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Astrocyte morphology

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Abstract

Astrocytes are predominant glial cells that tile the central nervous system (CNS). A cardinal feature of astrocytes is their remarkably complex and visually enchanting morphology, referred to as bushy, spongy, and star-like. A central precept of this review is that such complex morphological shapes evolved to allow astrocytes to contact and signal with diverse cells at a range of distances in order to sample, regulate, and contribute to the extracellular milieu, and thus participate widely in cell-cell signaling during physiology and disease. The recent use of improved imaging methods and cell-specific molecular evaluations have revealed new information on the structural organization and molecular underpinnings of astrocyte morphology, the mechanisms of astrocyte morphogenesis, and the contributions to disease states of reduced morphology. These insights have reignited interest in astrocyte morphological complexity as a cornerstone of fundamental glial biology and as a critical substrate for multicellular spatial and physiological interactions in the CNS.

Keywords

glia; Sholl; territory; neuropil; morphology; electron microscopy; imaging

Astrocytes and their multicellular interactions

Astrocytes are fascinating, ubiquitous, and predominant CNS glial cells. They make extensive contacts with neurons and perhaps all other central nervous system (CNS) cell types. Astrocytes represent around 20–40% of the cells in the brain [1, 2], serving a multitude of functions, including ion homeostasis, neurotransmitter clearance, lipid homeostasis, synapse formation/removal, synaptic modulation, and contributions to neurovascular coupling [3, 4]. Astrocytes contribute to the regulation of synapses, neurons, neuronal circuits, and ultimately behaviour [5] and are widely implicated in CNS disease [6–

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11]. Importantly, astrocytes change their molecular programs in specific ways that portend altered functions in a context-dependent manner [12–14]. Furthermore, astrocytes display diversity between and within CNS regions [15, 16]. These studies raise many exciting new questions that are being explored with renewed vigor. Chief among these is how astrocytes perform such varied physiological and pathological responses. We tackle this topic through the lens of their complex and intricate morphology.

Astrocytes were identified historically as a separate cell type because of their unique appearances [17]. Initially called star-like cells, it has become apparent that this descriptor is simplistic and that astrocytes in fact display highly complex morphologies. A central tenet of this review is that complex morphological shapes of astrocytes have evolved to allow them to contact and signal with other cells and cell components at multiple distances (e.g., somata, dendrites, synapses, axons etc.). In this way, astrocytes are able to sample, regulate, and contribute to the extracellular milieu [18], and thus participate widely in cell-cell signaling for physiology and during disease.

In this review, we summarize recent findings concerning astrocyte morphology. The review is broken down into sections that broadly cover (*i*) astrocyte structural organisation at the micro and nanoscale, (*ii*) astrocyte morphogenesis, (*iii*) progress on unmasking the molecular basis of astrocyte morphology, and (*iv*) recent studies concerning astrocyte morphology and mouse models of disease. We restrict ourselves to work from the last few years that has provided new structural or mechanistic insights on these topics. We hope that our review will stimulate discussion, new experiments, and coordinated efforts among researchers to understand astrocyte morphology, its molecular basis, and contributions to pathophysiology.

Although human astrocytes are larger than those in mice, this topic has been reviewed already and is not considered here [19, 20]. We also do not consider astrocyte morphological changes in post mortem tissue of human CNS diseases, because such studies from the last few years do not contain pertinent structural or mechanistic data. Furthermore, several reviews on human astrocytes in disease already exist [6, 7, 9, 10, 21] and there have been few advances germane to our review since. The topic of real time dynamics of astrocyte processes is not considered, because this has been reviewed [22–24].

Astrocyte morphology at the microscopic scale

Each individual mature protoplasmic astrocyte residing in the gray matter comprises a cell body, six or more major branches that emanate from the soma, a thick blood vessel-associated endfoot, and myriad finer branchlets and leaflets that contact synapses (often simply called processes, sheets, or interchangeably as perisynaptic or peripheral astrocyte processes (PAPs; Box 1). In this way, astrocytes take on extraordinarily complex “bushy” or “spongy” appearances (Figure 1). Rodent hippocampal astrocyte territories display diameters of ~40–60 μm and volumes of ~6.6×10^4 μm^3 [25, 26]. About 90–95% of an astrocyte’s surface area is formed by branches, branchlets and leaflets [27]; at best only ~15% can be estimated by GFAP immunostaining [25, 27]. Although diffraction-limited light microscopy is frequently used, it has not been possible to determine how branchlets

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and leaflets emerge from the larger branches or to accurately measure their numbers or dimensions, and thus the organisation of astrocyte morphology has remained mysterious. The reason for this is twofold. First, conventional light microscopy does not have the required spatial resolution necessary to measure objects smaller than the point spread function of the microscope or the ability to resolve them as separate [28]. Second, astrocyte branchlets and leaflets are between 10–100 nm in diameter and thus smaller than the resolving power of light microscopes. However, even with light microscopy it is evident that fine astrocyte processes interdigitate extensively between cellular elements of the neuropil and astrocytes likely contact all CNS cell types in their vicinity [12, 29, 30].

Although astrocytes are highly complex structures, they are not obviously polarised like neurons and their complexity is largely equivalent [31] throughout their shapes that form territories [25]. A territory of a protoplasmic astrocyte has a flattened 2D area of ~1000–2600 μm^2, depending on the brain area [32]. Despite their complexity, astrocyte territories overlap by less than 5% at their edges [25, 33], and in this way astrocytes appear to tile in 2D, and presumably tessellate in 3D, the entire CNS (Figure 1). The only observable polarised astrocyte compartments appear to be endfeet-bearing thicker somatic branches that terminate on blood vessels such that by ~P21 the entire vasculature is enwrapped [34]. In the mouse cortex, 99.8% of astrocytes display at least one such blood vessel contact, with most displaying three [35], meaning that *de facto* vascular contact and highly complex morphology are defining features of protoplasmic astrocytes. Given such anatomy, it is widely held that endfeet and astrocyte processes perform important functions for CNS physiology. In accord, mRNAs and proteins enriched and unique to these compartments have been reported [36, 37].

A variety of methods are available to image and study astrocyte morphology using light microscopy, including intracellular dye iontophoresis [38], diolistics [39], and genetic approaches employing viruses and mice to express membrane-tethered and cytosolic fluorescent proteins [21, 40] and spaghetti monsters [41]. The membrane-tethering Lck domain is often used [31, 42, 43]. However, the widely used marker GFAP should be used with caution, as it only labels the intermediate filaments of some astrocytes and does not reveal their complex morphology [25, 27]. An added advantage of the newer methods is their ability to enable sparse labelling of astrocytes, which is preferable for detailed morphological assessments.

Several metrics to assess astrocytes can be obtained with light microscopy such as to quantify territories (Figure 2A), branching (Figure 2B), neuropil infiltration volumes (Figure 2C) and territory volumes (Figure 2D). None of these measure the 3D structural organisation of astrocytes. Each of these measurements has a distinct purpose, but they all come with caveats. In these regards, 2D projection images from confocal microscopes are the easiest to accomplish (Figure 2A), though the ultimate goal remains accurate high-resolution 3D maps of entire astrocytes. Since the highest achievable lateral X-Y resolution for light microscopy (typically ~200–350 nm) is better than the axial z-axis resolution (typically 500–800 nm) [28, 44], 2D projections avoid greater ambiguities in the z-dimension and instead provide several detailed metrics about the shape of astrocyte territories that can be obtained unambiguously (Figure 2A). Knowledge of the limitations of 2D imaging is important, but

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this does not negate the use of this approach for carefully chosen questions concerning astrocyte territories and their shapes.

3D reconstructions of astrocytes can be used for visualizing astrocyte territory volumes (Figure 2D) and for investigating astrocyte major branching organization and astrocyte tiling in space [45]. However, for measuring astrocyte volumes in 3D we caution that 3D reconstructions can give rise to significant errors in the off-optical axis lowest resolution plane, which are readily observed as “stretching” in the z-axis of the reconstructions. Thus, 3D astrocyte volume estimates from light microscopy (Figure 2C, D) are likely contaminated by the addition of neuropil volume, which is a potential confound when evaluating how astrocyte volumes change experimentally or during disease. Sholl analysis from imaging stacks is useful to capture the overall branching complexity of astrocytes (Figure 2E), but owing to resolution limits mentioned above Sholl analysis cannot assess branching complexity of finer processes such as branchlets and leaflets.

Given these considerations with the use of light microscopy, we advise that the analysis method should be chosen following careful consideration of the underlying question at hand and tailored for the specifics of the experiment and the available instrumentation. Nonetheless, we advocate that all four methods illustrated in Figure 2 are useful to quantify territories, branching, and neuropil infiltration volumes. With the availability of further data, it may be possible to provide a consensus or “recipe” on how to assess astrocyte morphology using light microscopy.

Astrocyte morphology at the nanoscale

While much has been learned about the anatomical properties of neurons and specific axonal, dendritic, and synaptic structures that underlie their function, it remains unclear how astrocytes are anatomically built and structurally organized at the nanoscale [46]. This knowledge is critical to understand fully how these cells communicate with myriad cell types and structures within the CNS including neurons, microglia, oligodendrocytes, and the vasculature [15]. The challenge of studying astrocytic nanostructure and architecture relates to the extreme morphological complexity of astrocytes and the limited approaches currently available to acquire, handle, and analyze large anatomical datasets capable of revealing the nanostructural features and organizing principles of these cells. Astrocytes display astonishing complexity, with thousands of nanoscopic parts giving rise to a bushy, heterogenous appearance when visualized at the light microscopic level (Figure 1). Recent advances in super-resolution light microscopy have improved the ability to visualize small structures of astrocytes in both fixed and living tissues [47]. However, finer resolution methods that go beyond the limits of optical microscopy are needed for more accurate shape and architectural analysis of astrocytes that possess parts that can be less than 10 nm in size. The application of three-dimensional (3D) electron microscopy, or volume EM (vEM) approaches over the last several years has helped to establish a series of ground truths for understanding astrocytic nanostructure. We focus on two recent studies using serial block face scanning electron microscopy (SBF-SEM) [48] or focused ion beam scanning electron microscopy (FIB-SEM) [49] that reveal astrocyte complexity at the nanoscale. A study optimizing FIB-SEM in relation to other electron microscopy methods, including

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SBF-SEM, is available for background information [50]. FIB-SEM is superior in relation to other electron microscopy methods in terms of high isotropic resolution.

SBF-SEM

Unlike neurons that have generally tubular-shaped structures with distinct point-to-point connectivity involving axons, dendrites and synapses, early SBF-SEM showed that astrocytes have a seemingly unpredictable shape throughout their territories and an abundance of nanoscale-sized processes that invade tight spaces between CNS microanatomy [31, 46]. Utilizing SBF-SEM, Aten and colleagues made a series of observations to improve our understanding of astrocytic nanostructure [48]. They accomplished this through examining large portions of astrocytes using SBF-SEM, a vEM approach that offers the advantage of imaging relatively large landscapes of brain tissue at the nanoscale. Aten and colleagues analyzed three partially reconstructed hippocampal CA1 astrocytes from postnatal day 45 with a voxel size of 7.7 nm x 7.7 nm x 75 nm. The striking astrocytic models generated revealed a complex, dendrite-like patterning of astrocytic arbors and an abundance of self-contact points or ‘reflexive’ loops that enable astrocytes to encompass nearby structures such as axons and dendrites. Reconstruction of three neighboring astrocytes allowed for visualization of mutually exclusive territories of astrocytes along with analysis of astrocytic structures at their interface. This demonstrated that the processes from a single astrocyte can make intimate contact with more than one adjacent astrocyte, thereby enhancing the potential for gap junctional coupling. Modeling this multi-astrocytic juxtaposition showed that increased contact points can boost coupling efficiency across an astrocytic network. The near complete reconstruction of a whole astrocyte (~97% completion) revealed that astrocytic coverage of single synapses can be achieved by two separate, neighboring astrocytes, thus indicating that synaptic governance need not be exclusively performed by a single astrocyte. The remarkably few vesicle-like structures in synaptic and non-synaptic areas of astrocytes (~2 vesicle-like structures/μm^3) suggested the absence of clear vesicular release sites within astrocytic compartments that are akin to neurotransmitter release sites in neurons (~612 vesicles/ μm^3 in presynaptic boutons). Astrocytes also contained a dense tubular array of mitochondria that spanned most cellular compartments with exception of the smallest processes, thus potentially affording astrocytes with a network of mitochondria that can produce/distribute ATP and release/buffer Ca^{2+} [51]. This mitochondrial organization agrees with an earlier study that partially reconstructed four astrocytes from Layer VI of somatosensory cortex from postnatal day 14 rat using SBF-SEM (voxel size of 20 nm x 20 nm x 50 nm) and found that astrocytes accumulated long mitochondria and dense endoplasmic reticulum networks near their somata [52]. Thus, studies using SBF-SEM have helped to resolve the nanostructural features of astrocytes, including the specific arrangement of their processes and organelles.

FIB-SEM

One limitation of SBF-SEM is the need to cut relatively thick sections commonly in the 30–75 nm range for vEM. This thickness can be problematic for resolving finer structures that may go undetected or be perceived as disconnected components in reconstructions. Recently, Salmon and colleagues applied high resolution FIB-SEM to identify astrocytic nanostructures from postnatal day 50 mouse somatosensory cortex [49] (Figure 3). While

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FIB-SEM is constrained to smaller specimen blocks, it can more finely section a volume of brain tissue using an ion beam (i.e. < 10 nm resolution in x, y, and z-axes). Salmon and colleagues showed that extremely thin sectioning intervals were needed to reconstruct astrocytic nanoarchitecture with high fidelity. Using a voxel size of 4.13 nm x 4.13 nm x 8 nm, they demonstrated the impact of section thickness in resolving astrocytic nanoarchitecture, with coarser z-sectioning (i.e. >30 nm) leading to loss and disconnection of astrocytic parts. Extending the analysis, they applied 3D quantitative methods to uncover fundamental parts that give rise to astrocytic nanoarchitecture. Astrocytes were deconstructed into constituent parts comprised of connected thin ‘constrictions’, thicker ‘expansions’, and thick ‘core’ regions. These parts were found in structural motifs, with constrictions frequently flanked on both ends by expansions. These motifs may serve to impose structural constraints on intracellular signaling events or Ca^{2+} flux associated with microdomains in astrocytes [53, 54]. Importantly, constrictions, expansions, and cores formed patterned connectivity, ultimately producing wide, but shallow hierarchical networks that initiate at thick cores, bridge to expansion/constrictions, and finish in terminal constrictions. This connectivity pattern was observed across multiple Layer II/III astrocytes from different mice, thus indicating structural consistency among astrocytes. Within the hierarchical structural framework of astrocytes, discrete connectivity hubs were formed by cores and expansions that gave rise to large numbers of connected parts (up to 45). These hubs may represent signal integration sites within astrocytic nanoarchitecture (Figure 3).

Insights on astrocyte morphology from SBF-SEM and FIB-SEM

Astrocyte morphology is often referred to as ‘spongiform’ because of its sponge-like appearance and the perceived abundance of holes and loops when visualized with light microscopy. The potential for holes and loops in astrocytic nanostructure may have functional implications for astrocytes, allowing them to more completely fill spaces between CNS microanatomy and encapsulate or isolate structures such as axonal boutons and dendritic spines. However, whether or not astrocytes form *bona fide* loops and holes with continuous cytoplasm has been debated for decades. Recent super-resolution light microscopy studies demonstrated that astrocytes form ‘O-ring’ like structures, which may affect how Ca^{2+} signals propagate along astrocytic processes near synapses [55–57]. However, vEM results suggest that astrocytes are not truly spongiform. Aten and colleagues showed with SBF-SEM that astrocytic processes contacted themselves without fusing, creating ‘reflexive’ loops. This was consistent with an earlier study investigating small fragments of hippocampal astrocytes (60–70 nm intervals; ~100 total sections) that also did not detect holes and loops [58]. Perhaps most convincingly, Salmon and colleagues applied FIB-SEM and a finer sectioning interval (8 nm) to minimize merge and split errors that can occur in vEM reconstructions and rarely found loops and holes [59]. Astrocytic nanoarchitecture in three separate volumes was characterized as hierarchical, with major connectivity hubs, and importantly, a scarcity of holes and loops. Thus, astrocytes have a branched rather than a true sponge morphology [49].

Insights on PAPs from SBF-SEM and FIB-SEM

There is also a need to understand the complex 3D architecture of astrocytes with respect to other CNS microanatomy especially near synapses, where astrocytes form PAPs and

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are believed to modulate their function [60, 61]. PAP coverage of synapses appears to vary and different approaches have been used to estimate these interactions at the EM level [62–65]. PAPs may also structurally rearrange to modify synaptic function [66, 67], behavior [68], and response to disease states [13, 30, 32, 36, 69, 70]. PAPs are presumed to be located at astrocytic process tips that contact pre- and/or postsynaptic sites. The PAP is believed to be equipped for detection of synaptic activity, reuptake of ions and neurotransmitters, and release of neuroactive molecules to regulate synaptic transmission and plasticity. Despite this, a clear structural definition of a PAP is lacking. Aten and colleagues found that 86% of synapses (including those with asymmetric and symmetric structure) have some form of astrocytic contact at the cleft, higher than earlier estimates [71] from rat CA1 hippocampal stratum radiatum at ~57%. More recently, Salmon and colleagues used 3D geodesic path measurements to identify structural attributes of PAPs. They analyzed thousands of PAPs across several brain volumes and found that synaptic interactions with the astrocytic surface were concentrated on terminal constrictions, which are more protrusive and thinner compartments on astrocytes. However, contact points were found across the entire distributions of astrocytic parts, suggesting that PAPs do not occur solely at astrocytic process tips but can be ‘en-passant’ on internal structures composed of constrictions, expansions, and even thick core regions. While constrictions form the majority of PAPs, expansions form PAPs associated with synapses containing larger postsynaptic densities (PSDs). Expansions, which have larger surface area and volume than constrictions, may have specialized PAP functions near larger synapses. Analyzing all synapses interacting with astrocytes in their volumes, Salmon and colleagues discovered a consistent relationship between astrocytic parts, clusters of synapses, and astrocytic mitochondria. They found that astrocytes structurally partitioned the neuropil and had specific associations with discrete clusters of synapses that can be organized into astrocyte-defined synaptic clusters (ADSCs). Astrocytic mitochondria were localized closer to larger ADSCs, suggesting that astrocytes localize their mitochondria to subcellular compartments associated with specific connectivity and neural circuits (Figure 3).

Astrocyte morphogenesis

Our mechanistic understanding of astrocyte morphogenesis during brain development has improved substantially in the past decade, but remains incomplete and lags behind our understanding of neuronal morphogenesis. In the mouse cortex, astrocytes are derived perinatally from radial glial stem cells [72, 73]. During the first postnatal week, astrocytes migrate, proliferate, and begin to elaborate their branches and establish endfeet [74, 75]. Morphological maturation occurs largely during the second and third postnatal weeks, in parallel with synapse formation and maturation. During this time, astrocytes increase in size and complexity and refine their territories to minimize overlap with neighboring astrocytes [76]. We highlight recent studies that have identified secreted factors and contact-mediated mechanisms that regulate different aspects of astrocyte morphogenesis during this critical developmental window.

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Secreted factors

Early studies of astrocyte morphogenesis identified neuron-secreted factors that bind astrocyte-expressed receptors to regulate astrocyte morphogenesis (Figure 4). In Drosophila, neuron-secreted fibroblast growth factor (FGF) ligands bind the FGF receptor Heartless on astrocytes to promote astrocyte outgrowth and infiltration into the neuropil [77]. More recently, *fgfr3* and *fgfr4* were found to be essential for astrocyte morphogenesis in zebrafish [78]. In mice, neuronal glutamate regulates astrocyte arborization and synapse association via astrocyte-expressed metabotropic glutamate receptor 5 (mGluR5) [79]. Brain-derived neurotrophic factor (BDNF), expressed by both neurons and astrocytes, promotes astrocyte morphological maturation *in vitro* via the truncated TrkB receptor (TrkB.T1), the predominant TrkB isoform in astrocytes. Loss of TrkB.T1 from astrocytes in the mouse cortex impaired astrocyte morphogenesis and altered gene expression [80]. Sonic hedgehog (Shh), secreted by layer V neurons, binds to astrocyte-expressed Patched 1 (Ptch1) to promote astrocyte morphogenesis in a layer-specific manner [81]. Thus, neuron-secreted factors not only shape astrocyte morphogenesis, but may represent a mechanism underlying astrocyte morphological heterogeneity within brain areas.

A recent study by Cheng and colleagues found that inhibitory neuron activity promotes astrocyte morphogenesis during development via astrocytic $GABA_B$ receptors ($GABA_BRs$) [82]. Chemogenetic activation of inhibitory interneurons between P7–21 increased astrocyte complexity, branch points, and process length. Conversely, chemogenetic reduction of inhibitory interneuron activity decreased astrocyte complexity [82]. Astrocytic $GABA_BR$ expression was increased during morphogenesis and by chemogenetic activation of inhibitory interneurons. Moreover, astrocyte-specific $GABA_BR$ deletion impaired astrocyte morphogenesis and the ability of chemogenetic activation of interneurons to increase astrocyte complexity [82]. Although $GABA_BR$ represented a mechanism operating throughout the brain, it was regulated in a brain-region-specific manner by different transcription factors (*NFIA* for cortex, *Sox9* for olfactory bulb). Deletion of specific transcription factors altered astrocyte morphology in a region-specific manner. Thus, Cheng and colleagues demonstrate how astrocyte morphogenesis is regulated mechanistically by GABAergic signaling and how this mechanism relates to transcription factors that control region-specific astrocyte development. The picture that emerges is that astrocyte morphogenesis is regulated cell autonomously and by neuronal activity during development [82], which potentially implicates astrocyte morphology dysregulation in a variety of neurodevelopmental disorders associated with altered neuronal function.

Elegant experiments by Rosenberg and colleagues show that adrenergic signaling, particularly that mediated by β1 adrenergic receptors, regulates morphology of gray matter astrocytes in female mice [83]. These experiments are significant, because although it is known that noradrenergic signaling can affect astrocyte calcium dynamics, the possibility that this mechanism also affects astrocyte development and morphology with marked behaviorally consequential effects in adult female mice is both intriguing and new. These data add to the appreciation that astrocyte morphology is regulated by neuronal activity and implicate such mechanisms in the effects of noradrenergic signaling on brain function.

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Contact-mediated mechanisms

Recent studies have focused on the role of astrocyte-synapse contact in astrocyte morphogenesis (Figure 4). Stogsdill and colleagues found that astrocyte-expressed Neuroligins (NL) 1, 2, and 3 interacted with neuronal Neurexins to regulate astrocyte morphogenesis via direct contact *in vitro* [84]. In the developing mouse cortex, sparse knockdown of individual astrocytic NLs at P1 impaired different stages of astrocyte morphological maturation, with NL1 and NL2 knockdown astrocytes showing reduced NIV at P7, and NL2 and NL3 knockdown astrocytes showing reduced NIV at P21. Genetic deletion of NL2 from astrocytes similarly reduced astrocyte NIV and impaired excitatory synapse formation and synaptic function at P21. Of note, a recent preprint by Golf and colleagues questioned the role of astrocytic neuroligins in regulating astrocyte morphology and synaptic properties. Using a different mouse genetic approach to simultaneously target NL1–3, the authors found no change in astrocyte morphology or synaptic properties at the later time point of P35 [85]. Further investigation is needed to determine whether the findings of Golf and colleagues represent a recovery of the phenotypes Stogsdill and colleagues observed at P21 or if the differences between these studies reflect the methods used to reduce NL expression. Irrespectively, these two studies call for further exploration of NLs as they are highly expressed within astrocytes [29, 86]. In contrast to the role of astrocytic NLs in promoting astrocyte neuropil infiltration during development [84], astrocyte-expressed NRCAM restricts neuropil infiltration via homophilic transcellular interaction with neuronal NRCAM [87]. Deletion of NRCAM from astrocytes or neurons robustly increased neuropil infiltration and impaired astrocyte association with inhibitory synapses, resulting in impaired inhibitory synaptic function. In addition to NLs and NRCAM, other cell adhesion molecules (CAMs), such as Connexin 30 and Necl2 are proposed to help to fine-tune astrocyte-synapse association through contact-mediated mechanisms [88, 89].

Contact with different neuronal subpopulations may also represent a source of morphological heterogeneity. Tan and colleagues recently discovered a contact-mediated mechanism that regulates astrocyte morphogenesis in a layer-specific manner in the cortex [90]. Using primary cultures of astrocyte and neurons, as well as *in vivo* manipulation of gene expression, Tan and colleagues found that δ-catenin in both astrocytes and neurons regulates astrocyte morphogenesis by controlling the surface expression of cadherins, including Chd2/N-cadherin. Reduction of δ-catenin in sparse populations of cortical astrocytes significantly reduced astrocyte territory volume and branching complexity during early postnatal development, though astrocyte territory volume recovered by P21. Knocking down δ-catenin in upper layer neurons only altered the complexity of upper layer astrocytes, indicating that layer-specific differences in neurons may facilitate layer-specific differences in astrocytes. In the mouse cortex, astrocytes in all layers express high levels of N-cadherin, while its expression in neurons is restricted to lower layer neurons. Knockdown of N-cadherin in astrocytes only reduced the complexity of lower layer astrocytes, and not upper layer astrocytes, suggesting that *trans* interaction of astrocytic and neuronal N-cadherin promotes astrocyte morphogenesis in a layer-specific manner.

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Astrocyte-astrocyte interactions are also essential for astrocyte morphogenesis. *In vitro*, astrocyte-astrocyte contact promotes morphological maturation [91]. *In vivo*, astrocytes form highly organized networks by establishing non-overlapping territories to tile the brain [25, 76] and communicating with their neighbors via gap junctions. Disruption of functional astrocyte networks via deletion of astrocyte-expressed connexins impairs synaptic plasticity and spatial learning [92] and hampers metabolite distribution in response to neurodegenerative stress [93], indicating that formation and maintenance of functional astrocyte networks is essential for proper brain function. During development, hepatic and glial cell adhesion molecule (hepaCAM) regulates both tiling and coupling by controlling astrocyte branching organization and localization of the gap junction protein Connexin 43 [94]. Deleting *Hepacam* from individual astrocytes reduced astrocyte territory volume and permitted territory invasion by neighboring wild type astrocytes, and global deletion of *Hepacam* from astrocytes impaired gap junctional coupling. HepaCAM is also localized to inhibitory synapses, and loss of *Hepacam* from astrocytes impaired inhibitory synaptic function. Unlike NLs and NRCAM, loss of *Hepacam* did not impact astrocyte neuropil infiltration. How these and other molecules work in parallel to regulate astrocyte morphogenesis remains to be explored and will likely require in depth understanding of upstream and downstream signaling mechanisms.

The molecular mechanisms that link the aforementioned cell adhesion molecules (CAMs) and cell surface receptors to morphological changes in astrocytes are largely unknown. Studies in cultured astrocytes and *in vivo* injury models have linked the regulation of actin dynamics and Rho GTPase activity to astrocyte morphology [95]. However, none of these mechanisms have been studied in astrocytes in the context of development or in relation to specific CAMs or receptors. Proper regulation of mRNA localization and translation have also been implicated in astrocyte maturation [96]. Astrocytes locally translate mRNAs in their synapse-associated processes [97] and in endfeet processes [37]. Whether this phenomenon occurs during development as astrocyte branches navigate the tissue microenvironment remains to be explored, as do the molecular mechanisms that regulate the early stages of astrocyte polarization and endfeet development. For an in-depth discussion of astrocyte endfeet, we suggest a recent review article [34].

Molecular underpinnings of regional variation in astrocyte morphology

Unlike neurons, which are extremely diverse, astrocytes have historically been viewed as homogeneous. However, recent studies show that astrocytes are separable between and within CNS regions[15, 16]. This has led to the realization that astrocytes are brain region specific, attributes that together with their highly complex morphology may allow them to mediate their multiple roles *in vivo.* However, until recently there had been no broad molecular assessment of astrocyte morphology across the CNS.

To quantify the morphology of astrocytes across the CNS, Endo and colleagues expressed a reporter sparsely in astrocytes of adult mice [32]. They captured diffraction-limited images of single astrocytes from confocal volumes using immunohistochemistry and subjected them to evaluations of ten morphological parameters that represented distinct aspects of their complex shapes at the cellular scale (Figure 2). They found clear evidence for region-

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specific astrocyte morphological features, *e.g.,* astrocytes in the motor cortex displayed the largest territory sizes and showed a relatively round shape, whereas astrocytes in the cerebellum showed the most elongated shapes [32]. However, although the number of major branches per astrocyte from the 13 CNS regions examined was the same (on average at 4.0–5.6 per cell), the morphological complexity was significantly different, implying that key differences exist with the finer structures that comprise astrocyte territories. Since astrocytes showed distinct morphological features, the authors performed weighted gene co-expression network analysis (WGCNA) using astrocyte-specific RNA sequencing data of the same regions and identified 62 module eigengenes that were correlated with astrocyte morphological parameters. Such analyses provided a set of genes within distinct modules (named as distinct colors) correlated to astrocyte morphology. Of these modules, those named as “darkmagenta” and “turquoise” displayed the highest correlation with astrocyte territory size [32]. By analyzing genes within these modules using stringent criteria and by focusing only on the top 5% of genes displaying the highest significance *p* value, Endo and colleagues identified several genes that may contribute to astrocyte territory sizes.

From the analyses of astrocyte territory size-related modules, proteins encoded by *Fermt2* and *Ezr*, i.e. FERMT2 (FERM Domain Containing Kindlin 2) and ezrin were assessed, because they are known putative actin interacting proteins. Both were expressed within astrocytes at the protein level, prompting the authors to use an astrocyte-specific CRISPR/Cas9-based gene knockdown approach to reduce expression of these genes in astrocyte reporter mice. Significant reduction (>70%) of *Fermt2* and *Ezr* resulted in ~20% reduced territory sizes of sparsely labeled hippocampal CA1 astrocytes. These candidate gene evaluations thus supported predictions from WGCNA where *Fermt2* and *Ezr*-containing core modules were correlated with astrocyte territory sizes [32]. Furthermore, the magnitude of the reductions in astrocyte morphology were consistent with expectations from past single gene evaluations (mentioned in preceding sections) and suggest that multiple molecular mechanisms contribute to mature astrocyte morphology, perhaps during development and to its maintenance in adults. Furthermore, Endo and colleagues provided a valuable resource to explore additional molecular mechanisms and their relation to the molecular underpinnings of astrocyte morphology.

Astrocyte morphology and models of disease states

Early anatomists observed that astrocytes change their shapes in disease. In 1858, Rudolf Virchow noted “*…..this very interstitial tissue of the brain and spinal marrow is one of the most frequent seats of morbid change…*” and then later in 1873 Theodor Meynert commented “*…..these cells swell up under certain pathological conditions and assume grotesque forms”* (for a historical summary, see [98]). Astrocyte morphology changes can occur in parallel with the underlying disease (correlative changes), in response to it (reactive changes), or orthogonally due to astrocyte-specific mechanisms owing to the underlying cause of disease that may or not contribute causally to pathology (cell autonomous mechanisms). It is probable that all three types of changes occur in different diseases or even in the same disease at different stages of severity [11]. The topic of how astrocyte changes contribute causally to distinct aspects of disease are now being explored with cell-specific and genetic approaches. We comment on three recent topics pertinent to morphology.

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Huntington’s disease (HD)

Huntington’s disease (HD) is a severe, fatal, monogenic, autosomal dominant neurodegenerative disease caused by a polyglutamine encoding CAG expansion in the huntingtin (Htt) gene that results in the expression of mutant Huntingtin proteins (mHTT) in cells throughout the body. The striatum undergoes marked atrophy in HD. Astrocytes are abundant within the striatum [99] and contain mHTT in HD [100]. Traditionally, astrocyte morphological changes in HD have been studied using GFAP immunostaining. However, astrocytes in the striatum express low levels of GFAP at the RNA and protein level [29, 32] and low GFAP levels were found in controls and HD mice [101, 102]. Furthermore, detailed analyses of GFAP in the astrocytes where immunostaining was observed showed no differences between HD and control mice [103]. Because complex morphology is a defining feature of astrocytes, the possibility that morphology may change was therefore missed by GFAP immunostaining and was explored in HD model mice using different methods.

A variety of methods have recently been used to measure astrocyte morphology more directly by using Lucifer yellow iontophoresis, plasma membrane targeted fluorescent proteins, and with sophisticated EM approaches. Intracellular Lucifer yellow iontophoresis and cell-specific fluorescent proteins were used to examine astrocyte territory sizes [30]. There were no significant differences in the size of astrocyte somata, but the territory areas were significantly smaller in HD model mice [30]. An optical assay was used to measure the proximity of astrocyte processes to cortical inputs onto striatal medium spiny neurons (MSNs): the number of the most proximate astrocyte processes to cortical inputs decreased in symptomatic HD mice, whereas this parameter increased for thalamic inputs. The changes in cortical and thalamic pathways occurred before loss of cortical and thalamic presynaptic immunostaining, implying that astrocyte morphological changes and process withdrawal from cortical excitatory synapses may precede excitatory synapse loss in HD model mice. These data suggest that diminished astrocytic homeostatic support could be a trigger for the HD-associated dysfunction. Based on analyses of gene expression changes in HD model mice and in human HD tissue that identified astrocyte Gi-GPCRs as potential targets [13, 104], this hypothesis was tested using a Gi-GPCR chemogenetic approach. Activation of Gi-GPCRs restored astrocyte morphology in HD model mice as assessed with plasma membrane targeted GFP, with the result that excitatory synapse function as well as early behavioural phenotypes associated with HD were improved [13].

A recent study employing SBF-SEM markedly expanded the findings from light microscopy that astrocytes in HD display constricted domains and that astrocyte process coverage of mature dendritic spines onto striatal medium spiny neurons is reduced in HD [70]. However, in this study astrocytic coverage of immature thin spines was enhanced [70]. This study and the work discussed in the preceding paragraph supports the hypothesis [13, 30, 70, 101, 105] that altered astrocyte morphology in HD contributes to manifestations of the disease.

Lengthy general anaesthesia

Pediatric general anesthesia may cause adverse behavioural and cognitive effects in humans, but the mechanisms are incompletely understood. Comprehensive work discovered that lengthy exposure (4 h) of neonatal mice (but not adults) to the general anesthetic sevoflurane

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reduced astrocyte intracellular calcium signaling and led to the down regulation of ezrin, a key astrocytic cytoskeletal protein [106]. Such changes were associated with reduced astrocyte morphogenesis and aberrant synaptic structures and functional properties (reduced fast synaptic excitation), as well as reduced social interactions in mice when they reached maturity (P27–30). Intriguingly, deficits across biological scales were remedied when ezrin was overexpressed within astrocytes using AAVs, indicating that the loss of ezrin expression within astrocytes and the subsequent morphological changes following sevoflurane exposure were causal for synaptic and behavioural deficits [106]. These studies therefor exemplify how drugs (and perhaps environmental agents and toxins) can affect brain function by altering astrocyte morphology at a key developmental stage. Furthermore, since astrocyte morphology during development is regulated by GABAergic signaling [82], these findings raise the possibility that sevoflurane may converge on GABAergic regulation of cortical astrocyte morphogenesis. A role for ezrin in regulating astrocyte morphology (and the consequences of its loss) is supported by studies in adult mice [32, 68]. However, we emphasize that at this stage we are unaware of data showing that anesthesia used during laboratory procedures in adult mice such as surgeries impacts astrocyte morphology. Such studies, with a range of anesthetics, may be insightful.

Obsessive-compulsive disorder (OCD)

OCD is characterized by persistent intrusive thoughts (obsessions), repetitive mental or behavioral actions (compulsions), and severe anxiety. OCD is a chronic, disabling psychiatric condition affecting ~2–3% of the population [107]. A recent study mapping astrocyte and neuron subcompartment-specific proteins within the striatum found that SAPAP3 protein (gene; *Dlgap3*) associated with OCD in humans was detected at equivalent levels in striatal astrocytes and MSN plasma membranes [36]. SAPAP3 anchors neurotransmitter ion channels in postsynaptic densities of cortical synapses onto striatal MSNs, and SAPAP3 knockout mice display OCD phenotypes, anxiety, and facial lesions likely due to repetitive self-grooming [108] and hind paw scratching [109]. However, the mechanisms giving rise to OCD phenotypes remain to be understood.

Among the proteins that interacted with SAPAP3 within astrocytes were those related to the actin cytoskeleton, and actin cytoskeleton proteins were also disrupted in astrocytes from SAPAP3 knockout mice [36]. In accord with these data, astrocytes from SAPAP3 deletion mice displayed reduced territory sizes and disrupted actin cytoskeletal organization, findings that prompted exploration of whether astrocytic SAPAP3 contributed to OCD phenotypes in these mice. This was addressed using astrocyte and neuron-specific rescue of SAPAP3 in the knock out mice followed by detailed behavioral and cellular analyses [36]. These experiments revealed that astrocytes and neurons both contribute to the compulsive phenotypes in SAPAP3 deletion mice, but that neurons largely contribute to the anxiety phenotypes. Of relevance to this review, astrocytic rescue of SAPAP3 restored their actin cytoskeleton and morphology, as well as protein-protein interactions with ezrin and Glt1 glutamate transporters [36]. Taken together, these studies provide strong evidence that astrocyte morphological complexity is required for normal physiology and that its reduction in SAPAP3 deletion mice contributes causally to the emergence of complex psychiatric phenotypes related to OCD in humans.

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Interestingly, in relation to psychiatric phenotypes, a recent study has suggested that changes in cortical astrocyte morphology are associated with phenotypes related to depression in mouse models [110]. Furthermore, pioneering studies in the lateral habenula have shown that the special morphological arrangement of astrocytes relative to neuronal somata in this brain region are central to their contributions to depression behaviors in mice [111]. However, neuroinflammation-evoked depression-like behaviors in mice occurred independently of notable astrocyte morphological changes in the prefrontal cortex, although there were subtle changes within processes [12].

Concluding remarks and future directions

Compact morphological complexity is a defining feature of astrocytes that sets them apart from other CNS cells. Recent and exciting progress is summarized in this review, but much work remains in order to understand astrocyte morphology and its physiological and pathological implications. We highlight four key areas below and refer the reader to the **Outstanding Questions** box which highlights major open questions with regards to astrocyte morphology.

At the molecular level, we need to understand the full extent of the mechanisms involved in astrocyte morphogenesis and in the maintenance and stability of astrocyte morphology in adulthood. Recent multiomic and developmental studies provide valuable insights, but further hypothesis-driven studies are needed. Astrocytes are replete with cell adhesion molecules that may be the substrate for their interactions with other cells or neighboring astrocytes, but our biophysical understanding of which molecules are used, when and where they are expressed, and what they do in terms of intracellular signaling remains limited.

At the cellular level, we need to understand how astrocytes interact with other cells, how they tile, and how they acquire their complex morphology. Such studies would need to employ quantitative light microscopy, but the use of FIB-SEM will be critical to understand fine astrocyte processes at the necessary resolution. It would be a boon for the entire field to systematically document the structure of astrocytes at ~4 nm resolution in their entirety which would help determine structural signatures of specific astrocytic nanostructures. Comprehensive nanoscopic analysis may reveal structural features of astrocytes that change early in disease processes and that can be used to construct testable hypotheses. For physiology, we need much improved methods capable of imaging signaling events in entire astrocyte volumes in 3D and at fast tens or hundreds of millisecond frame rates. In particular, we need detailed studies on astrocytes using correlated light and electron microscopy in order to be able to compare and contrast these two approaches with rigor.

At the circuit level, we need to understand how withdrawal or approach of astrocyte PAPs relative to synapses affects synaptic transmission and the functions of circuits. We also need to understand how astrocyte leaflet dynamics and morphological changes affect the physiology of other cell types and whether changing vascular coverage by endfeet alters BBB function. A major open question is whether factors secreted from astrocytes are released at specific cellular sites or are released equivalently or diffusely with signaling specificity being conferred by appropriate expression of the cognate receptors on the

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receiving cells. For such experiments to be fruitful there is much need to develop tools to measure biochemical signaling events in electrically non-excitable cells simultaneously with astrocyte leaflet motility and signaling. We also need tools to image the proximity and interactions of multiple cell types simultaneously.

In the case of disease, emerging evidence suggests that astrocyte morphological changes contribute in ways that are causal for the phenotypic manifestations of disease. Of course, it has been known for a long time that astrocytes change shape in disease, but is it possible that this has been simplistically included in the increasingly catch all term “reactivity”. Is it possible that astrocyte morphology changes are disease-specific, occur at multiple stages, and are causal to subsequent synaptic pathophysiology and possibly drive reactivity by virtue of altered cell-cell interactions? If so, might strategies that restore astrocyte morphology in disease be broadly beneficial? Do anesthetics in general, perhaps even in adulthood, alter astrocyte morphology? If so, what are the implications of such phenomena?

The development and use of reliable and cell-selective genetic and imaging methods will be vital for progress in all of the areas mentioned above. We have discussed in detail astrocyte morphology based on microscopic and nanoscopic evaluations, but are cognizant of the fact that it is not possible to compare and contrast between such studies in a rigorous manner. Thus, there is a pressing need to implement and use correlated light and EM approaches. The next few years hold great promise as key questions are addressed (**Outstanding Questions**), with the promise to lead us into areas of cell biology hitherto not considered and, expectantly, into novel mechanistic understanding and therapeutic strategies for human disease.

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Box 1:

Terminology

We suggest that the general term astrocyte process, although useful, should evolve into several related terms that convey meaningful information about the particular astrocyte subcompartment. We provide some working definitions that develop further from a previous review [112] and recent progress summarized herein.

Branches are the major processes emanating from the astrocyte soma. These probably do not number more than eight per astrocyte and display diameters on the micrometer scale and have also been called “stem processes” in the literature.

Branchlets are the finer secondary, tertiary, and higher order structures that emanate from branches. The precise number of these is presently unknown, but they display diameters on the submicrometer scale. Conventional diffraction limited light microscopy is not ideal to study the finest branchlets, although they can be seen as distinct from branches.

Leaflets are the thinner regions of astrocyte branches and branchlets that contact synapses and exist throughout territories. The precise numbers of these per astrocyte is currently unknown, but based on electron microscopy studies they display dimensions on the tens of nanometer scale and their numbers could run into the tens of thousands. Leaflets would be the same structures as those variously termed “astrocyte lamellae,” “astrocyte sheets,” “veillike lamellae,” “peripheral astrocyte processes,” and “astrocyte fingers”. Leaflets cannot be imaged with diffraction-limited light microscopy [113].

Perisynaptic astrocyte processes (PAPs) are the extensions of astrocyte branches and branchlets that contact pre and postsynaptic elements of the synapse. Peripheral astrocyte process and perisynaptic astrocyte process are often used interchangeably. However, truly “peripheral” astrocyte processes do not exist, because fine processes exist throughout astrocyte territories and are not located preferentially at the periphery.

Endfeet are specialized distal extensions of astrocytes that contact the vasculature. Each astrocyte bears at least one branch with enlarged endfeet. Endfeet appear to represent a truly polarized compartment of astrocytes with a well-accepted function.

Astrocyte loops are structures observed with some forms of super resolution microscopy whereby astrocytic processes commonly contact themselves without fusing, creating ‘reflexive’ loops. See text for discussion of the evidence for and against their existence.

Process expansions were observed by FIB-SEM when astrocytes were deconstructed into constituent parts comprised of connected thin ‘constrictions’, thicker ‘expansions’, and thick ‘core’ regions. These parts existed in motifs, with constrictions frequently flanked on both ends by expansions.

Terminal constrictions are synaptic landing points on the astrocytic surface recently discovered by FIB-SEM. Terminal constrictions are the more protrusive and thinner compartments on astrocytes. Such structures may be the anatomical basis of leaflets, a loose term used to encapsulate fine astrocyte processes (see above).

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The more general term of **astrocyte process** is useful and could be used generally, for example, to describe astrocytes that have lost their native morphology in cell culture or organoids or when the location of a particular signal or event does not matter or encompasses all processes.

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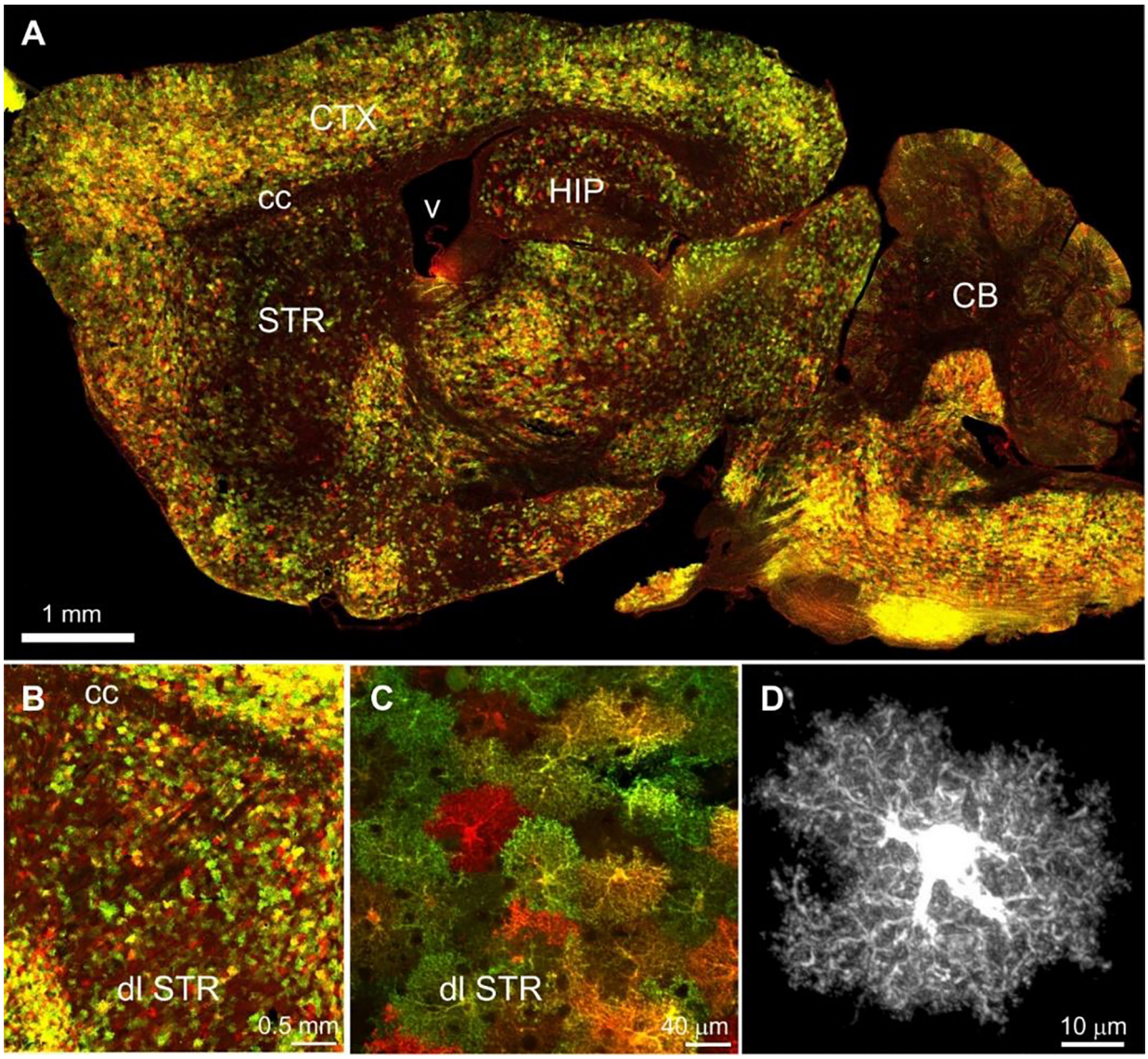

Figure 1: Views of astrocytes at the microscopic scale.

A. Sagittal brain section of an adult mouse expressing GFP and tdTomato from two separate AAV 2/5 constructs using the astrocyte specific GfaABC$_1$D promoter, illustrating astrocyte specificity and tiling of the CNS by astrocytes. B. Higher magnification view of the striatum from panel A. C. Higher magnification view of a region from panel B, showing astrocyte tiling and elaborate morphology. D. A single striatal astrocyte loaded with Lucifer Yellow with intracellular iontophoresis. Panels A-C were captured by Baljit Khakh and Ling Wu. Panel D is reproduced from a published study [29].

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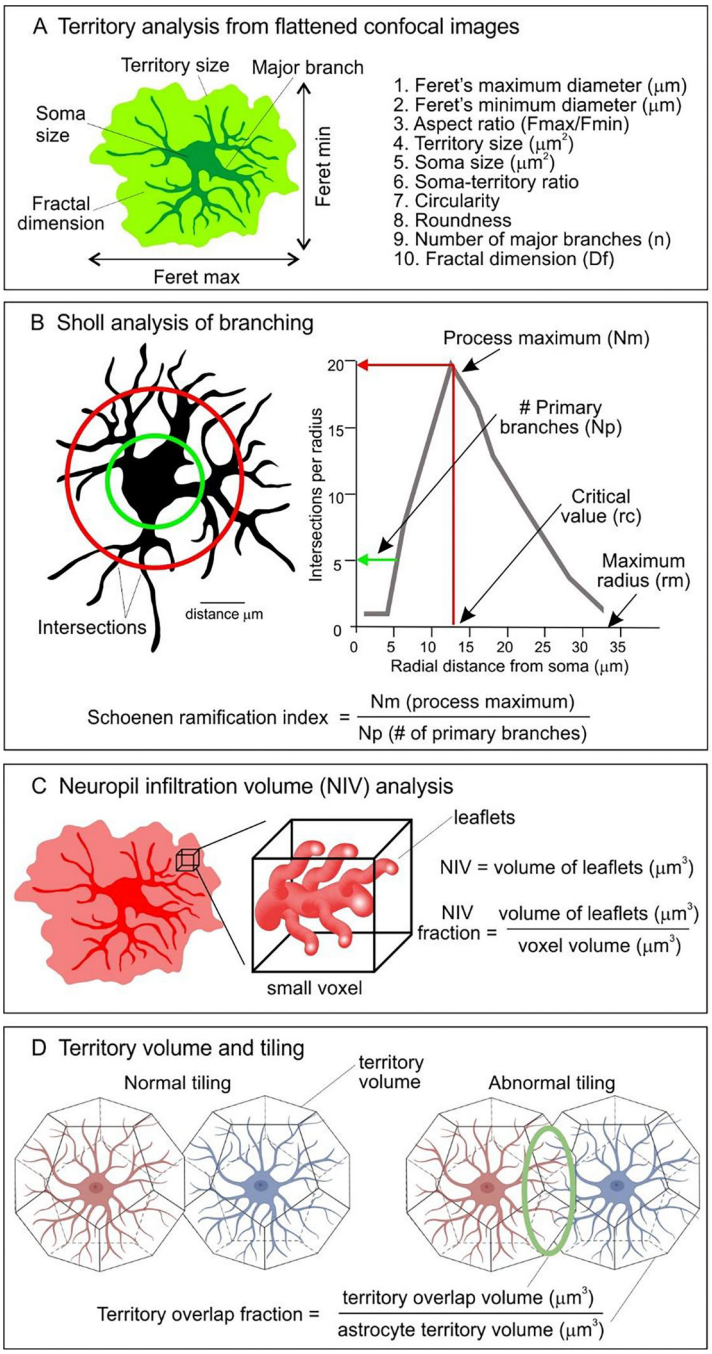

Figure 2: Metrics used to assess astrocyte morphology using light microscopy.

A. Cartoon illustrates various morphological parameters that can be measured from confocal images of astrocytes expressing a reporter protein such as GFP. These numbers provide metrics by which to assess astrocyte morphology and its changes. The panel is adapted from a published study [32]. A stated in the text, these 2D measurements do not accurately reflect 3D morphology, which is impossible to image with light microscopy. However, they do quantify astrocyte territories. B. Illustrates the method of Sholl analysis that is used to assess cellular complexity. From such Sholl plots, the process maximum (Nm) represents

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the highest number of intersections an astrocyte makes, the critical value (rc) is the distance from the soma at which Nm occurs, and the maximum radius (rm) is the maximum width of the Sholl plot. In addition, the number of primary branches emanating directly from the soma of each astrocyte (Np) can be used to calculate the Schoenen ramification index, which quantifies overall branching. These numbers provide metrics by which to assess astrocyte morphology and its changes, as shown by Sholl [114] and Shoenen [115]. Standard imaging programs can be used and the mentioned analysis methods are available or easily implemented in ImageJ (https://imagej.nih.gov/ij/). However, we note that while methods exist to preform Sholl analysis on 3D images, this analysis is subject to the caveat of z-axis "stretching" and, like 2D analysis, lacks the resolution to resolve astrocyte leaflets. C. Schematic illustrating the neuropil infiltration volume and how the neuropil infiltration volume fraction can be measured using small voxels placed within astrocyte territories. In this measurement too, the absolute volume of leaflets is not possible to measure with light microscopy. D. Schematic illustrating astrocyte territory volume, and how this method can be used to examine astrocyte tiling behavior.

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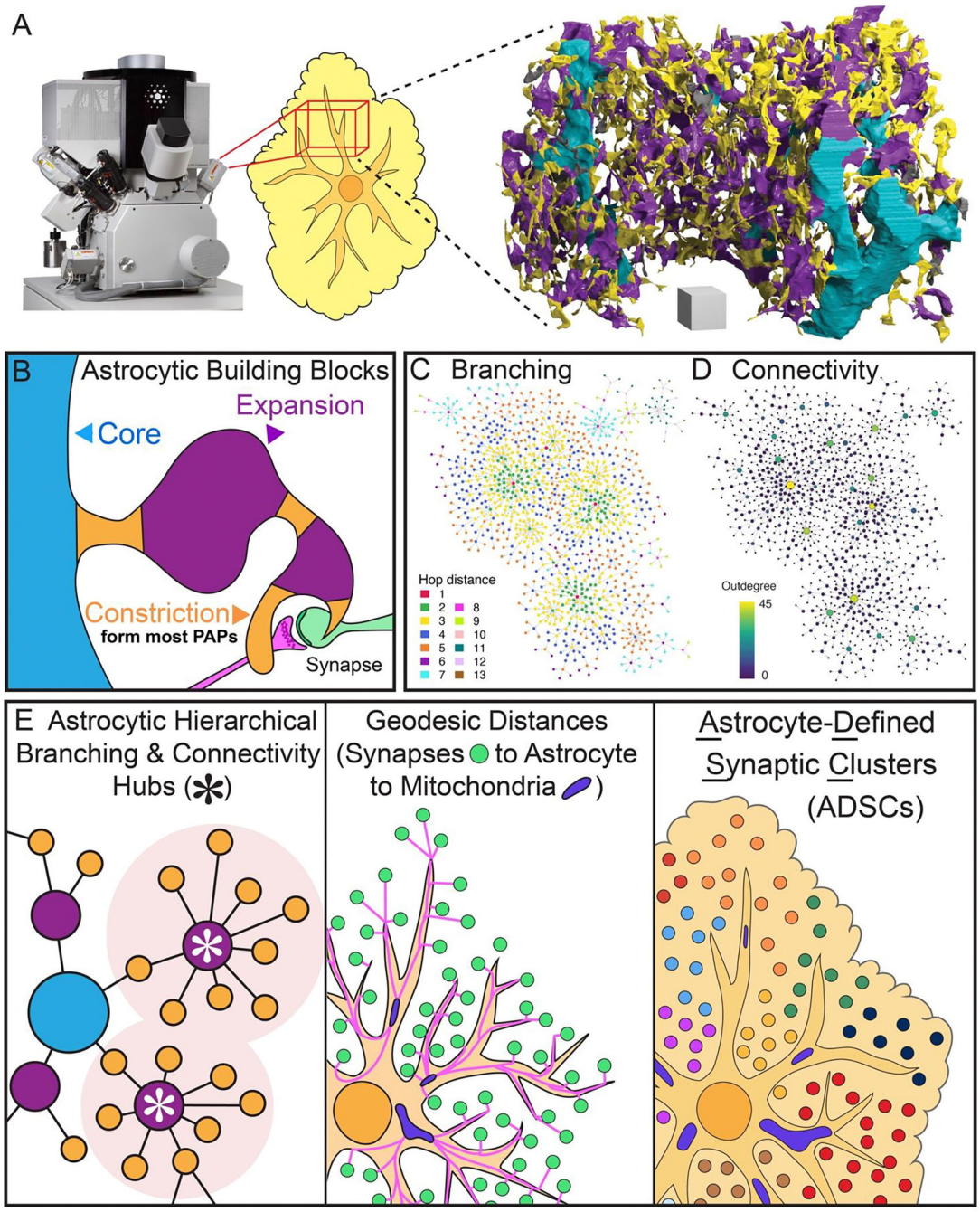

Figure 3: Astrocytes at the nanoscale.

Complexity of astrocytic nanoarchitecture revealed by ultra-high resolution focused ion beam scanning electron microscopy (FIB-SEM). A. FIB-SEM instrumentation (left) used to image a portion of an astrocyte (schematized in the center) using scanning electron microscopy and reconstruction in three dimensions (right; portion of mouse Layer 2/3 cortical astrocyte shown). B. Three specific nanostructural building blocks can be identified in astrocytes including cores (cyan), expansions (purple), and constrictions (orange). Most perisynaptic astrocytic processes (PAPs) are formed by constrictions. C-D. Astrocytic

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building blocks can be used to examine the hierarchical branching (C) and connectivity (D) of astrocytic nanoarchitecture using directed graphs. E. Astrocytic nanoarchitecture can be described as having a wide, but shallow hierarchical branching organization with major connectivity hubs (asterisks, left), signature distances between astrocytic processes, mitochondria, and synapses (magenta lines; center), and distributed sets of astrocyte-defined synaptic clusters (ADSCs; multi-color synaptic clusters; right). Scale cube in A, $1\mu m^3$. Figure reproduced and adapted from Ref [49]. Thank you to Dr. David Polcari (Systems for Research) and Dr. Chris Salmon (McGill University) for the image of the FIB-SEM instrument and the schematic of astrocyte in Panel A, respectively.

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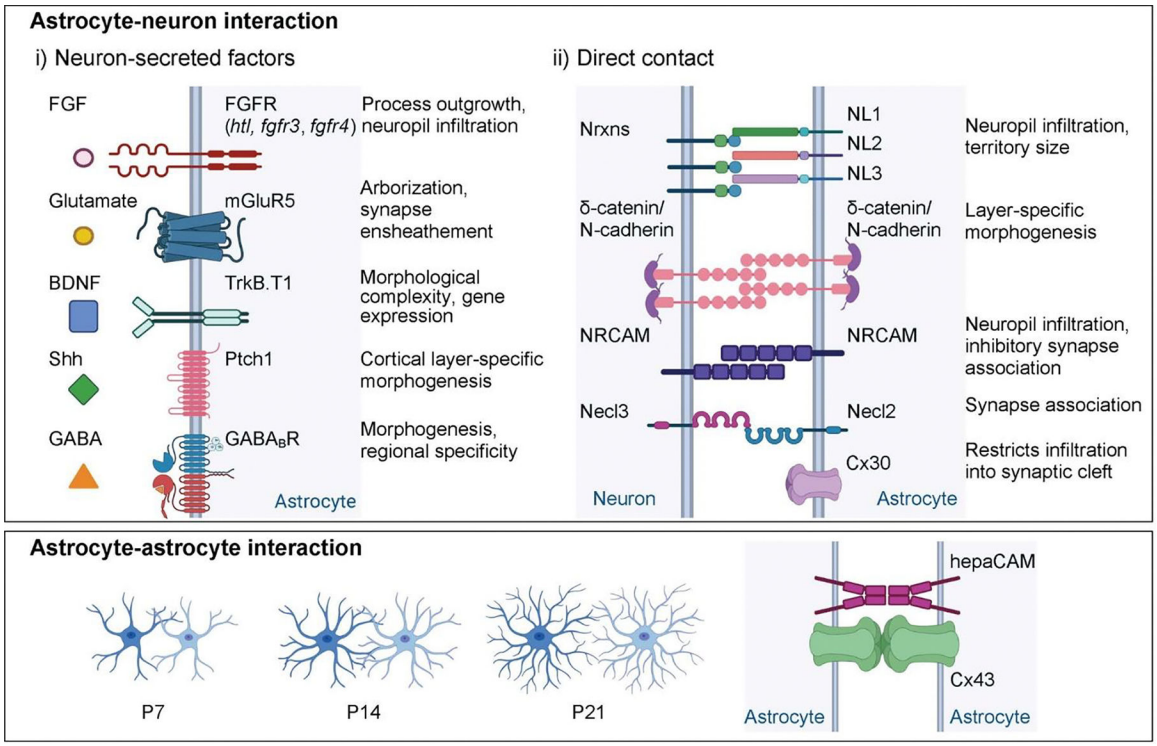

Figure 4: Molecular regulators of astrocyte morphogenesis.

A number of cell surface receptors on astrocytes play key roles in astrocyte morphogenesis via their interaction with neuron-secreted factors, neuronal membrane proteins, and astrocytic membrane proteins. **Astrocyte-neuron interaction:** Several members of the FGFR family, including *heartless* [77] in Drosophila and *fgfr3* and *fgfr4* [78] in Zebrafish, promote astrocyte outgrowth and neuropil infiltration. Glutamate promotes astrocyte arborization and synapse ensheathment via mGluR5 [79], GABA promotes astrocyte morphogenesis via GABAB receptors [82], while BDNF induces morphogenesis via the TrkB isoform, TrkB.T1 [80]. Expression of Shh by layer V cortical neurons in enhances astrocyte morphological complexity in a layer-specific manner [81]. Astrocytic Neuroligins promote astrocyte territory size and neuropil infiltration via interaction with neuronal neurexins during early postnatal development [84] (see text for discussion of recent findings by Golf and colleagues at later time points [85]). δ-catenin regulates surface expression of N-cadherin in both astrocytes and neurons to regulate cortical layer-specific astrocyte morphogenesis [90]. Homophilic NRCAM interaction restricts astrocyte neuropil infiltration. Both NRCAM [87] and Necl2 [89] promote astrocyte-synapse association, while Cx30 [88] restricts the extent of astrocyte process infiltration into the synaptic cleft. **Astrocyte-astrocyte interaction:** During the first three postnatal weeks, astrocytes grow in size and complexity and establish non-overlapping territories with neighboring astrocytes. Transcellular hepaCAM interaction plays a key role in astrocyte territory establishment and gap junction coupling through regulation of Cx43 [94]. This figure was created with Biorender.com.