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ORIGINAL ARTICLE

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Size and number of binucleate and mononucleate superior cervical ganglion neurons in young capybaras

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Abstract The total number of neurons in the superior cervical ganglion (SCG) of adult capybaras is known from a previous study, where a marked occurrence of binucleate neurons (13%) was also noted. Here, distribution, number and fate of binucleate neurons were examined in younger, developing capybaras, aged 3 months. The mean neuronal cross-sectional area was 575.2 μm^2 for mononucleate neurons and 806.8 μm^2 in binucleate neurons. Frequency of binucleate neurons was about 36%. The mean ganglion volume was about 190 mm^3 in young capybaras and the mean neuronal density was about 9,517 neurons/mm^3. The total number of neurons per ganglion was about 1.81 mill. Neuronal cell bodies constituted 22.5% of the ganglion volume and the average neuronal volume was 23,600 μm^3. By comparing the present data with those previously published the conclusion is drawn that the maturation period was characterized by the following points: a 26% remarkable decrease in neuronal density which was significant ($P < 0.05$) and a significant 16% ($P < 0.05$) decrease in the total number of SCG neurons accompanied by a 23% decrease in the total number of SCG binucleate neurons.

Keywords Binucleate neurons · Disector · Stereology · Rodents · Capybara

Introduction

In mammals ageing (which is part of the post-natal development) is associated with decrements in cellular and physiological functions and a major incidence of degenerative diseases. These alterations are the result of an interaction among many factors and one may say that ageing is therefore a multifaceted phenomenon (Szweda et al. 2003). In the nervous system, the changes most frequently related are neuron loss, atrophy and hypertrophy (Cabello et al. 2002). Nevertheless, these claims are discussed because there are related differences between various components of the nervous system among animal species (Finch 1993; Vega et al. 1993).

From large mammals little is known about the superior cervical ganglion (SCG), although in the last 20 years they have been studied frequently with the help of pharmacology, electrophysiology, immunohistochemistry and biochemical techniques mostly in rats, mice and guinea pigs, especially because their innervation territory includes intracranial organs such as cerebral vessels, pineal and eye (Miolan and Niel 1996).

The total number of neurons was estimated in SCG of adult rats, capybaras and horses in a previous study by Ribeiro et al. (2004). Further, an unexpected high frequency of binucleate neurons, i.e. about 13%, was found in capybaras.

Mammalian sympathetic ganglion neurons are usually mononucleate, but cells with two nuclei are also found (Huber 1899; Smith 1970; Filichkina 1981; Dalsgaard and Elfvin 1982; Macrae et al. 1986; Purves et al. 1986) and the frequency in which they appear changes considerably from one species to another.

Hence, in the present study we had several objectives and the main questions investigated were therefore the distribution, number and fate of both binucleate and mononucleate neurons in an early stage of post-natal development, i.e. in young capybaras, and to compare these data to that obtained from adult capybaras (Ribeiro et al. 2004).

In order to achieve that, we have used a reliable preparation procedure available (vascular perfusion, resin embedding, semi-thin sections), applying an unbiased stereological method, i.e. the disector method (Sterio 1984; Pakkenberg and Gundersen 1988, 1989)

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that does not require the application of correction factors and any assumption of the object form whatsoever.

It is expected that the current results may shed light on some neuronal quantitative aspects during the postnatal development (maturation and ageing) in large mammals which have not been frequently investigated as well as give support to the veterinary neurology as to the treatment of some SYMPATOPATHIES such as Horner's syndrome (Panciera et al. 2002).

Materials and methods

Animals

This study was carried out on four left cranial cervical ganglion (SCG) of 3-month-old young female capybaras (*Hydrochaeris hydrochaeris*).

For quantitative work, four capybaras were used 1, 2, 3 and 4, weighing 21.5, 20, 20.5 and 22 kg, respectively. The capybaras were from Profauna Farm at Iguape (São Paulo, Brazil) which is licensed by the Brazilian Institute of Environment (IBAMA: 1-35-93-0848-0).

Methods

Histology

Capybaras were killed with an overdose of anaesthetic (Na pentobarbitone 80 mg/kg, i.v.) in connection with other experiments. With a skin incision along the neck, the common carotid artery and jugular vein were exposed. A bulbed cannula was inserted into the left common carotid artery and about 100 ml of pre-wash fluid (PBS, Na nitrite and heparin) was perfused, followed by about 200 ml of fixative (5% glutaraldehyde and 1% formaldehyde, 100 mM Na cacodylate) used at room temperature. Drainage was obtained by slitting the left jugular vein.

In all animals, the neuro-vascular bundle was isolated at the base of the neck. Vagus nerve and cervical sympathetic trunk were dissected away from the common carotid artery. The cervical sympathetic trunk was followed cranially along the common carotid artery, while removing surrounding muscles, up to the cranial cervical ganglion (SCG).

All the nerve trunks issuing from the ganglion were cut and the ganglion was dissected out in its entirety and all ganglia were weighed afterwards.

Long and short axes were recorded from each ganglion using a digital pachymeter and a microfilm reader (Zeiss) at 9–13.5× enlargement. The length or long axis was taken as the distance between the two poles and the width or short axis as the major transverse distance. The ganglia were placed in fresh fixative for 1–2 weeks before embedding.

The ganglia from capybaras were cut transversely with a tissue slider into 6–7 slabs comprising the whole ganglion. The slabs were of approximately equal thickness, i.e. on average 2 mm which is approximately 1/6 or 1/7 of the ganglion length (Fig. 1). The ganglion slab images were captured using the digital camera DC 150 Leica® coupled to a S6 D Leica® Stereomicroscope and projected on computer screen. Afterwards all the slab cross-sectional areas were calculated using a Q-Win Leica® Image Analysis Program coupled to a DMR Leica® light microscope.

The ganglion slabs were washed thoroughly in Na cacodylate buffer, post-fixed in 2% osmium tetroxide in cacodylate buffer, block-stained with a saturated solution of uranyl acetate, dehydrated in graded ethanols and propylene oxide and embedded in resin (Araldite). The resin was cured at 70°C for 3 days.

For light microscopy qualitative study, 2 μm sections were cut with glass knives, collected in glass slides, dried on a hot plate, stained with Toluidine Blue and mounted under a coverslip with a drop of Araldite and photographed on a DMR Leica light microscope equipped with phase contrast optics with 5×, 10×, 20×, 40× and 100× objective lenses.

Morphometry

To obtain without bias a population of neurons for the study of distribution of cell sizes, an identical area was selected in each section. Forbidden and acceptance lines were applied to the four sides of the area, and inclusion and exclusion planes were applied to the beginning and end of the series (Gundersen 1977; Howard et al. 1985).

After sampling the reference and the look-up section (see Stereological study) a full serial sectioning was performed, so that 60–80 serial and consecutive sections were cut at 2 μm thickness.

The sections were mounted on glass slides, numbered and stained with Toluidine Blue. After applying forbidden and acceptance lines from the beginning to the end of each serial sectioning set, images were captured using a Q-Win Leica® Image Analysis Program coupled to a DMR Leica® light microscope equipped with digital camera DC 300F Leica® and projected on a computer screen.

Next, a test system comprising six small frames was overlapped on the screen and the total area of the section examined measured 31,300 μm^2.

In each section the neuronal profiles were numbered individually and the same number was given to all the profiles of a given neuron through the series. Then, of each neuron and its nucleus, the largest profile was identified and measured. Thus, cross-sectional areas and maximum and minimum diameters were also measured.

It has to be stressed that the identification of binucleate neurons was based on an appreciation of the full three-dimensional shape of the nucleus or nuclei on serial sections and not only in a single section.

The frequency of binucleate neurons was achieved through the full series of sections covering all ganglia length, simply calculating the percentage of them in the

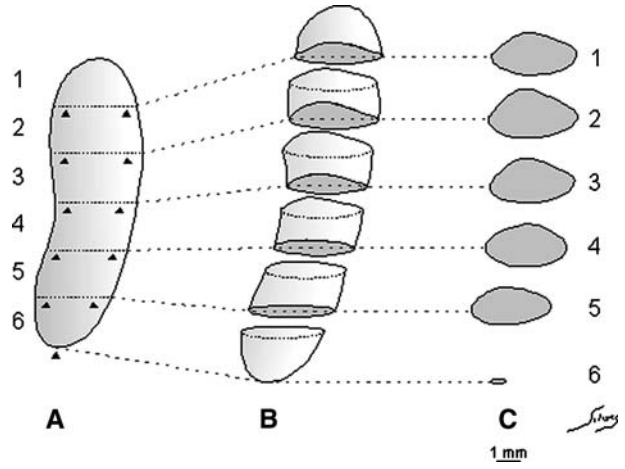

Fig. 1 Schematic drawing (*A*, *B*, *C*) showing a capybara's SCG transversely cut with a tissue slider into six slabs comprising the whole ganglion. The slabs were of approximately equal thickness, i.e. on average 2 mm which is approximately 1/6 of the ganglion length. Slabs were used for both ganglion volume and neuronal number estimation. *Scale bar*: 1 mm

total of 408 (case 1); 396 (case 2); 360 (case 3) and 372 (case 4) neurons analysed. Therefore, the total of neurons (mononucleate and binucleate) examined in the morphometric study was 1,536 (considering all four ganglia together).

Stereological study

Neuronal counting

For the neuronal counting a combination of the physical disector and Cavalieri principle was pursued.

The stereological parameters investigated in this paper were: (1) total number of ganglion neurons using the physical disector method (Sterio 1984; Pakkenberg and Gundersen 1988, 1989; Mayhew and Gundersen 1996; Howard and Reed 2004); (2) numerical density; (3) volume of the reference space or ganglion volume; (4) volume density; (5) mean neuronal volume.

The disector method consists of counting the number of particles, in this case the transects of ganglion neurons (n), that are present in the reference section but not in the look-up section, or vice versa. This value gives the numerical density (N_v) of neurons or neuronal density within the volume of each disector.

Afterwards, the total number of particles (N) is obtained multiplying the numerical or neuronal density (N_v) by volume reference (V_r) or ganglion volume.

Disectors were then systematically, uniformly and randomly applied to cover all slabs of the capybara's ganglia: in case 1, 34 disectors (=34 section pairs); in case 2, 33 disectors (=33 section pairs); in case 3, 30 disectors (=30 section pairs); and in case 4, 31 disectors (=31 section pairs). The number of neurons counted in these disectors was 130 in case 1, 128 in case 2, 110 in case 3 and 124 in case 4.

A constant disector height (12 μm, a third of the average particle size) was considered when applying the method, and the total effective area examined per disector was 31,300 μm^2.

Neuronal density (numerical density)

Numerical density (N_v) is the ratio of the total number of discrete objects (in our case ganglion neurons) and the total reference volume.

For obtaining this data, we have used the formula

$$N_v = \sum Q^- \Big/ \sum ta,$$

where t is the height of each dissector, a the area of unbiased two-dimensional frame used for sampling of particles to be counted and therefore ta the volume of each disector. Q^- is the number of particles counted (seen in a reference section but not in a look-up section).

Ganglion volume (V_g)

In order to estimate the volume of the reference space (ganglion volume), the Cavalieri principle was used before and after histological processing. The procedure was performed on ganglion slabs. The ganglia were fully sliced using a tissue slider (see Histology). This procedure generated a set of 6–7 slabs of mean thickness T = 2 mm on average (which was obtained by dividing the length of the ganglion by the number of slabs made) and the location of the first slab was taken as an uniform random number between 1 and T as with the procedure adopted for the human brain by Mayhew and Olsen (1991) and for the capybara's and horse's SCG by Ribeiro et al. (2004).

The following formula was used for the ganglion volume before histological processing:

$$V_g = T \sum A,$$

where T is the mean ganglion slab thickness and A the ganglion slab cross-sectional area.

After histological processing, however, the formula was applied as below:

$$V_g = T \times \mathrm{ma} \times k \sum A,$$

where T is the section thickness, ma the ultramicrotome advance, k the interval between sections used for the estimate and A the section area.

The accuracy of the volume estimate was evaluated through the coefficient of error of the Cavalieri principle (Gundersen and Jensen 1987). The formula used to assess the error coefficient (CE) was: $CE = 1/\sum \mathbf{A} \times [1/12(3\mathbf{a} + \mathbf{c} - 4\mathbf{b})]^{1/2}$, where $\sum \mathbf{A}$ is the sum of all section or slab areas; **a** the sum of all products $a \times a$; **b** the sum of all products $a(a + 1)$; and **c** the sum of all products $a(a + 2)$.

The tissue shrinkage effects were calculated using the formula: shrinkage = volume before processing − volume after processing/volume before processing. The tissue shrinkage using Araldite embedding was 15%.

Therefore, the volume of the reference space estimated using the Cavalieri principle was corrected as follows: V_{ref} (corrected) = $V_{(ref)}$(1 − shrinkage) (Braendgaard et al. 1990; Howard and Reed 2004).

Total number of neurons (N)

The total number of neurons (N) was obtained by multiplying the numerical or neuronal density (N_v) by the ganglion volume (V_g):

$$N = N_v.V_g.$$

The error coefficient was estimated as (Gundersen et al. 1999)

$$CE(N) = 1 \Big/ \sqrt{\sum Q^{-}}.$$

Estimation of volume density or volume fraction (V_v)

The volume fraction is the ratio of total volume occupied by ganglion neurons and the total reference volume, i.e. the volume of the SCG.

In order to estimate V_v a point grid system was randomly thrown over the same reference sections used for numerical density estimate. Next, the total number of points falling within the reference space (rs) was counted, P(rs). Also the total number of points landing in the nerve cell bodies, P(ncb), which consist of both cytoplasm and nucleus was counted.

Hence,

$$V_v(\text{ncb}, \text{rs}) = \sum P(\text{ncb}) \Big/ \sum P(\text{rs}).$$

Volume fraction ranges from 0 to 1 but is often expressed as a percentage.

Indirect estimation of mean particle volume (V_N) from stereological ratios

The mean ganglion neuron volume was calculated from estimates of V_v (volume density) and N_v (neuronal or numerical density) (Mayhew 1989; Howard and Reed 2004). Hence,

$$V_N = V_v/N_v.$$

Statistical analysis

Stereological data were analysed using the Statistical Analysis System (SAS 1995). The analysis of variance (ANOVA) was performed between age groups in all stereological parameters to assess the effect of age, especially on the total number of neurons (N). When the results were considered significant ($P < 0.05$) the T test was pursued.

For the morphometric data (cell and nuclear sizes) the Fisher's exact test (a non-parametric test) was used ($P < 0.05$).

Results

Anatomy

In all capybaras, the cranial cervical ganglion (SCG) was located in the most cranial region of the neck. Dorsally the ganglion was in contact with the vagus nerve and ventrally it was close to the occipital artery. The caudal pole of the ganglion continued into the cervical sympathetic trunk.

The ganglia were roughly spindle-shaped and length and width were 14.1 × 5.5 mm (case 1), 12.3 × 5.0 mm (case 2), 14.5 × 6.1 mm (case 3) and 12.2 × 4.8 mm (case 4). The four ganglia of capybaras weighed 0.18, 0.19, 0.2 and 0.18 g, respectively (mean ganglion weight: 0.19 g ± 0.009).

Structure

In semi-thin section, the ganglion consisted of an agglomeration of clusters of neurons separated by nerve fibres, blood vessels and prominent septa of collagen. The capsule of the ganglion was conspicuous, 30–50 μm thick and was composed of several layers of collagen fibres and flattened fibroblasts.

The ganglion neuron profiles were circular or more commonly oval shaped and surrounded by 1–3 glial cell nuclei (satellite cells) whose processes formed the neuron's glial capsule. The percentage volume occupied by nerve cell bodies, determined stereologically, amounted to 22.5% on average (Table 1). Thus in the capybara approximately 1/4 of the ganglion volume is constituted by nerve cell bodies (perikarya). The remaining 3/4 of ganglion volume were occupied by neuropil (neurites and glial cells) and connective tissue and blood vessels (Fig. 2).

In mononucleate neurons, some nuclei were located in the centre of the perikaryon, while the majority was eccentric, but none resided at the periphery of the neuronal profile. On the other hand, in binucleate neurons the nuclei occupied the two respective poles of the cell having a very distinct and defined position in the cell.

Binucleate neurons appeared to be very common (Fig. 3). In addition, some neurons that seemed to be mononucleate in one neuronal profile in one single section turned out to be binucleate when their subsequent profiles were looked at in adjacent sections. Conversely, some neuronal profiles appeared to contain two nuclei,

but these turned out in subsequent sections to be parts of the same nucleus (bean-shaped nucleus).

Thus, the identification of a binucleate neuron was based on an appreciation of the full three-dimensional shape of the nucleus or nuclei on serial sections. Neurons with three or more nuclei were not observed.

Frequency of binucleate neurons

Of the total number of neurons investigated for the morphometric study, i.e. 408 (case1), 396 (case 2), 360 (case 3) and 372 (case 4), the frequency of binucleate neurons was 48.3% (case 1), 32.5% (case 2), 35% (case 3) and 30% (case 4). On average the frequency of binucleate neurons was about 36% (SD = 7.0).

Neuron cell size

The neuron size was expressed as the area of the maximum profile of a ganglion neuron in an unbiased sample. The cross-sectional area ranged from 111.4 to 1,543.1 μm^2 in mononucleate neurons (average = 575.2 μm^2) (n = 977; SD = 291.8) and between 637.2 and 1,404.3 μm^2 in binucleate neurons (average = 806.8 μm^2) (n = 559; SD = 90.1) (Figs. 4, 5).

Nuclear size

The nuclear cross-sectional area ranged between 32 and 117.8 μm^2 in mononucleate neurons (Fig. 5). The mean nuclear area was 80.7 μm^2 (n = 250; SD = 30.6). As to binucleate neurons the largest nuclear area pair was 159.3 (A); 129.3 (B) μm^2 and 54.9 (A); 33.4 (B) μm^2 for the smallest one, A and B being the largest and smallest nucleus, respectively.

For the mononucleate neurons, the nuclear area represented on average about 14% of the cell body area.

With regard to the binucleate neurons, the largest nucleus area (nucleus A) represented on average about 10.6% of the cell body area. As to the smallest nucleus area (nucleus B), it represented on average about 6.1% of the cell body area.

The two nuclei area (nucleus A + nucleus B) represented together on average about 16.7% of the cell body area.

Ganglion volume

The volume of the SCG was estimated stereologically by means of the Cavalieri principle (Table 1). The mean ganglion volume was about 190 mm^3 in young capybaras. The volume estimates for the ganglia were closely matched by the wet weight obtained (see Anatomy) and showed a small variability between individuals.

The coefficient of error (CE) of the Cavalieri estimate of the volume was 6, 8, 9 and 8% (cases 1, 2, 3 and 4, respectively).

Number of neurons

Based on a large number of disectors distributed at all levels through the ganglia (128 disectors) (Fig. 6) and comprising an overall volume of 0.048 mm^3 an average neuronal density was obtained: 9,517 neurons/mm^3 (Table 1). From these values and from the volume of the ganglia the total number of neurons per ganglion was estimated as 1.81 million on average in the young capybara (n = 4; SD = 48,846; CV = 0.02).

The error coefficients for N estimate, CE(N) were 8.8% (case 1), 8.8% (case 2), 9.5% (case 3) and 8.9% (case 4).

Neuron volume density and mean neuronal volume

From the neuron volume density and from the stereological data obtained with the disectors, i.e. the neuronal density, it was possible to calculate the mean neuronal volume, i.e. mean neuronal volume as the ratio between volume density and neuronal density. Thus, the neuronal cell bodies constituted 22.5% of the ganglion volume

Table 1 Neuronal density, ganglion volume, total number of neurons, neuronal volume density and mean neuronal volume in superior cervical ganglion of young capybaras

Species	Neuronal density (per mm^3)[a]	Ganglion volume (mm^3)	Number of neurons in whole ganglion	Neuronal volume density (%)[b]	Mean neuronal volume (μm^3)
Capybara 1	9,735	180	1.75 mill.	25	25,600
Capybara 2	9,852	190	1.87 mill.	25	25,300
Capybara 3	8,830	205	1.81 mill.	19	21,500
Capybara 4	9,650	188	1.81 mill.	21	21,700
Mean	9,517	190	1.81 mill.	22.5	23,525
SD	465.2	10.4	48,846	3	2,227.7
CV (%)	5	5.5	2.7	13	9.5

[a]The ratio of total number of ganglion neurons and the total reference volume
[b]The ratio of total volume occupied by ganglion neurons and the total reference volume

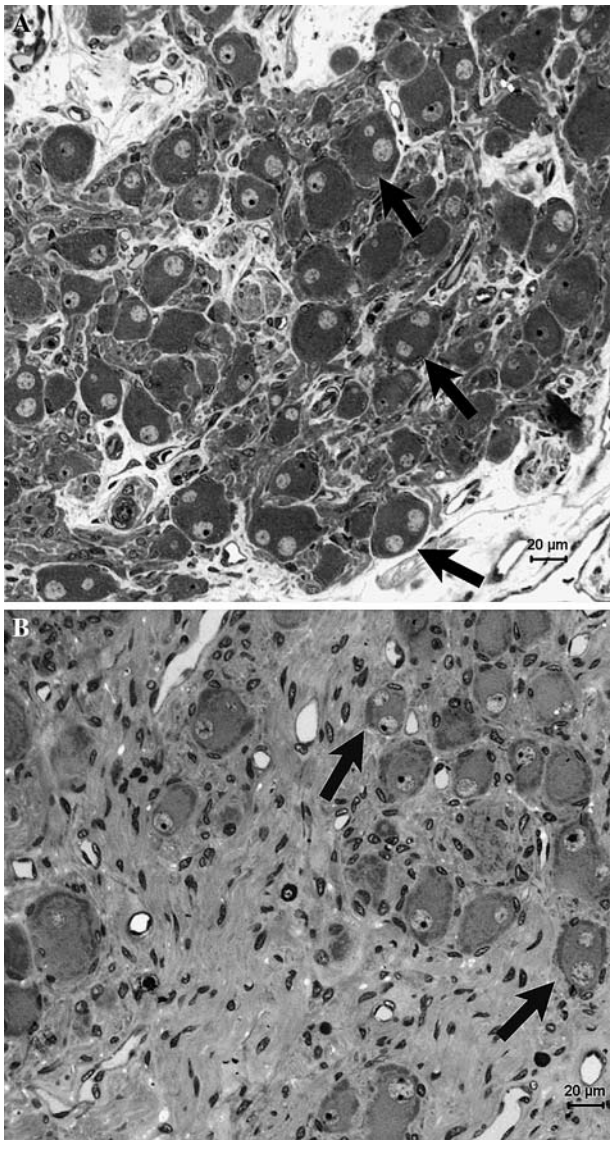

Fig. 2 Fine structure (2 µm) of the superior cervical ganglion of a young capybara (**a**) and adult capybara (**b**) in thin sections at the same magnification. The young capybara shows the highest packing density and in adults nerve cell bodies are separated by large spaces occupied by connective tissue and small blood vessels. Note also the packing density differences in binucleate neurons (*black arrows*) in young and adult subjects. Toluidine Blue. *Calibration bar*: 20 µm

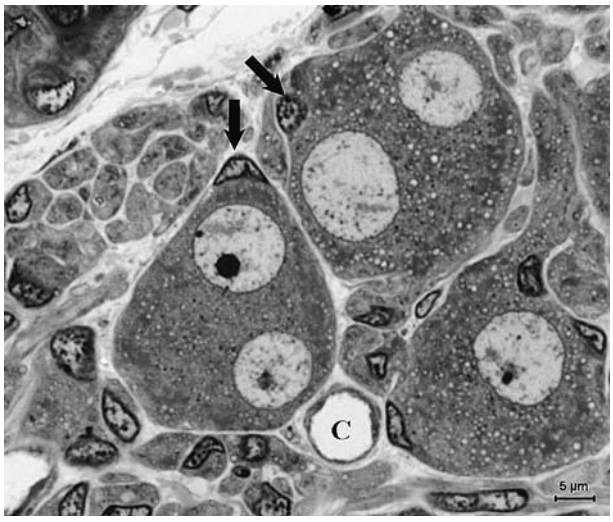

Fig. 3 High magnification view of the superior cervical ganglion of a young capybara. Two large binucleate neurons are visible and two glial cells nuclei are pointed by *two black arrows*. A small blood vessel (*C*) is observed between three large nerve cell bodies. Toluidine Blue. *Calibration bar*: 5 µm

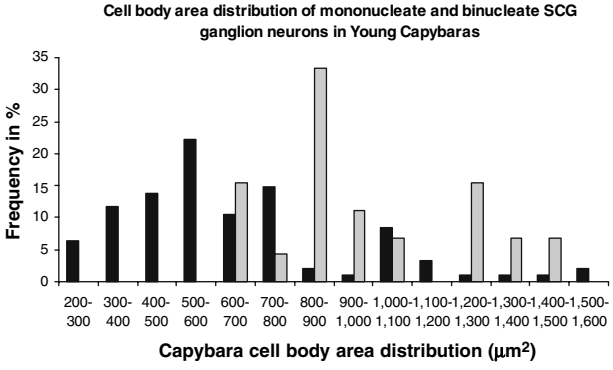

Fig. 4 Histogram documenting the distribution of mononucleate and binucleate Capybara's SCG nerve cell body sizes (cross-sectional area of the largest profile of a neuron) measured from 559 binucleate and 977 mononucleate neurons. The percentage distribution of mononucleate neuron sizes was divided into classes of the same size and ranging from the lowest (200–300 µm^2) to the largest (1,500–1,600 µm^2). The percentage distribution of binucleate neuron sizes was also divided into classes of the same size and ranging from the lowest (600–700 µm^2) to the largest (1,400–1,500 µm^2)

and the ganglion volume is 190 mm^3; the total volume of all neuronal cell bodies in a ganglion was 42.8 mm^3; as there were 1.81 mill. neurons, the average neuronal volume was (42.8 mm^3)/1.81 mill. or about 23,600 µm^3 (Table 1).

Discussion

Frequency of binucleate neurons

From the technical point of view, one must take into account that the appearance of a binucleate neuron can be generated by the superimposition of two cell profiles or due to their very close contact. Further, in a true binucleate neuron it should be possible to see two nucleoli (King and Coakley 1958).

In order to be sure of a binucleate cell, one can perform two different technical approaches, i.e. full serial sectioning or optical sectioning. Using optical sections which are more common in these days, the observer should be able to see two separated nuclei and verify that these two nuclei belong to the same cell. On the other hand, in physical sections (especially in semi-thin sections, 1–2 µm) it is absolutely necessary to pursue a

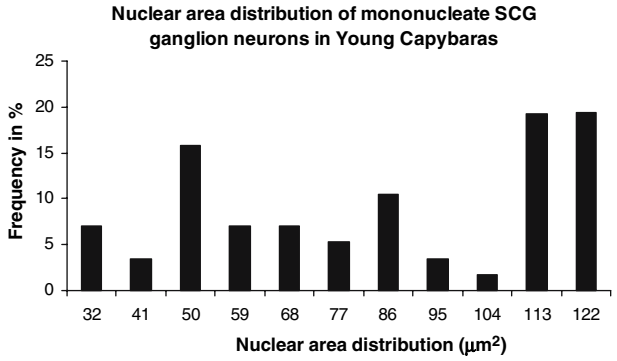

Fig. 5 Histogram documenting the distribution of nerve cell nuclei sizes (cross-sectional area of the largest profile) obtained from 250 nuclei. The histograms illustrate mononuclear SCG neurons showing the percentage distribution of nuclear sizes in 11 classes evenly spread between the maximum and the minimum values for that species

full serial sectioning through the whole extent of the cell in order to ensure its binucleate characteristic.

Binucleate cells have been detected in the hearts from a series of human fetuses ranging in age from 12 weeks (50 mm crown-rump length) until term (Smith 1970). At the 55 mm stage of development binucleate cells are occasionally present in the fusiform ganglia lying in the adventitia of both the aorta and pulmonary artery. Slightly later (65 mm) they are found in ganglia situated close to the superior atrial walls and still later (120 mm) they are present in about 2/3 of the intrinsic cardiac ganglia. The frequency of occurrence of binucleate neurons reaches a peak at the 120 mm stage and then declines so that, at term, only a few of these cells can be identified in the atrial ganglia.

Therefore, binucleate cells make their appearance at the stage in the development of the innervation of the heart when the immature neuroblasts seen in early stages are becoming fewer in number and when their migration along nerve bundles passing from the cardiac plexus has almost ceased. On this basis binucleate nerve cells are found when cell division is occurring and it may represent a stage in a mechanism to increase the number of ganglion cells in the heart, by a process of active division, at a time when migration of neuroblasts into the heart has virtually ceased (Smith 1970).

One explanation for the occurrence of two nuclei in the guinea pig sympathetic ganglion cells is that a mitotic arrest late in the differentiation process has occurred. There is no known reason for this, but it has been suggested that it might be due to a response to nerve growth factor (NGF) stimulation of the ganglion cells in their final differentiation to adrenergic cells. It is, however, unclear why only these cells respond in such a manner and not other adrenergic cell populations. NGF is also known to affect cell size and dendritic complexity by increasing the cell size and terminal arborization (Levi-Montalcini and Aloe 1983; Purves et al. 1988).

The functional significance of the phenomenon with the binucleate cells is not known. It is possible that the

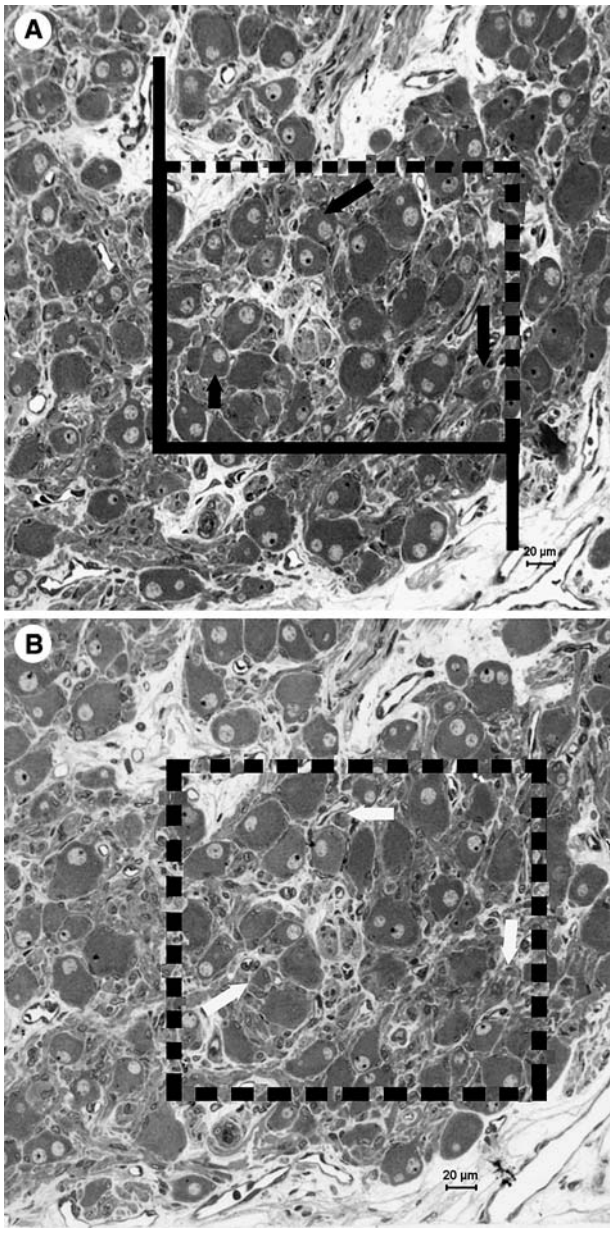

Fig. 6 Micrographs of a pair of sections (one disector) (2 μm thick and stained with Toluidine Blue) used for estimating nerve cell numbers in a young capybara. In each pair the reference section is at the *top* (**a**) and **b** is the look-up section. The separation between reference section and look-up section is 12 μm. *Black arrows* show three neuronal transects seen in the reference section which no longer exists in the look-up section (*white arrows*). *Scale bar*: 20 μm

occurrence of the two nuclei is an adaptation to some specific demand imposed on the cells with respect to protein synthesis. It is also doubtable that the binucleate cells genome is broader, as there is no indication that one of the nuclei has a larger proportion of euchromatin than the other or that the nuclei are different from those of the mononucleate cells in their hetechromatin/eurochromatin ratio.

However, these speculations are contradicted by the study of Filichkina (1981), where the binucleate's rabbit

SCG cells did not show an increase in the total protein synthesis or axonal transport of proteins compared to the mononucleate SCG cells.

The ultrastructural aspects of the guinea pig's cardiac neurons were investigated by Kobayashi et al. (1986) both in cell culture and in situ. Binucleate neurons were similar to mononucleate neurons in size either in situ or in culture and the authors have attributed the presence of binucleate neurons as peculiar in the heart intrinsic innervation.

Mammalian sympathetic ganglion neurons are usually mononucleate, but cells with two nuclei are also found (Huber 1899; Smith 1970; Filichkina 1981; Dalsgaard and Elfvin 1982; Macrae et al. 1986; Purves et al. 1986) and the frequency in which they appear changes considerably from one species to another and during the post-natal development. For instance, in 9 to 10-month-old adult capybaras, Ribeiro et al. (2004) have reported that 13% of SCG neurons are on average binucleate. However, in 3-month-old young capybaras the percentage of binucleate neurons in SCG is on average 36%. Hence, during the post-natal development 23% of binucleate neurons disappear for some inexplicable reason until the present moment.

From a genetic point of view the relative proportion, ultrastructure and DNA content of the binucleate cells in the guinea pig's celiac superior mesenteric ganglion was studied by Forsman et al. (1989). The number of mono- versus binucleate cells was found to vary with the stage of development with about 40% of cells being binucleate in adult animals and 50% in the late prenatal stage (60-day-old fetuses). Further, no differences in ultrastructure were observed between the nuclei of the two cell types. Using the Feulgen method the authors have stated that binucleate cells contain twice the amount of DNA found in the mononucleate cells.

However, our knowledge of the binucleate nerve cells and their functional significance is far from complete, as there is no plausible explanation for the large occurrence of binucleate sympathetic neurons and for their 10 and 23% loss during the post-natal development in guinea pigs (Forsman et al. 1989) and now in capybaras as well.

Apart from nervous system, certain other cell systems also show bi or even multinucleate cells. The vertebrate skeletal muscle cells are multinucleate (20–40 nuclei) and each nucleus contains diploid quantity of DNA and is unable to replicate its DNA. These cells derive from fusion of myoblasts and manufacture specialized proteins (Bruce et al. 1983). Smooth muscle cells of ascidian are also multinucleate. The cells have also been suggested to derive from cell fusion, as the cell length is proportional to the number of nuclei (Terakado and Obhinata 1987).

In the liver, polyploidy, however, develops through another mechanism, probably by a disturbance of the cyto- or karyokinesis (Wilson and Leduc 1948). The doubling of chromatin material has, however, no functional limitations (Romen et al. 1980).

Furthermore, binucleate cells occur in villus growth of goat placentomes (Lee et al. 1986). Two different types of binucleate cells are present, they have equivalent ultrastructural appearance but different granule content (determined by immunocytochemistry) and functions in the goat placenta (Lee et al. 1986).

Nerve cell size

Our findings show that there is a clear significant difference between mononucleate neuron sizes from young to adult animals ($P < 0.05$). Furthermore, a significant difference was verified in binucleate neuron sizes between young and adult capybaras ($P < 0.05$). For instance, a 70.7 and a 56.1% increase in the size of mononucleate and binucleate neurons size, respectively, was recorded at these two ages. Further, the majority of mononucleate neurons (23%) in young animals had their cell size between 500 and 600 μm^2 whereas in adult animals it ranged from 800 to 900 μm^2.

Most of binucleate neurons (35%) had cell sizes ranging from 800 to 900 μm^2 in young capybaras and from 1,200 to 1,300 μm^2 in adults. Hence, these data show an increase in cell size during the maturation period (from young to adults) and binucleate neurons in young animals are equivalent in size to mononucleate neurons in adults.

Additionally, the sizes of both mononucleate and binucleate neurons are significantly different within the same age group, i.e. in either young capybaras or in adult capybaras, separately ($P < 0.05$).

Numerous studies in the literature have shown that at least in small mammals mature ganglion neurons are capable of further growth, when challenged with an expansion of their innervation territory or when rats were submitted to physical exercise as reported by Alho et al. (1984) for the rat SCG with an increase in the diameter of neurons.

Additionally, the observation indicates that allometric relations in the nervous tissue of different species at different ages, such as they exist, are complex because several morphogenetic factors are at work and interact. When trying to figure out morphometric correlations, more than one parameter should be considered at the same time, for example size and number of neurons.

Ganglion volume

Except Ribeiro et al. (2004) data on the volume of the capybara's SCG are not available in the literature and we have now provided estimates in young capybaras. On average the SCG volume was 190 mm^3 in young capybaras, whereas it was 226 mm^3 in adult capybaras (Ribeiro et al. 2004). It represented a 19% non-significant increase ($P > 0.05$) in the ganglion volume.

Within the sample of four ganglia, there is little inter-individual variation, the largest ganglion examined

being 13.8% larger than the smallest one. However, in adult capybaras the inter-individual variation (between the largest and the smallest ganglions) was larger, i.e. about 50% (Ribeiro et al. 2004).

It should be stressed that ganglia contain, in addition to nervous tissue, a significant amount of non-neuronal tissue, i.e. connective tissue and blood vessels. The differences in ganglion volume reflect in part a variation in the volume of the nervous tissue and in part a variation of the vascular and connective tissue. As to the former, the proportion of neuronal material is 6.7% smaller in the ganglia of adult capybaras than in young animals. As a result, the proportion of non-neuronal tissue is 1.9% larger in adults than in young capybaras.

Total number of neurons

In the former paper we reported that the total number of neurons in the SCG of adult capybaras is on average 1,520,000 with a small inter-individual variation (about 13%) (1,413,700 vs 1,608,000). Further, 13% on average of the total number of neurons is made of binucleate neurons, e.g. 197,600 (Ribeiro et al. 2004).

In the present study we estimated the total number of SCG neurons of young animals, which is on average 1,812,132 with smaller inter-individual variation than in adults (about 6.8%) (1,752,300 vs 1,814,200). Furthermore, on average 36% of this total number of neurons is made of binucleate cells, e.g. 652,367 neurons.

Taking these two ages into account, i.e. young and adult capybaras, the current data show that there was a significant ($P < 0.05$) 16% decrease in the total number of SCG neurons during part of the post-natal development which is called maturation (from young to adult animals) accompanied by a decrease of 23% in the total number of SCG binucleate neurons though there was a 98.5% increase in the body weight from young to adult capybaras.

Generally speaking, the differences in the total number of ganglion neurons are related to the difference in body weight—the largest species having the largest number of neurons—but they obviously are not proportional to body weight variation (if the number of neurons were proportional, horse should have more than 20 million neurons, more than six times as many as were actually found). Actually, Mayhew (1991) using the fractionator method has shown that the weight of the mammalian cerebellum affords a satisfactory way of predicting the total number of Purkinje cells. Further, Gagliardo et al. (2005) have reported that both the caudal mesenteric ganglion volume and dog's body weight allow an accurate prediction of neuronal density and total number of ganglion neurons during the post-natal development.

These data are in line with those already published (Purves et al. 1986 present data on several species and discuss the works in the literature) and confirm the trend even for species as large as the horse or for rodents as large as the capybara.

In the nervous system, the effects of ageing are verified by a functional decline that involves the central and peripheral nervous systems. The changes most frequently related are neocortical neuron loss, atrophy and hypertrophy, reduction in white matter and in the mean volume of Purkinje cell body, loss of both Purkinje and granular cells in the anterior lobe of cerebellum and loss of myelinated fibres in the human brain (Tang and Nyengaard 1997; Cabello et al. 2002; Andersen et al. 2003; Marner et al. 2003; Pakkenberg et al. 2003). Nevertheless, these claims are controversial because there are related differences between various components of the nervous system and amongst animal species (Finch 1993; Vega et al. 1993).

Farel (2003) reported that the increase in the number of neurons in the dorsal root ganglia of rats is not associated to a possible neurogenesis, but perhaps a late maturation or incomplete differentiation.

In agreement with the results of Farel (2003), a greater number of neurons in the hypogastric ganglion of rats of 24 months than in rats of 4 months was reported by Warburton and Santer (1997) and a greater number of neurons in the dorsal root ganglia of rats of 80 days than rats of 11 days was also stated by Popken and Farel (1997). Similarly, Gagliardo et al. (2005) have reported a significant 17× increase in the total number of dog's inferior mesenteric ganglion neurons during the post-natal development, i.e. from pups to middle-aged dogs.

In all these studies the physical disector method was used. In contrast, a reduction in the number of neurons with ageing was shown in the SCG, where rats of 4 months presented a "not significantly" larger number of neurons than rats of 24 months. In that study the physical fractionator method was pursued instead (Santer 1991). Similar results were also reported in our present study (16% decrease in the capybara's SCG ganglion neurons) and by Bergman and Ulfhake (1998) where a reduction in the number of neurons was observed in the dorsal root ganglia of rats of 3 months when compared to rats of 30 days using either physical or optical dissector, respectively.

Neuronal density

We studied the number of nerve cells in a total reference volume (mm^3) in young capybaras' ganglia. Again, when comparing young and adult capybaras, i.e. the latter was reported by Ribeiro et al. (2004), there was a 26% remarkable decrease which was significant ($P < 0.05$). A cubic millimetre of tissue contains 9,517 neurons in a young capybara and 7,050 neurons in adults. This difference is accounted for the larger nerve cell bodies in adult capybaras and the greater separation between nerve cells in adult animals.

A good inverse correlation between neuronal density and body weight has been observed in other regions of the nervous system. In the cerebral and cerebellar cortex, for example, Tower (1954), Barasa (1960), Haug (1967), Lange (1975), Mayhew (1991) and De Felipe et al. (2002) observed that the density of pyramidal neurons was inversely related to body size, ranging from 95,000 neurons/μm^3 in the mouse to 50,000 in the rat, 20,000 in the dog and 11,500 in the horse (Barasa 1960). DeFelipe et al. (2002) have reported 24,186 neurons/mm^3 for human temporal cortex, 54,468 neurons/mm^3 for rat hindlimb somatosensory cortex and 120,315 neurons/mm^3 for mouse barrel cortex. A similar correlation was found for SCG between squirrel monkey (32,528 cells/mm^3) and humans (4,455 cells/mm^3) (Ebbesson 1968a, b).

Volume density and mean neuronal volume

When the volume density was comparatively studied, i.e. between young and adult animals, the outcome was a non-significant 6.7% ($P > 0.05$) decrease in this parameter accompanied by an also non-significant 36% ($P > 0.05$) increase in the mean neuronal volume.

These results suggest that during maturation neurons occupy a smaller fraction of the ganglion volume, though they increase in size. Simultaneously, the non-neuronal tissue (vessels, connective tissue, neuropil) would represent a larger fraction of the ganglion volume.

Remarks for future studies

In conclusion this paper sheds light on some aspects of the capybara's SCG post-natal development. The main aspects seen here were the total number of neurons, the high frequency of binucleate neurons, neuronal density and neuron size.

Although there are still controversies regarding what happens to the total number of neurons in ageing (specially in large mammals) and in different parts of the nervous system (enteric nervous system and central nervous system), the results presented in this investigation, which were obtained by using modern and current unbiased stereological methods (Baddeley 2001) for number estimation, revealed a significant reduction in this parameter during maturation.

In addition, due to the fact that only two life stages were looked at, i.e. young and adult capybaras (maturation period), future studies should concentrate on more age groups (fetus, newborn and middle-aged) in order to figure out what happens, for instance, to the total number of binucleate neurons on ageing (both prenatal and post-natal development periods) in an attempt to elucidate the neurogenesis mechanisms namely during the post-natal development whose features are still unclear to most neurobiologists. For instance, what might happen to 23% of binucleate neurons which totally disappeared? Do they die? Do they divide?

Hence, further studies using either mitosis markers (BrdU, Activin, KI-67) or apoptosis markers (caspases) may help us to figure out what really happens to binucleate nerve cells during the post-natal development. Furthermore, there is a need for investigations focusing on whether or not binucleate neurons differ from mononucleate neurons with respect to biochemical and physiological properties.

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