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ORIGINAL ARTICLE

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Estimate of size and total number of neurons in superior cervical ganglion of rat, capybara and horse

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Abstract The superior (cranial) cervical ganglion was investigated by light microscopy in adult rats, capybaras (*Hydrochaeris hydrochaeris*) and horses. The ganglia were vascularly perfused, embedded in resin and cut into semi-thin sections. An unbiased stereological procedure (disector method) was used to estimate ganglion neuron size, total number of ganglion neurons, neuronal density. The volume of the ganglion was 0.5 mm^3 in rats, 226 mm^3 in capybaras and 412 mm^3 in horses. The total number of neurons per ganglion was 18,800, 1,520,000 and 3,390,000 and the number of neurons per cubic millimetre was 36,700, 7,000 and 8,250 in rats, capybaras and horses, respectively. The average neuronal size (area of the largest sectional profile of a neuron) was 358, 982 and 800 μm^2, and the percentage of volume occupied by neurons was 33, 21 and 17% in rats, capybaras and horses, respectively. When comparing the three species (average body weight: 200 g, 40 kg and 200 kg), most of the neuronal quantitative parameters change in line with the variation of body weight. However, the average neuronal size in the capybara deviates from this pattern in being larger than that of in the horse. The rat presented great interindividual variability in all the neuronal parameters. From the data in the literature and our new findings in the capybara and horse, we conclude that some correlations exist between average size of neurons and body size and between total number of neurons and body size. However, these correlations are only approximate and are based on averaged parameters for large populations of neurons: they are less likely to be valid if one considers a single quantitative parameter. Several quantitative features of the nervous tissue have to be taken into account together, rather than individually, when evolutionary trends related to size are considered.

Keywords Rodents · Sympathetic ganglia · Stereology · Disector · Morphometry

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Introduction

Characteristically and uniquely among cell types, there is some correlation between the size of certain neurons and the body size of the animal. This correlation is sometimes obvious, but it is hard to define. First of all, a correlation is noticeable only in certain populations of neurons, and there is no sign of it in many others. Among the former are the neurons that project their axons into peripheral tissues: motoneurons of brain stem and spinal cord, sensory neurons of dorsal root ganglia and cranial ganglia, autonomic ganglion neurons. In each case a wide range of sizes extending over at least an order of magnitude is found even within a discrete population, making it difficult to say whether a body size correlation exists for all neurons within a certain population or only with some. Because all the best examples of a correlation between neuron size and body size are from neurons projecting into peripheral tissues (although there are studies on pyramidal neurons showing similar correlations), the idea naturally arises (already reviewed by Levi 1925) that it is the peripheral tissue that influences the neuronal size or, more precisely, that it is the extent of the peripheral tissue innervated by a given neuron that influences its size.

A second consideration is that even when a correlation is best noticeable, the variation in neuronal size is much smaller than that of body size. There is perhaps a form of allometric growth, where a 1,000-fold increase in body size, for example, is matched by a 10-fold increase in neuron size.

Along these lines, a third point to consider is that while the notion of body size can be reduced, for convenience,

to that of body weight, the notion of neuronal size is more difficult to handle. On the one hand, there are some problems related to the fact that what is measured is some linear parameter (the diameter, for example) of the neuronal cell body or its cross-sectional area: from this value, an approximate value of the cell volume can be calculated. On the other hand, there are difficulties with the very notion of cell size, because the neuronal cell body is only part of the cell, whose axon and dendrites, i.e. neurites, indeed constitute the more voluminous portions.

Regarding total number of neurons, there are many data in the literature, but also discrepancies. For the rat, which is the species most extensively investigated, and its superior cervical ganglion, estimates of the total number of neurons range between 12,500 and 53,000. For instance, 32,000 neurons at 7 days of age (Levi-Montalcini and Booker 1960), 17,000 at 7 days of age and 12,500 at 3 months (Hendry and Campbell 1976), 25,000 (Dibner and Black 1978), from 15,000–20,000 after birth to 35,000 or 45,000 at 1 month (Davies 1978), 53,500 (Gorin and Johnson 1980) and 26,369 (Purves et al. 1986b). The differences between these experiments include the use of rats of different strains, gender and age, and the use of different methods and correction factors; none of the estimates is based on an unbiased method.

In sympathetic ganglia of primates, including man, Ebbesson (1968a) observed interesting morphometric correlations and showed that the ranking of neuronal number was the same as the ranking of body weights. More recently, Purves et al. (1986b), working on the superior cervical ganglion of mouse, hamster, rat, guinea pig and rabbit, confirmed the existence of a relationship between number of neurons and average body size, but documented that a 65-fold increase in body size is accompanied by no more than a 4-fold increase in the number of ganglion neurons (and no more than a 2-fold increase in the number of the relevant preganglionic neurons).

In the present study we had several objectives. We wanted to examine structural quantitative aspects of the SCG (superior cervical ganglion or cranial cervical ganglion), a discrete, easily dissected and well understood ganglion, by using the most reliable preparation procedure available (vascular perfusion, resin embedding, semi-thin sections), and by applying an unbiased stereological method, i.e. the disector method (Sterio 1984; Pakkenberg and Gundersen 1988, 1989) that does not require the application of correction factors. The aim of these choices has been to overcome the technical criticisms that can be raised against all the older studies and the existing estimates. By using the rat as our reference species we compared its ganglion with that of a very large mammal, the horse (more than a thousand times the weight of a rat), and with the ganglion of the capybara, a member of the Cavioidae sub-order and the largest extant rodent, some 200 times heavier than a rat. For horses and capybaras there are no previous data regarding neuronal quantitative aspects, and the range of body sizes that we investigated is broader than in previous studies on this topic. By looking at the nerve cell volume, ganglion size, total number of neurons and neuronal density, we explored the possible correlation of various structural parameters in animal species of very different body sizes.

Materials and methods

Animals

This study was carried out on the left cranial cervical ganglion (superior cervical ganglion,SCG) of adult female Sprague-Dawley rats, adult female capybaras (*Hydrochaeris hydrochaeris*) and adult female horses.

For the quantitative work three animals of each species were used: rat 1, 2 and 3, weighing 170, 172 and 192 g, respectively; capybara 1, 2 and 3, weighing 45, 40 and 40 kg, respectively; and horse 1,2 and 3 weighing 315, 125 and 200 kg, respectively.

The rats were from an inbred colony in the Biological Services of University College London. The capybaras were from Profauna Farm at Iguape (São Paulo, Brazil), which is licenced by the Brazilian Institute of Environment (IBAMA: 1-35-93-0848-0). The horses were from the Veterinary Hospital of the Department of Surgery of the University of São Paulo, Brazil (FMVZ-USP).

Methods

Histology

The rats were killed with an overdose of anaesthetic (Na pentobarbitone, Sagatal 10 mg/100 g body weight injected i.p.). The chest was opened and a bulbed cannula was inserted into the left ventricle of the heart and into the ascending aorta. First, 100 ml of phosphate-buffered saline (PBS), containing 0.1% Na nitrite (vasodilator) and 1% heparin (1,000 IU/ml) (anticoagulant), was injected. The right atrium was open to allow the drainage of blood and perfusate. Then about 200 ml of fixative was injected, consisting of 5% glutaraldehyde and 1% formaldehyde in 100 mM Na cacodylate buffer (pH 7.4) used at room temperature.

The capybaras and horses were killed with an overdose of anaesthetic (Na pentobarbitone 80 mg/kg, i.v.). With a skin incision along the neck, the common carotid artery and the jugular vein were exposed. A bulbed cannula was inserted into the 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 jugular vein.

In all three species, the neuro-vascular bundle was isolated at the base of the neck. The 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 (superior cervical ganglion, SCG).

All the nerve trunks issuing from the ganglion were cut and the ganglion was dissected out in its entirety; the ganglia from capybaras and horses were weighed.

The ganglia were placed in fresh fixative for 1–2 weeks before embedding. 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 of the long axis was taken as the distance between the two poles and the width or short axis as the major transverse distance.

The ganglia from rats were embedded whole. The ganglia from capybaras were cut transversely with a razor blade and a calibrated ruler into 4 or 5 slabs comprising the whole ganglion. The slabs were of approximately equal thickness: one-fifth of the ganglion length in capybara 1, that is on average 3.02 mm thickness and one-fourth of the ganglion length in capybaras 2 and 3 that is on average 4.05 and 4.08 mm, respectively. The ganglia from horses were sliced with a razor blade and a calibrated ruler into 4–5 slabs

comprising the whole ganglion. The slabs were approximately equal thickness: one-fifth of the ganglion length in horse 1, that is on average 4.96 mm and one-fourth of the ganglion length in horses 2 and 3, that is on average 4.3 and 4.28 mm, respectively. In capybaras, the cranial cervical ganglion were cut into 5 slabs of 3.02 mm on average comprising the whole ganglion (case 1), into 4 slabs of 4.05 mm on average (cases 2 and 3, respectively). Outlines of the 4–5 slabs of ganglia of capybaras and horses were enlarged ×9 using a microfilm reader and projected on drawing paper and traced by hand.

The whole rat ganglia and all the ganglion slabs from horses and capybaras 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, 1–2 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 in a DMR Leica light microscope and in an Zeiss Axiophot microscope equipped with phase-contrast optics and orange filter with ×20, ×40 and ×100 objective lenses.

Morphometry

Neuronal size. Between 50 and 80 consecutive sections were cut at 1 μm from each ganglion across its long axis. The sections were mounted on glass slides, numbered and stained with Toluidine Blue; selected matching areas were photographed in all the sections of each series. The photographic negatives were projected to obtain a total magnification of ×325 or ×660. 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 the largest profile was identified and measured. Maximum and minimum diameters were measured with a calibrated ruler, and the form factor (or shape factor) was calculated as the ratio of minimum over maximum diameter (the ratio is 1 in a circular profile). The area of the largest profile of a neuron was measured on a digitising tablet and a morphometric program run on an Acorn Archimedes computer. Axes and area of the nucleus of the same neuron were measured with a similar approach.

To obtain without bias a population of neurons for the study of distribution of cell sizes, an identical rectangular area was selected in each section of a series, 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). In this manner a population of 213 neurons was obtained from the ganglion of three rats, 1 of 157 from the ganglion of three capybaras and 184 from the ganglion of three horses.

Stereological study

Neuronal counting. 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; Pover and Coggeshall 1991; Mayhew and Gundersen 1996 and Howard and Reed 1998); (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 (Nv) 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 (Nv) by volume reference (Vr) or ganglion volume.

Disectors were then systematically and randomly applied to cover all the slabs of the capybara's ganglia. In case 1, 25 disectors were applied in 5 ganglion slabs (5 disectors / ganglion slab); in case 2, 14 disectors were applied in 4 ganglion slabs (about 3–4 disectors/ ganglion slab) and in case 3, 16 disectors were applied in 4 ganglion slabs (4 disectors / ganglion slab). The number of neurons counted was 100, within 25 disectors (case 1), 103 within 14 disectors (case 2) and 101 within 16 disectors (case 3).

In a pilot study different disector heights were used, ranging between 6 and 16 μm. However, a constant height (11 μm, a third of the average particle size) was considered when applying the method.

A test system composed of eight small frames was placed over each section-field image. In this system the frames were sampled at a fraction 1/2 using a random start between 1 and 2, and the total effective area (considering all frames chosen) was 0.0638 mm^2 = 63,800 μm^2.

Therefore, dissectors were systematically and randomly applied to cover all the slabs of the horse's ganglia. For instance, 20 disectors (case 1) were applied in all 5 slabs of the ganglion, so that 4 disectors were applied in each ganglion slab. In case 2, 13 disectors were applied in all 4 ganglion slabs (about 3–4 disectors/ ganglion slab). Finally, in case 3, 16 disectors were applied in all 4 ganglion slabs (about 4 disectors/ ganglion slab).

The number of neurons counted was 101, within 20 disectors (case 1), 101 within 13 disectors (case 2) and 119 within 16 disectors (case 3).

In a pilot study different disector heights were used, ranging between 6 and 16 μm. However, a constant height (11 μm, a third of the average particle size) was considered when applying the method.

In rats, we adopted a different approach, given the smaller size of the ganglia. In case 1, the ganglion was fully sectioned at 1 μm thickness, giving a total of 3,130 sections. Afterwards, 23 disectors were applied and every 136th section (k=136) was considered as the reference-section. In case 2, the ganglion was also fully sectioned (1 μm thickness), giving a total of 3,143 sections. Then, 24 disectors were applied and every 130th section (k=130) was taken as the reference-section. Case 3 was also fully sectioned at 1 μm thickness, giving a total of 2,894 sections. Then, 19 disectors were taken into account and every 152th section (k=152) was considered as the reference-section.

The number of neurons counted was 102, within 23 disectors (case 1), 100 within 24 disectors (case 2) and 100 within 19 disectors (case 3).

In a pilot study different disector heights were used, ranging between 5 and 12 μm. The height used in practice was 7 μm, which is about a third the average particle size in this species.

A test system composed of eight small frames per section image was used for counting. The frames were sampled at a fraction 1/2 using a random start between 1–2 and the total effective area examined was 0.0154 mm^2=15,400 μm^2.

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

To obtain these data we used this formula:

$$N_v = \Sigma n / \Sigma\, txa$$

where *t*=the height of each disector and *a*= the area of unbiased two-dimensional frame used for sampling of particles to be counted. Therefore *t*×*a*= the volume of each disector, and *n*= the number of particles counted (seen in a reference section, but not seen in a look-up section).

Ganglion volume

In order to estimate the volume of the reference space (ganglion volume, Vg), the Cavalieri method was used. The method was applied with two different approaches, i.e. it was applied on ganglion slabs (for horses and capybaras) and on fully sectioned gan-

glia for rats. In horses and capybaras, the ganglia were fully sliced using a razor blade and calibrated ruler (see Histology section). This procedure generated a set of slabs of mean thickness *T* (which was obtained dividing the length of the ganglion by the number of the 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 brain by Mayhew and Olsen (1991).

The following formula was used for the ganglion volume:

$$Vg = T \times \Sigma A$$

where *T*= mean ganglion slab thickness and *A*= ganglion slab cross-sectional areas.

In rats the ganglia were fully sectioned. In rats 1 and 2, 10 sections (every 300th section) were taken into account and photographed. The negatives were viewed in a microfilm reader and enlarged ×9–13.5. Afterwards, the section profiles were traced by hand on drawing paper, and their areas were measured. In case 3, 10 sections were considered for area estimate, taking every 289th section. In the three cases the first section was taken as a uniform random number between 1 and 300 (cases 1 and 2) or between 1 and 289 (case 3). The following formula was used for the ganglion volume:

$$N = N_v x Vg$$

Estimation of volume density or volume fraction (Vv)

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

In order to estimate *Vv* 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, $Vv(\text{ncb, rs}) = \sum P(\text{ncb}) / \sum P(\text{rs})$. 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 *Vv* (volume density) and *Nv* (neuronal or numerical density) (Mayhew 1989; Howard and Reed 1998). Hence:

$$V_N = Vv/Nv$$

Results

Anatomy

In the three species studied, the cranial cervical ganglion (superior 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 internal carotid artery in rats and horses and to the occipital artery in capybaras. The caudal pole of the ganglion continued into the cervical sympathetic trunk.

In rats and in capybaras the ganglion was roughly spindle-shaped, while it was ovoidal in horses (Fig. 1). In rats, the length and width of the ganglion were: 3.3× 0.7 mm (case 1); 3.4x0.9 mm (case 2), and 3.1×0.9 mm (case 3). In capybaras, the length and width were: 15.1x6.1 mm (case 1); 16.2×5.5 mm (case 2), and 16.3x 5.5 mm (case 3). The three ganglia of capybaras weighed 0.29, 0.20 and 0.21 g, respectively (mean ganglion weight: 0.23 g±0.05). In horses, the ganglion length and width were: 24.8×11.1 mm (case 1); 17.2×6.5 mm (case 2), and 17.1×5.6 mm (case 3). The three ganglia of horses weighed 0.44, 0.41 and 0.42 g (mean ganglion weight: 0.42 g ± 0.01).

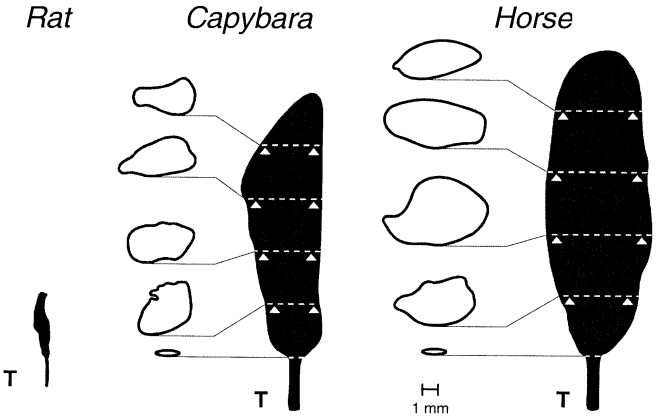


Fig. 1 Outline of the superior cervical ganglion of a rat, a capybara and a horse seen from the dorsal side, with the caudal end (leading to the cervical sympathetic trunk: *T*) at the bottom, all three at the same scale. Outlines of cross sections of the ganglia at the level indicated appear to the left of the larger ganglia. Calibration bar: 1 mm

Structure

Rat

The structure of the ganglion studied in semi-thin sections of plastic-embedded specimens was less complex than that observed in horses and capybaras. Sections of the ganglion showed agglomerations of ganglion neurons separated mainly by nerve fibres and intraganglionic capillaries (Fig. 2). The ganglion was surrounded by a thin connective capsule (15–30 μm) composed of collagen and fibroblasts (Fig. 3). There were no distinct intraganglionic septa. The ganglion neuron profiles were circular or more commonly oval; about two-thirds of the neuronal profiles had a form factor of between 0.5 and 0.8 (Fig. 5). Some of the nuclei were located eccentrically, but the majority were in the centre of the perikaryon. All the neurons were mononucleate. (Fig. 4). Neurons were assembled more closely to each other than in the ganglia of the other species. The percentage volume of the rat ganglion occupied by nerve cell bodies (perikarya), determined stereologically, amounted to 33% (Table 1). The remaining two-thirds of ganglion volume were occupied by neuropil (neurites and glial cells) and connective tissue and blood vessels.

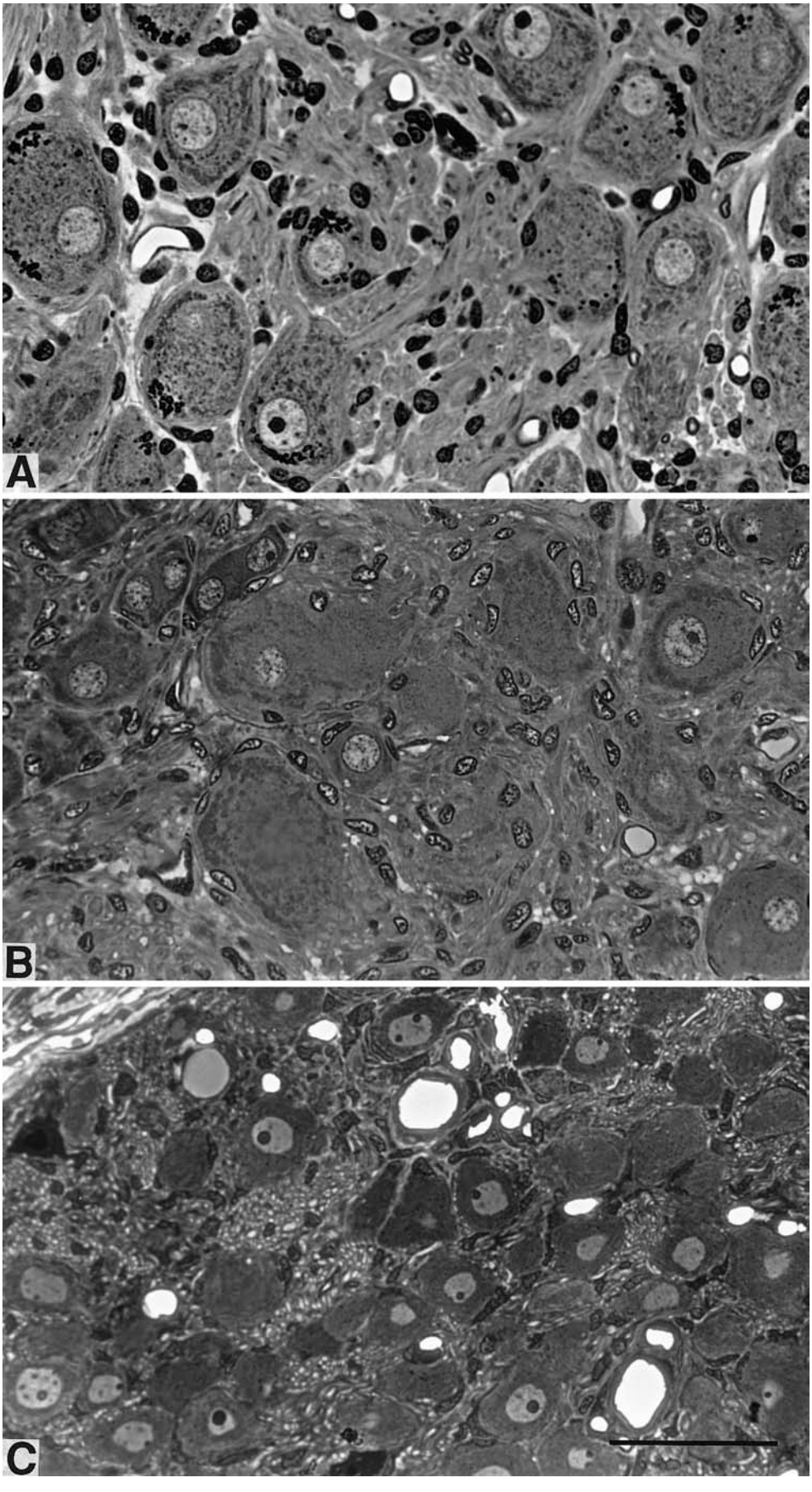


Fig. 2 Fine structure of the superior cervical ganglion of a horse (**a**), capybara (**b**) and rat (**c**) in thin sections photographed at the same magnification. The rat ganglion shows the smallest neurons and the highest packing density. In the ganglion of the capybara, and even more in that of the horse, the nerve cell bodies are separated by large spaces occupied by satellite cells (glial cells), nerve processes, connective tissue and small blood vessels. Toluidine Blue. Calibration bar: 50 μm

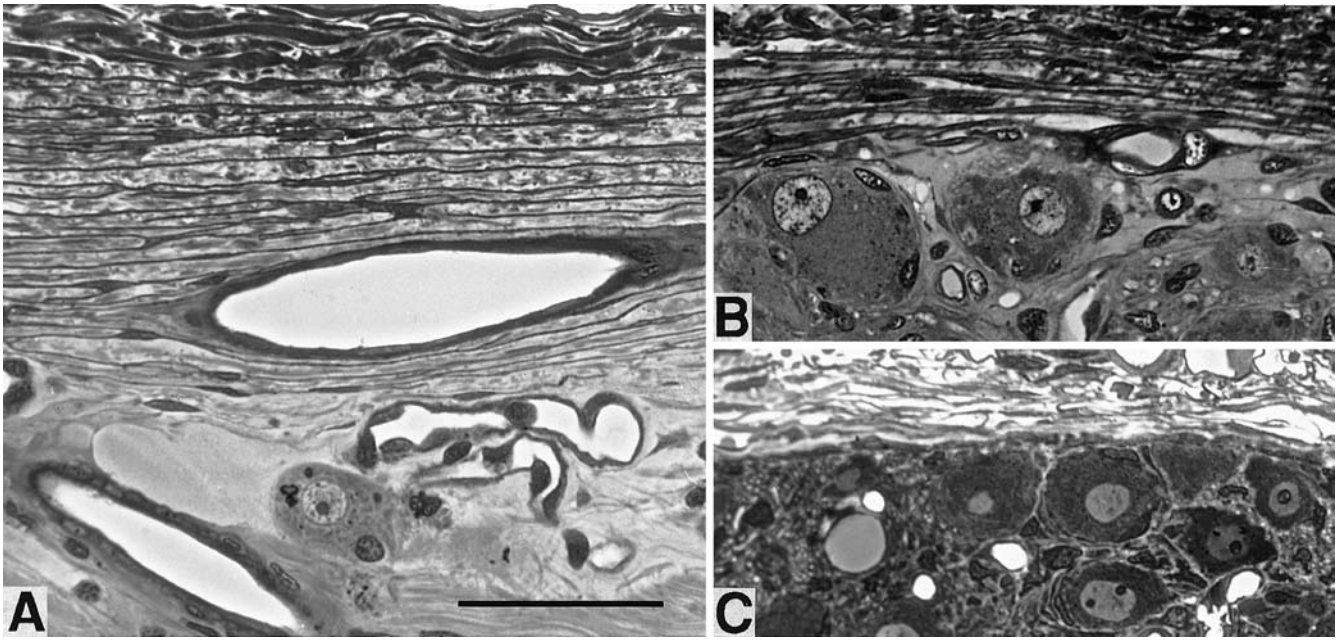


Fig. 3 Views of the capsule at the surface of ganglia of a horse (**a**), capybara (**b**) and rat (**c**), at the same magnification. In the horse (**a**) the capsule consists of several layers of collagen, which become thicker towards the outer surface of the ganglion (*top*). Some large blood vessels are visible. In the capybara (**b**) the capsule shows fewer layers of collagen. In the rat (**c**) the capsule is markedly thinner than in the other species. Toluidine Blue. Calibration bar: 50 μm

Table 1 Neuronal density, ganglion volume, total number of neurons, neuronal volume density and mean neuronal volume in superior cervical ganglion of rat, capybara and horse

Species	Neuronal density (per mm^3)[a]	Ganglion volume (in mm^3)	Number of neurons in whole ganglion	Neuronal volume density (%)[b]	Mean neuronal volume (in μm^3)
Rat 1	40,300	0.42	16,900	27.5	6,800
Rat 2	29,500	0.48	14,300	39.6	13,400
Rat 3	40,200	0.63	25,300	32.2	8,000
Mean	36,700	0.5	18,840	33	9,400
Capybara 1	5,480	280	1,534,400	22.5	41,000
Capybara 2	8,600	187	1,608,000	21.1	24,500
Capybara 3	6,700	211	1,413,700	20.5	30,500
Mean	7,050	226	1,520,000	21	32,000
Horse 1	7,600	422	3,207,000	17	22,300
Horse 2	9,360	404	3,782,000	18.2	19,400
Horse 3	7,780	410	3,190,000	14.8	19,000
Mean	8,250	412	3,393,000	17	20,230

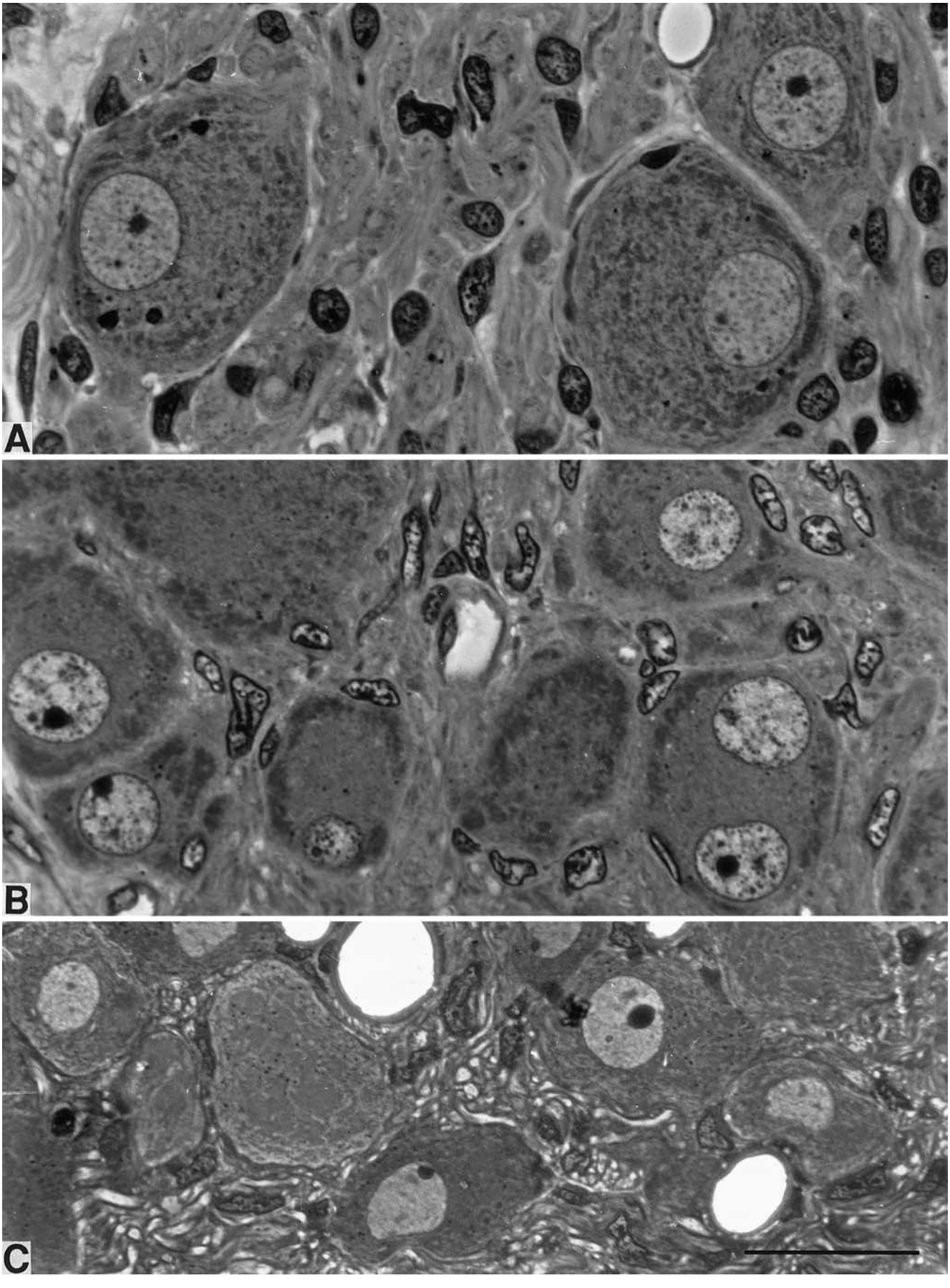
[a] The ratio of total number of ganglion neurons and the total reference volume
[b] The ratio of total volume occupied by ganglion neurons and the total reference volume

Horse and capybara

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 (Fig. 2). The capsule of the ganglion was conspicuous, 60–80 μm thick in horses, 30–70 μm thick in capybaras, and was composed of several layers of collagen fibres and flattened fibroblasts (Fig. 3). Within the ganglion, the stroma consisted of bundles of collagen fibrils isolated or gathered into intraganglionic septa. The septa included fibroblasts and blood vessels, mainly capillaries, but also arterioles and venules.

The ganglion neuron profiles were surrounded by 1–3 glial cell nuclei (satellite cells). The glial cell processes formed the ganglion neuron's glial capsule (Fig. 4). In capybaras, and even more in horses, the neuronal perikarya occupied a smaller proportion of the sectional area

Fig. 4 High-magnification view of ganglia of a horse (**a**), capybara (**b**) and rat (**c**) at the same magnification. In the rat (**c**), the spaces between nerve cell bodies are limited; glial cell nuclei and myelinated axons can be recognised. In the capybara (**b**) a large binucleate neuron is visible at the *bottom right*. Many nuclei of glial cells are visible in the large spaces between nerve cell bodies. In the horse (**a**), the spaces between the large nerve cell bodies are wider than in the other species. Toluidine Blue. Calibration bar: 25 μm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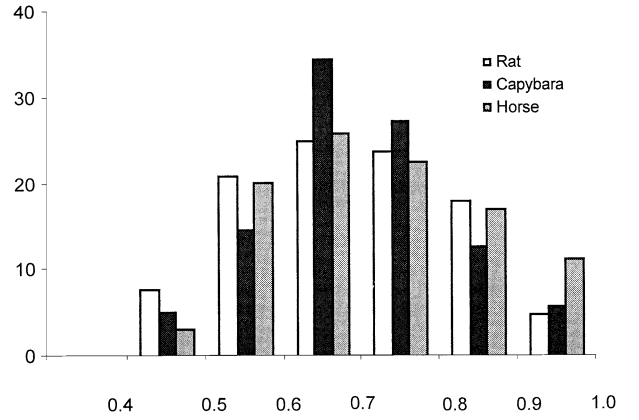

Fig. 5 Histogram showing the distribution of form factor in a sample of neurons from ganglia of the three species. In the *vertical axis* are the percentage values and in the *horizontal* axis the form factor, which ranges from 0.4 to 1

that in the rat, and the neuropil was proportionally more extensive. The percentage volume occupied by nerve cell bodies, determined stereologically, amounted to 21% in capybaras and to 17% in horses (Table 1). Thus in the horse less than one-fifth of the ganglion volume is constituted by nerve cell bodies (perikarya). The remaining four-fifths or more (in horses) of ganglion volume were occupied by neuropil (neurites and glial cells) and connective tissue and blood vessels.

The ganglion neuron profiles were circular or more commonly oval shaped; in both species about two-thirds of neuronal profiles had a form factor of between 0.5 and 0.8 (Fig. 5).

Some nuclei were located in the centre of the perikaryon, while the majority were eccentric, but none resided at the periphery of the neuronal profile. In the ganglia of horses all the neurons were mononucleate; in contrast, 13% on average of the neurons in ganglia of capybara were binucleated (7%, case 1; 24.4%, case 2; 7.4%, case 3). No neurons had more than two nuclei.

Nerve cell size

Rat

The cell body size was measured in 213 ganglion neurons from three ganglia. The size of neurons was expressed as the area of the maximum profile of a neuron, and the maximum (or largest) profile was obtained by examining neurons through serial sections. An unbiased sample of neurons was used, based on stereological criteria (see Methods).

The cross-sectional area of the ganglion neuron profiles ranged from 207 to 661 μm^2 (Fig. 6). The average neuronal cross-sectional area was 358 μm^2 (n= 213, SD=99.7). There was no correlation between neuron size (expressed as its cross-sectional area) and form factor. As

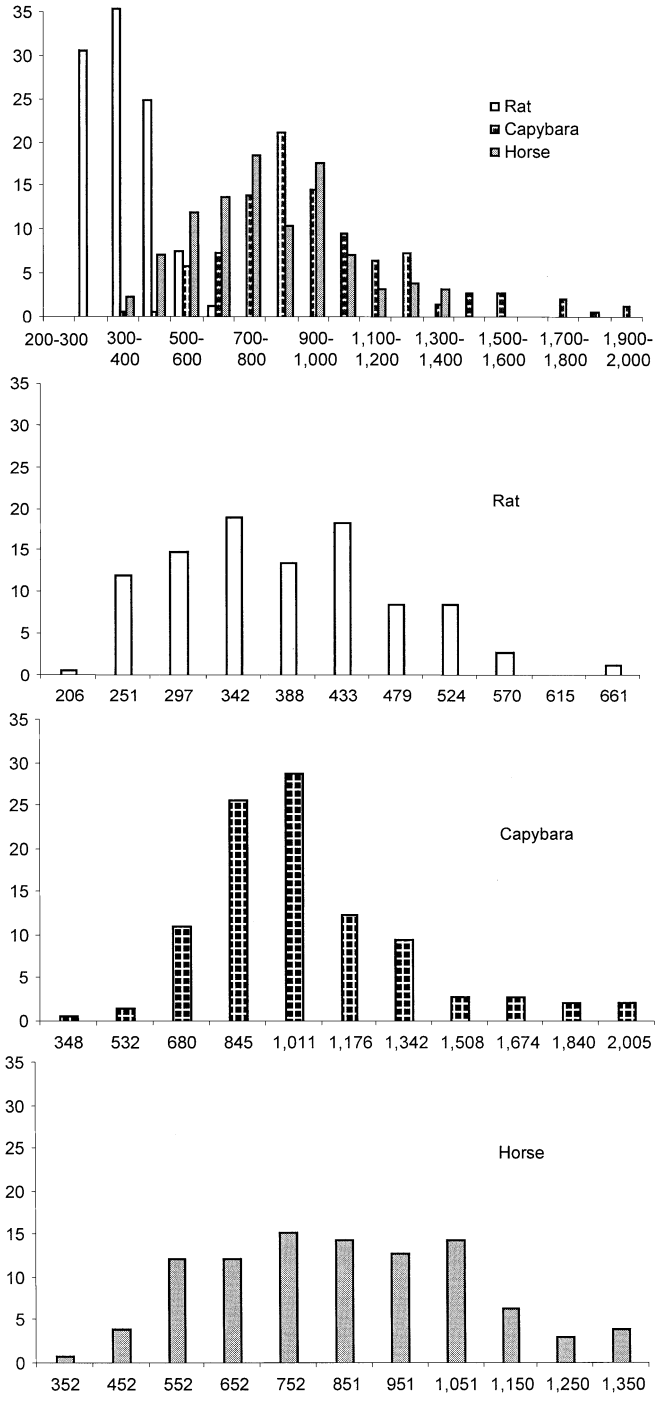

Fig. 6 Histograms documenting the distribution of nerve-cell-body sizes (cross-sectional area of the largest profile of a neuron). The *top histogram* shows the percentage distribution of sizes divided into classes of the same size for the three species and ranging from the lowest (200–300 μm^2) to the largest (1,900–2,000 μm^2). The *other three histograms* illustrate a single species each and present the percentage distribution of cell body sizes in 11 classes evenly spread between the maximum and the minimum values for that species

to nuclear size, the neuronal nuclear area varied from 15 to 132 μm^2. The mean nuclear area was 71.4 μm^2 (n=147, SD=40.4). On average, the nuclear area represented about 20% of the neuronal area in rats.

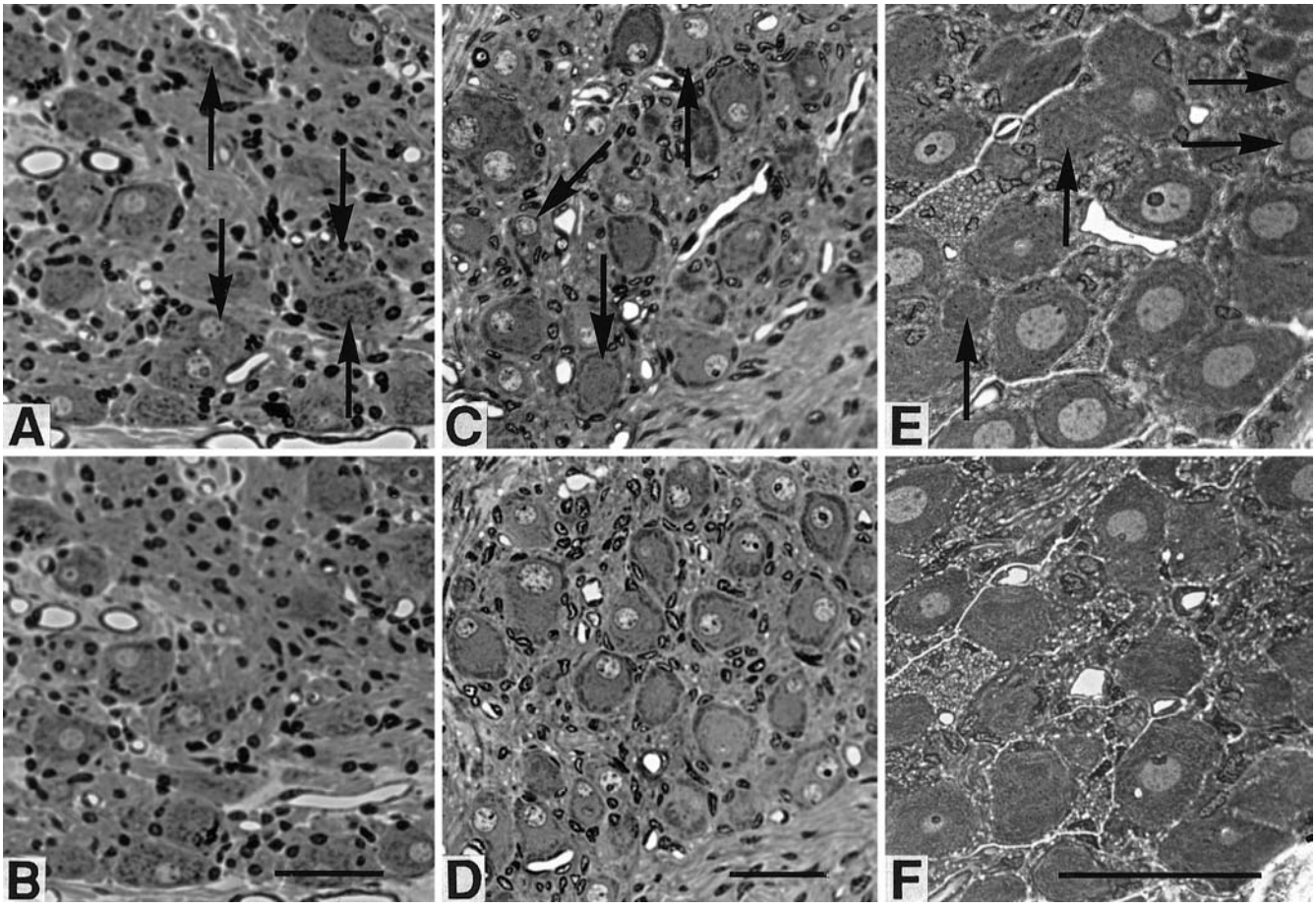

Fig. 7 Micrographs of pairs of sections (1 μm thick for rats and 2 μm thick for a horse and capybara stained with Toluidine Blue) used for estimating nerve-cell numbers in a horse (**a**, **b**), capybara (**c**, **d**) and rat (**e**, **f**). In each pair the reference section is at the top. The separation between reference section and look-up section is 10 μm (**a**, **b**); 14 μm (**c**, **d**) and 8 μm (**e**, **f**). *Arrows* show neuronal transects seen in the reference section that no longer exist in the look-up section. Calibration bars: 50 μm for a horse (**a**, **b**) and capybara (**c**, **d**); and 50 μm for the rat (**e**, **f**)

Capybara

The cell body of 157 ganglion neurons from three ganglia were measured. Of these, 21 (13%) were binucleate. The neuron size was expressed as the area of the maximum profile of a ganglion neuron in an unbiased sample. The neuronal cross-sectional area of the ganglion neuron ranged from 348 to 2,005 μm^2 for mononucleate neurons (Fig. 6). The average neuronal area was 982 μm^2 (n=136, SD=306.3). With regard to binucleate neurons the cross-sectional area ranged from 612 to 2,120 μm^2, the average area being 1,260 μm^2 (n=21, SD=357.0). There was no correlation between neuron size (expressed as its cross-sectional area) and form factor. The nuclear area of mononucleate neurons varied from 23.4 to 206 μm^2, with a mean of 66.0 μm^2 (n=64, SD=37.9). For the mononucleate neurons, the nuclear area represented on average about 7% of the neuronal area in capybaras. In binucleate neurons the smallest nuclear area combination was 31.4/16.0 μm^2 and the largest was 177/95.2 μm^2.

Horse

The perikaryon of 184 ganglion neurons from three ganglia was measured. The neuron size was expressed as the area of the maximum profile of a ganglion neuron in an unbiased sample. The neuron size ranged from 352 to 1,350 μm^2 with an average of 800 μm^2 (n=184, SD=233) (Fig. 6). There was no correlation between neuron size (expressed as its cross-sectional area) and form factor. The nuclear area varied from 38 to 175 μm^2 with a mean of 79.3 μm^2 (n=82, SD=45.6). On average, the nuclear area represented about 10% of the neuronal area in horses.

Ganglion volume

The volume of the superior cervical ganglion was estimated stereologically by means of the Cavalieri method (Table 1). The mean volume was 0.5 mm^3 in rats, about 226 mm^3 in capybaras and about 412 mm^3 in horses. The volume estimates for the ganglia of capybara and horses were closely matched by the wet weight obtained (see

section on Anatomy). The ganglia of the rat showed the highest variability between individuals and the ganglia of the horse the smallest variability.

The coefficient of error (CE) of the Cavalieri estimate of the volume for three rats was 5.7%. In capybaras the coefficient of error was 10%, 10.9% and 4.2% (cases 1, 2 and 3, respectively). In horses the coefficient of error of the Cavalieri estimate was 10.5%, 8.9% and 10.2% (cases 1, 2 and 3, respectively).

Number of neurons

Based on a large number of disectors distributed at all levels through the ganglia (66 in rat's ganglia, 55 in capybara's and 49 in horse's and comprising an overall volume of 0.0097 mm^3, 0.042 mm^3 and 0.050 mm^3, respectively, these average neuronal densities were obtained: 36,700 neurons per mm^3 in the rat, 7,050 per mm^3, in the capybara and 8,250 per mm^3 in the horse (Table 1; Fig. 7). From these values and from the volume of the ganglia the total number of neurons per ganglion was estimated as follows: 18,840 on average in the rat (n=3; SD= 5,700; CV= 0.3); 1,520,000 in the capybara (n=3; SD= 98,000; CV= 0.06) and 3,390,000 in the horse (n=3; SD=337,000; CV= 0.09).

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, in the rat, the neuronal cell bodies constituted 33% of the ganglion volume and the ganglion volume was 0.5 mm^3; the total volume of all neuronal cell bodies in a ganglion was 0.33×0.5 mm^3; since there were 18,840 neurons, the average neuronal volume was (0.33x0.5 mm^3) / 18,840, or about 9,000 μm^3. In the same way we calculated the mean neuronal volume in the capybara as 32,000 μm^3 and in the horse as 20,200 μm^3 (Table 1).

Discussion

Technical approach

Autonomic ganglia of the size of the superior cervical ganglion need to be sectioned in order to estimate cell size and number. For practical reasons, counts and measurements have to be done on samples of sections, rather than on the entire set of sections which the ganglion could generate. Of the morphometric methods applicable to section samples, the stereological methods and the use of disectors are at the moment the most accurate (Sterio 1984; Pakkenberg and Gundersen 1988; 1989; Mayhew and Gundersen 1996; Howard and Reed 1998), and we have used them. Many quantitative data in the literature were obtained with methods that would not be accepted today (Abercrombie 1946; Ebbesson 1968a, 1968b; Davies 1978; Gorin and Johnson 1980; Purves et al. 1986b). Of course, those data should not be disregarded, but the doubts about the techniques should also not be ignored while we are waiting for confirmations and the production of more reliable data.

In perusing the relevant literature, one is struck by the scarcity of photographic documentation of the material investigated. The emphasis of our work was on carrying out the morphometry on histologic preparations which were as free as possible from artefacts and which allowed us to measure and count with some confidence. Three technical steps were important to this effect: they are now standard histological practice, but they were rarely used in previous studies in this field (Davies 1978; Gorin and Johnson 1980; Purves et al. 1986b; Pover and Coggeshall 1991; Santer 1991). First, our ganglia were fixed by vascular perfusion, a procedure which delivers the fixative quickly and close to every neuron and prevents the collapse of the vascular space. Second, the ganglia were embedded in Araldite, rather than in wax, a procedure which, although unable to avoid some overall shrinkage of the tissue, minimises other distortions, thermal damage and cracking of the tissue. Third, sections were cut with glass knives at 1 or 2 μm thickness, an approach that gives sharp cell outlines, making it easy to identify neuronal profiles and to measure them. Moreover, the same block used for light microscope in the present study can also be used for additional studies by electron microscopy. By cutting, collecting and photographing continuous series of sections, rather than single sections, cell sizes can be measured accurately.

Comparative microstructural organization

Regarding the connective organization, in the rat a thin capsule of connective tissue sheaths sympathetic ganglia, occasionally sending septa between groups of neurons (Elfvin 1983). In contrast, in the ganglia of capybara and horses the capsule is conspicuous and composed of numerous layers of collagen and fibroblasts. In the capybara and to a greater extent in the horse septa of connective tissue, mainly extending longitudinally, penetrate deep into the ganglion. The effect of this is to subdivide the neurons into clusters, and the possibility exists that clusters have distinct projections to peripheral targets. This point was not investigated here, but a similar suggestion has been made in previous studies (Uddman et al. 1989; Flett and Bell 1991; Andrews 1996). In autonomic ganglia of mammals of medium and large size (rabbits, cats, dogs, sheep and buffaloes) the ganglion appeared organised in "clusters of cells" (Gabella et al. 1988) or "ganglion units" (Ribeiro et al. 2000a, 2000b; Ribeiro et al. 2002; Gagliardo et al. 2003; Sasahara et al. 2003). These authors have suggested that there are variations in the general architecture of the ganglion in larger mammals

when compared with the structure in small laboratory rodents.

Ganglion volume

Data on the volume of superior ganglion are not available in the literature, and we have provided estimates in the three species studied: 0.5 mm^3 in rats, 226 mm^3 in capybaras and 412 mm^3 in horses. The volumetric data matched those obtained by weighing the ganglia. The average volume of the ganglion varies within the three species in a way similar to body weight. Within the small sample of 3 ganglia for each species, the rat and the capybara show the largest inter-individual variation, the largest ganglion examined being 50% larger than the smallest one. In contrast, in the horse the largest and the smallest ganglion differed by only about 5%.

It should be stressed that ganglia contain, in addition to nervous tissue, a significant amount of 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 latter, the proportion of non-neuronal material (although we did not measure it) appears larger in the ganglia of the horse and capybara than in those of the rat. As to the nervous tissue, the difference in volume, for example between rat and horse, is due to three factors: first, a larger number of neurons in the larger species; second, larger nerve cell bodies; third, a greater extension of dendritic processes and axons.

Even with the reservation that the ganglion contains abundant connective tissue and blood vessels, it appears that the evolutionary growth of the ganglion follows the growth of the body size more closely than most parts of the CNS do. Actually, the cerebellum and the cerebral hemispheres show little or no correlation in weight with body size (Pakkenberg and Gundersen 1997; Andersen et al. 2003).

The horse weighs ×1,200 more than a rat, its ganglion is ×825 larger than that of a rat, according to our data, while its brain weighs about ×250 that of a rat (Nieuwenhuys et al. 1998): the ratio of the autonomic ganglion is intermediate between that of the body weight and that of the brain size. Likewise, the capybara weighs ×230 more than a rat, its ganglion is 450 times larger than that of a rat, but its brain weighs only about ×45 that of a rat (the brain of a capybara weighs about 92 g for females and 96 g for males, our personal data).

Nerve cell size

Among all cell types, neurons are the cells with the widest range of sizes. The soma of a Purkinje neuron of the cerebellum has a diameter that is about seven times that of an adjacent granule neuron; hence its volume is about 350 times larger. Unlike all other cell types, there is some pattern in the way the size of homologous neurons varies in different species and, at least for certain neurons, there is some direct correlation between neuron size and body size of the animal (Levi 1925).

Our findings show that there is a clear difference between the size of the smallest neurons in the three species. In the rat the smallest neurons are about 200 μm^2 in a cross-sectional area. In the capybara and horse there are no neurons smaller than about 350 $\mu m^{2;}$ in contrast, in the rat only about 40% of the neurons are larger than 350 μm^2. Likewise, the largest neurons in the ganglia of the rat measure about 660 μm^2, whereas in the capybara and horse 87% and 70% of the neurons are larger than 660 μm^2 and they reach a maximum of 2,000 and 1,350 μm^2, respectively.

When the volume is calculated from the maximum sectional area (by the assumption that neurons did extend on the z axis as much as on the x and y axes), the smallest and the largest neurons of the rat are 2,230 μm^3 and 12,760 μm^3 in volume, respectively: for a rat neuron to grow from the lowest to the highest value, its volume has to double some two and a half times. By the same token, the smallest neurons in the horse are about 4,840 μm^3 and the largest about 37,130 μm^3: during the growth from the smallest to the largest, the volume has to double about three times. In capybaras, the smallest and the largest neurons are 4,840 and 67,000 μm^3, respectively, and during the growth from the smallest to the largest, the neuron cell volume has to double a lot more than three times.

However distinct these patterns are, our data and those in the literature are too sparce to allow us to discuss how and when in development these differences arise. Presumably the precursor cells of these neurons are all similar in size; some mechanisms at some time during development bring about these huge differences in size. How the nerve cell size is regulated is not known. However, it is well documented that the size of certain neurons is related to the extent of their peripheral projections. Within the population of somatic motoneurons in the spinal cord, the soma size of a motoneuron is correlated with the diameter of its axon, with the conduction velocity and with the extent of peripheral branching (number of motor end plates: Henneman et al. 1964). It is possible that the variations in neuronal size in a given autonomic ganglia and the difference between ganglia of animal species of different body size are in part accounted for by variations in the extent of the innervation territory.

Consistent with this idea—but not excluding the role of other peripheral and central factors—is the difference in the average size of neurons projecting to different targets. For example, in the superior cervical ganglion of the rat the neurons projecting to the iris and salivary glands have larger perikarya (Dail and Barton 1983) than those innervating cerebral vessels (Hendry 1977; Gibbins 1991; Luebke and Wright 1992; Andrews 1996).

Evidence supporting a role of the peripheral innervation tissue in the neuronal growth in size is provided by the experiments on neuronal hypertrophy. Enlargement or reduction in the size of the innervated organ (a salivary

gland) is followed by an increase or reduction of the sympathetic neurons projecting into it (Voyvodic 1989a). In the rat bladder, hypertrophy of the musculature with outlet obstruction is followed by a marked enlargement of the related ganglion neurons (neuronal hypertrophy) (Steers and De Groat 1988; Steers et al. 1990; Gabella et al. 1992). An increased production of nerve growth factor (NGF) by the hypertrophic bladder muscle is a major factor in the growth in size of the nerve cell body (Steers et al. 1991). Most of the neurons return to the control size when the hypertrophy is reversed (Gabella and Uvelius 1998).

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 have to undergo physical exercise, as reported by Alho et al. (1984) for the rat superior cervical ganglion with an increase in the diameter of neurons.

It remains unclear whether the additional growth (in hypertrophy, for example) observed in rats would also occur in larger mammals where the neurons are already markedly larger in control conditions by comparison with those of the rat. Secondly, it is not clear whether in the experiments of hypertrophy in the rat an upper limit for neuronal size is reached, or whether these neurons could be stimulated to grow even further. Thirdly, while the role of diffusible trophic factors is undeniable, it is not clear which other factors play a role in the process, including fixed elements in the peripheral tissue of innervation or descending neuronal influences. These considerations apply also to comparative data, even if the differences in neuronal size between species are likely to involve many additional factors.

An unexpected finding is provided by the capybara where the average neuronal size is substantially larger than in the horse. This is a case of an animal species that has an average neuronal size larger than a much larger species. The observation indicates that allometric relations in the nervous tissue of different species, 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 the size and number of neurons. Furthermore, the capybara, which is a rodent like the rat, has other unusual features, which are not discussed here: one of them is the high proportion of binucleate ganglion neurons. In the adult capybara examined in this study, 13% of the neurons were binucleate and we have found that in young capybara (1–2 months-old) binucleate neurons were about 40% of the total (unpublished data).

Total number of neurons

In this study the total number of neurons in the superior cervical ganglion from two rodents (rats and capybaras) and from horses could be estimated with a certain accuracy because of the use of the disector method for determining neuronal density per unit volume and the use of the Cavalieri method for determining the volume of the same ganglion.

The total number of neurons in the ganglion of the rat is on average 18,800. Several investigations have been carried out before to establish this value and the results published by various authors vary substantially (see Introduction). The only study applying an unbiased method produced a figure of about 22,000 neurons for Wistar rats (not significantly different at 4 and 24 months of age) (Santer 1991), and our figure for female Sprague-Dawley rat is close to it.

Although the differences in the literature are based on data obtained with different stereologic methods, it seems unlikely that the variation in the figures published could be accounted for entirely on technical grounds. The literature seems to suggest that in the rat SCG there is a very considerable variability (even between individuals of the same strain) in the number of neurons. Our figure of just under 19,000 on average is well within the range of figures obtained by other authors. However, even within the same strain and the same sex and the same antimere of the body, and when the same stereological method was applied, we found a large difference in neuron number between three individual rats, an observation that is at variance with what we found in the capybara and horses. The ganglion of the rat with the largest number of neurons had about 80% more neurons than the ganglion that had the fewest (14,300 vs 25,300). This represents a true interindividual variability.

The total number of neurons in the ganglion of the capybara is on average 1,520,000 and 3,393,000 in the horse. In contrast to the figure reported in the rat, there is only a small inter-individual variation between the three ganglia of the capybara (about 13%) (1,413,700 vs 1,608,000) or between the three ganglia of the horse (about 18%) (3,190,000 vs 3,782,000).

These large differences in 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, the horse should have more than 20 million neurons, more than 6 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. These data are in line with those already published. Purves et al. (1986b) have presented data on several species in comparison with the literature); they confirmed the trend even for species as large as the horse or for rodents as large as the capybara.

Neuronal density

We studied the neuronal density in the ganglia of the three species, namely the number of neuronal cell bodies in a

unit of volume (mm^3). Again, when comparing the largest with the smallest species, there is a remarkable difference. A cubic millimetre of tissue contains 36,700 neurons in the rat, 8,250 neurons in the horse and 7,050 in the capybara.

This difference is accounted for by the larger species having larger nerve cell bodies, and by the large nerve cell bodies being further apart from each other than the small nerve cell bodies. The greater separation between nerve cells is, in turn, accounted for by more extensive dendritic arbours issuing from the neurons and by larger and more numerous axons and glial cells.

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 and Olsen (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 per 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 the human temporal cortex; 54,468 neurons neurons/mm^3 for the rat hindlimb somatosensory cortex and 120,315 neurons/mm^3 for the mouse barrel cortex. A similar correlation was found for SCG between the squirrel monkey (32,528 cells/mm^3) and humans (4,455 cells/mm^3) (Ebbesson 1968a, 1968b).

In conclusion, these experiments provide additional data and some advance in the techniques employed on quantitative parameters of an autonomic ganglia in three mammals of very different body size. The quantitative differences in this region of the nervous system in the different species are less substantial than the differences in the body weight, and they affect all the parameters studied, namely total number of neurons, size of nerve cell bodies and extent of neurites around the neurons (as indicated by the amount of space between nerve cell bodies). From these data and from those in the literature correlations between the average size of neurons and body size or between the total number of neurons and body size emerge. However, these correlations are approximate and are limited by the fact that they involve the use of averaged parameters for large populations of neurons. We suggest that it is unlikely that there are distinct and widespread correlations between body size and any single one of these parameters. These parameters have to be considered together rather than individually, and different quantitative pattern may emerge in the structural adaptation of a ganglion to the variation of the body size as a whole. Average neuronal size and neuron number both contribute to the expansion of the nervous tissue, and an understanding of how these two factors are linked to one another would be crucial for explaining evolutionary adaptations. A whole set of quantitative features of the nervous tissue has to be taken into account rather than individual parameters when one considers evolutionary trends related to size.

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