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Growth Descriptors for Brain Development

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Brain growth is achieved mostly by adding and extending axons and dendrites, connecting neurons and glial cells. But the increase in fibre mass is a very crude parameter to describe selforganization in this complex system. Therefore, other parameters are regarded as more specific descriptors for growth. Developmental changes in the neuropil were analyzed by an automatic image processing system (IBAS 2, Kontron). The neuropil pattern was transformed to its skeleton, and length, density, orientation and branching of the obtained network were used to describe the development of structural organization of rat cerebral cortex. This approach provides us with quantitative morphological data on the time course of complex changes in the tissue architectonics (lamination, heterogeneity, topology).

INTRODUCTION

In neuroanatomy and neurobiology there is a growing interest not only in the numeric relations of different tissue components, but also in their spatial arrangement and its change in time of development or reorganization (plasticity). Arrangement of cellular components and neuronal connectivity are possibly correlated and determine the complex function of nervous tissue. In the cerebral cortex the number of neurons remains constant during growth and development. The growing component is the so-called neuropil which consists of, among other components, axons and dendrites connecting the neuronal cells. During ontogenetic development the neuropil varies in its arrangement. Bundle-like structures with different orientations and thickness as well as more or less isotropic intercallations of cell bodies in the neuropil phase may be observed (Plate 1).

From a neuroanatomical point of view different fibre systems are defined according to their laminar position in the cortex as well as by the source and destination region in the central nervous system. To study the question, how such systems develop under normal and experimental conditions, we tried in a pilot study to find an appropriate technique for the characterization of such neuropil patterns in different layers of one selected

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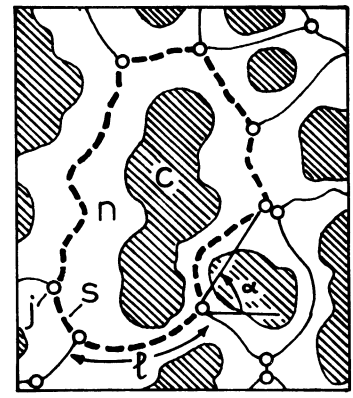


Fig. 1: Part of the neuropil skeleton. The following structural features are demonstrated: c: cell bodies (single or clusters); n: neuropil; s: skeleton line segment; j: junction (node) of skeleton lines. l designates the length of skeleton line segments between two nodes. α is the inclination of the connecting line between two nodes at the ends of one line segment. The border of the central mesh is emphasized by a thick broken line.

region - the senso-motor cortex.

MATERIAL AND METHODS

For each developmental stage of rat (1, 5, 10 days and adult) one cranial section of two to four animals was studied. The Nissl-stained sections of 12 µm thickness were selected from complete series of the rat brain and included corresponding parts of the senso-motor cortex.

A routine has been developed for image processing equipment (IBAS 2, Kontron, Munich). Without going into details, the fundamental steps of the processing and measuring routines were as follows: The image was obtained by a TV-camera adapted to a microscope. The digitized and stored image was preprocessed which included frame setting, removal of shading and contrast enhancement. At the present stage of our study these operations were done interactively. Using the pan or zoom capabilities of the instrument different preprocessed images could be seen in parallel or superimposed with the original to check on serious deviations between the original and the shade corrected and contrast enhanced image. The best result was selected for further treatment. Again, the result of different segmentation procedures was judged by comparison with the original gray image and the best binary image selected for postprocessing. This included removal of background debris, automatic filling of pale cell bodies, suppression of neuropil or unstained zones with a width smaller than half of an average nerve cell diameter (6 µm) and finally the skeletonization of the remaining neuropil phase (Fig. 1). In contrast to preprocessing and segmentation the postprocessing steps ran without interaction of the operator because the result (skeleton) was an abstract structure without a clearly visible relation to the original image. Therefore, we standardized this part of the procedure.

To separate the skeleton into lines and nodes a Laplace operator was applied. Sometimes more distant nodes of the skeleton are connected by rather curved line segments (Fig. 1). In such cases the orientation will be calculated by the image processing equipment as shown in Fig. 1, which is an average of all actually observed orientations of that skeleton line segment. To describe its orientation more precisely all segments were cut into shorter lineal elements of appropriate length as indicated by the broken

line in Fig. 1 (Eins: 1986).

RESULTS

According to the applied image transformations three structural features are available for analysis (Fig. 1):

1. the binary image of the neuropil;
2. the neuropil skeleton, which is the reduction of the neuropil phase to lines of only one pixel width;
3. the skeleton lines form meshes around the profiles of single cells or fused groups of them. The mesh is defined as the smallest continous neuropil space in the section plane. Inside the meshes the soma- and neuropil space may be correlated.

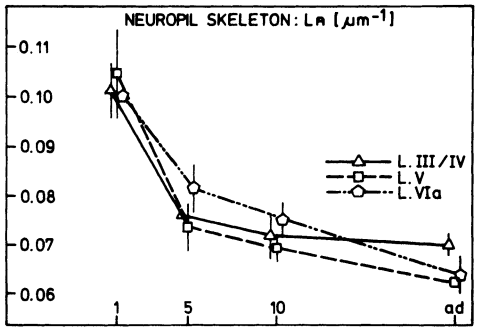


Fig. 2: Length density of neuropil skeleton lines in cranial section planes as measured at postnatal age of 1, 5, 10 days and adult.

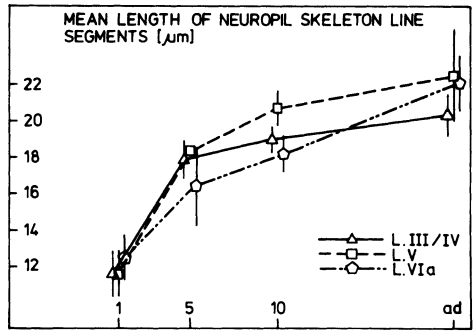


Fig. 3: Average length of neuropil skeleton line segments connecting two nodes (1 in Fig. 1) during postnatal development.

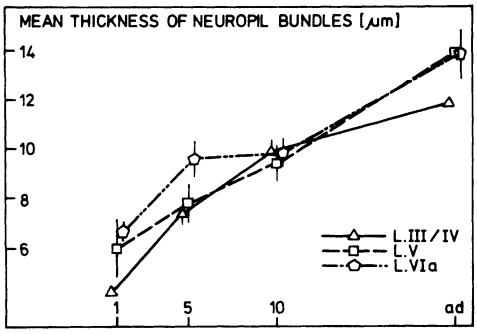


Fig. 4: Neuropil bundle thickness depends on the developmental stage (postnatal days 1, 5, 10 and adult). Average bundle thickness was calculated as the quotient of neuropil area- and skeleton length densities (A_{An}/L_{As}).

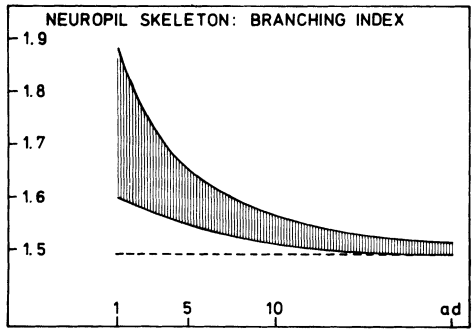
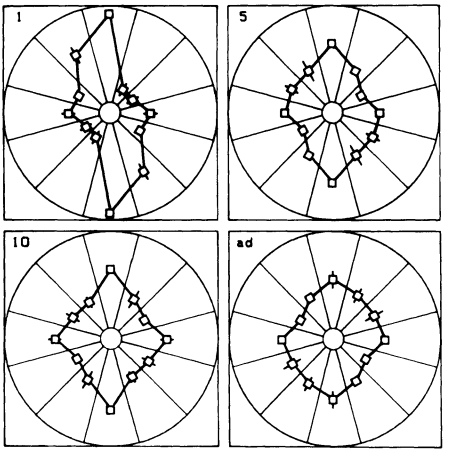


Fig. 5: Range of branching index of the neuropil skeleton in all measured layers (lamina III, IV, V, VIa). The average branching index was calculated as the quotient of numerical densities of skeleton line segments and junctions (N_{As}/N_{Aj}).

The present investigation will focus on the skeleton which may be regarded as a linear structure in a two-dimensional space. Stereological estimators and mean geometrical parameters may be calculated for this structure. Clearly, the length density of neuropil skeleton decreases during development (Fig. 2), and the average length of the single skeleton line segment between two nodes (Fig. 1) increases (Fig. 3). In agreement with these findings, the average thickness of the neuropil bundle profiles increases during growth (Fig. 4). A different information is



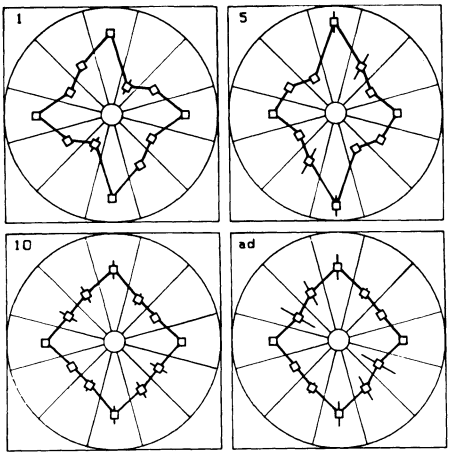


Fig. 6 (left): Roses of orientation for skeleton lineal elements in lamina III/IV at four developmental stages (1, 5, 10 days and adult). Roses of orientation are relative frequency distributions of the orientation index in a polar coordinate system. The orientation index is defined as the fraction of lineal elements belonging to a certain orientation class.
Fig. 7 (right): The same as Fig. 6 but for a deeper lamina (lamina VIa).

obtained from the analysis of skeleton type. The branching index tells us how many skeleton lines belong to one node. The value and the range of this index are high for a younger cortex and it approximates 1.5 in the adult state (Fig. 5), which is the theoretical value for triple junctions.

The skeleton is also a powerful tool for analyzing orientations. The frequency distributions of the direction of skeleton lineal elements are plotted in Fig. 6 and 7 as roses of orientation for two different layers of rat neocortex. Laminar differences are clearly demonstrated. The more superficial layers (lamina III/IV) change from pronounced vertical organization postnatally to isotropy in the adult state (Fig. 6). On the other hand a deeper layer (lamina VIa) shows a more or less pronounced bidirectional organization throughout development (Fig. 7).

If we finally consider the neuropil skeleton meshes, so the mean areal fraction of neuropil in these meshes is expressed by the occupation index, and its average shows a lamina-specific increase during development (Tab. 1). For the single mesh the area of the soma and surrounding neuropil phase can be correlated and the data show the degree of proportionality of cell clustering

and neuropil accumulation (Tab. 1).

DISCUSSION

The neuropil in central nervous tissue may be regarded as a continous phase instead of being composed of discrete particles. This fact makes orientation studies more difficult because there is obviously no unique preferential direction. Therefore, by skeletonization the neuropil was transformed to a structure showing a higher degree of discreteness. The skeleton of an image component is an artificial structure without clearly visible correspondence to the original image or even to a biological structure. Nevertheless, the skeleton contains different information about the structural arrangement in materials composed of several components (Fig. 1):

1. The skeleton consists of lines and it is very easy to measure the orientation of a line as an anisotropy parameter.
2. The skeleton network is built up of line segments and nodes or branching points connecting these segments. The relation of line segments and nodes describes the compactness of a structure or the arrangement of a specific component.
3. The space between the skeleton lines (meshes) may be used as distance descriptors of adjacent structures, similar to the zones of influence discussed by Prewitt (1978) for the skeleton of the extracellular space (which was called exoskeleton by this author).

These manifold advantages of the above mentioned points led us to favour the use of skeleton parameters over other approaches in anisotropy studies, e.g. lineal analysis with a rotated line grid (Weibel: 1980) or autocorrelation of the binary image with its linear transforms in different directions. Furthermore, the skeletonization algorithm is available as a standard routine on most image processing machines and there are no restrictions concerning the width of angular classes of orientation histograms nor the number and directions of anisotropy axis in the section plane. Only line segments which are too long or too short may cause some errors (Eins: 1986). Another effect is caused by the section thickness which must be kept constant to obtain comparable results. Thus, our approach is not the application of stereological methods in anisotropic systems (Oberholzer: 1983) but we use anisotropy parameters, measured in a given reference plane, to characterize changes during development.

The integral and average parameters - length density of neuropil skeleton lines (Fig. 2), mean length of neuropil skeleton line segments (Fig. 3) and mean thickness of neuropil bundles (Fig. 4) - clearly show the structural changes during development. The growing amount of neuropil dilutes the skeleton network: its lines and nodes are more apart from each other and each line represents a thicker layer of neuropil in the more adult stages of development. Laminar differences are not prominent, but the bundle thickness (Fig. 4) is a more sensitive parameter to demonstrate the earlier prenatal development of neuropil in deeper cortical layers and their higher content of neuropil (myelin) in the adult state (Raedler & Sievers: 1975).

Obviously existing laminar differences are possibly masked by the huge increase of neuropil mass which determines the above mentioned skeleton parameters. These changes are accompanied by

a change of the skeleton type from those with multiple junctions, which are more probable in dense skeletons of young cortices, to the purely triple junction type in the more open skeleton of the adult state. Fig. 5 shows the wide range of possible branching indices in younger states as compared to the focussing of all values near to 1.5. In a closed skeleton network 1.5 is the theoretical value of the branching index for triple junctions.

Roses of orientation are more sensitive to demonstrate varying prefered directions and degrees of anisotropy. Two examples (lamina III/IV and lamina VIa) which are known for their unequal

Table 1: Correlation of skeleton mesh size and neuropil.

	Lamina III/IV				Lamina V				Lamina VIa			
	1	5	10	ad	1	5	10	ad	1	5	10	ad
OI	0.26	0.48	0.66	0.78	0.53	0.52	0.60	0.82	0.61	0.74	0.70	0.84
$\bar{A}_n$	94	254	355	485	141	255	417	670	166	297	388	617
r	0.71	0.93	0.82	0.78	0.81	0.91	0.88	0.57	0.82	0.87	0.82	0.61
a	0.16	0.60	1.08	1.99	0.73	0.79	0.96	1.30	0.99	1.77	1.27	2.22
b	49	90	159	211	50	70	152	485	61	108	176	360
N	25	135	171	152	148	100	129	119	172	110	234	130

All data are for different developmental stages (postnatal days 1, 5, 10 and adult) and for three cortical layers, units in μm^2;
OI: occupation index = mean fraction of neuropil in meshes;
$\bar{A}_n$: mean profile area of neuropil in meshes;
a,b: parameters of the linear regression line of the profiles of neuropil area (n) and the corresponding cell somata or its clusters (c) according to the equation $\bar{n} = a \cdot c + b$;
r: correlation coefficient of n and c;
N: number of meshes.

differentiation (Raedler & Sievers: 1975, Jacobson: 1978) show laminar differences (Fig. 6, 7). Each measuring point represents the relative frequency of lineal elements (Eins: 1986) having a direction falling in the chosen 30 degree angular sector.

The neuropil mesh is like the original skeleton itself, an abstract and artificial structure. As stated by Serra (1982) for any morphological operation the transformed structure may be used to quantitatively characterize the original structure. The meshes in this way define and can be used to calculate an occupation index as a mean proportion of neuropil area included in such mesh and the overall mesh area surrounded by the nearest set of skeleton line segments (Fig. 1). This index may vary between zero (no neuropil) and one (no cell somata). Its mean for a given cortical layer (Tab. 1) increases according to new and extending fibres, showing that an increased amount of neuropil corresponds to an unchanged amount but differently arranged somatic phase. This correspondence is expressed by the inclination of the regression line of the area of neuropil and soma included in a single mesh (Tab. 1). There are layers with continous postnatal

neuropil development (lamina III/IV) and others with an earlier prenatal development and a reduced growth in the early postnatal state, which is known for the deeper cortical layers (Raedler & Sievers: 1975).

In summary we may state that skeletonization and analysis of the correlated structural parameters is a useful technique for characterization of growth processes in tissues composed of several spatially distributed phases. In selected regions of the neocortex of rats some known or supposed arrangements of neuropil could be confirmed and expressed in quantitative terms. But this analysis has not reached the final stage. First of all the laminar pattern of one selected region should be completed and extended to other regions. Furthermore, the sensitivity of the above mentioned parameters to structural changes has been proved only with different model structures (not published) but not in the case of developmental abnormalities as they may appear under experimental conditions.

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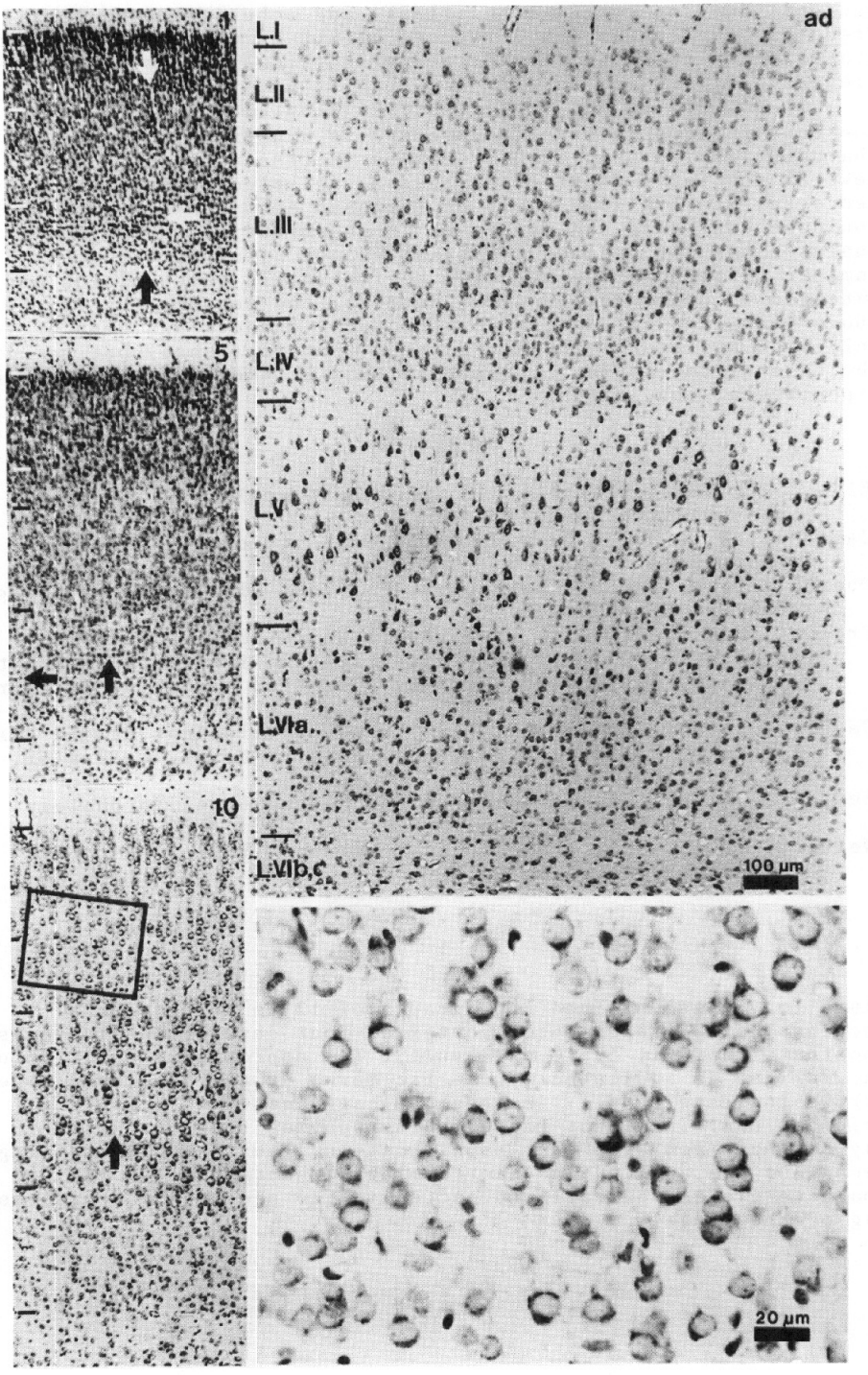
Plate 1: Nissl-stained photographs of 12 µm thick cranial sections of rat senso-motor cortex at four developmental stages (postnatal days 1, 5, 10 and adult). The approximate position of the laminae is indicated by the tic marks at the left hand side. Arrows indicate bundle-like neuropil aggregates of different orientations. The lower right photograph was taken from the 10 day old specimen at higher magnification. The standard staining technique was applied and shortcomings concerning the original image were electronically corrected in the pre- and postprocessing of the image.


1
5
10
L.I
L.II
L.III
L.IV
L.V
L.VIa
L.VIb,c
ad
100 µm
20 µm

8-3
Q: How do you "detect" neuropil on the sections you analyse -in particular, do you take care of extracellular space which is not part of the neuropil? (G. Bernroider)

A: Our main interest in this analysis is not the amount of neuropil but anisotropic arrangement and their change in time. Therefore another component superimposed either isotropically or in close topographical relation to the original phases will not influence the skeleton orientation seriously.
Costained small nonneuronal structures were rejected by a size filter as one step in the postprocessing of the binary image.

Q: 1. Have you examined the anisotropy of the "skeleton" in anything other than the coronal plane? For e.g. the para sagittal plane could give you sections through the full thickness of the cortex.
2. You mention the measurement of length density of the skeleton -in a developmental through series the reference volume (i.e. of the cortex) will be changing -have you controlled for this?
3. This is a somewhat "artificial" skeleton, produced by image analysis, in 2-D, in a single orientation. How much confidence do you have in the biological conclusions that you draw? (V. Howard)

A: 1. Not yet. We are planning to do this for selected area with the aim to construct a three-dimensional representation of the orientation index.
2. For the standard preparation procedures used in our laboratory to have an image analysis check to follow the volume changes appearing during the different steps of preparation(*). By this method we are able either to adapt the procedure to zero volume change or to calculate a correction factor for inavoidable volume changes.
3. I agree with you that there is no strong correspondence of the planer skeleton and three-dimensional neuropil structure. We have proven that the quality of orientation does not change inside a narrow range (adjacent sections). Therefore, the obtained neuropil skeletons are, in our opinion restricted descripters of the three-dimensional structure and may be helpful in the analysis of time depending processes.

* Reference
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