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# VITAMIN E DEFICIENCY AS A MODEL OF PRECOCIOUS BRAIN AGING: ASSESSMENT BY X-RAY MICROANALYSIS AND MORPHOMETRY

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## Abstract

Vitamin E ($\alpha$-tocopherol) is a known biological antioxidant able to quench the lipid peroxidation chain and to protect the cellular structures (e.g., plasma membranes) from the attack of free radicals which are reported to play a primary role in aging. To assess whether the absence of $\alpha$-tocopherol from the diet of young laboratory animals may be considered a reliable model of precocious brain aging, intracellular ionic content of brain cortex pyramidal cells, ultrastructural features of synaptic contact zones, synaptic mitochondria and perykarial mitochondria positive to the succinic dehydrogenase (SDH) histochemical reaction with copper ferrocyanide have been investigated by X-ray microanalysis and computer-assisted morphometry in young, adult, old and 11-month-old vitamin E deficient rats. Our data document significant alterations of intracellular ionic content, synaptic contact areas and synaptic and perykarial mitochondria in aging. Vitamin E deficiency caused similar alterations in adult animals. Taking into account the known role of $\alpha$-tocopherol in protecting the cellular membrane structure, we support that the common process underlying the changes found in aging and vitamin E deficiency is an excessive deterioration of the neuronal membrane.

**Key Words**: X-ray microanalysis, morphometry, brain aging, vitamin E deficiency, succinic dehydrogenase activity, aging of synaptic membranes, mitochondrial aging, synaptic mitochondria, perykarial mitochondria, free radicals and aging.

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## Introduction

Aging has been defined as a time-related loss of the cellular capacity to maintain homeostasis. This is particularly true in postmitotic cells, like neurons, which must substitute promptly their damaged molecules with newly synthesized "spare parts". As a consequence of this ongoing turnover of nerve cell basic constituents, aging of the brain is a subtle process and may be thought of as a particular condition in which specific pathological changes are found without clinically evident manifestations: deterioration of function occurs when the number of neurones and neurones' connections decrease below a critical reserve level and when coping with environmental stimulations becomes difficult (Meier-Ruge, 1990; Meier-Ruge *et al.*, 1994). Primary causes of the aging process, as defined above, have been claimed to be free radical attacks originating physiologically from cellular oxidative phosphorylation (Harman, 1981).

Vitamin E ($\alpha$-tocopherol) is a well known biological antioxidant able to quench the lipid peroxidation chain (Burton and Ingold, 1989) and to stabilize the molecular composition of cellular membranes (Giasuddin and Diplock, 1981; Kagan, 1989). Recently, vitamin E has been reported to play a specific neurological function in human beings (Muller *et al.*, 1983; Nelson, 1989; Sokol, 1989) and over the past decade, an isolated vitamin E deficiency syndrome, in absence of fat malabsorption, has been associated with neurologic dysfunction (Sokol *et al.*, 1988). In addition to the biochemical evidence of vitamin E deficiency, the affected patients show many hallmarks typical of aging (e.g., swollen dystrophic axons, lipofuscin accumulation in dorsal root sensory neurons, reduction of axonal transport) which have also been found in rats and rhesus monkeys fed a vitamin E deficient diet (Stumpf *et al.*, 1987; Yokota *et al.*, 1987, Southam *et al.*, 1991). This similarity of alterations suggested to us that vitamin E deficiency in laboratory animals may be considered a model of precocious brain aging; the present paper reports the results of our experiments performed to assess the reliability of our assumption by X-ray microanalysis and computer

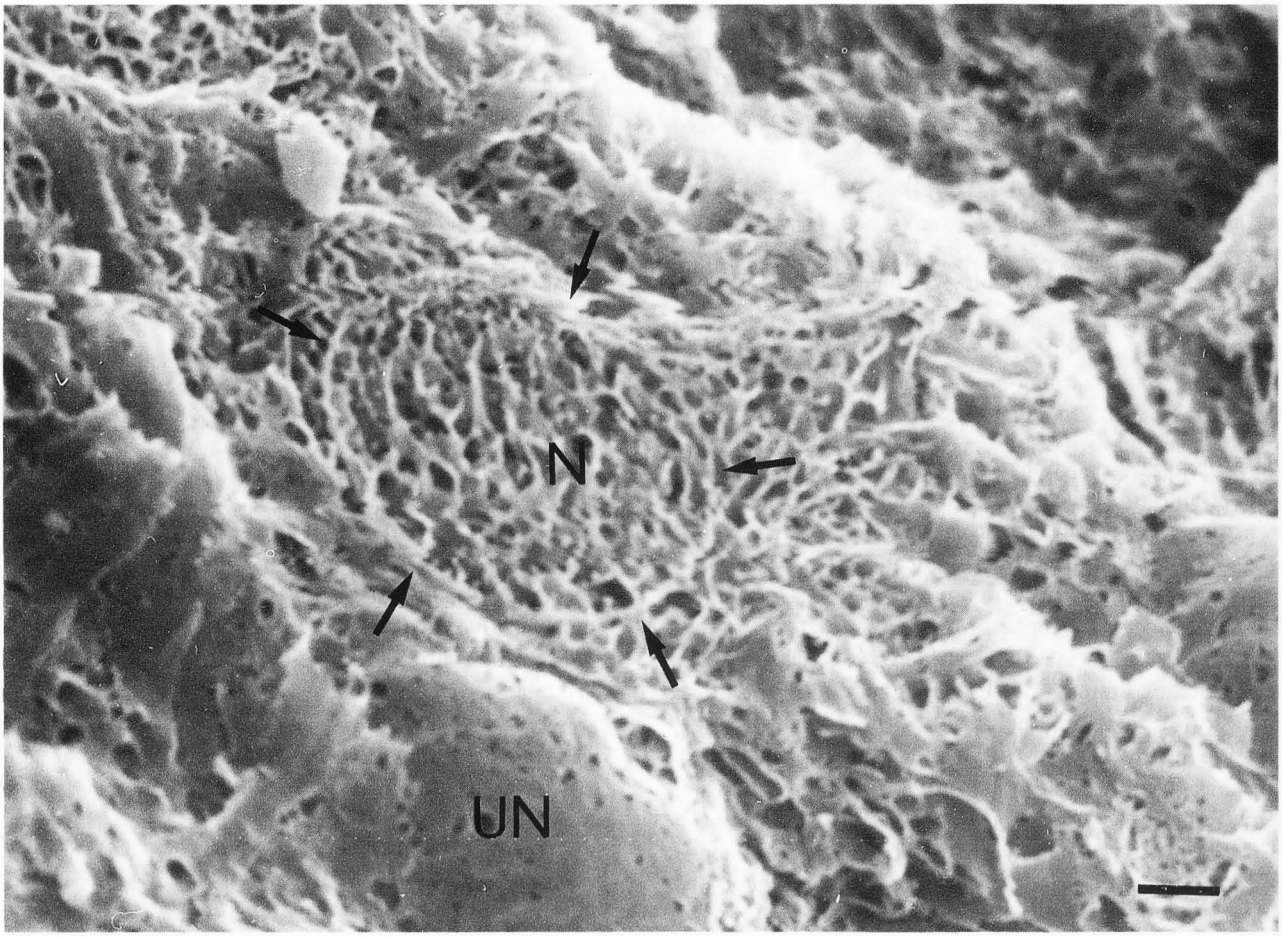


**Figure 1.** Scanning electron image of a freeze-fractured, freeze-dried BCPC prepared according to Zs-Nagy *et al.* (1977). The nuclear membrane is easily identifiable (arrows). N = nucleus; UN = unfractured cell. Bar = 3 $\mu$m.

assisted morphometry in the central nervous system (CNS) of young, adult, old and vitamin E deficient adult rats.

## Material and Methods

### Animals and diet

Female Wistar rats of our breed were used for the present study. Rats from 1 to 3 months of age were considered young. The age of the adult groups ranged between 11 and 12 months. The old groups were composed of 24- to 30-month-old animals. Each group that was analysed consisted of at least 5 rats. Young animals of 1 month of age were fed for 10 months with a vitamin E deficient diet of the following composition: 58% maize starch and sucrose, 18% extracted casein, 10% torula yeast, 8% melted filtered fat, 5% Osborne-Mendel salts and 1% vitamin supplement lacking vitamin E. A long period of vitamin E deficiency was necessary to induce exhaustion of the CNS stores of this vitamin according to the data reported by other authors (Muller and Goss-Sampson, 1989). Loss of weight since the age of 4 months and progressive loss of hair were the macroscopic signs of this diet.

### X-ray microanalysis

The brains of 1-, 12- and 24-month-old animals were quickly exposed from the skull after decapitation. The tissue samples were excised and processed according to a method elaborated in our laboratory (Zs-Nagy *et al.*, 1977). Briefly, these are the main steps of our technique: (I) deep freezing of the tissue samples in isopentane cooled by liquid nitrogen; (II) fracturing of the frozen tissue by a pair of scissors; (III) freeze-drying in a vacuum evaporator; (IV) analysis of the dried samples by X-ray microanalysis in secondary electron image mode. Twenty brain cortex pyramidal cell (BCPC) nuclei were measured per animal and this yielded 100 measurements per group of age. Our X-ray microanalytic method enabled us to measure light elements, i.e., $Na^+$, $Cl^-$, $K^+$, within the dried cells. Quick freezing and dehydration of the tissue samples at increasing temperatures from -100°C up to +24°C in a vacuum

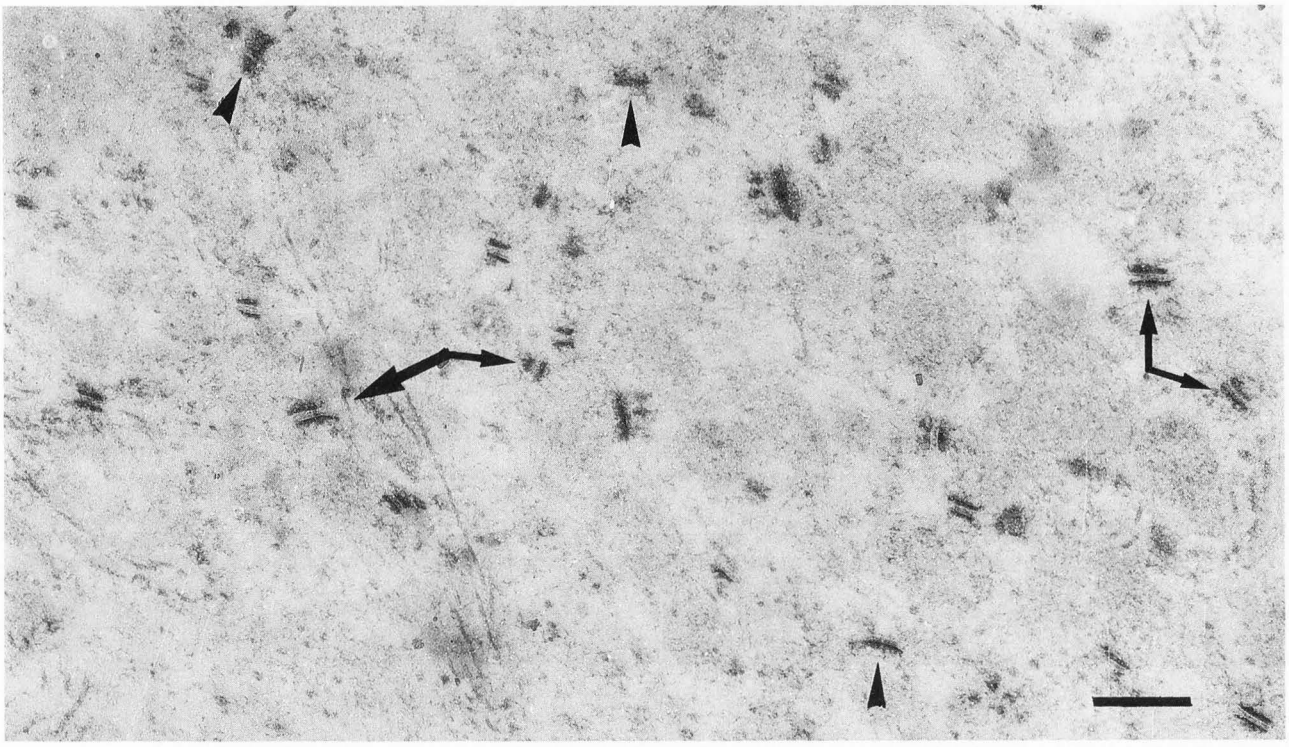

**Figure 2.** Electron micrograph of E-PTA stained synaptic junctions appearing as parallel black lines or as a dotted and full line against a faint background (longer arrows). The single synaptic densities (arrowheads) were automatically eliminated by the computer program of our image analysis system. Bar = 0.5 $\mu$m.

evaporator were carried out in order to avoid ion displacements from one cell compartment to another. Although fine ultrastructural details of the nerve cells could not be seen by means of our procedure, we were always able to recognize neuronal nuclei and measure their ionic content (Fig. 1). As described elsewhere, measurements were carried out in dried material: we first calculated the intranuclear electrolyte content as a peak to background ratio and then as a percent of biological dry mass (Zs.-Nagy *et al.*, 1977). White counts were taken always from the 4 to 6 keV range where no characteristic peaks are present.

### Synaptic morphometry

Rats of 3, 11 and 28 months of age were used for synaptic investigations in the cerebellum. In our hippocampal studies, we used animals of 3, 12 and 30 months of age. The vitamin E deficient group consisted of the same 11-month-old animals used for the above studies. Hippocampal and cerebellar tissue samples freshly excised from the skull were fixed in 2.5% phosphate buffered glutaraldehyde for 2 hours and kept overnight in the same solution at 4°C. According to our previous papers (Bertoni-Freddari *et al.*, 1986, 1988, 1990, 1994), the ethanol phosphotungstic acid (E-PTA) preferential technique was used to evidence selectively the synaptic paramembranous material (Fig. 2); following the dehydration steps in ethanol, the tissue samples were stained for 1 hour in 1% ethanol solution of PTA at 60°C. Embedding and sectioning of the tissue samples were carried out according to conventional electron microscopic procedures. Morphometry of synaptic contact areas was performed on 8,000x electron microscopic negatives. Twenty micrographs were taken per animal and this yielded 100 fields to be measured per age and vitamin E deficient group. The number of synapses/$\mu m^3$ (numerical density: Nv), the total area of the synaptic contact zones/$\mu m^3$ (surface density: Sv) and the average synaptic area (S) were semiautomatically calculated by a computer assisted image analyser properly programmed by ourselves applying the morphometric formulas reported in our previous paper (Bertoni-Freddari *et al.*, 1988).

### Mitochondrial morphometry

Rats of 3, 12 and 24-26 months and the 11 vitamin E deficient animals were used for mitochondrial investigations. Anaesthetized rats were perfused through the left ventricle with saline followed by fixation solution {2% glutaraldehyde, 4% formaldehyde, 5 mM $CaCl_2$ in 0.1 M cacodylate buffer (pH 7.4)}. The fixative was allowed to flow for 30 minutes after the animal's death, then the cerebellar cortex was excised. Tissue samples were processed for electron microscopy according to

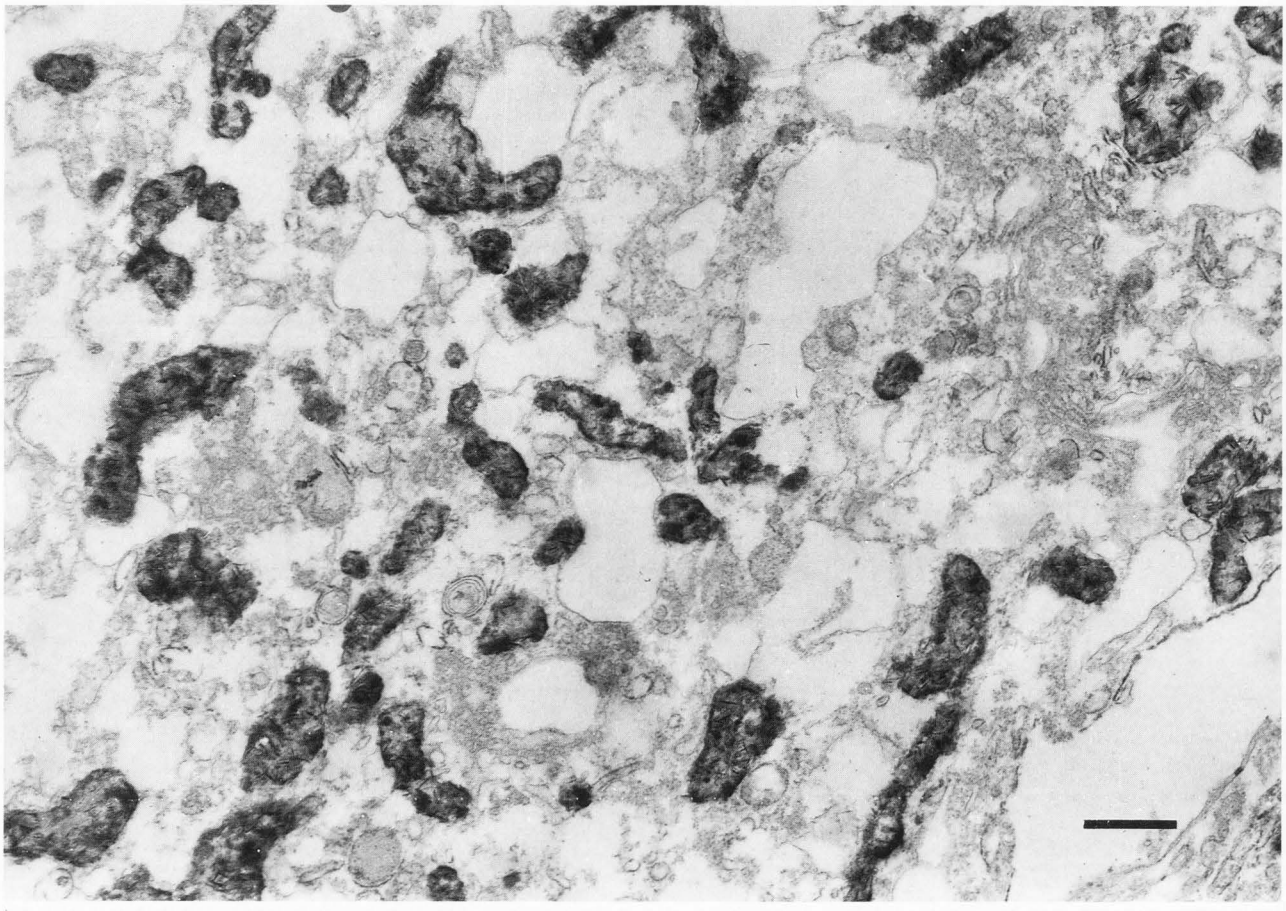

**Figure 3**. Purkinje cell perykarial mitochondria positive to the copperferrocyanide histochemical reaction evidencing the activity of succinic dehydrogenase. The surrounding background is rather faint since the reaction was performed in unfixed tissue samples. Bar = 1 $\mu$m.

conventional procedures. Twenty electron micrographs (EM) taken at 8,000x from the cerebellar cortex were analysed per animal in order to obtain 100 micrographs per group. As for synaptic morphometry, the EM negatives were analysed by the semiautomatic image analyser applying the formulas reported in our previous paper (Bertoni-Freddari *et al.*, 1993). The number of mitochondria/$\mu m^3$ (numerical density: Nvm), the total mitochondrial volume/$\mu m^3$ (volume density: Vv) and the average length (skeleton: Sk) of the single organelle were the parameters taken into account.

### Morphometry of succinic dehydrogenase (SDH)-positive mitochondria

Rats of 3, 12 and 24 months of age and the 11 vitamin E deficient animals were used for the present investigation. Freshly excised cerebellar cortices were sectioned into 100 $\mu$m thickness slices by means of a McIllwain tissue chopper. The histochemical identification of SDH activity was carried out by incubating the cerebellar samples in potassium ferricyanide as electron acceptor as reported by Kerpel-Fronius and Hajos (1968) (Fig. 3). By using the same formulas applied for the organelles investigated in the cerebellar glomerulus, we measured Vv and Nvm on Purkinje cell SDH-positive mitochondria. At variance with the investigation carried out on glomerular mitochondria, by the ratio Vv/Nv, we calculated automatically the average volume (V) of the single positive mitochondrion: since SDH activity is directly related to the total surface area of the inner mitochondrial membrane, this parameter better reports on the size of active organelles. Statistical comparisons were performed by the Student's t-test in each experiment reported here.

## Results

### Intranuclear ionic content of BCPC

First of all, it must be pointed out that the intranuclear measurements of electrolyte content performed by us should be considered as reporting on the actual ionic concentration of the whole nerve cell since the

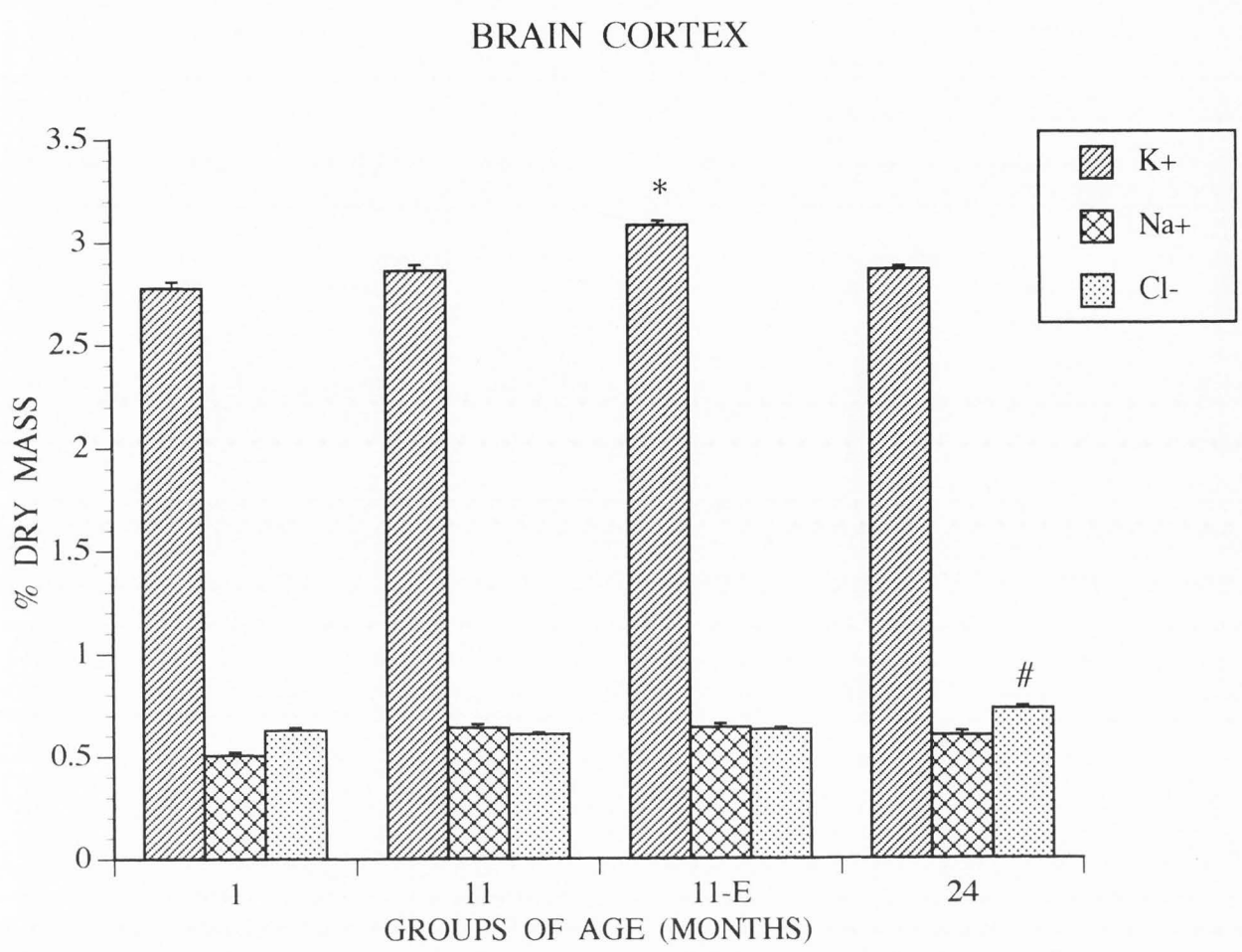


**Figure 4**. Intracellular ionic content of BCPC. The values are expressed as percent of dry mass; * $p < 0.001$ vs. 1-, 11- and 24-month-old controls; # $p < 0.001$ vs. 1-, 11-, and 11-month-old vitamin E deficient group.

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nuclear membrane represents only a virtual permeability barrier between nucleoplasm and cytoplasm. In the nucleus, the ionic content appears to be higher because of the higher amount of water. The results of our microanalytic investigation on BCPC are summarized in Figure 4. In our previous paper (Bertoni-Freddari *et al.*, 1981), we reported that the $K^+$ content significantly increases with age at 11 and 24 months. $Na^+$ content is unchanged between 11 and 24 months of age, but it is significantly higher in the 11-month-old rats than the 1-month-old rats. The $Cl^-$ content significantly decreases at 11 months of age and is significantly higher in old animals than in the other groups investigated. Vitamin E deficiency resulted in an increase of the total intracellular ionic content due to $K^+$ ions. In a comparison with the old group of animals, the adult vitamin E deficient group showed a significant increase of $K^+$, while $Cl^-$ appeared to be significantly lower and $Na^+$ remained unchanged.

**Ultrastructure of cerebellar and hippocampal synaptic junctional areas**

The synaptic ultrastructural features were measured in two discrete CNS zones: the cerebellar glomerulus and the dentate gyrus supragranular layer. While the former is innervated by fibers using different neurotransmitter substances, the latter is reported to receive cholinergic inputs from the septal nuclei. Both of these two areas met Weibel's (1979) basic morphometric criteria and were considered reliable anatomical models to perform our investigations. Table 1 reports the results we got on the cerebellar synapses. Nv was significantly increased and Sv was unchanged between 3 and 11 months of age, but both these parameters were significantly decreased in the 28-month-old group. S was significantly lower in adult animals than in both young and old rats, while it was the same in the latter two groups. Vitamin E deficient animals unlike normally fed littermates showed significant decreases of Nv and Sv, while

**Table 1**. Morphometry of synaptic junctions in the cerebellar glomerulus. Mean ± S.E.M..

| AGE (months) | Nv No. syn./$\mu m^3$ | S $\mu m^2$ | Sv $\mu m^2/\mu m^3$ |
|---|---|---|---|
| YOUNG (3) | 1.6499 ± 0.0624 | 0. 0858 ± 0.0047 | 0.1207 ± 0.0039 |
| ADULT (11) | 1.8001 ± 0.0428 | 0.0747 ± 0.0022 | 0.1226 ± 0.0019 |
| OLD ( 28) | 1.3869 ± 0.0 734 | 0.0863 ± 0.0051 | 0.0961♠ ± 0.0034 |
| -VIT.E (11) | 1.4440♣ ± 0.0354 | 0.0787 ± 0.0026 | 0.1045♦♣ ± 0.0022 |

♦ $p < 0.05$ vs. the old group;
♣ $p < 0.001$ vs. control littermates;
♠ $p < 0.001$ vs. normally fed animals.

**Table 2**. Morphometry of synaptic junctions in the dentate gyrus supragranular layer. Mean ± S.E.M..

| AGE (months) | Nv No. syn./$\mu m^3$ | S $\mu m^2$ | Sv $\mu m^2/\mu m^3$ |
|---|---|---|---|
| YOUNG (3) | 0.4723 ± 0.0091 | 0.1574 ± 0.0029 | 0.0720 ± 0.0012 |
| ADULT (11) | 0.5392♣ ± 0.0105 | 0.1369 ± 0.0031 | 0.0718 ± 0.0013 |
| OLD (30) | 0.3810 ± 0.0073 | 0.1738♣ ± 0.0029 | 0.0650 ± 0.0012 |
| -VIT.E (11) | 0.4127 ± 0.0070 | 0.1507♦ ± 0.0032 | 0.0611 ± 0.0012 |

♣ $p < 0.001$ vs. the other groups;
♦ $p < 0.01$ vs. the adult group;

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S was not significantly increased. Comparing the vitamin E deficient group with old rats, we found that Nv and S were not significantly different, but Sv was higher in the deficient animals. Table 2 shows the results of synaptic morphometry in the hippocampal dentate gyrus. Nv significantly increases in the normally fed adult animals, in contrast with the other age groups. Sv is unchanged between 3 and 11 months of age, but significantly decreases in the adult animals. S is significantly smaller at 11 months of age and is larger in the old rats than in the other age groups analysed. Vitamin E deficiency, unlike control condition, resulted in significant decreases of Nv and Sv, while S was not significantly increased. In a comparison with 30 month-old animals, the vitamin E deficient group showed a significant decrease of Sv and S, while Nv was significantly increased. Figure 5 reports a percentage distribution of S in both the CNS areas investigated. It is clearly evident that, with advancing age, the percentage of larger contact areas markedly increases. Vitamin E deficient animals, when compared with control littermates, showed higher percentages of larger junctional areas, particularly in the hippocampus; in the cerebellar glomerulus, only a trend towards larger contact zones was envisaged. Old animals, in contrast to adult vitamin E deficient rats, showed a higher percentage of larger synapses in the cerebellum; in the hippocampus, the percent of synaptic population accounting for contacts larger than 0.16 $\mu m^2$ was around 70% in the deficient group, whereas in the old normally fed rats, it was 40%.

### Ultrastructure of synaptic mitochondria

It must be pointed out that the categorization of the mitochondria present at nerve terminals with the term "synaptic" does not refer merely to the anatomical location of the organelles, but it also identifies a well compartmentalized population of mitochondria from a morphofunctional point of view (Lai *et al.*, 1977). Namely, it has been reported by several authors that the mitochondria present at synaptic regions, while being closely coupled with actual synaptic performances, are also structurally adapted to the energy needs of the neural network they subserve. This is evidenced also by the different molecular composition of mitochondrial membranes between perykarial and synaptic organelles within the nerve cells (Harmon *et al.*, 1987; Villa *et al.*, 1989). Table 3 reports the results of our morphometric studies on synaptic mitochondria in the cerebellar glomerulus. Our investigation shows that Vv is constant throughout the whole life-span. Nv increases significantly between

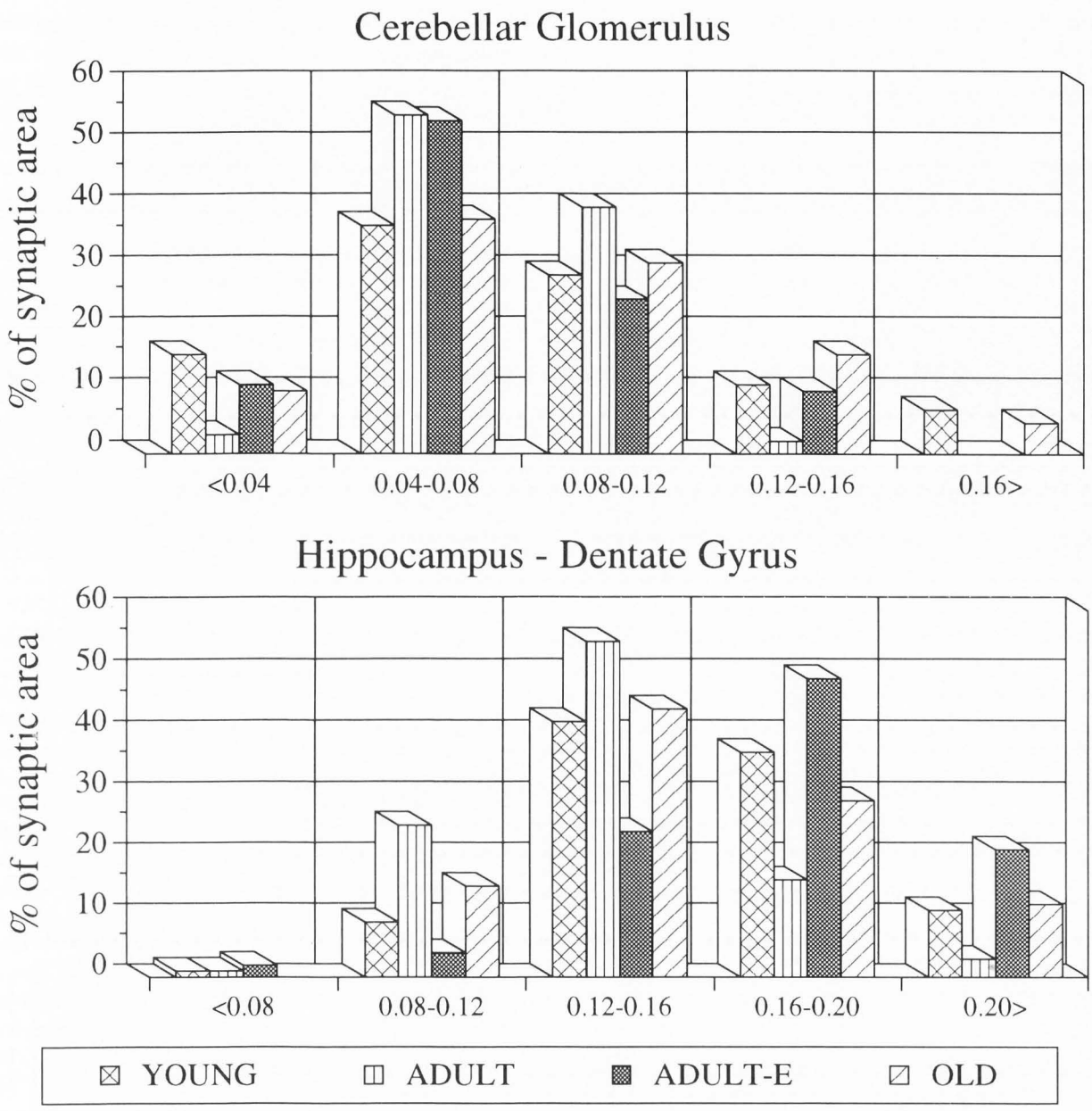


**Figure 5**. Percentage distribution of the synaptic average area (S) in the cerebellar glomerulus and hippocampal dentate gyrus. In the former CNS area, the size range of synaptic area spans from < 0.04 to 0.16 > $\mu m^2$, while in the latter, it ranges from < 0.08 to 0.20 > $\mu m^2$. It appears evident that different CNS zones show different synaptic size.

3 and 11 months of age, while it is significantly decreased in old animals. Sk decreases significantly, by 30%, in adult rats when compared to the young ones, but is 44% greater in old animals than in the young group and 64% greater in old rats than in the adults. Vitamin E deficiency, in a comparison both with control littermates as well as with old animals, resulted in no change of mitochondrial Vv, a significant increase of Sk and a significant decrease of Nv. Figure 6 reports a percentage distribution of Sk: aging appears to be characterized by a higher percentage of longer mitochondria. In the vitamin E deficient group, the percentage of elongated organelles (from 3.75 $\mu m$) accounts for 44% of the whole mitochondrial population, while in old, normally fed animals this type of organelles is 31%.

### Ultrastructure of SDH-positive perykarial mitochondria

Although SDH histochemical detection by the copper-ferrocyanide method cannot be taken as a sign of the

**Table 3**. Morphometry of synaptic mitochondria in the cerebellar glomerulus. Mean ± S.E.M..

| AGE (months) | Vv $\mu m^3/\mu m^3$ | Nv No.mit./$\mu m^3$ | Sk $\mu m$ |
|---|---|---|---|
| YOUNG (3) | 0.1334 ± 0.0077 | 0.7268♣ ± 0.0301 | 1.9012♣ ± 0.0512 |
| ADULT (11) | 0.1142 ± 0.0071 | 0.9911♣ ± 0.0368 | 1.3221♠ ± 0.0432 |
| OLD (24-26) | 0.1311 ± 0.0073 | 0.5000 ± 0.0297 | 2.9041 ± 0.0713 |
| -VIT.E (11) | 0.1153 ± 0.0074 | 0.3745♣ ± 0.0172 | 3.2712♦ ± 0.0107 |

♣ $p < 0.001$ vs. all the other groups;
♦ $p < 0.01$ vs. the old group;
♠ $p < 0.02$ vs. the old group

**Table 4**. Morphometry of SDH-positive perykarial mitochondria in Purkinje cells. Mean ± S.E.M..

| AGE (months) | Vv $\mu m^3/\mu m^3$ | Nv No.mit./$\mu m^3$ | V $\mu m^3$ |
|---|---|---|---|
| YOUNG (3) | 0.0679♦ ± 0.0029 | 0.3771♦ ± 0.0115 | 0.2041♦ ± 0.0110 |
| ADULT (12) | 0.0662 ± 0.0029 | 0.4111 ± 0.0123 | 0.1742♠ ± 0.0092 |
| OLD (24) | 0.0558 ± 0.0034 | 0.3226 ± 0.0114 | 0.1587 ± 0.0092 |
| -VIT.E (11) | 0.0444♣ ± 0.0028 | 0.3701 ± 0.0275 | 0.1304♥ ± 0.0068 |

♣ $p < 0.001$ vs. all the other groups;
♦ $p < 0.01$ vs. the old group;
♥ $p < 0.001$ vs. 3 months
♠ $p < 0.01$ vs. vitamin E deficient group

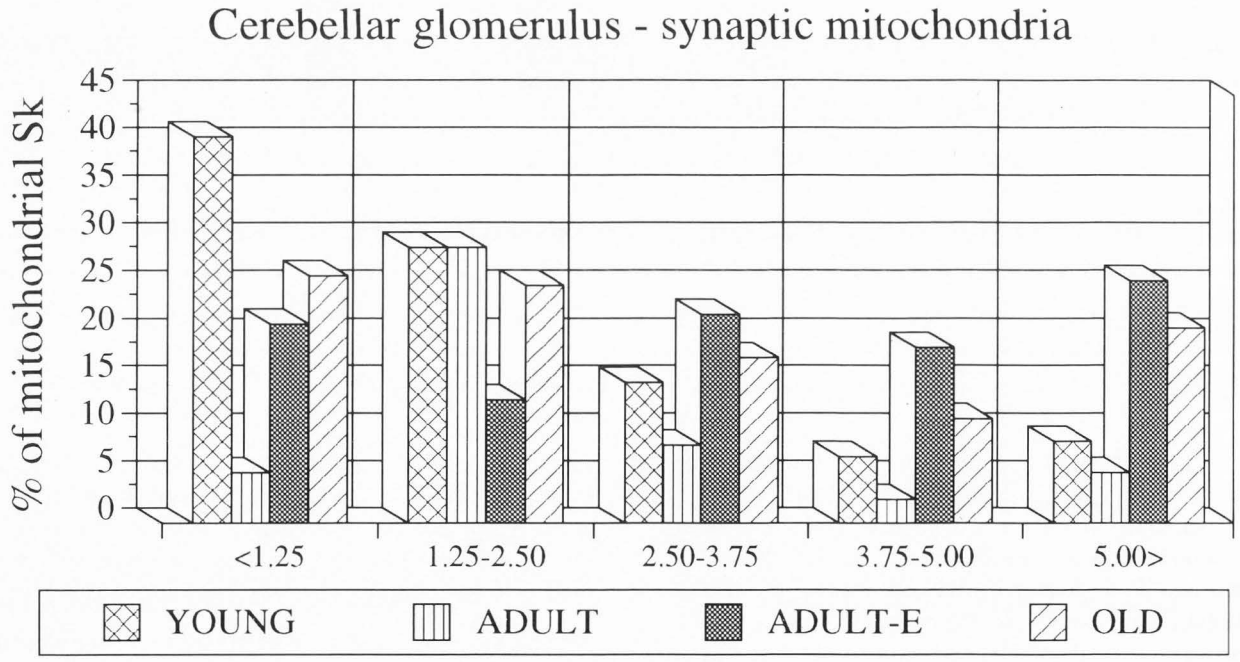


**Figure 6**. Percentage distribution of synaptic mitochondrial Sk from the cerebellar glomerulus. Higher percentages of longer organelles are found both in aging and $\alpha$-tocopherol deficiency.

actual activity of this important key-enzyme of the Krebs' cycle, nevertheless, the positive mitochondria are to be taken as active units of the neuronal metabolism. Table 4 show the results of our study. Vv is constant between 3 and 12 months of age, but in old rats, it is significantly decreased. Nv increases in adult, and not young, animals, but in 24-month-old rats, it is significantly lower then in the other groups of age. V is significantly less in adults than in the young group and it further decreases in old animals. The vitamin E deficient rats showed no change in Nv and a marked decrease of Vv in a comparison with all the age groups investigated. In the vitamin E deficient animals, V is significantly less than in the 3- and 12-month-old normally fed animals, but it is unchanged when compared with the 24-month-old group. Actually, the sampling procedures regarding the vitamin E deficient group are still in progress, thus the present findings are to be regarded as preliminary.

## Discussion

Alterations of the intracellular ionic content, deterioration of synaptic contact areas, and synaptic and perykarial mitochondria are the changes due to age documented by the present study. Vitamin E deficiency from 1 to 11 months of age resulted in changes very similar to those found in old animals, thus, it is reasonable to assume that, both in aging and $\alpha$-tocopherol deficiency, common mechanisms are operating to carry on similar neuronal deteriorations. While no clear-cut process can be hypothesised to give proper explanations for the many impairments occurring in old nerve cells, vitamin E is reported to be involved in several defense processes, the final aim of which is to protect and stabilize cellular membranes (Erin *et al.*, 1984; Kagan, 1989; Niki *et al.*, 1989). Conceivably, a consistent deterioration of neuronal membranes may be hypothesised as an unfavourable condition able to play a pivotal role in the progressive, age-dependent decline of brain performances, and in some pathological degenerative states typical of the senile brain, such as Alzheimer's disease (Bertoni-Freddari, 1988). The present findings appear to support this contention.

### Intracellular ionic changes

The remarkable result of this study is represented by the overall increase of the intracellular ionic concentration in aging and in vitamin E deficiency. This appears to be mainly due to the significant increase of $K^+$. This electrolyte has been reported to contribute to a large extent in the regulation of the intracellular ionic strength. It is well proven that histones neutralize the negative groups of DNA, but within the nucleosomes only 15% to 50% of the charges are neutralized and the result is that chromatin is negatively charged (Brust, 1986). Neutralization of chromatin negative groups is very important for DNA stabilization and it is accomplished also by maintaining, in a physiological range, the intracellular concentration of positive ions (Brust, 1986; Higgins *et al.*, 1987; von Zglinicki and Bimmler, 1987). In old animals and adult vitamin E deficient rats, the reported increase of $K^+$ may affect the physicochemical properties of chromatin through sterical hindrance of enzymes, displacements of histones, abnormal DNA unfolding, etc. In turn, altered nuclear functions (e.g., template activity) may also impair the ongoing supply of "spare parts" to the nerve cell terminal regions and contribute to the degeneration of the synaptic junctional areas. In addition to these changes specifically relevant to DNA performances, an increased concentration of ions in the cytoplasm may, in turn, alter the physicochemical properties of the cellular colloidal system, which results in a longer lifetime of the enzyme-substrate complexes (Damjanovich *et al.* 1983). The increased intracellular $K^+$ content of BCPC can find a possible explanation in the relevant changes of lipid composition of the cellular membranes (decreased unsaturation of their fatty acids) reported both in aging and vitamin E deficiency (Sun and Sun, 1979). As a matter of fact, if cellular membranes are more rigid, the $K^+$ passive flow to the extracellular space is markedly impaired. In addition, the proper functioning of the $Na^+$ - $K^+$ pump is tightly coupled with the surrounding lipid environment and any decrease in membrane fluidity has been reported to stimulate its activity (Sun and Sun, 1978). In both these conditions, it can be assumed that $K^+$ ions are pumped into the cells at a high rate, but they are slowly eliminated with the final result of an increased intracellular $K^+$ concentration.

### Changes of the synaptic ultrastructural features

In summation of our present findings on synaptic ultrastructure, it is evident that, both in aging and in vitamin E deficiency, the number of contacts decreases and, despite a significant increase of the average size of the persisting junctional zones, the final outcome is a significant reduction of the total synaptic contact area. An analysis of the size composition of the synaptic population in both the CNS areas, considered as anatomical models for our investigation (Fig. 5), shows that, from adulthood to old age, the percent of enlarged contact areas increases. This significant increase of the synaptic size has been reported by several investigations, not only in aging and time-related pathologies (Bertoni-Freddari *et al.*, 1988, 1990; DeKosky and Scheff, 1990), but also in experimental stimulation paradigms of the synaptic performances (Siekevitz, 1985; Geinisman *et al.*, 1988,

1990). On the basis of reliable morphological findings from different research groups (Carlin and Siekevitz, 1983; Greenough and Bailey, 1988; Calverley and Jones, 1990), it is currently supported that synaptic enlargement should be considered as a well-categorized step of synaptogenetic processes aiming at modulating the morphology of the terminal areas as an adaptive response to repeated environmental stimulations. Namely, the cycle to improve the neural hardware for a better and proper reply appears to involve the following steps: I) enlargement of the contact areas, II) perforation and III) fragmentation and splitting into several smaller synaptic clods which, upon stimulation, can undergo a new cycle of this functional synaptogenetic process or be deafferented and die. This is a still debated remodelling hypothesis, nevertheless, it reasonably accounts for a dynamic mechanism of synaptic morphofunctional rearrangements. The trend to synaptic enlargement (Fig. 5) can be seen also in the cerebellum of vitamin E deficient rats when compared to their littermates up to 0.16 $\mu m^2$, but over this size we found only a small percent of contacts in young and old rats. At variance with these data, in the hippocampal dentate gyrus of the adult vitamin E deficient rats, we found that, from 0.16 $\mu m^2$, the percent of larger synapses is higher than in any other age group considered. A possible explanation of this observation may be found in the different vulnerability of these two areas to aging and support the idea that an increased peroxidative stress, carried out in absence from the diet of the biological antioxidant $\alpha$-tocopherol, may cause an impaired synaptic resharpening of the synaptic contact zones following reactive synaptogenetic phenomena.

Synaptic junctions are functionally differentiated areas of the neuronal membrane very abundant in double bonds, as clearly evidenced by osmium tetroxide staining. While the molecular composition of these peculiar sites of the nerve cell membrane entails them a high degree of fluidity, which is a necessary prerequisite for their proper performances and dynamic plasticity, it also constitutes the structural feature determining their high vulnerability to peroxidations, as appears to be supported by our present findings.

## Changes in the ultrastructure of synaptic mitochondria

The synaptic mitochondria in the cerebellar glomerulus decrease in number, while their size increases and the resulting total neuropil volume occupied by these organelles is maintained constant throughout the whole life-span. The vitamin E deficient group showed a decrease in the number of organelles and a paired increase in their average length, while the total mitochondrial volume/$\mu m^3$ of tissue appears unchanged from that of the other age groups. As for synaptic average size, the percentage distribution of mitochondrial length shows that, from the beginning of adulthood on, there is a consistent increase of the percentage of longer organelles; this is also remarkably evident in the vitamin E deficient group when compared to the adult littermates: the histograms regarding old and vitamin E deficient groups are fairly paired in any mitochondrial size class considered (Fig. 6). As mentioned above, synaptic mitochondria provide, in a timely manner, proper amounts of ATP actually requested by synaptic activity, thus they represent very sensitive metabolic units, and even mild impairments in their efficiency may lead to functional deteriorations of the neural network they subserve. Synaptic mitochondria, in comparison to perykarial ones, show marked differences in the molecular composition of their membranes (Lai *et al.*, 1977; Villa *et al.*, 1989) and, in addition, are continuously recycled from the nerve cell body to the nerve terminals via the axonal flow, thus any delay in the prompt substitution of the damaged synaptic mitochondria may represent a possible cause of functional impairment. The constancy in Vv supports the idea that the potential hardware for energy production at terminal regions is preserved even in unfavourable conditions, but the mitochondria, as discrete functional units of the synaptic metabolism, are considerably altered as regards their number and size. Many determinants are involved in the biogenesis of mitochondria, therefore, the alterations reported by us in aging and vitamin E deficiency appear to be due to multiple causes. Mitochondrial DNA is very vulnerable to mutagens because of its supercoiled structure, moreover, the lack of protection from histone and/or non-histone proteins, the high frequency of point mutations associated with limited DNA repair mechanisms and the presence of respiratory enzymes which can activate chemical carcinogens are some of the mitochondrial features which support the marked vulnerability of these organelles (Attardi and Schatz, 1988). In addition to this, alterations of the inner mitochondrial membrane originating from impaired control of the respiratory chain could contribute to mitochondrial DNA damage by producing very harmful electrophyles such as peroxides, epoxides and nitroxides (Miquel and Fleming, 1984; Bandy and Davison, 1990; Miquel, 1991). Changes occurring at the inner membranes can be supposed to be due to free radicals generated by a defective control of oxygen which produces reactive molecules (Sohal and Sohal, 1991, Ferrandiz *et al.*, 1994). When biological defense mechanisms are impaired, as in vitamin E deficiency, the above vicious cycle may be accelerated and lead to a progressive mitochondrial deterioration and metabolic decay.

The observed increased percentages of elongated

mitochondria in old and vitamin E deficient rats deserve a particular comment. It is well known that mitochondria increase in mass and number by accretion and integration of newly synthesised material into preexisting organelles. The elongation of mitochondrial size found by us in old and vitamin E deficient animals may be regarded as the preceding step of organelle division into two smaller units. Thus, our present data, while seeming to support that this process is impaired in both these conditions, account also for a kind of compensatory mitochondriogenesis which is not carried out to the final splitting step, for the reasons discussed above.

### Changes in SDH activity

As evidenced by our histochemical reaction, SDH activity in perykarial Purkinje cells mitochondria decreases in aging because of the significant reduction of the number and size of the positive organelles. The vitamin E deficient group showed decreases of the three parameters taken into account, although Nv does not appear statistically different because of the high standard error of the mean at the present level of sampling. Before going into details with the discussion of the data on SDH positive mitochondria, some considerations should be made on this key-enzyme of the Krebs' cycle. The SDH molecule is synthesized by nuclear genes (Tzagoloff and Myers, 1986), then it is transported into the mitochondria and metabolically activated by mitochondrial DNA intervention. Conceivably, quantitation of SDH activity, besides providing information on metabolically activated organelles, reports also on interactions between nuclear and mitochondrial genome. A clear age-dependent impairment in the activity of this enzyme can be envisaged by our present findings, which report that long lasting vitamin E deficiency results in similar alterations in the adult animals. The SDH molecule is inserted into the mitochondrial inner membrane and its activity is closely coupled with the structural integrity of the surrounding molecular environment where it is located (Kugler *et al.*, 1988). Thus, as for the previous alterations, an increased membrane deterioration can be hypothesised to explain our findings, but also a defective interaction between nuclear and mitochondrial DNAs may contribute to a significant extent.

## Conclusions

Aerobic organisms need oxygen for survival. The brain has a high oxygen consumption and it is rich in oxidizable substrates, mainly unsaturated lipids. Thus, biological processes, e.g., cellular respiration, involving oxygen must be properly controlled to avoid free radical production (Halliwell and Gutteridge, 1985). Living cells, through repeated evolutive events, have improved their defense mechanisms to inactivate free radicals and it has been suggested that the life-span of a given species depends on the balance between free radical production rate and the efficiency of cellular protecting mechanisms (Ku *et al.*, 1993; Sohal *et al.*, 1993; Ku and Sohal, 1993). Thus, any impairment and/or decrease of compounds involved in free radical quenching may result in an acceleration of aging and in a potential threat to longevity. Brain performances are characterized by a constant high metabolic rate, conceivably nerve cells appear to be particularly sensitive to peroxidations. Our present findings lend further support to the concept that the proper control of harmful molecules, as free radicals, plays an important role in brain aging processes. In this context, vitamin E deficiency as a model of precocious brain aging can help to envisage the most vulnerable sites to free radical attacks in nerve cells.

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## Discussion with Reviewers

**J. Miquel**: How do the mitochondrial changes observed in the old and vitamin E deficient rats compare with those previously seen in other tissues? My own observations on the liver of old mice suggest that the aged hepatocytes contain less mitochondria per cytoplasmic volume and that the remaining organelles are larger.

**Authors**: Your data on hepatocyte mitochondria are very interesting. The good similarity of mitochondrial changes in the liver and brain of old animals supports that these organelles are to be considered very sensitive to the aging process independently of the type of cell or cellular compartment where they are located. If the enlargement of mitochondria is considered as a sort of compensation for their reduced number, then the morphological plasticity of these organelles appears to have a ubiquitous character.

**J. Miquel**: Have the authors performed or do they intend to do some behavioral studies in the vitamin E deficient rats?

**Authors**: We have not performed behavioural studies on our vitamin E deficient rats. Recently, Sokol (1989) and Southam *et al.* (1991) have published interesting data on vitamin E deficiency and neurologic function in laboratory animals and human beings: in their reviews, these authors do not report results on learning and memory tests in vitamin E deficient rats, thus we plan to perform these studies in the future.

**G.M. Roomans**: While the composition of the vitamin E-deficient diet is mentioned, no data are given about the diet of the control animals. Was the actual vitamin E content of both diets measured, and if so, what was the result? Was vitamin E measured in the animals? If not, how do the authors know that the animals are vitamin E-deficient?

**Authors**: The composition of the diet supplemented to control animals was the same of the vitamin E deficient rats, the only difference was in the content of $\alpha$-tocopherol which was 86.5 mg/kg. We did not measure the vitamin E content in the deficient animals because, according to J.G. Bieri and P.M. Farrel (1976; Vitamin E. Vitamins and Hormones **34**, 31-75) a 10 month treatment with vitamin E deficient diet is sufficient to cause a marked oxidative stress on brain structures (for further details, see Muller *et al.*, 1983).

**G.M. Roomans**: The brain region used for X-ray

microanalysis was different from those used for morphometry. I suppose that there are practical reasons for this choice, but can we automatically assume that changes in BCPC are also present in other parts of the brain?
**Authors**: To fulfill Weibel's morphometric criteria, we had to chose discrete anatomical models for our studies on synapses and mitochondria, however, as reported also by several studies on brain aging and on synaptic alterations in the old CNS, we can suppose that the reported changes have a ubiquitous character.

**T. von Zglinicki**: The authors draw two far-reaching conclusions, namely that the proper control of free radicals is a key determinant of brain aging and that vitamin E deficiency as applied here can help to single out sites vulnerable to free radical damage. These conclusions are not supported by the present data. It is simply presupposed that all measured effects are due to increased antioxidative capacity, in fact, increased membrane peroxidation, in the very same cells under study. Although systemic effects are observed (the authors mention in **Materials and Methods** under the subheading **Animals and Diet**, weight and hair-loss in rats fed a vitamin E-free diet), no results are presented which might exclude systemic effects as a significant cause of the observed brain cell changes. The possibility of systemic effects is not even discussed.
**Authors**: The fact that "the proper control of free radicals is a key determinant of aging" and that vitamin E is a biological antioxidant able to protect plasma membranes from lipid peroxidation are two concepts currently supported by a wealth of research data from different laboratories. The present investigation lends further support to these well grounded assumptions. We conclude our manuscript supporting that the absence of $\alpha$-tocopherol from the diet of young animals appears to cause an increased oxidative stress which may be of help in identifying early alterations similar to those found in postmitotic nerve cells of old normally fed animals. This rationale seems to us at least logical, however we have "smoothed" our conclusions. We have adopted an experimental paradigm of vitamin E deficient diet used by several investigators. As reported in the text (**Animals and Diet**), we fed the animals for 10 months in order to cause a deficient situation, but not a pathological condition which may cause systemic effects (see also Muller and Goss-Sampson, 1989).

**T. von Zglinicki**: With regard to the ion content of cells, it is not understandable whether new information is presented as compared to the 1981 paper (see the first paragraph in **Results** and Figure 4). If not, the whole complex can easily be omitted. If there is new material, its bearings to the old paper should be stated. It should be clearly shown to what extent the increase in cell ion concentrations is due to higher ion content or to dehydration. For that reason, results should be given as concentrations per dry mass first, and water content should be measured in the same cells or at least in well comparable cells. In addition, measurement errors should be indicated.
**Authors**: The present review manuscript is expected to report some data from our previous studies: this is the case for the microanalysis results which constitute an important bearing to the argument discussed in this article. In Figure 4, the values of the single measurements are given in % of dry mass $\pm$ S.E.M.

**T. von Zglinicki**: Nv and Sv of synaptic junctions, as well as Nv of mitochondria, were measured using methods which lead to biased results, especially if more complicated shapes are considered. This is clearly here the case. Any bias will be amplified in the tertiary data (S and V). Although Nv and Sv might still be measured using the old techniques as a first estimate, tertiary data should be calculated from unbiased measurements. At least, the data are probably not robust enough to warrant the detailed discussion of the frequency distribution of S (Fig. 5).
**Authors**: Measurements of synaptic Sv and Nv, as well as mitochondrial Nv, were calculated by applying morphometric formulas entered into the common usage several years ago (see Weibel, 1979). Although new methods have been introduced to evaluate the same parameters calculated by our image analyser, in a comparison between our previous results (obtained by "old methods") with the results from other authors using new methods (e.g., the dissector), we found a difference lower than 5% (Bertoni-Freddari *et al.*, 1990; Calverley and Jones, 1990)

**T. von Zglinicki**: The reference volume for all morphometric measurements has to be defined. Is it tissue volume or cell volume? With or without nuclei? Does it change with age or vitamin E deficiency, and how much?
**Authors**: The reference volume of our morphometric studies is 1 cubic micrometer of the CNS area where sampling has been carried out (i.e., cerebellar glomerulus and dentate gyrus supragranular layer). If shrinkage of the tissue due to age, vitamin deficiency, or technical mistakes of the procedures has occurred, then our data are an underestimation of the true situation in the tissue, but in all the samples analysed.

**T. von Zglinicki**: Is the ordinate in Figure 6 really % of Sk, or is it rather the number of measurements in the indicated range?
**Authors**: The % in ordinate of Figure 6 is the % of mitochondria falling within the indicated range.