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Towards obtaining unbiased estimates of the total number of synapses in a brain region: problems of primary and secondary importance

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[Response to the letter of C. Schmitz ''Towards more readily comprehensible procedures in disector stereology'']

Our paper (Geinisman *et al.*, 1996) was intended to demonstrate that the hallmarks of meaningful quantitation (uniform three-dimensional sampling at *all* levels and in the *entire* organ or anatomical region) can be readily implemented to count synapses at the electron microscopic level in well defined regions of the nervous system. We explicitly state in the Conclusions of the paper (p. 817) that the paper reports two results which are of biological importance: ''the mean number of synapses in CA1 stratum radiatum of young female rabbits, $N(\text{syn}) = 2.40 \times 10^{10}$; and the observed variability of that estimate among five rabbits, $\text{OCV}_a[N(\text{syn})] = 0.17$'' (i.e., $\text{OSD}_\alpha[N(\text{syn})] = 0.41 \times 10^{10}$). The estimate of the mean is based entirely on unbiased estimation principles, and the *observed* standard deviation, OSD_a, is the only measure of variability in synapse number that is permissible for statistical comparisons of groups (e.g. with a *t*-test).

In the absence of any critique of these main points by Dr Schmitz, we assume that he agrees that the main objectives of our paper have been realized. He then raises a number of questions regarding estimation of sampling variances and the strategy that was used to optimize the sampling scheme. Before addressing Dr Schmitz's questions, it is important to point out that while calculations of the simple mean and standard deviation reported in the paper are based on elementary and very well known statistical principles, comparable principles do not exist for the calculation of sampling variances in *systematic*, uniform random designs. In practice, however, the absence of a rigorous theoretical basis for variance estimation in efficient, systematic designs is of little concern. The only reason for computing the variances at the lower levels of the

sampling scheme is simply to avoid having to work too much. In contrast, the reason for using unbiased principles for the estimation of the group mean and its variance is to produce reliable results that are biologically meaningful. It was emphasized in the paper that variances at the lower levels of the sampling scheme are of ''secondary interest'' (p. 817). We feel that the issues dealt with in the letter by Dr Schmitz are of secondary importance.

The estimation of sampling variances of *dependent* samples is a topic of continued research in statistics. At present, only rules of thumb are available, and they admittedly are not very well defined mathematically. Ordinary common sense and a bit of fantasy are still in fashion when it comes to evaluating systematic sampling designs. Therefore, most of Dr Schmitz's questions do not have unambiguous and straight answers. The approach used in our paper and the following comments are based on our opinions and experiences.

(1) Contrary to the statement of Dr Schmitz, recent stereological studies that report unbiased estimates of neuronal numbers (including those of West, 1993; 1994; West *et al.,* 1996) do not make general recommendations about how many neurons should be counted per disector and how many disectors should be used per individual. The recommendations that have been made are those for a pilot sampling scheme that could or should be adjusted after preliminary data have been collected. We believed that a sample size of a few hundred synapses per animal was a reasonable starting point for a pilot study of five animals to find out more about the data structure. The number of disectors should reflect the inhomogeneity of the tissue at the level of sections *and* the relative cost of disectors.

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The cost of an extra optical disector in light microscopic histological sections is the effort that it takes to push a button, whereas the cost of an extra physical disector pair of ultrathin sections is orders of magnitude greater. While we have deviated in this study from 100–200 neuron counts in 50–100 optical disectors per animal (suggested as a starting point for light microscopic studies), our strategy for designing the sampling scheme for counting synapses was based on exactly the same considerations as those used to design sampling schemes for estimating neuron number.

(2) There is no substantial difference between the estimates of $OCE_a[N]$ in our paper and that by West and Gundersen (1990), and a total uncertainty of 0.089 in an estimate of synapse number per animal cannot be considered ''relatively high''. The amount of bung (precision) one gets per buck (effort) depends on (i) the real biological variability of the primary sampling items (sections, blocks, stacks of disectors, or whatever) and (ii) how close one is to an optimal sampling scheme at other sampling levels. Different organs/animals/studies are therefore generally not directly comparable. If one, nevertheless, does compare quite disparate studies, the nicely inverse relationship of (number of sections, OCE_a): (21, 0.044), (13, 0.077), and (11, 0.089) quoted by Dr Schmitz does not contradict the above statement.

(3) We referred to the papers by Pakkenberg and Gundersen (1988), Brændgaard and colleagues (1990) and West and Gundersen (1990) as explicit examples of how to calculate parameters A, B and C that are entered in the formula for determining $Var[\Sigma a]$ (p. 813). It is true that previous stereological papers, with the exception of that by West and colleagues (1996), did not include consideration of the Nugget variance, hence the difference between Dr Schmitz's equations 3 and 4. The so-called Nugget-term in sampling variances is a complicated topic of statistical research that is still in progress. On behalf of Gundersen, Baddeley and Vedel-Jensen we may apologize for the fact that this particular piece of research is not finished yet.

(4) The approach of Cruz-Orive (1993) to the calculation of the variance in the Cavalieri volume estimate is less realistic, in our experience, than the one used in our study.

(5) The independent variable used to calculate the $OSD_f^2[\bar{Q}^-]$ was the mean number of synapses sampled with disectors in a counting field. It was stated in the text that ''the lowest level of synapse sampling was that of counting fields and not of disectors since synapses were sampled with five disectors from the same counting field'' (p. 815). In Table 3, the $OSD_f^2[\bar{Q}^-]$ was defined as ''the total intra-animal variance of $\bar{Q}^-$ among $n_f = 6$ counting fields''. This definition obviously involves the use of $\bar{Q}^-$ per counting field, not per disector. The reported $OCE_f[\bar{Q}^-]$ of 0.072 (Table 3) is the relevant estimate of the uncertainty with which the synaptic numerical density was determined in an animal. Unfortunately, the note to Table 3 contains an error: ''The variance of N_V was estimated, below the group sampling level, as variance of the mean number of synapses, $\bar{Q}^-$, counted per disector'' should have read ''counted per field''.

(6) *Estimates*, including those reported in our paper, are always random in the strict statistical sense. The paper describes a methodological *pilot* study in which there are rather few observations. Therefore, the estimates of variances, in particular among only five animals, must be interpreted with some caution. Note that irrespective of the large uncertainty in a pilot estimate of animal variability, it is much better than no estimate (and no other procedure for making the estimate is available). We are unaware of any rules that make it ''incorrect'' to use the only available estimate of a central quantity to optimize the design. Our methodological conclusion is simply that the sampling scheme, with only 28% of the overall variance due to subsampling, seems promising. As mentioned above, only the observed mean and observed total variance can be used in statistical tests. The estimate of the real biological variation in the number of synapses among rabbits was not of primary interest. Fortunately, nowhere in the editorial by Saper (1996) is there any mentioning of the prerequisite that an ''analysis of the estimation procedure used must guarantee and demonstrate that the $OCE_a[N]$ was much smaller than the real $CV_a[N]$'' as stated by Dr Schmitz. This is also fortunate because the statement cannot be true in general for *estimates*. The OCE_a may actually be *larger* than the OCV_a (of which there are several published cases).

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