ACTA STEREOL 1993; 12/2: 271-276
PRO 6ECS PRAGUE, 1993
ORIGINAL SCIENTIFIC PAPER

TOTAL NUMBER OF NEURONS IN SPECIFIC CORTICAL LAYERS IN THE HUMAN BRAIN

Bente Pakkenberg & Hans Jørgen G. Gundersen

Neurological Research Laboratory, Bartholin Instituttet, Copenhagen;
Stereological Research Laboratory, Aarhus University, Denmark

ABSTRACT

The neurons in the human neocortex are organized in layers which are distinguished on the basis of variations in cell density, size, shape, and orientation. The human neocortex is conventionally divided into six layers. Both the number of neurons and the thickness of the different layers vary a great deal, and are also a function of which region they belong to. In order to estimate the total neuron population in each layer we have applied two different sampling designs in which we have tried to overcome the problems involved in such an estimate. In one design, a "uniform" sample of neocortex is initially taken, the other, the "arbitrary" design, is close to commonly designs for studying the cortical layering. Neither method will provide an unbiased result but an approximation to the true total neuron number in each layer. The conclusion is that both methods have the same efficiency, and that the "uniform" method therefore is preferred in most cases, since the sampling can be performed without further handling of the brain.

Key words: brain cortex, Cavalieri, cortical layers, neurons, optical disector.

INTRODUCTION

The neuron counting method developed some years ago for human neocortex provides an estimate of the total neuron number in a well-defined region (Pakkenberg et al. 1989, Brændgaard et al. 1990), not an estimate of the total neuron number in the different neocortical layers. In some brain diseases such as Alzheimer, Down Syndrome, and schizophrenia localized patho-anatomical changes in specific regions have been reported in specific cortex layers (Hyman 1992, Cabalka et al. 1993, Benes and Bird 1987).
The methodologically fundamental problem is that neocortex in mammals is very complex and highly curved. In a uniform sample not all layers are visible in all sampled tissue blocks. Moreover, because of the curvature, the layers are not necessarily represented according to their true volume in each tissue block. Two different designs were applied to estimate the total number of neurons in the six different neocortical layers in an illustrative sample of three human brains.

MATERIAL

Brains from three normal individuals, age 40, 64, and 78, were used for the study. The patients presented had no prior neurological or psychiatric disorders, no medical record of disease that may affect the central nervous system, and none had any alcohol or drug abuse.

METHOD

The brains were fixed in 0.1M sodium phosphate buffered formaldehyde, (pH 7.2, 4% formaldehyde),the meninges removed and the cerebellum and brain stem detached at mid pons. The frontal, temporal, parietal, and occipital lobes were delineated and painted on the pial surface in different colours as described elsewhere (Brændgaard et al. 1989, Pakkenberg et al. 1990), the hemispheres embedded in 6% agar, sliced coronally at 7 mm intervals and the neocortical sectional area estimated by point counting (Regeur and Pakkenberg 1989). From every second slice, starting randomly, wedges were sampled systematically from each neocortical region, each wedge cut into 2 mm wide parallel bars providing 25 to 50 bars per region. These were subsampled systematically so that each region was represented by 6 to 10 bars which were embedded in 4% agar, randomly rotated round their long axis so that they could be used for vertical section measurements, (see Gundersen et al. 1988a, 1988b). Each agar block was embedded in LKB-Historesin from which one 35 μm thick section was cut and stained with Wolbach's Giemsa stain and used for counting in an optical disector in a modified Olympus BH-2 microscope. The optical disector equipment consists of a BH-2 Olympus microscope with motorized XY-stage, and an electronic microcator with digital readout for measuring movements in the Z-direction. High image resolution and a thin focal plane were obtained using a high numerical aperture (NA 1.4) 100x oil-immersion objective for cell counting. Using the GRID program (Olympus Denmark) and an AMIGA 2000 computer, a counting frame was superimposed on a color monitor where counting took place. An **unbiased** estimate of the total number of neurons in neocortex in each neocortical region of the hemisphere was then calculated by multiplying the Cavalieri-estimate of the regional neocortical reference volume, by the regional numerical density. The area of the frame was 20.0·19.2 mm^2, (at 3900X), and the height of the disector was 15 μm. Changes in the tissue volume between the volume estimate and the numerical density estimate were quantified and found to be a negligible few percent.

To estimate the total neuron population in each **layer** of the cortex, two designs were applied. In design #1, all sampled bars from the uniform sampling design were further analyzed. Only bars in which all six cortical layers were represented in their natural sequence were subsampled. These bars do not constitute an unbiased sample of the whole neocortex but for practical purposes hereafter this will be termed the "uniform" design. The total neuron number was counted in a known volume in columns perpendicular to the trace of the pial surface, using optical disectors. The sum of all neurons counted in each layer, divided by the sum of all neurons counted in the whole column provides the numerical fraction per layer. This fraction is multiplied with the estimate of the absolute, total neuron number in each cortex region, providing the total neuron number in each neocortical layer in the four specified regions.

Design #2 is termed the "arbitrarily selected" design. In the same three brains used for the first estimate, four complete gyruses, one from each neocortical region, was cut out from the brain slices. The 3-D distortions at various levels of the gyrus due to convex and concave curvature of the cortex was now taken into consideration. Choosing five different positions, given the numbers from 1 to 5, from the four selected gyruses, columns were sampled perpendicular to the pial surface (see Fig. 1).
One bar from each position, including the full cortical thickness with all six cortical layers in natural sequence, was cut perpendicular to the 3-D pial surface, and embedded in LKB-Historesin from which one 35 μm thick section was cut and stained with Wolbach's Giemsa stain. The total neuron number was now estimated in a known volume perpendicular to the pial surface, using optical disectors. The volume of the column was the width of the counting frame, multiplied by the depth of the disector and the full cortical thickness. Again: the sum of all neurons estimated in each layer, divided by the sum of all neurons counted provides the numerical fraction per layer. This fraction was now multiplied with the estimate of the absolute, total neuron number in cortex, providing the total neuron number in each neocortical layer in specific regions.

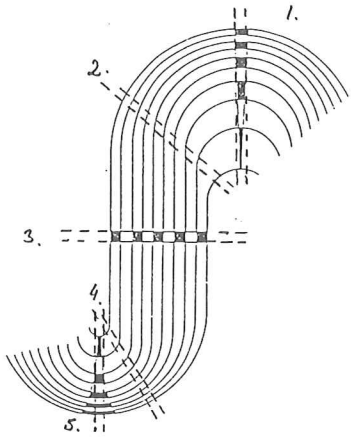

Fig. 1. The five different positions in cortex in which bars were selected for further processing in design #2. Distortions at various levels of the gyrus due to convex and concave curvature of the cortex is indicated.

RESULTS

In design #1, the "uniform" design, an average of 233 neurons was counted in the frontal lobe in an average of 155 disectors; 325 neurons in the temporal lobe in an average of 194 disectors; an average of 322 neurons in the parietal lobe in an average of 172 disectors, and an average of 193 neurons in the occipital lobe in an average of 148 disectors. An average of 9 bars were sampled per region. Of these an average of 1/3 contained all six cortical layers in natural sequence.
The number of neurons per layer in percent was almost the same in all four regions, a few percent in layer I, and approximately 20% in the other five layers (see Fig. 2.).
Since the height of the individual layers is very different it would be expected that the density per layer was also very different, which is indeed the case (see Fig. 3.).

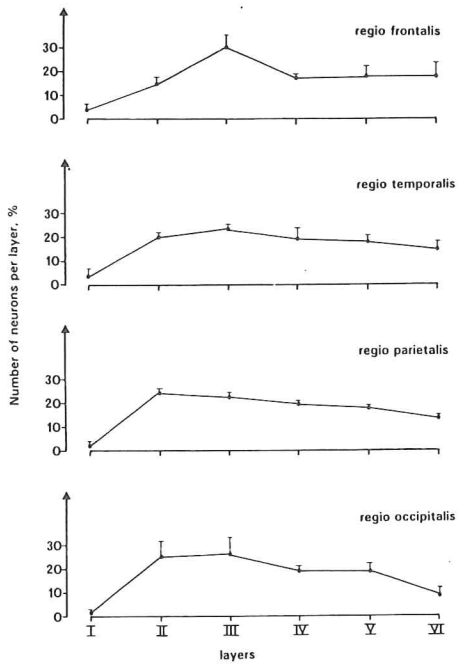

Fig. 2. The number of neurons per layer in percent in the "uniform" design.

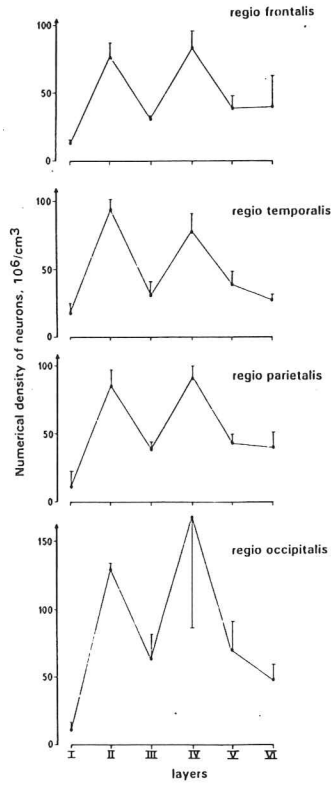

Fig. 3. The numerical density of neurons in the four neocortical regions in the "uniform" design.

In design #2, the "arbitrarily selected" design, an average of 375 neurons was counted in the frontal lobe in an average of 233 disectors; 413 neurons in the temporal lobe in an average of 274 disectors; an average of 453 neurons in the parietal lobe in an average of 256 disectors, and an average of 315 neurons in the occipetal lobe in an average of 269 disectors.

The relative number of neurons per column in layer I+II and layer V+VI and corresponding cortical thickness in the five different positions selected is shown in Fig. 4. In this study the relative number of neurons per column varies almost symmetrically around 100% and is closely related to cortical thickness. The relative neuron number is also closely related to the position in the gyrus: In the convex cortex at the top of the gyrus (position 1) layers I and II are "wide" and thin with few neurons in a column of constant width. Layers V and VI are "compressed" and thick with many neurons in a vertical column. Note that the five positions represent five qualitatively different situations in the gyrus, they do not in any way constitute a **uniform** sample of the gyrus. It is therefore an artifact that the middle position (position 3) of the five is close to 100%; that does not mean that it is necessarily close to the true average.

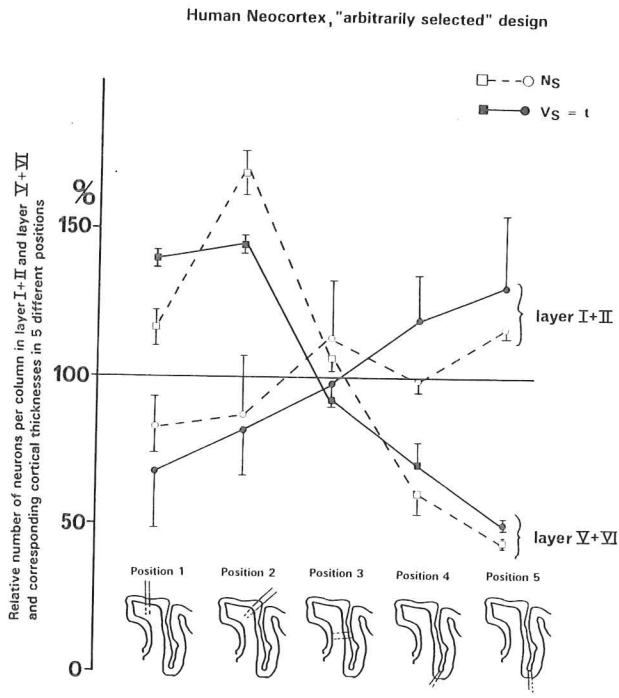

Fig. 4. The relative number of neurons per column in layer I+II and layer V+VI and corresponding cortical thickness in the five different positions selected.

Tbl. 1 shows the efficiency of the two designs. The standard deviations of neurons per column in percent in layers I to VI are roughly the same.

EFFICIENCY OF UNIFORM AND ARBITRARILY SELECTED DESIGN

layer	I	II	III	IV	V	VI
From "uniform" design	2.62	12.43	8.21	4.93	7.75	7.03
From "arbitrarily selected" design	2.93	10.50	7.97	5.42	6.81	6.23

Standard deviations of neurons per column in %, in layer I to VI in two different designs

DISCUSSION

It is rather difficult to obtain an unbiased estimate of the total neuron number in the six different cortical layers using ordinary Nissl or similar staining techniques. Apart from quantitative problems the distinctions between the different layers is not clear cut, the layers change appearances in different regions, and a global count in whole neocortex can easily overlook small, but localized cell losses in subdivisions of different neocortical areas. The aim of the present study, however, was to try to overcome some stereological problems involved in an approximate estimate, which can then be applied to any subregion and/or subpopulation of specific cell types of interest. None of these two methods provides an unbiased result but an approximation of the true total neuron number in each layer. The conclusion is that both methods provide the same approximation and one method can not really be recommended to the other. In most laboratories the first method, the "uniform" design, will be preferred since the sampling design for the unbiased estimation of total neocortical neuron numbers can be extended to include cortical layer estimates without further tissue sampling and technical work and with less counting.

ACKNOWLEDGEMENT

The economic support provided by the Hartmann Foundation, The Ferd. and Ellen Hindsgaul Foundation, København Kommunehospitals Jubilæumsfond and Director Emil C. Hertz and wife Inger Hertz´ Foundation is highly appreciated.

REFERENCES

Benes FM, Bird ED. An analysis of the arrangement of neurons in the cingulate cortex of schizophrenic patients. Arch Gen Psychiatry 1987; 44: 608-16.

Hyman BT. Down Syndrome and Alzheimer disease. Prog Clin Biol Res 1992; 379: 123-42.

Cabalka LM, Hyman BT, Goodlett CR, Ritchie TC, Van Hoesen GW. Neurobiol Aging 1992; 13(2): 283-91.

Brændgaard H, Evans SM, Howard CV, Gundersen HJG. The total number of neurons in human neocortex unbiasedly estimated using optical disectors. J Microsc 1990; 157: 285-304.

Gundersen HJG, Bendtsen TF, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sørensen FB, Vesterby A & West MJ. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. Review article. APMIS 1988a; 96: 379-94.

Gundersen HJG, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR, Pakkenberg, B, Sørensen FB, Vesterby A & West MJ. The new stereological tools: Disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. Review article. APMIS 1988b; 96: 857-81.

Pakkenberg B, Evans SM, Møller A, Brændgaard H, Gundersen HJG: Total number of neurons in human neocortex related to age and sex estimated by way of optical disectors. Acta Stereol 1989;8: 251-6.