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# DIETARY RESTRICTION MODULATES SYNAPTIC STRUCTURAL DYNAMICS IN THE AGING HIPPOCAMPUS

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## ABSTRACT

**A computer-assisted morphometric study has been carried out on the synaptic ultrastructural features in the hippocampus of 14-month old (DR14) and 27-month old (DR27) dietary restricted (-50% lipids and -35% carbohydrates) rats. Age-matched controls were maintained on an *ad libitum* (AL) feeding schedule. Synaptic numeric density (Nv), surface density (Sv) and average area (S) were the parameters measured. In old AL vs. adult AL animals, Nv decreased to a not significant extent, while S increased and Sv decreased significantly. In DR14 rats vs. AL littermates Nv increased significantly, but S and Sv were unchanged. DR27 rats vs. age-matched AL controls showed a significant increase of Nv and Sv while S was significantly decreased. Comparing DR14 vs. DR27, no significant difference due to age was documented. Both in DR14 and in DR27 groups the percent distribution of S showed a marked increase of smaller contact zones. Despite reporting on discrete aspects of synaptic ultrastructure, Nv and S are supported to be in an inverse relationship which aims at maintaining Sv constant. Thus, these three ultrastructural parameters when taken together per experimental group, appear to provide information on synaptic morphological rearrangements. In this context, the percent increase of smaller synapses in DR animals is consistent with the idea of a marked remodelling process. Considering previous data from the same groups of rats reporting significant changes in neuronal membrane lipid composition and fluidity, we interpret our findings to account for a positive modulation of dietary restriction on the synaptic structural dynamics.**

## INTRODUCTION

Dietary restriction (DR) is currently reported to have beneficial effects in prolonging the life time of several species of laboratory animals, mainly rodents (1-3). Despite many hypotheses, so far all have failed to explain the mechanism of action of different food restriction schedules, it remains well proven that the function organ systems are better and longer preserved in dietary restricted animals. Namely, the immunological response is markedly improved (4), renal and myocardial diseases (5) as well as the incidence of tumor mediated by free radicals (6, 7) are reduced in food restricted animals.

With regard to the central nervous system (CNS), recent studies have documented that a reduction of food intake in laboratory animals has beneficial effects on behavioural tests (8-10). DR attenuates the age-related loss of striatal dopamine receptors (11); suppresses age-related changes in dendritic spines (12); reduces the production of reactive oxygen species and it is able to counteract age-dependent changes in lipids of cortical membranes (13). This wealth of current literature data supports the conclusion that reduced food intake not only can extend life, but also it can improve significantly its quality by delaying those aging alterations affecting the postmitotic nerve cells.

Previous studies from our group on adult and old CD-COBS dietary restricted rats have documented that a hypocaloric diet resulted both in significant improvements of motor and cognitive performances as well as in maintaining a good fluidity of neuronal membranes by modulating their lipid composition (10, 13, 14). Continuing these behavioural and biochemical investigations, we conducted a computer-assisted morphometric study on the synaptic contact zones in the dentate gyrus supragranular layer of adult and old dietary restricted CD-COBS rats.

## RESULTS

The male animals investigated in the present study were taken from the same CD-COBS colony used for the experiments described in refs. 10, 13, 14. Our hypocaloric diet resulted in a decreased body weight and in an increased survival of the underfed animals. Cognitive behaviour (10), memory performances (14) and the biochemical composition of brain membranes (13) in these rats resulted significantly improved.

The results of the synaptic numeric density (Nv) are reported in Fig. 2. Nv decreased by about 18% in AL27 vs. AL14 animals, but this difference was not significant. A significant increase of the number of synapses is clearly evident both in DR14 and DR27 groups compared with AL age-matched controls. No significant difference was envisaged comparing DR14 and DR27 groups of rats. In AL27, Nv was significantly lower than in DR14 animals.

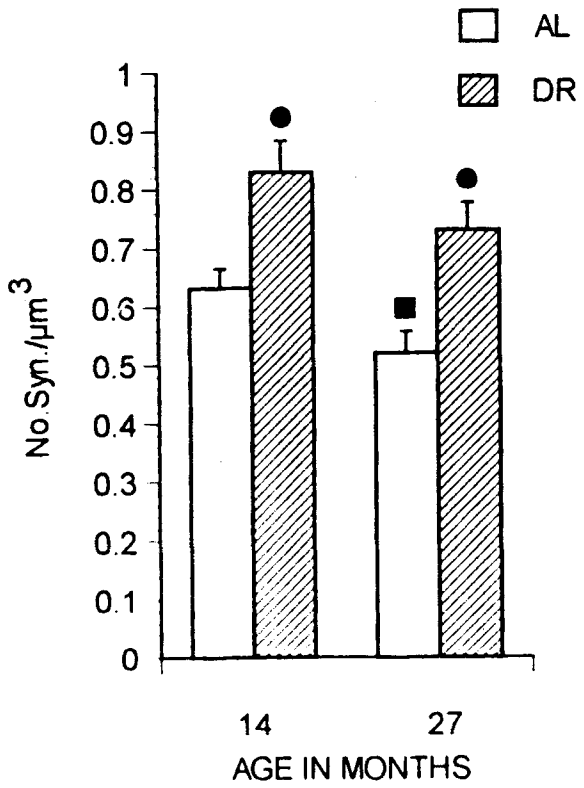


**Figure 2.** Synaptic numeric density (Nv). Dietary restriction resulted in significant increases (• $p < 0.001$) of this parameter both in DR14 and DR27 vs. AL age-matched rats. ■ $p < 0.001$ vs. DR14 rats.

The synaptic average area (S) significantly increases with age in AL27 vs. AL14 animals (Fig.3). DR14 and DR27 rats show a decrease of this parameter vs. their AL littermates, however only in DR27 rats vs. the AL27 ones the difference was significant. DR14 and DR27 animals showed the same values of S.

In AL27 rats the value of the synaptic surface density (Sv) is significantly lower than in the other groups investigated (Fig. 4). No change in the values of Sv was found comparing DR14, DR27 and AL14 rats.

A percentage distribution of S is shown in Fig. 5. In DR14 and DR27 rats, the junctional areas smaller than 0.08 $\mu m^2$ are markedly increased and account for 72 and 48%, respectively. Conversely, in AL14 and at a higher extent in AL27, the percent of enlarged contact zones (i.e. larger than 0.12$\mu m^2$) is markedly higher than in the respective age-matched food restricted groups.

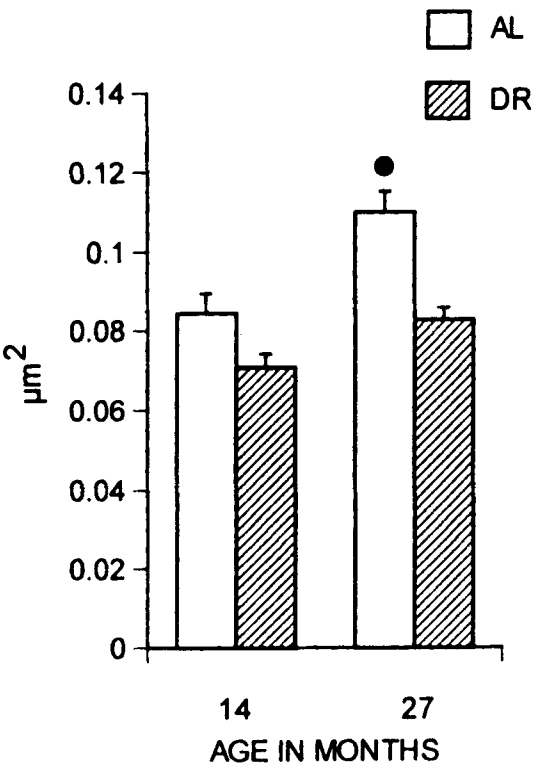


**Figure 3.** Average area of the synaptic contact zones (S). Dietary restriction prevented the increase in synaptic size reported in AL27 rats. • $p < 0.001$ vs. the other groups investigated.

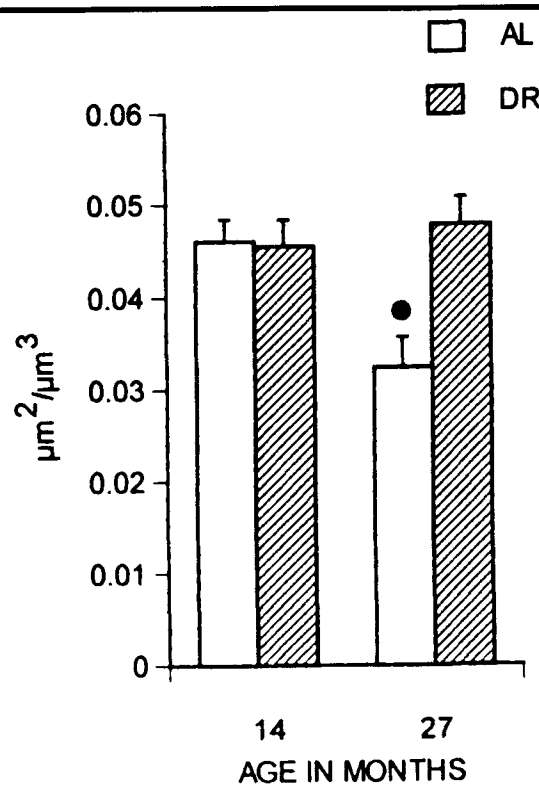


**Figure 4.** Synaptic surface density (Sv). DR14 and DR27 rats showed unchanged Sv values when compared with AL14 rats. • $p < 0.001$ vs. the other groups investigated.

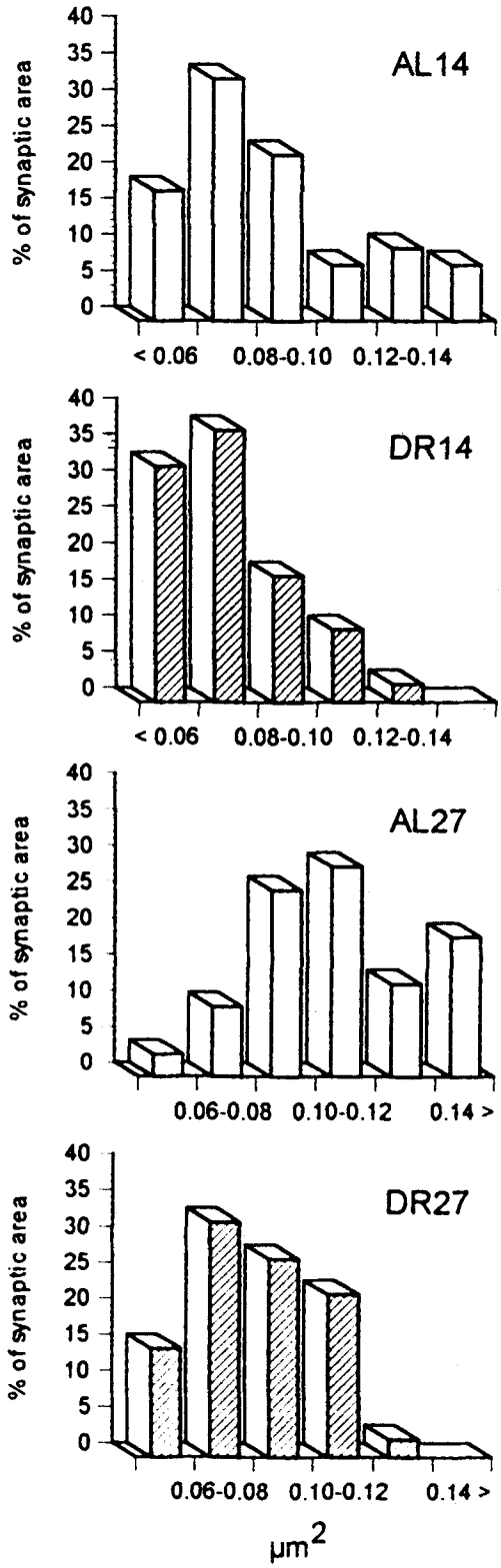


**Figure 5.** Percent distribution of the synaptic average area (S). Comparing DR14 and DR27 vs. their respective AL control groups, a marked increase of the percent of the contact areas smaller than 0.08 µm², together with a decrease in the amount of enlarged junctional areas (i.e. larger than 0.12 µm²), are clearly evident.

## DISCUSSION

The main finding from the present investigation is that dietary restriction results in a positive and functional modulation of the synaptic structural dynamics both in adult and in old rats. Namely, DR14 and DR27 animals *vs. their respective AL control groups*, showed higher Nv values together with increased percentages of the junctional areas of smaller size. With specific reference to aging, the final outcome of dietary restriction is represented by an increase of the total synaptic contact area (Sv) in DR27 rats so that the age-related decrease of this parameter is no longer apparent.

An interpretation of these findings is in order. Despite well differentiated zones of the nerve cell terminal regions, synaptic junctions are widely reported to be very plastic structures capable of relevant structural remodelling processes (15-19). Quantitative estimations of the dynamic morphology of the synaptic junctional areas are currently carried out by evaluating functional ultrastructural features. While individually reporting on particular aspects of synaptic plasticity, and considered per experimental group, these estimations account for the reciprocal rearrangements occurring in the physiological responsive adaptation to environmental stimuli. In this context, Nv and S are supported to be in an inverse correlation which aims at maintaining a constant value of Sv. Although these assumptions are currently controversial they are supported by data from different experimental paradigms including physiological and pathological aging, vitamin E deficiency, malnutrition and experimental lesions (20-25). On the basis of these concepts, dietary restriction resulted in a significant counteraction of the age-dependent decrease of Sv through a positive Nv and S balance. The biological significance of the inverse relationship between these two parameters is still debated, however recent experimental data support that higher synaptic densities are associated with better CNS performances (17, 19, 26) while reductions in synaptic number, together with enlargement in size of the surviving contacts, are reported to occur in adverse and pathological conditions (22, 27). Conceivably, maintenance of a high number of small junctional zones appears to represent a functional task not only for routine CNS performances such as learning and memory, but also for a proper response to environmental stimuli (16, 26, 28).

Considering tenable explanations of these effects of dietary restriction on the synaptic adaptive potential, it must be mentioned that current knowledge of synaptic *functional morphology hypothesizes an ordered* sequence of steps occurring in the structural rearrangements of the junctional areas (15, 16, 19, 29). As a consequence of stimulation, the sequence is as follows: synaptic contact zones (I) enlarge, (II) perforate and (III) split to yield an increased number of smaller contacts. This cycle of events is supposed to lead to an increase of the number of contacts (i.e. Nv) within those discrete CNS zones undergoing stimulation and this, by improv-

ing cell-to-cell communication, is proposed to lead to a reinforcement of the information processing among nerve cells. The increase of the percent of smaller synapses in dietary restricted animals (Fig. 5), suggests two hypotheses: I) the smaller junctions are better preserved by the reduced caloric intake; II) the cycle of steps purported to remodel the synaptic network is better accomplished in a dietary restricted regimen. In our opinion, these two claims do not exclude each other, on the contrary, both may act positively on the synaptic potential for plasticity if the many beneficial effects of a reduced food intake and the physico-biochemical features of the synaptic contact zones are taken into account.

The modulation of synaptic structural dynamics is a multifactorial task and this implies that the maintenance of the experience-driven junctional network involves the actual performances of several cellular processes, e.g. protein synthesis, axonal transport, energy metabolism. The up regulation of these determinants of cellular health, as it is reported to occur in DRs (1, 2, 4-8, 14), may help to preserve the physiological pattern of synaptic density and size through continuous repairs and reinforcement of the synaptic contact areas. With specific reference to aging, the maintenance of synaptic connectivity is of critical and functional importance since neurone number is reported to undergo a not significant decrease both in physiological and pathological aging (30). In agreement with these literature data, we found a substantial stability of cerebellar and hippocampal granule cell density both in normal aging and Alzheimer's disease (21, 31), however we also reported that the number of synapses per neurone was significantly lower in old and demented vs. adult patients because of the significant decrease of synaptic Nv. These findings document a marked synaptic vulnerability in the senile as well as demented CNS and support that the early deterioration of the synaptic contact zones is to be regarded as a primary alteration preceding the eventual decrease in neurone number (32-34). On the basis of this rationale, neurone loss does not appear to represent a necessary prerequisite for the occurrence of age- or pathology-related impairments since these involve first the consistent decay of synaptic functions. On tackling the problem of CNS decline in aging, important items appear to be represented by precocious interventions aimed at protecting the pattern of synaptic connections established by the peculiar experiential framework of each individual: in this context, our present findings clearly document that the synaptic dynamic morphology is positively modulated by DR.

Although the mechanism(s) by which a reduced caloric intake retards many age-related alterations is yet unknown (35-39), there is consensus proposing that dietary restriction triggers a multifactorial process responsible of a decreased cellular load of labile and damaged proteins, lipids and DNA (35). With specific reference to the present findings, reasonable explanations can be inferred from previous papers reporting consistent changes in the lipid composition and physicochemical properties of the neuronal membrane of rats fed with the same reduced calorie-high fibre diet (13, 36). Namely, it has been found that old food restricted rats show significant reductions in the cholesterol/phospholipid and in the sphingomyelin/phosphatidylcholine ratios suggesting that these modifications oppose the age-related decrease in the fluidity of brain membranes. As clearly documented by brain tissue samples processed according to conventional electron microscopy procedures (i.e. those using $OsO_4$, uranyl acetate and lead citrate), synaptic junctions are very abundant in unsaturated lipids. This biochemical feature plays a key role in the dynamic morphology of these discrete zones of the nerve terminals and, while being of critical importance from a functional point of view, it is also the main determinant of the prominent vulnerability of synaptic membranes to free radical lipid peroxidation (5). On the basis of several experimental data, caloric restriction is currently claimed to modulate positively free radical metabolism by reducing the generation of reactive oxygen species and thus exerting its anti-aging effect (37-39). This action is of critical functional significance for the postmitotic nerve cells which are characterised by high lipid content and high oxygen consumption (40). On the basis of these concepts, a higher percentage of smaller junctional areas may be the outcome of the splitting of larger contact zones or of an improved preservation of the persisting contacts. In both cases, the balance between Nv and S in DR14 and DR27 can be supposed to act at reinforcing nerve cell connectivity. Although this suggestion is highly speculative, previous results reporting significant improvement of learning and memory as well as of behaviour in the same dietary restricted rats (10, 13, 14) can be regarded as well-grounded evidences supporting our tenet.

In conclusion, the present findings document that dietary restriction is capable of a positive modulation of the synaptic dynamic morphology which may be due to the many reported effects of a reduced caloric intake on free radical generation and/or control (4, 6, 7, 35-39, 41).

## EXPERIMENTAL PROCEDURES

A total of 20 male CD-COBS rats were used for the present investigation. The animals were divided into four groups, each consisting of 5 animals: I) 14-month-old control rats fed *ad libitum* with the standard diet (14AL); II) 27-month-old control rats fed *ad libitum* with the standard diet (27AL); III) 14-month-old rats fed a hypocaloric diet from weaning (14DR); IV) 27-month-old rats fed a hypocaloric diet from weaning (27DR). Housing, animal care and diet composition have been described in previous papers (10, 13, 14). With specific reference to the hypocaloric regimen, at weaning 50% of the lipids and 35% of carbohydrates of the standard pellet have been replaced by fibres.

Anaesthetised rats were perfused through the left ventricle with saline followed by 2.5% phosphate buff-

ered glutaraldehyde (pH 7.4). The hippocampi were rapidly excised, sectioned in 500μm thickness slices by a Mac Illwain tissue chopper and stained for synaptic junctional areas by means of the ethanol phosphotungstic acid preferential technique (E-PTA) (Fig. 1) according to our previous papers (15, 20-22).

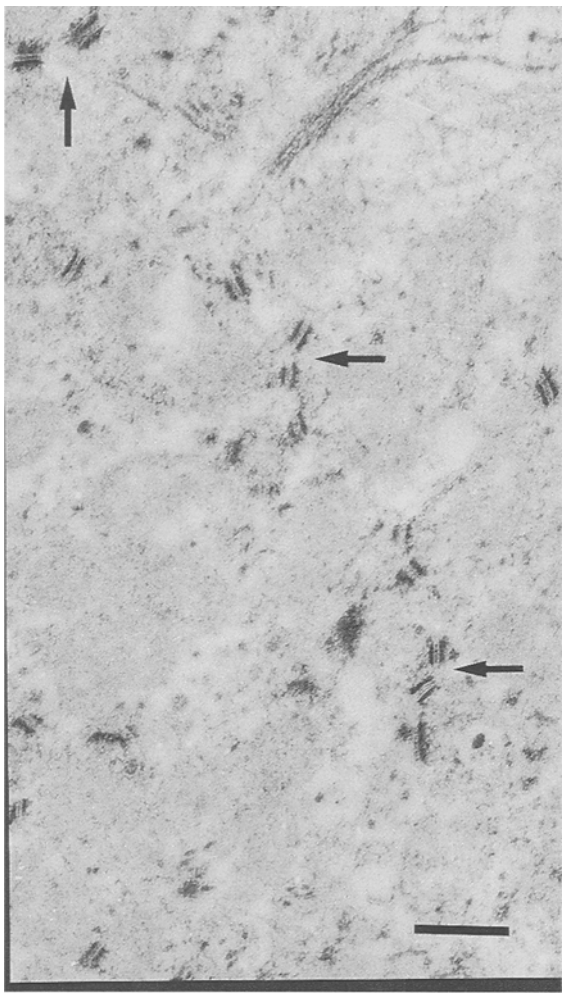

**Figure 1.** Electron microscopic picture of E-PTA stained synapses in the rat dentate gyrus supragranular layer. The cross-sectioned junctional zones are clearly evidenced as parallel black lines against the faint background (arrows). Bar: 0.5 μm.

*Morphometry.* A systematic random sampling was carried out by means of a Kontron KS300 computer-assisted image analysis system equipped with a TV camera directly connected with the electron microscope image plate. In details, starting from a section randomly chosen in the section ribbon, synaptic morphometry was systematically performed at a fixed 10 μm rule distance in the neuropil just above the granule cell layer (considered as the reference structure) (42). Synaptic measurements were carried out on the video of the KS300 image analyser at a final enlargement of 75,000x: this allowed us to sample a surface area of 14,755 $\mu m^2$/field. The actual number of fields/group of animals was determined by the progressive means method (43): collection of the data was terminated when a representative sample of each group of animals was obtained, i. e. when the standard error of the mean (SEM) for each parameter was less than 5%. This procedure yielded 50 to 60 fields/group of rats and a total of 3,000 to 3,600$\mu m^2$ of the neuropil analysed area in the dentate gyrus supragranular layer. By applying current morphometric formulas (44, 45), the following ultrastructural parameters were semi-automatically evaluated on coded samples unknown to the operator: 1) the number of synapses/$\mu m^3$ of tissue (numeric density: $Nv = 8Na/L\pi^2$), where Na is the number of synapses/$\mu m^2$ and L is the average length of the synaptic contact zones (37); 2) the synaptic average area: $S = (\pi/4L)^2\pi$; 3) the total synaptic area/$\mu m^3$ of tissue (surface density of the synaptic contact zones: $Sv = 4La/\pi$), where La is the length of synapses/$\mu m^2$ of tissue.

Statistical comparisons were performed by analysis of variance (ANOVA) followed by the Student-Newman-Keuls' test.

## ACKNOWLEDGEMENTS

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## ABBREVIATIONS

**ANOVA** = analysis of variance; **AL14** = ad libitum fed rats of 14 months of age; **AL27** = ad libitum fed rats of 27 months of age; **CNS** = central nervous system; **DNA** = deoxyribonucleic acid; **DR14** = dietary restricted rats of 14 months of age; ***DR27*** = *dietary restricted rats of 27* months of age; **E-PTA** = ethanol phosphotungstic acid; **L** = synaptic average diameter; **La** = synaptic length per unit area; **Na** = synaptic number per unit area; **Nv** = synaptic numeric density; **$OsO_4$** = osmium tetroxide; **S** = synaptic average area; **SEM** = standard error of the mean **Sv** = synaptic surface density; **μm** = micron; $\pi$ = Greek p: 3.14.

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