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STEREOLOGICAL METHODS FOR QUANTITATING SYNAPSES

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Chemical transmission in the nervous system occurs between nerve cells at junctions called synapses. Morphologically, synapses can be recognized under standard transmission EM, using a variety of staining methods, by three distinctive features as shown in Figure 1: a) a presynaptic profile (A) containing synaptic vesicles clustered at the presynaptic membrane; b) a cleft of 150-300 Å in width separating the pre- and post-synaptic profiles; and c) a postsynaptic profile (B) with an area of increased electron density (between black arrows) or postsynaptic density. Within the last few years, changes in the number and complexity of synapses have been shown to occur in the cortex of animals raised in enriched environments (Turner and Greenough, 1983), and may underlie some of the deficits in learning and memory which occur during aging.

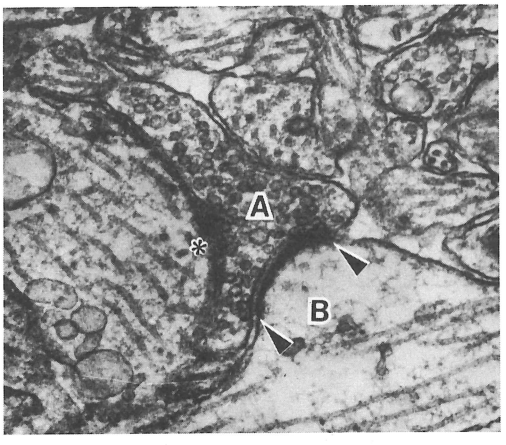

Figure 1. An axo (A)-dendritic (B) synapse in the optic tectum of the frog. An obliquely sectioned synapse (*) is also indicated.

We are interested in how the number of synapses is regulated in the CNS. We have studied the development of retino-tectal synapses in the optic tectum, the major nucleus receiving input from the retina in lower vertebrates,

in normal and in 3-eyed frogs in which a supernumerary eye primordium has been transplanted during embryogenesis. Common to these studies has been the need for an accurate estimate of the number of synapses in a volume of tissue. Because a structure as small as the tectum ($\simeq 10mm^3$) may contain 10^9 or more synapses, adequate sampling has been difficult to achieve using the common method of counting the number of synapses from photographs. In addition, there are complex gradients in the number of synapses present in the immature brain, making it necessary to sample large areas in a number of animals at different developmental stages.

For these reasons, we have developed rapid and efficient quantitative EM methods for estimating the number of synapses in a volume of tissue, and in a structure. In order to estimate the number of synapses accurately, three parameters must be known: the areal density (N_A) of synapses, the size of synapses (which requires an understanding of the 3-dimensional geometry of the synapse), and the volume of the tissue of interest. While N_A can be obtained using a variety of methods, we have developed a new method based on sampling theory which allows the number of synapses in a large area to be estimated accurately without the use of micrographs. The number of synapses within individual EM grid squares of known area is counted "on-line" at the microscope using a systematic serpentine sampling method. This method proved to be more efficient than a true random sampling. The number of synapses per EM viewing window (e.g., the fluorescent screen of the EM) is recorded and enough windows are sampled until the true number of synapses can be estimated from the sample mean within a confidence limit (α) set by the investigator, according to the formula given below:

$$n \geq \frac{N\left[s^2 + \left(\frac{\alpha M}{1+\alpha}\right)^2\right]}{s^2 + N\left(\frac{\alpha M}{1+\alpha}\right)^2} \qquad (1)$$

where n = number of windows sampled to estimate the true number of synapses present in an area within a confidence interval, set by α; N = total number of windows necessary to scan the entire grid square at a given magnification; s^2 = sample variance; α = confidence limit; and M = sample mean number of synapses per window.

The number N_A of synapses per grid square of area A is then given by $N_A = (\overline{M})(N)$, where $\overline{M}$ is the true mean number of synapses per

window, $N = A/W$ is the number of windows in A, and W is the area of a window at a given magnification. Enough grid squares are then sampled until the S.E. of the counts is reduced to an acceptable level determined by the degree of accuracy desired and the natural variation inherent in the tissue.

Control measurements and 3-dimensional reconstructions revealed that the great majority of tectal synapses are shaped like flat or slightly curved circular randomly oriented disks. Using this model, we derived an iterative subtraction method to calculate the number and size distribution of synapses from measurements of the lengths of postsynaptic densities seen in thin sections. Knowledge of the true size of synapses is important since the number and size of synapses may change independently.

Slicing synaptic disks of various sizes into portions which are visible in ultra-thin sections, has important consequences for determining the actual number of synapses present in a volume of tissue. First, not all of the synapses present in an ultra-thin section will be counted either because a) the orientation of the synapse is such that the angle between the plane of the section and the synaptic disk is not large enough to resolve a cleft between them (an "obliquely" sectioned synapse (*) is shown in Figure 1); or b) because the size of a portion of a synapse cut in a grazing section is too small to be resolved by the EM or recognized by the viewer. Finally, some synapses will overlap in adjacent sections since the average size of the synapse (as computed from serial reconstruction) is larger than the average thickness of an ultra-thin section. The following correction factors have been derived to correct for the effects of overlap, truncation and finite minimum recognizability:

$$F_t = \frac{\sqrt{r^2 - l_m^2}}{2r} + \frac{r\sin^{-1}}{2l_m}\frac{l_m}{r} \qquad (2)$$

is equal to the fraction of synapses appearing in section (i.e., whose profile in section exceeds a minimum recognizable length, l_m); r = radius of synapse (truncation correction factor);

$$F_o = \frac{2}{\pi t}(d - c) \qquad (3)$$

is equal to the expected number of sections a given synapse overlaps, where d= diameter of synaptic disk, c = width of synaptic cleft, and t = section

thickness (overlap correction factor); and

$$F_r = \frac{2}{\pi}\sin^{-1}\left(\frac{c}{t}\right) \quad (4)$$

is equal to the fraction of synapses whose membrane appositions are resolvable (orientation correction factor).

If the volume of the structure of interest is known, the total number of synapses N_T in a volume V_T is then given by:

$$N_T = (\overline{N}_A)(V_T/tF_tF_oF_r) \quad (5)$$

where $\overline{N}_A = N_A/A$ is the mean areal density.

Recently it has been shown that some large synapses contain perforations or holes and when such "complex" synapses are viewed in ultra-thin sections, they can be mistaken for two smaller synapses because a zone devoid of postsynaptic density material occurs where the perforation is present (deGroot and Bierman, 1983). We have treated the rare occurence of a single presynaptic profile making two appositions onto the same postsynaptic profile as a "complex" synapse. This distinction between a simple and complex synapse is facilitated by the requirement for a visible cleft in our strict definition of a synapse. Thus the techniques we have described should be equally applicable to systems in which synapses are simple (Figure 1) or complex.

Use of these techniques has allowed us to provide the first definitive evidence that the number of synapses formed by a given afferent system in the CNS is regulated by postsynaptic cells. Quantitative EM analysis of normal and hyperinnervated tecta in 3-eyed frogs indicates that the number of retinotectal synapses is dependent not on the number of retinal cells projecting to the tectum, but on some property of the postsynaptic tectal neurons (Norden and Constantine-Paton, 1982).

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