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Neuronal Lipofuscin Accumulation in Ageing and Alzheimer Dementia: A Pathogenic Mechanism?

J. H. DOWSON

Summary: The amount of intraneuronal lipofuscin in the parietal cortex and in the inferior olivary nucleus was measured in post-mortem tissue affected by Alzheimer dementia and in specimens from non-demented individuals. The results indicate that there is a linear relationship between the accumulation of cell body lipofuscin and advancing age, both in neuronal populations of the non-demented group, and in the olivary neurones of the demented group. However, in the demented group, the lipofuscin fluorescence intensity of the parietal neurones was not significantly correlated with age. The estimated amount of lipofuscin in the olivary neurones in the demented group was significantly higher than in the non-demented group, when age has been taken into account ($P < 0.01$). The possible pathogenic role of lipofuscin accumulation in ageing and Alzheimer dementia is discussed.

The pigment lipofuscin contains a complex and variable mixture of substances (Brizzee *et al*, 1975; Glees and Hassan, 1976). It has no clear definition, but in the present paper it is considered to be a tissue pigment which exhibits yellow to yellow-orange autofluorescence under specified conditions (Dowson and Harris, 1981).

A relationship between degree of intraneuronal lipofuscin accumulation and ageing has been recognized since the early 19th century, and has been reported to be linear in the inferior olivary nucleus, dentate nucleus, pyramidal cells of the hippocampus, Purkinje cells, and in the myocardium (Strehler *et al*, 1959; Mann and Yates, 1974; Mann *et al*, 1978).

It has also been reported that the rate of increase of intraneuronal lipofuscin is correlated with the rate of reduction of cytoplasmic RNA in inferior olivary neurones and anterior horn cells of the spinal cord, and it was suggested that there is a critical volume of intraneuronal lipofuscin, above which further increases in pigment may be causally related to a more rapid rate of reduction of cytoplasmic RNA, such as was found in the olivary neurones in the 8th and 9th decades (Mann and Yates, 1974). (Reduced intracellular RNA may be associated with impairment of the functional capacity of the cell.)

However, another study has reported that the rate of decrease in cytoplasmic RNA, and also in nucleolar volume, is similar in Purkinje cells, neurones of the dentate nucleus, and in pyramidal cells of the hippocampus, despite the fact that these three cell populations show different rates of lipofuscin accumulation. Thus, although the rate of cytoplasmic RNA reduction and of nucleolar volume decrease, was more marked in the inferior olivary neurones which contained more lipofuscin compared with the other three cell populations, the ageing process which is shown by a reduction in cytoplasmic RNA and nucleolar volume, is not consistently correlated with the degree of lipofuscin accumulation (Mann *et al*, 1978).

It has been claimed that variations in the volume of intraneuronal lipofuscin are correlated with a variety of disease processes other than the ceroid-lipofuscinoses, including progeria, Jakob-Creutzfeldt dementia, Huntington's chorea and Alzheimer dementia (Brizzee, 1975; Brizzee *et al*, 1975; West, 1979). Several studies have reported that Alzheimer dementia is associated with an increased amount of lipofuscin in various populations of neurones, including spinal cord neurones (Kent, 1976; Malamud, 1972; Yamada, 1978), although it has also been claimed that there are no such increases (Mann *et al*, 1977; Mann and Sinclair, 1978; Scholtz and Brown, 1978; Torack, 1978). Mann and Sinclair (1978) reported that although Alzheimer dementia was associated with decreased amounts of intraneuronal cytoplasmic RNA, together with reduced nucleolar volume, in Purkinje cells, dentate nucleus, hippocampus and inferior olivary nucleus, there was no increase in intraneuronal lipofuscin in Alzheimer dementia in any of these cell populations.

Various methods have been used to quantify neuronal lipofuscin in cell bodies, including estimation by eye and counting grid interactions overlying the pigment (Brizzee and Cancilla, 1972; Brizzee *et al*, 1974; Nandy, 1978; Riga and Riga, 1974; West, 1979). In contrast with these indirect methods, measurements of the intensity of lipofuscin autofluorescence enables a value to be obtained which is directly related to the amount of pigment. Four publications have reported such measurements, but many of the methodological problems were not discussed (Mann and Sinclair, 1978; Mann and Yates, 1974; Mann *et al*, 1978; Wing *et al*, 1978). The present paper reports data derived from a recently developed method for the quantification of the autofluorescence derived from neuronal lipofuscin. This technique has been used to estimate the volume of intraneuronal lipofuscin in the parietal cortex and inferior olivary nucleus from the brains of two diagnostic groups; one group was composed of patients who had Alzheimer dementia, while the other consisted of individuals who had no history of dementia.

The aim of this study was to investigate the possible pathogenic role of lipofuscin in both normal ageing and Alzheimer dementia.

Materials and Methods

The details and validation of the technique which was used for the measurement of lipofuscin fluorescence intensity, have been reported elsewhere (Dowson and Harris, 1981). Post-mortem brain tissue from specified regions of the parietal cortex and inferior olivary nucleus were taken from two groups of individuals over the age of 60. The first group had a history which clearly indicated that there had not been any significant impairment of cognitive functions due to a chronic dementing process. In addition, the brains of the first group showed an apparent absence of ventricular enlargement, sulcal widening, gyral atrophy, or significant infarction. The second group had a history of severe dementia which suggested Alzheimer's disease, together with an apparent marked ventricular enlargement, sulcal widening and gyral atrophy. Brains from demented patients were excluded if there were significant macroscopic or microscopic signs of infarction. Although senile plaques were not used for diagnostic purposes, no plaques were found in the sections examined from 8 of the 13 non-demented brains, and the remaining 5 had a relatively modest density of plaques compared with all the 10 Alzheimer brains, in which many plaques were seen.

Tissue blocks were embedded in paraffin wax and unstained 20 μm sections were examined using a Leitz Ortholux II microscope with an MPV I photometer and an EMI 9558B photomultiplier (Ploem, 1977). The light source was a mercury lamp (HBO 100 w) and each specimen was irradiated from above. The exciting light passed through a filter which allowed the passage of violet and blue light (390–490 nm), and a mirror, which only reflected certain wavelengths, reflected light below 510 nm towards the specimen. The resulting tissue autofluorescence above 510 nm was transmitted by the mirror before passage through a barrier filter which transmitted wavelengths above 515 nm. This filter combination gave the maximum intensity of yellow lipofuscin autofluorescence, although it also produced a significant degree of unwanted green 'background' tissue autofluorescence, as did all other possible filter combinations.

In the parietal cortex, neurones from the inner third of layer III were examined. To be selected for measurement, the neuronal body (or section of the neuronal body) had to have a maximum dimension of at least 20 μm in each of the two axes which were perpendicular and parallel to the long axis of the neurone, in the plane of the section. These criteria ensured that at least the greater majority of the selected cells were pyramidal neurones (Gray's Anatomy, 35th edition, pp. 948–949). Inferior olivary neurones have a characteristic appearance and seem to form a relatively homogeneous population. An olivary neurone was eligible for selection if the neuronal body, (or section of the neuronal body), had a maximum dimension, in each of any two axes perpendicular to each other, in the plane of the section, of at least 12 μm.

A 400 square grid was superimposed over a field of the region to be examined, and a grid intersection was selected using random numbers. The neurone which was nearest to this grid intersection, and which was large enough to meet the selection criteria, was identified for measurement. Only one neurone was measured from each randomly selected field. The chosen neurone was then placed within that central part of the field from which the intensity of autofluorescence was measured. Each measurement gave a value, in arbitrary units, of the intensity of the autofluorescence, which was mainly derived from the yellow lipofuscin autofluorescence and a surrounding region of green 'background' autofluorescence.

After measurements had been obtained, several correction procedures were applied, to give an estimate of that part of the total measured fluorescence which was derived from lipofuscin. The constancy of the arbitrary unit of fluorescence intensity was ensured by the use of a fluorescent standard of non-fading Uranyl Glass (GG 17, Leitz).

Measurements were obtained from 80 neurones

from each of the two regions of each brain, and each set of 80 values consisted of 10 measurements from each of 8 consecutively alternate sections. The mean of each set of 80 measurements was taken as the estimate for the volume of intraneuronal lipofuscin for each tissue sample.

Results

For the olivary neurones the demented group contained 10 brains, while the non-demented group consisted of 13 brains. Some additional specimens were excluded from the analysis because the intensity of the green 'background' fluorescence was considerably raised when compared with that of the majority of the specimens. This would probably have distorted the estimate of that part of the measured fluorescence intensity which was derived from lipofuscin, despite the correction procedures (Dowson and Harris, 1981). (The reason for such an increase in 'background' fluorescence is unknown, but may have been due to the degree of autolysis before fixation of the tissue.) In addition, one of the parietal cortex specimens from each of the two diagnostic groups had to be discarded, again because of markedly increased 'background' fluorescence, so that, for the parietal cortex, the demented group consisted of 9 brains, while the non-demented group contained 12 brains. Twenty-one of the 23 brains were from females, and the 2 brains from males consisted of the youngest member of each diagnostic group.

The estimated regression lines for the relationship between intraneuronal lipofuscin intensity and age are shown in the Figure, which demonstrates significant linear correlations between lipofuscin fluorescence intensity and age in the inferior olivary neurones of both demented and non-demented groups, and in the parietal neurones of the non-demented group. However, in the demented group, the lipofuscin

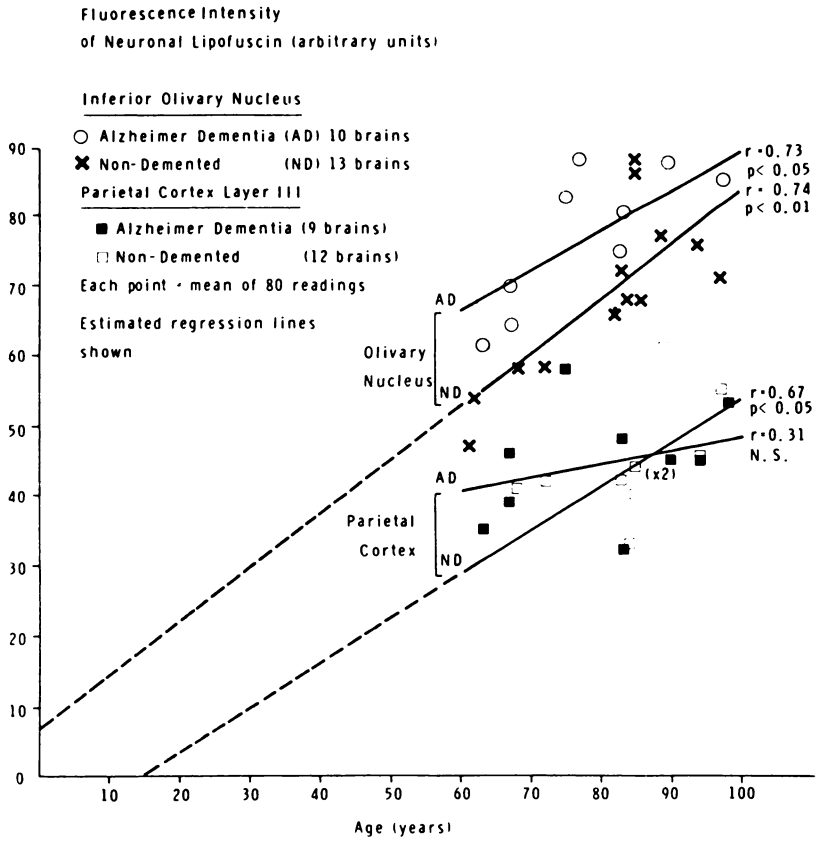

FIG.—The relationship between intensity of intraneuronal lipofuscin fluorescence and age, in Alzheimer dementia and in non-demented human brains.

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fluorescence intensity of the parietal neurones was not significantly correlated with ageing.

For the olivary neurones, an analysis of covariance demonstrates that the slope of the regression lines for the two diagnostic groups did not differ significantly ($F = 2.1$, df 1, 19; $P > 0.05$) but that the elevation (intercept) of the regression line of the demented group was significantly higher than that of the non-demented group ($F = 8.7$, df 1, 20; $P < 0.01$) (Snedecor and Cochran, 1976). Analysis of covariance of the data derived from the parietal neurones did not yield any significant differences between the demented and non-demented groups.

The significantly greater lipofuscin fluorescence intensity in the olivary neurones of the demented group, relative to the non-demented group, can also be demonstrated by comparing the 8 pairs of brains, one member of each pair from each diagnostic group, which can be closely matched for age to within three years (see Table) (Wilcoxon matched pairs test, 2 tailed, $P = 0.01$; Siegel, 1956).

In the non-demented group, the lipofuscin fluorescence intensity of the inferior olivary neurones was correlated with that of the parietal neurones, (Spearman rank correlation coefficient $rs = 0.6$, $P < 0.05$, 2 tailed; Siegel, 1956). However, a similar correlation between the two brain regions could not be demonstrated in the demented group.

It should be noted that no differences could be detected between the (uncorrected) emission spectra of the olivary neuronal lipofuscin of demented and non-demented brains; any difference would have distorted the relationship between volume of pigment and fluorescence intensity (Dowson and Harris, 1981).

TABLE

Intraneuronal lipofuscin fluorescence intensity in the inferior olivary nuclei of 8 age-matched pairs of brains, from demented (AD) and non-demented (ND) groups

Pair	Fluorescence intensity (arbitrary units)		Ages	Years
	ND	AD	ND	AD
1	54.4	61.4	62.3	63.0
2	58.2	70.2	67.6	67.3
3	57.7	82.8	72.3	75.2
4	72.2	75.1	82.9	82.8
5	67.9	80.4	84.3	83.5
6	77.4	87.7	89.4	89.9
7	75.5	79.0	93.8	93.8
8	70.8	85.4	97.3	97.7

Each value for intensity = mean of the measurements from 80 neurones.
(AD = Alzheimer dementia).

Discussion

If a relationship between lipofuscin fluorescence intensity and volume of lipofuscin is assumed, these findings demonstrate changes in intraneuronal lipofuscin in Alzheimer dementia and in the ageing process. (Although all but one of each group of brains were from female individuals, it has been reported that the rate of lipofuscin accumulation in both demented and non-demented brains does not differ significantly between the sexes (Mann *et al*, 1978).)

However, there are two possible factors, other than an increase of intraneuronal lipofuscin, which could have produced or contributed to the present findings. The first factor is that any reduction of neuronal volume in relation to ageing or Alzheimer dementia might have modified the sampled population so that it included a higher proportion of cells which were relatively large in relation to the mean population size and which tended to contain more lipofuscin than relatively small members of the population; Brizzee and Knox (1980) have described a reduction of neuronal volume with ageing in the rat. The second factor is that any selective loss of cells which contained relatively small amounts of lipofuscin, in ageing or dementia, might also have weighted the sampled population in favour of cells containing relatively large amounts of pigment.

In the present study, an examination of estimated neuronal body size was carried out for the olivary neurones by counting grid intersections overlying a series of randomly selected cell bodies which fulfilled the selection criteria. This technique did not show any significant difference in mean cross-sectional area of the neuronal bodies between the demented and non-demented groups, or between younger and older brains in either diagnostic group. It should also be noted that the selection criteria for the olivary neurones led to the inclusion of the majority of the cells, or sections of cells, which could be identified. Thus, it is unlikely that changes in cell body size of inferior olivary neurones were responsible for, or contributed to, the increases of intraneuronal lipofuscin with increasing age or in Alzheimer dementia. Although the possibility of a selective loss of relatively sparsely pigmented olivary neurones with ageing or with Alzheimer dementia cannot be ruled out completely, this is also unlikely for two reasons. Firstly, the most heavily pigmented olivary neurones in older brains exhibited more lipofuscin fluorescence than the most heavily pigmented cells in the younger brains, in both diagnostic groups, and secondly, the olivary neurones which contained the most pigment in the 8 demented members of the age-matched pairs contained more lipofuscin than the most heavily pigmented cells in their age-matched counterparts in

the non-demented group. Therefore, a selective loss of relatively sparsely-pigmented olivary neurones is unlikely to have been responsible for the increase in olivary neuronal lipofuscin in ageing or in Alzheimer dementia. It should be noted that Mann and Sinclair (1978), who claimed that there was no increase in olivary neuronal lipofuscin in Alzheimer dementia, used different selection criteria for their population of olivary neurones. These authors required the presence of a visible nucleolus, and this procedure may not have delineated equivalent samples of olivary neurones in both diagnostic groups or at different ages, as the nucleus and nucleolus become more difficult to visualize as the amount of intraneuronal lipofuscin increases. (If a cell contains relatively small amounts of pigment, the nucleus and nucleolus, if present in the section, should always be visible, but if relatively large amounts of lipofuscin are present, the nucleus and nucleolus are often hidden from view.)

In the parietal cortex, the cell body volume of the neuronal population which was defined by the selection criteria was not estimated, because a continuous cross-section of a cell body was often difficult to identify using fluorescence or phase contrast microscopy. So, it is possible that a decrease in cell body size contributed to the finding of a linear increase of lipofuscin fluorescence with ageing in the parietal neurones of the non-demented group. However, as with the olivary neurones, the parietal cells with the most lipofuscin fluorescence in the older brains exhibited more lipofuscin fluorescence than their counterparts in the younger brains. Therefore, as with the inferior olivary neurones, the linear increase of lipofuscin autofluorescence with ageing in the non-demented parietal neurones is likely to have been a reflection of the accumulation of pigment.

In the so-called 'normal' elderly, there have been many reports of various cerebral changes, such as cortical atrophy, synaptic changes, loss of dendrites, impairment of cognitive functions, and a reduction in the numbers of various populations of neurones (Brizzee and Knox, 1980; Earnest *et al*, 1979). However, in the case of the inferior olivary nucleus, the evidence regarding loss of neurones with ageing is conflicting; one study of 18 subjects up to the age of 89 found an apparent lack of cell decrease with age (Monagle and Brody, 1974), while a more recent investigation reported a 20% decrease in the number of olivary neurones between the ages of 20 and 80 (Sandoz and Meier-Ruge, 1977). However, whatever the degree of cell loss, it is possible that the accumulation of lipofuscin contributes to functional impairment and cell death in many neuronal populations in the normal ageing process.

Previous reports have claimed that pathological changes in Alzheimer dementia can be found in hippocampal pyramidal neurones, Purkinje cells, neurones of the dentate nucleus, cells of the lateral ventral nucleus of the thalamus, cells of the locus coeruleus and substantia nigra, Betz cells, cells of cranial nerve motor nuclei, and neurones of the inferior olivary nucleus (Mann *et al*, 1977; Mann and Sinclair, 1978). The present finding of increased lipofuscin in inferior olivary neurones in Alzheimer dementia is consistent with previous reports of a greater reduction of cytoplasmic RNA, and of nucleolar volume, in inferior olivary neurones in this disease. It is possible that pathogenic effects of this increase in lipofuscin, which may result from an underlying pathological process or some other variable such as diet or drug treatment, may summate with the lipofuscin accumulation which is found in normal ageing.

Although the amount of lipofuscin in the parietal neurones in the demented group is not significantly different from that of the non-demented group, the present results suggest that, at the terminal stage of dementia, a linear relationship between lipofuscin accumulation and ageing had been destroyed by the disease process. It is possible that, in the earlier stages of Alzheimer dementia, intraneuronal lipofuscin is also increased in the cerebral cortex and that lipofuscin accumulation may contribute to the development of impaired cortical function in both normal ageing and Alzheimer dementia. The critical threshold, above which an increase in lipofuscin is harmful, may vary between cell groups and may also depend on any concomitant disease process. For example, in Alzheimer dementia, the effects of relatively modest amount of intraneuronal pigment may summate with the effects of other pathogenic mechanisms.

One possible means of altering the amount of intraneuronal lipofuscin is by dietry manipulation. In animal studies a protein-deficient maternal diet has been associated with increased intraneuronal lipofuscin in the neonate (Sharma and Manocha, 1977), and lack of antioxidants such as vitamin E, selenium, sulphur-containing amino-acids and chromium, has also been claimed to cause an increase of intraneuronal lipofuscin and a reduction in brain weight (Siakotos and Armstrong, 1975). Also, dietry supplements of vitamin E and other antioxidants have been reported to reduce testicular, cardiac and retinal lipofuscin in mice, and to increase the life-span of experimental animals (Katz *et al*, 1978; Ledvina and Hodanova, 1980). However, in another study, a vitamin E supplement led to a marked increase in rat mortality (Blaauboer *et al*, 1979). Dietry pro-oxidants such as polyunsaturated fatty acids may also affect lipofuscin accumulation. This area of research may

have implications for reducing the degree of intraneuronal lipofuscin accumulation in man, in both normal ageing, and in diseases in which amounts of pigment are increased.

Another method of changing neuronal lipofuscin content is by administration of centrophenoxine. There is histochemical evidence that this drug increases the activity of the pentose phosphate pathway, and other animal studies have reported that centrophenoxine alleviated the ill effects of cerebral oxygen deprivation, which were produced by lowering the atmospheric pressure, or by cyanide administration. The drug has been reported to cause a reduction in the amount of intraneuronal lipofuscin in several cell populations, after daily administration for between 30 and 90 days (Nandy, 1978; Spoerri and Glees, 1975), while a clinical study has claimed that centrophenoxine shortened the post-anaesthetic recovery period after thiopentone anaesthesia (Kugler *et al*, 1973). Two double-blind placebo controlled studies have reported that centrophenoxine administration led to a significant improvement in the performance of a task involving new learning, in elderly individuals (Gedye *et al*, 1972; Marcer and Hopkins, 1977).

In conclusion, the present data indicate that in non-demented individuals over 60, ageing is correlated with a linear increase of intraneuronal lipofuscin in two populations of neurones, and that, in the inferior olivary neurones, the amount of intraneuronal lipofuscin is increased in Alzheimer dementia, when age is taken into account. Lipofusion accumulation may impair cellular function by mechanical effects such as displacement or disruption of endoplasmic reticulum and other cellular constituents, or impairment of transport mechanisms. In view of the possible pathogenic effects of intraneuronal lipofuscin, both in cell bodies and cell processes, research into the factors which reduce the rate of lipofuscin accumulation or the amount of intraneuronal lipofuscin which has already accumulated, may eventually provide treatment for one possible contributory factor to the development of cognitive deficits in both normal ageing and Alzheimer dementia.

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J. H. DOWSON, M.A., M.B., B.Chir., Ph.D., F.R.C.Psych., *Honorary Consultant Psychiatrist and University Lecturer in Psychiatry, University of Cambridge Clinical School, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ*

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