The neuroglia of the optic nerve. Part II. Fractal morphometry of astrocytes

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SUMMARY

The astrocytes of the optic nerve form a network of cells around the nerve fibers and - in particular - establish contacts with the meningeal envelope of the nerve. The morphology of these cells has been analyzed by applying fractal methods (FracLac software) to camera lucida (two-dimensional) drawings of astrocytes, taking the following three parameters into consideration: fractal dimension (D_B), lacunarity (lambda) and density. These parameters provide information on the morphological complexity, heterogeneity and size of the cells respectively.

All three parameters demonstrate that differences exist between astrocytes located in the central region of the optic nerve and those in a marginal position. The former are larger than the latter, which are closely associated with the meningeal envelope of the nerve.

Our results suggest that peculiarities in the morphology of astrocytes are related to the anatomical region in which they are located, where they adapt to the gaps that remain between other neural elements, as is the case of the myelinated nerve fibers of the white matter. This theory is supported by the results of a study in which the morphology of the astrocytes of the corpus callosum were analyzed by applying the same fractal geometry methods.

Keywords: Optic nerve – Astrocytes – Fractal dimension – Lacunarity – Density

INTRODUCTION

The morphology of rat optic nerve astrocytes has been described in analogue microscopy in part I of this study (Mestres and del Rosario, 2022). This second part focuses on examination of these cells by applying fractal geometry analysis.

Fractal geometric analysis is a relatively new tool available to the microscopist interested in quantitatively measuring the dimensions of objects of irregular and complex shape, such as cells, which, as experience shows, are less accessible to analytical scrutiny, such as Euclidean geometry (Cross, 1994). The application of fractal geometric analysis requires digital imaging of the cells to be analyzed (Cross, 1994).

This approach has received much attention in the field of neuroscience. The shapes of neurons are both complex and highly variable (cell body, dendrites and axon), even within one and the same type of neuron, such as, for example, in the case of retinal ganglion cells. With fractal geometry, subtle differences can be defined

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within that group of cells, for example with regard to activity or to functional and pathological reactions (Fernandez and Jelinek, 2001). Studies have been carried out on astroglial cells which display a variety of different shapes in the same species, depending on their anatomical location in the brain. However, morphological differences have also been detected when comparing astroglial types from a different animal species. Fractal geometry enabled the quantification of such differences (Reichenbach et al., 1992).

Another interesting case are the microglia cells, which constitute the autochthonous macrophage population of the central nervous system. Inherent to their nature as macrophages is the fact that they react quickly to external attacks (infections, wounds...) or internal ones (necrosis of the nervous tissue, degeneration, inflammations, etc.), with considerable changes in shape. The application of methods based on fractal geometry has made it possible to document the changes and responses of these cells in a precise and elegant way (Karperien et al., 2013; Young and Morrison, 2018).

As already noted in Part I of this study (Mestres and del Rosario, 2022), hypotheses have been presented which postulate the existence of different types of astrocytes —fibrous and protoplasmic— in the optic nerve (Raff, 1989). However, it remained unclear as to whether these differences in the morphology of this singular population of neuroglial cells are due to their location in the optic nerve. In view of the fact that the Golgi method provides complete impregnations of the astrocytes of the rat optic nerve, we surmised that the application of fractal geometry methods could provide an answer to the question as to whether there are two different morphological types of astrocytes in this anatomical location.

MATERIALS AND METHODS

Tissue preparation and staining of astrocytes

As already described in part I, the animals were sacrificed and processed according to the Golgi-Hortega method, which selectively stained the astrocytes.

Image acquisition

The optic nerve was examined under an M20 WILD optical microscope (Heerbrugg, Switzerland), equipped with a WILD drawing tube and a 50X LWD (long working distance) Olympus objective. For more panoramic examinations, a 20X LWD Olympus objective was used. A fine-tipped (0.2 mm) pigment liner (black water-resistant ink) was used and a total of 60 astrocytes of the optic nerve were traced.

Scale invariance analysis and selection of lenses and magnification

This analysis was performed in order to verify that the selected conditions enabled the definition of a linear relationship between the magnification of the images and the fractal dimension (Sandau, 1996).

Image processing prior to fractal analysis

The determination of the invariance of the scale was a basic step towards understanding the behaviour of the fractal morphology of our cells. Three different magnifications were used (10X, 20X and 50 X), and the linear relationship between fractal dimension and magnification was monitored. Subsequently, the images of the cells obtained from different regions of the optic nerve were digitally processed prior to performing fractal analysis.

This pre-treatment includes the following steps:

1. Digitalization of each cell drawing in black and white with high resolution. The 8-bit grayscale digital image thus obtained was then converted into a binary one using ImageJ's Binary function. This method of generating images is carried out in order to minimize loss and distortion of information during pre-processing. Finally, the background was converted to black pixels, while the lines corresponding to the outline of the cell and its interior were changed into white pixels.
2. All of the images analysed were adjusted to 1000 x 1000 pixels. This was done in order to ensure that neither the cell size nor the length of the drawing traces influences the fractal analysis.

Fractal analysis

The software used for fractal analysis was *FracLac for Image J* (Karperien and Jelinek, 2015). The parameters of interest were: fractal dimension (D_B), lacunarity (λ) and density.

The fractal dimension supplies information on the complexity of a morphological pattern; the higher the value, the higher the complexity.

Lacunarity is associated with changes in the cell body and its extensions, and this parameter measures the heterogeneity of the cell shape (Karperien et al., 2011). A low value of lacunarity indicates homogeneity. Conversely, high values of this parameter imply heterogeneity.

The lacunarity calculated with the FracLac box counting software is nothing other than the pixel mass distribution of the measured astrocytes. The value of lambda obtained is a coefficient of variation expressed as pixel density per box and as a function of box size (Karperien et al., 2011).

The calculation of density involves parameters such as the area of the measured cell (total number of pixels filling the image of the cell under examination, which, if desired can be transformed into square microns) and the so-called convex hull area (CHA), in which the convex hull is the smallest polygon in which the cell is completely included in it (Karperien et al., 2011; Young and Morrison, 2018). The density parameter gives us a measurement of the cell size, understood not only as the cell soma but also including the field occupied by the cell extensions.

Analysis of data

The data were numerically analyzed (polynomic adjustments) and a descriptive statistical analysis was also performed. All algorithms are included in the FracLac software package (Sandau, 1996).

RESULTS

Figure 1 shows examples of optic nerve astrocytes stained according to the Golgi-Hortega method and drawn with the aid of a camera lucida.

Astrocytes located in the central part of the optic nerve exhibit numerous quite thin processes with a smooth surface. Additionally, in some cases cell bodies –somewhat tuberous in shape– have been observed, as well as more robust processes than usual (Fig. 1A).

The cells located in the marginal zone of the nerve have prolongations that are directed towards the surface and enter into a proximity relationship with the meningeal sheaths (Fig. 1B). The astrocytes located in the central part of the nerve display processes in contact with blood vessels and Ranvier nodes, as well as the pial surface.

On the whole, these cells repeat the same morphological pattern in both zones. They do, however, show subtle differences which have been quantified by fractal morphometry methods.

Fractal dimension (D_B)

On average, the fractal dimension of astrocytes in the central zone of the nerve was 1.45303 (SD=0.01822), the minimum value measured in

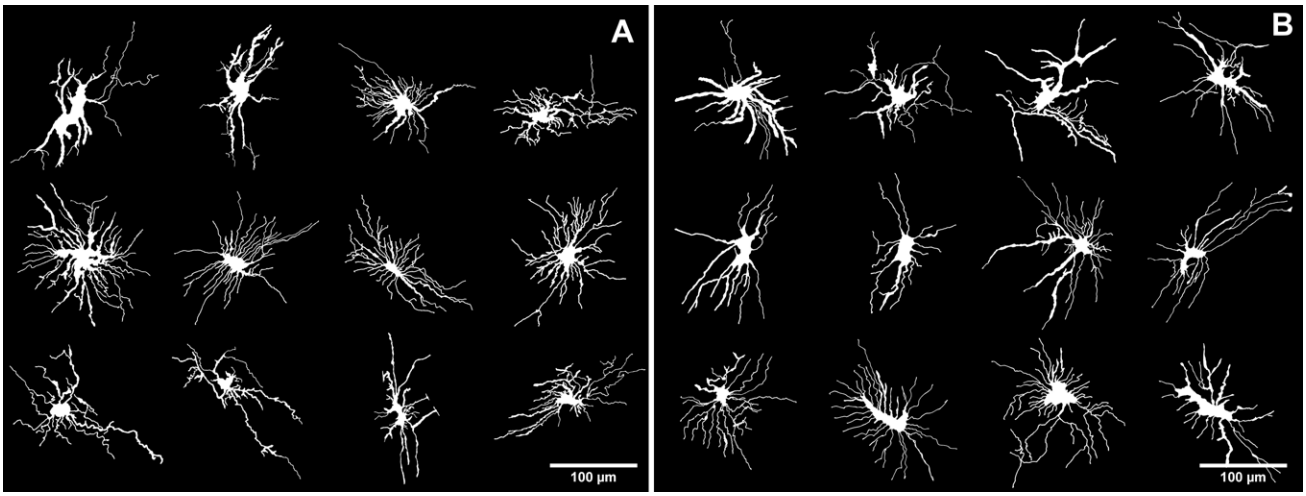

Fig. 1.- Digital images in binary format optimized for the analysis of the fractal dimension, contours and branched structures belonging to astrocytes of the intra-orbital portion of the optic nerve. **A:** Central location. **B:** Marginal location.

our material being 1.3079 (SD=0.0057), while the maximum value is 1.5946 (SD=0.0369). See Fig. 2A.

The morphological complexity of the cells was defined according to the following formula: $(D_{B\,max} - D_{Bmin})/ D_{Bmin}$. This formula expresses the growth rate, or rate of variation, which is a positive change of a variable, and in general terms is defined as: [(final value - initial value) / initial value] x 100 %.

Applying this formula to our data, we obtained a growth rate of 0.22 for the group of central

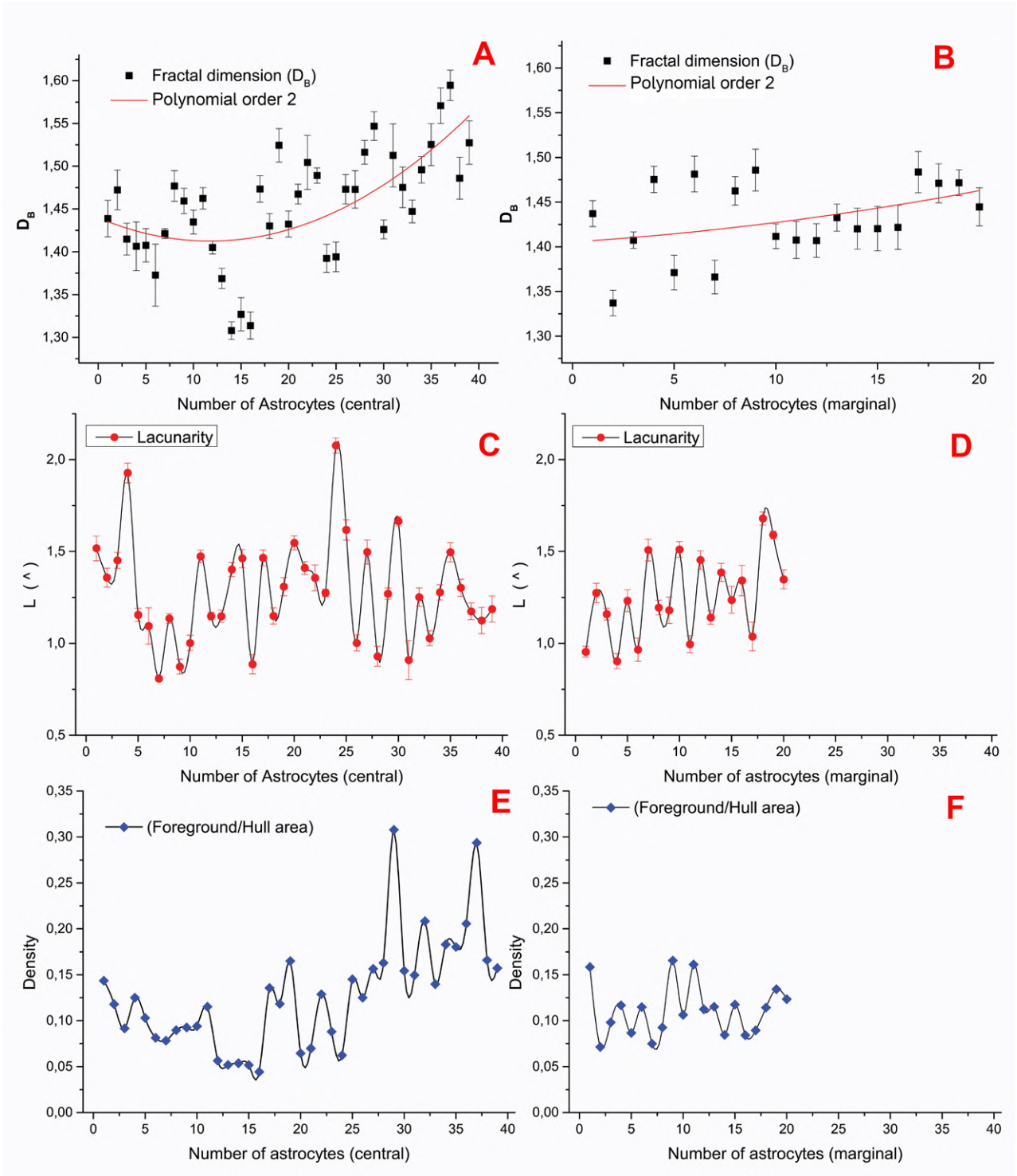

Fig. 2.- Fractal analysis of the complexity of the astrocytes of the optic nerve.

1. Graphic representation of the calculation of the fractal dimension (D_B) of astrocytes of central and marginal location, graph **2A** and **2B**. Polynomial fitting describes details of a similar trend complexity pattern.
2. The lacunarity obtained from the standard box counting calculation is presented in graphs **2C** and **2D**. On the left, lacunarity is shown for central astrocytes that show greater variation and gaps than those obtained from the marginal astrocytes on the right. Consequently, the heterogeneity and complexity registered on the left is higher.
3. The density of pixels in the foreground as an indicator of the degree of occupancy of astrocytes in the optic nerve presents a higher mean value for the central location in comparison with the marginal one, graphs **2E** and **2F**.

astrocytes, which indicates that, within this group, there are variations of the fractal dimension in the order of 22%.

In the population of astrocytes of the marginal zone of the optic nerve, the mean fractal dimension was 1.4308 (SD= 0.0186), while the minimum value measured in this population was 1.3370 (SD=0.0093) and the maximum value 1.4858 (SD=0.0249). See Fig. 2B.

The morphological complexity of this population, which was calculated with the above-defined expression, shows a value of 0.11, which indicates that the variations of the fractal dimension in this group is in the order of 11%.

The values of D_B in each of the populations are represented graphically in Figs. 2A and 2B. Through the application of the polynomial fitting function, we can recognize a clear difference in the behavior of the two populations.

As can be seen from a comparison of the distribution of the measured values with the polynomial fitting curve, D_B is lower in the marginal population than in the central population. In the marginal group, the curve is almost rectilinear, but the central population shows a very marked curve (Fig. 1A, B).

Lacunarity (λ)

The astrocyte population of the central zone of the nerve displays mean values of this parameter of 1.2861(SD = 0.2763), with a coefficient of variation (CV) of 0.0473 (Figure 2C). In this cell population, minimum values of 0.8084 (CV= 0.0152) were measured, while the maximum values were 2.0762 (CV= 0.1060). It is noteworthy that there is a considerable variance between the minimum and maximum values in lacunarity in this region of the nerve.

In the marginal zone, there is less variation in the value of the lacunarity parameter of the cells than in the central zone, which would seem to indicate reduced heterogeneity in comparison with the central part. In this zone, the mean value of lacunarity is λ =1.2544 (CV= 0.0509), while the minimum value measured was λ = 0.9032 (CV=0.0210), with a maximum value of 1.6790 (CV=0.0831). See Fig. 2D.

Density

The measurements of this fractal parameter are based on the convex of Hull and bounding circle, as well as on the pixel density of the image.

In the central region of the nerve, the density shows stochastic values. The mean value was 0.1271 (SD= 0.0605), while the lowest value was 0.0442 and the maximum one was 0.3078 (Fig. 2E).

In the marginal region, variations in this parameter were more discrete with a mean value of 0.1111 (SD= 0.0276). The minimum and maximum values of density were 0.0713 and 0.1655 respectively (Fig. 2F). Accordingly, the variation of density was 1.32 times higher than the minimum value measured for this parameter.

Data Normalization

In order to compare the three parameters measured in the two cell populations, the data were normalized (normalize [0,1]) by applying the following mathematical formula: Y=(Y-Ymin)/(Ymax-Ymin) [source: Origin 9.0 analysis program (64 bit)]

The alignment of the values to a normal distribution enables comparison of the normalized values with those of data sets in a way that eliminates external influences. The results of this operation are shown in Fig. 3.

The normalized distribution of points belonging to the fractal dimension D_B of the central astrocyte population fitted with a Gaussian function is symmetric with respect to a value identified in Figure 3 A as Xc, which coincides with the mean of the D_B values calculated in this population (mean D_B = 1.45303). Similarly, the fitting of the normalized point distribution of the marginal astrocyte population shows a smaller variance and deviates to the right of the mean obtained in the central population (Fig. 3A).

Fig. 3B shows the behavior of the lacunarity parameter in both astrocyte populations. The two curves appear a little shifted to the left, but the marginal astrocyte population still appears to the right of the central astrocyte curve.

This shift of the two curves to the left is even more marked in the density data (Fig. 3C).

DISCUSSION

The optic nerve is a unique tract of cerebral white matter composed of myelinated nerve fibers and neuroglia cells (Sandell and Peters,

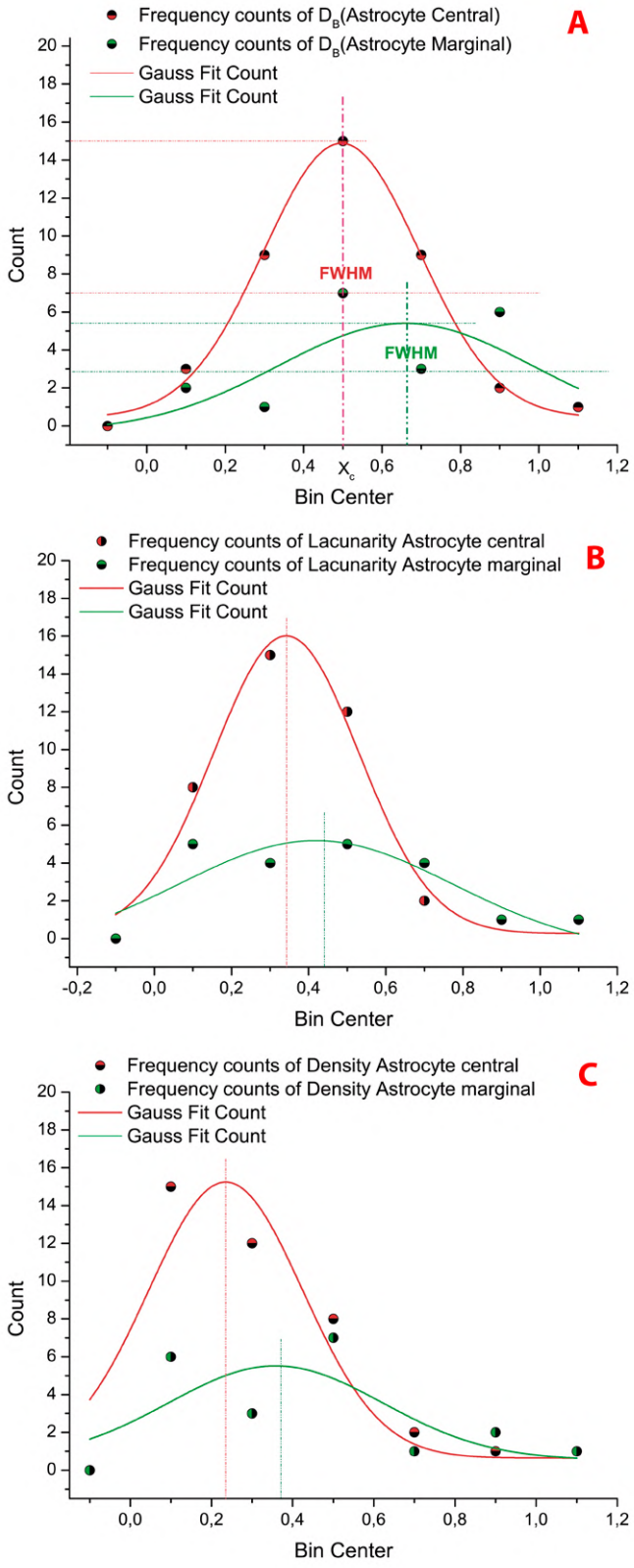

Fig. 3.- Comparative analysis of the normalized data of three calculated fractal parameters (D_B, λ, Density) for both populations of astrocytes, central and marginal. The fitting of the values measured on different scales represented on a common dimensionless index scale. The adjustment using a Gaussian distribution function for both populations allowed us to describe the general behavior of the morphological patterns displayed by the central astrocytes in contract to the singular patterns described by the marginal astrocytes.

2002). Astrocytes, which, in the optic nerve are of the fibrous type, are neuroglia cells that form a braid that embraces the nerve fibers of the optic nerve in bundles or fascicles. However, a part of them maintains a close relationship with the pial sheath of the nerve through terminations of their prolongations.

In this study, we addressed the question as to whether these neuroglia cells –the astrocytes– in the optic nerve of the rat display differences according to their location within the nerve. This is an eminently morphological question and fractal geometry is a very useful tool as it enables us to quantify the morphology of the astrocytes (Jelinek and Fernandez, 1998).

According to Jelinek and Fernandez (1998) "the Euclidean dimension describes objects in space as an integer". Thus, a straight line has a dimension of one (DE=1), a plane a dimension of two (DE=2), and a volume a dimension of three (DE=3). Df, as a dimension, is simply a number that reflects a particular aspect of a geometric form. The dimension value is called fractal because it is a fraction and not a whole number. It is called dimension because it provides a means of measuring how completely an object fills a space. Since a cell represented in two dimensions is not a straight line and does not completely cover a two-dimensional area, it cannot be adequately characterized by Euclidean geometry.

These considerations justify the use of fractal geometry to determine as exactly as possible whether cells with irregular shapes, such as the astrocytes of the optic nerve, show differences in shape.

Fractal geometry is based on several parameters, three of which we have considered in our study: fractal dimension (D_B), lacunarity (λ), and density (Karperian et al., 2013).

The fractal dimension (D_B) summarizes the complexity of the cell, while the lacunarity (lambda) describes its heterogeneity of the cell, such that the higher the value of the lacunarity the greater the heterogeneity. Conversely, lower values of this parameter indicate that the population in question is more homogeneous in morphological terms (Young and Morrison, 2018). The density parameter gives an idea of the size of the cell; this value corresponds to the field occupied by a cell with its soma and all its extensions within the confines of this field (Young and Morrison, 2018).

Fractal geometry has been applied to various types of glial cells such as, for example, Bergmann cells in the cerebellum, also known as Golgi epithelial cells. Using fractal geometry, it was determined that, in terms of fractal geometry, there are differences between these cells, depending on the vertebrate species from which they originate (Siegel et al., 1991). The differences described are very subtle, and generally escape simple examination with an optical microscope. After having studied the fibrous astrocytes of the cat optic nerve (Reichenbach et al., 1992), Professor Reichenbach's group examined in a later study the differences between astrocytes from different locations within the cat brain. This was a comparative study determining the D_B of protoplasmic astrocytes and that of fibrous astrocytes. It especially highlighted that the marginal astrocytes of the cerebral cortex are in contact with the pial envelope, a situation that also occurs in the optic nerve.

Fractal dimension (D_B)

Our results indicate that there is a difference in terms of fractal dimension between the astrocytes in a central position within the optic nerve and the marginal ones, with the D_B being higher in the former. Normalization of the data further supports this interpretation.

Lacunarity (λ)

This parameter also shows differences between central and marginal astrocytes, the former being more heterogeneous than the latter. This further supports the hypothesis that there are two populations of astrocytes in the optic nerve according to their anatomical localization.

Density

Finally, density indicates that the size of the field in the tissue occupied by central astrocytes is significantly larger than that of marginal astrocytes.

On the normalization of the data

The parameter known as "full width at half maximum" (FWHM) is defined as a variance and explains why the three curves of the central population shown in Fig. 3 A, B, C present a more uniform (homogeneous) behavior than those of the marginal one.

This means that the marginal population in the three parameters (Fractal dimension, Lacunarity and Density) presents a higher FWHM than the one corresponding to the central population. However, the fact that the marginal population maintains its maximum (maximum height of the curve) within the representation of the function belonging to the central population indisputably relates them, since it can be affirmed that there are variables which both populations share, to the order of 55 to 70 %. Nevertheless, that differences do exist between them can be deducted from the curves shown in Fig. 3.

Final considerations

The data presented show that, morphologically, central and marginal astrocytes differ to a high degree. They are not distinct cell types, since the morphology of their prolongations in particular corresponds to that of fibrous astrocytes, with

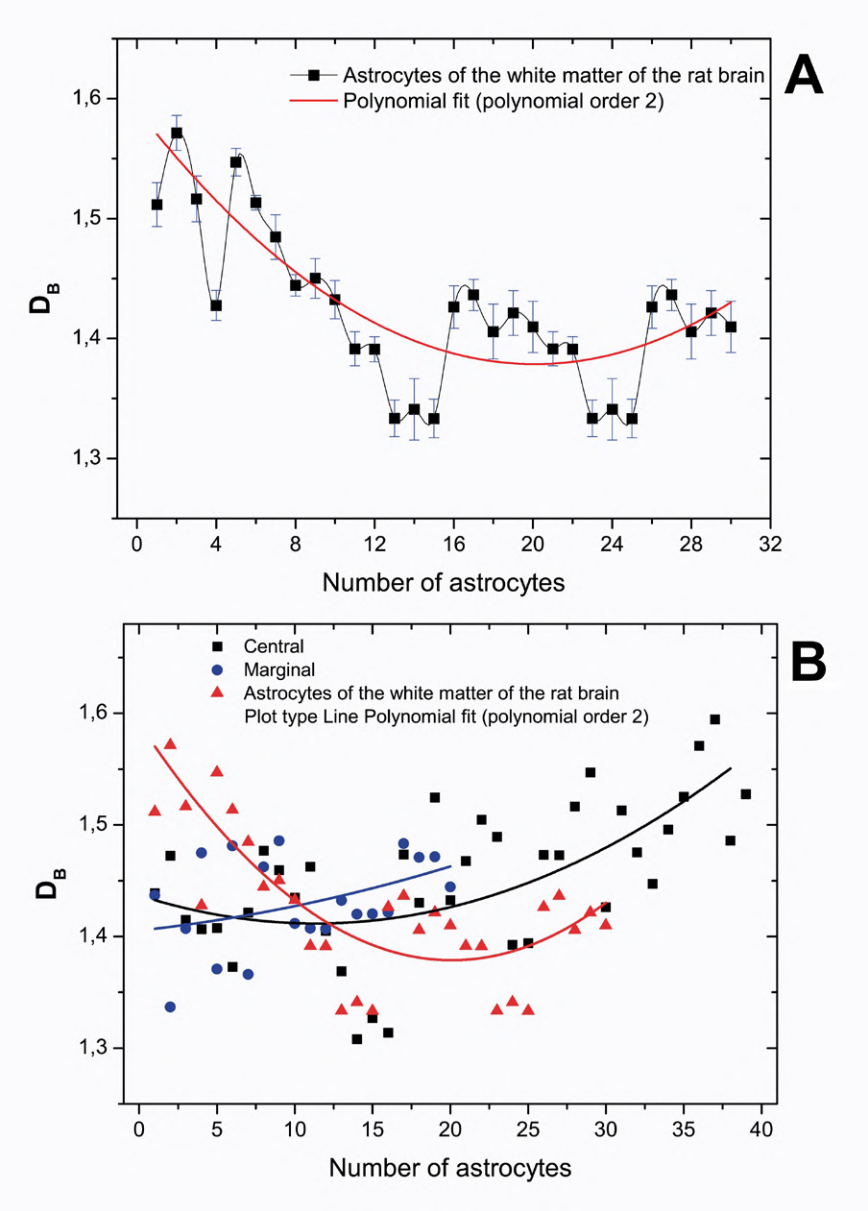

Fig. 4.- Graphical representation of the calculated values of fractal dimension of astrocytes of the white substance **(A)**. The polynomial line of fitting describes a trend contrary to that observed in central and marginal astrocytes of the optic nerve. The behavior described by the three populations is described by polynomial regression methods. This is a procedure of applying the optimal degree to a quadratic model, which provides an explanation of the relationship between both types of interconnected cell populations **(B)**.

their typical thinness and smooth surface. They thus differ from protoplasmic astrocytes, which have shorter prolongations and exhibit a very rough surface (Siegel et al., 1991). The shape changes exhibited by optic nerve astrocytes when cultured in vitro is a sign of great plasticity in that they adopt other shapes as they adapt to the geometric conditions of the culture surfaces (Smith and Behar, 1994). Following this line of thought, it seems plausible to contend that optic nerve astrocytes adapt to the anatomical conditions of the nerve. This also correlates with the differences that can be observed in the cells presented in the work of Reichenbach et al. (1992).

In a parallel study of owr own (unpublished) using the brains of the same animals from which the optic nerves were extracted, it has been shown that the fibrous astrocytes of the corpus callosum (Atlas of König and Klippel, 1963), approximate level A 7470 microns show fractal dimension values different from those measured in the optic nerve, although their cell characteristics (microscopical images) are analogous, if not identical. These differences in D_B after polynomial fitting can be clearly seen in the graph below (Fig. 4).

These findings, although based on a smaller set of cells (30), speak in favor of the interpretation that fibrous astrocytes display morphological differences in the optic nerve in relation to their anatomical environment, i.e., in relation to their location. This seems to be a general pattern of behavior in such cells in the central nervous system.

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