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NUMBER AND SIZE DISTRIBUTION OF CEREBELLAR NEURONS ESTIMATED BY THE OPTICAL FRACTIONATOR AND THE VERTICAL ROTATOR

Jytte Overgaard Larsen, Trine Tandrup, Hans Brændgaard

Stereological Research Laboratory, Institute of Experimental Clinical Research, University Institute of Pathology and Second University Clinic of Internal Medicine, Department of Neurology, University of Aarhus, Denmark

ABSTRACT

This study describes how two recently developed design based stereological methods can be used to obtain estimates of the number and size of Purkinje cells and granule cells in rat cerebellum: The optical fractionator has been used to count the cells and the vertical rotator has been used to make estimates of the volumes of the Purkinje cells. Both techniques were highly efficient in that the coefficients of error (CEs) of the individual estimates of the total numbers of Purkinje cells and granule cells were about 10 % when 100 cells of each type were counted in each rat and the CEs of the individual estimates of mean cellular volumes were 3-4 % when ~ 100 Purkinje cells were analysed with the rotator.

Key words: mean volume, number, optical fractionator, rat cerebellum, size distribution, vertical rotator.

INTRODUCTION

Although the cerebellar cortex is a highly regular, laminate neural structure, the density of the constituent cells vary greatly within the folia (Armstrong, 1970) and the notable amounts of neuroglia perikarya located in the Purkinje cell layer, which are especially prone to swelling, compromise interpretations of quantitative structural changes observed in two-dimensional sections. The new stereological tools described here make it possible to obtain precise unbiased quantitative information about cerebellar cells that can be used to evaluate experimental and pathological conditions. Through the use of the optical fractionator neurons are sampled uniformly with a three-dimensional probe, the disector. In addition to permitting estimates of total number unbiased estimates of the size of the sampled neurons can be made with the vertical rotator technique. In a study to be published (Larsen *et al.*, 1993), cerebellar neurons of acrylamide intoxicated rats were quantified with the methods described here.

MATERIALS AND METHODS

In 12 rats that had been exposed to acrylamide and 9 controls (Larsen *et al*, 1993, to be published) the total numbers of Purkinje cells and granule cells were estimated with the

optical fractionator technique and the volumes of the Purkinje cells were estimated with the vertical rotator technique.

Sectioning

Vertical sections (Baddeley, 1986) were generated by ensuring that the plane of sectioning was oriented perpendicular to an arbitrarily chosen horizontal plane and randomly rotated around the vertical axis. To achieve this the cerebellum was divided by a sagittal cut through the vermis and the cut surface was defined as the horizontal plane. Both hemispheres were embedded in the same block of glycolmethacrylat with the cut surface facing down. The left hemisphere was randomly rotated and the right hermisphere was rotated 90 relative to the left hemisphere. The block with the embedded cerebellum was cut into a set of 150 - 200 sections on a LKB Historange Microtome with a microtome setting of 40 μm. The plane of sectioning was normal to the horizontal plane. The sections used in the analysis were selected at 12 section intervals after randomly selecting the first of these from the first 12 section interval.

The optical fractionator

The optical fractionator analysis was carried out on video images of microscopic fields that were merged with graphic representations of stereological probes and grids generated by an Amiga 2000 computer that was interfaced with the video image through a genloc. A Heidenhain MT-2-microcator was used for measuring the distance that the plane of focus was moved through the tissue along the focal axis. Two motors connected to the microscopic stage were used to move the section in a raster pattern of steps under the microscope objective.

The numbers of Purkinje cells and granule cells were estimated with the optical fractionator method, which involves fractionator sampling and optical disector counting. Reference neurons were counted in a systematic sample of optical disectors that comprised a known fraction of the volume of cerebellar cortex. The total number of neurons, N, was estimated as the product of the number of neurons counted with the disectors, Q^-, and the reciprocal of the aggregate sampling fraction. The sections sampled during sectioning the block with the embedded cerebellum provided the first sampling fraction, $F_1 = 1/12$.

The second sampling fraction was the ratio of the area of the counting frame, $a(frame)$, used with the disector to the area of the rasted step movements. The microscopic stage and thereby the section was moved systematically in steps of length dx and dy along the x and y-axes, $F_2 = a(frame)/dx \times dy$. F_2 was 1094 / (430 × 450) when counting Purkinje cells and 37 / (997 × 900) when counting granule cells. The third sampling fraction was defined by the distance that the disector was moved through the section along the axis of focus, h, and the thickness of the section, dz. F_3 was 25 / 40 when Purkinje cells were counted and 10 / 40 when granule cells were counted. Neurons, which are coming into focus are counted giving the number of neurons in the optical disector of volume $a(frame) \times h$. The sampling fractions resulted in approximately 100 - 150 neurons of both kinds being counted.

The combined, aggregate sampling fraction was $1/12 \times a(frame)/dx \times dy \times h/dz$. The total numbers of neurons, N, were estimated as the number of particles counted, Q^-, multiplied by the reciprocal of the sampling fraction.

$$N = Q^- \times 1/F_1 \times 1/F_2 \times 1/F_3 = Q^- \times 12/1 \times dx \times dy/a(frame) \times dz/h \qquad (1)$$

In determining F_3 it is necessary to know the true thickness of the section. The thickness of the section was measured at four locations of each section. The mean of these measurements was 40.4 μm. This was within the accuracy of measurements, why the microtome setting of 40 μm was used in the calculations.

The vertical rotator, theoretical background

The vertical rotator is a two-stage procedure for estimating particle volume (Jensen and Gundersen, 1993; Cruz-Orive 1987). Disectors are used to make uniform samples of the neurons to be analysed. The rotator measurements are made on vertical sections.

The vertical rotator is based on the Pappus-Guldinus theorem: "If a planar area be revolved about an axis in its plane, but not intersecting the area, the volume of the solid of revolution so formed is equal to the product of the area and the length of the path traced by the centroid of the area" (Beyer, 1981).

In situations in which only two-dimensional profiles (planar area) of the three-dimensional object (particles) are available for analysis, as in histological sections, the particle volume can be estimated, using this principle, in the following way. The vertical axis is drawn through a unique sampling point in the profile of the sampled particle in order to generate two half-planes. The volume of the particle is estimated from the product of the area of one half-plane, a, and the distance between the axis and the center of gravity of the area, designated dc, multiplied by 2π:

$$V = a \times dc \times 2\pi \qquad (2)$$

$2\pi \times dc$ correspond to "the length of the path traced by the centroid of the area" in the Pappus-Guldinus theorem. In irregularly shaped particles only estimates of the particle volume can be obtained. This is accomplished by rotating the vertical section around the vertical axis to map the volume of the particle. An analysis of a uniformly sampled set of vertical profiles provides an unbiased and efficient estimate of the mean particle volume.

To optimise the estimate, estimates of volume of each particle are made from each half of each particle profile.

The vertical rotator, practical procedure

Estimates were made of the volumes of Purkinje cells that were uniformly sampled by the optical disectors. The section was rotated on the microscopic stage so that the edge that represented the cut surface was oriented horizontally on the TV-screen. This was done so that the vertical axis had a vertical orientation on the screen. The unique reference point, the nucleolus, was interactively defined and a software generated vertical axis, which separated the two half-planes, was displayed on the screen. The upper and lower limits of the cell body were interactively defined on the monitor; neither need to be on the vertical axis. These limits are used to determine the projected height, H, of the cell on the vertical axis. Perpendicular to the vertical axis, the computer generated a predetermined number, m, of randomly positioned, equidistant lines. The first line is positioned randomly in the first $1/m$ of the height, subsequent lines are displayed at intervals of $t = H/m$. Sets of three horizontal lines were used in the study. The points of intersection between the cell boundary and the horizontal lines were marked in both half-planes and the distance between the vertical axis and the points of intersection, l_i, was calculated by the computer (The rotator software averages two estimates of volume for each particle: one in each half-plane). The estimate of the particle volume is obtained as:

$$V = \sum l_i^2 \times \pi \times t \quad (3)$$

The estimate of the volume was presented on the screen.

CALCULATIONS AND RESULTS

The Purkinje cell volumes were transformed to logarithmic values because, the intra-animal distributions were skewed to the right in probability plots. The geometric mean from each animal was used in the calculations. Student's t-test was used to compare the two groups of animals. The intra-animal coefficient of error for the rotator estimates was calculated as:

$$"CE" = exp(SEM[log\ volume]) - 1 \quad (4)$$

and the intra-animal coefficient of variation as the tolerance factor - 1:

$$"CV" = exp(SD[log\ volume]) - 1 \quad (5)$$

The inter-animal CV of the estimated geometric means = SD/mean.
The inter-animal CE of the fractionator estimates were calculated with the quadratic approximation formula (Gundersen and Jensen 1987) modified to take the nugget effect into consideration (Gundersen *et al*, 1993, to be published).
The total numbers of cerebellar Purkinje cells and granule cells in the two groups were the same, approximately 370.000 Purkinje cells and 145.000.000 granule cells. The geometric mean volume of the Purkinje cells was significantly reduced from 3500 μm^2 in the controls to 3140 μm^2 in the group of animals that had received acrylamide (Larsen *et al*, 1993). Because the number of Purkinje cells was the same in both groups, the reduced mean volume can be explained in at least two different ways. For example all cells could have been reduced in volume or a sub-population (e.g. the largest cells) could have been preferentially decreased. In order to determine which of these alternatives was the case here, the volumes of the cells in the treated animals were scaled so that they had the same mean value as the controls. The size distributions of the two groups were then compared (Fig 1). This analysis indicates that all the cells were equally reduced in size.
The size distributions of the controls are presented on a linear scale in Fig. 2.
The intra-animal "CE" of the rotator estimate was 3 - 4 % and "CV" was 45 %.
The inter-animal CV of the estimated geometric means was 9 %.
The mean CEs of the estimates of the total numbers of Purkinje cells and granule cells were both 10 %. The inter-animal coefficient of variation, CV, was 13 % for Purkinje cells and 8 % for granule cells.

Fig.1.
The average size distributions of Purkinje cells in acrylamide intoxicated animals (hatched curve) and controls (fully drawn curve). The ordinate shows frequency and the abscissa is logarithmic. The values from the treated animals were shifted to the right by a value equal to the difference in the geometric means of the two distributions in order to compare directly the shapes of the two distributions. The shapes of the two distributions were the same indicating that Purkinje cells of all sizes are equally reduced in volume by exposure to acrylamide

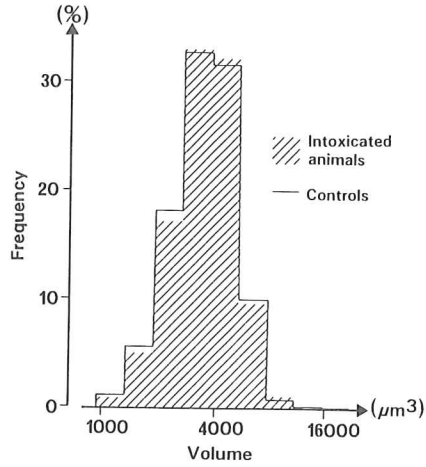
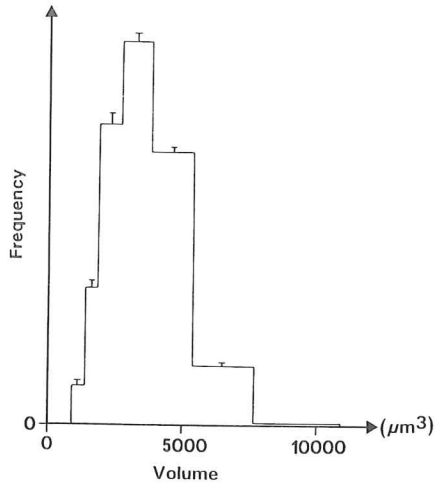


Fig. 2.
The average size distribution of the Purkinje cells in the controls presented on a linear abscissa. SEM for each class is shown.



DISCUSSION

Only a small number of requirements need to be met in order to use the applied methods described here.
To use the optical fractionator, one must be able to exhaustively section the organ of interest, and to stain the cells of interest in relatively thick sections.
In order to use the vertical rotator, it must be possible to make vertical sections and the cells of interest must contain a unique reference point.
The optical fractionator has been described in detail by West et al, 1991. The combination of fractionator sampling and optical disector counting has some unique virtues. The estimates of numbers of neurons are unaffected by swelling or shrinkage of the tissue during processing. The use of optical disectors greatly facilitates counting; makes the identification of neurons easier (of great importance, when counting granule cells) and requires no assumptions about the number of nucleoli in the neuron. The nucleolus is a structural feature which has often been used as a basis for neuronal counts under the assumption that there is a one to one relationship with neurons. This relationship may, however be altered by toxic substances.

Another important feature is that the neurons that are counted can be considered to be a uniform sample for rotator analysis, with the consequence that sampling and size estimation of neurons can be combined into one procedure.

The vertical rotator is a recently developed method for estimating cell volume. It has a number of advantages over previously available estimators of particle volume. The fact that estimates are made at the cellular level provides us with the ability to examine the size distributions of the cells. This provides more information to a study than that obtained in simply comparing the mean neuronal volumes of the different groups. The vertical rotator has been shown by Jensen and Gundersen, 1993 to have a smaller variance than the other main method for making unbiased estimates of neuron volume, the nucleator.

The estimates of the cell volumes are sensitive to shrinkage or swelling of the cells during processing. In these studies it is assumed that these changes are of equal magnitude in the different groups. Some situations leading to differential shrinkage/swelling of the cells during processing, any change in cell volume, whether present *in vivo* or arisen from a differential change of cell volume during processing does however reflect functional and structural changes in that particular cell type.

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