Corpora amylacea in the brain form highly branched three-dimensional lattices

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Abstract
Corpora amylacea (CA) are glycoprotein-based depositions that accumulate in the normal aging brain or consecutively to different neurodegenerative diseases. Although controversies still exist in what it regards their origins and functions, their morphology is described simply as round basophilic entities based on bi-dimensional observations. The aim of the present study was to evaluate the three-dimensional morphology of these bodies in the brain in normal aging. We utilized here brain tissue from six aged patients, and performed an in-depth stereological analysis of CAs based on series of thin serial sections, and 50 μm-thick sections. The thin seriate sections have been counterstained with Hematoxylin and Eosin, while the thick sections have been immunostained in fluorescence for ubiquitin and GFAP/collagen IV or aquaporin 4. Three-dimensional renderings have been obtained after aligning the serial sections, while high-resolution z-stacks have resulted after deconvolution on the thick sections. More than 70% of all the identified CAs proved to be in fact parts of larger aggregates, where the flattened individual spheroids branched and communicated with other bodies in a complex pattern, and budding of small CAs from larger CAs could be observed. There was a direct correlation between the diameter of the vessels and the number of associated CAs. Astrocyte GFAP and aquaporin 4 signals surrounded CAs, but without any colocalization with the ubiquitin areas, while perivascular CAs were sometimes enclosed in pockets of the basement membranes. In conclusion, as far as we know, this is the first study to describe the three-dimensional branching complexity of corpora amylacea in the brain.

Keywords: corpora amylacea in the brain, three-dimensional architecture, aging, ubiquitinated bodies.

Introduction
Corpora amylacea (CA) in the brain are glycoproteinaceous depositions that accumulate in the glial, neuronal and extracellular compartments of the aging central nervous system, and largely, in different neurodegenerative diseases [1–4]. A great deal of efforts has been dedicated to elucidating the origin and functions of these structures. CAs are composed of mucopolysaccharide matrices encasing ubiquitinated proteins that are thought to accumulate after degeneration of neurons, glial cells or in chronic vascular diseases [1, 5–7]. Although both cellular and vascular origins have been supported and exemplified, the exact source of CAs is still to be elucidated. It has even been suggested that CAs occurring in normal aging are mostly found at the interface of the parenchyma with the cerebrospinal fluid or blood, as opposite to those that occur in neurodegenerative brain diseases [8]. They could be involved in accumulation of inorganic materials from the blood and cerebrospinal fluid, or in shielding immunogenic residues after neuronal and glial degeneration against an autoimmune response.

However, not the same amount of data exists about the morphological structure of CAs themselves, which have been classically described as round basophilic entities based on bi-dimensional observations only [4, 5, 9]. A more thorough morphological evaluation of any structure might add helpful data toward a better understanding of its roles and origins, yet, there are no studies investigating the three-dimensional morphology of CAs on thick brain tissue specimens [10].

The aim of the present study was to elucidate the three-dimensional architecture of CAs in the aged human brain, to evaluate if they represent individual entities or if they belong to more complex architectural designs.

Materials and Methods
Formalin-fixed paraffin-embedded archived brain tissue blocks were selected from six patients deceased from non-neurological causes with ages of 72, 89, 77, 81, 80 and 79 years. A written informed consent was obtained for each patient from their relatives. All the tissue presented here was prelevated in the Clinic of Neurology and Department of Pathology, University of Medicine and Pharmacy of Craiova, Romania.

At necropsy, general neuropathological evaluation revealed different degrees of cortical atrophy, ventricle enlargement and athermanous changes on the arteries of the circle of Willis. Frontal, temporal, parietal and
occipital tissue blocks were fixed in neutral buffered formalin and further processed for paraffin embedding. Upon sectioning on a rotary microtome with a waterfall-based section transfer system (Thermo Scientific, Waltham, MA, USA), 4 μm-thick serial tissue sections were cut. Representative slides from each case and location were routinely stained for Hematoxylin and Eosin. Histopathology confirmed mild to severe cortical neuronal loss, perivascular, subleptomeningeal and intraparenchymal accumulation of CAs together with different degrees of vascular hyalinization and atheromatosis. Stacks of 40 thin (4 μm) and two thick (50 μm) serial sections were further cut from 15 tissue blocks showing the highest densities of CAs (Research Center for Microscopic Morphology and Immunology, University of Medicine and Pharmacy of Craiova).

Single immunohistochemistry was next performed with an anti-GFAP antibody (mouse anti-human, ABIN125137, Antibodies Online, dilution as 1:300) on the series of thin seriate sections. As in our experience, CAs tend to break apart after boiling or microwaving the slides in citrate buffer, we have utilized here instead an overnight incubation at 70°C in the buffer. Briefly, after antigen retrieval, endogenous peroxidase block and blocking of unspecified binding sites, the sections were incubated with the primary antibody for 18 hours at 4°C, and the next day the signal was amplified for 30 minutes utilizing a species specific peroxidase polymer-based system (Nikirei-Bioscience, Tokyo, Japan). The signal was then detected with 3,3'-diaminobenzidine (DAB) (Dako, Glostrup, Denmark) and the slides were cover-slipped in DPX (Sigma-Aldrich, St. Louis, MO, USA) after a Hematoxylin and Eosin staining.

In order to study the relationship between CAs and astrocytes or basement membranes, double immunofluorescence was performed on the 50 μm-thick sections, utilizing an anti-ubiquitin antibody (rabbit anti-human, Z0458, Dako, diluted as 1:1,000) together with either the anti-GFAP antibody, an anti-aquaporin 4 antibody (mouse anti-human, MA1-34259, Thermo Scientific, diluted as 1:500), or an anti-collagen IV antibody (mouse anti-human, MA1-34259, Thermo Scientific, diluted as 1:1,000) together with either the anti-GFAP antibody, an anti-aquaporin 4 antibody (mouse anti-human, MA1-34259, Thermo Scientific, diluted as 1:500), or an anti-collagen IV antibody (mouse anti-human, MA1-34259, Thermo Scientific, diluted as 1:1,000). The sections were blocked for one hour in 3% skim milk, and incubated overnight with the pair of primary antibodies. Next day, the sections were incubated with a mixture of goat anti-rabbit Alexa Fluor 594, and goat anti-mouse Alexa Fluor 488 – labeled secondary antibodies (Invitrogen, Carlsbad, CA, USA; 1:300, one hour at room temperature).

For consistency, whole slides needed for transmitted light microscopy and analysis were scanned with a 40× objective magnification under a specialized slide scanner-capable microscope (Leica Ario DM6000, Laboratory for Experimental Medicine and Fundamental Research, “Prof. Dr. Matei Balș” National Institute for Infectious Diseases, Bucharest, Romania). After reviewing the whole images, selected areas rich in CAs were cropped and aligned as tiff-format image stacks with multiple serial layers in order to allow the volumetric assessment of CAs (Adobe Photoshop CS2, Adobe Systems Inc., NY, USA).

Fluorescent images were grabbed utilizing a Nikon Eclipse 90i motorized microscope (Research Center for Microscopic Morphology and Immunology, University of Medicine and Pharmacy of Craiova) equipped with dedicated narrow band filter cubes, a 1.4-megapixel monochrome Rolera-XR cooled CCD camera (Q-Imaging, Surrey, BC, Canada), together with the Image ProPlus AMS 7 image analysis software (Media Cybernetics, Bethesda, MD, USA). Image stacks were collected from the full thickness of the slides utilizing a piezoelectric z-stepper at 0.5 μm intervals. All image stacks were originally stored in Image ProPlus’s proprietary format, then they were subjected to a blind deconvolution algorithm based on a multi-pass adaptive point spread function (PSF) subtraction of diffraacted light (AutoDeblur, Image ProPlus), and lastly three-dimensional models were rendered utilizing the 3D Constructor module (Image ProPlus). Ubiquitin-positive small dots, neuritis and perikaryons were not considered here, as they are part of the general neuritic pathology in the elderly [11].

All counting and manual distance measurements have been carried out on the archived images utilizing the Image ProPlus measurement tools and exported for analysis in Excel sheets (Microsoft Office 2003, Microsoft Corporation, Redmond, WA, USA). Continuous data for variate assessments have been averaged and compared utilizing a Student’s t-test, and correlations were assessed based on the Pearson’s correlation coefficient. In all cases, p<0.05 was used to indicate statistical significance.

**Results**

We have first followed CAs on 3 μm-thick serial sections counterstained with Hematoxylin and Eosin and immunostained for GFAP (Figure 1, A–C). As classically described in the literature, most of the CAs were identified as round disks with different degrees of basophilia giving the appearance of onion-like lamination, and more or less aggregated to particular neuropil sites. No inflammatory response, neuronal shrinkage or reactive glia could be identified preferentially around these structures.

More than 70% of all the identified CAs proved, after three dimensional rendering, to be in fact parts of large aggregated material, where the flattened individual spheroids branched and communicated with each other in a complex but still very compact pattern (Figure 1, D–J). Usually, the complexes from within the neuropil had less elements and less complexity compared to those from around the blood vessels. On occasion, elongated or fused spheroid-like structures could be identified and three-dimensional analysis also revealed clear-cut bridges between them. Large compact aggregates could be identified spanning across the whole thickness of the stacks, but also small groups of two or three fused spheroids in the middle of the stack could be identified, thus revealing small isolated aggregates. On enzymatic immunodetection, GFAP tended indeed to aggregate around blood vessels (representing probably perivascular astrocytes’ end-feet), but not particular around CAs.

More than 2500 chosen CAs have been assessed around 50 vessels for the three-dimensional renderings following serial section-based reconstructions. The average size of the considered CAs was of 12.03 (±5.17) μm, and the vessels diameters’ range averaged at 180.13 (±45.2) μm. There was a strong direct correlation between the diameter of the vessels and the number of associated CAs [r(48)=0.82, p<0.01], as well as with the number of branching points in the CAs’ structural architecture [r(48)=0.75, p<0.01].
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Figure 1 – Three-dimensional rendering of corpora amylacea based on transmission light microscopy on serial sections. Consecutive serial sections (A–C) were manually segmented for corpora amylacea and blood vessels, together with an automated RGB-based segmentation for GFAP immunohistochemistry (visualized with DAB) (D–F). Numbers in the right lower corner indicate the order of the serial sections, and the arrows clearly show branching CAs. Three-dimensional rendering of the z-stack obtained by overlaying the serial sections clearly identify a highly branched complex (G–J).

We next evaluated 50-μm thick-sections immunostained for ubiquitin/GFAP, ubiquitin/aquaporin 4, respectively ubiquitin/collagen IV. On immunofluorescence, on ubiquitin-GFAP-stained thick slides, with a higher objective and after deconvolution, clear budding of small CAs from larger CAs could be observed without the errors possible induced by the planes alignment algorithm used during the processing of transmission-light microscopy stacks (Figure 2). It was thus clear that many CAs observed classically as discs are not just isolated spheres upon three-dimensional reviewing. On occasion, GFAP signal clearly showed astrocytes extensions that completely surrounded CAs, but without any colocalization between GFAP and ubiquitin (Figure 3). Although not in all instances, we identified isolated CAs completely wrapped in GFAP signal.
On ubiquitin/collagen IV immunohistochemistry, CAs appeared located mostly very close around blood vessels [with a distance of 4.44 (±2.92) μm for the first line of CAs closest to the vessels], around subpial regions, beneath the ependyma, or in the neuropil itself. Around the vessels, CAs sometimes were enclosed in pockets of the basement membranes, but without any colocalization between collagen IV and ubiquitin (Figure 4, A–E). More detailed analysis of isolated CAs tightly surrounded by collagen IV revealed no overlapping between the two signals, and like for GFAP, sometimes collagen IV signal completely unsheathed some CAs.

An antibody raised against the astrocyte membrane protein aquaporin 4 showed this marker to exist in the immediate proximity of CAs, but again not overlapping with the ubiquitin signal (Figure 4, F–J).

Figure 2 – Three-dimensional rendering of corpora amylacea based on fluorescence microscopy and deconvolution. Consecutive optical planes of a thick section imaged for ubiquitin (red) and GFAP (green) show a small CA budding from a larger one (arrows). Numbers in the right lower corner indicate the order of the optical planes (A–E). Three-dimensional rendering illustrating branching of CAs (F).

Figure 3 – GFAP enwraps CAs but it does not colocalize with ubiquitin. An optical plane from a z-stack shows no colocalization of the two markers after deconvolution (A–C). The corresponding three-dimensional rendering reveals an astrocyte process covering the CA without entangling with its inner structure (D and E). For more clarity, a transparency effect is added to the CA in figure E.
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Figure 4 – Relationships of corpora amylacea with the vascular basement membrane and the astrocyte membrane. On occasion, perivascular CAs could be seen enveloped in a dedublation of the basement membrane, but without any colocalization with the ubiquitin-based structure. This is illustrated through both an example of an optical plane (A–C) and the rendering of the complete optical stack (D and E). Although aquaporin 4, an astrocyte membrane marker, is closely associated with CAs, they also do not colocalize (F–J).

Discussion

Much has been written on the origin and composition of CAs in the brain, and almost all reports discussing these entities refer to them as spherical glycoprotein-based structures [12–15]. CAs are composed of a mucopolysaccharide matrix encasing ubiquitinated proteins accumulating after degeneration of neurons, glial cells and myelin [1, 5]. There are two main theories that have attempted to tackle with the mechanisms that lead to formation of CAs: (i) the vascular, and (ii) the metabolic hypotheses [4]. The vascular hypothesis incurs that in chronic vascular diseases CAs develop especially in the proximity of structures possessing a barrier function, one of the proposed functions of CAs being thus to isolate these glycoproteins and protein fragments from being recognized as immunogenic determinants by lymphocytes and microglia [6, 7]. This is the case of the CAs appearing in the perivascular spaces, or the subpial and the subependymal regions. The second theory postulates that degeneration and oxidative stress-related pathways lead to the formation of CAs and seem to be more adequate to explain increased and early CAs’ apparition in neurodegenerative diseases [16, 17]. The exact cellular source involved in generation of CAs is another source of controversy, both neuronal and glial origins being suggested and supported by different authors [18–20].
The present report was not intended to solve these long-standing conundrums regarding the mechanisms and the origins of CAs, but mostly to assess the morphology of these bodies in the brain. First, on Hematoxylin and Eosin staining, we have followed CAs on consecutive serial sections, and we have attempted to resolve their real three-dimensional structure. This analysis revealed that, while most of the individual CAs are indeed spherical or flat-like structures, they are not isolated elements but highly branched and connected with each other. More than 70% of the CAs we have analyzed on serial sections had this complex architecture, which given their relative large diameters, would show branching or fused CAs only rarely on individual sections. Three-dimensional counting techniques have been used before to assess CAs densities in the brain, but without resorting to serial sections or immunofluorescence on thick sections, and thus their complex architecture was never before described [10]. Our serial section-based approach covered an average total thickness of 160 μm, and the fluorescence studies have been carried-out on 50 μm-thick sections, surpassing thus in both instances other evaluation procedures reported till now for continue tissue depth analysis [10].

Electron microscopy studies showed that these are not-membrane bound bodies, a fact that would either confirm them as resulting from degenerative neuronal or glial processes, or that they are indeed extracellular-born structures [13, 21, 22]. Although astrocyte membrane and cytoskeletal markers envelop CAs, our study revealed no overlapping between them, supporting a rapid development toward an extracellular end phase that could be identified practically in all the analyzed instances.

We have also showed that some perivascular CAs are located in dedublations of vascular basement membranes, which would point also towards the extracellular origin of these elements. While juxtavascular astrocyte proliferation has been shown to occur in acute injury models, it remains to be determined if these astrocyte perivascular end-feet can indeed be found in dedublations of the vascular basement membranes, or our observation sheds light on a new type of vascular intramural-type of CAs [23]. No colocalization could be found between ubiquitin in CAs and the astrocyte membrane-specific target, aquaporin 4. Aquaporin 4 and GFAP are known not to colocalize on brain tissue since one is a marker of astrocytes’ membrane and the other is a marker of their cytoskeleton [24]. In our data, however, antibodies raised against aquaporin 4 and GFAP showed both very close proximity to CAs, probably indicating these areas as spots where astrocytes’ branches distort and their structure entangles losing the membrane-cytoskeleton interface. Whatever these origins might be, we could find no morphology differences between purely parenchymal CA networks and perivascular CAs.

Regardless of their origin, giving the present data describing CAs as a net and nodes-like appearance that seems rather difficult to originate from initial neuronal and glia meshwork, this looks more like a shielding structure at different interfaces that builds up further on based on cellular and extracellular components.

Conclusions

Altogether, to the best of our knowledge, the present study is the first to describe the three-dimensional branching complexity of corpora amylacea in the brain, regardless of their perivascular or pure intraparenchymal disposition.

Acknowledgments

This paper is supported by the Sectoral Operational Programme Human Resources Development (SOP HRD), financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/159/1.5/S/137390.

Author contribution

All authors have contributed equally to this work.

References

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Received: May 12, 2014
Accepted: October 10, 2014