

## **Analysis and estimation of synaptic densities and their spatial variation on the motoneuron surface**

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In order to understand the integration of the inputs to a motoneuron, detailed knowledge is required of the location and numbers of the various types of synapses on the entire somatodendritic surface<sup>1</sup>. Such knowledge permits the testing of hypotheses made from the results of electrophysiological investigations<sup>3,14,15</sup> and is helpful in establishing estimates of numbers of connections made between cells of one population and another.

Earlier quantitative studies of the synaptology of cat spinal motoneurons have led to widely differing estimates of the total numbers of synapses or boutons. In a light microscopic study<sup>19</sup>, this number was estimated at 2000; another reference placed the number between 2000 and 20,000<sup>13</sup>. A summary of many early estimates of synaptic density was compiled by Illis<sup>6</sup>. It is expected, of course, that some variability among these studies is due to different locations of cells in the cord and different sizes and ages of animals investigated. Nevertheless, it should be possible to refine these estimates further in the light of recent experimental findings, as we shall show.

An extensive electron microscopic examination of cat spinal motoneurons has been made by Conradi<sup>5</sup>, and a detailed light microscopic examination of the soma and dendritic trees of several spinal motoneurons in the same region of the cord has been made with iontophoretic injection of Procion dye by Barrett and Crill<sup>2</sup>. Barrett<sup>1</sup> has combined the results of these two studies to estimate the total number of synaptic endings as between 20,000 and 50,000. We shall improve upon Barrett's estimate upon adopting a simple model for the distribution of boutons and then using this model to estimate numbers of the various types of boutons per unit area (densities). Our adoption of this model is in part justified by the excellent agreement between predictions based on it and the corresponding experimental findings of Conradi<sup>5</sup>. With this model, we shall also investigate variations in synaptic densities with regard to distance from the cell body (which is held to be an important anatomical variable

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— Conradi, private communication), thereby obtaining estimates of the total numbers of the various types of boutons on various parts of the motoneuron surface.

In the detailed electron microscopic examination<sup>5</sup>, 20 motoneurons in the L7 region of the spinal cords of adult cats were examined in transverse sections. The regions of the neuron are named SO (cell body), DA (defined as that part of the dendrite such that, counting from the cell body, 20 boutons were encountered on one side of the section), and DB (more distal than DA). In addition, 30 dendritic fragments in the neuropil were examined; these were labelled 'd'. The origins of these fragments could not be traced to any given cell body, but it was inferred that many of these fragments probably belonged to motoneurons. Boutons were classified according to the morphology of their synaptic complexes as: (i) S, containing spherical vesicles; (ii) T, containing spherical vesicles, and with postsynaptic dense bodies; (iii) F, containing flattened vesicles; (iv) FD, containing flattened vesicles, with dense bodies and large synaptic cleft; (v) C, containing spherical vesicles with a subsynaptic cistern (vi) M, large boutons containing spherical vesicles, and with postsynaptic dense bodies. (Data on P type (presynaptic) boutons were not presented at length in ref. 5, so will not be considered here.) For each region, various data, including total membrane length and counts for each type of bouton, were given. In order to obtain a sharper classification of distances from the cell body, we subdivided the DA data into SDA (short DA, with membrane length less than 100  $\mu\text{m}$ ) and LDA (long DA, with membrane length greater than 100  $\mu\text{m}$ ). Data on this latter group are weighted in the direction of DB, so that in particular the average distances of its boutons from the cell body are greater than that of the SDA group.

It would be misleading to compare directly the numbers of each type of bouton across the different locations, because the membrane lengths are unequal. Accordingly, we obtained for each location and for each type of bouton are score of the number of boutons per unit length, which we denote  $m_S$ ,  $m_T$ ,  $m_F$ ,  $m_C$  and  $m_M$ . (The subscripts denote each type of bouton. We follow Conradi's convention in that F and FD boutons are counted together, as are M and big S boutons.) The total number of boutons per unit length is designated  $m_{TOT}$ . We did not wish to make any parametric assumptions concerning the underlying probability distributions of these scores for each bouton type; nevertheless, a well-known statistical procedure, the Kruskal–Wallis test<sup>10,17</sup>, can be used in the present situation to determine for each bouton type whether the scores remain constant, regardless of the locations of the membranes on the motoneuron surface.

The Kruskal–Wallis test were performed on the  $m$  counts using a BMD statistical routine furnished by the Health Sciences Computing Facility of UCLA, as implemented on the University of British Columbia IBM-370 computer system. The values of the test statistics are reported in Table I. Under the null hypothesis that the scores remain constant regardless of location, the test statistics are approximately distributed as chi-squared random variables with 4 degrees of freedom. The corresponding levels of significance are also given in Table I. It is clear that the scores are not at all constant relative to location with respect to the cell body. For the individual bouton types the size distributions are not significant here. However, the result for

TABLE I

*Test statistics and levels of significance of the Kruskal–Wallis and trend tests of the constancy of the numbers of boutons per unit length over the various locations*

<i>Bouton type</i>	<i>K.–W. test statistic</i>	<i>P value</i>	<i>Trend test statistic</i>	<i>P value</i>
S	26.9682	0.0000	3.574	0.0004
T	17.3708	0.0016	3.696	0.0002
F	28.1943	0.0000	0.904	0.3660
C	35.3096	0.0000	—4.666	0.0000
M	14.8480	0.0050	0.252	0.8088
Total	30.3933	0.0000	4.305	0.0000

$m_{TOT}$  contains a small departure from the true situation, because the probability of observing a large bouton is greater than that of observing a small one. Still, the  $P$ -value is such that this source of error is likely insignificant. The size distributions will be taken into account later.

The hypotheses of the constancy of the  $m$  scores over the various locations being rejected, it is of interest to detect the presence of either increasing or decreasing linear trends in these scores as distance from the cell body increases. We cannot infer from the Kruskal–Wallis test results whether these alternatives are indeed true. However, a simple modification of the Kruskal–Wallis procedure specifically designed to detect trend is available<sup>9</sup>. The test statistics for detecting linear trend are also given in Table I; these statistics have standard normal distributions if trend is not present, so that large positive values indicate increasing trend, large negative values, decreasing trend. The  $P$ -values are also reported.

We conclude from these results that the  $m_S$  and  $m_T$  values increase, and the  $m_C$  values decrease with increasing distance from the cell body. For  $m_F$  and  $m_M$ , there is no significant *linear* trend in their scores; this does not preclude the possibility of, say, a quadratic trend. The total counts,  $m_{TOT}$ , show a positive linear trend. If the distributions of the various bouton types obtained by considering the average (ranked) scores determined by the Kruskal–Wallis procedure are graphed, the trends are readily discernible.

It was realized by Conradi<sup>5</sup> that bouton counts obtained from single sections may not give a complete quantitative picture of the distributions of the various types of boutons. Thus, in estimating the true proportions of each type of synaptic endings, the different mean sizes (lengths of apposition) were taken into account, because, as Conradi reasoned, smaller boutons were less likely to be encountered. There is an additional factor that also ought to be taken into account, namely, the spacing between like boutons: boutons which are more widely separated have less chance also of being encountered in single sections. This factor can be incorporated in the following, albeit simple, way. (It is realized that the problem of considering randomly distributed boutons of varying sizes and shapes is difficult; nevertheless, the following

TABLE II

*Estimated expected numbers of boutons of various types/sq.  $\mu\text{m}$  at various locations on the motoneuron surface*

<i>Bouton density</i>	<i>Location</i>				
	<i>SO</i>	<i>SDA</i>	<i>LDA</i>	<i>DB</i>	<i>d</i>
$n_s$	0.0479	0.0697	0.0784	0.0813	0.0778
$n_T$	0.0014	0.0030	0.0028	0.0071	0.0076
$n_F$	0.0614	0.0946	0.0909	0.0936	0.0676
$n_C$	0.0034	0.0043	0.0027	0.0016	0.0003
$n_M$	0.00006	0.0004	0.0010	0.0006	0.0001
$n_{TOT}$	0.1142	0.1720	0.1758	0.1842	0.1534

model agrees quite well with experimental evidence. For a different approach, see Palkovits<sup>12</sup>.)

Over reasonable distances, the dendrites in a small volume of nervous tissue are approximately cylindrical. Then, the surface of a dendrite of reasonable length may be projected onto a strip, as illustrated by Conradi (ref. 5, Fig. 4 therein). Boutons counted on a single sections will lie along lines parallel to the axis of the cylinder if the section follows the dendrite for a considerable length. Given a large number of sections, we may imagine that their corresponding strips are placed alongside each other to form a planar region. To obtain a quantitative picture of the synaptic densities, we can picture the average bouton arrangement to correspond to boutons occurring a lattice points on the plane. This lattice will in reality be random; still, we find that an average regular lattice model predicts densities extremely well.

To translate this picture so as to relate to experimental measurements, we suppose that a particular type of bouton has mean radius  $r$ , and that the average spacing between nearest neighbors is  $k$  (with  $2r \leq k$ ). Then the probability of encountering a bouton with a line perpendicular to the grid lines of the lattice is  $2r/k$ . The expected or average number of boutons per unit length will therefore be

$$\bar{m} = 2r/k^2.$$

Moreover, the expected number of boutons per unit area, the synaptic density, is

$$n = 1/k^2.$$

Since the experimental results of Conradi include the values of  $\bar{m}$  for the various bouton types, we can find  $k^2$  by using for the value of  $r$ , one-half of the reported mean length of apposition of the boutons. This procedure can also be adopted for the cell bodies.

The values of  $r$  were obtained from the tables of Conradi<sup>5</sup>; note that the standard errors of these mean values are very small because of the relatively large sample sizes. (For SDA we took the same values of  $r$  as reported for all the DA data.) The reported  $m$  values were used in conjunction with the  $r$  values to find the mean spacings,  $k$ ; the corresponding densities  $n_s$ ,  $n_T$ ,  $n_F$ ,  $n_C$ ,  $n_M$  and  $n_{TOT}$  were then obtained at various locations on the motoneuron surface. The numerical values so obtained are given on

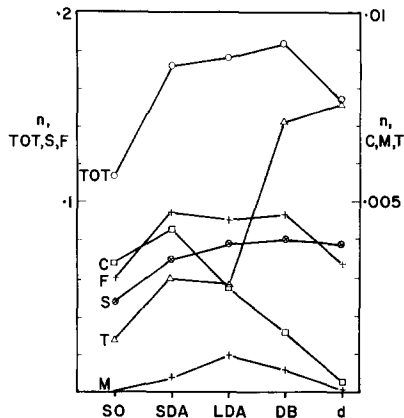


Fig. 1. Estimated bouton densities at various locations on the motoneuron surface.

Table II. We may remark that shrinkage and swelling, if they affect both postsynaptic and presynaptic membranes about equally, will not affect these densities.

To test the reliability of this simple procedure for estimating densities from single section data, we compared predictions based on this model with the values obtained by Conradi<sup>5</sup> in his thorough quantitative study by serial sections of a restricted part of a proximal dendrite of a motoneuron. This part of the surface corresponds with the region that we have denoted SDA. In the experimental study of region A (Conradi's designation), the total density of boutons was  $0.175/\text{sq.}\mu\text{m}$ , whereas our predicted value is  $0.172/\text{sq.}\mu\text{m}$ , an error of only 1.7%. Our estimated proportions of each type of bouton are as follows, with corresponding experimental proportions in parentheses: S + T, 42.26% (41.5%); F + FD, 55.00% (56.5%); C, 2.5% (1.5%); M + big S, 0.24% (0.5%). The expected proportions of each type of bouton at the various locations were calculated from the estimated densities of Table II. A statistical test of the null hypothesis that the proportions do not differ across the different locations yields a chi-squared value of 23.422 with 8 degrees of freedom, corresponding to a *P* value of 0.0029. We conclude that the proportions are decidedly different.

The estimates of the densities for each type of bouton are plotted against distance from the cell body via the classification SO, SDA, LDA, DB and d in Fig. 1. A similarity was seen to the trends for the numbers of boutons of each type per unit length. This supports the assertion<sup>5</sup> that data on bouton counts in single sections can give a reasonably accurate picture of the relative numbers of the various bouton types. Nevertheless, allowance should be made for both the sizes and the mean separation of the boutons.

From Fig. 1 it can clearly be seen that the total density of boutons,  $n_{TOT}$ , is minimal on the motoneuron cell body, reaches a maximum in the region DB (approximately 100–150  $\mu\text{m}$  from the cell body), then declines on thin dendrites. The density of S boutons slowly increases from the cell body towards distal dendrites; for F boutons, a peak is apparent on proximal dendrites. Significant spatial variation

TABLE III

*Mean numbers and standard deviations for the numbers of boutons on lumbosacral cat motoneurons estimated from electron microscopic and light microscopic data*

Location	Bouton type					
	S	T	F	C	M	Total
SO	569 ± 226	17 ± 7	729 ± 289	40 ± 16	1 ± 4	1356 ± 538
SDA	923 ± 366	40 ± 16	1252 ± 497	57 ± 23	5 ± 2	2277 ± 904
LDA	930 ± 369	33 ± 13	1079 ± 428	32 ± 13	12 ± 5	2086 ± 828
DB	968 ± 384	84 ± 33	1114 ± 442	19 ± 8	7 ± 3	2192 ± 870
d	7455 ± 2959	728 ± 289	6477 ± 2571	29 ± 12	10 ± 4	14698 ± 5835
TOT	10845 ± 4304	902 ± 358	10651 ± 4227	177 ± 72	35 ± 14	22610 ± 8975

also occurs for the other bouton types. Type T boutons undergo a dramatic increase in density; contrast this with type C boutons, which undergo a large decrease in density as distance from the cell body increases, after a slight increase on SDA. Type M boutons have a well-defined maximal density on the region we have called LDA, decreasing in both distal and proximal directions.

We can combine our estimates of the densities of the various boutons with the data on the sizes of motoneuron cell bodies and dendrites obtained in the detailed light microscopic examination<sup>2</sup>, as the neurons in both studies were at similar locations in the cord. From data on a typical motoneuron furnished by Barrett (private communication), the percentages of the motoneuron surface area corresponding to SO, SDA, LDA, DB and d were estimated. The mean surface area of the motoneurons (lumbosacral) investigated by Barrett and Crill was 144,690 sq.  $\mu$ m, with a standard deviation of 57,425. In Table III we show the expected numbers and standard deviations for each bouton type at the various locations of the motoneuron surface, obtained by using our estimates for the densities with the surface area data.

The total predicted number of boutons is 22,610; this is considerably higher than most earlier estimates, and probably reflects the fact that the recent staining technique reveals more of the dendritic tree than had been seen previously. The estimated total number of S, T, C and M boutons is 11,959; the corresponding F bouton figure is 10,651. Since the former group contained rounded vesicles and the F types flattened vesicles, then, if the hypothesis that vesicle shape correlates with the nature of the synapse is tenable, we see that the total number of excitatory connections is about equal to, but possibly slightly greater than, the number of inhibitory connections to a motoneuron.

The M type boutons are believed, on the basis of dorsal root sectioning, to belong to Ia afferent monosynaptic pathways. Our estimate of the expected total number of such boutons is 35, with a standard deviation of 14. This is compatible with a previous estimate<sup>1,8</sup> of 10–20, if account is taken of the greater portion of the dendritic tree being observed in more recent light microscopic studies. The small number of Ia monosynaptic endings has been questioned on the basis of the strength of the excitation which results from stimulation of Ia afferents<sup>7</sup>.

It is interesting to ask how many terminals each Ia fiber supplies to a motoneuron<sup>8,11</sup>. The numbers of Ia fibers from medial gastrocnemius, lateral gastrocnemius and soleus muscles have been reported as 62, 35 and 56 respectively<sup>4</sup>. The neurons in Conradi's study<sup>5</sup> were in L7, where there is a large proportion of lateral gastrocnemius motoneurons<sup>16</sup>; Barrett and Crill's study<sup>2</sup> included motoneurons from the medial gastrocnemius and neighboring nuclei in the lumbosacral cord. Bearing in mind that there is overlap of the various columns of motoneurons which supply particular muscles, and that Ia afferents from a muscle form connections, with practically all the motoneurons innervating that muscle<sup>11</sup>, we see that the most likely number of terminations of a Ia fiber on a motoneuron is one, and certainly not greater than two. This is precisely the range of values given by another study<sup>8</sup>, and is less than that quoted by other workers<sup>11</sup>.

The distribution of Ia endings on the motoneuron surface was deduced from electrophysiological investigations<sup>8</sup> to be greatest at about 0.4 space constants from the soma. This agrees very closely with our estimate that places the greatest density of M type boutons as being on the region LDA, which is between approximately 50 and 100  $\mu\text{m}$  from the soma. The spatial variation of the density of M boutons corresponds very closely with the predicted variation in density of Ia synapses with increasing electrotonic distance from the soma. The distributions of the other types of boutons raise several questions as to the origin of their preterminal axons. The Ia inhibitory disynaptic pathway and recurrent collateral pathways via Renshaw cells have been postulated to have endings preferentially located on proximal dendrites, with the recurrent inhibitory postsynaptic potentials generated somewhat more distally<sup>3</sup>. The only type of synapse which fits this scheme is that of the C type bouton; this type has an expected number of 177, with a standard deviation of 72. The C type boutons had, however, spherical vesicles, so that, if one adheres to the classification of synaptic type according to vesicle shape, then the hypothesis that C types belong to these inhibitory synapses must be abandoned. The T type boutons show a definite increase with distance from the cell body, and have an expected number of 902 (S.D. 358). It would be interesting if the nature of these inputs could be established, as they represent a significant proportion of the total number of boutons.

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