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Astrocytic complexity distinguishes the human brain

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One of the most distinguishing features of the adult human brain is the complexity and diversity of its cortical astrocytes. Human protoplasmic astrocytes manifest a threefold larger diameter and have tenfold more primary processes than those of rodents. In all mammals, protoplasmic astrocytes are organized into spatially non-overlapping domains that encompass both neurons and vasculature. Yet unique to humans and primates are additional populations of layer 1 interlaminar astrocytes that extend long (millimeter) fibers, and layer 5–6 polarized astrocytes that also project distinctive long processes. We propose that human cortical evolution has been accompanied by increasing complexity in the form and function of astrocytes, which reflects an expansion of their functional roles in synaptic modulation and cortical circuitry.

Functional pleiotropy of astrocytes

Astrocytes have become the focus of much attention in the past decade. In addition to their roles in many of the supportive functions of the brain – such as ion and water homeostasis, neurotransmitter production, removal and breakdown, and blood–brain barrier maintenance [1–4] – new functions are beginning to emerge. Astrocytes have been shown to be involved in the regulation of blood flow, proliferation of stem cells, and determination of synaptic number [5–10]. Furthermore, abundant evidence now supports the notion that astrocytes are actively involved in synaptic transmission in most brain regions. Although astrocytes are not themselves electrically excitable, they release transmitters, triggered by increases in cytosolic Ca^{2+} concentrations ($[Ca^{2+}]$), that modulate the activity of neighboring cells, including both neurons and other glia [11]. Astrocytes express a large number of primarily metabotropic receptors that mobilize intracellular Ca^{2+} stores in a phospholipase C (PLC)- and inositol (1,4,5)-trisphosphate [IP_3 or Ins(1,4,5)P_3]-dependent fashion [1]. Astrocytes are thereby able to respond to neuronal activity in a receptor-dependent fashion, and in return can modulate synaptic transmission by transmitter release, thereby permitting feedback control of neuronal activity levels [1]. Astrocytes release glutamate, serine and ATP, and possibly other transmitters, that might regulate the activity of surrounding neurons [12–15]. However, the nature and net effect of such astrocytic regulation can be difficult to predict. For instance, whereas ATP is typically an excitatory transmitter linked to opening of cation channels [16], its metabolic product adenosine is a powerful inhibitor of excitatory transmission; this suggests that astrocytes might exert both excitatory and inhibitory effects upon neurons within their functional domains [13,17]. Similarly, glutamate can be released from astrocytes in response to environmental stressors such as hypoosmolarity and reduction of interstitial $[Ca^{2+}]$, and as such can contribute not only to the regulation of neuronal firing by astrocytes, but also to glial-initiated excitotoxic neuronal death in ischemia and head trauma [18–22]. Thus, the influence of astrocytes upon neuronal function might extend to encompass entire neural networks, such that astrocytic activity is intimately associated with long-term potentiation (LTP) and long-term depression (LTD), in addition to synaptic scaling, in which the strengths of all synapses on a single cell are adjusted [12–15].

In addition to modulating local intracortical neuronal activity, astrocytes can also regulate the effects of sensorimotor neurotransmission. For instance, astrocytes in the barrel cortex responded to whisker stimulation with dynamic, oscillatory increases in intracellular $[Ca^{2+}]$ [23]. This sensation-associated astrocytic activation seemed to be mediated by the synaptic spillover of glutamate, which mobilized intracellular astrocytic Ca^{2+} stores in a metabotropic glutamate (mGlu)-receptor-dependent fashion [23]; this triggered the release of metabolic products of cyclooxygenase 1 (COX-1), which initiated vasodilatation and increased blood flow to the barrel cortex [7,23]. It remains unclear whether sensation-associated astrocytic Ca^{2+} signaling leads to the release of glial transmitters; if so, then astrocytic signaling might prolong local synaptic activation, and thereby directly modulate sensory processing.

Besides the wide range of functions in normal physiology, astrocytes take on additional roles in and as a consequence of disease. Reactive astrocytosis, as manifested by characteristic alterations in glial filament expression and organization, is associated with a wide variety of brain pathologies, including traumatic, ischemic, neurodegenerative and genetic disorders in humans [24]. Functional changes in reactive astrocytes have been implicated in both primary and secondary epilepsies; these include changes in K^+ buffering, aquaporin 4 expression and localization, and AMPA receptor expression and

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function [25]. In fact, astrocytes can release glutamate, triggering spontaneous depolarizations of groups of neurons resulting in hypersynchronous firing or seizure activity in several experimental seizure models [22]. Astrocytes also seem to have prominent roles in the neurodegenerative disorders, which are often characterized by distinctive local astrocytosis. In Alzheimer's disease, reactive astrocytes accumulate amyloid β peptide (Aβ) to an extent proportionate to the severity of the disease [26]. Recent studies suggest that astrocytes overburdened with Aβ actually undergo lysis, leading to astrocyte-derived senile plaques [27]. Similarly, amyotrophic lateral sclerosis (ALS), which is caused by the selective degeneration of motor neurons, has been linked to the dysfunction of astrocytic glutamate transporters [28]. In each of these cases, current data suggest that reactive and diseased astrocytes might directly contribute to the neuronal loss that typifies these disorders.

Astrocytes are organized in domains

Cajal first observed the microarchitecture of human cortical astrocytes, using Golgi-stained neocortical sections that revealed protoplasmic astrocytes [29]. He noted the close relationship of astrocytes to both the vasculature and neuronal cell bodies and dendrites. Cajal also observed that the processes of two adjacent cells typically overlapped only slightly, suggesting that what we now know as protoplasmic astrocytes – initially termed independent stellate cells – delineated distinct domains within the neuropil [29]. This was in contrast to his original observations of fibrous astrocytes of the white matter, whose processes he noted interdigitated extensively yielding dense networks of intertwined fibers [29]. Cajal's original drawings from 1897 suggested that the organization of protoplasmic astrocytes distinguished the cortical gray matter. More recently, two groups used dye injection in adult rodents to show that the expression of glial fibrillary acidic protein (GFAP), which gave the astrocytes its name 'star-like', reflected only 15% of the total volume of the cell [30,31]. Protoplasmic astrocytes contain numerous GFAP-negative leaflet processes that together subtend a polyhedral space of remarkable uniformity. These leaflet processes wrap and envelop blood vessels and synapses alike. Dye injection of two adjacent astrocytes revealed that the percentage of overlap between two astrocytes is 4.6% of the total volume of the cell [31] and that rodent astrocytes in the hippocampus are organized in essentially non-overlapping domains, with limited interaction solely at the borders between cells. The fact that the astrocytic domains are most clearly defined in areas of high synaptic density, including hippocampus and cortex, suggests that the domain organization might be important for astrocytes as modulators of synaptic transmission [13,32]. An astrocyte domain defines a contiguous cohort of synapses that interacts exclusively with a single astrocyte. Synapses within a particular territory are thereby linked via a shared astrocyte partner, independent of neuronal networking. It remains to be defined how the strict organization in domains affects the function of astrocytes, but the ability of astrocytes to sense neuronal activity and in turn release gliotransmitters creates a new dimension of communication that might participate in processing of local activity independent of synaptic transmission.

Diversity of human astrocytes

The manifold functions of mammalian cortical astrocytes suggest the opportunity for functional specializations unique to given groups of species. We have found that this is indeed the case, because human astrocytes exhibit both structural specializations and functional competencies not shared by their counterparts in other primates. Indeed, more than a century after Cajal's work, new imaging technology enables refined and extensive appreciation of how the structure and organization of human astrocytes compare with those of other mammals [33–35]. Human cortical astrocytes are characterized by high cortical GFAP expression that increases with age [36] (N.A. Oberheim *et al.,* unpublished; Figure 1). There are at least four distinct morphologies of GFAP-positive cells within the human cortex, compared with two in the rodent.

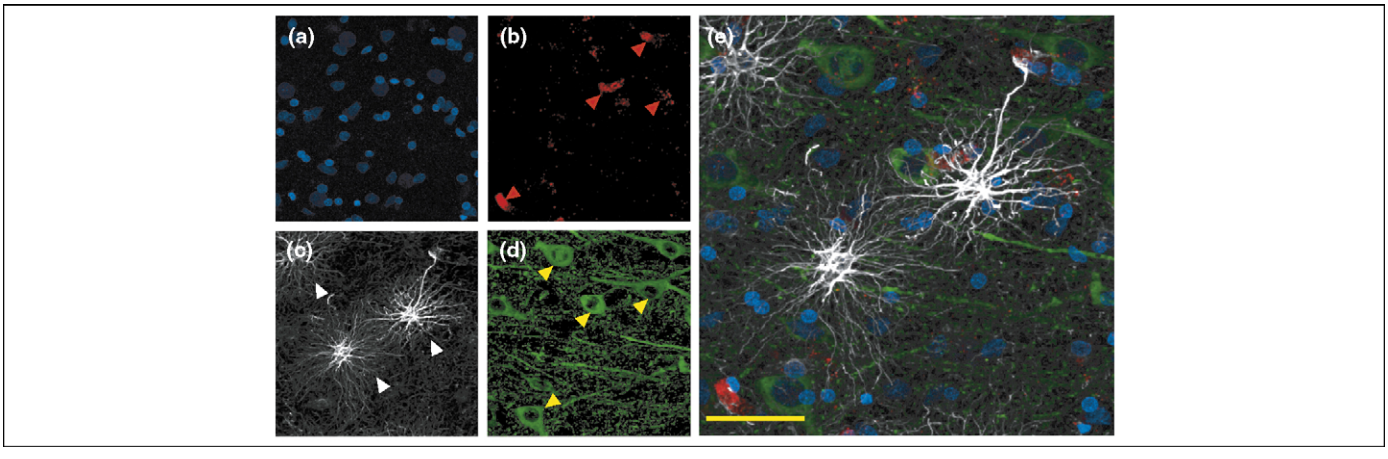

Figure 1. Human cortical astrocytes are organized in domains. Human temporal lobe cortex is shown immunostained for **(a)** nuclei using 4′,6-diamidino-2-phenylindole (DAPI, blue), **(b)** vasculature using antibodies to aquaporin 4 (AQ4, red), **(c)** astrocytes using antibodies to GFAP (white), and **(d)** neurons using antibodies to microtubule-associated protein 2 (MAP2, green); these four images are overlaid in **(e)**. Red arrowheads indicate four small vessels (AQ4), white arrowheads indicate astrocytes (GFAP) and yellow arrowheads indicate neuronal cell bodies (MAP2). The protoplasmic main GFAP-positive processes of astrocytes do not overlap. Each astrocyte occupies its own anatomical space, with neuronal cell bodies and capillaries organized within the processes or domain of the human astrocytes. Some vascular processes do not respect the domains, owing to the sharing of vessels between astrocytes. Scale bar, 50 μm.

Human protoplasmic astrocytes

The most abundant type of astrocyte in humans, the protoplasmic astrocyte, resides in the deeper layers of the cortex (layers 2–6) and, as in rodents, the majority of the GFAP-positive processes of protoplasmic astrocytes in the human cortex do not overlap (Figure 1). This cytoarchitectural organization in which there is minimal contact between cells indicates a domain organization. For example, within one domain of a human cortical astrocyte processes can extend and cover five different blood vessels, eight neuronal cell bodies and numerous synapses [29] (Figure 1). The exact number of neurons and blood vessels within the domain of a protoplasmic astrocyte (based on GFAP morphology) depends on the density of neurons and vasculature within the specific cortical layer. This organization places the protoplasmic astrocyte in a central position for coordination of, for example, increased blood flow in response to increased synaptic activity [7]. Furthermore, astrocytes are highly coupled by gap junctions composed primarily of connexin 43 [37,38]. An interesting feature of astrocytic gap junctions is the large number of autocellular junctions, which are gap junctions that link processes from the same cell [38,39]. The function of these junctions is not clear, but connexins have adhesive properties and autocellular gap junction might stabilize the complex network of astrocytic processes [39]. Autocellular gap junctions might also facilitate intracellular diffusion of energy metabolites and possible signaling molecules, such as IP_3, between fine astrocytic processes [39]. Another important aspect of connexin function in astrocytes is the presence of unopposed hemichannels [18,38]. Astrocytes are able to communicate and coordinate activity within and around their domain by regulated release of ATP and glutamate through hemichannels, in addition to the other pathways of gliotransmitter release [13,18,20,38].

Humans have evolved protoplasmic astrocytes that are not only larger but also far more elaborate than their rodent counterparts [29] (Figure 2). Rodent cortical protoplasmic astrocytes are ~30–60 μm in diameter, consisting of three or four primary GFAP-positive processes that branch distantly. These cells can be polyhedral with their cell body centrally located, or more polarized with their processes oriented in one direction. These astrocytes usually reside near blood vessels, with the majority of their processes contributing to the blood–brain barrier. In rodents, the GFAP skeletons of protoplasmic astrocytes are rarely symmetrical. By contrast, human protoplasmic astrocytes are highly symmetrical, and remarkably larger and more complex (Figure 2). Although the cell body of human astrocytes is only ~10 μm in diameter, their processes span 100–200 μm, giving them a 27-fold greater volume than their rodent counterparts. On average, human astrocytes extend 40 large GFAP-positive processes radially and symmetrically in all directions from the soma (Figure 2). The tenfold increase in number of processes not only increases the size of the astrocyte domain, but also permits the radial symmetry of protoplasmic human cortical astrocyte. It has been estimated that the volume of a domain of a rat astrocyte is 66 000 μm^3 [30] and the synaptic density within the cortex of a rat has been estimated to be 1397 million synapses mm^{-3} [40].

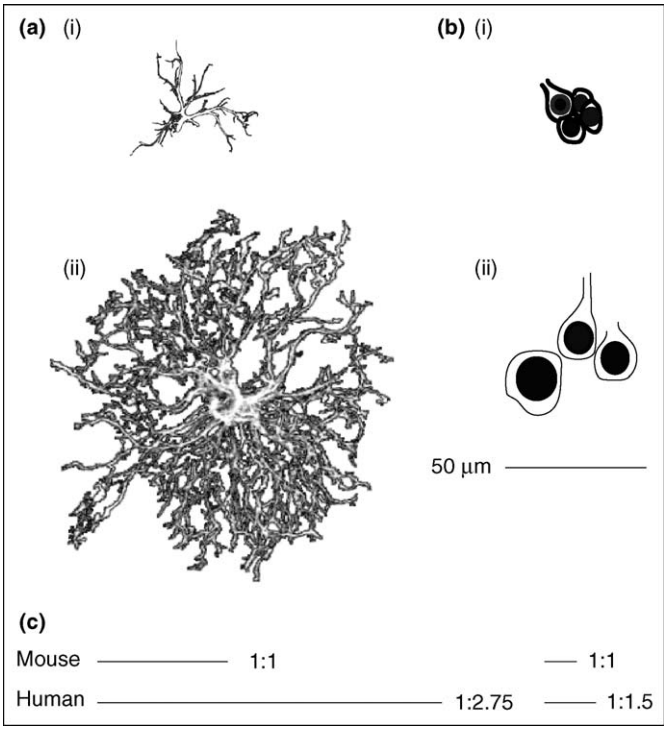

Figure 2. Evolution of astrocytes and neurons. **(a)** Graphical representation and GFAP immunostaining of mouse (i) and human (ii) cortical astrocytes. **(b)** Graphical representation and MAP2 immunostaining of mouse (i) and human (ii) cortical neurons. **(c)** Bars illustrating the sizes of human astrocytes (left) and neurons (right) relative to the sizes of these cells in mice. Human cortical astrocytes are almost threefold larger, have approximately tenfold more GFAP-positive processes, and are more symmetrical than mouse astrocytes. The increase in complexity and size of astrocytes from mouse to man is disproportionate to the evolution of neuronal structure, possibly reflecting the increasing importance of astrocytes in the brain function of higher organisms.

Therefore, within the domain of a single rodent astrocyte, there are ~90 000 synapses, all covered by the processes of that astrocyte. The synaptic density of the human cortex is ~1100 million synapses mm^{-3} [40]. Taking into account the increase in size of protoplasmic astrocytes that accompanies this increased synaptic density, we can estimate that each astrocyte supports and modulates the function of roughly two million synapses. That being said, the importance of domain organization to the coordination of activity among these synapses remains unexplored. In addition, it is unclear whether a higher level of organization among astrocytic domains exists within functionally defined regions, such as within the barrel cortex.

Several authors have examined the relative numbers of glia and neurons in adult cortices, and have reported that the ratio of glia to neurons differs between species. In general, the ratio increases as a function of brain size, and humans have a higher ratio of glia to neurons than most other species [41–44]. These classic histological assessments were based on nuclear counts, which typically included microglia and glial progenitors, in addition to protoplasmic astrocytes [41–45]; thus, the ratio of glia to neurons is greater in humans than in rodents, irrespective of the disproportionate increase in the size of human protoplasmic astrocytes.

Human interlaminar astrocytes

Protoplasmic astrocytes are not the only glia that have evolved with phylogeny. In layer 1 of the primate cortex, an

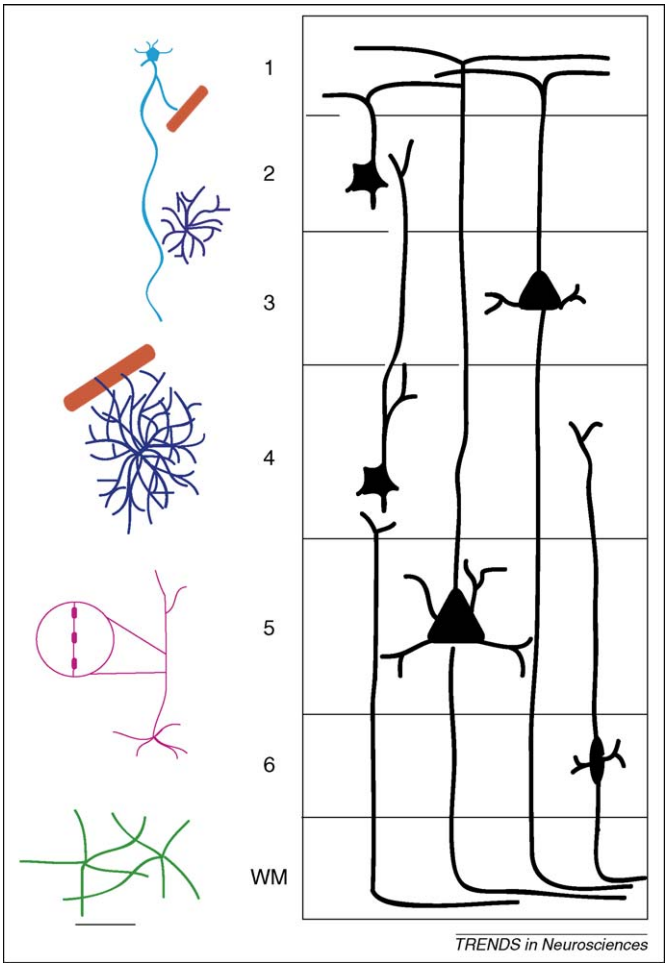

Figure 3. Distinct classes of human astrocytes (left) are located within different layers of the cortex (right). Primate-specific interlaminar astrocytes (light blue) are located in layer 1 and send long fibers that extend throughout the cortex terminating in layers 3 and 4. Protoplasmic astrocytes (dark blue) characteristically inhabit layers 2–6 and vary in size (shown here in layers 2 and 4). Protoplasmic astrocytes are organized into domains associated with neurons and blood vessels (red). Polarized astrocytes (pink) also extend long processes, but are found in layers 5–6 rather than near the pia and have varicosities along their processes (inset). Fibrous astrocytes (green) reside in the white matter (WM) and are not organized into domains. Scale bar, 100 μm.

area devoid of neuronal cells bodies but highly enriched with synapses, there are interlaminar astrocytes first described by Andriezen and Retzius in the 1890s [46,47] (Figure 3). These primate-specific cells extend striking long, frequently unbranched processes throughout the layers of the cortex, terminating in either layer 3 or 4 [35] (Figure 4). The cell bodies of these astrocytes are ~10 μm in diameter and extend two types of processes: three to six fibers that contribute to the astrocytic network near the pial surface, and another one or two that penetrate deeper layers of the cortex (Figure 4). The latter have a constant diameter, and can extend up to 1 mm in length [35] (N.A. Oberheim *et al.*, unpublished). These processes are tortuous and, although largely unbranched, occasionally send collaterals to the vasculature [35] (Figure 4). Work by Colombo and colleagues demonstrated that the endings of these interlaminar fibers deep in the cortex might be in the form of a 'terminal mass' or end bulb, containing a multilaminar structure and mitochondria [48] (Figure 4). Colombo *et al.* found that in the *Caboidea* monkey, the interlaminar fibers are short and sparse at one month of age, and increase in length and number throughout development [49]. These investigators also noted analogous astrocytes with long and numerous interlaminar fibers in a ten-day-old human infant [49], indicating the early appearance of this phenotype in humans.

The interlaminar astrocytes comprise one of the most striking differences between the cortices of primates and those of other species; nonetheless their function remains unknown (Box 1). Their interlaminar fibers clearly violate the domain organization and might serve as a non-synaptic pathway for long-distance signaling and integration of activity within cortical columns. Interestingly, the interlaminar fibers are altered in several pathologies including Down's syndrome and Alzheimer's disease, in which the number of the interlaminar processes decreases with disease progression [50,51]. Additional studies are needed to define more clearly both the developmental history and normal roles of these interesting astrocytes.

Human polarized astrocytes

A third type of human astrocyte is the polarized astrocyte (Figure 3). These essentially unipolar cells reside in the deep layers of the cortex, near the white matter, and

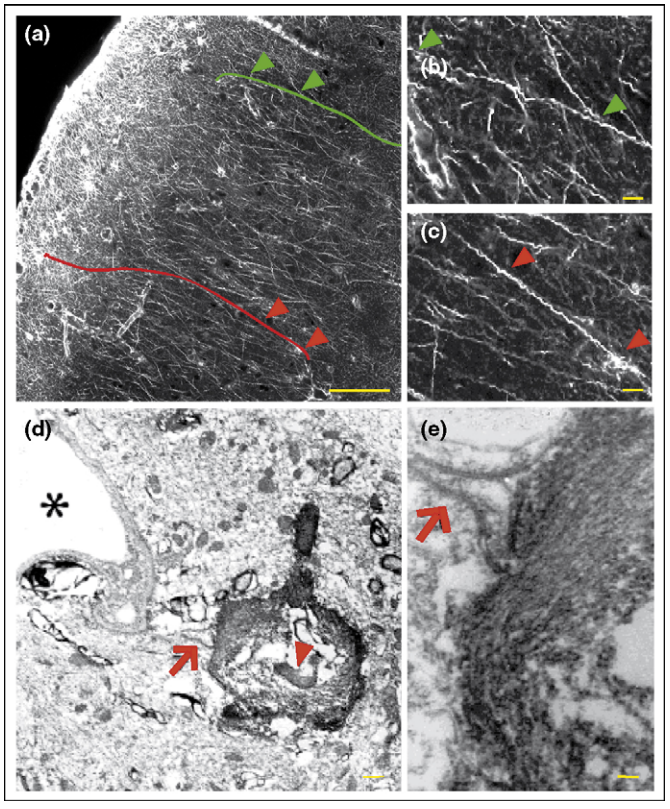

Figure 4. Primate-specific interlaminar astrocytes. **(a)** Layers 1 and 2 of human cortex stained with GFAP. Numerous GFAP-positive interlaminar astrocytes in layer 1 extend long fibers into the deeper layers of the cortex. Two examples of interlaminar fibers are traced in green and red. Arrowheads indicate landmarks for orientation in panels (b,c). Scale bar, 100 μm. (b,c) Higher magnification images of the fibers traced in green **(b)** and red **(c)** in (a). Scale bar, 10 μm. These fibers are characterized by their tortuosity; they do not respect domain organization and terminate both in the interstitial space and on blood vessels. **(d)** Electron microscopy illustrating a club-like ending of an interlaminar fiber in close proximity to a blood vessel (asterisk). The arrowhead indicates a mitochondrion; the arrow indicates the bridge between a bulbous ending and perivascular elements. Scale bar, 2 μm. **(e)** Higher magnification of (d); scale bar 200 nm [51].

Box 1. Unanswered questions

- What is the functional significance of the astrocyte domain organization?
- What is the evolutionary advantage of the interlaminar fibers?
  Long-distance communication?
  A role in columnar organization?
- What is the function of the polarized astrocyte?
  Rapid communication?
  A connection between astrocyte networks?
  A link between gray and white matter?
- What is the significance of the loss of astrocyte domain organization in pathology?

extend one or two long (up to 1 mm in length) GFAP-positive processes away from the white matter [52] (N.A. Oberheim *et al.,* unpublished; Figure 3). These long processes are frequently unbranched or branch once, are a constant diameter of ~2–3 μm, and have numerous 'beads' or varicosities. The long fibers of polarized astrocytes are, as opposed to the tortuous astrocyte interlaminar fibers, straight, and the overall appearance of the cell is more neuronal than astrocytic, despite their intense GFAP-labeling. Occasionally, polarized astrocytes extend processes to the vasculature, but most terminate in the neuropil [52]. Another noticeable difference is that the shorter GFAP-positive processes that emerge from polarized astrocytes are straighter and less branched than the fine laminate processes of neighboring protoplasmic astrocytes, perhaps indicating that polarized astrocytes make only sparse synaptic contacts. The polarized astrocytes do not respect the domain boundaries of their neighbors, because the long processes from these cells travel directly through other protoplasmic astrocyte domains. These cells are relatively uncommon, and tend to reside near the white matter in layers 5 and 6; their functions are unknown [52] (Box 1). The electrophysiological properties of polarized astrocytes have not been investigated, but these cells might, similar to interlaminar astrocytes, serve as an alternative pathway for long-distance communication across cortical layers, perhaps forming links between functionally related domains in different laminae, or between white and gray matter.

Human fibrous astrocytes

Fibrous astrocytes of the white matter represent a fourth major class of astrocytes that are arguably the least distinguished between primates and non-primate mammals [52] (Figure 3). Fibrous astrocytes are less complex than protoplasmic astrocytes, and have fewer primary GFAP-positive processes. Their fibers are straighter and less branched than those of other glia [52,53]. In contrast to the non-overlapping domain structure of protoplasmic astrocytes, and the respect exhibited by their processes for those of their neighbors, the processes of adjacent fibrous astrocytes intermingle and overlap [29,52,53]. Yet despite the overlap of their fiber arbors, the cells are found roughly equidistant from one another, suggesting that signals dictate the relatively homogeneous dispersal of these cells within the white matter parenchyma. Fibrous astrocytes are perhaps those that best fit into the traditional supportive role of astrocytes; their simple morphology and relative uniformity suggest that their functions might be limited to metabolic support, and not extend to information processing and modulation of neural activity.

Phylogenetic changes of human neurons

The increased complexity of cortical astrocytes contrasts with the relatively limited changes to individual cortical neurons during phylogeny [54,55] (Figure 2). Nonetheless, some differences in the relative representations of cortical neuronal phenotypes have been observed. For instance, the proportion of inhibitory interneurons among all neurons is 15% but 25% in some areas of the primate cortex [40]. This in part could be due to the emergence of the double-bouquet cells in carnivores, primates and humans [40]. Double-bouquet cells are neocortical GABAergic interneurons, characterized by a long axon that descends across several cortical layers and extends many inhibitory synapses onto pyramidal neurons in multiple layers within a small cortical column [56]. They are absent in rodents and relatively sparse in carnivores but abundant in primates, occurring in a regular pattern resulting in what is thought to be a microcolumnar organization [57]. Their relationship to the primate-specific astrocytes that traverse cortical layers remains to be elucidated.

Another relatively subtle difference is the presence of spindle-shaped projection neurons in the anterior cingulate cortex of humans and great apes, but not other primates [58]. These cells are larger than surrounding pyramidal cells and have only a single basal dendrite, giving them a spindle shape [58]. These neurons are localized in small clusters in layer 5 only, and their function is unknown [58]. They might contribute to the increased processing power of the human brain, but their limited representation does not argue for key role.

It has also been suggested that species that are more evolutionarily advanced have a greater number of dendritic spines, thereby increasing connectivity [59]. However, the tree shrew, a non-primate mammal, has a larger number of dendritic spines in corresponding cortical areas than humans [60]. In fact, the density of synapses in the cortex of an adult rat is ~1397 million synapses mm^{-3} [40]. Interestingly, using similar techniques, the synaptic density of human frontal, temporal and parietal cortex is calculated to be 1094 million synapses mm^{-3} [40]. Furthermore, there is no difference in the percentages of asymmetric versus symmetric synapse per neuron, or in the density, proportion and sizes of synapses, in mice, rats and humans. Neither do the numbers of synapses per neuron increase across these species [40]. Therefore, it is unlikely that the synaptic density itself has contributed to the increased capacity of the human brain.

In the human brain, axons are clearly longer and dendritic arbors can be more complex than in the brains of other species [40,54]. However, electrophysiological analyses show that neuronal properties are remarkably similar across species; such properties include the resting potential, the input resistance, the rise time, fall time and duration of action potentials, the current characteristics and the after potential [61,62]. Synaptic

mechanisms, such as generation of excitatory and inhibitory postsynaptic potentials are also highly conserved, with glutamate as the principle excitatory transmitter throughout evolution [62]. By contrast, in one study the electrophysiological properties of human astrocytes were found to be highly variable, although a direct comparison with rodent astrocytes was not possible owing to a limited number of samples [63].

Evolution of the functional glioneuronal unit

Although the intellectual capacity of humans exceeds that of other species, it has proven difficult to define the structural basis for the unique properties of the human brain. Size no doubt matters, but it is clearly not the defining factor, as is apparent from the larger brains of several other mammals [64]. The diversity of cortical neurons also fails to address the unique features of the human brain, because the variety and density of cortical neuronal phenotypes have remained largely constant throughout mammalian evolution [40]. Indeed, it seems unlikely that the increased functional competence of the human brain can be attributed to any discrete aspect of neuronal number, form or function. In that context, the highly conserved morphological and electrophysiological properties of neurons throughout evolution present a stark contrast to the remarkable increase in complexity and size of human protoplasmic astrocytes. On that basis, we propose here that the domain encompassed by a cortical protoplasmic astrocyte creates and defines a glioneuronal functional unit, the complexity and functional importance of which has increased with mammalian evolution. By integrating the activity of a larger contiguous set of synapses, the astrocytic domain might extend the processing power of human brain beyond that of other species. By the same token, one could postulate that the loss of astrocytic domains contributes to pathology in the adult CNS. Reactive astrocytes obey no domain organization, and are associated with a panoply of both acute and chronic neurological diseases. Their overlapping astrocytic processes and loss of exclusive synaptic regulation might profoundly disturb neural function, in ways suggested by the association of reactive glial scars with the genesis of epileptogenic foci. As such, the increasingly complex astrocytic organization and domain architecture of the adult human brain might have vastly increased its functional competencies, albeit perhaps at the expense of providing increased opportunity for structural disruption and concomitant dysfunction.

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