

Age-related quantitative changes in the organelles of rat neocerebellar Purkinje cells

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Summary. A quantitative study regarding the age-related changes occurring in the somatic organelles of the neocerebellar Purkinje cell is carried out, using female rats aged 2 to 24 months. Standard manual morphometric techniques are used to calculate the following parameters: somatic volume, volumetric fractions and absolute volumes of the intracellular structures as well as the membrane profile concentration, the membrane surface concentration and the mean surface of the rough endoplasmic reticulum cisternae per cell (RER-S).

From a statistical point of view, all the cell components significantly modify their volumetric fractions (except the multivesicular bodies and nucleolus; the latter in relation to the nucleus) and their absolute volumes (except the mitochondria and the multivesicular bodies); the parameters regarding the reticulum are also modified during ageing. There is a linear trend between the age and either the somatic volume of the RER-S or the absolute volumes of the following structures: mitochondria, dense bodies, ground substance and total cytoplasm. A linear correlation is also observed between the cell volume and either the RER-S or the absolute volume of intracellular structures (the Golgi apparatus, the multivesicular bodies and nucleolus being excluded).

Anatomophysiological considerations about the findings are discussed. The role of the ground substance as the major modulator of the volumetric plasticity of the Purkinje cell during ageing, is emphasized as a conclusion.

Key words: Morphometry, Purkinje cells, Cerebellar cortex, Age-changes

Introduction

The capacity of adaptation of animals contributes to

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increase longevity and is dependent upon the activity of the central nervous system. Because this system coordinates several functions, it is expected that as it is affected by the ageing process, it promotes alterations in the physiology and structure of the organs it commands. Because the neuron is a fixed postmitotic cell, it is more susceptible to modifications by ageing mechanisms than other ordinary dividing cells; for instance, the neuron cannot release its growing contents of lipofuscin by karyokinetic dilution.

The Purkinje cell is a prototype of a nerve cell with a high level of either external or internal organization. Because of its supreme importance in connection with motor coordination, its contribution to the overall quality of motor dignity, which is distressfully handicapped in a number of dysfunctions, is beyond any discussion.

In spite of an excellent account on qualitative cytological variations of ageing in Purkinje cell (Nosal, 1979), quantitative studies are indispensable when regarding the apparent immutability of some organelles during the ageing process. Some attempts have been made with respect to this matter; thus, Hinds and McNelly (1978) verified the dispersion of cisternae of rough endoplasmic reticulum and Rogers et al. (1984) evaluated other histological senescent modifications occurring in the neuron under analysis. To the best of our knowledge, however, a detailed study of the organelles in quantitative terms has not been published so far, at least in relation to neocerebellar areas. This is why the present study to seek possible age-related organelle changes occurring in the soma of the Purkinje cell, at intervals, over a period of 22 months (M) was undertaken.

Materials and methods

Preparation of tissue for transmission electron microscopy

Female Sprague-Dawley rats of 2, 6, 9, 12, 15, 18, 21 and 24-month-old (five specimens per age) were perfused, through the heart, with 2.5% glutaraldehyde in Sørensen phosphate buffer (pH = 7.3), after being

anaesthetized with 3.5% aqueous solution of chloral hydrate (35 mg per 100 g of body weight, intraperitoneally). Samples from Crus I and Crus II were removed and processed for transmission electron microscopy as described in a previous study (Monteiro, 1983).

Morphometric analysis

The cells were cut with no special orientation. A total of 100 negatives per age (only one from each block) were obtained at a primary magnification of $\times 2,000$ or $\times 3,000$. The final print magnification was determined for calculation purposes. Only profiles of Purkinje cells with a complete outline were photographed.

The cell volume (V) was determined with the formula of Lindberg and Vorwerk (1970); $V = \beta \cdot a$ in which β is a dimensionless coefficient depending on cell shape, which was considered to be a prolate ellipsoid (Aherne, 1984), and a is the mean transection area estimated by point counting with a multipurpose test system, widely known as a standard morphometric technique (Weibel et al., 1966; Weibel and Bolender, 1973), with 84 lines, each 1.35 cm long. This manual test enables one to determine the volume densities or fractional volumes (V_v) of the organelles as well. The multiplication of the mean volume of V by the individual volume density of the organelles gives their absolute volumes (v).

A grid consisting of 17 parallel lines spaced 1 cm apart was used to perform the method of Loud (1962), which allowed the determination of two parameters in connection with the rough endoplasmic reticulum (RER): a) the membrane profile concentration (MPC), i.e., the average number of micrometres of RER profile per square micrometre of cytoplasm: $(MPC = \pi \cdot C \cdot M) / (2000 \cdot L)$; b) the membrane surface concentration (MSC) or surface density (S_v), i.e., the average number of square micrometres of RER per cubic micrometre of cytoplasm: $MSC = (2 \cdot C \cdot M) / (1000 \cdot L)$. In both formulae, C is the number of crossings between the profiles of RER and the grid lines, M is the individual print magnification and L is the total line length that lays over the total cytoplasm. The value of the mean surface of RER per cell (RER-S) was calculated by multiplying the MSC by the mean volume of the total cytoplasm.

As the aim of this study is to compare neurons with different ages, morphometric corrections were ignored, since the error in the various age groups is in the same direction. This fact had been stated by Abercrombie (1946) and was more recently stressed by Loud (1987).

Statistical analysis

Standard errors (SE) of percentages (V_v) were calculated according to the system of Bradbury et al. (1975). The data regarding absolute values were presented, in graphical form, as mean \pm SEM (standard error of the mean). The comparison between values was

carried out by studying the standard error of the difference and the critical ratio examined by a «two-tailed» test with a Z probability table. The results under comparison were considered not significant for $P > 0.05$; this analysis is summarized in latticed statistical tables (no.1 to no.6). As the number of pairs of observations is small ($n = 8$), the significance of the coefficients of correlation (r) was obtained from a table of probabilities of r values (Allan, 1982).

Results

The volumetric fractions of the intracellular structures are condensed in Table 1. Their absolute volumes, as well as the Purkinje cell volumes, are represented in Graphs (no.1 to no.11) none of the represented parameters shows any clear-cut temporal pattern, except to some extent, the graph in connection with the variation of the absolute volumes of dense bodies.

Graph no.1 exhibits the variation of the mean cell volume. When the cells at 2 or 12 M, on the one hand, are compared with the cells at 18 or 24 M, on the other hand, a statistically significant difference is found ($P < 0.05$); the latter possess a larger volume. There is no linear correlation statistically different from zero when the cell volume is studied versus the body weight; that correlation, however, exists between cell volume and age ($r = 0.80 - P < 0.05$). Additionally, there are positive linear correlations between the cell volume and the absolute volumes of the following structures: mitochondria ($r = 0.80 - P < 0.05$), dense bodies ($r = 0.84 - P < 0.01$), ground substance ($r = 0.97 - P < 0.01$), total cytoplasm ($r = 0.99 - P < 0.01$) and nucleus ($r = 0.90 - P < 0.01$). The relationship is also real in that which concerns the mean surface of RER cisternae per cell ($r = 0.91 - P < 0.01$).

Regarding the volumetric fractions of mitochondria, there is a significant decrease at 24 M ($P < 0.05$) when the value is compared with those of 2, 9, 12 and 15 M. These findings do not match the absolute values (Graph no.2) in which statistically significant comparisons are not detected, despite the peak exhibited at 9 M and the occurrence of a positive linear correlation between age and absolute volumes of mitochondria ($r = 0.74 - P < 0.05$).

As far as the Golgi apparatus is concerned (Fig. 1), a volumetric fraction is found to be significantly different between 15 and 24 M and the values relative to all the other ages which display preponderant volumetric fractions. Concerning the absolute values (Graph no.3) there is a prominent decrease between 12 and 15 M to the extent that the absolute volume of the latter age significantly differs from the absolute volumes of all other ages, 24 M being an exception. The organelle does not show any linear correlation statistically different from zero between its absolute volume and age.

With respect to the multivesicular bodies, neither the volumetric fractions nor the absolute volumes (Graph no.4) present significant alterations during ageing, in spite of the peak relative to the absolute volume at 18 M. The absolute volume of these organelles shows no linear



Fig. 1. Detail of a Purkinje cell perikaryon (2 M) showing a typical and exuberant Golgi apparatus (arrows). The stack of densely packed cisternae is surrounded by a number of vacuoles and small vesicles. $\times 45,000$

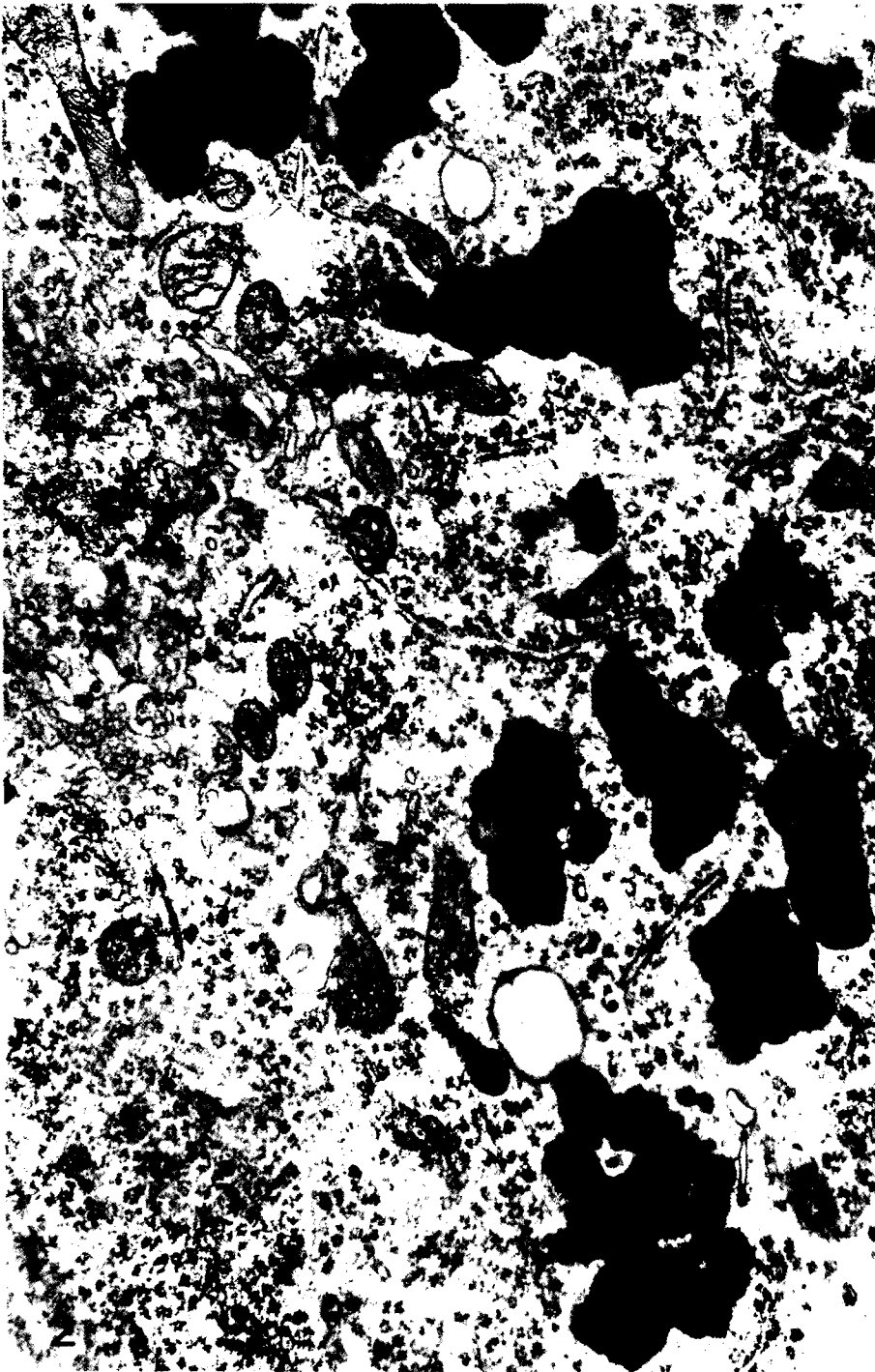


Fig. 2. Part of a Purkinje cell perikaryon (24 M) exhibiting gathered dense bodies (lipofuscin). $\times 24,000$

correlation with age statistically different from zero.

The dense bodies (including lipofuscin) display the most expressive ultrastructural age changes (Fig. 2). Remarkable quantitative differences exist during the ageing processes as well; those which are highly significant from the statistical point of view predominating ($P < 0.001$), with a few exceptions. Moreover, there is a greater number of significant comparisons at the range of the absolute volumes (Graph

no.5), which show a quasi perfect positive linear correlation with age ($r = 0.97 - P < 0.01$).

The ground substance (perikaryon, exclusive of the measured major organelles, but including the ergastoplasm) and the total cytoplasm significantly modify their volumetric fractions as well as their absolute volumes (Graph no.6 and no.7, respectively). Both parameters are positively correlated with age ($r = 0.72$ and $r = 0.81$, in the same order, $P < 0.05$).

Concerning the nucleus, the differences in connection with the absolute volumes (Graph no.8) are most frequently observed and of greater statistical significance when compared with the values in reference to the volumetric fractions. The organelle reveals the smallest absolute volume at 2 M and the biggest one at 18 and 24 M; these extreme values are significantly distinct from all the remaining ones. Between 6 and 15 M the volume decreases so gradually that the variation within that interval is not statistically significant. In contrast, the nucleolus does not exhibit significant changes in its volumetric fractions, something which does not happen regarding the absolute volumes (Graph no.9); indeed, there is a maximum peak at 18 M, the value of which is significantly different in relation to the values of 9, 15 and 24 M. Neither the nucleus nor the nucleolus displays a linear correlation statistically different from zero when their absolute volumes are studied versus age.

The membrane profile concentration (Graph no.10), the membrane surface concentration (Graph no. 10) and the mean surface of RER per cell (Graph no. 11), in connection with either the typical Nissl bodies (Fig. 3) or the dispersed cisternae seen throughout the cytoplasm, vary during ageing. Both types of concentration show high peaks at 6 and 24 M and an indentation at 12 M. Furthermore, the peak value at 24 M is significantly greater than that found for all the other ages, 6 M being an exception. With regard to the mean surface, the greatest value is also achieved at 24 M and is significantly distinct from the ones seen for the



Fig. 3. Detail of a Purkinje cell perikaryon (24 M) displaying a Nissl body. The cisternae possess clear contents, are somewhat tortuous and present irregularly attached ribosomes. A large population of free ribosomes, clustered into rosettes, agglomerates between the cisternae. $\times 60,000$

Age changes in Purkinje cell organelles

Table 1. Volume densities (Vv - %) of organelles of Purkinje cells of rat neocerebellum (*Crus I* and *Crus II*) from 2 to 24 months old (\pm SE).

| AGE | 2 | 6 | 9 | 12 | 15 | 18 | 21 | 24 |
|-----------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Mitochondria | 9.8 \pm 0.3 | 9.3 \pm 0.25 | 9.6 \pm 0.3 | 9.8 \pm 0.3 | 9.6 \pm 0.3 | 9.1 \pm 0.25 | 9.4 \pm 0.3 | 8.8 \pm 0.25 |
| Golgi apparatus | 5.0 \pm 0.2 | 4.6 \pm 0.2 | 4.4 \pm 0.2 | 4.8 \pm 0.2 | 3.7 \pm 0.2 | 4.7 \pm 0.2 | 4.6 \pm 0.2 | 3.8 \pm 0.2 |
| Multivesicular bodies | 0.2 \pm 0.05 | 0.2 \pm 0.05 | 0.2 \pm 0.05 | 0.2 \pm 0.05 | 0.2 \pm 0.05 | 0.3 \pm 0.05 | 0.2 \pm 0.05 | 0.2 \pm 0.05 |
| Dense bodies | 0.8 \pm 0.1 | 1.4 \pm 0.1 | 2.2 \pm 0.15 | 2.4 \pm 0.15 | 2.8 \pm 0.15 | 3.3 \pm 0.15 | 3.1 \pm 0.15 | 3.4 \pm 0.15 |
| Ground substance | 69.3 \pm 0.45 | 68.7 \pm 0.45 | 68.4 \pm 0.45 | 66.9 \pm 0.45 | 68.4 \pm 0.45 | 66.4 \pm 0.45 | 67.9 \pm 0.45 | 68.2 \pm 0.45 |
| Total cytoplasm | 85.1 \pm 0.35 | 84.2 \pm 0.35 | 84.8 \pm 0.35 | 84.1 \pm 0.35 | 84.7 \pm 0.35 | 83.8 \pm 0.35 | 85.2 \pm 0.35 | 84.4 \pm 0.35 |
| Nucleus | 14.9 \pm 0.35 | 15.8 \pm 0.35 | 15.2 \pm 0.35 | 15.9 \pm 0.35 | 15.3 \pm 0.35 | 16.2 \pm 0.35 | 14.8 \pm 0.35 | 15.6 \pm 0.35 |
| Nucleolus (*) | 2.7 \pm 0.4 | 2.2 \pm 0.35 | 1.9 \pm 0.35 | 2.6 \pm 0.4 | 1.9 \pm 0.35 | 2.9 \pm 0.4 | 2.7 \pm 0.4 | 1.8 \pm 0.3 |

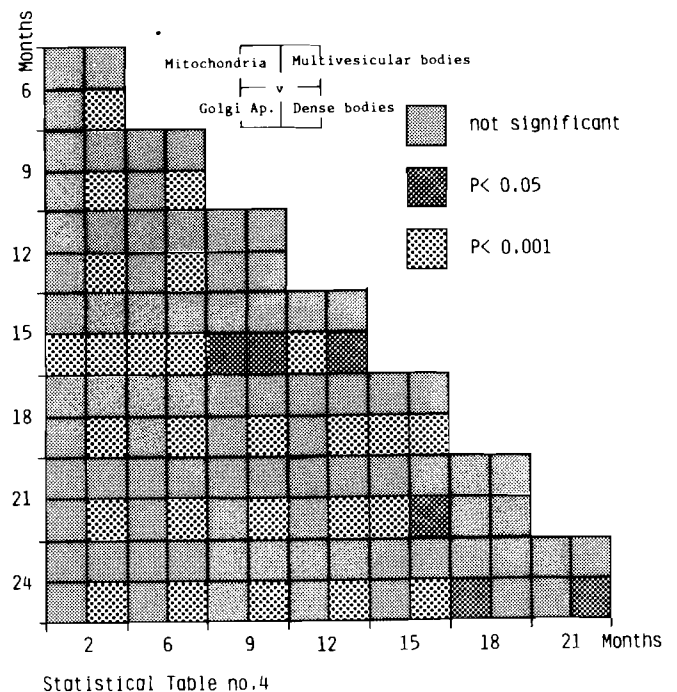
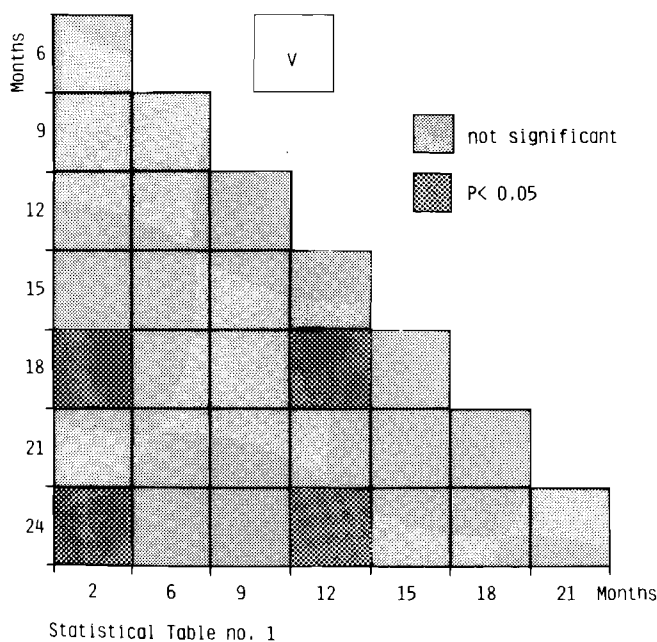
n = 100 profiles per age
 (*) - in relation to the nucleus

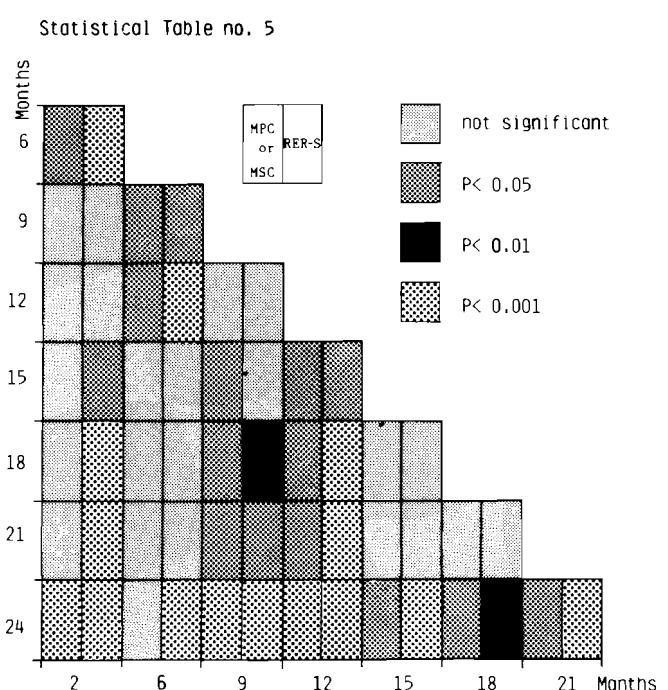
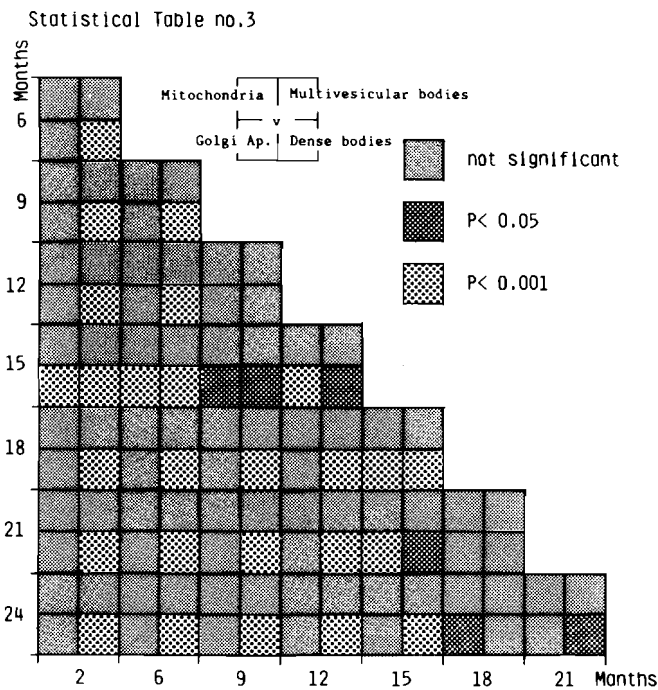
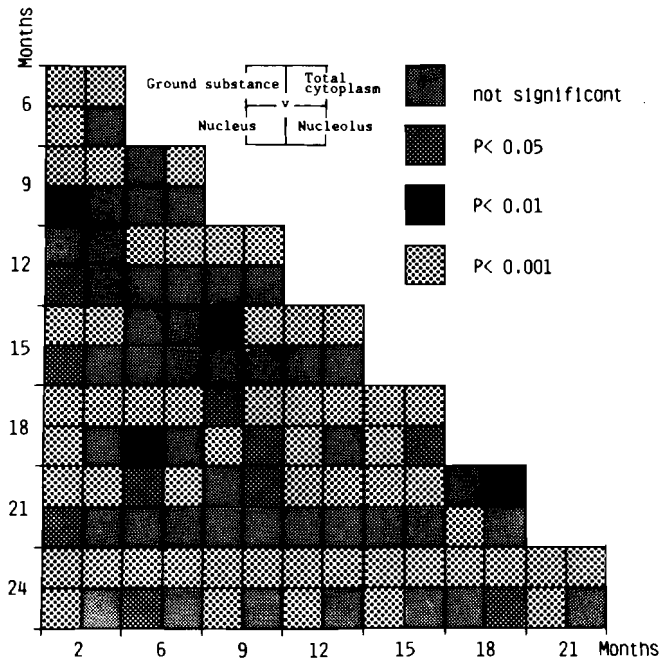
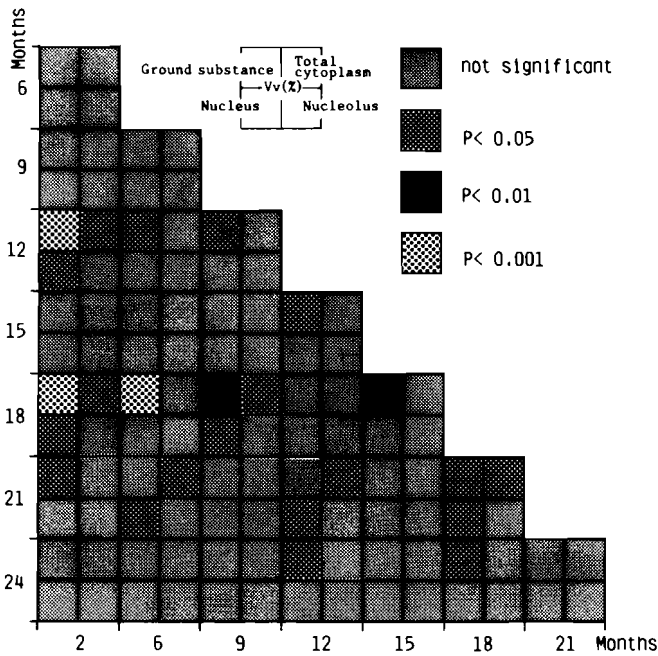
remaining ages, with no exception. The lowest surface values are observed at 2 and 12 M which are not statistically significant between themselves; the opposite is true when any of those values are compared with those concerned with 6 M or with the series of ages ranking from 15 to 24 M. The parameter keeps a positive linear correlation with age ($r = 0.77 - P < 0.05$); this characteristic is not maintained by any of the above mentioned concentrations.

Discussion

From the analysis of a great number of publications a general conclusion may be drawn: the inexorable ageing process is a physiological phenomenon affecting the cells

of all individual of all species. The Purkinje cell is no exception; it is a large neuron with a very complex structure, being considered a paradigm of a nerve cell with a high level of differentiation (Palay and Chan-Palay, 1974). Furthermore, being of paramount importance for motor coordination and because it has a special susceptibility to certain toxics (Chan-Palay and McCrosky, 1976), to hypoxia (Hoff et al., 1945), to malnutrition (Bedi et al., 1980; Bernocchi and Scherini, 1981) and to trauma (Andreoli et al., 1973) its study is of great significance either for neurologists or neuropathologists. Effectively, the increase in life expectancy and the improvements of geriatrics imply that the fixed postmitotic Purkinje cell is prone to more intense age changes which progressively affect the





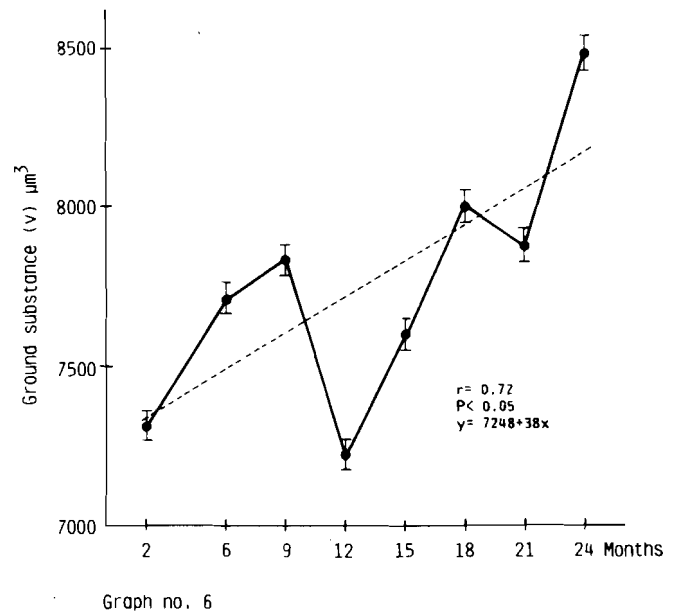
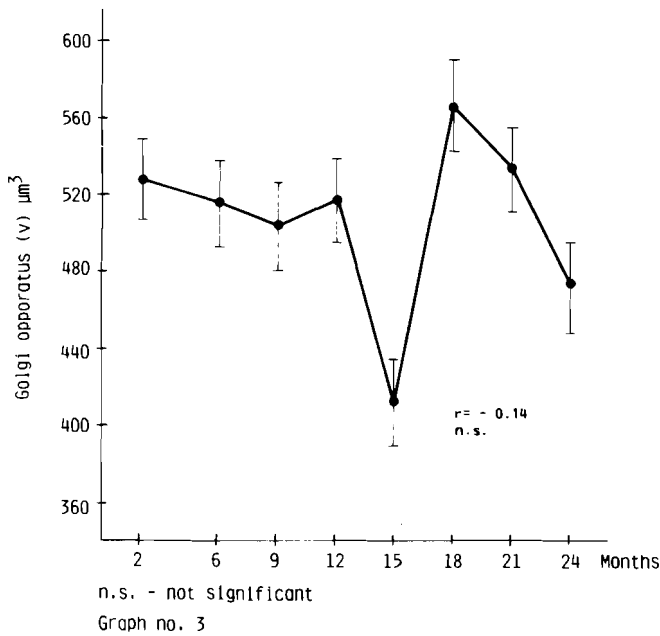
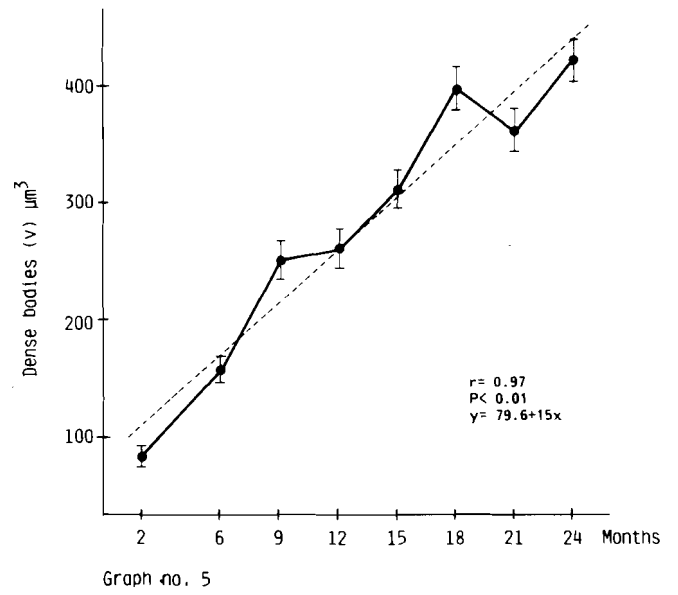
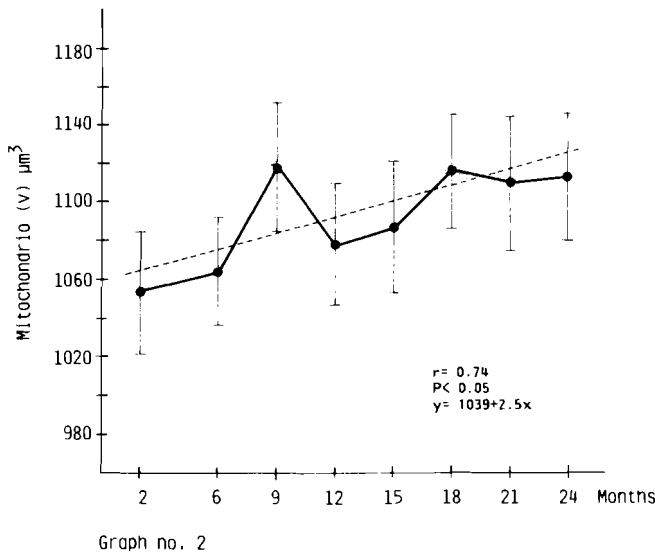
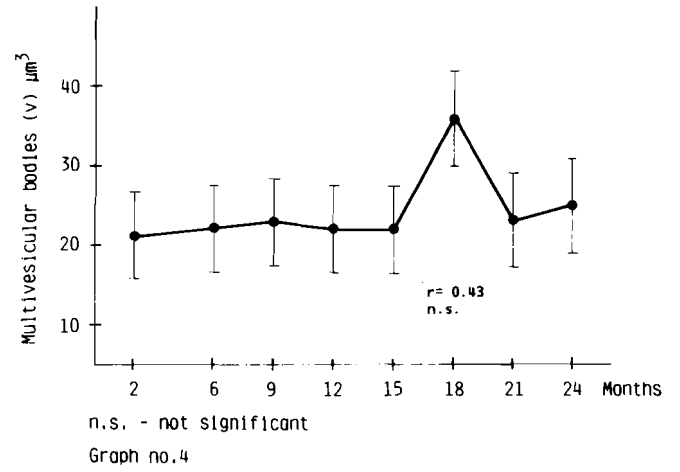
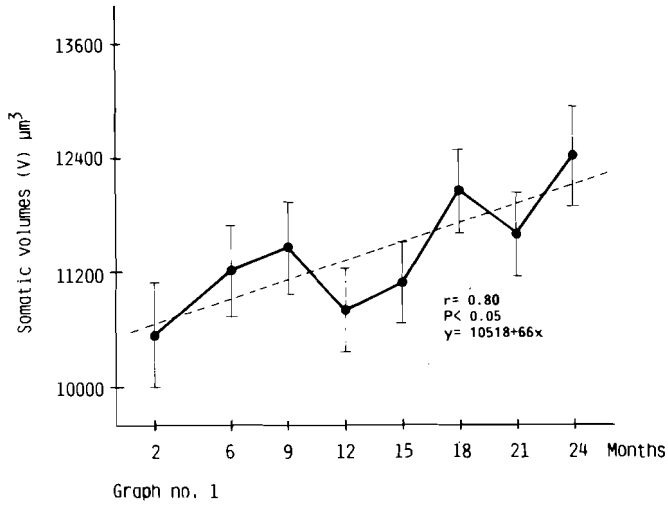
harmony of motor coordination; thence the extreme interest of the study of its microanatomy and the modifications occurring along ageing.

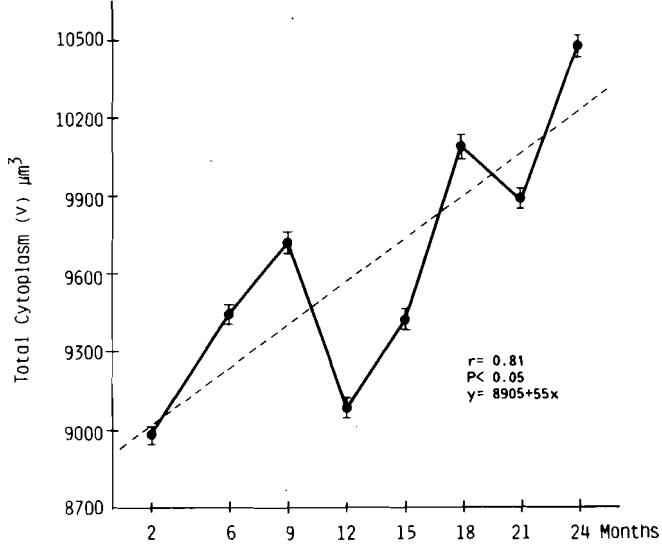
In virtue that the Purkinje cell is not spheroid it was prudent to calculate its volume with the formula of Lindberg and Vorwerk (1970). According to Aherne (1984) the Purkinje cell is a prolate ellipsoid; in fact, this can be easily demonstrated with the test described by Aherne and Dunnill (1982). Because there is no linear

and positive correlation statistically different from zero between the body weight and the mean somatic volume of the neuron, it was interferred that the volumetric fluctuations were not dependent on the body size variations of the animals.

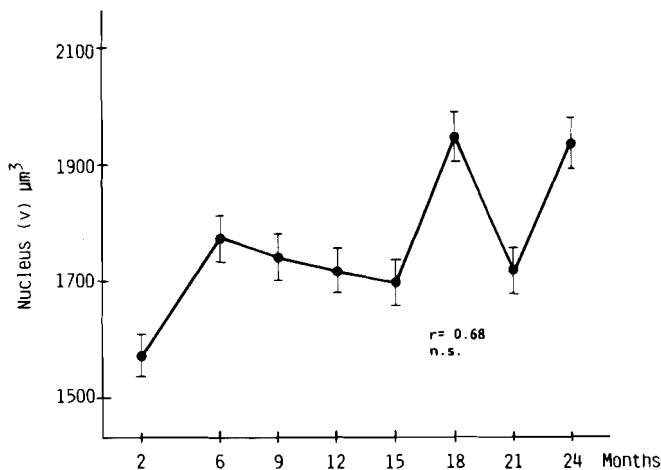
Ruela et al. (1980) found a significantly smaller value for the Purkinje cell volume in the vermis of the Wistar rat as did Hinds and McNelly (1978) in the same zone of the Fisher 344 rat. As Parma (1969) and Parma and

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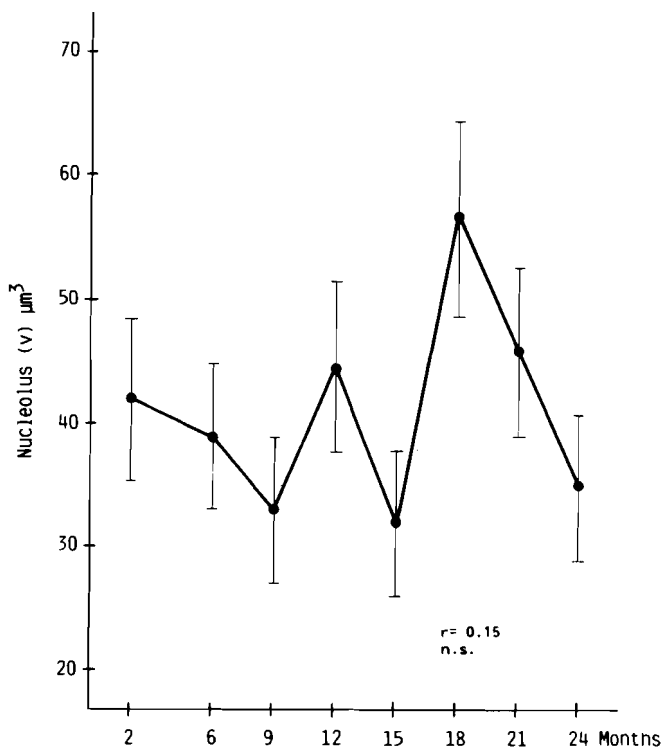


Graph no. 7



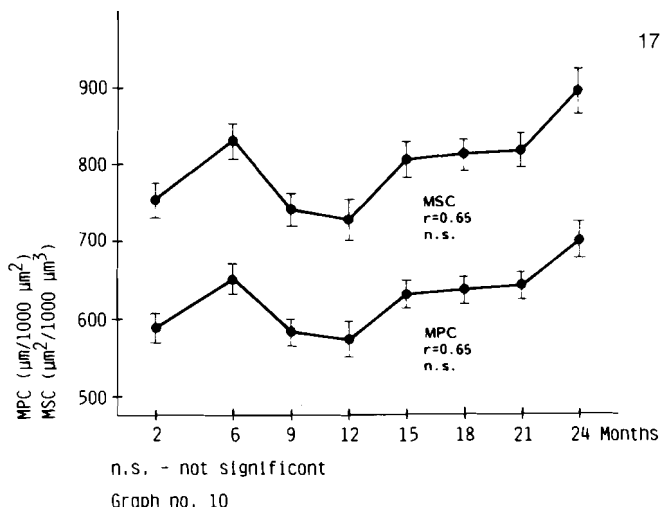
n.s. - not significant

Graph no. 8



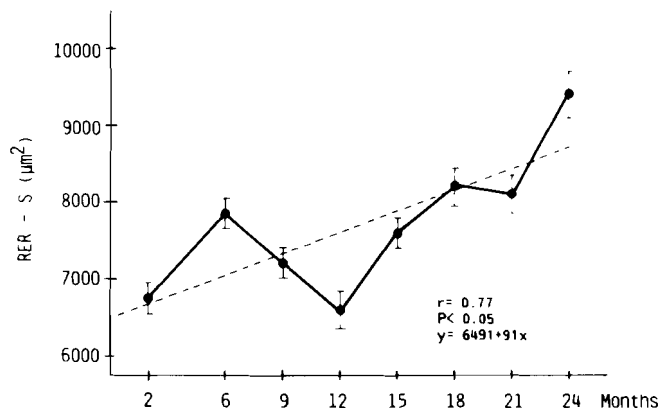
n.s. - not significant

Graph no. 9



n.s. - not significant

Graph no. 10



Graph no. 11

Baldini (1969) came to the conclusion that the paleocerebellar Purkinje cell is larger than its neocerebellar counterpart, it was expected that the cells of the present study (Crus I and Crus II) would then present a smaller volume, a fact which was not so. The discrepancy is feasibly supported by diversities regarding the strain and the methods as well as the regional (or even subregional) differences of the cortical architecture which have been expressed in a number of publications (Lange, 1972a,b, 1974a,b, 1976, 1982; Heinsen, 1981). Moreover, comparing our results with those achieved by several authors in other areas of the encephalon (Hinds and McNelly, 1977; Uemura and Hartmann, 1978, 1979; Vaughan and Vincent, 1979) or even in a distinct zone of the same structure (Hinds and McNelly, 1978) it is concluded that the volumetric variations during ageing does not follow a universal pattern.

It must be stressed that the somatic volume graph displays a bimodal-like design (perhaps even trimodal if the age spectrum had been larger). This must maintain some connection with compensatory hypertrophy phenomena referred to by several workers (eg., Hinds and McNelly, 1977; Frolikis and Bezrukov, 1979) in an attempt to substitute the age-dependent neuronal fall-out observed either in the cerebellar cortex (Ellis, 1920; Inukai, 1928; Boya et al., 1974; Hall et al., 1975; Nandy, 1981) or elsewhere (Maleci, 1934; Brody, 1970; Hinds and McNelly, 1977; Ball, 1977; Bugiani et al., 1978; Hsu and Peng, 1978; Bowen et al., 1979; Sabel and Stein, 1981).

Because the somatic volume is linearly and positively correlated with age, it is important to seek the responsible factors for the occurrence. The mitochondria have their absolute volumes correlated with age ($r = 0.74 - P < 0.05$) and with the cell volume ($r = 0.80 - P < 0.05$). The absolute volumes, however, exhibit a rather slow increase during ageing; in fact, the comparison between any pair of ages is not statistically significant. Therefore, the condrioma does not seem seriously responsible for suggestive variations of the somatic volume, despite the above mentioned relationship.

Neither age nor the cell volume presents a linear correlation with the absolute volumes of the Golgi apparatus. The same is true as far as the multivesicular bodies are concerned. On the contrary, the absolute volumes of dense bodies comparatively establish the most perfect linear correlation during ageing ($r = 0.97 - P < 0.01$). This relationship has been noted by a number of researchers (Strehler et al., 1959; Samorajski et al., 1968; Mann and Yates, 1974; Hinds and McNelly, 1978, 1979; Vaughn and Vincent, 1979). Because the lipofuscin constitutes the largest part of the dense bodies, it is reasonable to infer that there is also age-dependent linear increase of the lipofuscin in the Purkinje cell. The lipopigment content leads to an apparent drastic congestion superior to 400% between the limit ages of the present study, whereas the cell volume only increased about 18%; this reveals the contribution of the absolute volume of the dense bodies for the expansion of the cell volume with which is linearly and positively correlated ($r = 0.84 - P < 0.01$). Nevertheless, the accumulation rate is not constant, presenting a maximum between 2 and 6M (approximately 87%) and a minimum between 9 and 12 M (approximately 3%) with a general average of about 33% from age to age. In addition, it must be emphasized that the increasing absolute volumes of dense bodies seem to be more related to age than to the expansion of the cell volume.

The volumetric fraction of the nucleus significantly modifies during ageing, evidencing that the somatic volumetric variations are neither due to a proportional increase nor to a proportional decrease of the cytoplasm and nucleus. Between the cell volumes and the nuclear absolute volumes there is a linear and positive correlation statistically different from zero ($r = 0.91 - P < 0.01$); nonetheless, looking at their graphic designs, it can be easily deduced that they do not correspond between 6 and 15 M, a time during which the nuclear evolution is independent from the cell volume fluctuations. The slow decrease of the nuclear volume between the above lapse as well as the large decrease seen between 18 and 21 M may explain why the nuclear volume is not correlated with age.

The two parameters in connection with the concentration of RER cisternae (MPC and MSC) exhibit graphical designs with an exact match, owing to the perfect linear correlation ($r = 1$) that exists between them; as a matter of fact, the MSC can be derived from the multiplication of the MPC by $4/\pi$.

Particular details regard the mean surface of RER

cisternae per cell (RER-S) are linearly and positively correlated with age ($r = 0.77 - p < 0.05$); except for the growth from 6 to 9 M. The other age transits follow, in general lines, the volumetric variations of the cell body with which it is also correlated ($r = 0.91 - P < 0.01$). In fact, the highly significant increases ($P < 0.001$) in the parameter coincide with the age transitions in which the somatic volume attains significant increases, too. In virtue that the volumetric fractions of the cisternae (in relation to the total cytoplasm, calculated by multiplying the MSC by the mean thickness of the cisternae) are, however, of a somewhat low value (between 2 and 24 M it only varies from 2.63 to 3.13%), the RER cisternae are not excessively involved in volumetric modifications of the cell body; nevertheless, bearing in mind the magnitude of the correlation, a mechanism of mutual self-regulation in the binary RER/cell volume, probably exists.

In spite of the well stated importance of both the RER and nucleolus as fundamental requisites for protein synthesis, there is no linear correlation between RER-S and the absolute volume of the nucleolus - the coefficient is even negative ($r = -0.005$). A contrary aspect was noted by Mann et al. (1978) when they observed that the quantity of ribosomal RNA and the nucleolus dimensions were proportionally correlated. Some plausible explanations can be advanced concerning the present study: the relative scarcity of the nucleolus as seen in the sections; the lack of a differential morphometric study which might discern whether or not a specific part of the nucleolus (that acting on the production of ribosomal RNA) has indeed its absolute volume correlated with the RER-S; and finally, the reticulum was evaluated by studying their cisternae and not taking into account the quantity of ribosomes attached to their membranes. Thus, the high correlation coefficient obtained between age and RER does not forcibly mean that the ribosomal component of the RER has undergone a real increase. To stress the veracity of the point, the linear density of the attached ribosomes to the reticulum membranes has to be studied. Therefore, it can only be affirmed that there is really an increase in the canaliculi, perhaps in an effort to lessen the difficulties of intracellular transport of proteins, the production of which becomes progressively impaired with the advent of old age.

Because the RER is included as part of that which was designated as ground substance in this study, it might be thought that the volumetric fluctuation of the latter, which presents a high linear trend with the cell volume ($r = 0.97 - P < 0.01$), would be very dependent on the variation of the RER contents. Effectively, that does not seem to be so: the RER occupies a small fraction of the ground substance, which only varies from 3.19 to 3.84% between the limit ages of the spectrum; thus, if its absolute volume is subtracted, the remaining volume (which includes the cytosol, the free ribosomes, the smooth endoplasmic reticulum and the cytoskeleton structures) exhibits the same graphical variations. Therefore, taking into account the considerable proportional contribution of the ground substance and

the match of its volumetric variations with those of the somatic volume, that intracellular component is without doubt the principal modulator of the volumetric plasticity of the Purkinje cell.

This work completes a former study carried out to seek the age-related quantitative changes in axo-somatic synapses around the neuron in discussion (Monteiro, 1990). These could help to integrate microanatomical findings with physiological data (Marwaha et al., 1980; Rogers et al., 1980; Rogers et al., 1981a,b) and biochemical data (Marwaha et al., 1981) in order to reach a comprehensive analysis about the biology of the Purkinje cell. Moreover, the particular cytological data would contribute to explain Purkinje cell physiology up to a certain age; more specifically, they help to understand the changes in inhibitory power which is the ultimate order emitted by the cerebellar cortex via the Purkinje cell axon.

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