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EXPERIENCES ON THE USE OF THE DISECTOR PRINCIPLE IN NEUROPATHOLOGY

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ABSTRACT

We used the disector principle in studying the pars compacta of the substantia nigra in control cases, following the principles of Pakkenberg et al. (1991). On the theoretical side we were much helped by the formula of Ebbeson and Tang, which is a mathematical representation of the disector principle. In comparing the images of the test (the disector proper) and the reference section we used two microprojectors; the images were projected side by side on a table. We noted a number of potential problem areas in applying the system. The embedding of the tissue in and cutting of the sections from several blocks necessarily results in some, but probably insignificant, loss of material. Outlining of the pars compacta was problematic because it was not easy to do this accurately, and the human hand was shaky. If the area measurement is made with the help of the counting frames, one can determine the border of the counted area accurately, but overlaps or gaps between the frames need be carefully avoided. After a training period for an unexperienced user the disector principle works quite well in practice. Biased results in this kind of disector can be caused by the overlap of cells, especially in tissues with high cell density.

Key words: disector, stereology, substantia nigra, Parkinson's disease, neurons.

INTRODUCTION

The disector principle (Sterio 1984) has helped in counting the numbers of cells in brain nuclei both in humans and in experimental situations (Pakkenberg and Gundersen 1989, Pakkenberg et al. 1991, Nagele et al. 1991, Nurcombe et al. 1991, Pover and Coggeshall 1991, West et al. 1991, Coggeshall 1992). Because of our interest in Parkinson's disease, we have specially been interested in the substantia nigra and applied the method in that connection. From the conceptual side we have been much helped by the formula of Ebbeson and Tang (Ebbeson and Tang 1965, Weibel 1979) which - surprising as it may sound - is the mathematical representation of the disector principle (Collan

1991, Collan 1992). For practical reasons we had to create our own solutions in applying the method and while doing it several aspects emerged that we find potentially valuable for those willing to apply the principle.

MATERIAL AND METHODS

We studied samples from one side of the mesencephalon and pons which in several paraffin blocks contained the right or the left half of the substantia nigra through its whole length. The other half was originally sampled for neurochemical studies. The first control sample (Table 1) was sliced and embedded in 7 paraffin blocks, the second control in 4 paraffin blocks. As suggested by Pakkenberg et al. (1991) we cut the pars compacta of the substantia nigra in series and numbered the sections consecutively. In the two control cases studied, pars compacta of the substantia nigra was present in 720 and 1160 sections. Thickness setting of the microtome was 11 ums. When estimating the total number of cells in the pars compacta of the substantia nigra it was not necessary to know the exact thickness of the sections. The exact thickness would, however, be necessary in determining the absolute volume of the substantia nigra.

Theory. The principle of the disector was thoroughly explained by Sterio (1984) and later reviewed in a number of studies (Gundersen 1986, Gundersen et al. 1988). It has later turned out (Collan 1991, Collan 1992) that the formula of Ebbeson and Tang (1965, Weibel 1979) in its traditional form is a mathematical representation of the disector, especially in a situation in which the tissue is probed at two focal levels, the depth of focus being t_2, and the distance between the upper surface of the upper focal level, and the lower surface of the lower focal level t_1. In the application at hand, used in neuropathology, the surface of the section (thickness=0) corresponds to an optical section by the focal plane, and the formula for the situation is $N_V=(N_{A1}-N_{A2})/t$. The formulae derive from the formula which relates the number of cells seen per area in the histological section and the number of cells per volume of tissue (DeHoff and Rhines 1961, Underwood 1970, Weibel 1979, Aherne and Dunnill 1982, Collan et al. 1983, Oberholzer 1983).

Instrumentation. We did not have two identical projection microscopes available to us when we decided to build the system which we apply for counting cells with the disector (Fig. 1). Two projection microscopes were placed on a long table and between them two mirrors which reflected the projected images side by side on the white table. The distances from the mirrors to the corresponding projectors were different because the magnification of the projection lenses differed. However, the final sizes of the fields projected on the table were equal, and so was the magnification (317 times), which was checked with a stage micrometer (1 mm divided into 100 parts). For counting we used the objective magnification of 25x, the projector lenses had magnifications of 2x and 4x. The non-biased counting frame (Gundersen 1977, Gundersen et al. 1988) was fixed on the image of

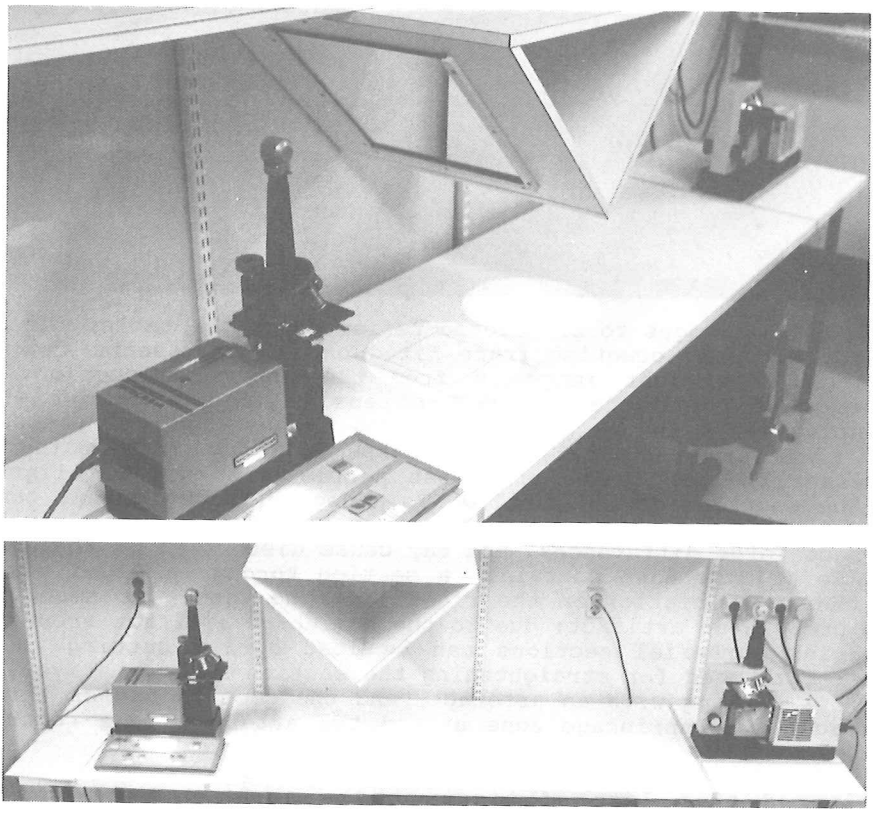

Fig. 1. The system used for application of the disector principle. Two microprojectors are used to project the images of the test and the reference slides side by side on a white table with the help of reflecting mirrors. In this system the distances from the mirrors are different because the magnification of the projection lenses was different in the two microprojectors. The distances of the projectors were adjusted so that the magnifications on the table were identical. In this case also the field sizes were identical at the same magnification, which made matching of images easy.

the test section, and we also used a corresponding frame on the reference section. The latter was by no means necessary, but it helped in finding the corresponding areas. The latter frame was not fixed on the table and it was especially helpful when the alignment of the sections on the slide was not identical.

Cutting the sections. Cutting of the sections was done most carefully. The technician was adviced to cut the individual blocks from the surface to the very bottom of the sample. The idea was to cut all material available for cutting. Necessarily some material was lost between paraffin blocks. At the most we

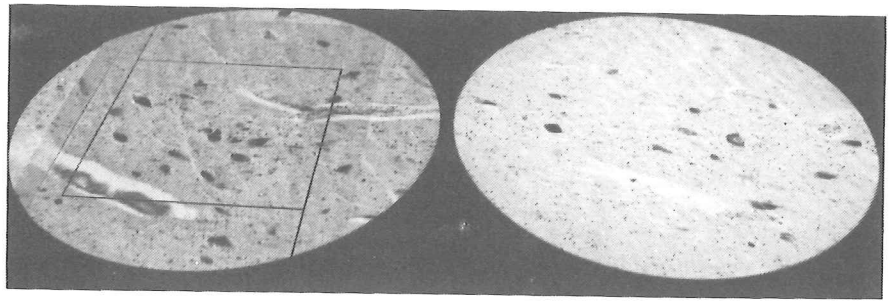

Fig. 2. The images to be matched reflected on the table. The left image, with the counting frame fixed on it, represents the test section. The right image is from the reference section. The photograph demonstrates two problems which can be met while counting cells in the samples. In the images the orientation is not perfectly identical, which makes matching difficult. This stresses the importance of almost perfectly identical alignment of the sections during cutting. To a degree the alignment can be adjusted by moving the microprojectors, but this is not enough to correct large differences, and may cause distorsion of the image. The left image also contains a section through a blood vessel. At the same location in the right image no vessel is seen. This can present an artifact: due to the shrinkage artifact around the arteries, arterial sections can be lost during cutting in the water bath used for straightening the sections. Alternatively, we are not dealing with an artefact, and the section passes through the acellular shrinkage zone around the artery in the reference image.

estimated this loss of tissue to be about 1 % of the cut material. Obviously the most optimal solution would use the substantia nigra or one half of it embedded in a thick paraffin block. The cutting of such blocks, however, would be difficult.

Matching the sections. There were two problems related to cutting which affected the count of cells. First the alignment of the samples on the slides was not always identical, i.e. the samples were in slightly different orientations on the slides. This made it a bit difficult to find the corresponding areas on the two projected images. Here a free counting frame was most helpful because it could be turned in a position which corresponded to the position of the corresponding frame on the test tissue. The other problem was related to the potential loss of material during cutting. Because there was some shrinkage, especially around blood vessels, cross sections of blood vessels could potentially be lost from the section, probably during the water bath phase immediately after cutting (Fig. 2). Alternatively, the differences described were caused by sectioning parallel or nearly parallel to the long axis of the blood vessels. After we understood that this kind of differences could occasionally be seen we no longer had big problems in matching the cells in the two sections. If artifacts were created during cutting in the test or reference sections additional section pairs were

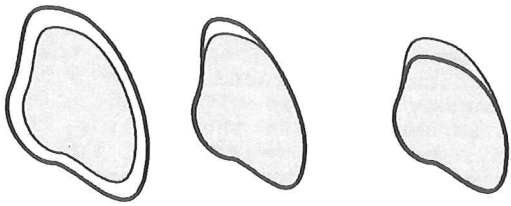

Fig. 3. Outlining of a brain nucleus on histological sections. Outlining is done with a felt tip pen. On the left the type of outlining recommended by the authors for situatins in which the studied cell group is well separated from other cell groups. The outline does not exactly follow the borders of the sectioned nucleus, but there is a no man's land between the nucleus and the drawn outline. When the area of the sectioned nucleus is estimated with point counting on the basis of the outline the result is bigger than the true area. When the total number of cells is aimed at, this type of outlining, however, does not affect the result, if the sampling is done within the same outlined area which was measured by point counting. This approach assumes that no other brain nuclei are in immediate connection with the nucleus under study. If the latter is the case the inner surface of the outline at such locations should follow the outline of the nucleus as keenly as possible. In the middle the outlining is done with the idea to follow the true border of the nucleus accurately. This is not easily achieved, however, because the outlining cannot be done with high magnification which would allow one to see the borders accurately. Also, the outline is usually shaky - a human factor difficult to avoid. The end result is that some cells of the nucleus will be seen under the ink outline, and at places a space is left between the nucleus and the surroundings. Under these circumstances the area estimate may be inaccurate, and this also applies to the estimate of the total number of cells in the nucleus. On the right the outlining excludes part of the nucleus altogether. In such a case, the cell number estimate will be too small. This also applies to the area estimate.

available, and we also used these in this study. Because of artifacts, the disectors could not be always selected in a perfectly systematic fashion (see sample 1 in Table 1).

Outlining of the pars compacta of the substantia nigra on section. Outlining of the pars compacta of the substantia nigra with a felt pen was problematic. This was to be done with low power which made it that the details of the nucleus could not be seen clearly. Also the drawing hand often gave a rough outline, which sometimes overlapped the peripheral cells of the compacta. This type of overlap of the border should be avoided because it may result in false estimation of the total number of cells in the compacta (Fig. 3), if the count is made within the outline. The optimal outlining would be that of exactly following the border of the compacta. In brain nuclei in general this is often

difficult. As a general rule we recommend outlining which allows for a zone of no man's land between the border of the nucleus and the outline made by the felt tip. Even though the estimation of the volume of the nucleus, when based on the outline, would be biased, the total number of cells within the nucleus will not change if sampling is done within the outline. In the special case of this study, however, we have to realise that pars compacta at the periphery is surrounded by pars reticularis. The latter part is less dense and if a no man's land is left around pars compacta, the result will not represent compacta accurately. So it turns out that it is best to try accurate outlining of the pars compacta of the substantia nigra. When area estimation is done on the basis of the number of counting frames successively fitted over the section much of the problems associated with outlining are avoided. On the other hand, the general rule is applicable to the whole substantia nigra which includes the compacta with the dopaminergic cells, and the pars reticulars.

Estimation of the area by pointcounting. The volume estimation of substantia nigra was based on the so called Cavalieri principle: the nucleus was serially sectioned and at the levels of the sections of the disectors (every 40th section) the area of the pars compacta on the section was estimated. As mentioned above the compacta was outlined with a felt tip pen. The outlined area was projected at low power on a white table and a point grid was laid on the image. If the area was so large that the whole nucleus could not be projected as a single image, first one half of the outlined area was projected, then the other half. After the distance between the points was estimated with a micrometer scale projected on the transparent point grid, the area represented by each point could be estimated (Underwood 1970, Weibel 1979, Collan et al. 1983). In our test system this was 0.189 mm^2. The total number of points within the outlined area multiplied by the area represented by each point was the area of the compacta at that level. As this method estimated only the area within the outline, it paid no attention to the potential problems in outlining the compacta. When the outlining was not successful, and the outline overlapped the cells of the compacta, there was the danger that also the estimate of the total number of cells within the compacta was biased (Fig. 3).

Estimation of the area by counting frames. The second type of measurement applied the counting frame which was used for counting cells in every 15th field of the test section. The frames were moved from one counting frame to the next so that there was as little overlap as possible. The total number of counting frames needed to cover the whole area of the substantia nigra was available after the whole section was covered. By multiplying the area of the counting frame with the number of frames the area of substantia nigra on the section could be estimated. In our system the area of each counting frame was 0.212 mm^2. This method gave slightly higher values than estimation by point counting. The reason for this was quite obvious: while counting the number of nerve cells it often turned out that the outlining was not perfect. In many cases cells belonging to the nucleus could be found outside the outlined area, especially under the ink from the felt tip pen. On the

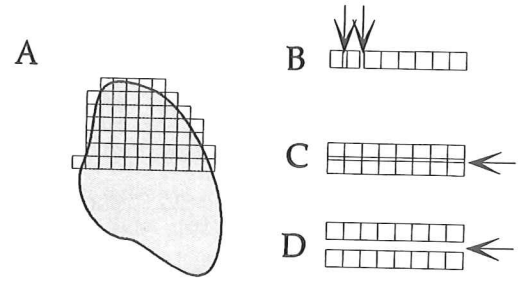

Fig. 4. Estimating the volume of the brain nucleus (e.g. substantia nigra) by applying the counting frame which is used in counting the number of cells. A. The counting frames should accurately follow each other and contain all the cells of the nucleus in the section. Here the nucleus is seen partly covered with counting frames used in the count, at about the middle of counting. It is obvious that the area of the nucleus based on this method will be slightly larger than the true area of the nucleus on section. When the total number of cells in the nucleus is aimed at, this biased area, however, does not affect the end result, if the cell count is made exactly over the same area, and if there are no neurons outside the immediate border of the nucleus. For accurate estimation of area, which is necessary for true volume estimates of the pars compacta, area estimation should be based on pointcounting. Pointcounting can be done separately with a point grid. Alternatively, a point of the counting frame (e.g. center, one corner) is followed during the disector count, and only those frames are counted the point of which falls within the borders of the brain nucleus. Those frames are also included in the area estimate. At B - D problem areas are shown. **B.** Overlaps or spaces between adjacent frames should be avoided. This is why the sample stage should be moved carefully to make a perfect match with the left border of the new frame and the right border of the former frame. **C.** There should be no overlaps between the lines of frames. **D.** There should be no spaces between the lines of frames either. Overlaps tend to increase the estimated total cell number, spaces to decrease it.

other hand, estimation with frames resulted in some overlaps or spaces between adjacent counting frames of sectioned substantia nigra. The overlaps may have contributed to slightly higher area figures than with the former method. The spaces between the lines of frames had an opposite effect (Fig. 4). The obvious benefits of this method were in the fact that with it the margins of substantia nigra could be detected at high power. The best way is to select a point within the frame (e.g. the center of the frame), and count only those frames the point of which falls within the borders of the pars compacta. The area can also be estimated by counting the number of frames with their point falling on the pars compacta.

Definition of cell type. In making comparisons between matched fields we soon found that all cells that appeared pigmented in

one section did not necessarily appear pigmented in the other section. This made us wonder whether there was an ovelap of a pigmented cell with a non-pigmented cell. Because of the reasonably long distance between cells, however, we concluded that the overlap was generally not probable, and that our finding was due to uneven distribution of pigment in the cytoplasm of the neural cells. Finally we defined the pigmented and non-pigmented cells as follows:

- pigmented cells show pigment in the cell cytoplasm at the same location in both sections (test section and reference section) or in one of them
- non-pigmented cells show no pigment in cell cytoplasm in either of the sections

In estimating the cells with the disector the above had little meaning because the cell type among cells counted when applying the disector principle was decided in the test section only. Of course, this slightly underestimates the true number of pigmented cells, but the bias is probably of little significance.

Potential sources for mistakes in counting. If a new cell emerges in the reference section at the same location at which there is a cell in the test section, the cell in the test section may be lost from the count. In the test system we have used one overlap per 100 cells will cause a mistake of about 1% in the total number of cells in the pars compacta, and will tend to decrease the result from the optimal result.

Evaluation of the final result. When we compared our first results with the data of Pakkenberg et al. (1991) we were astonished because the results were clearly smaller than theirs. Of course, our results were based on counts made on pars compacta on one side of the brain only, and the results of Pakkenberg et al. were from the whole substantia nigra, from both sides of the brain. So, in fact, our results were in complete agreement with theirs. However, because we had only one side studied, we could not make estimates on side differences in individuals, a point which has not been studied so far. Another difference in our approach, in comparison with theirs, was that we outlined the pars compacta, not the whole nucleus. There are biological reasons for doing this, e.g. the uniformity of the dopaminergic neurons in the compacta.

RESULTS

Measurements done on two control cases are shown in Table 1. First it is obvious that the number of sections cut of the nuclei may remarkably vary between individuals, as may the total number of sections in which the pars compacta of the substantia nigra is present. This is the reason for the variable number of disectors: the more sections, the higher number of disectors will be counted. The same difference is reflected in the mean area of the sectioned pars compacta: the more sections cut containing the nucleus, the smaller is the area of the nucleus on the section. When the volumes of the pars compacta are estimated with the Cavalieri principle, they will be equal within the limits of biological variation.

The variation in the total number of neurons in the pars compacta

Table 1. Details of samples, area measurements, selection of disectors, and disector counts on two serially sectioned brain nuclei (pars compacta of the substantia nigra).

Characteristic	Sample 1	Sample 2
Age and sex of the control subject	21, female	82, female
Number of paraffin blocks	7	4
Total no. of sections cut (microtome setting 11 um)	2160	1240
Sections with substantia nigra present	960-1680	1-1160
Number of sections with substantia nigra present	720	1160
Mean no. of counting frames (area 0.212 mm^2) covering the pars compacta of the sectioned substantia nigra in the disectors	111	64
Mean no. of points (corresponding to the area of 0.189 mm^2) falling on pars compacta of the substantia nigra	106	62
Mean area of the sectioned pars compacta (based on point counts)	20.03 mm^2	11.72 mm^2
Volume of the pars compacta (t=thickness of section)	14422t mm^3	13595t mm^3
Number of disectors counted	4	6
Levels of disector (section numbers)	1000, 1160, 1400, 1560	40, 240, 440, 640, 840, 1040
Total number of cells counted in the disectors		
- pigmented cells	91	70
- nonpigmented cells	37	36
Cell density estimate based on disector counts (t=thickness of section)		
- pigmented cells	17.04/(t mm^3)	14.93/(t mm^3)
- nonpigmented cells	6.93/(t mm^3)	7.68/(t mm^3)
Number of cells in pars compacta of the substantia nigra (on one side of the brain)		
- pigmented cells	245751	202973
- nonpigmented cells	99944	104410

among control cases varied within wide limits. In most cases it was within the range of 600000 - 890000 cells per pars compacta. In one of our studied control cases the value was remarkably lower, 445 000, suggesting that healthy persons may vary in the

number of neurons originally present in the pars compacta. Based on the available data on 6 controls (including those in Table 1) the medial part of the pars compacta contained 50-65% of all cell and the lateral part 35-50%. The distribution appears to be equal among pigmented and nonpigmented cells: the ratio pigmented/nonpigmented cells does not appear to differ markedly between the medial and lateral parts of the nucleus. In the studied controls, of all cells in the pars compacta of the substantia nigra 60-70% of cells were pigmented and 30-40% nonpigmented.

DISCUSSION

The results in Table 1 seem to show the great difficulty that there is in sampling brain nuclei. Because of topographical differences, brain size differences, and variations in fixation, the direction of sectioning in respect to the long axis of the nucleus cannot be perfectly standardized. However, theoretically this sampling variation should not affect the final estimates of the total number of cells within the brain nuclei, because the whole nucleus is scanned with sections, of which a systematic sample is taken for area and cell density estimates. At the moment very little is known about the size and 3-dimensional shape variation of the nucleus. It is probable that larger brains have larger nuclei, but how this reflects in the total number of neurons in the substantia nigra is not known.

The disector, which is so well applicable to studies on brain nuclei, is based on the principles relating the number of cells per area of histological section to the number of cells in tissue volume (DeHoff and Rhines 1961). We now know that the formula of Ebbeson and Tang (Ebbeson and Tang 1965, Weibel 1979) decribes the disector principle especially well (Collan 1991, Collan 1992). This formula also makes the disector easily understandable, also to persons who are not well acquainted with stereology. In principle the formula of Ebbeson and Tang (and the disector) gives a method to make reliable estimates of cell density within a volume of tissue, in a manner which is independent of cell size or shape. However, to be able to reflect reliably the total number of cells within a brain nucleus, the principle has to be associated with systematic sampling of the nucleus. The values in Table 1 are for one side of the brain and - for clarity - they are based on means and the fact that every 15th counting frame was counted. E.g. 91/4 estimates the mean disector count of pigmented neurons in sample 1. The latter value multiplied by 15 and divided by the mean area of the sectioned pars compacta estimates the disector count per area of sectioned pars compacta. By dividing the latter figure by the thickness of section one gets the cell density per volume of pars compacta.

The Cavalieri principle was a couple of years ago called "volume determination by serial sectioning", and we still prefer the latter, because it beautifully tells the reader what the technique is all about. This method has allowed volume estimates after microreconstruction of organs and tissue structures (Gaunt

1971), and the volume variation estimates after serial sectioning of cells and their nuclei, e.g. lymphocytes (Collan 1972). Without the volume estimation the total number of cells can not be estimated with the disector. It is especially valuable that both the cell density and the volume can be expressed in terms of the thickness of the sections. This results in that the absolute value of the section thickness need not be known. This is quite comfortable because determination of section thickness is problematic. However, section thickness would be especially helpful in tissues with high cell density and nuclear overlaps, and in situations in which the sections are thick in relation to the objects studied. Because, in those cases, matching of sections in the disector count may be problematic (Collan and Collan 1970).

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