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COMPARISON OF EMPIRICAL AND ESTIMATED EFFICIENCY IN NEURON COUNTING BY THE FRACTIONATOR METHOD AND IN VOLUME MEASUREMENT BY CAVALIERI´S METHOD

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ABSTRACT

Reconstructing the volume and the nerve cell number of the facial and hypoglossal nucleus of one Wistar rat with Cavalieri´s estimator and a fractionator design we determined the mean empirical error of systematic sampling probes through these nuclei depending on the sample size as reference values. We compared these empirical values of the error to the mean estimated values obtained by error estimators of Gundersen and Jensen (1987; J. Microsc. 147: 229-263) for the Cavalieri and fractionator design and to the error estimator by Cruz-Orive (1990; J. Microsc. 160: 89-95) for the fractionator design. Using the emperical approach, the mean error of the volume determination does not exceed 10%, i.e. the range of interest of most stereological studies, using 4 equidistant sections through the brainstem nuclei. The mean error of the neuron number estimation does not exceed 10% using about 8 sections of the facial or 10 sections of the hypoglossal nucleus in the investigated rat. The error estimator by Gundersen and Jensen (1987) overestimates the error for the volume calculation using small sample sizes ≦ 16 sections, but correlates nearly exact with the empirical error of the nerve cell count using the fractionator design. The error estimator by Cruz-Orive (1990) underestimates the error of the fractionator design for sample sizes ≦ 16 sections in both nuclei. In conclusion, about 2% of the total number of possible 6 µm sections are enough to estimate the volume of cranial nerve nuclei and 5% of the sections to estimate the neuron number with an intra-individual precision less than 10%. In the range of efficient sample sizes the error predictor of Gundersen and Jensen (1987) is very reliable for the neuron number error estimation, but overestimates the error of the neuron number. In our example the error predictor of Cruz-Orive (1990) underestimates the neuron number error in the range of interest.

Key words: Fractionator, Cavalieri´s principle, error estimator, neuron counting, volume measurement

INTRODUCTION

An important aspect of stereological studies with systematic sampling estimators like the Cavalieri´s principle for the volume or the disector estimator (Sterio, 1984) and the fractionator (Gundersen, 1986) for the total number of particles in arbitrary objects is the efficiency, e.g. the coefficient of error (CE) of these estimators. Using random sample probes in serial sections over- or underestimations are made, the estimate is always an approximation to the true volume of number of particles depending on the number of sections that have been taken into account (Zilles et al., 1982; Gundersen and Jensen, 1987).

Basing on Matheron´s theory (1971) on the efficiency of systematic sampling, Gundersen and Jensen (1987) developed a formula to predict the error variance of the Cavalieri and fractionator

estimator. This predictor has been applied to many stereological investigations (e.g. Pakkenberg and Gundersen, 1988; Braendgaard et al., 1990; West et al., 1991). Cruz-Orive (1990) presented an different error estimator for the fractionator design basing on an idea in Gundersen (1986). This second method has been applied by Geiser et al. (1990).

In order to investigate the efficiency of our simplified method for particle counts with the physical disector using a drawing-microscope (Guntinas-Lichius et al., 1993) we counted in one rat the number of neurons and calculated the volume of the left hypoglossal and facial nucleus using all sections through these nuclei, i.e. reconstructed the whole nuclei (Alternative method for total reconstruction, see: Pover and Coggeshall (1991)). In the following we determined the average error of the volume and neuron number calculation depending on the enlargement of the interval between the measured sections and compared these values with the results of the error estimator by Gundersen and Jensen (1987) and of the estimator by Cruz-Orive (1990).

MATERIAL AND METHODS

Sections

One untreated Wistar rat was fixed by percardial perfusion with Bodian´s solution, the brainstem removed and embedded in paraffin. Using a motor-driven rotary microtome the brainstem was cut in a complete series of sections at a nominal thickness h, h = 6 µm. Out of this series, starting slightly cranial and finishing slightly caudal of the left facial and the left hypoglossal nucleus respectively, all sections were mounted and Nissl-stained for quantitative investigation, yielding as total of m_F = 170 sections through the left facial and m_H = 228 sections through the left hypoglossal nucleus.

Drawings

For the estimation of the number of neurons we applied the physical disector method with a drawing microscope described in detail elsewere by Guntinas Lichius et al. (1993):

Using a binocular Leitz Laborlux S with calibrated drawing apparatus at magnification 200x we evaluated all cross-cut profiles of the left facial and hypoglossal nucleus in the 170 respectively 228 sections. The boundary of the respective nucleus was delineated on transparent drawing paper and all perikarya of motoneurons that contained a cross-cut profile or cap of a cell nucleus within the plane of the section, were marked with a cross, and all perikarya without recognizable part of the cell nucleus were marked with a circle in each drawing. The drawings were numbered from *F1* to *F170* for the facial and *H1* to *H228* for the hypoglossal nucleus in cranio-caudal direction.

Data from drawings

Beginning with *F1* of the facial nucleus, this drawing was placed on top of the immediately following drawing *F2* and aligned (cf. Guntinas-Lichius et al.,1993) in order to construct a physical disector with the reference section *F1* and the look-up section *F2*. According to Sterio (1984) the number of nerve cells Q_s^- (s = 1 for *F1*, *H1*; s = 2 for *F2*, *H2*; ...) within this physical disector were determined counting all perikarya marked with a cross in the reference section *F1* but not in the look-up section *F2*. Nerve cells with a cross in both drawings or only in *F2* must not be counted.

Next step we used *F2* as reference and *F3* as look-up section, then *F3* up to *F170*. The whole counting procedure was repeated for the hypoglossal nucleus.

We estimated the area a_S (s = 1 for *F1*, *H1*; s = 2 for *F2*, *H2*; ...) of the cross-cut profile of the nucleus on every section by planimetry of the areas we had delineated on the transparent paper using an ATARI STE computer with digitizing tablet. Problems of area measurement with a tablet (Gundersen et al., 1981; Haug, 1981) were not important for our investigation.

All nerve cell counts Q_s^- (s = 1, 2, .. m_F for the facial nucleus and s = 1, 2, .. m_H for the hypoglossal nucleus) and profile areas a_S (s = 1, 2, .. m_F for the facial nucleus and s = 1, 2, .. m_H for the hypoglossal nucleus) provide the database for all subsequent calculations.

Calculation of neuron number and volume of the nuclei using all sections

The nerve cell number N can be calculated easily by

$$N = \sum_{s=1}^{m} Q_s^- , \qquad (01)$$

i.e. using the data of all sections through the facial or hypoglossal nucleus.

The volume V can be calculated by

$$V = h * \sum_{s=1}^{m} a_S . \quad (02)$$

Sampling

In the following we constructed systematic random samples out of the m_F = 170 sections of the facial nucleus and m_H = 228 sections of the hypoglossal nucleus with different equidistant intervals t, t = 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 30, 40, 50, 60. For every interval t there exist j, j = 1, ..., t, possibilities to construct a sample. Every sample contains contains $n(j)$ sections, j = 1, ..., t, when $n(j) = [m/t]$ or $n(j) = [m/t] + 1$ depending on j ($[m/t]$) is the largest integer less than m/t. For example, for t = 4 exist j = 4 samples: {*F1, F5, F9, ..., F169*} or {*F2, F6, F10, ..., F170*} or {*F3, F7, F11, ..., F167*} and {*F4, F8, F12, ..., F168*}.

For each of these samples the respective Q_S^- and a_S values, i.e. for {*F1, F5, F9, ..., F169*} the data subsets { Q_1^- , Q_5^- , Q_9^- , ..., Q_{169}^- } and { a_1 , a_5 , a_9 , ..., a_{169} } were extracted from the database for the estimation of volume and neuron number. In the following calculations the data of the subsets are referred to as Q_i^- and a_i with i = 1, 2, 3, ..., $n(j)$ separately for each sample.

For all j different samples for each interval t we estimated the nerve cell number $N_F(t,j)$ by the fractionator technique (Gundersen, 1986):

$$N_F(t,j) = t * \sum_{i=1}^{n(j)} Q_i^- \quad (03)$$

According to Cavalieri 's principle we calculated the volume $V(t,j)$:

$$V(t,j) = t * h * \sum_{i=1}^{n(j)} a_i \quad (04)$$

The means of the subsets of each sample j of each interval t, $\overline{N_F(t,j)}$ and $\overline{V(t,j)}$, are equal to the result obtained by the whole data set, N, respectively V. Therefore the mean emperical error $\overline{E}$ of the subsets of each interval t, calculating the standard deviation SD of each interval, is:

$$\overline{E}[V(t)] = SD[V(t)] / V \quad (05)$$

$$\overline{E}[N_F(t)] = SD[N_F(t)] / N \quad (06)$$

These calculations were repeated for every interval t in both nuclei. Table I shows an example for the calculation of the empirical errors.

Error estimators

The systematic nature of our samples does not only increase the efficiency of the sampling compared to random independent sampling (Gundersen and Jensen, 1987), but it also allows to estimate the efficiency of the volume calculations, CE[$V(t,j)$], for every sample j, j = 1, ..., t, of any interval t according to the formulae by Gundersen and Jensen (1987) derived from Matheron (1971) using the following sums:

$$A = \sum_{i=1}^{n(j)} (a_i * a_i) \quad (07)$$

$$B = \sum_{i=1}^{n(j)-1} (a_i * a_{i+1}) \quad (08)$$

$$C = \sum_{i=1}^{n(j)-2} (a_i * a_{i+2}) \quad (09)$$

$$CE[V(t,j)] = \sqrt{(3A+C-4B)/12} \; / \sum_{i=1}^{n(j)} a_i \quad (10)$$

Table I. Example for the calculation of the emperical and predicted errors for the identical random (j= 3) data-subset with distance t = 50 in the hypoglossal nucleus according to equation (01) – (18):

Section	Q_i^-	a_i mm^3	$a_i * a_i$	$a_i * (a_{i+1})$	$a_i * (a_{i+2})$	$Q_i^- * Q_i^-$	$Q_i^- * Q_{i+1}^-$	$Q_i^- * Q_{i+2}^-$
H3	2	0.03	0.0009	0.0042	0.0048	4	32	28
H53	16	0.14	0.0196	0.0224	0.0168	256	224	192
H103	14	0.16	0.0256	0.0192	0.0224	196	168	168
H153	12	0.12	0.0144	0.0168		144	144	
H203	12	0.14	0.0196			144		
$\sum$	50	0.59	A=0.0801	B=0.0626	C=0.044	D=744	E=568	F=388

Q_o = 28 Q_c = 28

$$N_F(t,j) = N_F(50,3) = t * \sum_{i=1}^{n(j)} Q_i^- = 50 * 50 = 2500$$

$$V(t,j) = V(50,3) = t * h * \sum_{i=1}^{n(j)} a_i = 50 * 0.006 * 0.59 = 0.177 \text{ mm}^3$$

The calculations of the other samples j of the interval t = 50 are not shown. The standard deviations of the interval were $SD[V(50)]$ = 0.02 and $SD[N_F(50)]$ = 430. V = 0.18 mm^3 and N = 2053 in the hypoglossal nucleus.

$$\overline{E}[V(t)] = \overline{E}[V(50)] = 0.02/0.18 = 0.11 = 11\%$$

$$\overline{E}[N_F(t)] = \overline{E}[N_F((50] = 430/2053 = 0.21 = 21\%$$

$$CE[N_F(t,j)] = CE[N_F(50,3)] = \sqrt{(3D+F-4E)/12} \;/\; \sum_{i=1}^{n(j)} Q_i^- = 5.385/50 = 0.107 = 10.7\%$$

$$CE_{II}[N_F(t,j)] = CE_{II}[N_F(50,3)] = (1/\sqrt{3}) * (|Q_o-Q_e|/Q_o+Q_e) = (1/\sqrt{3}) * (|28-28|/28+28) = 0 = 0\%$$

$$CE[V(t,j)] = CE[V(50,3)] = \sqrt{(3A+C-4B)/12} \;/\; \sum_{i=1}^{n(j)} a_i = 0.0531/0.59 = 0.09086 = 9.001\%$$

The calculations of the CE of the other samples j and of the mean CE are not shown for the example.

The mean efficieny $\overline{CE}$ for all j samples of an interval t with j= 1,..., t is:

$$\overline{CE}[V(t)] = \sqrt{(1/t) * \sum_{j=1}^{t} CE^2[V(t,j)]} \tag{11}$$

Likewise it is possible to estimate the efficiency of the fractionator estimate for every sample, $CE[N_F(t,j)]$ constructing the sums:

$$D = \sum_{i=1}^{n(j)} (Q_i^- * Q_i^-) \tag{12}$$

$$E = \sum_{i=1}^{n(j)-1} (Q_i^- * Q_{i+1}^-) \tag{13}$$

$$F = \sum_{i=1}^{n(j)-2} (Q_i^- * Q_{i+2}^-) \tag{14}$$

$$CE[N_F(t,j)] = \sqrt{(3D+F-4E)/12} \;/\; \sum_{i=1}^{n(j)} Q_i^- \tag{15}$$

So the mean CE, $\overline{CE}$ is:

$$\overline{CE}[N_F(t)] = \sqrt{(1/t) * \sum_{i=1}^{t} CE^2[N_F(t,j)]} \tag{16}$$

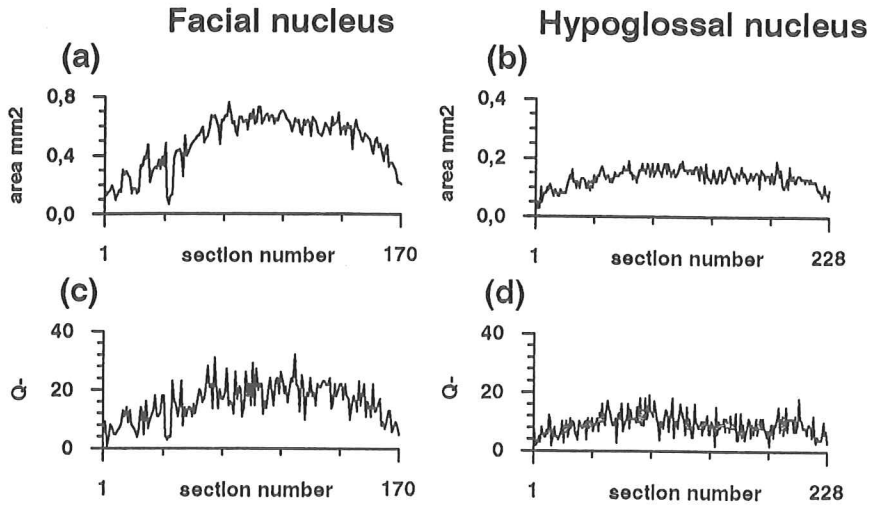

Fig. 1. Original data, showing the area measurements *(a, b)* and the cell count *(c, d)* for each of the 170 sections through the facial nucleus *(left side)* and of the 228 sections through the hypoglossal nucleus *(right side)*. The section-to-section variability of the data is lower in the hypoglossal than in the facial nucleus.

Cruz-Orive (1990) developed another way to calculate the efficiency of the fractionator estimate, $CE_{II}[N_F(t,j)]$:

For each sample the reference sections respectively the drawings were numbered successively 1, 2, 3, ... in their cranio-caudal order. Then, the total number of nerve cells counted in the odd-numbered drawings, Q_o, and the even-numbered drawings, Q_e, were determined separately (details in: Geiser et al., 1990). Now the CE can be calculated:

$$CE_{II}[N_F(t,j)] = (1/\sqrt{3}) * (|Q_o - Q_e| / Q_o + Q_e) \quad (17)$$

The mean CE_{II}, $\overline{CE}_{II}$ for a group of j samples of an interval t is:

$$\overline{CE}_{II}[N_F(t)] = \sqrt{(1/3t) * \sum_{j=1}^{t} [(Q_{oj} - Q_{ej}) / (Q_{oj} + Q_{ej})]^2} \quad (18)$$

The $\overline{CE}[V(t)]$, $\overline{CE}[N_D(t)]$ and the $\overline{CE}[N_F(t)]$ had been calculated for the intervals t = 4, 6, 8, 10, 12, 20, 25, 30, 60 and the $\overline{CE}_{II}[N_F(t)]$ for t = 1, 2, 3, 4, 6, 8, 10, 12, 20, 25, 30, 60. These calculations of the 4 different $\overline{CE}$ have been repeated for both nuclei.

In table I the predicted errors are calculated for an example and compared to the empirical error.

RESULTS

Reference values

In the left facial nucleus we counted by total reconstruction (see above) N = 2671 motoneurons in a volume of V = 0·50 mm³ and in the left hypoglossal nucleus N = 2053 motoneurons in a volume of V = 0·18 mm³. Figures 1a-1d illustrate section-to-section variability of the profile areas a_S and the nerve cell counts Q_S^- for both nuclei. In the facial nucleus the mean area of all a_S is 0.49 mm² [range: 0.07-0.76] with a standard deviation of 36%, the mean count of Q_S^- per section is 16 [range: 1-32], standard deviation of 41%. In the hypoglossal nucleus the mean area is 0.13 mm² [range: 0.03-0.19] with a standard deviation of 23%, and a mean cell number Q_S^- per section of 9 [range: 2-19] with standard deviation of 39%. Figures 2a-d show the results of the empirical mean volume

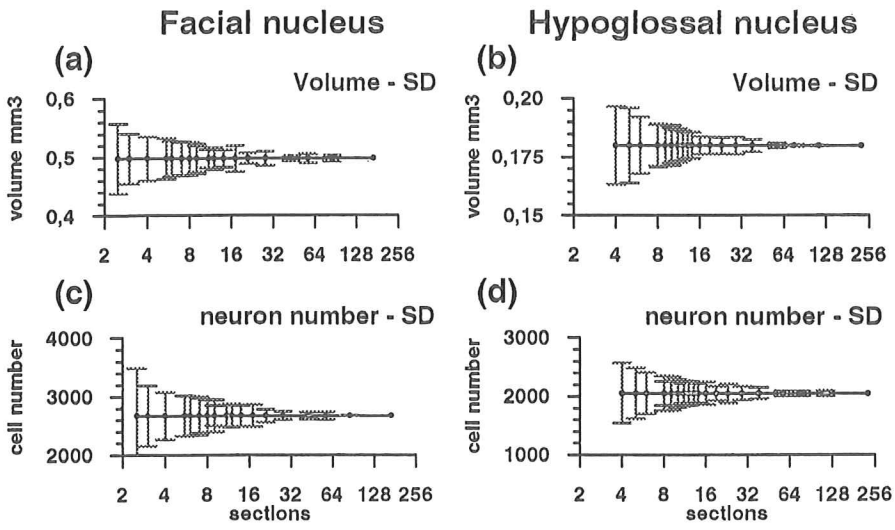

Fig. 2. Original data, presenting the mean empirical volume *(a, b)* ± standard deviation (SD) and the mean empirical neuron number *(c, d)* ± SD versus the section number in the facial nucleus *(left side)* and in the hypoglossal nucleus *(right side)*. The variability of the subsets is lower in the hypoglossal than in the facial nucleus.

and neuron number (± standard deviation (SD)) depending on the sample size for both nulcei. Generally, the variability within the subsets is lower in the hypoglossal (Fig. 2b, 2d) than in the facial nucleus (Fig. 2a, 2c), and the variability of the subsets is lower for the volume than for the cell number measurement in both nuclei. In the facial nucleus the maximal standard deviation using just 3 equidistant sections is 12% for the volume measurement and 30% for the cell count. In the hypoglossal nucleus the maximal standard deviation using the minimum of our example, 4 sections, is 9% for the volume and 25% for the cell number calculation.

Empirical error of estimate depending on sample size

Figures 3a and 3b show the mean empirical error of the volumetric determination of both nuclei. The mean empirical error slowly increases for both nuclei with greater section number n. This general tendancy is modified by a sequence of increasing and decreasing values of the error. In the facial nucleus the mean empirical error is greater than 10% beginning with an section number of about 3 sections, and in the hypoglossal nucleus the mean empirical error does not reach 10% up to a section number n = 4 sections, i.e. just ≦ 2% of all possible 6 µm sections..

The mean empirical error of the the neuron number determination, $\overline{E}\,[\,N_F\,(t)\,]$ is shown in the Figures 3c-3d . As described for the volumetric determination the error of the neuron number calculation increases with greater intervals and the sequence of increasing and decreasing of the mean empirical error is also seen. The 10% barrier is reached with a section number n = 8 sections (= 5% of all section) in the facial nucleus, i.e. 4-5 equidistant section pairs, and with a section number n = 10 (also = 5% of all sections) in the hypoglossal nucleus, i.e. 5 equidistant disector pairs.

Predicted error of the measurement depending on sample size versus empirical error

In figures 3a and 3b the estimated errors of the volume determination, $\overline{CE}\,[\,V(t)\,]$, are compared with the mean empirical errors, $\overline{E}\,[\,V(t)\,]$. In difference to the empirical error the estimated error increases steeper for the volume estimate in both nuclei and overestimates the empirical error beginning with a section number n = 11 in the facial and about n = 12 sections in the hypoglossal nucleus. The dicrepancy between empirical and predicted error is more obvious in the facial than in the hypoglossal nucleus in the phase of small sample sizes.

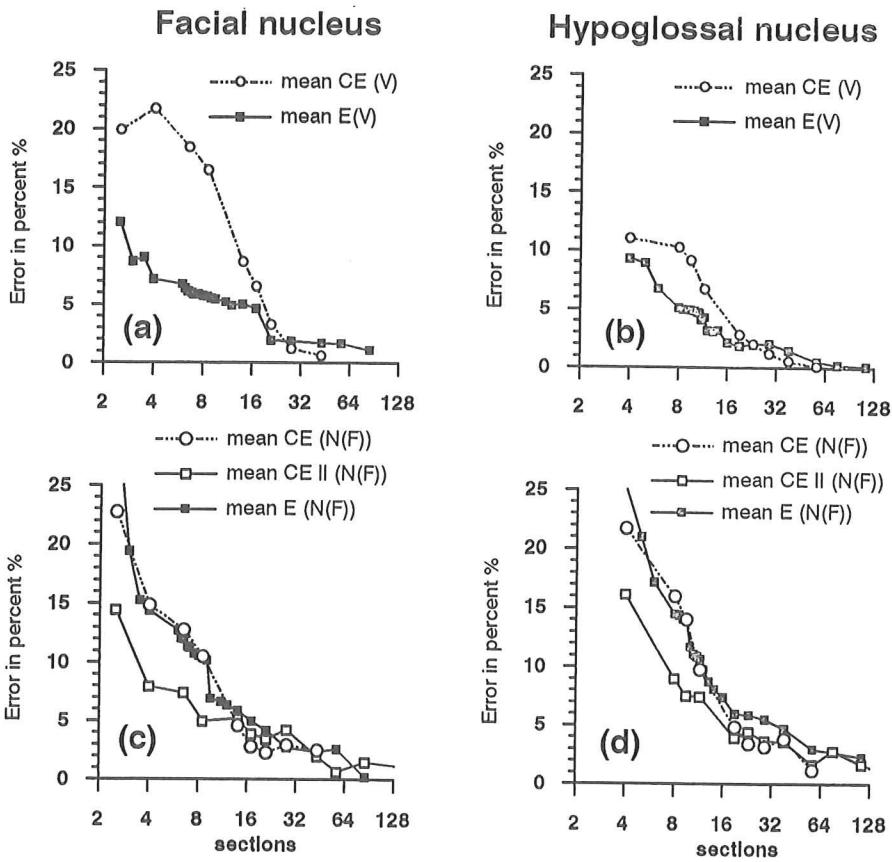

Fig. 3. Comparison of the empirical and predicted efficiency of the volume (*a, b*) and the neuron number calculation by the fractionator (*c, d*) in the facial (*left side*) and in the hypoglossal nucleus (*right side*). The error estimator by Gundersen & Jensen (1987) overestimates the mean error of the volume measurement in the phase of efficient small sample sizes, but is in good accordance with the empirical error of the neuron count. In contrast the estimator by Cruz-Orive underestimates the mean empirical error for small sample sizes.

The figures 3c-3d show the comparison of the empirical error of the neuron count depending on the sample size with the results of the error estimators, $\overline{CE}\,[N_F\,(t)\,]$ and $\overline{CE}_{II}\,[N_F\,(t)]$. Using the fractionator in the facial nucleus the estimated error according to Gundersen and Jensen (1987) is nearly equal to the mean empirical error independent of the sample size, whereas the estimated error according to Cruz-Orive (1990) underestimates the mean empirical error with a section number $n \leq 9$ in the facial nucleus and $n = 11$ in the hypoglossal nucleus.

DISCUSSION

Efficiency and its prediction is an important aspect of sampling procedures in modern stereological tools like the disector (Sterio, 1984), the fractionator (Gundersen, 1986) or recent developments (Cruz-Orive and Roberts, 1993; Vedel Jensen and Gundersen, 1993). Therefore, estimators of the efficiency are useful guidelines in the precision of the estimate. This prediction is a non-trivial problem (Cruz-Orive, 1990), on the one hand because of the systematic nature of the samples and on the other hand because exact calculation of the efficiency depends on the object itself and can

only be easily derived for artificiel models that are unlike to empirical biological conditions (Gundersen and Jensen, 1987).

Because stereological investigations are frequently used in neurobiological studies, we tested and compared the error estimators by Gundersen and Jensen (1987) and Cruz-Orive (1990) against the empirical error of volumetric and neuron number determination in the facial and hypoglossal nucleus of the rat. We just investigated one animal, i.e. our results are a small variance contribution to the unknown total variance among individuals but we investigated two different and undependent structures: Two independent brainstem nuclei of different shape (cigar-shaped hypoglossal and nearly spheric facial nucleus), of different neuronal density (Guntinas Lichius et al., 1992; in our example 5342 neurons per mm^3 in the facial versus 11405 neurons per mm^3 in the hypoglossal nucleus), and of different cytology (Paxinos, 1985).

For all that and in spite of the calculated different section-to-section variability of the facial and hypoglossal nucleus we show for both nuclei the same results, that 2% of the total number of possible 6 µm sections for the volume estimation and 5% for the neuron estimation are sufficient to stay below a empirical error of 10%, i.e. the usual range of neurobiological stereological investigations, that means less than 10 sections per object as Gundersen and Jensen (1987) propose for a adequate sample for the Cavalieri estimator.

That means for the determination of the neuron number 8 sections in the facial and 10 sections in the hypoglossal nucleus are sufficient to stay bellow the 10%-barrier. Braendgaard et al. (1990) stated that normally less than 200 particles need to be counted for a CE between 5 and 15%. In our example this is confirmed: We counted on average $Q^- = 16$ neurons per section in the facial and $Q^- = 9$ neurons per section in the hypoglossal nucleus, that means having counted about 128 neurons in the facial and 90 neurons in the hypoglossal nucleus the mean empirical error does not exceed 10%. Because we obtained the same results in all investigated parameter for both, independent nuclei in spite of the different section-to-section variability, we propose that the results are of general relevance for any anaatomical sites in the brian of the Wistar rat

In this methological study we have exclusively employed single disectors, i.e. the second section of each disector was used as look-up section only. In any applied work the use of double disectors, i.e. the exchange of reference and look-up section doubles the reference space and accordingly improves sampling efficiency (e.g. Pakkenberg and Gundersen, 1988; Neiss et al., 1992; Guntinas-Lichius et al., 1994). For volume determination of the hypoglossal nucleus 6 double disectors yield a mean empirical error of 3.96% (Neiss and Guntinas-Lichius, unpublished data), whereas the mean empirical error amounts to 6.73% with 6 single sections. In principle,the same results were obtained for the other parameters.

Another possibility to decrease the error in systematic sampling, to count more sections or measure them by planimetry, is too time-consuming, i.e. also not efficient : With a section number ≥ 28, i.e. every 8-9th section, a mean empirical error of less than 5% is reached with certainty in both nuclei. Therefore, it is more effective to enlarge the number of investigated animals or to cut thinner slices (Braendgaard et al., 1990).

In most cases the formulae of Gundersen and Jensen (1987) for the error estmation is in excellent accordance with the empirical error (cf. Figure 3). Only for small sample sizes the prediction overestimates the empirical error for the volume estimation. This result means that the error predictor by Gundersen and Jensen (1987) derived from Matheron (1971) is as robust as described by the authors. Mattfeldt (1989) tested the estimator by Gundersen and Jensen (1987) empirically for volume estimates in a variety of geometrical models and in rat hearts with equally good results.

The error estimator of Cruz-Orive (1990) leads to different values than does the estimator of Gundersen and Jensen (1987): In our example the former underestimates the mean error (cf. Figs. 3c and 3d) in the ranges of stereologically interesting sample sizes with ≤ 16 section, respectively section intervals ≥ 10-14. In some cases the approximation of Cruz-Orive (1990) presents the only available choice. An example for this is provided by the study of Geiser et al. (1990). These authors

had initially splitted the material on very small slices and hence obtained zero counts in some subsamples. Under such conditions Gundersen and Jensen's formulae (1987) are meaningless (Cruz-Orive, 1990).

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