

# Astroglial anatomy in the times of connectomics

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**Abstract** In contrast to neurons, the role of astrocytes has been matter of debate since their discovery, and mostly because of misconceptions about their role. As a consequence, technologies to study brain physiology have been designed around neurons, to answer one specific question, leaving glia experts with the only possibility to “hack” these techniques to describe astrocytes. As questions to answer about astrocytic functioning are based on factual observations, conclusions are often vague and cryptic, no matter how technically sound the work is. For instance, compelling evidence on calcium elevations has been provided, their dynamics have been studied in detail, but their role is still open for interpretation. Another astrocytic feature that carries a lot of mysteries is their complex morphology. The use of three-dimensional electron microscopy (3DEM) would most certainly be the best approach to unveil hidden features of such complex cells, nevertheless so far 3DEM hasn’t been fully exploited in that sense, nor techniques has been adapted for astrocytic observations in particular. One of the most ambitious neuroscience projects, the connectome, is pushing to their limits electron microscopy, image segmentation and 3D reconstruction and analysis, making it a very good candidate to adapt pipelines and methodologies to the study of astrocytic morphology. Here, we briefly review our current knowledge and technical state of art on 3D glia morphology, and speculate about its future directions.

**Key words** astrocytes, connectome, three-dimensional (3D), glia morphology

## 1 The history of astrocytes: from Nervenkitz to the tripartite synapse

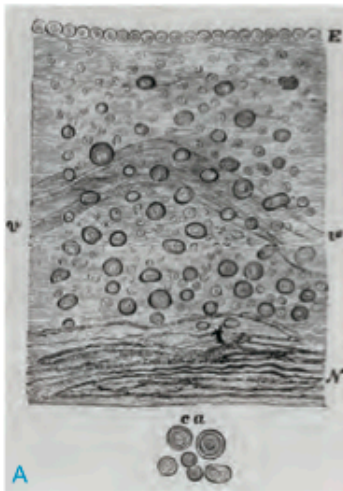
### 1.1 Early observers

The first observation of glial cells can be dated back to 1824, by a french physician, Henri Rene Dutrochet, one of the fathers of the cell biology, who also investigated and described the osmosis process. He indeed noted in the mollusk nervous system some “globules” adhering with the nerve fibers.

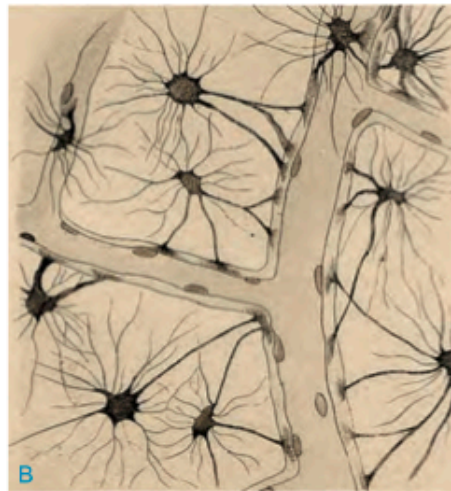
It was only in 1856 that the german neuroanatomist Rudolf Virchow fathered the term we use nowadays for the other half of the brain, *neuroglia*, from the german “Nervenkitz”, whose literal translation is nerve glue. Indeed, although he dared to name first this cell population, he thought, similarly to Dutrochet, that he was observing some kind of connective tissue (Fig. 1A).

### 1.2 The Golgi staining revolution

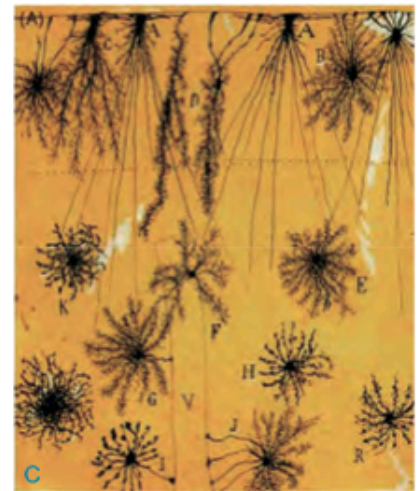
Then imaging and staining techniques evolved, and so did our knowledge of the CNS. The *reazione nera*, discovered by the italian scientist Camillo Golgi, better known today as “Golgi staining”, was the basis of thousands of works in the years to come and still to date, but in particular of another father of the modern neuroscience, Santiago Ramón y Cajal, to whom we owe a magnificent collection of hand-drawn tables of brain cells. Camillo Golgi first hypothesized the astrocytic involvement in metabolic support of neurons, amid their morphological relation with neurons and blood vessels (Fig. 1B), but



Wirchow, 1858  
*Ependyma and Neuroglia in the floor of the fourth ventricle*



Golgi, 1903  
*Astrocytes forming numerous contacts (the end feet) with brain capillaries*



Ramón y Cajal, 1903  
*Human cortical neuroglial cells of the plexiform layer (a–d), cells of the second and third layers (e–h and k, r) and perivascular glia (i, j).*

Fig. 1 Early observers of astrocytes

Note: A: Drawing of Neuroglia from Wichow, 1858. Adapted from "Astrocytes and Epilepsy", Chapter 1, History of astrocytes; B: Drawing from Camillo Golgi, 1903. Adapted from Parpura V and Verkhratsky A. Astrocytes revisited: concise historic outlook on glutamate homeostasis and signaling; C: Drawing from Ramón y Cajal, 1903. Adapted from "Astrocytes and Epilepsy", Chapter 1, History of astrocytes

Cajal's contribution in the morphological assessment of glial cells is definitively recognized as major, as he managed to modify Golgi's protocol to stain astrocytes specifically (Fig. 1C). Others, like Del Rio-Hortega for instance, followed, but Golgi and Cajal's pioneering studies in the last two decades of the 19th century shed a first light on these complex cells.

The term "astrocytes" though was first introduced in 1893 by another German scientist, Michael von Lenhossek, to refer to these star shaped cells, although this name was misused at least until 1913 for all glial cells, until Cajal was able to distinguish the different types of non-neuronal cells by modifying the Golgi technique to specifically recognize different categories of glial cells. Overall, these and other early observers of glia left as intellectual heritage to the future generation of neuroanatomists that "the form of astrocytes is important, in thinking about their function"<sup>[1]</sup>.

### 1.3 The debate on Gliotransmission: a symptom of neuronal sciovinism in neuroscience?

Although the better acknowledged role of astrocytes

is the metabolic support of neurons, it most certainly is not the only one. Their role in the clearance of neurotransmitters, such as glutamate and GABA, to limit the transmission in space and time and to recycle transmitters, is well established<sup>[2,3]</sup>. Ion homeostasis is also among their better characterized roles: one extremely important among all being  $K^+$  and the capacity of astrocytes to buffer it. Indeed excessive increase during intense neuronal action potential discharge could result to diminish the threshold of activation of neurons to non-physiological levels, leading to pathological consequences like epileptic seizures<sup>[1]</sup>. All the aforementioned cases are well supported by biochemical and ultrastructural evidence, but most importantly they do not imply any direct communication or modulation path from the astrocytes to the neurons.

Recently many works have suggested a role of astrocytes in the direct communication with neurons, by releasing neuroactive molecules (so called "gliotransmitters"), such as glutamate<sup>[4]</sup>, GABA<sup>[5]</sup>, ATP<sup>[6,7]</sup> or monoamines<sup>[8]</sup> (although this latter is limited to a non-murine model), or co agonists such as d-serine<sup>[9]</sup>.



#### 1.4 Lack of solid ultrastructural evidence for gliotransmission

Nevertheless, scientists are still in large disagreement about the cellular and molecular mechanisms of trigger and release of these compounds, mostly revolving around  $\text{Ca}^{2+}$  signalling<sup>[10]</sup> and ultrastructural evidence of subcellular machinery (vesicular compartments) responsible for the release<sup>[11]</sup>, which makes this a matter of debate in the glia field for almost 20 years, to date.

It can be safely argued that the debate became harsh after the first evidence for the presence of the so called “synaptic-like microvesicles”, expressing *in vivo* a similar exocytotic machinery to neurons, in 2004<sup>[11]</sup>(Fig. 2A). But behind the history of this debate, lies a much bigger problem; indeed, ultrastructural characterization of the astrocytic machinery is largely lacking in literature, and detailed analysis are limited to a few aspects revolving around neuronal physiology. Although the few, pioneering extensive works centered on astrocytes and aimed mostly to characterize astrocytic escheatment of synapses under different conditions<sup>[12-14]</sup>(Fig. 2B) helped to starting decipher astrocytic involvement in the synaptic transmission<sup>[15]</sup>, other basic morphological features (such as total synapses under control of a single astrocyte, total

mitochondrial volume, to name a few), that their finest 3D arrangement could reveal, are still lacking. Possibly, this could be due to the fact that unlike neurons that show clear functional subunits that could be classified and studied individually aside their soma (axons, boutons, dendrites, spines), astrocytes does not show such morphological diversity, making it difficult, or impossible, to find a rational to select cellular processes and explore them individually, and compare findings with similar structures found in another cell of the same type. The only exception is the astrocytic vascular endfoot, which indeed has been better characterized, not only from a molecular, but also from a purely morphological point of view<sup>[16,17]</sup>.

## 2 A brief history of 3DEM dedicated to astrocytes

### 2.1 Why we need more astrocytes morphology in EM

High-resolution electron microscopy (EM) is the best technique to observe details of brain parenchyma which are order of magnitudes below light microscopy resolution, such as synaptic contacts, synaptic vesicles, as well as endoplasmic reticulum or mitochondria. Gross neuronal morphology doesn't suffer much from such

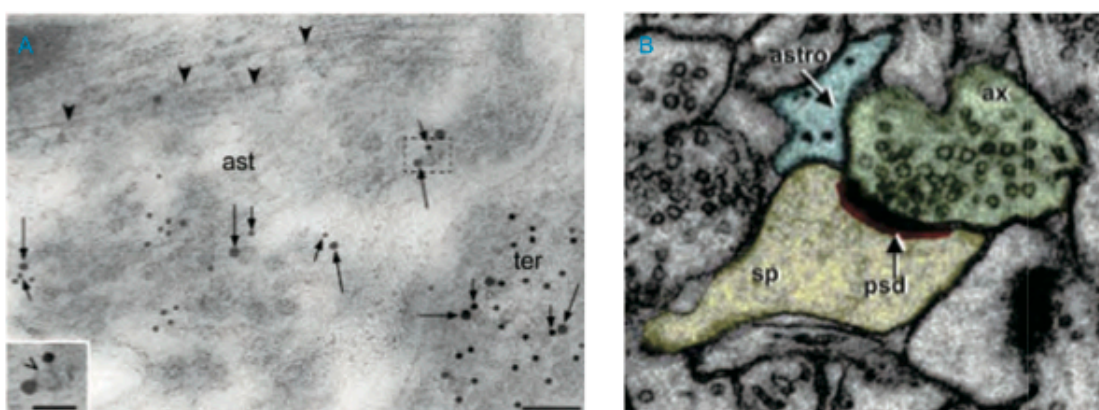


Fig. 2 Perisynaptic astrocytic processes and their physiological relevance

Note: A: VGLUT1 (small gold particles; short arrows) and cellubrevin (large gold particles; long arrows) localize to small vesicular organelles in an astrocytic process (ast) containing GFAP filaments (filled arrowheads). The astrocyte is close to a terminal expressing VGLUT1 and cellubrevin on synaptic vesicles. Magnification: colocalization of VGLUT1 and cellubrevin within the same vesicle (open arrowhead). Adapted from the reference 11; B: Electron micrograph representative of the “tripartite synapse”. Astroglial processes at the axon-spine interface (ASI) Astroglial process (blue); psd, postsynaptic density (red); sp, dendritic spine head (yellow); ax, axonal bouton (green). Adapted from the reference 14

limitation, although the morphology of certain dendritic spines with very short or thin necks hasn't been classified properly in the past<sup>[18]</sup>.

This is not true for astrocytes. Light microscopy provided important information about morphological hallmarks in correlative morphological studies of astrocytes (such as their territorial coverage in murine models shown in Fig. 3A, which is not conserved in higher mammals, or the presence of types of cortical astrocytes with processes extending throughout many layers, only in human astrocytes, to name a few). Nevertheless, it is to date a well accepted fact that small lamelliform processes, important because they interface structurally and possibly functionally with synapses, cannot be imaged simply using light microscopy, and need electron microscopy to be visualized properly.

A better spatial resolution (to about 1 nm per pixel size) can be obtained by transmission electron microscopy (TEM), a technique that always helped to fill the gap between the “gross” light microscopy observations, and the speculations based on functional data linked to details too small to be observed directly. One example

comes from speculations of the astrocytic involvement in the energy storage through glycogen, a phenomenon first observed directly using EM by Phelps<sup>[19]</sup>(Fig. 3B).

The first electron microscope though dates four decades before that, built by Max Knoll and Ernst Ruska in 1931<sup>[21]</sup>; three-dimensional reconstructions kicked off no longer later than that, and has been around since the first half of last century. The first methods papers appeared between the 50s and the 70s<sup>[22-24]</sup>. In addition to the challenge of obtaining serial sections and printing on a photographic paper hundreds of images, one had to think about how to reproduce a three-dimensional picture, either by drawing stereograms on paper, or by manually producing models from piles of papers drawn and pasted and cut<sup>[25,26]</sup> or from wax or plastic<sup>[27]</sup>, making the whole procedure extremely long and tedious.

## 2.2 3D reconstructions of astrocytes in the 20th century

Early work focused on a range of volumes of neuropil, depending whether the focus of the study was on the astrocyte, its intracellular content or the synaptic connection, astrocytes' most important partner. The first paper

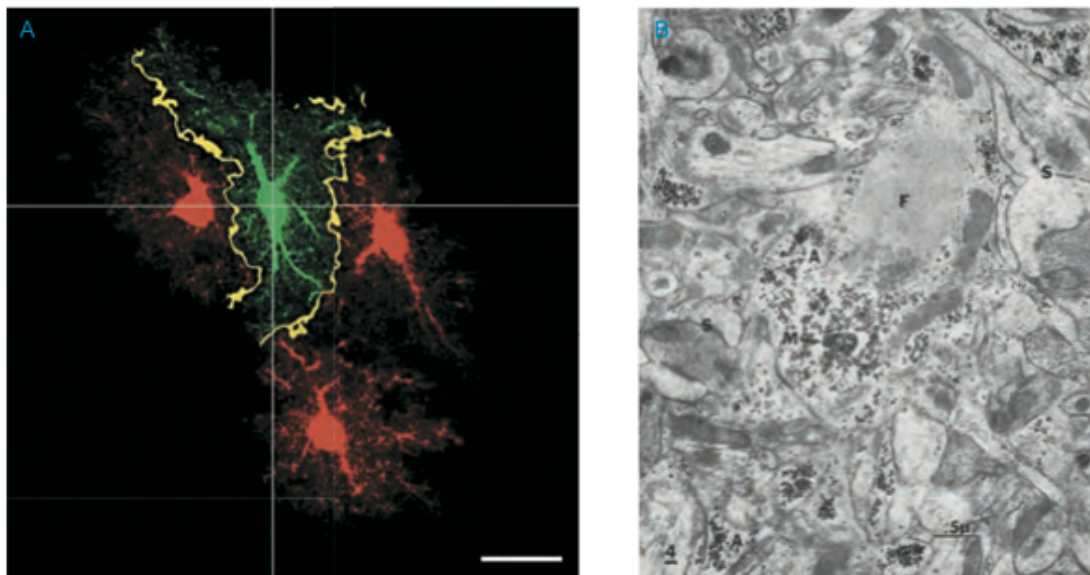


Fig. 3 Astrocytes, from light microscopy to the electron microscopy

Note: A: Astrocytes in the CA1 region of hippocampus filled with Lucifer yellow or Alexa488 imaged under confocal microscope. Yellow pseudocolor highlight the fraction of processes overlapping between the astrocytic territories(Scale bar, 20  $\mu$ m). Adapted from reference 20; B: Synaptic neuropil in the area dentata of the mouse hippocampus; large astrocytic processes (A) densely filled with glycogen granules, among axo-dendritic synapses (S). In the large astrocyte process in the center is a collection of gliofilaments and tubules (F)



showing a three-dimensional reconstruction of an astrocytic process, from brain cortex, was published in 1963 by Wolff<sup>[28]</sup>(Fig. 4). In this paper, he investigated in cortical and subcortical areas of rabbits and rats brains the finest structure of unstained astrocytic processes, that he recognized by their morphological traits, acknowledging the necessity for such complex cells the need to use serial sections and three-dimensional reconstructions to avoid mistake in the interpretation of the data<sup>[29]</sup>. Similarly to Wolff, Stensaas also attempted the identity of astrocytic processes, as well as oligodendrocytes and microglial cells, by morphology<sup>[30]</sup>, in the spinal cord of the toad. He tried for the first time to formalize criteria to distinguish these types of cells based on their appearance in the electron micrographs, and stated how important three dimensional reconstructions are to correlate EM micrographs with observations based on light microscopy, at that time still based on impregnation protocols based on the Golgi staining and adapted by Ramón y Cajal and Del Rio-Hortega for glial cells<sup>[31]</sup>.

Only one year later, 1969, the first paper where the word glia (clearly referring to astrocytes) and the term “three-dimensional ultrastructure” were present together

in the title was published by Poritsky<sup>[32]</sup>. In this very elegant report, he described the presence, and distribution of GFAP, and speculates about the three-dimensional reconstruction being a tool revealing the presence of pocket-like compartments, that we could define nowadays as microdomains, as ways to probably increase the surface-area to volume ratio to increase astrocytic efficiency to redistribute energy.

Another important contributor was Joseph Spacek, to whom we owe an important collection of works where astrocytic processes were reconstructed and analysed in 3D, between the 70s and the 80s<sup>[33-35]</sup>. Inspired by the beautiful work of his predecessors, he focused his qualitative and quantitative analysis of so called “glial sheets” (a.k.a. perisynaptic astrocytic processes), in cortex and cerebellum, either using golgi impregnation or naive tissue. Importantly, he highlighted for instance how astrocytic protrusions facing axonal boutons might appear similar to dendritic spines in single section electron micrographs<sup>[33]</sup>, hence the relevance of observing such structures over their three-dimensional morphology.

### 2.3 Technological advancements of 3DEM

As the technology advanced, producing and repre-

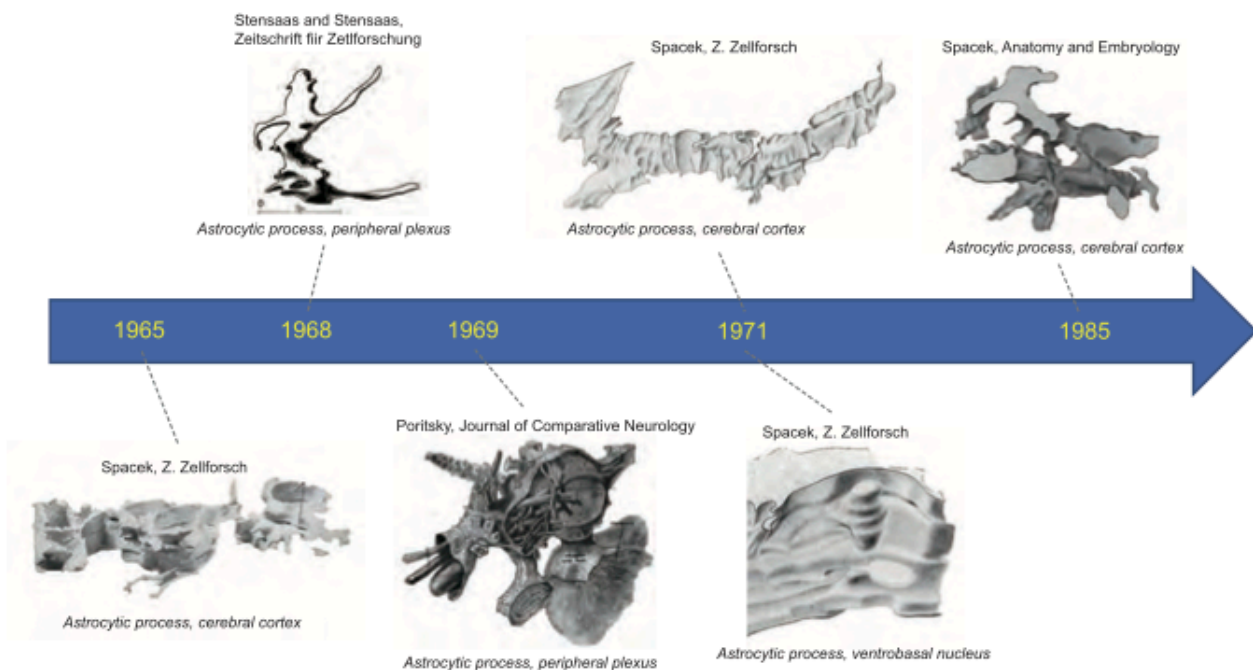


Fig. 4 Timeline with references of papers showing 3D reconstructions of astrocytic processes from 1965 to 1985

senting 3D reconstruction with the aid of software dedicated to image processing and segmentation, together with the improvements of digital microscopy, made the technique more accessible. As a consequence we are witnessing to date the generation of impressive datasets including thousands of objects, the so called “dense reconstructions”, used to date for connectomics purpose for instance<sup>[36]</sup>. Indeed, in the last two decades the number of works showing three-dimensional ultrastructure of astrocytes grew considerably, starting 1999 (Fig. 5), at the end of a decade where seminal observations were made about calcium waves, as well as release of glutamate from astrocytes<sup>[37,38]</sup>, making rethink of the glial sheets serving not only as an energy reservoir, but also as an active synaptic partner<sup>[15]</sup>.

## 2.4 3D reconstructions of astrocytes in the 21th century

In 1999 The Kettenmann group<sup>[39]</sup> published a rendering of a 3D reconstruction from a ssTEM sample of a large astrocytic process surrounding axons and dendrites, in order to structurally highlight the existence of astrocytic calcium microdomains around cerebellar synapses.

The last attempt dated back 1985 (Fig. 1), and the use of modern image processing software thanks to 15 years of computer science advancements resulted in a spectacular series of 3D renderings. Cerebellar astrocytes (a.k.a. Bergmann Glia) in this case were first injected with Lucifer Yellow, a morphological marker, and then photoconverted to be recognized under EM. The volume reconstructed covered a fairly large area (about 20  $\mu\text{m}$  in x, y, z), enough to envelop a rather complex astrocytic process with a large dendrite.

In the same year, another paper from the team of Kirsten Harris, one of the pioneers of recent 3DEM, published a work<sup>[13]</sup> showing to which extent hippocampal astrocytic processes enwrap CA1 synapses. In order to better highlight the escheatment of synapses by glia, and speculate on their impact on glutamate reuptake and spillover, perisynaptic processes were reconstructed in 3D from serial section electron micrographs. In this case though, astrocytic processes in single section TEM micrographs were not stained, and recognized by morphological features. The team of Kirsten Harris has been very prolific, and published many other works using 3D reconstructions of

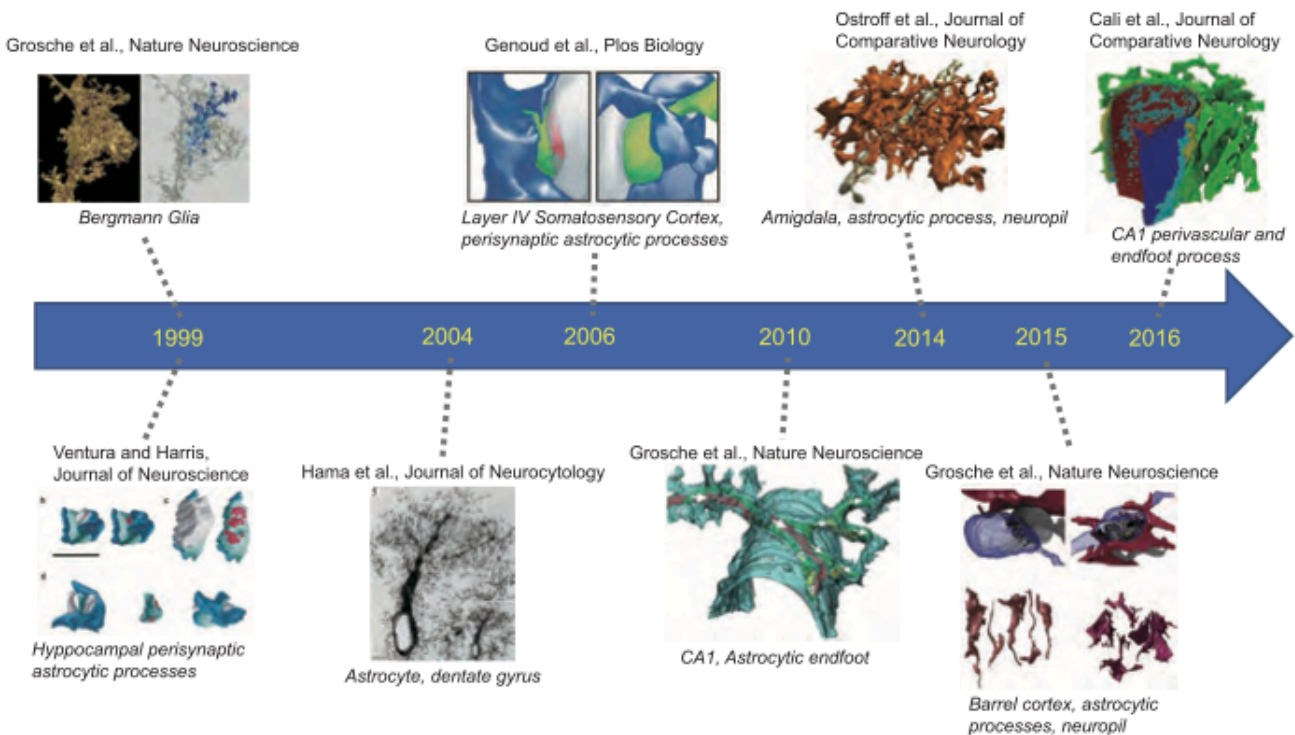


Fig. 5 Works showing three-dimensional ultrastructure of astrocytes in the last two decades



astrocytes to assess their function<sup>[40-42]</sup>.

Another milestone was placed in 2004, when the group of Mark Ellisman showed a 3D rendering of an entire astrocyte<sup>[43]</sup>, reconstructed from an EM tomography of a thick Golgi preparation, in order to attempt an accurate evaluation of morphometric parameters such as total perimeter, surface area and volumes.

Clearly, astrocytic analysis during this century is centered on its relationship with synapses, and whether they mutually impact each other, not only functionally but also structurally. In a very elegant study in 2006, the team of Graham Knott<sup>[12]</sup> assessed the changes of astrocytic processes coverage of barrel cortical synapses by comparing brain samples from mice in resting state with mice undergoing a protocol of whisker stimulation, which is known to potentiate synaptic transmission. The use of 3D reconstruction was key to demonstrate an activity dependent increase of the number of covered synapses, as well as the amount of covered surface area.

Another important aspect to take into account about the astrocytic structure is their intimate relationship with the blood vessel, one of the hallmarks of these cells, and as mentioned before, the only clearly functional identifiable subunit of this cell type. In 2010, the team of Ottesen from the university of Oslo<sup>[17]</sup> published an extensive morphometric analysis, based on 3D data, of a large perivascular astrocytic process, including its mitochondria, and also the contact with pericytes surrounding the blood vessel.

Compared to older datasets, that were coming from models such as rabbits, cats and toads, that are less and less common to date, recent observations are mainly carried out in murine models and humans, although to a lesser extent<sup>[42]</sup>. Also, the vast majority of these studies are carried on, depending certainly on the main interest of the investigators, in the hippocampus or in the cortex. In contrast, one paper from the group of Joseph LeDoux in 2014<sup>[44]</sup>, using an approach reminiscent of Genoud et al.<sup>[12]</sup> compared the extent and the effect of astrocytic coverage on density and size changes of a population of spines of the lateral amygdala during a fear conditioning task. Surprisingly, astrocytic coverage seemed to inhibit spine growth, and indeed newly formed spines, as well as size-

grown spines, didn't show any astrocytic coverage.

A very well known and evident artifact of brain electron micrographs is the shrinkage and expansion of the tissues, due to the aggressive protocols allowing for the infiltration of resin at room temperature. One way to overcome this is to use the water present in the sample as a fixative by cooling it down at very low temperature, and then infiltrate the resin and raising the temperature gently, with special resins. In order to avoid the formation of ice crystals that might destroy the ultrastructure, the process (so called "vitrification") should be fast, and performed at high pressure. This so-called "high-pressure freezing" (HPF) has been performed in a provocative work by Graham Knott in 2015<sup>[45]</sup>. Serial sections from the recently developed focussed ion beam SEM (FIB-SEM) technology<sup>[46]</sup> (a scanning electron microscopy with automated sectioning and imaging capabilities) has been used for three dimensional reconstructions of neurites, astrocytic processes and extracellular space (ECS); comparison between conventional and HPF samples revealed a decrease of more than 10% in the extracellular space, and while neuronal morphology remains relatively untouched, astrocytic morphology is particularly affected, leaving a big question about the accuracy of the many quantitative studies performed on astrocytes to date, based on electron microscopy.

Finally, although the relationship between astrocytic process and synapses are the main topic, a paper from the lab of Pierre Magistretti<sup>[46]</sup> investigated to which extent the glycogen content present within a CA1 astrocytic process was facing pre- compared to post- synaptic processes. The analysis revealed a preference of glycogen clusters towards presynaptic terminals, although more recent visual analysis tools might highlight different patterns<sup>[48]</sup>.

### 3 Conclusions and perspectives

The history of scientists dedicating time and efforts in reconstructing glial morphology in 3D is fascinating, from both a historical and a technical point of view. But, aside the beauty of the technique, an interesting picture arises by observing what kind of information scientists were interested to extract from this kind of morphological

analysis.

### 3.1 The rationale behind the first works

The first, pioneering works investigating the ultrastructure of astrocytes seemed to have a very vague rationale, which is demonstrated by the fact that analysis were carried out in different species, such as cats, toads and rabbits, and comparing them. Despite such gross approximation, they were still able to find common traits worth comparing; notably, the fact that astrocytic membranes were extremely convoluted, and presented “sheets” and “pockets”, whose role we still don’t fully understand, although functional imaging makes us think they might be relevant to space-limiting functional microdomains, although this is still matter of speculation<sup>[4,49]</sup>. If indeed one wants to investigate a phenomenon, the first question to answer is: where is this happening? If the answer was known, then one could design experiments accordingly, and focus his attention to that particular compartment. For instance, if one is interested in how synapses grow depending on sensory stimuli, then Layer IV of barrel cortex is the place to look, whisker stimulation is the experiment to be done, and synapses in that region can be studied. This seem pretty straightforward for neurons in general, as we have a general good knowledge of networks and pathways, and, as mentioned before, neurons have very well characterized compartments with peculiar morphologies adapted to their function. One can then think of using synapses as a focal point, and study the dynamics of astrocytes around them, like the Genoud et al.’s paper<sup>[12]</sup>. But what about astrocytes?

### 3.2 Studies on astrocytic morphology revolve around synaptic structure

In the last two decades, scientists have been struggling to demonstrate that astrocytic calcium waves are, to make it very simple, the language that astrocytes use to communicate; the glial parallel of action potential. This phenomenon became, then, something of a reference point to look at. The paper from Grosche was indeed investigating structurally sites of calcium elevations, that they correlated to the presence of synapses, whose activity is supposedly the trigger. Many studies followed this rationale, but still the shape of the astrocyte doesn’t help,

visually, to understand how, and whether, structural microdomains exist. Also, if they exist, understanding the meaning of calcium oscillations is still an open challenge. We can speak “neuron”, but we cannot speak “astrocyte” yet, therefore until the language of calcium is not decoded, the task is comparable to an italian mother tongue trying to translate a book written in arabic using a russian dictionary.

### 3.3 Metabolism, an easier, exclusively astrocytic language

Maybe one exception can be made for the perivascular process, because it does exactly what its morphology suggests<sup>[17]</sup>: exchanging nutrients, and redistribute them within the neuropil. This was hypothesized at the very beginning of last century by Camillo Golgi, and confirmed by the presence of large cluster of glycogen right at the perivascular processes of astrocytes, when imaged with electron microscopy<sup>[19]</sup>(Fig. 3B). As astrocytes “speak” another “dialect”, less complex than calcium and easier to understand, *i.e.* metabolism, this could represent an easier direction to investigate in the near future; compared to calcium, the metabolic support of neurons have an easy readout, the glycogen granules<sup>[47]</sup>, that can be identified under EM, and can be used as a visual clue to investigate the ultrastructure of astrocytes and identify functional microdomains<sup>[48]</sup>.

In conclusion, many have attempted to properly characterize the stars of the brain, but the problem still remains: what is the right question?

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