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STEREOLOGYCAL ANALYSIS OF THE DENTATE NUCLEUS IN DEVELOPING HUMAN BRAIN*

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ABSTRACT

Stereological analysis of the dentate nucleus was performed at various stages of fetal development and one brain of a newborn. The cells of the first and the second stage of maturation were observable during the 13th week. In later stages (14-20 weeks) the cells of the second stage were noticed. Parallelly with the cells of the second stage of maturation in the 20th week, the cells of the third stage were found. In the 24th week only the cells of the fourth stage were present, remaining conspicuous up to birth. The diameters of the nerve cell nuclei were measured in some stages of development and their volumes were calculated. In certain stages of development numerical density of the nerve cell nuclei was determined. The value of the numerical density was decreasing in the course of development the most probably due to growth and the shrinkage of the tissue, impossibility to distinguish neuroblasts from the spongioblasts in the earlier stages of development and due to the increasing volume of the dentate nucleus. The decrease in numerical density was statistically significant up to the 24th week. Between the 24th and 31st week the decrease of the numerical density was statistically insignificant, while between the 31st week and a six-day-old newborn it was again statistically significant.
KEY WORDS: dentate nucleus, man, development, stereology.

INTRODUCTION

Parallelly with the phylogenetic development of the neocerebellum and neocortex, the dentate nucleus develops into a dominant and the most convoluted structure. For this reason it is obvious that it reached the highest degree of development in man. Its convoluted pattern provides an optimal design for storing a large number of cells, fibers and synapses (Heidary and Tomasch, 1969). The dentate nucleus is a very important structure for the regular functioning of the neocerebellum. In other words, it is a necessary element in

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the coordinating control of the visuo-oculo-manual tracking system (Vercher and Gauthier, 1988). The occurrence of the intentional tremor is also a sign of functional disturbance on the dentate nucleus level (Siegfried et al. 1970).

However, the role of the dentate nucleus is still not elucidated. This was the reason why the dentate nucleus was the subject of investigation in man (Gans, 1924; Fazzari, 1933; Nistri and Fabiani, 1956; Verbitskaya, 1969; Rakić and Sidman, 1970; Gudović et al., 1987) as well as in animals (Korneliussen, 1968; O'Leary et al., 1970; Fix, 1975; Chan-Palay, 1977; Altman, 1978). There is a small number of papers dealing with the determination of the number of the dentate nerve cells (Höpker, 1951; Heidary and Tomasch, 1969; Chan-Palay, 1977). Cytomorphometric and stereological analysis of the dentate nucleus in man at various stages of development has not been performed yet. The aim of this study was to shed more light on this nucleus during prenatal development by using a stereological methodology.

MATERIAL AND METHODS

The cytomorphometric and stereological analysis of the dentate nucleus was performed on 20 human fetal brains aged from the 12.5th to the 31st week (Kostović, 1979) and on a brain of a newborn. The brains were fixed in 10% formalin solution and embedded in celloidin and paraffin. The brains were cut in frontal plane (Gudović et al. 1984) in 15 and 30 µm thick sections. For counting the nerve cell nuclei each third section in rostrocaudal direction was used. They were counted in each section of the 20 test fields; ten on the left and ten on the right side. In younger stages test fields were placed in one linear row, but in older stages, where the dorsomedial and ventrolateral lamina could be distinguished, 5 test fields were counted in dorsomedial and 5 test fields in ventrolateral lamina. For this purpose the sections were stained with cresyl violet. The nerve cells were drawn by cammera lucida. In each stage of maturation 240 test fields were counted, using light microscopy (occ. 10; obj. 100).

The stage of maturation of the nerve cells was established by the modified criteria of Rakić (1968). To determine the diameters of the nerve cell nuclei the eye-piece graticule was used. Since there is an obvious linear relationship between the radius (R) of the sphere and its mean profile size, the following formula to estimate the true radius from the mean profiles was used (Kališnik, 1985):

$$R = \frac{4}{\pi} \cdot \bar{r} \qquad (1)$$

The volume of the nerve cell nuclei was calculated according to the following formula:

$$V = \frac{4}{3} \cdot \pi \cdot R^3 \qquad (2)$$

The nerve cell nuclei were counted in light microscopy at one level of a thick slice (using the multipurpose test system M42 according to Weibel) and therefore, in formulas for esti-

mating numerical density, the thickness of slices is replaced by the subjective depth of focus (DF). It can be calculated by the formula (Pajer and Kališnik, 1986):

$$DF = T + T' \qquad (3)$$

The physical depth of focus (T) can be calculated by the formula:

$$T = n_o \left(\frac{4\lambda}{A^2}k + \frac{L}{AM}\cdot\omega\right) \qquad (4)$$

and the individual enlargment due to visual adaptation (T') by the next formula:

$$T' = n_o \frac{L^2}{M^2}\left(\frac{1}{P} - \frac{1}{R}\right) \qquad (5)$$

where n_o is the refractive index of intermediate material (for biological material under the cover slip its value is 1.45), λ is the wave of the light, (0,55 μm) A - the numerical aperture of the objective , k is a constant number (1/8), L is a usual optical distance (25 cm), M is the total magnification of the microscope, ω is a constant number (0.00136), P is punctum proximum and R punctum remotum.

Thus, numerical density can be calculated by the formula (Pajer and Kališnik, 1984):

$$N_V = \frac{N_A}{DF + \bar{D}} \qquad (6)$$

where DF is the subjective depth of focus, $\bar{D}$ means average diameter and N_A number of the nerve cell nuclei per unit test area. The data obtained were calculated by a PCXT. The results were statistically evaluated using Student's t-test and graphically presented. The numerical density of the dentate nerve cell nuclei was expressed on the ordinate. The age of the fetuses were presented on the abscissa as $\log_\Delta (t) . k$, where t is a number of days counting from the 12.5 postovulatory week and k is a freely choosen number (in our case 27) by which the expression $\log_\Delta (t)$ was multiplied in order to englarge the distance between the weeks.

RESULTS

The cells of the dentate nucleus appear in the 12.5 postovulatory week and correspond to the first and the second stage of maturation (Fig. 1). Apart from the difference in size, there is a difference in staining intensivity of nuclear inclusions. The difference between the size of the nuclei of the first and the second stage of maturation was statistically highly significant ($p<0.001$). The cells of the second stage of maturation were present up to the 20th postovulatory week. Parallelly with the cells of the second stage of maturation,

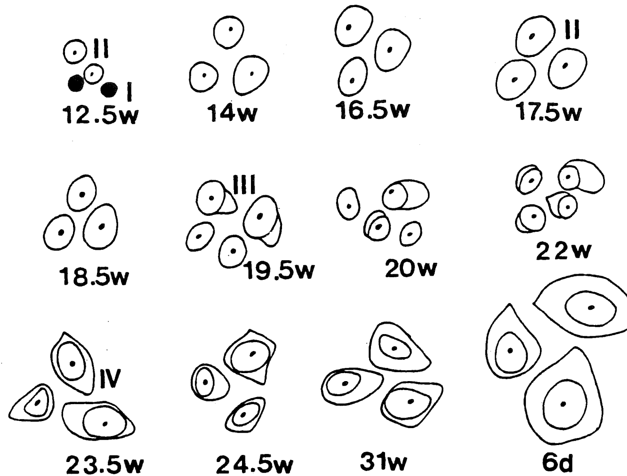

Fig 1. Graphical reconstruction of the dentate nerve cell nuclei at various stages of development drawn by camera lucida: obj. x 100

I first stage of maturation, II second stage of maturation , III third stage of maturation, IV fourth stage of maturation

in the 20th postovulatory week, the cells of the third stage were noticed (Fig. 1). The maturation of the cells was accompanied with their nuclear augmentation, which was statistically significant up to the 17.5 postovulatory week. The cells of the second stage of maturation in the 19.5 postovulatory week are smaller than in the previous stage, and this decrease was statistically significant (Tab. 1). The difference in size between the nerve cell nuclei of the second and the third stage in the 20th postovulatory week was statistically highly significant ($p<0.001$). This augmentation indicates that in this period of development the cells growth is very intensive.

In the stage of the 22nd postovulatory week only the cells of the third stage of maturation were found (Fig. 1). The cells of the fourth stage of maturation were present in the 24th postovulatory week, remaining conspicuous up to birth (Fig 1). The growth of the nuclei from the 19.5th postovulatory week until birth was statistically significant ($p<0.001$; $p<0.05$, respectively - Tab. 1).

The analysis of the values obtained for numerical density of the nerve cell nuclei indicates a constant decrease. The decrease of numerical density of the nerve cell nuclei was statistically highly significant up to th 24th postovulatory week ($p<0.001$; $p<0.01$, respectivelly, (Fig 2), and from the 31st postovulatory week to birth ($p<0.001$; Fig. 1). The decrease in numerical density of the nerve cell nuclei between the 24th and the 31st postovulatory week was small and statistically insignificant ($p<0.4$; Fig. 2), although the rise

Table 1. AVERAGE NUCLEAR VOLUMES OF FOUR STAGES DURING DEVELOPMENT OF NERVE CELLS IN μm^3

		12.5 w		14 w		16.5 w		17.5 w		19.5 w	24 w		31 w		6 d
S I	$\bar{X}$	65.72													
	SEM	0.96													
S II	$\bar{X}$	135.17	ooooo	154.86	ooooo	231.06	ooo	303.38	oooo	262.96					
	SEM	1.47		0.02		3.37		10.74		6.05					
S III	$\bar{X}$									850.55					
	SEM									19.20					
S IV	$\bar{X}$										987.63	oo	1019.11	ooooo	1238.92
	SEM										23.76		29.62		36.22

Table 2. THE NUMERICAL DENSITY (N_v) OF THE DENTATE NERVE CELL NUCLEI

	12.5 w		17.5 w		19.5 w		24 w		31 w		6 d
$\bar{N}_V$ mm^{-3}	273000	oooo	28000	oooo	16000	ooo	14000	o	13600	oooo	11500
SEM	5000		550		400		500		300		300

w - week
d - day
S - stage of maturation
$\bar{X}$ - mean
SEM - standard error of mean

$p<0.001$ ooooo
$p<0.005$ oooo
$p<0.01$ ooo
$p<0.05$ oo
$p<0.4$ o

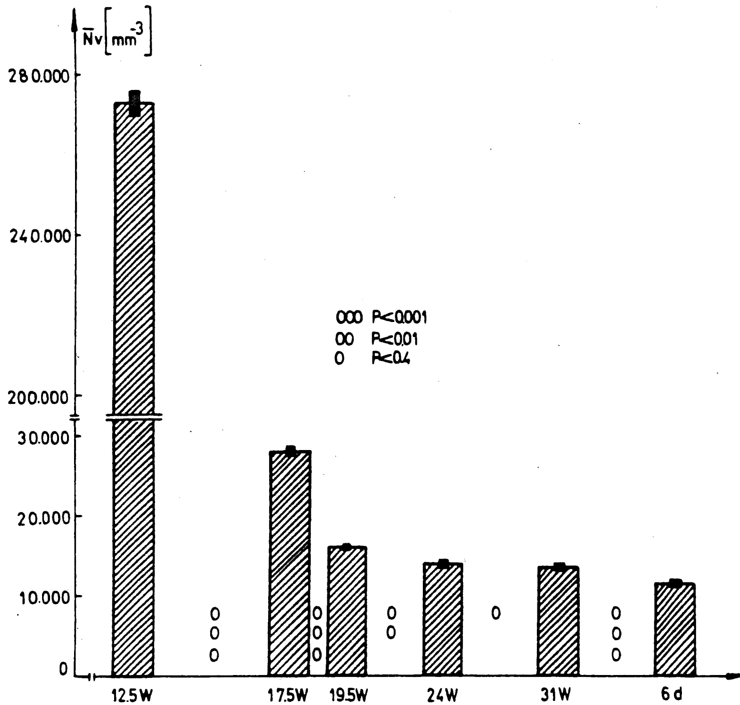

Fig. 2. The numerical density (N_v + 2 SEM) of the dentate nerve cell nuclei at various stages of development (w-weeks, d-days).

in average nuclear volume in the same period was statistically significant ($p<0.05$).

DISCUSSION

In our paper the term "stage of maturation" of the nerve cells has been used as one of the parameters of development. It was introduced in neuroanatomy by Rakić (Rakić, 1968). The differences in the stages of maturation of the nerve cells was based on their visible characteristics when stained with aniline dyes. On the basis of the degree of basophilia, metachromatic characteristics of the nucleoli and colouring of the cytoplasm, as well as the occurrence of the Nissl bodies, it was possible to distinguish four stages of maturation.

For the first stage of maturation (S-I) a dark, round and extremely chromafin nucleus with a granular basophilic substance was characteristic. During development, the nuclei became larger, pale, with a prominent nucleolus and a very poor chromatin substance. These cells were considered to be cells of the second stage of maturation (S-II). In these stages, the cytoplasm was invisible. This is so because the

visibility of the Nissl bodies, stained with aniline dyes, is a result of colouring the crowed ribosomes on the membrane of endoplasmic reticulum. It means that the granular endoplasmic reticulum, in these stages, has not been sufficiently morphologically and functionally differentiated. Also, it should be mentioned that in the first and the second stage of maturation it was impossible to distinguish the neuroblasts from the spongioblasts. As a result of the further development and maturation of the cells, particularly of the granular endoplasmic reticulum, the pale sickle-shaped cytoplasm occurred in some cells. These cells belonged to the third stage of maturation (S-III). The cells of the fourth stage of maturation (S-IV) were characterized by a morphologically and functionally developed granular endoplasmic reticulum. These cells had a large, pale nucleus with a prominent nucleolus. The light blue cytoplasm, having Nissl bodies differing in fineness, entirely enveloped the nucleus. The axon hill was also evident. In the third and the fourth stage of maturation, the differences between the neuroblast and spongioblast were obvious (Rakić, 1968).

Comparing the present results with those from one of our previous papers on the same subject (Gudović et al., 1987), we may conclude that the period of the most intensive growth of the dorsomedial lamina (20-22nd postovulatory week) corresponds to the cells of the third stage of maturation. The cells of the fourth stage of maturation appeared at the beginning of the ventrolateral lamina wrinkling-24th postovulatory week (Gudović et al., 1987). These facts indicate that, in the period of the most intensive wrinkling of both laminae, the differentation of the cells commenced, more exactly of the granular endoplasmic reticulum, which differentiate gradually during development (Miller, 1966). At the same time, together with the cell maturation, manifested in the growth of the cell nuclei (Tab. 1), as well as in the occurrence of more prominent and more rude Nissl bodies, a decrease in the numerical density of the nerve cell nuclei took place (Tab. 2; Fig. 2), probably for the reasons stated below. First, the choise of fixative and embedding media must be careful. For our quantitative study, formalin as a fixative and paraffin as an embedding medium were employed. During the fixation in aldehydes, to which formalin belongs , probably the most important reactions are those which stabilize proteins. Formalin has the ability to estabilish cross-links between proteins thereby forming a gel (Hopwoord, 1982). For this process, a loss of water is characteristical. It occurs also during dehydration in graded seria of alcochol. In other words, these processes reduce the amount of water in the tissue and elicit shrinkage. The shrinkage after fixation of the brain with formalin amounts to about 50% in volume (Haug, 1972). The degree of shrinkage depends on the amount of water supply. Therefore, it is understable that the shrinkage is higher in the younger fetuses than in the older ones. This influences the value of numerical density, too. Also, it is known that the counting error in large neurons is high, because their diameters are usually greater than the thickness of the sections. To avoid this error, the counting of nucleoli is recommended (Haug, 1972). In the present study , we could

not follow this advice for the reasons mentioned above, and the counting was done as described in material and methods.

On the basis of this consideration we may conclude that, in the period between th 12.5th to the 17.5th postovulatory week, the neuroblasts and the spongioblasts were counted together, i.e. the numerical density in this period was presented as a sum of their numerical densities. In the later stages, this was not the case, because these two groups of the cells were easily distinguishable. This could explain why the nuclei of the cells of the second stage of maturation in the 19.5th postovulatory week were smaller than the ones in the 17.5th postovulatory week. Namely, it would be logical to suppose that the cells of the third stage of maturation arose from the part of the cells of the second stage of maturation, while the cells of the second stage of maturation, which were found in the fetuses of the 19.5th postovulatory week, most probably represent the cells later to differentiate into glia. We were led to this conclusion by the fact that the nuclei of the cells of the second stage of maturation in the 19.5th postovulatory week were smaller than in the 17.5th postovulatory week, the decrease being statistically significant ($p<0.001$). But, the possibility that some still immature neuroblasts exist in the cells of the second stage of maturation, besides spongioblasts, may not be excluded. The assumption is still hypothetic because it seems unlikely that some neuroblasts directly differentiate from the second to the fourth stage of maturation.

In addition to the facts described, one should not forget that, during development, the processes of proliferation, migration, differentiation and death of some amount of cells take place. These processes finally result in a fall of the numerical density in later stages. The reduction in the numerical density of the nerve cell nuclei could be accounted for by the total growth of both laminae of the dentate nuclei including the development of neuropil, too.

Although there are several papers dealing with numerical analysis of the dentate nucleus in man (Höpker, 1951; Heidary and Tomasch, 1969), it is not possible to compare them with ours because of the different methodology and the fact that the brains of the adults were investigated. Stereological and morphometric analyses of the human dentate nucleus at various stages of development have not been carried out so far. Although special attention was devoted to the stage of maturation of the nerve cells when morphometrically analysed, this fact was neglected when numerical density was calculated. Namely, all nerve cells were counted regardless of the stage of their maturation. From the beginning, it was taken into consideration that laminae of the dentate nucleus were of a different phylogenetic age (Gans, 1924), but the stereological analysis revealed statistically insignificant results between them. Therefore, the numerical density of both laminae is presented in this way.

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