A stereological evaluation of synaptic diversity on spinal motoneurons in the rat

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Summary. We compare 2 methods ('planar' and stereological) for quantifying different types of synapse on the surface of spinal motoneurons. Stereological results suggest that the planar approach - wholly confined to counting synapses visible on electron micrographs - can produce valid data. Stereological results are also compared with estimates obtained by other techniques (light microscopy and serial microreconstruction).

The vertebrate spinal motoneuron is capable of integrating information from diverse afferents converging upon its surface. There is a corresponding variety in synapse morphology and topography and this may be related to known differences in their functioning[3-6]. Combined ultrastructural/morphometric studies of this synaptic diversity have been performed for several mammalian genera, including rat, cat and monkey[7-11]. Most have confined themselves to counts made directly on electron micrographs.

This 'planar' approach fails to allow for the fact that the number of synapses appearing on an ultrathin section is influenced not only by their real number but also by section thickness and by synapse size and shape[12]. Bigger synapses have a greater probability of being sectioned than smaller ones: accordingly, their relative frequency may be overestimated.

Though a previous investigation[7] has suggested that size alone may produce small systematic errors in the frequencies of occurrence of synapses, the combined biases introduced by size and section thickness have not been evaluated. As a prelude to quantifying rat ventral horn synaptology during development, we therefore decided on a preliminary study to assess what errors if any, might arise by neglecting the observed variation in synapse size[7-11] and section thickness.

For this purpose, a stereological model suitable for counting synapses[12,13] was applied to motoneurons in the cervical spinal cord of normal adult rats. In general, the frequencies of synaptic apposition zones appearing on electron micrographs were in good accord with stereological findings, i.e. planar estimates were not affected seriously by systematic errors.

Materials and methods. Details of preparative stages will be found in our earlier reports[14,15]. Briefly, 3 adult male rats from an inbred University of Sheffield strain were anesthetized with Nembutal and perfused with buffered aldehyde solutions. Cervical enlargements were excised and fixed overnight in perfusate. Each was sliced transversely at about 1 mm intervals and each slice divided into right and left sides which were trimmed to retain the most ventral, ventromedial and ventrolateral regions of grey matter. Tissue was postfixed in osmium tetroxide, dehydrated in graded ethanols and embedded in Araldite. Up to 6 tissue blocks per animal were selected at random. A single semithin section (about 1 µm) cut from each block and stained with toluidine blue provided a systematic random sample of 8 light micrographs, printed at a final magnification ×935. Altogether 96 micrographs were recorded and these were used to estimate the plasma membrane surface area of the average motoneuron soma, $\bar{S}_{ms}$. Full details of relevant stereological procedures are published elsewhere[15]. Profiles of motoneuron somata were identified on the basis of relative size, nuclear shape, cytoplasmic granularity and the presence of prominent aggregates of Nissl substance.

A similar strategy was employed to obtain a randomized sample of 150 micrographs from ultrathin sections (about 70 nm) using an AEI-Corinth electron microscope. These were taken from the same areas sampled by light microscopy and printed at a final magnification ×20,000. The pooled micrograph sample was used to estimate the numbers of different synaptic types at 3 topographical sites: soma, proximal and distal dendrite. Motoneurons, their dendrites and 5 morphological classes of synapse (designated S, F, C, T and M) were identified using established criteria[5,7,11,16]. The term 'synapse' is here synonymous with 'total apposition zone of pre- and post-synaptic membranes'[12]. This constituted the basic unit of measurement, though boutons on which these synapses were differentiated were classified primarily on the basis of vesicle size and shape and on the nature of paramembrane specializations. The term 'distal dendrite' refers to dendrites found in the immediate vicinity of motoneuron somata but apparently not projecting from them. Some of these may belong to interneurons. We compared synaptic frequencies calculated by 2 methods. The 1st (planar) method merely involved counting synaptic apposition zones appearing on a constant reference area of each electron micrograph. A total of 900 appositions was counted. For the 2nd method, these and other planar measurements were converted to stereological parameters using appropriate formulae[12,13]. In this case, measurements of synaptic appositions were made by overlaying each micrograph with a simple quadratic test lattice of spacing 1 cm. The main parameter estimated was N_S, number of synaptic apposition zones per unit of postsynaptic membrane surface[12]. Synapses in a given class were considered to be flat circular discs of uniform size. Though not universally appropriate, this is a reasonable working approximation[12,17,18]. Values of N_S for each type of synapse were calculated from numbers of appositions per unit length of soma or dendrite plasma membrane (N_B) by the relation

$$N_S = N_B/((4/\pi)(\bar{B}+t))$$

where $\bar{B}$ is the average length of synaptic apposition sites of

Table 1. Comparison of 'planar' and stereological estimates of synaptic frequencies on the motoneuron surface

Synapse Type	Frequency of synaptic types by: Planar approach No.	%	Stereological approach N_S	%
A Soma				
S	154	38.4	10.6	42.0
F	238	59.4	14.4	56.8
C	9	2.2	0.3	1.2
Total	401	100	25.3	100
B Proximal dendrite				
S	72	42.4	10.3	42.7
F	92	54.1	13.4	55.5
C	5	2.9	0.3	1.2
M	1	0.6	0.1	0.5
Total	170	100	24.1	100
C Distal dendrite				
S+T	197	59.9	14.4	59.4
F	132	40.1	9.8	40.6
Total	329	100	24.2	100

No., number of apposition zones on the total micrograph sample; N_S, number of synapses per 100 µm² of postsynaptic membrane. Where a synaptic type is not listed, it was not observed in the present samples.

a given class seen on sections of thickness t. It is this factor ($\bar{B}+t$) which is directly proportional to the probability of sectioning synaptic 'discs'. Moreover, $\bar{B}$ is related to disc diameter ($\triangle$) by the formulation $\triangle = (4/\pi)\bar{B}$. All lengths were estimated in this study by counting intersections between membrane images and test lines[12,13].

Finally, numbers of synapses per average soma were calculated by multiplying relevant estimates of N_S (obtained from ultrathin sections) and $\bar{S}_{ms}$ (from semithin sections).

Results and discussion. Results for the comparison of methods are summarized in table 1. Values are given for synapses of different type and at different localities.

The 2 methods produced remarkably similar results. This suggests that systematic errors arising from synapse size variation and finite section thickness have only minimal influence on the relative numbers of synaptic appositions visible on electron micrographs. More detailed analysis reveals a differential effect on synapses of differing diameter. We estimated relative biases inherent in the planar approach in terms of the magnitude and direction of departure of planar from stereological data. It must be emphasized that these biases do not represent departures from the 'true' synaptic frequencies which are, of course, unknown. We found that S and F types (mean diameter 1.7–2.0 µm) had small relative biases of less than ± 9% but these biases were much greater (+ 83–142%) for the larger C synapses which have a mean diameter of 3.7–4.8 µm, the ranges reflecting the variation between topographical sites. As expected, therefore, the frequencies of occurrence of larger synapses tended to be overestimated. For this tissue at least, the overestimation was of little practical consequence when balanced against the overwhelming preponderance of S and F synapses which together accounted for more than 90% of total afferents projecting to the motoneuron surface.

These findings lend support to, and extend, results obtained for motoneurons at lower spinal levels[7] and for which biases of + 6–9% (F type), − 12–18% (S + T) and + 117–140% (C) may be computed. However, one should be wary of the planar approach when studying synapses in other regions of the CNS where the proportions of large and small synapses may differ from those on spinal motoneurons and where, in consequence, the biases may produce misleading information.

Previous investigations[5,7–11] have indicated also that F synapses tend to predominate on the motoneuron soma and proximal dendrites and S synapses on more distal parts of the dendritic tree. There is also evidence[6] that F synapses are inhibitory and employ glycine as neurotransmitter, whereas S, T and M synapses are expected to be excitatory. The preferential localization of synaptic types witnessed here is compatible with current ideas on the distribution of inhibitory synapses based on electrophysiological results[3,19]. Though one might conclude that stereology offers no benefits when quantifying frequencies in the present model, the technique is certainly preferable to the planar approach for studying other aspects of synaptic organization. For instance, it permits calculation of absolute data characterizing the synaptic complement of the average soma. Table 2 provides mean diameters and numbers of axosomatic synapses on the average motoneuron. The cell body (surface area 2900 µm^2) was estimated to bear some 730 synapses, the majority inhibitory. This estimate may be compared with one of 870 for Betz cell bodies in cat cerebral cortex[18]. Our value of total synaptic density (24–25 per 100 µm^2) is encouragingly close to figures obtained by other techniques. Combined light microscopy and silver techniques[20,21] have furnished density estimates of 15–20 per 100 µm^2 of cat motoneuron surface, some of which may be too low because of technical caprice[20] or inadequate resolution. Serial reconstruction studies have indicated values of 17–22 per 100 µm^2 of dendritic surface for 1 cat motoneuron[8] and 5–8 per 100 µm^2 of soma for 2 neurons in the lateral geniculate nucleus of the rat[22]. These figures are based on analysis of very few cells and this reflects the technical demands of serial sectioning.

On the assumption that total dendritic surface accounts for 80% of the total receptive surface of motoneurons[23], the average rat spinal motoneuron in this study would have a dendritic surface of some 11,500 µm^2 and a total surface of 14,500 µm^2. The complement of afferents on the total receptive area would then be in the order of 3500. This figure is not inconsistent with a light microscopic estimate of more than 2000 per cell[21] or with one of 16,000 for a cell of 80,000 µm^2 surface area[23], both being for cat motoneurons. We conclude that stereology provides a powerful alternative tool for studies in this area of quantitative neurocytology as in others. Indeed, it offers several advantages over serial sectioning and light microscopic techniques. It is superior to both in terms of the sample size which can be measured in a reasonable time and to light microscopy in terms of the resolution it affords and the accuracy with which synaptic counts may be made.

Table 2. Mean diameter and number of synaptic 'discs' on the surface of the average motoneuron soma ($\bar{S}_{ms}$ = 2886 µm^2)

Synapse type	Mean diameter, µm	Number/soma*
S	1.73	307
F	1.99	416
C	3.68	9

* Results expressed to nearest whole number. Total number of all types of synapse = 731.

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