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AN EFFICIENT METHOD TO ESTIMATE CELL NUMBER AND VOLUME IN MULTIPLE DORSAL ROOT GANGLIA

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ABSTRACT

The combined use of the optical fractionator and the rotator was used to estimate the number of neurons and the mean neuronal volume in multiple and/or individual celloidin-embedded dorsal root ganglia of adult macaque monkeys. The cervical ganglia C_4-T_2 from both sides were studied. Ganglia from the same side were embedded in a single block and isotropic orientation of each ganglion in the horizontal plane was ensured in order to obtain vertical sections. Sampling, counting and measuring were carried out with the help of an interactive computer system, and test grids were provided by the GRID® general stereological software package (Olympus Denmark). The mean number of neurons on each side of the C_4-T_2 set was 236,500, with a moderate variation among animals (CV_a =0.13). Of these neurons, 42% were A-type and 56% were B-type. Neuron numbers for each ganglion showed important segmental and side differences both within and between animals. Mean neuronal volume (uncorrected for shrinkage) was 29,862 μm^3 for A cells (CV_a=0.13) and 5,062 μm^3 for B cells (CV_a=0.08), and no side differences were found. Soma size distribution was heavily skewed and there was a substantial overlap between the volume distributions of the two cell types.

Key words: neuron counting, neuron size, primary sensory neurons, spinal ganglia, stereology.

INTRODUCTION

The obtention of cell numbers and sizes has been a major objective of many studies in neuroscience since the introduction at the turn of the century of staining methods that enabled a distinct identification of all cell bodies to be obtained (Hammarberg, 1895; Hatai, 1902). In addition to its descriptive biological value, these quantitative data are critical to understand processes in which the nervous system is involved, such as development and aging, as well as how neural tissue reacts to spontaneous or experimentally induced injuries.

The primary sensory neurons in the dorsal root ganglia (DRG) are well suited to study the cellular reaction to experimental manipulations or pathological conditions of the peripheral sensory nerves and receptors. Rodents have been the most frequently used species in DRG quantitative research, this being justified by the wide use of

them in nerve lesion studies, whereas few studies are available on humans (cf. Ygge et al., 1981), and just one in non-human primates (Avendaño and Lagares, 1996).
One of the major problems of quantitation in DRG studies is the important variation of the data presented by different authors, specially among DRG studies (see refs. in Tandrup, 1993, and Avendaño and Lagares, 1996). This variability could be partly due to substantial interindividual side and segmental differences (Ygge et al., 1981; Avendaño and Lagares, 1996), but the use of various and not always unbiased quantitative methods should undoubtedly have played a role. Controversy about the methodology used has been a cause of dispute between different groups, and we hope that stereological methods could help to settle it (see, for example, Saper, 1997, and Mayhew and Gundersen, 1996). The aim of this study is to describe how two stereological tools, the optical fractionator (Gundersen et al.,1988; West,1993) and the vertical rotator (Jensen and Gundersen,1993), can be combined to efficiently obtain unbiased estimates of the absolute neuronal number (*N*) and the mean neuronal body volume ($\bar{v}_N$) in single and multiple DRG in macaque monkeys. The individual volume estimates were also used to plot the distribution of perikaryal size in the DRG. A preliminary report was presented elsewhere (Avendaño and Lagares, 1995).

MATERIALS AND METHODS

Animals and fixation

Five adult male pig-tailed monkeys (*Macaca nemestrina*), weighing 5-8 kg, were used in this study. Under deep sodium pentobarbital anesthesia (40mg/kg i.p.), the animals were perfused through the ascending aorta using a peristaltic pump. After a brief wash with saline at room temperature, 3000 ml of cold 4% paraformaldehyde in 0.1M Na phosphate buffer (pH 7.4) were infused at a rate of 70-100 ml/min. Cervical DRGs C_4 through C_8 and the first 2 thoracic ganglia were exposed bilaterally. They were dissected free from the dura and excised by cutting the dorsal roots and the spinal nerves 5 mm away from the ganglia.

Embedding and vertical sectioning

After 1-2 weeks of storage in the same fixative, each ganglia was individually dehydrated in ethanol, defatted in ether and sequentially infiltrated in 2%, 4%, 6% and 12% celloidin (Fluka) dissolved in ethanol/ether (1:1). All ganglia from each side were then transferred to a glass container with new 12% celloidin solution; each ganglion was randomly rotated around its longitudinal axis and all were arranged in parallel at the bottom of the container to be included in the same block (Fig. 1). Sections were cut at a nominal thickness of 50 μm and Nissl-stained with 0.5% cresyl violet. These embedding and sectioning procedures were used in order to obtain vertical sections (Gundersen et al. 1988) using the longitudinal axis of the ganglia as the vertical axis. All sections were mounted, but only every third section was used for quantitation. The nucleoli were used as counting units.

Histology

Neurons were classified into two types according to widely accepted criteria (Andres, 1961; Lawson, 1992). A-type neurons were large, with irregular cytoplasmatic

staining due to the large clumps of Nissl substance interspersed with poorly stained areas, and had a large and lightly stained nucleus with a prominent and heavily stained nucleolus centrally located in the nucleus. B-type neurons were smaller in size, with a darker and more evenly stained cytoplasm, and a smaller and less prominent nucleoulus. A small percentage of neurons could not be ascribed to either type and were thus grouped as NC (non-classified).

Fig.1. Fifty µm-thick sections of C_4 (upper left) through T_2 (lower right) dorsal root ganglia, all ganglia being embedded in the same celloidin block, and aligned in parallel to each other.

Sampling procedure

N, $\bar{v}_N$, and the distribution of individual volume estimates were obtained at the same time by applying the optical fractionator and the vertical rotator to individual ganglia or the whole series of ganglia that were cut and mounted on the same slide. The combination of both methods with only one sampling scheme resulted in a very efficient procedure.

All measurements of this study were carried out with the help of an interactive computer system comprising a high-precision motorized microscope stage, a 0.5 µm resolution microcator (Heidenhain VZR 401), a solid stage video-camera and a high resolution video monitor. The objective used was a planapochromatic 100X oil-immersion lens with a n.a.=140 (Olympus, SPlan Apo100). The interactive test grids and the control of the motorized stage were provided by the GRID® general

stereological software package (Olympus Denmark) running on an Amiga 2000 computer.

The fractionator sampling scheme was applied at three successive stages:

a) All celloidin sections were mounted, but only every third section was used for counting (f_S=1/3).

b) The section of each ganglion was divided into fixed areas. The first of these areas sampled within each individual ganglion was positioned at random. A stepping motor moved the microscope stage systematically to other locations at pre-established and fixed distances, which varied depending on whether all ganglia or separate ganglia were the targets of the study. The area of the disector frame covered 1087μm^2, which was used to estimate the fraction, f_d, of the total sampled area.

c) The third fraction, f_h , was obtained by dividing the disector height (h = 20 μm) by the mean section thickness (56.6 μm).

Table 1. Numbers of neurons in the Macaque DRGs C_4-T_2 (in thousands)

Case		Cell type A		Cell type B		Cell type NC[a]		Total
Mn2	Left	112	(45)	134	(53)	6	(2)	252
	Right	114	(41)	157	(57)	4	(2)	274
	% left-right	99		85				92
Mn3	Left	99	(42)	129	(55)	7	(3)	235
	Right	88	(42)	118	(56)	6	(3)	212
	% left-right	112		109				110
Mn4	Left	91	(41)	122	(55)	7	(3)	219
	Right	87	(40)	124	(57)	8	(4)	219
	% left-right	104		98				100
Mn5	Left	103	(37)	167	(61)	5	(2)	275
	Right	118	(42)	157	(57)	2	(1)	277
	% left-right	88		106				99
Mn6	Left	93	(47)	105	(53)	1	(0)	199
	Right	97	(48)	104	(51)	3	(1)	203
	% left-right	96		101				98
All cases	Left	100	(42)	131	(56)	5	(2)	236
	Right	101	(43)	132	(56)	2	(1)	237
	% left-right	100		100				100
	CV	0.11		0.17				0.13

Note. Side percentages are shown in brackets. Figures are rounded to the nearest figure.

[a]NC, non-classified

Table 2. Numbers of Neurons in the Macaque DRGs C_4, C_5, C_6 and T_2(in Thousands)

Case	Cell type	C_4 Left	C_4 Right	C_4 %L-R	C_5 Left	C_5 Right	C_5 %L-R	C_6 Left	C_6 Right	C_6 %L-R	T_2 Left	T_2 Right	T_2 %L-R
Mn2	A	10.9	9.2	118.5	6.0	9.2	65.2	17.4	9.2	189.1	10.9	8.3	131.3
	B	16.6	22.0	75.5	4.9	7.3	64.1	12.4	15.1	82.1	18.1	23.9	75.7
	NC	0.4	0.5		0.4	0		0.8	0		1.1	0.9	
	Total	27.9	31.7	88.0	11.3	16.5	68.5	30.6	24.3	125.9	30.1	33.1	90.9
Mn3	A	7.2	10.0	72.0	4.8	4.1	117.1	14.3	12.3	116.3	8.6	6.8	126.5
	B	20.0	16.9	118.3	8.6	7.3	117.8	20.5	12.3	166.7	16.7	14.6	114.4
	NC	0	0.9		0	0.5		1.4	0.9		0	0	
	Total	27.2	27.8	97.8	13.4	11.9	112.6	36.2	25.5	142.0	25.3	21.4	118.2
Mn4	A	5.5	8.1	67.9	5.0	8.1	61.7	14.6	14.7	99.3	5.9	6.0	98.3
	B	16.8	12.5	134.4	11.4	11.9	95.8	15.0	16.3	92.0	15.0	16.3	92.0
	NC	0.5	0.5		0.9	0.6		1.8	0.5		0	0.5	
	Total	22.8	21.1	108.1	17.3	20.6	84.0	31.4	31.5	99.7	20.9	22.8	91.7
Mn5	A	8.0	10.4	76.9	9.0	6.0	150.0	16.4	16.9	97.3	8.5	9.9	85.9
	B	22.4	20.3	110.3	12.4	6.4	193.8	20.9	22.8	91.6	15.9	15.4	103.2
	NC	0	0.5		0.5	0		1.8	0.5		0.5	0.5	
	Total	30.4	31.2	97.4	21.9	12.4	176.6	39.1	40.2	97.3	24.9	25.8	96.5
Mn6	A	5.9	6.1	96.7	3.9	6.7	58.2	15.2	10.5	144.8	5.9	7.0	84.2
	B	17.7	19.2	92.2	6.9	5.7	121.1	11.3	12.7	89.0	8.4	9.6	87.5
	NC	0	1.3		0	0		0	0		0	0	
	Total	23.6	26.6	88.7	10.8	12.4	87.1	26.5	23.2	114.2	14.3	16.6	86.1

	Cell Type	Left Mean	Left SD	Right Mean	Right SD	%L-R Mean	%L-R CV	Left Mean	Left SD	Right Mean	Right SD	%L-R Mean	%L-R CV	Left Mean	Left SD	Right Mean	Right SD	%L-R Mean	%L-R CV	Left Mean	Left SD	Right Mean	Right SD	%L-R Mean	%L-R CV
All cases	A	7.5	2.1	8.8	1.7	86.4	0.24	5.7	2.0	6.8	2.0	90.4	0.45	15.6	1.3	12.7	3.1	129.4	0.30	8.0	2.1	7.6	1.5	105.1	0.21
	B	18.7	2.5	18.2	3.7	106.1	0.22	8.8	3.1	7.7	2.4	118.5	0.40	16.0	4.5	15.8	4.2	105.5	0.32	14.8	3.8	16.0	5.1	94.6	0.16
	NC	0.2		0.7				0.4		0.2				1.2		0.4				0.3		0.4			
	Total	26.4	3.1	27.7	4.3	96.0	0.08	14.9	4.7	14.8	3.8	105.8	0.40	32.8	4.9	28.9	7.1	115.8	0.16	23.1	5.9	23.9	6.1	96.7	0.13

*Note.*Cell numbers and percentages are rounded to the nearest decimal unit.

The total fraction (f_t) was the product $f_S \cdot f_d \cdot f_h$. The number of cells counted (ΣQ^-) on each side was, on average, 498 for the C_4-T_2 set, and 52 for individual ganglia.The total number of neurons was then estimated as: $N = \Sigma Q^- \cdot f_t^{-1}$

Measuring procedure

Every second neuron that was counted was measured using the vertical planar rotator (Jensen and Gundersen, 1993; Vestergaard et al., 1997). Although less than 3% of the neurons had two nucleoli (none had more), the presence of more than one nucleolus was explored in all nuclei whether cut by the upper or lower disector planes; if two nucleoli were present, only the uppermost in the nucleus was used for the count.

Table 3.Mean neuronal volumes in the Macaque DRGs C_4-T_2(in $\mu m^3 \times 10^{-3}$)

		Side				
Case	Cell type	Left	CV	Right	CV	%left-Right
Mn2	A	32.4	0.89	32.1	0.84	101
	B	4.9	0.71	5.5	0.65	90
Mn3	A	26.9	0.81	24.7	0.97	104
	B	4.8	1.27	4.6	0.63	105
Mn4	A	26.9	0.73	26.8	0.70	100
	B	4.7	0.59	4.5	0.70	103
Mn5	A	35.4	0.77	34.5	0.72	103
	B	5.2	0.69	5.6	0.58	93
Mn6	A	31	0.71	28.7	0.70	108
	B	5.5	0.64	5.4	0.53	104

		Left		Right		Both sides	
	Cell Type	Mean	CV[a]	Mean	Cv[a]	Mean	CV[a]
All cases	A	30.4	0.13	29.3	0.13	29.9	0.13
	B	5	0.07	5.1	0.10	5.1	0.08

[a] CV, Coefficient of variation of the distribution of mean volume estimates among animals.

Statistics

The precision of the estimates of N was evaluated by computing the CE of ΣQ^- (CE_N) as described by West and Gundersen (1990). With the sampling scheme used, the value of CE_N was kept at or below 10%. The variability among animals was expressed by the coefficient of variation (CV). The mean values of CE_N and CV

among animals were obtained by extracting the square root of the corresponding mean of squared values.
Individual values were computed for each monkey and each cell type. Also, cell volume histograms were prepared dividing individual measurements into 15 classes using a logarithmic scale. The percentage of cells belonging to each class was calculated as the average of the percentage of cells belonging to that class in the different monkeys.
The precision of the estimates of $\overline{v}_N$ was evaluated by computing the CE using ordinary statistics for independent observations. The mean CE was 7% (range: 5-11%).

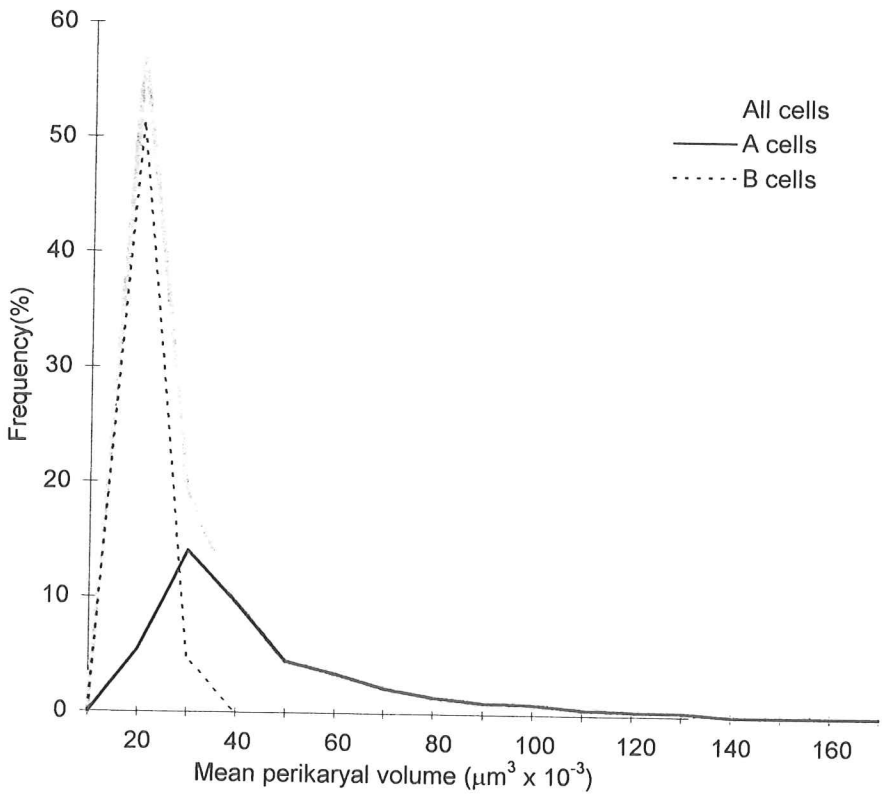

Fig.2. Linear Neuronal volume estimates distribution in the Macaque DRGs C_4-T_2. Y axis represents the average percentage of cells.

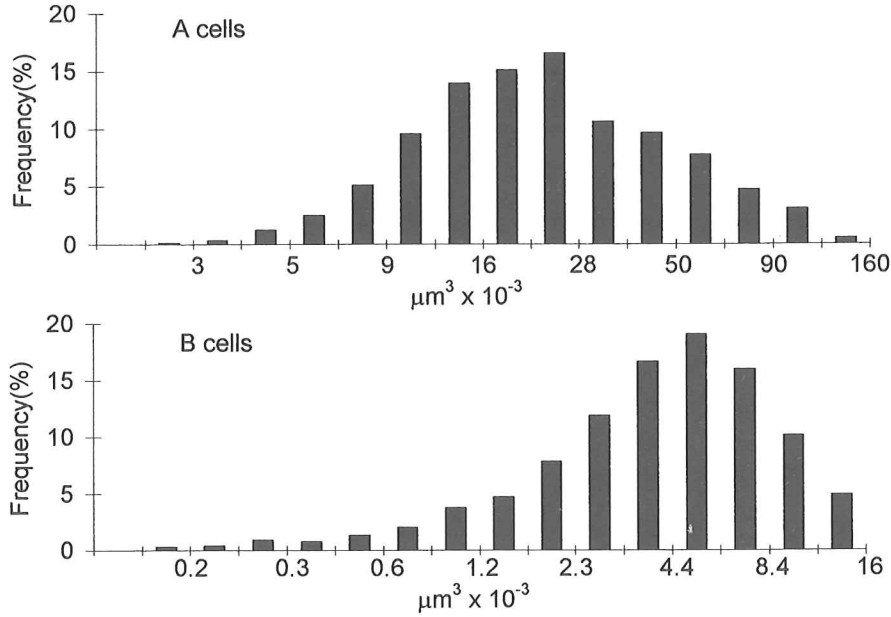

Fig.3. Logarithmic converted Neuronal estimates Volume Distribution of A-type and B-type cells in the Macaque DRGs C_4-T_2. Class heights represent the average percentage of cells.

RESULTS

Cell number

The number of neurons in the whole set of DRG C_4 through T_2 in each case is shown in Table 1. The mean total number of neurons was found to be 236,500 with a moderate variability among animals(range=199,000-277,000; CV=0.13). A-type cells represented 42% of these neurons, while B-type cells represented 56%. There were only 2% of non-classified cells. Variability between sides was low when the whole range of ganglia was considered. A more detailed analysis of cell numbers in ganglia C_7-T_1 has been published elsewhere (Avendaño and Lagares, 1996). Cell numbers in ganglia C_4-C_6 and T_2 are shown in Table 2.

Cell volume

Mean neuronal volumes were estimated for A and B cells as shown in Table 3. There is an important variability in the distribution of neuronal volume estimates within each monkey (CV from 0.53 to 1.27). This variation is seen in both types of cells.
The mean neuronal volume was found to be 29,862 μm^3 for A cells (range=24,749-35,391; CV=0.13), and 5,062 μm^3 for B cells (range=4,669-5,563; CV=0.08), thus expressing a low variation among animals. No side differences were found.
The linear distribution of neuronal volume estimates is heavily skewed, as shown in Figure 2. There is an overlap between the volume distribution of A and B cells.

Logarithmic conversion of A and B cells volume distributions are shown in Fig. 3. They both have a wide unimodal distribution.

DISCUSSION

"Classical" methods for counting cells are often based on assumptions about cell distribution, size, or shape, and their application entails the use of different correction factors in order to diminish the possible effect of artifacts in tissue sectioning (particularly truncation and overprojection) and in the recognition of the target (nuclei, nucleoli) profiles. Also, sampling schemes did not always guarantee unbiasedness, thus adding more reasons for results obtained on the same structure to differ widely (see Avendaño and Lagares, 1996). Regarding neuronal size, most studies report the profile area or the diameter of the sectional profiles of cell bodies rather than actual volume data. These parameters are affected by cell shape and orientation, and so assumptions about them have to be made. Cell sampling is also a cause of bias in producing histograms of neuronal sizes (Pover et al.,1993), as most histograms are constructed without using a correct sampling scheme, thereby producing an over-representation of large cells.

The method described in this study is intended to provide an efficient solution to many of these problems. The combination of the optical disector, the vertical rotator and the optical fractionator provides a procedure which assures an unbiased sampling scheme and the estimation of cell counts and volume independent of cell characteristics. However, some considerations to this method have to be made.

Dimensional distortions introduced by histological processing could be an important source of imprecision in the estimation of both *N* and $\overline{v}_N$. Although *N* is independent of volume changes when using the fractionator, as it is independent of the reference volume, it is still dependent on sectional thickness changes, as *N* is directly proportional to section thickness. Therefore a precise and unbiased estimation of section thickness is important. The mean thickness of the coverslipped sections is estimated with more than 5% precision by means of depth readings with the microcator. The estimation of $\overline{v}_N$ is affected by tissue shrinkage. The effect of this phenomenon on celloidin-embedded sections has been found responsible for a 55% overall reduction in volume of the cat's diencephalon (Avendaño and Dykes, 1996), and a 60% mean reduction in the rat brain stem (Avendaño and Lagares, unpublished). However this factor can not be freely applied to every celloidin-embedded structure, since it is not known whether all nervous structures undergo the same degree of shrinkage after embedding. No shrinkage calculation was done in this study. Finally, the possibility of a double counting of cells with more than one nucleoli was eliminated by adhering to a strict criterion about which nucleolus should be used for counting (see methods above).

The results of the estimation of cell numbers in this study shows that when a group of ganglia is considered, differences between sides are low, while when individual ganglia are studied these side differences rise. Thus, side and segmental asymmetry could preclude the use of paired ganglia at a single level to compare effects of unilateral lesions or treatments (Avendaño and Lagares, 1996). The two subpopulations of cells, A and B cells, differ widely when comparing their mean neuronal volume. However, both subpopulations substantially overlap in the distribution of individual volume estimates, thus making it difficult to distinguish between the two cell on the basis of size alone. The only existing study on neuronal

volume of DRG cells using stereological techniques (Tandrup, 1993) found in L_5 of rats a mean neuronal volume of 53,400 μm^3 for A cells and 8,500 μm^3 for B cells. Certainly, rat DRG neurons are NOT larger than DRG neurons in monkey (A. Lagares and C. Avendaño, unpublished observations), and therefore the discrepancy between Tandrup's and our data should find an explanation elsewhere. The use of different embedding media may account for a large part of this difference, given the marked shrinkage produced by celloidin embedding, and the absence of shrinkage (or even mild swelling) accompanying glycol methacrylate embedding. However, other so far undetected factors could have played a role in these differences.

In conclusion, the method described in this study enables precise unbiased estimates of neuronal numbers and volumes to be carried out on single and multiple DRG's in a single sampling sweep. This adds badly needed efficiency to a quantitative procedure that zeroes in on a heterogeneous population of discrete clumps of cells with substantial segmental, side and subject differences.

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