THE EFFECT OF CALORIC RESTRICTION ON LIPOFUSCIN ACCUMULATION IN MOUSE BRAIN WITH AGE

W.A.L. Moore[a], V.A. Davey[b], R. Weindruch[c], R. Walford[d] and G.O. Ivy[b]
[a]Office of Post Grad Education, Clarke Institute of Psychiatry, 250 College Street, Toronto, ON, Canada M5S 1R8; [b]Life Sciences Division, University of Toronto, 1265 Military Trail, Scarborough, ON, Canada M1C 1A4; [c]University of Wisconsin and VA GRECC, Madison, WI 53705; [d]Department of Pathology, University of California, Los Angeles, CA

Key Words
Lipofuscin, caloric restriction, diet, aging, brain, mouse

Abstract

Caloric restriction (CR), has been shown to extend average and maximum lifespan in rodents and other animals as well as to delay a wide variety of manifestations of aging. The purpose of this study was to further elucidate the relationship between lipofuscin (LF) accumulation and the aging process by examining the effect of lifelong CR on LF accumulation in brain cells. Specifically, 1) we include age groups of CR (CR1 ≅ 90 kcal/wk and CR2 ≅ 58 kcal/wk) and ad libidum fed (AL; ~ 120 kcal/wk) mice including groups at maximum lifespan; 2) CR was the major dietary manipulation; 3) LF was identified using EM; 4) LF was quantified by areal measurement; and 5) the results were analyzed by inferential statistics.

We have found that 1) LF increased with age and 2) that animals in the CR2 group had significantly less overall LF in the perikarya of the granule cells of the dentate gyrus when compared to CR1 or AL animals at equivalent ages. In addition, CR2 mice at maximum lifespan (45 mo.) had slightly less LF than did CR1 or AL mice at their maximum lifespans (36 mo.). Our results clearly demonstrate that CR (at 52%, but not 25% of AL diet) retards the overall accumulation of LF with time and, further, suggest that LF accumulation is not simply a linear function of age.

Introduction

Lipofuscin (LF) has long been thought to accumulate approximately linearly with age in most tissue types and organisms, and is thought by many to be a biological marker of aging. Thus, the

cells of long lived species accumulate the pigment relatively more slowly than do those of short lived species and, by analogy, if we could extend the lifespan of a species, we should be able to slow the rate of LF accumulation proportionately. The best way to test this theory is to measure cellular LF in a relatively homogeneous population of post mitotic cells in an animal on a caloric restriction (CR) regime compared to an animal fed ad libidum (AL). CR has been shown to extend mean and maximum lifespan in a variety of species, as well as to drastically reduce a number of age related phenomena (see 1 for review). These include reducing the age-related decline in T and B cell function and maintaining thymic function (which results in reduced ability to induce diseases in old animals, as well as in reduced spontaneous diseases such as diabetes and cancers), maintaining hormone levels (resulting in sexual longevity), and maintaining more youthful DNA repair and protein turnover levels. Two studies [2,3] have previously reported that CR has no effect on LF accumulation, one [4] that CR increases LF, and seven [5-10] that CR retards LF accumulation in various cell types. However, a close examination of these studies reveals that eight of these studies used a non-isonutrient CR regime in their diets, thus potentially confounding the results. Also, four of the studies measured lipofuscin by spectrofluorometry of chloroform-methanol extracts, a method now known to be unreliable [11,12]. Finally, three of the studies failed to analyze their results by inferential statistics, leaving the significance of their conclusions difficult to interpret. The present study is an attempt to analyze the relationship between CR and LF accumulation while avoiding the above problems. In addition, we incorporate two control groups, one fed AL (at ~ 120 k/cal/wk) and one restricted to 25% of the AL diet (at ~ 90 k/cal/wk) in order to prevent any possible confounds of obeisity in this group.

Methods

Twenty six brains were obtained from a long lived F1 hybrid strain (C3B10RF1) of female mice from the animal facility of the University of California, Los Angeles [see 1 for details of rearing].

Females were weaned at 21-28 days of age, individually caged in plastic cages on wood chip bedding, assigned to one of 3 basic diet regimes and sacrificed at 9-10, 17-20, 36, or 45 months of age [for details of diets see 1]. The diets were maintained at isonutrient levels. Figure 1 provides the experimental design with the individual treatment groups coded so as to specify which of the three standard diets animals consumed.

EXPERIMENTAL DESIGN

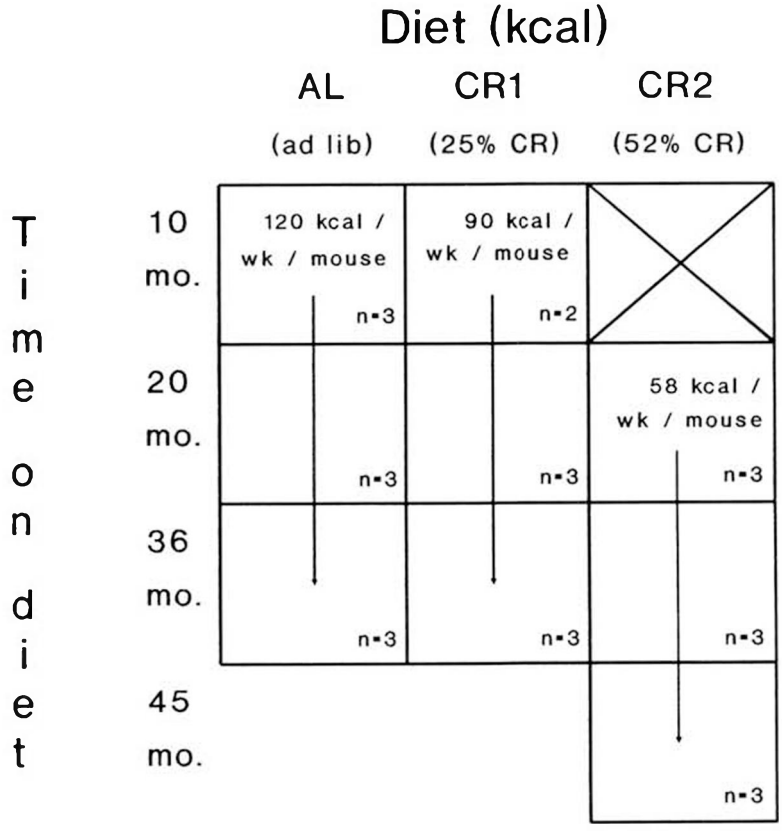

Fig. 1

Animals were anesthetized and perfused intracardially with a fixative containing 2.5 percent each of paraformaldehyde and glutaraldehyde in 0.1M phosphate buffer, pH 7.4. The animals' brains were dissected and left in the same fixative overnight at 4°C. The following day, brains were sectioned in their sagittal plane at 100 μm with a vibratome, osmicated and embedded in EPON Araldite.

Appropriate dissections of the dorsal leaf of the dentate gyrus (DG) from the embedded tissue were mounted on pellets and sectioned at 1 μm on an ultramicrotome. Sections were stained with toluidine blue and viewed under a light microscope to confirm the location of granule cells in the dorsal leaf of the DG. Five to nine ultrathin sections were taken from each brain.

Electron micrographs were taken at magnifications of 100x and 500x for each brain, to verify standard location of the sample, and for general qualitative comparison of brain tissue between animals. At a magnification of 5000x, a series of forty to fifty electron micrographs were taken to form a complete montage of granule cells in the dorsal leaf of the DG for each brain. In those sections with grid bars, unavoidable artifacts or unwanted tissue components such as blood vessels, more than one montage was taken, as necessary to ensure a homogenous area including a minimum of fifty whole (containing a portion of the nucleus) granule cells from each brain. An electron micrograph of a standard calibration specimen (cross grating replica 2160 lines/mm) was taken for each series of photographs. Negatives were then photographically enlarged at 3.2x and printed. Large montages of the photographed areas were carefully assembled for quantitative analysis.

Using a specially developed software program, an IBM XT/PC computer was interfaced with a Panasonic X microplan digitizing pad to collect areal measurements of cellular components within the EM montages of brain sections. Raw data were stored in dBASEIII standard format and converted to standard ASCII files for statistical analysis using the statistical software program SPSS/PC.

A computer linked digitizing pad was used for tracing three basic components of granule cells: cell membrane perimeter excluding neurites, nuclear membrane perimeter, and LF granule perimeter. From these data, areal measurements for all three components were automatically calculated and recorded as primary data. An additional measurement of total tissue area was taken for each brain by summing successfully digitized portions of the montage(s). This was recorded separately.

LF was identified on the electron micrographs as electron dense bodies in accordance with the classification scheme outlined by [12]. This classification does not exclude lysosomes, the organelles from which LF is formed and it is recognized in this study that some electron dense bodies were primary lysosomes. Measurements, tissue preparation, and EM photography were done "blind", with the identification code for the animals being revealed when all data collection had been completed.

Intracellular LF measurements were calculated using a computer generated random sample of 50 granule cells per animal. Absolute amounts of intracellular LF per animal were calculated by summing the amount of LF in 50 granule cells per animal. The four specific calculations are listed below:

LF Area:
Total LF area within
50 g.c. (sqr. microns)

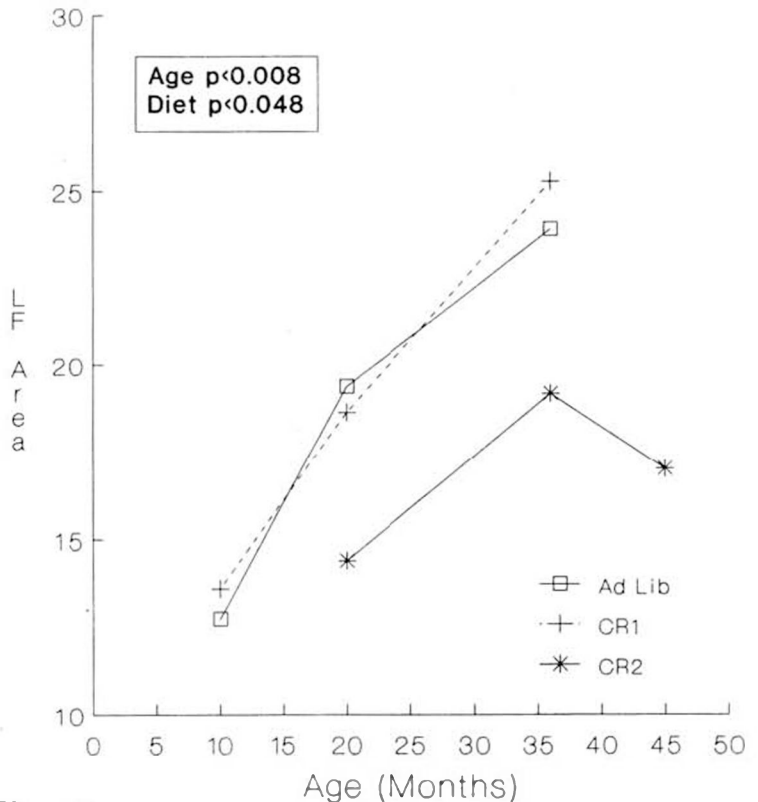

Fig. 2

LF Area:
Percent LF within cytoplasmic area
of 50 granule cells

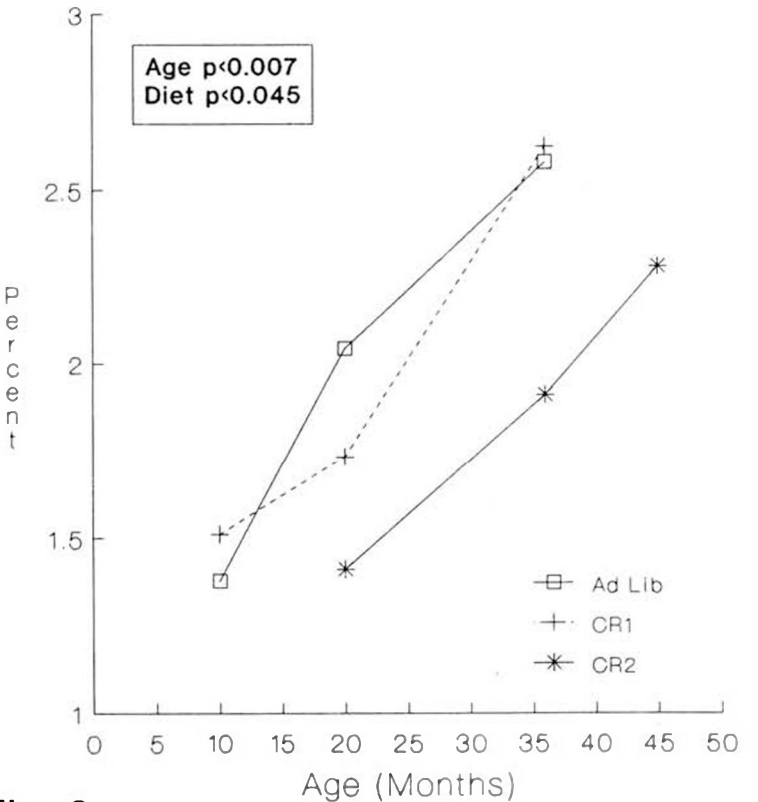

Fig. 3

LF Area:
Percent total neuropil area
with LF

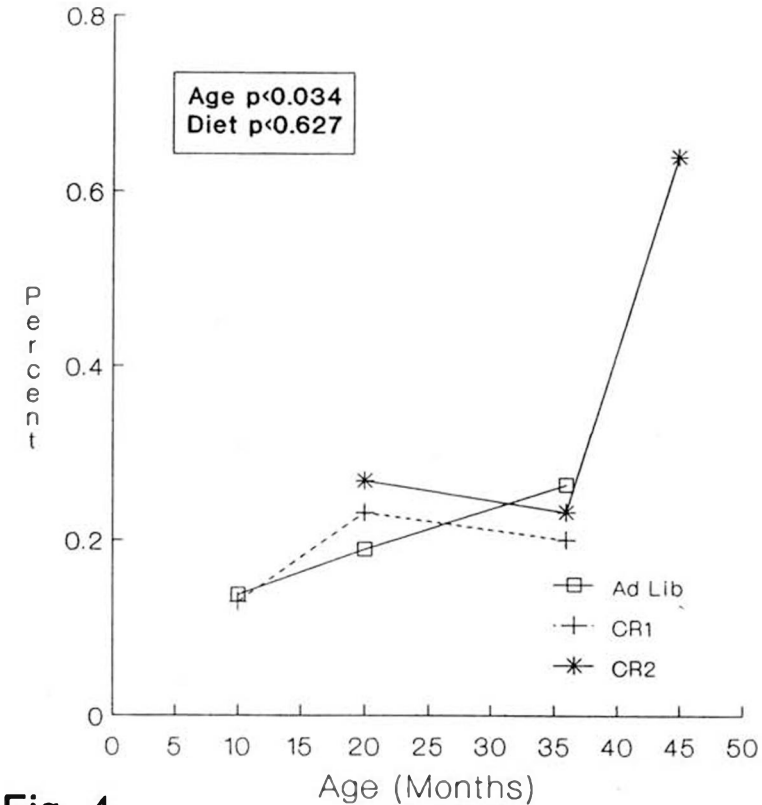

Fig. 4

LF Area:
Percent LF in total tissue area
(excluding glia + b.v.)

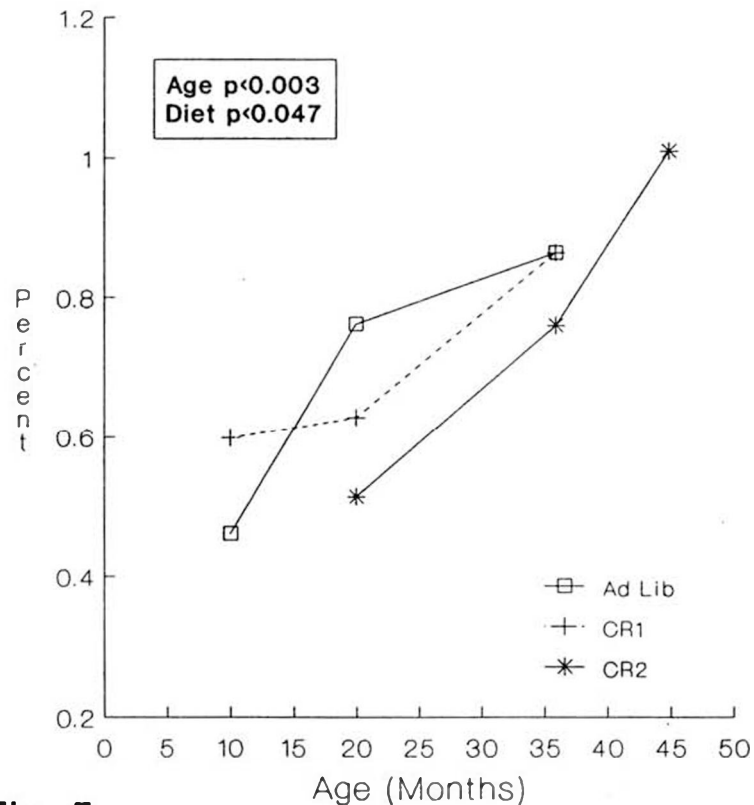

Fig. 5

1) Total amount of LF area in 50 nucleated granule cells of the DG
2) Percentage of total cytoplasm containing LF in 50 nucleated granule cells of the DG
3) Amount of LF in neuropil of montage, measured as a percentage (excluding obvious glial cells, but not small processes that could not be identified with certainty, and blood vessels).
4) Total amount of LF in montage (exclusion as in 3, above).

These obtained values were analyzed by ANOVAs for diet and age effect, and further testing with one way analysis, trend analysis and T tests were carried out as indicated.

Results

We have found that LF accumulation increased significantly with age in all diet groups in nucleated granule cells ($p<.008$), in neuropil ($p<.034$), and in total tissue area ($p<.003$) (figs. 2-5). However, while there were no significant differences between the two control groups, AL and CR1, on any LF parameter, there were significant differences between controls and the 52% calorically restricted CR2 group. The total LF area in 50 granule cells of CR2 was significantly less than that in controls in both absolute measurements in square microns ($p<.048$) and in percent of cytoplasmic area in 50 cells ($p<.045$) (figs. 2 and 3, respectively). The percent of LF in total neuropil area in CR2 was not significantly different from control levels ($p<.627$) (fig. 4). However, in a one way ANOVA for diet effect at maximum ages for groups AL, CR1 and CR2, there was a significant effect for percent of neuropil with LF in the CR2 group ($p=.046$), with CR2 having more LF in neuropil than did the controls. The percent of LF in total tissue area was significantly lower in the CR2 than the control groups ($p<.047$) (fig. 5). Interestingly, in "super-aged" mice on the CR2 diet, the amount of LF in 50 granule cells appeared to decrease, or at least level off, between 36 and 45 months of age, although the apparent decrease was not statistically significant (fig. 2).

Discussion

We have found that the total LF area within 50 granule cells of the dentate gyrus, whether measured by actual area (in μm^2) per cell or as percent of cytoplasm, increases significantly with age in mice fed an AL diet, a 25% CR diet (CR1) or a 52% CR (CR2) diet. However, the amount of LF was significantly less in the CR2 group at all ages examined. These findings are also true when amount of LF in total tissue area of the DG containing the 50 granule cells is

analyzed (this analysis excludes glial cells and blood vessels, see Methods). This indicates that, as is well known in many tissues, LF increases with age. Further, the amount of LF in brain tissue, as measured by percent of total tissue area containing LF showed a significant linear trend for all three diet groups. A linear trend was also found for intracellular LF in the two control groups, as measured both by absolute amount of LF in 50 granule cells and as percent of their cytoplasm containing LF. However, while intracellular LF as measured by percent of cytoplasm containing LF showed a significant linear trend in the CR2 group, when actual amount of LF was analyzed (in μm^2), there was no significant linear increase with age. Indeed, as indicated in figure 2, there appears to be a slight decrease in the amount of intracellular LF in these super-aged animals.

In order to understand this finding, it is useful to examine changes in the distribution of LF with age. The amount of LF in the neuropil increased significantly with age in all diet groups, but there was not a significant diet effect, overall. However, if one compares the amount of LF in the neuropil among the three diet groups at maximum age only (AL=36 mo., CR1=36 mo., and CR2=48 mo.), the CR2 group is found to have significantly more LF in the neuropil ($p=.046$). Thus, while LF appears to move out of the perikaryon and into cell processes with increasing age, this phenomenon is amplified in CR2 animals at their maximum age. This movement of LF from perikaryon to neuropil may represent an attempt by the cell to "push" the intracellular "garbage" away from the nucleus and the protein synthesis and postranslational modification machinery.

Brunk et al (this volume) present evidence that LF may actually be "toxic" to RPE cells in vitro, since these cells die when they accumulate a certain amount of LF in their cytoplasm. Unlike neurons, RPE cells do not have extensive processes, or "spare rooms", in which to store excess garbage. Brunk and colleagues (this volume) have argued that LF may become "leaky" for lysosomal enzymes which could wreak havoc in the cytosol. Others have argued that LF is "passive", but may merely accumulate to such an extent that it clogs the cellular machinery. In either case, the fact that neurons have extensive dendritic arbors may provide a mechanism for keeping the perikaryon relatively free of LF, at least if the cell maintains relatively youthful metabolic processes.

This movement of LF away from the perikaryon is much less dramatic in the granule cells of AL and CR1 animals by 36 months of age (their maximum lifespan) (fig. 4). At 36 months of age, the three diet groups have LF neuropil values that are not significantly different. While the

control mice are rapidly dying off, the CR2 mice continue to live quite healthy lives for the next nine months. It seems likely that the various mechanisms controlling cellular (and organ) homeostasis, which have been shown to be maintained by CR [1] may also include a mechanism(s) for moving excess LF out of neuronal perikarya.

There is increasing evidence from a number of laboratories that CR attenuates free radical damage to a wide range of cellular components and maintains anti-oxidant defense systems that normally decline with age [reviewed in 1,14]. In particular, a number of studies have shown that the mitochondria may play a key role in aging since the mitochondrial DNA becomes oxidatively damaged with age, leading to greatly perturbed mitochondrial function and thus to cellular metabolic dysfunction [15,16]. CR appears to maintain mitochondrial integrity by suppressing mitochondrial DNA deletions [17]. Further, CR has been shown to attenuate the age related increases in serum total iron and ferritin concentrations, likely by its maintenance of anti-oxidant defense systems. A recent report [18] shows that CR reduces the number of iron and ferritin containing microglial cells in brain tissue. These microglia normally produce reactive oxygen species via iron mediated pathways, leading to increased lipid peroxidation levels, and likely to other cellular and molecular damage with age.

It is well known that iron in the Fenton reaction can produce free radicals. In this volume, I.Zs.-Nagy and colleagues demonstrate that when rats are injected with ferrous ammonium sulfate in their CSF, LF rapidly accumulates in neurons in several brain regions (including hippocampus) in young rats. Thus, a mechanism by which CR reduces the accumulation of LF with age may include its reduction of the number of iron and ferritin containing microglial cells in the brain, and/or by reducing these compounds in the circulatory system.

Finally CR also appears to maintain levels of the neurotransmitter nitric oxide in the brain, which may be important in maintaining neuronal viability and function [18]. Nitric oxide is known to be important in hippocampal function, and it's maintenance by CR may allow neurons in the super-aged (48 mo, CR2) category to maintain the metabolic functions that may underlie the neurons' abilities to move lipofuscin from their perikarya and out into the neuropil.

While the precise mechanism(s) of CR's effect on reducing lipofuscin accumulation in mouse brain with age remains to be determined, it is likely that one or more of CR's growing number of antioxidative influences may be involved. We have clearly demonstrated that CR attenuates LF accumulation in a population of neurons, thereby maintaining a more "youthful" metabolic state of these cells. The results of this study further validate LF as a true biomarker of aging and

suggest that understanding the mechanisms of lipofuscinogenesis will provide insight into the aging process.

Acknowledgements

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Discussion after the talk of Dr. Ivy

Jolly: Would you not have been better to calculate volume of lipofuscin rather than area?

Ivy: Because our sample size is so high, we can easily calculate volume area of lipofuscin from our data, altough we did not take serial EM sections. Indeed, earlier in our analysis, we did calculate volume and found that the results paralelled those reported here. In order to calculate volume at this point, we would have to completely reformat our data, and I don't think that this effort would give us any different conclusions.

Brunk: Wouldn't it be very difficult to discriminate between a lysosome containing lipofuscin and one containing autophagocytosed material under degeneration? Both would contain electrophilic, amorphous material. I think your way of estimation gives a definite overestimation of lipofuscin.

Ivy: We seem to disagree on terminology. Lipofuscin has not yet been confirmed to be "residual" - i.e., undigestable material.. Therefore, it is not really distinguishable from a lysosome containing material that is under degradation. What we have measured are secondary (and some primary) lysosomes. These secondary lysosomes are usually referred to as autophagic vacuoles and they result from the fusion of a primary lysosome with an "autophagosome", an organelle which does not contain digestive enzymes. The autophagosomes are almost never seen in our material, probably because they fuse so rapidly with lysosomes. If they have fused with lysosomes, we define them as "lipofuscin" because no one can know how long the material inside this vacuole will remain there. We view it as an early form of lipofuscin. But, as you know, lipofuscin itself is often heterogeneous in morphology and thus may contain undigesti-ble as well as digestible material, even in young animals. I agree that we may have a slight over estimation of lipofuscin in our data, but this is due to our acknowledged inclusion of primary lysosomes in our measurements.

Alho: Have you done so called mega-analysis of all the previous studies?

Ivy: Yes, of the studies I reviewed briefly in this talk, I assure you that we have carefully analyzd each of them according to the criteria I outlined. There simply was not time to go into more detail in my brief talk.

Alho: Have you also measured other cell populations than granule cells? The result could be different in different brain areas.

Ivy: I agree that other cell populations may well yield different results. This particular study has been going on for 12 years. I do have lots of tissue from other brain regions and I would be extremely happy to have you analyze it!

von Zglinicki: By morphometry you are measuring essentially volume fractions in relative terms. How did you obtain absolute reference values?

Ivy: By knowing the exact magnification of our electron micrographs, and correcting for small variations due to technical factors in the electron microscope and in printing of the negatives, we were able to determine exact areas in microns.

Reznick: Have these experimental groups been followed by survival curves and weights of the animals?

Ivy: These animals are part of a larger study by Drs. Walford and Weindruch which showed larger survival rates for the 52% caloric restricted group. Many other parameters were also measured including body weights, immune responses, etc. Animals looked much healthier in the 52% caloric restricted groups even at the very old age of 45 months.

Reznick: You are looking at a very selective and unique group in your very aged rats, since most of your animals have died by then.

Ivy: Correct, but there is no other way of doing it. This is why we have used four different age groups (3 per diet group).