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Nerve cell loss in the thalamic mediodorsal nucleus in Huntington's disease. II. Optimization of a stereological estimation procedure

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Abstract This study provides the theoretical background of the decision to count approximately 750–1,300 neurons per individual in the preceding study of Heinsen et al. [6] finding a significant ($P < 0.05$) nerve cell loss in the thalamic mediodorsal nucleus in Huntington's disease with the so-called $V_{Ref} \times N_V$ method. Using a computer simulation of the study of Heinsen et al., it was shown that the legitimation for counting only 100–200 neurons per individual in previous studies comparable to that carried out by Heinsen et al. was based on incorrect assumptions. In this context it was of particular importance to confirm the theoretical prediction in the literature that the random error of total neuron number estimates obtained with the $V_{Ref} \times N_V$ method is actually greater than assumed in current stereological studies. In summary, this study revives the question of how many individuals need to be investigated and how many neurons (or other cell types, respectively) need to be counted per individual in studies comparable to that carried out by Heinsen et al.

Key words Computer simulation · Counting methods · Disector · Morphometry

Introduction

In the preceding study of Heinsen et al. [6] a significant ($P < 0.05$) nerve cell loss of approximately 24% was found in the thalamic mediodorsal nucleus (MD) of patients suffering from Huntington's disease (HD) by counting approximately 750–1,300 neurons per MD and using the so-called $V_{Ref} \times N_V$ method. Since in current studies ([11–13], among others) comparable to that presented by Heinsen et al. [6] only 100–200 neurons per individual were counted, a computer simulation – based on the data presented in [6] as well as on the detailed description of simulating estimates of total numbers of biological particles such as neurons, cells, synapses etc. in [16] – was carried out to demonstrate what effects the two methods (i.e., counting of 100–200 vs counting of approximately 750–1,300 neurons per MD) would have had on the results of the study of Heinsen et al. [6]. As the results are of general importance, the computer simulation is presented here as a separate report.

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Materials and methods

For each left thalamic mediodorsal nucleus (MD) investigated in [6] – i.e., 7 MDs of patients suffering from Huntington's disease (HD1–HD7) and 7 MDs of age- and sex-matched controls (C1–C7) – one virtual left thalamic mediodorsal nucleus (MD*) was generated here, named HD*1 to HD*7 (or C*1 – C*7, respectively). Concerning size and shape the 14 MD*s were similar to the 14 MDs investigated in [6]. The total reference volume of each MD* was shaped like an ellipsoid with $r_X = 0.66 \times r_Y = 0.4 \times r_Z$. The estimated total reference volumes ($\hat{V}_{MD}$) of the 14 MDs reported in [6] were used here as the true total reference volumes (V_{MD*}) of the MD*s. For example, for C*3 r_X was 3.41 mm, r_Y was 5.11 mm, r_Z was 8.51 mm, and V_{C*3} was 620 mm^3, according to $\hat{V}_{C3}$ of 620 mm^3 as found in [6] for C3. Each MD* contained a fixed number of virtual neurons (neurons*), that were shaped as points to represent so-called 'characteristic points' [7] of biological particles in biological specimens such as centroids of nuclei of neurons here. The estimated total numbers of neurons ($\hat{N}_{MD}$) of the 14 MDs reported in [6] were used here as the true total numbers of neurons* (N_{MD*}) of the MD*s. For example, for C*3 the true total number of neurons* (N_{C*3}) was 2,872,716, according to $\hat{N}_{C3}$ of 2,872,716 as found in [6] for C3. Thus, the true mean total number of neurons* of the HD* cases ($\hat{G}_{HD*}$) was 2,275,321 with a coefficient of variation ($CV_{N–HD*}$) of 0.109, whereas the true mean total number of neurons* of the C* cases ($\hat{G}_{C*}$) was 2,985,188 with a coefficient of variation ($CV_{N–C*}$) of 0.059. The relative difference between $\hat{G}_{HD*}$ and $\hat{G}_{C*}$ [i.e., $1-(\hat{G}_{HD*}/\hat{G}_{C*})$] was 23.7%. The spatial

distributional patterns of the neurons* in the MD*s were generated from a so-called 'homogeneous Poisson process' (see, e.g., [2, 16] for details) which corresponded to complete spatial randomness.

According to [6] the $V_{Ref} \times N_V$ method was simulated here for estimating the total numbers of neurons* of the MD*s. For a detailed description of the $V_{Ref} \times N_V$ method see [20]. Two estimation procedures (named EP*1 and EP*2) were modelled, which are described in the following.

EP*1 was adjusted similarly to those estimation procedures that have been described in contemporary literature (see [17, 20] among others). It was identical to the estimation procedure used for the pilot experiment in [6]. For estimating the total numbers of neurons* of the MD*s using EP*1, the MD*s were placed in a Cartesian coordinate system $\Omega = \{0, X, Y, Z\}$ to the effect that there was an angle α_x of 10° between r_x of the MD*s and the X-axis of Ω, an angle β_x of 87° between r_x and the Y-axis of Ω, and an angle γ_x of 81° between r_x and the Z-axis of Ω. Furthermore, there was an angle α_y of 90° between r_y of the MD*s and the X-axis of Ω, an angle β_y of 20° between r_y and the Y-axis of Ω, and an angle γ_y of 70° between r_y and the Z-axis of Ω, as well as an angle α_z of 80° between r_z of the MD*s and the X-axis of Ω, an angle β_z of 70° between r_z and the Y-axis of Ω, and an angle γ_z of 22° between r_z and the Z-axis of Ω. By orientating the X-axis of Ω parallel to a latero-lateral line through a thougt head (positive values of x to the right, negative ones to the left), the Y-axis of Ω parallel to a caudo-cranial line through a thougt head (positive values of y cranially and negative ones caudally positioned), and the Z-axis of Ω parallel to a occipito-frontal line through a thougt head (positive values of z frontally and negative ones occipitally positioned), the positions of the MD*s in Ω were similar to the positions of left MDs in the human brain. Afterwards the MD*s were dissected virtually in a plane of section with a direction vector parallel to the Z-axis of Ω to a series of parallel sections, modeling the dissection of MDs in the human brain with a frontal plane of section. The thickness of each section was 560 µm except the first one, whose thickness was selected randomly between 0 µm and 560 µm. Every third section was investigated, starting either with the first, second, or third one. Neurons* were counted with cuboid-shaped 'counting spaces' that corresponded regarding their base of 5,625 µm², their height of 29.7 µm, and their position of 20 µm below the surface of the sections exactly to those optical disectors that were used for the pilot experiment in [6]. The distance of the counting spaces in both directions X and Y was 1,300 µm, whereas the distance of the points used for estimating the total reference volumes of the MD*s with Cavalieri's principle and point counting was 1,725 µm (see [6] for details). Only those neurons* were counted which were situated in the counting spaces. For estimating, e.g., the total number of neurons* of C*3, the use of EP*1 resulted – on average – in investigating 9 sections of the MD*, counting of 169 neurons* with 219 counting spaces for estimating the neuron* density within C*3, and counting of 124 points for estimating the volume of C*3.

EP*2 was identical to the estimation procedure described in [6]. The section thickness was 560 µm; every third section was investigated. Neurons* were also counted with counting spaces (base: 15,625 µm², height: 29.7 µm from 20 µm to 49.7 µm below the surface of the section). The distance of the counting spaces in both directions X and Y was 865 µm; the point distance for estimating the total reference volumes of the MD*s was 1,725 µm. For estimating, e.g., the true total number of neurons* of C3, the use of EP*2 resulted – on average – in investigating 9 sections of the MD*, counting of 1,066 neurons* with 494 counting spaces for estimating the neuron* density within C*3, and in counting of 124 points for estimating the volume of C*3.

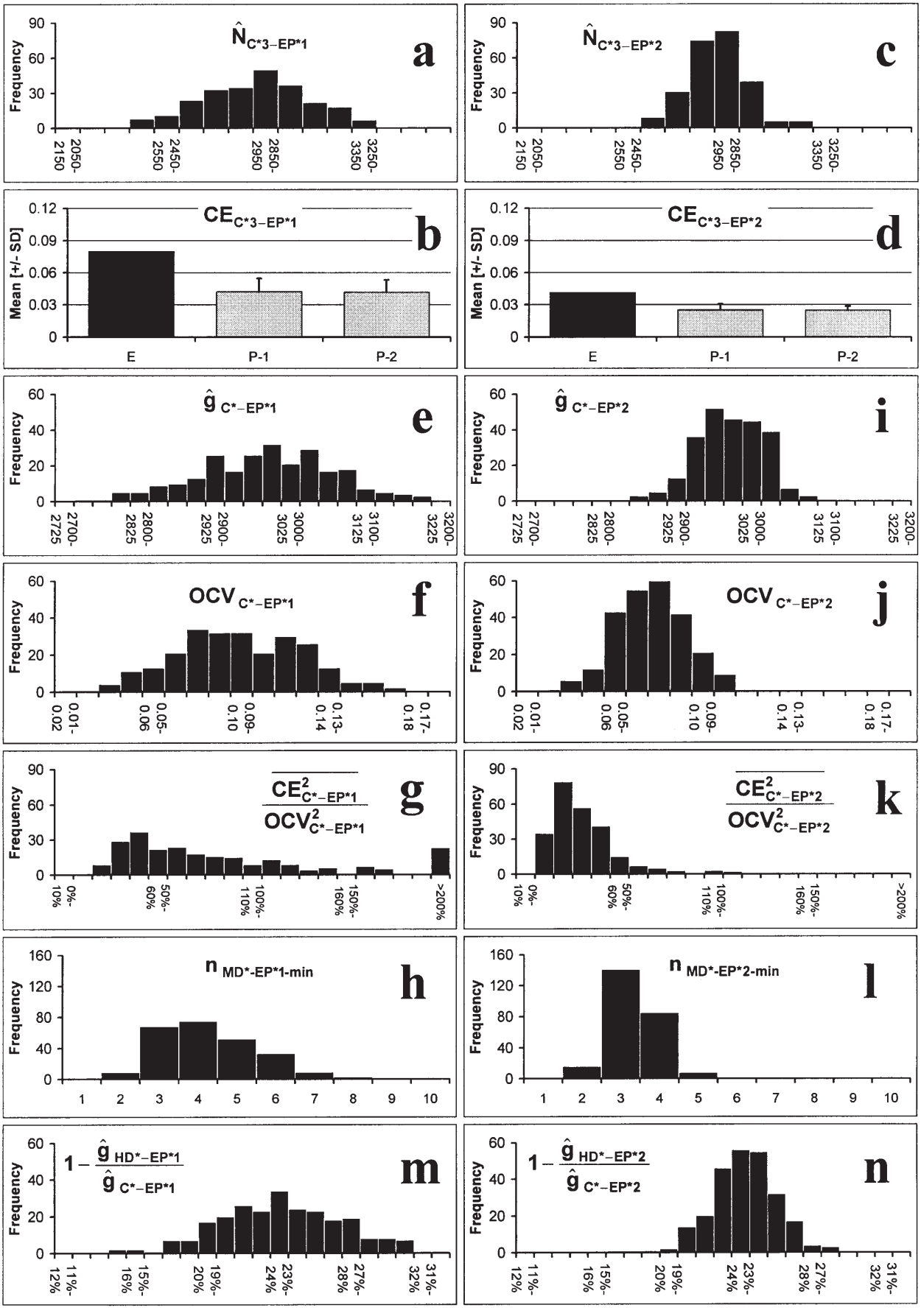
Both EP*1 and EP*2 were applied 250 times to each MD*. Thus, 2×250 mutually independent estimates of the true total number of neurons* of each MD* were obtained. From these data 2×250 mutually independent estimates of the mean total numbers of neurons* of the HD* cases or the C* cases were calculated. All random variables in the process of simulation were controlled by a pseudorandom number generator that was proposed by L'Ecuyer [8].

Results

To demonstrate the results of the computer simulation concerning repeated estimates of the true total number of neurons* of an individual MD*, C*3 was selected here as an example (for the other MD*s similar results were found; data not shown). Using EP*1, the arithmetic mean of the 250 estimates of the total number of neurons* of C*3 (N_{C*3}) was 2,866,005, which was 99.8% of N_{C*3}; 95% of these estimates were found within a range of approximately ± 16% around N_{C*3} (Fig. 1 a). This range was similar to that found in the pilot experiment in [6]. The coefficient of variation of these estimates – which for itself was an empirical estimate of the square root of the relative stereological sampling variance for estimating N_{C*3} using EP*1 (i.e., of $CE_{\hat{N}*-C*3-EP*1}$; CE is coefficient of error) – was 0.07992. As shown in Fig. 1 b, $CE_{\hat{N}*-C*3-EP*1}$ was underestimated by approximately 50% when applying the methods proposed in [17] or [20] for predicting CE. Using EP*2, the arithmetic mean of the 250 estimates of N_{C*3} was 2,861,324, which was 99.6% of N_{C*3}; 95% of these estimates were found within a range of approximately ± 8% around N_{C*3} (Fig. 1 c). As shown in Fig. 1 d, $CE_{\hat{N}-C*3-EP*2}$ – which was 0.04119 –, was underestimated by approximately 40% when applying the predicting methods described in [17] or [20].

To demonstrate the results of the computer simulation concerning repeated estimates of the true mean total number of neurons* of a sample of MD*s, the C* cases were selected here as example (for the HD* cases similar results were found, data not shown). Using EP*1, the arithmetic mean of the 250 estimates of the mean total number of neurons* of the C* cases ($\hat{G}_{C*}$) was 2,982,229, which was 99.9% of $\hat{G}_{C*}$; 95% of these estimates were found within a range of approximately ± 6% around $\hat{G}_{C*}$ (Fig. 1 e). The observed coefficient of variation among the estimates

Fig. 1 a–n Simulation results for estimating the relative difference ▶ between the mean total number of neurons* of seven virtual models of left thalamic mediodorsal nuclei of patients suffering from Huntington's disease (HD*) and seven virtual models of the left thalamic mediodorsal nuclei of age- and sex-matched controls as described in text. The simulation was repeated 250 times. Results obtained using the virtual estimation procedure EP*1 are shown on the *left*; corresponding results obtained using EP*2 are shown on the *right*. The figures show the frequency distributions obtained for the following variables. **a** and **c** $\hat{N}_{C*3}$; i.e., estimated total number of neurons* of C*3; $\times 10^3$. **b** and **d** CE_{C*3}; i.e., square root of the relative stereological sampling variance for estimating N_{C*3} using EP*1 as found empirically *(E)* or predicted as described in [20] *(P-1)* or in [17] *(P-2)*. **e, i** $\hat{g}_{C*}$; i.e., estimated mean total number of neurons* of the seven C* cases; $\times 10^3$. **f, j** OCV_{C*}; i.e., observed coefficient of variation among the seven $\hat{N}_{C*}$ values [$\hat{N}_{C*1}$ to $\hat{N}_{C*7}$] of a given repetition of the simulation. **g, k** $\overline{CE^2_{C*}}/OCV^2_{C*}$; i.e., ratio 'mean relative stereological sampling variance' vs. 'true interindividual variability of the total number of neurons* among the C* cases'. **h, l** $n_{MD*-min}$; i.e., minimal number of MD*s supposedly necessary to be examined to demonstrate that sample mean differences of 20% are significant at the 0.05 level. **m, n** $1 - (\hat{g}_{HD*}/\hat{g}_{C*})$; i.e., estimated relative difference between the mean total number of neurons* of the seven HD* cases and the seven C* cases. For detailed interpretation see Results


a
$\hat{N}_{C*3-EP*1}$
Frequency
2050-2150
2150-2250
2250-2350
2350-2450
2450-2550
2550-2650
2650-2750
2750-2850
2850-2950
2950-3050
3050-3150
3150-3250
3250-3350
c
$\hat{N}_{C*3-EP*2}$
b
$CE_{C*3-EP*1}$
Mean [+/- SD]
E
P-1
P-2
d
$CE_{C*3-EP*2}$
e
$\hat{g}_{C*-EP*1}$
2700-2725
2725-2750
2750-2775
2775-2800
2800-2825
2825-2850
2850-2875
2875-2900
2900-2925
2925-2950
2950-2975
2975-3000
3000-3025
3025-3050
3050-3075
3075-3100
3100-3125
3125-3150
3150-3175
3175-3200
3200-3225
i
$\hat{g}_{C*-EP*2}$
f
$OCV_{C*-EP*1}$
0.01-0.02
0.02-0.03
0.03-0.04
0.04-0.05
0.05-0.06
0.06-0.07
0.07-0.08
0.08-0.09
0.09-0.10
0.10-0.11
0.11-0.12
0.12-0.13
0.13-0.14
0.14-0.15
0.15-0.16
0.16-0.17
0.17-0.18
j
$OCV_{C*-EP*2}$
g
$\frac{\overline{CE^2_{C*-EP*1}}}{OCV^2_{C*-EP*1}}$
0%-10%
10%-20%
20%-30%
30%-40%
40%-50%
50%-60%
60%-70%
70%-80%
80%-90%
90%-100%
100%-110%
110%-120%
120%-130%
130%-140%
140%-150%
150%-160%
160%-170%
170%-180%
180%-190%
190%-200%
>200%
k
$\frac{\overline{CE^2_{C*-EP*2}}}{OCV^2_{C*-EP*2}}$
h
$n_{MD*-EP*1-min}$
1
2
3
4
5
6
7
8
9
10
l
$n_{MD*-EP*2-min}$
m
$1-\frac{\hat{g}_{HD*-EP*1}}{\hat{g}_{C*-EP*1}}$
11%-12%
12%-13%
13%-14%
14%-15%
15%-16%
16%-17%
17%-18%
18%-19%
19%-20%
20%-21%
21%-22%
22%-23%
23%-24%
24%-25%
25%-26%
26%-27%
27%-28%
28%-29%
29%-30%
30%-31%
31%-32%
n
$1-\frac{\hat{g}_{HD*-EP*2}}{\hat{g}_{C*-EP*2}}$

of N_{C*7} ($OCV_{C*\text{-}EP*1}$) varied for the 250 repetitions of the simulation between 0.03539 and 0.19116 (Fig. 1 f). The mean relative stereological sampling variance (i.e., $\overline{[CE^2_{N\text{–}C*X\text{–}EP*1}]}^{7}_{X=1}$ or $\overline{CE^2_{C*\text{–}EP*1}}$) was 0.00622; the 250 values obtained for the ratio $\overline{CE^2_{C*\text{–}EP*1}}/OCV^2_{C*\text{–}EP*1}$ (for details see below) varied between 17.0% and 496.4% (Fig. 1 g). Using the t statistic as proposed and demonstrated in [4] for calculating how many MD*s would have been to be examined to demonstrate that sample mean differences of 20% are significant at the 0.05 level (for details see below), the calculated number of MD*s ($n_{MD*\text{-}EP*1\text{-}min}$) varied between 2 and 8 (Fig. 1 h). Using EP*2, the arithmetic mean of the 250 estimates of $\hat{G}_{C*}$ was 2,983,913, which was 100.0% of $\hat{G}_{C*}$; 95% of these estimates were found within a range of approximately ± 3% around $\hat{G}_{C*}$ (Fig. 1 i). $OCV_{C*\text{-}EP*2}$ varied between 0.02533 and 0.11059 (Fig. 1 j). The mean relative stereological sampling variance (i.e., $\overline{[CE^2_{N\text{–}C*X\text{–}EP*2}]}^{7}_{X=1}$ or $\overline{CE^2_{C*\text{–}EP*2}}$) was 0.00158; the 250 values obtained for the ratio $\overline{CE^2_{C*\text{–}EP*2}}/OCV^2_{C*\text{–}EP*2}$ varied between 12.9% and 246.7% (Fig. 1 k). Using the t statistic [4] to calculate how many MD*s would have been to be examined to demonstrate that sample mean differences of 20% are significant at the 0.05 level (for details see below), the calculated number of MD*s ($n_{MD*\text{-}EP*2\text{-}min}$) varied between 2 and 5 (Fig. 1 l).

Using EP*1 for estimating the relative difference between the mean total numbers of neurons* of the HD* cases and the C* cases [i.e., $1 - (\hat{G}_{HD*}/\hat{G}_{C*})$], the estimates of this relative difference varied between 11.0% and 33.2% (Fig. 1 m). For 10 out of the 250 repetitions of the simulation – yielding estimated relative differences between $\hat{G}_{HD*}$ and $\hat{G}_{C*}$ of 11.0%, 14.2%, 14.3%, 15.6%, 17.5%, 18.8%, 18.9%, 19.7%, 20.3%, and 20.5% – this relative difference between $\hat{G}_{HD*}$ and $\hat{G}_{C*}$ was found to be not significant (i.e., $P > 0.05$; Mann-Whitney U-Test). Using EP*2, the estimates of the relative difference between $\hat{G}_{HD*}$ and $\hat{G}_{C*}$ varied between 18.1% and 28.9% (Fig. 1 n). For all 250 repetitions of the simulation the estimated difference between $\hat{G}_{HD*}$ and $\hat{G}_{C*}$ was found to be significant ($P < 0.05$; Mann-Whitney U-Test).

Discussion

In the following the results of the computer simulation will be discussed in the context of those methods that have been proposed and used in the literature for optimizing stereological estimation procedures in studies comparable to that carried out by Heinsen et al. [6]. For theoretical reasons these considerations are valid for estimates of total numbers of any biological particles (i.e., neurons, cells, synapses, etc.).

Recently, a method for deciding how many individuals are to be investigated in studies comparable to that carried out in [6] was proposed in a study evaluating the mean total number of synapses in the stratum radiatum of the hippocampal CA1 region of rabbits (henceforth abbreviated as synapses) [4]. The authors stated that differences of 20% between (i) the mean total number of synapses ($\hat{G}_x$) of a sample of rabbits (S_x) selected from a population P_X, and (ii) the mean total number of synapses ($\hat{G}_y$) of a sample of rabbits (S_y) selected from another population P_y would most likely have functional consequences. To decide how many individuals need to be examined per sample S_x and S_y to demonstrate that estimated sample mean differences between $\hat{g}_x$ and $\hat{g}_y$ of 20% are significant at the 0.05 level, the authors investigated a sample of five rabbits that were randomly selected from a population P_x. By counting approximately 250 synapses per individual on average, the authors found an estimated mean total number of synapses ($\hat{g}_x$) of 2.40×10^{10} with an observed coefficient of variation (OCV; see above) of 0.17 (i.e., an observed standard deviation of $0.17 \times 2.40 \times 10^{10}$). Using these data and the t statistic, and presuming the standard deviation among estimated total numbers of synapses of a sample of rabbits randomly selected from another population P_y also as $0.17 \times 2.40 \times 10^{10}$, the authors found a minimal number of eight individuals to be investigated per sample S_x and S_y (see [4], formula 3). However, when applying this method to the results of the computer simulation obtained with EP*1 and using the virtual C* cases as an example, the calculated number of MD*s supposedly to be investigated per sample varied between two and seven (Fig. 1 h). This was due to the fact that $OCV_{C*\text{–}EP*1}$ was a random variable varying in a broad range (Fig. 1 f). As both OCV and CV (see above) of samples of any individuals – when randomly selected from any population – are in principle random variables, the use of the t statistic as shown in [4] cannot serve as the basis for finding the minimal number of individuals to be investigated per sample S_x and S_y to demonstrate that estimated sample mean differences between $\hat{g}_x$ and $\hat{g}_y$ of a given magnitude are significant at the 0.05 level.

Another method has become the general basis for planning, performing and interpreting the results of stereological studies dealing with estimated total numbers of biological particles (i.e., neurons, cells, synapses, etc.) over the last decade (see, e.g., [4, 19, 20]. The essential aspect of this method consists in balancing random errors of estimated total numbers of particles (i.e., CEs) against interindividual variabilities of true total numbers of particles (i.e., CV_s) to the effect that an observed interindividual variability of estimated total numbers of particles (i.e., OCV) is mainly due to CV and not to CE. Based on the so-called 'analysis of variance for nested experimental designs' – details of which can be found in, e.g., [3, 10, 14] –, it is presupposed that an estimation procedure is appropriate when the ratio $\overline{CE^2}/OCV^2$ is smaller than 50%. For example, in the above-mentioned study [4], the authors found for the estimated mean total number of synapses of 2.40×10^{10} an OCV^2 of 0.17^2 and a mean predicted CE^2 of 0.089^2. As the ratio $\overline{CE^2}/OCV^2$ was approximately 28%, the authors characterized their estimation procedure as appropriate. However, the computer simulation demonstrates clearly that it is pointless to carry out such evaluations when only a limited number of individuals is investigated, as done in [6] as well as in most studies that have dealt with estimated mean total numbers of particles pub-

lished so far. This is due to the fact that the observed interindividual variability of estimated total numbers of particles (i.e. OCV) – and, thus, also the ratio $\overline{CE^2}/OCV^2$ – are random variables, that vary in broad ranges when investigating only a limited number of individuals (cf. [15]; see the results shown in Fig. 1 f, g, j, k). Note that using EP*1 and EP*2, the ratio $\overline{CE^2}/OCV^2$ was found both (i) smaller than 50% (supposedly indicating that the estimation procedures were appropriate) as well as (ii) greater than 50% (supposedly indicating that the estimation procedures were inappropriate). In consequence, it is not possible to decide on the basis of one value of the ratio $\overline{CE^2}/OCV^2$ whether or not an estimation procedure is appropriate. For details of the mathematical background of this important topic see, e.g., [3, 14].

Concerning the latter method, it is important to take into account that balancing of random errors of estimated total numbers of particles (i.e., CEs) against interindividual variabilities of true total numbers of particles (i.e., CVs) requires a precise prediction of CE. However, the predicting methods described in [17] or [20] underestimated the CEs of the virtual estimates of the total numbers of neurons* of the MD*s considerably (Fig. 1 d). This was not due to unrealistic results of the computer simulation but to a general invalidity of the predicing methods described in [17] or [20]. Obviously, it is beyond the scope of this study to offer the complete theoretical background of this topic. Nevertheless, a brief description will be given in the following. Both above-mentioned predicing methods are based on an adaption of the so-called 'transitive theory of regionalized variables' [9] to stereology as described in [5]. In terms of this theory the number of neurons* in a MD* may be interpreted as a so-called 'one-dimensional regionalized variable V defined on a domain D' (here, D may be interpreted as the reference volume of the MD*, and V is the number of neurons* in a given plane perpendicular to any given line L through D). If V is measured at any point x on L, the total amount Ω of V can be calculated according to the following formula (see also [18] formula 1):

$$Q = \int_D f(x)dx \quad \text{with} \quad f(x) = 0 \text{ if } x \notin D$$

For the use of the transitive theory of regionalized variables [9] to predict CEs of estimates of Q based on systematic random samples, it is crucial to analyze the structure of V and represent it globally by its covariogram (for details see [9, 18]). For theoretical reasons this covariogram must be modelled [9, 18]. It was an essential part in [5] to find a covariogram model for regionalized variables such as volumes of biological specimens or numbers of particles. However, the covariogram model given in [5] is just one among many possible, as pointed out in [18]. Furthermore, it was emphazised repeatedly that the covariogram model given in [5] may be invalid for regionalized variables such as numbers of particles contained in biological specimens, and that predictions of CEs of estimated numbers of particles that are based on this covariogram model are probably too low [1, 18]. Nothing other was meant to show with the computer simulation and the pilot experiment carried out in [6].

To summarize, the computer simulation has demonstrated that the use of EP*1 would not have guaranteed to estimate the difference between the true mean total number of neurons* of the HD* cases ($\hat{G}_{HD*}$) and the C* cases ($\hat{G}_{C*}$) as statistically significant ($P < 0.05$), although counting of all neurons* in all HD* cases and all C* cases would have resulted in just this finding. Furthermore, using EP*2 all repetitions of the simulation resulted in estimating the difference between $\hat{G}_{HD*}$ and $\hat{G}_{C*}$ as statistically significant. Based on these results it is reasonable to conclude that the estimation procedure used in [6] has guaranteed that counting of literally all neurons in all investigated MDs would also have resulted in finding a statistically significant nerve cell loss in the thalamic mediodorsal nucleus in Huntington's disease, whereas counting of only 100–200 neurons per MD would have not. As demonstrated here, this is due to the fact that the legitimation to count only 100–200 neurons per individual in previous stereological studies comparable to that carried out by Heinsen et al. [6] was not correct.

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