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LIPOFUSCIN—DOES IT AFFECT CELLULAR PERFORMANCE?

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INTRODUCTION

ONE OF the most consistent morphological observations made on tissues from ageing animals (both invertebrate and vertebrate) is the presence of the intracellular ageing pigment—lipofuscin (Timiras, 1972; Schofield and Davies, 1978). This material has been noted in a variety of cells over a period of a century or more of research (see references in Bourne, 1973; Samorajski *et al.*, 1964; Strehler *et al.*, 1959). It is thought that this accumulation of pigment is irreversible and that its presence will in some way interfere with cellular function (Samorajski *et al.*, 1964; Samorajski *et al.*, 1968), but to our knowledge there is very little evidence concerning either the reasons for the accumulation of this material with age, or whether or not the presence of lipofuscin adversely affects cellular function. Various cellular organelles have been implicated in the formation of age-pigment granules with evidence for the involvement of mitochondria (Bondareff, 1957; Duncan *et al.*, 1960; Kumamoto and Bourne, 1963), various elements of the endoplasmic reticulum and the lysosomal apparatus (Brunk and Ericsson, 1972). Exhaustive histochemical, enzyme cytochemical (Strehler, 1964), and biochemical investigations (Taubold *et al.*, 1975) have been carried out on the lipofuscin granule indicating a rather complex structure with a lipid core, combined with various protein and amino acid subcomponents. There is strong evidence that lipofuscin formation can be promoted by a vitamin E deficiency in rat adrenal tissue (Weglicki *et al.*, 1968), and the role of free-radical damage in the etiology of pigment manufacture has subsequently been discussed. In addition, considerable effort is being put into the removal of lipofuscin from cells using drug treatments (Hasan *et al.*, 1974; Nandy and Bourne, 1966; Nandy, 1978; Nandy *et al.*, 1978; Samorajski and Rolsten, 1976; Schneider and Nandy, 1977; Spoerri and Glees, 1975). Unhappily, the methods reported are not quantitative and are hence very difficult to interpret in functional terms. It is also not at all clear how these drugs influence the claimed removal of lipofuscin from cells, whether it be by prevention of cellular autophagy, physical dissolution of granules, or altering the fundamental ageing patterns of the cells concerned.

This paper presents quantitative data on lipofuscin accumulation with age and its reduction during the course of an experiment on the effects of physiological stress on neuroendocrine cells from aged mice. The regions of the brain where these cells are located are the magnocellular "nuclei" of the hypothalamus; the supraoptic (SON) and paraventricular nuclei.

Cellular processes from these regions extend through the median eminence to the posterior lobe of the pituitary which acts as a reservoir for the storage of their hormone products. The cells in these regions are involved in the synthesis, transport and release of at least two peptide hormones and their associated "carrier" proteins—vasopressin, oxytocin and the neurophysins (see references in the review by Morris *et al.*, 1978). An

osmotic challenge, in the form of sodium chloride in the drinking water, stimulates the release of vasopressin from the posterior pituitary and hence, synthesis of this hormone is initiated in the SON and paraventricular nuclei (review Morris *et al.*, 1978). This technique has become standard in the study of the dynamics of hormone synthesis and release in the SON, paraventricular nucleus and posterior pituitary lobe (Morris *et al.*, 1978). In this study, the above experimental procedure was used to induce SON cell activity in animals at two ages which was then measured by quantitative morphological techniques (Weibel, 1969). The results indicate that in these cells, the presence of lipofuscin does not limit the production of hormone containing organelles (the neurosecretory granules, NSG) in either the normally hydrated or the osmotically stressed old mice.

MATERIALS AND METHODS

C57BL/Icrfa[1] mice were kept under standard conditions in a colony of ageing animals maintained by the Geigy Unit for Research in Ageing. The maintenance conditions for these animals have been described previously (Davies and Fotheringham, 1980*a*).

Electron microscopy

Virgin male mice at 6, 16 and 28 months of age were used in these observations. All operations on these animals were carried out between 10.00 and 11.00 h in order to avoid diurnal fluctuations in hormone producing activity by these cells. The animals were anaesthetised by an intraperitoneal injection of sodium pentobarbitone (60 mg/kg body weight) and perfusion took place through the heart using a small peristaltic pump with a flow rate of approximately 2 cm^3/min (Davies and Fotheringham, 1980*a*). The perfusion procedure, together with the fixation, dehydration and embedding schedules have been described previously (Davies and Fotheringham, 1980*a*).

Osmotic loading

Only two sets of animals were used in this experiment; these were aged 6 and 28 months. Groups of these animals were administered an osmotic challenge consisting of 3% sodium chloride in the drinking water for a period of up to six days, with sampling of control and experimental groups at 1, 2, 3, 4 and 6 days. On each day one control (i.e. normally hydrated) and three experimental animals were killed and prepared for electron microscopy. The data in this paper refer to the six day salt loaded animals exclusively. In addition, a group of normally hydrated animals at 16 months of age was included in the experiment. All details of the experimental procedure used are described elsewhere (Davies and Fotheringham, 1981*a, b*).

Stereology

All details concerning the sampling of the SON cells and the quantitative methods used to assess the electron micrographs have been published previously (Davies and Fotheringham, 1980*a, b, c*). The relative volume fractions of lipofuscin and NSG were determined in the SON cells at the ages described.

Statistics

Observations from the individual animals within the control groups at each age were pooled to produce a mean of means for each age group. An analysis of variance was used to test between the three control groups. Subsequent testing between all possible pairs of observations was carried out using a multiple comparisons test (Scheffé test) with probability values set at 0.05 and 0.01 from *F*-distribution tables (Winer, 1971). Testing between the control and experimentally stressed animals employed the Students *t*-test. In addition, non-parametric tests were also used to assess the differences between the control and experimental groups with essentially similar results. All tabulated data are presented in the parametric form for convenience.

RESULTS

There is a progressive increase in the relative volume fraction of the SON cells occupied by lipofuscin between 6 and 28 months of age (Table 1). However, the only significant increases recorded by the multiple comparison test were between 6 and 28 months ($p < 0.01$), and 16 and 28 months ($p < 0.01$) of age. These observations indicate that the main period of lipofuscin accumulation was in the latter part of the lifespan.

The relative volume fractions of the NSG in the SON cells are given in Table 1. There is a significant decrease in the relative volume of NSG recorded between 6 and 16 months

TABLE 1. THE INFLUENCE OF AGE ON THE PERCENTAGE VOLUME FRACTION OF LIPOFUSCIN AND NSG IN NEURONES OF THE SON

Age (months)	n	Lipofuscin	NSG
6	5	1.02 ± 0.10	0.295 ± 0.04
16	4	1.43 ± 0.23	0.213 ± 0.02
28	5	2.46 ± 0.31	0.316 ± 0.03

Data presented as pooled means for n animals. Mean ± 1S.D. See text for statistical comparisons.

of age ($p < 0.01$) followed by a significant increase during the period 16 to 28 months of age ($p < 0.01$). However, there is no difference in the values recorded for 6 and 28 months of age.

There were no significant differences in the relative volumes of the cytoplasm, nuclei and mitochondria over the period investigated (analysis of variance, $p > 0.05$), although a decrease in the relative volume of the golgi-rich zone was recorded between 6 and 16 months of age (multiple comparison test, $p < 0.05$), followed by an increase between 16 and 28 months ($p < 0.05$). There were no significant differences in the data for the relative volume of golgi at 6 and 28 months of age.

Marked changes take place in the ultrastructure of SON cells of both young and old mice under osmotic load and these have been described elsewhere (Davies and Fotheringham, 1980*b*). Only the changes in the relative volumes of SON cells occupied by lipofuscin and NSG are reported in this paper. After six days of osmotic stress there is an approximate 50% reduction in the volume of the SON cells occupied by lipofuscin at both ages (Table 2). However, there is no significant difference in the relative volume of NSG in these cells at either age under these conditions (Table 3).

TABLE 2. THE INFLUENCE OF BOTH AGE AND OSMOTIC LOAD ON THE PERCENTAGE VOLUME FRACTION OF SON CELLS CONTAINING LIPOFUSCIN

	Age (months)		
Treatment	6	28	p
Control ($n = 5$)	1.02 ± 0.1	2.46 ± 0.31	0.001
Salt-load ($n = 3$)	0.58 ± 0.038	1.03 ± 0.11	0.001
p	0.001	0.004	

Data presented as pooled means for n animals. Mean ± 1S.D. Probabilities derived from Students t-tests (two-tailed).

DISCUSSION

It is generally claimed that lipofuscin is a waste-product which accumulates within cells and that its presence is an indicator of cellular inefficiency. There is some suggestion that the accumulation of lipofuscin in neurones in autopsy specimens of the human central nervous system is positively correlated with a reduction in cellular RNA content (Mann *et al.*, 1978). In this case there is at least a potential decline in cellular protein

synthetic function with age and pigment accumulation. However, a recent paper (Anthony and Zerweck, 1979) has indicated that neurones in the auditory cortex of old mice contained less RNA than those of young mice, but upon auditory stimulation neuronal RNA values were not significantly different between young and old animals. A recent paper by Brizee and Ordy (1979) showed that there were large increases in the amount of lipofuscin present in neurones from selected areas of the hippocampus and visual cortex of the rat. In addition, there is a strong intercorrelation between the accumulation of age-pigment, reduced neurone density and reduced short-term passive-avoidance retention behavior with age.

TABLE 3. THE INFLUENCE OF BOTH AGE AND OSMOTIC LOAD ON THE PERCENTAGE VOLUME FRACTION OF SON CELLS CONTAINING NEUROSECRETORY GRANULES

Treatment	Age (months) 6	28	p
Control ($n = 5$)	0.295 ± 0.04	0.316 ± 0.03	0.59
Salt-load ($n = 3$)	0.34 ± 0.03	0.34 ± 0.07	0.95
p	0.17	0.53	

Data presented as pooled means for n animals. Means ± 1S.D. Probabilities derived from Students t-tests (two-tailed).

In many of the investigations published so far it is implied (if not always stated) that age-pigment is detrimental to cellular function. However, to our knowledge, no one has attempted to critically evaluate this proposal. Indeed, others have recently voiced the same concern (Siakotos *et al.*, 1979). In this paper we confirm a significant increase in lipofuscin with age, but the relatively small volume of the SON cells occupied by this material leaves considerable doubt as to its potential for the disruption of cellular activity. Indeed, using the criterion of the volume of NSG in the SON cells as an indication of the functional potential at different ages, we can demonstrate a decrease in the NSG volume content between young mature and middle aged mice, with no significant increase in the volume of lipofuscin over this period. There is a significant increase in the relative volume of the NSG between mature and senescent mice, but also a significant increase in the lipofuscin content of the cells. In these cells therefore, there is no apparent connection between lipofuscin content and hormone content (as determined by these quantitative ultrastructural parameters). However, an elevated lipofuscin content does not preclude finding similar volumes of hormone granules in old as in young cells. In addition, the SON cells from old animals undergoing osmotic stress contain similar relative volumes of NSG to those from young mice. The decline in the volume fraction of lipofuscin under conditions of salt-stress suggests that these structures are not being maintained in some steady-state and are being allowed to deplete from the SON cell perikaryon. Hence active synthesis and turnover in the normally hydrated animal would seem highly likely. The reason for the decline of the lipofuscin organelles in osmotically stressed mice may be that cellular activity under these conditions is switched to a programme with NSG production as the "prime" objective. The suggestion has been made (Davies and Fotheringham, 1980*c*) that this programme is protected late into the lifespan, possibly at the expense of other cellular response and maintenance schedules. However, it should be noted that

measurements made on the size and numbers of NSG per unit volume of SON perikaryon and posterior pituitary neurosecretory nerve endings and swellings indicate that rather subtle changes are taking place in these structures also (Davies, Fotheringham and Wilkinson, in preparation). A critical, qualitative and quantitative evaluation of other cellular organelles in the SON cells of osmotically stressed old animals is presented elsewhere and it is evident that there are declines in the structure and function of a variety of cytoplasmic components under these circumstances (Davies and Fotheringham, 1980*b, c*).

As to the origin of these organelles in SON neuronal perikarya, a few comments can be made. Siakotos *et al.* (1979) proposed a modified Novikoff sequence (see Siakotos *et al.*, 1979 for references) which involves the fusion of lipid globules or cytolysosomes with primary lysosomes and microperoxisomes. This structure eventually becomes the residual body of the autophagic sequence, termed the "mature" lipofuscin granule by Siakotos *et al.* (1979). Siakotos bases his schema on sedimentation velocity isolation of lipofuscin from human brains with an age range of 0.36 to 78.8 years. In the age groups up to approximately 17 years of age the lipofuscin pigment is composed of electron lucent globular areas at first, with an increasing electron dense component added with time. After this age, the electron dense component increases. In the mouse SON cells (Davies and Fotheringham, 1980*b, c*) there is a proportionally greater increase in the vacuolated lipofuscin organelle with age. The source of the lipid globules or cytolysosomes in the proposed sequence suggested by Siakotos *et al.* (1979) is not stated. In the SON cells of young mice lipid globules arise during periods of osmotic stress (Davies and Fotheringham, 1980*c* and references therein) but to a significantly lesser extent in the older animals. In addition, it is possible to see these lipid bodies in young normally hydrated animals, but it is a *very rare event.* We have not encountered these lipid organelles in old mice of the same physiological status. In addition, using serial sectioning techniques at the electron microscope level, we have never seen a close association between lipid droplets and electron dense lysosomal structures in SON cells, even though the conjunction of these two structures was an attractive possibility in the formation of the vacuolated lipofuscin granule. Since our data suggest lipofuscin turnover, we can only conclude that the presence of these electron lucent vesicles is not a major source of potential lipofuscin material in these cells. Subsequent studies of osmotically stressed animals which have been allowed to recover and rehydrate may produce valuable information on the formation of the vacuolated lipofuscin granule.

In conclusion, the SON perikarya of ageing mice accumulate lipofuscin but this does not necessarily lead to a decrease in the volume content of NSG in these cells and there is no detectable decline in what has been termed the "prime" function of the SON cell in either normally hydrated or osmotically stressed mice. There is a period during the lifespan when the relative volume of the NSG decreases in the SON perikaryon, but no reasons for this are immediately apparent. Similar rather paradoxical observations were made on intranuclear inclusions in the nuclei of SON cells from aged female mice. In this case, there was a continuous increase throughout the lifespan until, in the oldest age group (32 months) there was an unexpected decline (Fotheringham and Davies, 1980). This was discussed with respect to selection of the "fittest" animals among extreme old age groups and may be applicable in this case (Fotheringham and Davies, 1980). No conclusions may be drawn with respect to other cell types because of the widely disparate relative volume content of lipofuscin in these various cells. For example, a recent publication

(Brizee and Ordy, 1979) reports the content of lipofuscin in neurones of the hippocampal CA1 zone as being 35.58% and in those of the visual cortex as 30.65% in 29-month-old rats. However, the results obtained in this study cast serious doubts on the role of lipofuscin as being deleterious; it seems more likely that it is an indication of active dissolution of redundant cellular organelles and as such is some form of protective mechanism for the cell. It is also evident that the accumulation of lipofuscin as an age-specific biological parameter (ASBP, as discussed in Donato *et al.*, 1979) is related to increasing intracellular damage with age. Therefore, it is of the utmost importance to understand what is happening with respect to the various drug treatments claiming to reduce lipofuscin accumulation. To reiterate; are these drugs preventing cellular autophagy, removing the granules or altering the fundamental ageing pattern of the cells? It is a matter of some urgency to establish which is the primary effect using critical and quantitative methods.

SUMMARY

Observations on the age-related volume composition of lipofuscin and hormone-containing organelles in the neurosecretory neurones of the supraoptic nucleus of the hypothalamo-neurohypophyseal system of the ageing C57BL mouse are presented. There is an age-related accumulation of lipofuscin in these neurones with age and although there are changes in the relative volumes of hormone containing structures as determined by quantitative ultrastructural techniques, these are not necessarily connected with lipofuscin accumulation. Under conditions of osmotic stress, the hormone containing organelles remain at a static level whereas lipofuscin pigment is significantly reduced. Evidence is put forward for a turnover of lipofuscin in these cells, and for the presence of this pigment being an indicator of intracellular damage. It is argued that there is insufficient knowledge available concerning the role of lipofuscin in ageing cells and that under these circumstances, it is a matter of urgency to determine this prior to the irresponsible use of drug treatments to reduce pigment content.

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