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Ultrastructural morphometry of the rat neurohypophysis

J. J. NORDMANN*
Department of Pharmacology, University of Cambridge

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INTRODUCTION

In order to produce a model for the secretion of oxytocin and vasopressin from the neurohypophysis it is essential to have quantitative information about the ultrastructure of the cells (Palay, 1957; Reinhardt, Henning & Rohr, 1969). This type of data should enable correlation to be made between physiological and ultrastructural changes during hormone secretion. Furthermore, it would be interesting to compare the data obtained in the resting state with those obtained after stimulation of oxytocin and vasopressin release. Stereological methods enable one to characterize the spatial characteristics of cellular organelles by simple counting and measuring procedures applied to electron micrographs of sectioned tissues (Elias, Henning & Schwartz, 1971).

This paper reports data obtained from unstimulated rat neurohypophyses.

MATERIALS AND METHODS

After decapitation of the animals neurohypophyses were isolated from albino rats of 300–350 g body weight and incubated for 40 minutes in modified Locke's solution (NaCl, 150 mM; $CaCl_2$, 2·2 mM; $MgCl_2$, 1 mM; $KHCO_3$, 5·6 mM; glucose, 10 mM) which was gassed continuously with 5% CO_2 in O_2. The glands were then fixed in 5% glutaraldehyde buffered with potassium bicarbonate 5·6 mM at pH 7·3 for 2 hours. The neurohypophyses were cut in four pieces and dehydrated in alcohol and propylene oxide, embedded in Epon resin and sectioned with a diamond knife on an L.K.B. microtome to give a section thickness of about 75 nm. The sections were placed on 200 mesh copper grids, stained with uranyl acetate dissolved in methanol and lead citrate, and examined in a Philips EM 300 electron microscope. Six neurohypophyses were used, giving a total of 24 blocks. Five sections were used at 1 μm intervals from each tissue block. Electron micrographs were taken from 120 sections and printed with a final magnification of 23 760, 35 640 and 47 520. Random sampling of the tissue section was achieved by using a specified corner of the copper grid as a reference system for each micrograph. The morphological compartments of the neurohypophysis were defined as follows: (1) Undilated axons containing neurotubules but no (or very few) microvesicles or 'synaptic' vesicles (*Ax*). (2) Endings (terminal dilatations) containing microvesicles but no neurotubules (*E*). (3) Swellings (non-terminal dilatations) containing neither microvesicles nor neurotubules (*S*).

* Present address: A.R.C. Unit of Invertebrate Chemistry and Physiology, Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ.

(4) Pituicytes (*P*). (5) Blood vessels (*BV*). (6) Extracellular space (*ECS*). (7) Unclassified structures.

Standard stereological procedures were used to estimate the volume, surface areas and numbers of structures in a specified volume of tissue (see Elias, 1967; Elias *et al.* 1971; Weibel, 1969). Point counting was used to calculate the volume occupied by an organelle per unit volume of tissue (volumetric density V_{vi}), by placing a lattice of P_t test points on a micrograph and obtaining the fraction $P_{pi} = P_i/P_t$ of those points enclosed within the profiles of these structures.

$$V_{vi} = P_{pi} \tag{1}$$

The surface area of a structure per unit volume of tissue (S_{vi}) was derived from counting the number of intersection points (I_i) of the surface profile with test lines of known total length L_t

$$S_{vi} = 2I_i/L_t \tag{2}$$

The number of swellings, endings or pituicytes per unit volume (N_{vi}) was calculated using the following formula

$$N_{vi} = \frac{n}{A - \bar{D}} \tag{3}$$

where $\bar{D}$ is the mean diameter of the structure (endings or swellings) or of the nuclei (pituicytes) and n the number of profiles counted in the total test area A.

Assuming that the structures are nearly spherical and that the distribution of their size approximates to the normal, the true mean diameter $\bar{D}$ can be calculated from the mean observed diameter $\bar{d}$

$$\bar{D} = \frac{4}{\pi}\bar{d} \tag{4}$$

Whereas if it is thought that the structure is a prolate spheroid (rotatory ellipsoid) of axes $2a \leqslant 2b$ where a is the rotatory axis, $\bar{D}$ can be calculated as follows:

$$\bar{D} = \frac{a+b}{\epsilon \operatorname{arc\,sinh} \epsilon} \tag{5}$$

where $\epsilon = [(a/b)^2 - 1]^{\frac{1}{2}}$.

The number of granules per unit of a given structure was calculated from the relationship

$$N_{vi} = \frac{N_{ai}}{(\bar{D} + t - 2h)} \tag{6}$$

where t is the thickness of the section, h is the height of optically or mechanically lost caps and n is the number of profiles counted in the total test area A.

The morphometric grids used were a coherent quadratic lattice, 225 points for volume and number measurements, and a 120 line lattice for surface measurements. In order to compare the results obtained by stereological methods and conventional geometric calculations the following formulae were used (Hilliard, 1967):

Table 1. *Percentage of total volume* (V_t) *occupied by the neurohypophyseal compartments defined in the text*

(The results are expressed in terms of mean ± S.E.M. and are calculated from 173 micrographs.)

Structures	Total volume %	Number/gland
Swellings	16·41 ± 1·05	$7{\cdot}98 \pm 0{\cdot}86 \times 10^6$
Endings	12·07 ± 0·75	$3{\cdot}37 \pm 0{\cdot}48 \times 10^7$
Axons	13·95 ± 1·02	—
Pituicytes	28·67 ± 1·45	$5{\cdot}85 \pm 0{\cdot}57 \times 10^5$
Blood vessels	7·44 ± 1·24	—
Extracellular space	15·08 ± 0·55	—
Unclassified structures	6·41 ± 0·44	—

(*A*) *Sphere*

$$\text{Surface area} = 4\pi r^2 \tag{7}$$

$$\text{Volume} = \frac{4\pi r^3}{3} \tag{8}$$

(*B*) *Prolate spheroid*

$$\text{Surface area} = 2\pi b^2 + 2\pi(a^2/\epsilon)\arcsin(b\epsilon/a) \tag{9}$$

$$\text{Volume} = \frac{4\pi a b^2}{3} \tag{10}$$

According to the work of Morris (1976), a neurohypophysial volume of 0·57 mm^3 was taken for calculation purposes.

RESULTS

The volume occupied by each compartment is given in Table 1. The extracellular space comprises 15 % of the total volume, which is very close to the data obtained with labelled extracellular markers such as inulin (Sunde, McElvy & Sachs, 1972 and mannitol (unpublished results). Axons, endings and swellings have been found to occupy 42 % of the total volume (Table 1). By subtracting the total volume of well-defined structures from the total volume of the gland, the unclassified structures have been found to represent approximately 6 % of the neurohypophysis.

The length axes of the swellings and endings having been calculated, the true diameter of these structures was estimated by two methods: *a*, assuming spherical proportions; *b*, assuming a prolate spheroidal structure. The observed diameter of the swellings, estimated from the mean axial length, was found to be 2·15 $\mu m \pm 0{\cdot}06$ (S.E.M., $n = 300$), corresponding to a real diameter of 2·74 $\mu m \pm 0{\cdot}08$ (equation 4) by method *A*. A mean axial ratio of $1{\cdot}52 \pm 0{\cdot}03$ (S.E.M., $n = 300$) resulted in a real diameter of 2·44 $\mu m \pm 0{\cdot}08$ (equation 5) by method *B*. From the diameter of the swellings the surface area and volume can be estimated from equations 7, 8, 9 and 10, by methods *A* and *B* (Table 2).

From equation 3 it is possible to estimate the number of swellings per mm^3. The density of these structures was found to be $1{\cdot}3 \times 10^7$ if a sphere would reflect the

Table 2. *Morphometric parameters of the swellings*

The numbers in brackets represent the number of studied micrographs (line 1, 2) or the number of analysed swellings (line 3–5). First column: parameters and method used for their determinations. Second and third columns: values of the parameters assuming a sphere on a spheroid shape. Mean diameter: $2{\cdot}7 \pm 0{\cdot}1$ μm (300). Axial ratio: $1{\cdot}5 \times 0{\cdot}3$ (300).

Parameters	Sphere	Prolate spheroid
Number/gland (equation 3)	$7{\cdot}1 \pm 0{\cdot}8 \times 10^6$ (120)	$8{\cdot}0 \pm 0{\cdot}9 \times 10^6$ (120)
Volume (μm^3) (% total volume and equation 3)	$13{\cdot}1 \pm 1{\cdot}4$ μm^3 (120)	$11{\cdot}7 \pm 1{\cdot}3$ μm^3 (120)
Volume (μm^3) (equations 8 and 10)	$7{\cdot}9 \pm 0{\cdot}2$ μm^3 (300)	$8{\cdot}1 \pm 0{\cdot}2$ μm^3) (300)
Surface area (μm^2) (equation 2)	$25{\cdot}6 \pm 1{\cdot}9$ μm^2 (100)	$22{\cdot}9 \pm 1{\cdot}7$ μm^2 (100)
Surface area (μm^2) (equations 7 and 9)	$23{\cdot}5 \pm 0{\cdot}7$ μm^2 (300)	$17{\cdot}9 \pm 0{\cdot}5$ μm^2 (300)

Table 3. *Morphometric parameters of the endings*

The numbers in brackets represent the number of studied micrographs (line 1, 2) or the number of analysed endings (line 3–5). First column: parameters and methods used for their determinations. Second and third columns: values of the parameters assuming a sphere or a spheroid shape. Mean diameter: $1{\cdot}58 \pm 0{\cdot}04$ μm (290). Axial ratio: $1{\cdot}80 \pm 0{\cdot}05$ (290).

Parameters	Sphere	Prolate spheroid
Number/gland (equation 3)	$3{\cdot}4 \pm 0{\cdot}5 \pm 10^7$ (40)	$5{\cdot}9 \pm 0{\cdot}6 \times 10^7$ (40)
Volume (μm^3) (% total and equation 3)	$2{\cdot}0 \pm 0{\cdot}2$ μm^3 (40)	$1{\cdot}1 \pm 0{\cdot}1$ μm^3 (40)
Volume (μm^3) (equations 8 and 10)	$2{\cdot}1 \pm 0{\cdot}05$ μm^3 (290)	$1{\cdot}3 \pm 0{\cdot}03$ μm^3 (290)
Surface area (μm^2) (equation 2)	$7{\cdot}1 \pm 0{\cdot}5$ μm^2 (100)	$4{\cdot}0 \pm 0{\cdot}3$ μm^2 (100)
Surface area (μm^2) (equations 7 and 9)	$7{\cdot}8 \pm 0{\cdot}2$ μm^2 (290)	$7{\cdot}4 \pm 0{\cdot}2$ μm^2 (290)

shape of the swellings and $1{\cdot}4 \times 10^7$ in the case of a prolate spheroid, corresponding to a volumetric density of $1{\cdot}6 \times 10^8$ $\mu m^3/mm^3$. These results are encouragingly close to the data obtained by a totally different method (e.g. using equations 8 and 10).

Using equation 2 the surface area of a swelling was calculated to be 44·9 μm^2 and 48·9 μm^2 for a sphere and a prolate spheroid respectively. The results are summarized in Table 2.

Table 3 summarizes the results obtained for the endings. The calculations were done in the same way as those used for the determination of swellings parameters.

Bandaranayake (1971) has shown that there are $1{\cdot}83 \times 10^4$ nerve cells projecting to the neural lobe; it is therefore possible to calculate the number of swellings and endings per nerve cell. The values obtained are $4{\cdot}36 \times 10^2$ swellings and $1{\cdot}84 \times 10^3$ endings per nerve cell.

In order to calculate the distribution of the neurosecretory granules (NSG) in the neurohypophysis equation 3 was used. Although it has been shown that the neurosecretory granules can be subdivided into different groups according to their diameters (Ishii, Thomas & Nakamura, 1973) an average of 160 nm was chosen according to the work of Morris (1976). To avoid any mistake due to magnification and definition of the structure, three magnifications were used (23760, 35640 and 47520). The Holmes (1927) correction was employed to compensate for overestimation of the point count due to the opacity of the granules. From the results (Table 4) it can be seen that the number of estimations was independent of the magnification

Table 4. *Number of neurosecretory granules in the neuronal compartments of the neurohypophysis*

First column: neuronal compartments. Columns 2–4: results obtained with different magnifications. Fifth column: mean results. Sixth column: % of the total neurosecretory granules in the different neuronal compartments of the neurohypophysis. Results are expressed in terms of mean ± S.E.M. The number in brackets represents the number of compartments analysed.

Compartments	Micrograph magnification			Mean	Total NSG %
	× 23 760	× 35 640	× 47 520		
Swellings	$8{\cdot}7 \pm 0{\cdot}2 \times 10^9$ (100)	$10{\cdot}3 \pm 0{\cdot}3 \times 10^9$ (50)	$10{\cdot}5 \pm 0{\cdot}5 \times 10^9$ (50)	$9{\cdot}8 \times 10^9$	59
Endings	$4{\cdot}5 \pm 0{\cdot}03 \times 10^9$ (100)	$5{\cdot}1 \pm 0{\cdot}3 \times 10^9$ (67)	$4{\cdot}8 \pm 0{\cdot}3 \times 10^9$ (50)	$4{\cdot}8 \times 10^9$	29
Axons	$2{\cdot}0 \pm 0{\cdot}02 \times 10^9$ (100)	$2{\cdot}0 \pm 0{\cdot}5 \times 10^9$ (65)	—	$2{\cdot}0 \times 10^9$	12

from which counts were made. Thus, the neurosecretory granules density was found to be $2{\cdot}9 \times 10^{10}/mm^3$, of which 59 % are contained in non-terminal dilatations and 12 % in axons. These values correspond to $2{\cdot}24 \times 10^3$ and $2{\cdot}58 \times 10^2$ NSG per swellings and endings respectively.

In order to calculate the number of pituicytes in the neurohypophysis, a 5016 magnification was chosen and the number of nuclei per cross section counted. The mean diameter of the nuclei (magnification × 23 760) has been found to be 0·197 μm. Assuming the pituicytes to be mononucleated, and using equation 1, the number of pituicytes in a neuronal lobe was found to be $5{\cdot}85 \pm 0{\cdot}51 \times 10^5$ ($n = 67$) and their mean cell volume 278 ± 14 μm^3. These values correspond to a density of $1{\cdot}02 \times 10^6/mm^3$ and a volumetric density of $2{\cdot}8 \times 10^8$ $\mu m^3/mm^3$.

DISCUSSION

The electron micrograph sections analysed in this study have been taken from rat neurohypophysis fixed and embedded in Epon. As with all studies of this nature, it is quite possible that the histological treatment can produce volume changes in the tissue. Thus it is difficult to assess the extent to which the volumetric densities of the ultrastructural components are affected by the histological procedure. Therefore, having these important reservations in mind, values for the morphometric parameters of the ultrastructural components of the neurohypophysis have been calculated. However, it is clear that the procedures used have not modified the structures to any great extent, since stereological measurements of the rat neurohypophysis fixed by perfusion of the animal give the same values as those reported in this paper (J. F. Morris, personal communication).

Stereological analysis is quite simply the application of the statistical geometry to samples of sectioned material randomly taken from a population of sections. Although I have observed that the number of axons in the distal part of the neurohypophysis is larger (but not significantly) than in the more proximal part of the

neural lobe, no attempt was made to divide the gland into topological compartments. Thus the results presented here are calculated on the assumption that the neurohypophysis is isotropic.

The morphometric parameters determined in this study are presented for the ultrastructural components of the neurohypophysis in the 'resting state'. It must be emphasized that the neural lobe has been incubated for 40 minutes in Locke solution before fixation, and that this procedure may have altered the morphology from its *in vivo* state. However, it should be possible to use this stereological information as a firm basis for comparison with known histochemical and biophysical properties of the neurohypophysis. Furthermore, a comparison between unstimulated and stimulated states (see Gerschenfeld, Tramezzani & DeRobertis, 1960) might be useful for studying the mechanism of hormone release, and thus building a model for oxytocin and vasopressin release.

SUMMARY

Standard stereological methods have been used to determine the morphometric parameters of the rat neurohypophysis. Sectioned material from six neurohypophyses was analysed at four levels of magnification in the electron microscope. Quantitative data was obtained about the volume, number and surface area of the various cytological components, and was expressed as density per mm^3 of tissue and as absolute dimensions for the average neurohypophysis.

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