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Methods of estimation of spine density – are spines evenly distributed throughout the dendritic field?

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INTRODUCTION

Dendritic spines are small finger-like protrusions, often terminating in bulbous expansions, which extend from the dendrites of many types of neurons. They vary in length between 0·5 and 4·0 μm depending on their type; the bulbous spine heads measure on average 0·6 × 1·4 μm (Peters & Kaiser-Abramof, 1970). Their function is much debated (Swindale, 1981; Crick, 1982; Gray, 1982) but they appear to be postsynaptic structures in that the majority of axons that form synapses with pyramidal cells do so on the spinous processes (Gray, 1959; Colonnier, 1968; Feldman & Dowd, 1975).

Spine density is the number of dendritic spines per micrometre of dendrite. Until quite recently these measurements were made by counting the number of visible spines along a designated length of dendrite which was estimated using an ocular micrometer. This method does not take into consideration the 'true' dendritic length since dendrites are rarely perfectly straight. A second problem is that the estimate only accounts for those spines which are visible due to their lateral extension from the dendritic shafts. The remaining spines, which probably constitute the majority of the total, are not included because they are obscured by the opacity of the Golgi-impregnated dendritic shafts. Assuming that the distribution of spines is similar throughout the dendritic field, the number of spines invisible to the counter will vary with the diameter of the dendrite. Therefore spine density estimates based on visible spine counts represent serious underestimates of the total number of spines (Chan-Palay, Palay & Billings-Gagliardi, 1974; Feldman & Dowd, 1975; McConnell & Berry, 1978). Feldman & Peters (1979) suggested that the magnitude of the underestimate correlates positively with dendritic shaft diameter and negatively with spine length. In an attempt to overcome the restrictions on the value of spine density estimates Feldman & Peters (1979) devised a geometrical equation (see Scheme 1) to take account of these shortfalls and to produce 'true' spine density estimates which they tested and declared to be accurate to within 10% of the actual spine numbers.

In this study, spine densities recorded at various loci on hippocampal (CA1) pyramidal cell dendrites are compared using both visible counts (Method 1) and the correction formula for dendritic diameter and spine dimensions (Method 2). The differences in recordings between Methods 1 and 2 are discussed in the light of the different dendritic diameters at each locus. The choice of method used in estimating spine density in quantitative studies is evaluated, together with the advantages and disadvantages of both assessments.

Scheme 1. Geometrical equation derived by Feldman & Peters (1979) for estimating 'true' spine numbers and equations for spine densities 1 and 2.

$$N = \frac{n\pi[(Dr+Sl)^2-(Dr+Sd)^2]}{[\theta/90\pi(Dr+Sl)^2]-2[(Dr+Sl)\sin\theta(Dr+Sd)]}$$

n/Dl = spine density 1.
N/Dl = spine density 2.

n, number of visible spines; Dr, dendritic radius; Dl, dendritic length over which spines are counted; Sl, spine length; Sd, diameter of spine head; N, estimate of 'true' total spine numbers as derived by equation.

MATERIALS AND METHODS

The material used for this study consisted of 120 μm coronal sections (n = 20 per rat) of rat hippocampus which had been perfusion-fixed with paraformaldehyde/glutaraldehyde mixtures under sodium pentobarbitone anaesthesia (Horner, O'Regan & Arbuthnott, 1991). The brains of 15 rats from three experimental groups (5 per group), which included an unhandled control group, a saline-injected control group and a drug-injected treatment group, were used. Blocks of hippocampus from each animal were impregnated and stained, using a modified Golgi–Kopsch technique (Riley, 1979), shelled in wax and sectioned at 120 μm.

Dendrites of CA1 pyramidal cells were viewed at $\times 10$ and $\times 40$ magnification. Those which were clearly visible with no excess precipitate and which were relatively straight were used for assessment of spine density at $\times 100$ oil immersion magnification. Segments of apical (50 μm), basal (25 μm) and oblique (25 μm) dendrites were used for calculation of spine density (Horner *et al.* 1991).

Spine density 1, which is based on visible spines only, was determined by counting all apparent spines along the segments of dendrites chosen and estimating the dendritic lengths with an ocular micrometer. Twenty recordings each, from different cells, for each particular locus, per animal were taken. Therefore 100 estimates of apical, basal and oblique spine densities 1 per experimental group were recorded.

On the same dendrites chosen for spine density 1 estimates, a further series of measurements including dendritic diameter, length of a typical spine, the diameter of the spine head and the specific length over which the visible spines were counted, were recorded using a digital mapping pen of a semiautomatic image analyser (Kontron Videoplan). These measurements were applied to the formula devised by Feldman & Peters (1979) and a 'true' estimate of spine density – spine density 2 – was calculated for 20 dendrites per locus per animal in each group. The group mean spine densities derived using Methods 1 and 2 were quantified and compared.

RESULTS

A total of 900 values of both spine density 1 and spine density 2 were recorded, 300 per experimental group. Although the values of spine density may vary between experimental groups, the ratio of spine density 2 to spine density 1 should be the same, regardless of treatment group, as the recordings for both methods were based on the same dendritic segments in the same cells in the same animals. Therefore the three experimental groups were used purely to provide greater numbers and to verify the consistency of the ratio.

Mean values of spine density 1 (visible spines/μm) and spine density 2 ('true' estimate), and the standard deviation of the means, are given in Table 1. In the

Table 1. *Mean spine densities* (±S.D.) *for apical, basal and oblique dendrites in drug-treated, saline-injected and unhandled control groups assessed by Method* 1 (*visible spine counts*) *and Method* 2 (*geometrical equation*).

	Drug	Saline	Control
Apical dendrites			
Spine density 1	1·27±0·33	1·15±0·30	1·07±0·25
Spine density 2	4·83±1·18	4·33±1·23	4·05±0·90
Basal dendrites			
Spine density 1	1·78±0·24	1·67±0·22	1·28±0·19
Spine density 2	5·71±0·75	5·17±0·69	3·97±0·70
Oblique dendrites			
Spine density 1	1·99±0·23	1·91±0·25	1·25±0·21
Spine density 2	6·30±0·76	5·77±0·80	3·87±0·56

Table 2. *Ratios of spine density* 2 *to spine density* 1 *for apical, basal and oblique dendrites in three experimental groups*

	Drug	Saline	Oblique	Group mean
Apical	3·80	3·77	3·79	3·79
Basal	3·21	3·10	3·08	3·13
Oblique	3·17	3·02	3·10	3·10

control group the spine density values are greatest for basal and lowest for apical dendrites, using visible spine counts. The correction formula (spine density 2) gave the greatest value on apical and the smallest value on oblique dendrites. This appears more realistic in that apical dendrites, having the greater diameter, may be expected to have more spines. However, these corrected spine density values do not reflect the differences in dendritic diameter between apical (2·6 μm), oblique (0·9 μm) and basal (1 μm) dendrites. Each value of spine density 1 recorded at each locus in the control animals correlates well with those recorded by Riley & Walker (1978) for basal dendrites, Rutledge, Duncan & Cant (1972) for apical dendrites and Feldman & Dowd (1975) for oblique dendrites. Therefore they appear to be reasonable estimates for the basis of this study. The saline and drug-injected groups have larger spine densities on oblique dendrites and the smallest values recorded were on apical dendrites using both Methods 1 and 2. The fact that the spine numbers do not necessarily relate to dendritic diameter in these groups is due to the experimental procedures (Horner *et al.* 1991). The results of spine density 2 are substantially greater than spine density 1 (Table 1). This is expected, in that it has taken into consideration the diameter and tortuosity of the dendrites and the possibility of visualising the spine by including both its length and head diameter. Ratios of spine density 2 to spine density 1 for each locus in each treatment group are given in Table 2. Apical spine density 2 was 3·8 times greater than spine density 1 for each treatment group. Differences in the ratio between experimental groups were negligible. Basal spine density 2 was on average 3·1 (3·08–3·21) times greater than spine density 1 as was the ratio of oblique spine density 2 to 1 (3·02–3·17).

Table 3. *Mean dendritic diameter (±S.D.) of apical, basal and oblique dendrites in each experimental group*

	Drug	Saline	Control
Apical	2·71±0·47	2·51±0·49	2·61±0·59
Basal	0·94±0·16	0·93±0·15	1·02±0·21
Oblique	0·90±0·15	0·90±0·15	0·89±0·16

Table 4. *Mean spine length (±S.D.) and diameter of the spine head (±S.D.) of typical spines located on apical, basal and oblique dendrites in each experimental group*

	Drug	Saline	Control
Spine length			
Apical	0·95±0·29	0·95±0·29	0·95±0·25
Basal	0·85±0·14	0·87±0·14	0·88±0·18
Oblique	0·83±0·11	0·85±0·13	0·80±0·13
Diameter of spine head			
Apical	0·55±0·17	0·55±0·17	0·55±0·16
Basal	0·54±0·11	0·54±0·09	0·53±0·13
Oblique	0·53±0·09	0·52±0·10	0·50±0·10

Differences in spine densities between different loci are probably due to variations in dendritic diameter or spine dimensions and the mean values for these measurements at the three loci chosen are presented in Tables 3 and 4 for comparison.

DISCUSSION

Spine density 1 is easily derived and less time-consuming. The disadvantages are that it is purely an estimate which indicates the number of visible spines. It totally under-represents the true figure since the complete circumference which bears spines is ignored. The tortuosity of dendrites also makes difficult the accurate estimation of the dendritic length over which spines are counted. Choice of relatively straight dendrites can minimise this although it is still a subjective assessment.

Spine density 2 is obviously more accurate since it is based on diameter of the dendrite which, assuming the distribution of spines is the same throughout, will be greater in thicker dendrites. The likelihood of spines being visible depends on their length, longer spines being more likely to protrude far enough laterally from the dendrite to be seen. Spines with large head diameters are also more noticeable, although Feldman & Peters (1979) do not mention this as a feature which correlates with the magnitude of underestimation of spine density based on visible spine counts. However, the diameter of the spine head is included in the formula and determines whether a process is included as a spine. The mean dendritic diameter (±S.D.) (Table 3) indicates that, due to the large variation in the diameter of dendrites between particular loci, differences in the ratio of spine density 2 and 1 are expected. The mean spine length and head diameter (±S.D.) (Table 4) for each locus in each group are similar and indicate that differences in these parameters are unlikely to be the cause

of any significant difference in spine density ratio. There is a small difference between spine length noted on apical as opposed to basal and oblique dendrites. The longer spines on apical dendrites would suggest that apical spine densities are less underestimated than either oblique or basal densities since the under-representation of spine density correlates negatively with spine length. Therefore the spine length does not explain the relatively small ratio of spine density 2 to spine density 1 for apical dendrites compared with basal and oblique dendrites.

The ratio of spine density 2 to spine density 1 is consistent for each locus, regardless of the experimental group (Table 2), and both basal and oblique spine density ratios are the same. The result is reasonable in that the basal and oblique dendrites have similar dendritic diameters that are quite small (0·9–1 μm) (Table 3). The apical spine density ratio is greater, as expected, because the apical dendritic diameters are larger and more varied. Therefore estimate 2 at this locus is proportionately greater than that for smaller dendritic diameters. However, the increase of spine density 2 over 1 is not as large as expected in that apical diameters are approximately two and a half times the basal and oblique diameters. A larger ratio would be expected considering the much greater diameter. It seems likely that the distribution of spines is not consistent throughout the dendritic tree.

In choosing a method of assessment of spine density, the time taken, accuracy required and the material being studied are all important factors. The second method of assessment is useful where absolute numbers are required, such as in comparisons between cell types, across species and between different locations in the same cell, since they may not all have similar spine distributions. With respect to studies of spine density within a single population of dendrites, although spine density 2 will always be more precise, the time and labour saved by using method 1 is substantial. The results of method 1 will be a good indicator of spine density and are sufficient for comparisons within the same dendritic type where there is a small range in the diameter of dendrites. Substantial differences in dendritic diameters do not permit comparative analysis. Even after correction for dendritic diameter the spine density values recorded in this study suggest an uneven distribution of spines over the dendritic field because large dendrites do not have the equivalent greater spine density.

SUMMARY

Dendritic spines are small protrusions extending from the dendrites of nerve cells, which bear the majority of synapses. In the past, researchers quantified spine density as the number of visible spines per estimated micrometre of dendrite. This estimate ignores all those spines hidden from view due to their position on the dendrite. Dendrites vary in diameter and the underestimation in some will be greater than others. Estimation of dendritic length is also subjective and difficult in those which are tortuous. The Felman & Peters (1979) geometrical equation takes account of these criteria and provides a method of estimating 'true' spine numbers which does not involve slow and laborious reconstruction. This study compares ratios derived from both methods of estimation (spine density 2:1) at three loci in three experimental groups.

Mean values of dendritic diameters and spine dimensions show the major cause for variation in the ratios between loci to be the shaft diameter of the dendrite. However, the greater ratio for apical as compared with basal and oblique dendrites is not as great as expected, bearing in mind that apical dendrites are approximately 2·5 times larger

than oblique and basal dendrites. Therefore the spine distribution may not be the same throughout the dendritic field.

Estimations of spine density based on visible spine counts are quicker, easier and sufficient for comparisons within the same locus. 'True' estimates (spine density 2) are more accurate and should be used when comparisons are being made between loci, cell types and species.

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