of its glial cells; the majority (75%) are

oligodendrocytes, while microglia amount to only 5% of glial cells in the grey matter (Pelvig et al., 2008). Thus, the increasing glia/neuron ratio that accompanies larger neurons presumably includes not only more astrocytes per neuron as the total size (including all arborizations) of the latter cells increase, but also, and predominantly, more oligodendrocytes per neuron, myelinating their axons. Such a predominance of oligodendrocytes in the glial fraction of grey and white matter alike could explain why the glia/neuron ratio shares the same relationship to neuronal density across structures that are rich in neuronal cell bodies (such as cortical grey matter), poor in neuronal cell bodies (such as the brainstem, which, together with diencephalon and basal ganglia, compose the “rest of brain” in Fig. 5a), and in the combined cortical grey + white matter and even the brain as a whole. In any case, the striking finding that all these brain structures share a similar

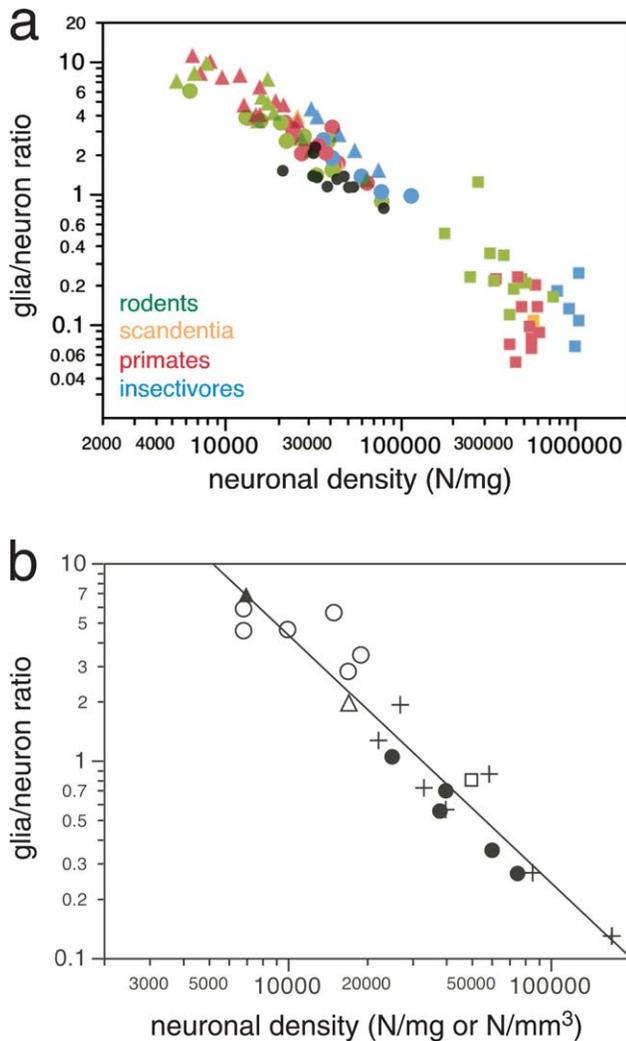


FIGURE 5: Uniform variation in glia/neuron ratio with neuronal density across brain structures, species, and mammalian orders. **a**, Each point represents the average glia/neuron ratio in the cerebral cortex (circles), cerebellum (squares), or rest of brain (triangles) for one species, obtained with the isotropic fractionator, plotted against the neuronal density in the same structure. Data from rodents, primates, scandentia, and insectivores from Herculano-Houzel et al. (2006, 2007, 2011), Azevedo et al. (2009), Sarko et al. (2009), and Gabi et al. (2010). All data represented by colored symbols combine grey and white matter. Black circles, grey matter of the primate cerebral cortex alone. **b**, Each point represents the average glia/neuron ratio in the cerebral cortex in six primate species (closed circles), six insectivores (crosses), five cetaceans (open circles), one marsupial (open triangle), one carnivore (open square), and one afrotherian (closed triangle). Data pooled from Friede (1954), Tower (1952), Hawkins and Olzeswki (1957), Haug (1987), and Stolzenburg et al. (1989), as in Fig. 1. Variation in glia/neuron ratio in **b** can be described as a power function of neuronal density of exponent -1.256 with a much better fit ($r^2 = 0.903$, $P < 0.0001$) than as a function of brain mass (see Fig. 1b; $r^2 = 0.558$, $P < 0.0001$).

relationship between *G/N* ratios and neuronal density (and not between *G/N* ratios and structure size, as formerly presumed) indicates that there are fundamental, and conserved,

rules that determine the *G/N* ratio according to average neuronal cell size in the structure, as explored below.

The Glia/Neuron Ratio in Humans

Our analysis of the human brain revealed a total average of 86.1 ± 8.1 billion neurons and 84.6 ± 9.8 billion nonneuronal cells in the whole brain, yielding a maximal glia/neuron ratio of 0.99, figures quite different from the common quotes of “100 billion neurons and 10 to 50 times more glia” (Allen and Barres, 2009; Bear et al., 2006; Kandel et al., 2000; Nedergaard et al., 2003; Nishiyama et al., 2005). As in other mammalian species, the glia/neuron ratio is very different across structures in the human brain. While nonneuronal cells

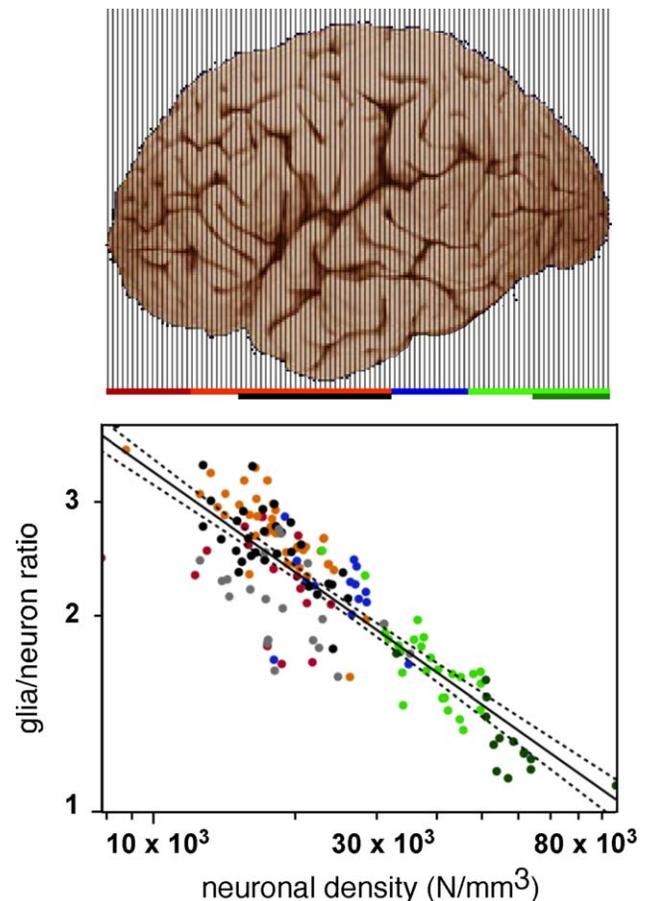


FIGURE 6: Uniform variation in glia/neuron ratio with neuronal density in the grey matter across sections of the human cerebral cortex. Each point represents the average glia/neuron ratio in the grey matter of one 2 mm coronal section of the human cerebral cortex, divided into prefrontal (red), frontal (orange), parietal (blue), occipital (green), and temporal (black) areas as indicated in the scheme (top). Glia/neuron ratios obtained with the isotropic fractionator are plotted against neuronal density found in the same part of each coronal section. Data from Ribeiro et al. (2013). The *G/N* ratio varies across all portions of the human cerebral cortex as a single power function of local neuronal density with an exponent of -0.515 ± 0.024 (95% CI -0.563 to 0.467 , $P < 0.0001$).

outnumber neurons by a ratio of 11.35 in the rest of brain (where non-neuronal cells represent 91.7% of all cells), in the cerebellum the glia/neuron ratio is only 0.23 (with non-neuronal cells amounting to 18.9% of all cells), and in the grey matter of the cerebral cortex, this ratio is 1.48 (that is, non-neuronal cells amount to 58.4% of all cells). These values are similar to those obtained previously with unbiased stereology in the human cerebellum (Andersen et al., 1992), the grey matter of the cerebral cortex (Pelvig et al., 2008; Sherwood et al., 2006), and in a handful of brainstem and diencephalic nuclei (Pakkenberg and Gundersen, 1988).

As pointed out above, the major source of these dramatic difference of as much as 49-fold in glia/neuron ratios within the human brain is not a variation in the distribution of non-neuronal cells, whose density varies little, by comparison, across structures in the human brain, by only 3.7-fold: from 27,446 cells/mg in the grey matter of the cerebral cortex to 69,850 cells/mg in the rest of brain, and 101,020 cells/mg in the cerebellum. Rather, the major contributor to the differences in glia/neuron ratio across the structures is the 72-fold difference in neuronal density across structures, from only 6,560 neurons/mg in the rest of brain to 19,541 neurons/mg in the grey matter of the cerebral cortex to 471,660 neurons/mg in the cerebellum (Azevedo et al., 2009). In line with this scenario, local variations in neuronal density across sites within the grey matter of the human cerebral cortex are also correlated with local variations in the glia/neuron ratio (Fig. 6; Ribeiro et al., 2013).

Remarkably, as illustrated by the arrows in Fig. 4, the neuronal densities found in the cerebral cortex, cerebellum, and rest of the human brain are not significantly different from those expected for a primate brain of its size. Similarly, the glia/neuron ratios found in these human brain structures follow the same trends with brain mass observed in other primates (Fig. 5a), and the glia/neuron ratio of 1.0 for the human brain as a whole is similar to the average overall glia/neuron ratio observed in the whole brain in other primate species (Fig. 2). Most importantly, glia/neuron values in the human brain vary as a similar function of neuronal density in the respective structures as in other species (Fig. 5a).

Why Larger Glia/Neuron Ratios with Larger Neurons? The Metabolic Argument

As reviewed above, we have found that the glia/neuron ratio increases as a single, uniform function of decreased neuronal density across brain structures and species (Herculano-Houzel, 2011a), a finding that suggests strongly that the glia/neuron ratio increases as a single function of increasing neuronal size. As originally proposed by Hawkins and Olszewski (1957), this should be the case because “larger neurons should have larger metabolic needs,” and thus require support from a larger number of glial cells each. Although astrocytes can no longer be

presumed to be the most numerous of glial cells in the brain (Pelvig et al., 2008), this expectation still fits the current understanding that both astrocytes and oligodendrocytes contribute to the metabolic support of neurons (Fünfschilling et al., 2012; Lee et al., 2012; Magistretti et al., 2006).

The expectation that larger neurons require more energy was formalized by Attwell and Laughlin in 2001, who estimated the distribution of the energy budget among the several energy-consuming processes within a neuron. These authors predicted that while nearly 80% of a neuron’s energy budget go toward glutamate-related neurotransmission, 13% are used to maintain the resting potential of the cell membrane (Attwell and Laughlin, 2001). The latter alone imposes a larger metabolic cost onto larger neurons because of the increase in the area of cell membrane to be repolarized. Later, Jan Karbowski estimated independently that, based on the supposedly homogeneous scaling of neuronal density with brain size across species, the average energy requirement per neuron increases with brain size (Karbowski, 2007). The average metabolic cost per neuron, however, had not yet been estimated directly.

Once numbers of neurons in the cerebral cortex and in the whole brain were determined directly with our method, it became possible to estimate the average metabolic cost per neuron (by dividing the measured metabolic cost in the cerebral cortex or whole brain of each species, compiled by Karbowski (2007), and dividing it by the respective number of neurons composing the structure) and to examine how this cost scales with numbers of neurons or neuronal density. This analysis revealed that total glucose use by the brain, cerebral cortex, and cerebellum scales linearly with the number of neurons in the structures (Fig. 7a), such that the estimated average glucose use per neuron is remarkably constant across species, including humans (Herculano-Houzel, 2011b). Most importantly, the small variations in the estimated average glucose use per neuron are not correlated with variations in neuronal density across species in any structure² (Fig. 7b), nor with the glia/neuron ratio (approximated as the ratio between

²The apparent scaling of glucose use per gram of brain tissue with brain size raised to an exponent of -0.127 can be explained by a similar apparent scaling of neuronal density in the whole brain with brain size raised to an exponent of -0.116 across the sample of three rodent and three primate species—apparent, only, given that neuronal density scales differently between rodent and primate species in the sample (Herculano-Houzel, 2011b). Similarly, the slightly larger exponent of -0.15 that relates specific brain metabolism to brain mass across larger mammalian samples (Karbowski, 2007) can be accounted for by an apparent scaling of neuronal density with brain mass raised to an exponent that varies depending on the choice of species. The scaling of brain metabolism, therefore, is best described as a function of the total number of neurons in the brain, regardless of how that relates to brain mass or neuronal density across species (Herculano-Houzel, 2011b).

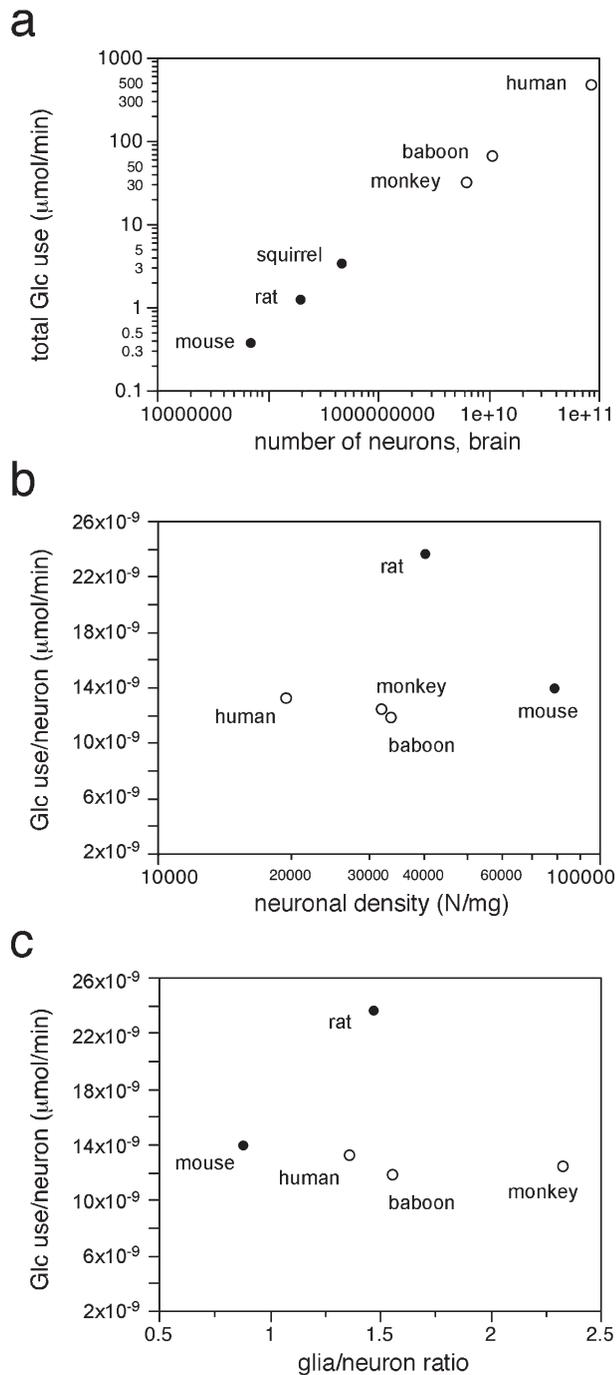


FIGURE 7: Total glucose use in the brain increases with number of brain neurons, and estimated average glucose use per neuron does not vary with neuronal density nor with glia/neuron ratio. **a**, Total glucose use by the whole brain scales linearly with the number of brain neurons (linear fit, $r^2 = 1.0$, $P < 0.0001$). **b**, No correlation across species between average glucose use per neuron in the cerebral cortex and neuronal density in the structure. **c**, No correlation across species between average glucose use per neuron in the cerebral cortex and glia/neuron ratio in the structure. Data from Herculano-Houzel (2011b).

non-neuronal and neuronal cells; Herculano-Houzel, 2011b; Fig. 7c). Considering that the inverse of neuronal density can be used to provide a direct estimate of how average neuronal size varies in the brain structures, these findings suggest that larger neurons do not have larger energetic requirements. Rather, there seems to be a fixed energy budget per neuron, independent of neuronal size (Herculano-Houzel, 2011b).

A fixed energy budget per neuron across brain sizes and neuronal sizes contradicts the notion that a rising glia/neuron ratio is related to increased metabolic needs of larger neurons. Further evidence against this relationship is the large metabolic rate of the cortical parenchyma in early postnatal development (Chugani, 1998), at a time when the glial/neuron ratio is vanishingly small (see below). An alternative activity-dependent mechanism to explain different glia/neuron ratios in the cerebral cortex across species has been proposed, based on the supposed accumulation of K^+ ions in thicker cortices, which would lead to local proliferation of glial cell progenitors and thus to larger glia/neuron ratios (Reichenbach, 1989). However, such a mechanism does not explain the very large glia/neuron ratios in thin cerebral cortical walls such as in cetaceans (Pillay and Manger, 2007). Instead, we have proposed that the single relationship between glia/neuron ratio and average neuronal size results from a single mechanism governing the addition of glial cells to the neuronal parenchyma during development, not only in the cerebral cortex but in all brain structures, as reviewed below.

Larger Glia/Neuron Ratios with Larger Neurons: The Developmental Argument

The adult glia/neuron ratio is established during postnatal development (Brizee et al., 1964), as the glial cell population proliferates, contributing to the postnatal expansion of brain volume. Indeed, we have shown that, in the rat, the brain is a predominantly neuronal structure at birth, when nonneuronal cells represent only 10% of all cells (Bandeira et al., 2009). The addition of massive numbers of glial cells to all brain structures begins on the second postnatal week and extends into the third postnatal week, after maximal numbers of neurons have been reached in all structures, and at the same time as neuronal cells are eliminated in large numbers (except for the cerebellum, which continues to gain neurons until the end of this period; Bandeira et al., 2009). Adult glia/neuron ratios are thus established simultaneously in postnatal development across all structures of the brain through the addition of large numbers of glial cells (Bandeira et al., 2009).

The addition of glial cells appears to happen in the same fashion during development not only to different brain structures but also to different mammalian species, with very little variation in glial cell densities, as mentioned above (Fig. 4b), such that there is a single relationship between the mass

of brain structures and their numbers of nonneuronal cells shared across structures and mammalian species (reviewed in Herculano-Houzel, 2011a; Fig. 4d, compare with 4c). This does not imply that average glial cell size is constant, but rather that it changes very little in comparison to the enormous variation in average neuronal size across structures and species. Indeed, using a simple mathematical model that links variations in neuronal density to variations in glial density in brain tissue, we have estimated that while average neuronal size (including soma and all arborizations) varies by as much as 260-fold across brain structures and species in our dataset, average glial cell size varies by only 1.4-fold (Mota and Herculano-Houzel, unpublished data).

Based on the relatively constant glial cell densities across structures and species (Haug, 1987; Herculano-Houzel, 2011a; Tower and Young, 1973), we have proposed (Herculano-Houzel et al., 2006, 2011a) that both the universal scaling of brain structure mass with number of glial cells and of the glia/neuron ratio with neuronal density result from the same mechanism: the generation of glial cells (with very little variation in average size across species) in numbers that are regulated by the size of the neuronal parenchyma, that is, the volume of tissue composed almost exclusively by neuronal cell bodies and arborizations, that is invaded by glial precursors in early postnatal development (Bandeira et al., 2009; Sauvageot and Stiles, 2002). Glial precursor proliferation is density-dependent and ceases once a steady-state glial density has been achieved, most likely by cell-cell contact inhibition (Hughes et al., 2013; Zhang and Miller, 1996), such that astrocytes, oligodendrocytes and microglia are tiled in territories that do not overlap with other cells of the same type (Hughes et al., 2013; Nedergaard et al., 2003). In this scenario, continued gliogenesis until confluency would yield similar numbers of glial cells in similar volumes of brain tissue, regardless of its number of neurons, neuronal density, location in the brain, or species. At the same time, because of the very small variation in average glial cell size in the face of very large variation in average neuronal cell size, the glia/neuron ratio in each structure would depend simply on the average size of the neurons that compose the initial parenchyma. As a result, those structures with large neurons will, by this mechanism, have large glia/neuron ratios, while those with small neurons will accordingly have small glia/neuron ratios, regardless of the structure or species (Fig. 8).

Implications for Brain Evolution

As reviewed above, the glia/neuron ratio is found to vary not with brain size, but most likely as a direct function of average neuronal size, with more glial cells per neuron as the latter become larger; however, this seems to happen not due to the supposed increase in the metabolic requirements of larger

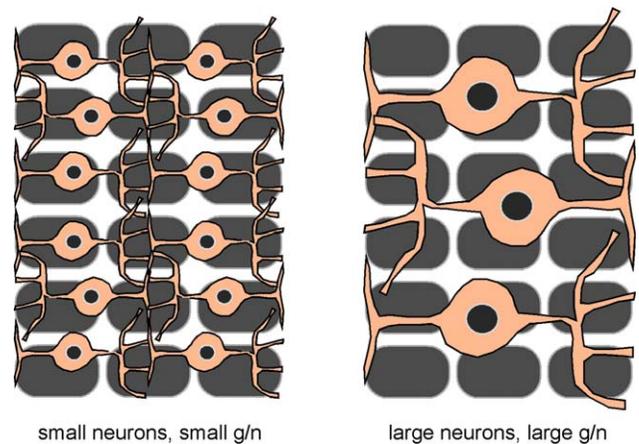


FIGURE 8: Glia/neuron ratio scales with average neuronal size. The scheme depicts two identical volumes of brain tissue which have similar glial cell densities (dark grey) and different neuronal cells densities owing to the different average neuronal cell size (orange). Because glial cells are proposed to occupy the tissue homogeneously and to vary little in average cell mass together with brain size, increases in average neuronal size either across structures or across species will result in corresponding increases in the glia/neuron ratio. Whether this principle applies to human or other large primate brains as well, remains to be determined (see text).

neurons (given that larger neurons were found not to cost more energy), but due to simple mechanical limitations to glial cell proliferation during development in the face of a large variation in average neuronal size. Remarkably, the glia/neuron ratio varies uniformly with neuronal density across brain structures and all mammalian species examined so far, which include a large number of rodents, primates, and insectivores, but also a handful of cetaceans, carnivores, and afrotheria (see Fig. 5). This uniform variation with neuronal density implies that the mechanism that regulates the addition of glial cells to the neuronal parenchyma, which gives rise to the glia/neuron ratio, has been preserved in evolution (Herculano-Houzel, 2011a). Indeed, the shared scaling of brain size with numbers of glial cells suggests that the glial characteristics that apply today to extant brains were present in the common ancestor to the current 28 species in our dataset, over 90 million years ago, and possibly already in the last common ancestor that gave rise to mammals, about 230 million years ago (Murphy et al., 2004).

The evolutionary implications of the clade (that is, evolutionary group)- and structure-specific neuronal scaling rules with putatively universal glial scaling rules are intriguing: in mammalian brain evolution, it appears that neurons have been largely free to vary in size across structures and species, while glial cells have not. Indeed, however variable in their morphology (Barres, 2008; Walz, 2000), astrocytes do not quite vary in size across species or even across structures,

maintaining very similar properties among mammals (Mishima and Hirase, 2010; Picker et al., 1981; but see Oberheim et al., 2009, below), and even in amphibia (Kuffler et al., 1966). We do predict a small 1.4-fold variation in average glial cell size across the species examined so far (in contrast to the 260-fold predicted variation in average neuronal cell size), and this variation should correlate with variations in average neuronal cell size (Mota and Herculano-Houzel, unpublished data).

Importantly, our findings do not imply that there is no variation in size in particular glial cell types, nor that average glial cell size is constant, but rather that, on average, glial cell size varies far less than average neuronal cell size. This possibility must now be addressed separately for astrocytes, oligodendrocytes, and microglial cells. As mentioned above, “glial cells” must no longer be read as “astrocytes,” given that oligodendrocytes turn out to be the large majority of glial cells in the human cortical grey matter (Pelvig et al., 2008). It remains to be determined whether this proportion also applies to the cerebral cortex and other brain structures in species other than humans. Given the very different proportions of astrocytes, oligodendrocytes, and microglia in the parenchyma, it remains possible that particular less common glial cell types increase more markedly in size than others, such that the end result is an only modest increase in average glial cell size. This would be the case, for instance, if oligodendrocytes retained a similar average across structures and species, while astrocytes (which are only 20% of all glial cells in human cerebral cortical grey matter; Pelvig et al., 2008) varied in size across structures and species.

Even if particular glial cell types (or even astrocytes as a whole) are found to vary in size more than others, the relatively small estimated variation (compared with neurons) in average glial cell size across brain structures and species found across mammalian brains of different species and spanning several orders of magnitude in size is compatible with the possibility that, in all structures and species, oligodendrocyte and their precursor cells are distributed in unique territories (Hughes et al., 2013), similarly to astrocytes, which are homogeneously distributed within the grey matter and occupy the entire parenchyma dividing it into polyhedral territories of similar volume (Bushong et al., 2002; Halassa et al. 2007; Nedergaard et al., 2003; Ogata and Kosaka, 2002). The small variation in glial cell density across structures and species is also consistent with the observation that grey matter astrocytes in the cerebral cortex and hippocampus are morphologically and electrophysiologically homogeneous (Mishima and Hirase, 2010). The overall evolutionary stability of glial structure and function proposed here is in agreement with the intricate functional and metabolic interactions between neurons and glia that have been found to apply to human and

rat brains alike (Magistretti et al., 1999; Shen et al., 1999; Sibson et al., 1998). Taken together, these observations indicate that glial cell evolution has been severely constrained, which in turn suggests that glial cells as a whole perform such a fundamental job that their structure and function can hardly be altered—or, at least, hardly in the same extent as neuronal structure and function are found to change.

Human-Exclusive Astrocytes?

In contrast to our prediction that glial cells as a whole vary little in size across structures and species, recent studies indicate that human astrocytes differ from astrocytes in other primates as well as in rodents. However, one must always be cautious to separate putative human-exclusive characteristics from those that might be expected for a large primate brain of comparable size (in other words, would a primate brain of the same weight and volume as a human brain be organized in the same manner as a human brain?). Human protoplasmic astrocytes were found to be dramatically larger in diameter, by a factor of 2.6, and more complex (10× more primary processes) than rodent astrocytes (Oberheim et al., 2009). The larger diameter and more numerous processes mean that human protoplasmic astrocytes occupy a 16.5-fold greater volume than their mouse counterparts, and suggests that human protoplasmic astrocytes have “domains” that cover up to 2 million synapses, *versus* 120,000 synapses in the mouse, a more than 10-fold increase (Oberheim et al., 2009). White matter astrocytes, or fibrous astrocytes, in human brain were also more than twofold larger in diameter than mouse fibrous astrocytes. However, great apes had protoplasmic and fibrous astrocytes that were also larger than in rodent cortex, although not as large as in the human cortex. Additionally, both human and great apes had astrocyte types not found in the rodent cortex, called interlaminar and varicose projection astrocytes. The finding of similar specialized astrocyte types in great apes and human suggests that these cells might be related to particular properties of large brains, primate or not, for example related to vascularization demands, although there is a growing suspicion that the differences portend the addition of novel functions (see below). It will be exciting to investigate the characteristics of astrocytes in other large, non-primate brains in the future, to clarify what features of astrocytes are related to brain size or to evolutionary group, and how, and to determine if any seemingly unique features in the human brain are indeed human-exclusive, or are expected for a primate brain of human proportions. A more recent study by the same group suggests that human astrocytes retain their different characteristics when grafted onto the mouse brain early in development, and actually improve LTP and learning in the recipient animals (Han et al., 2013). While this study clearly shows that human astrocytes have

different functional properties from mouse astrocytes, it remains to be determined whether a similar LTP improvement is also obtained with grafting astrocytes from other primate species, that is, whether LTP improvement is due to astrocytic features that are characteristic of primates as a whole, or of humans exclusively.

Implications for Brain Physiology

The lack of a correlation between the glia/neuron ratio and the metabolic requirement of neurons of different sizes (Herculano-Houzel, 2011b) does not mean that glial cells are not involved in the regulation of metabolism. Indeed, there is ample evidence that they are, both for astrocytes (Magistretti, 2006) and oligodendrocytes, which have been shown to support the energetic needs of axons in their territory (Fünfschilling et al., 2012; Lee et al., 2012). The lack of correlation between G/N and estimated average energy use per neuron only means that the function of glial cells is probably not to provide “more energy to larger neurons,” given that larger neurons on average were not found to use more energy. Moreover, the evolutionary preservation of the mechanism that regulates the addition of glial cells to the brain indicates that the functions of astrocytes and oligodendrocytes are not only fundamental for brain physiology, but also may impose constraints to it.

One likely such physiological constraint would result from the small variation in the density of glial cells, which are thus presumably organized into glial territories of similar size across brain structures and species. If glial cells not only instruct synapse formation (Ullian et al., 2001) but also provide metabolic support to synaptic activity (Magistretti, 2006), then a consequence of the small variation in glial cell density would be an also small variation in the density of synapses per volume of the parenchyma, irrespectively of neuronal density. The limited evidence in the literature suggests that synaptic densities in the cerebral cortex indeed vary little across species (Beaulieu and Colonnier, 1985; Cragg, 1967; Schuez and Demianenko, 1995; Schuez and Palm, 1989)³. In the case that synaptic densities are constant per volume of tissue, this would imply that the number of synapses per neuron increases with increasing neuronal size (Schuez and Demianenko, 1995)—which, given the constraint of a fixed energy budget per neuron, independent of neuronal size (Herculano-Houzel, 2011b), the obligatory metabolic cost of increased neuronal membrane, and the metabolic cost of synaptic activ-

³Additionally, in the case that synaptic density does not scale significantly with brain size, total numbers of synapses would scale proportionately with brain mass, and be for instance larger in the elephant than in the human brain—and thus would not explain the differences in cognitive abilities across species of similar brain mass (Herculano-Houzel, 2011c).

ity (Attwell and Laughlin, 2001), would result in a necessary reduction of rates of excitatory synaptic transmission in larger neurons, as suggested originally by Karbowski (2007, 2009), that is, the enforcement of sparse coding with increasing average neuronal size (Herculano-Houzel, 2011b). Additionally, synaptic homeostasis (Turrigiano, 2008) and elimination of excess synapses (including the decrease in synaptic markers during sleep; Gilestro et al., 2009), the bases of synaptic plasticity, might thus be necessary consequences of such a trade-off imposed by the constrained neuronal energetic expenditure (Herculano-Houzel, 2011b).

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