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Age-Related Changes in the Number of Lipofuscin- containing Neurons and Neuronal Histochemistry of Lipofuscin, and the Effect of Aging Reversal Drug Centrophenoxine on Senile Neurons in the Parietal Cortex of Rat

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This study, examined the alteration in the number of lipofuscin-containing neurons in the brain parietal cortex of rats aged: 4, 8, 16 and 24 months. Neurons containing: (i) scattered (ii) aggregated (iii) PAS-positive and (iv) NBS-positive lipofuscin were studied. Neurons devoid of lipofuscin were also studied. Statistical correlations were done for determining interrelationship between various types of neurons and to have some insight into the causal basis for histochemical changes involved in lipofuscinogenesis. The data showed that with ageing the number of neurons with lipofuscin increased. While the number of PAS-positive neurons declined with age that of NBS-positive increased. The statistical interrelationships between the neuronal number changes indicated that with advancing age the scattered lipofuscin aggregated; it acquired NBS-positivity and lost PAS-positivity. Centrophenoxine effect was studied in 24 month old rats to determine quantitatively the effect of the drug on neuron numbers and whether it acted on both PAS-positive and NBS-positive lipofuscin. The drug treatment caused a significant increase in the number of neurons devoid of lipofuscin.

Key Words: Age-related changes, Lipofuscin-containing neurons, Neuronal histochemistry, Centrophenoxine, Rats

Introduction

Ageing is associated with accumulation of neuronal lipofuscin (Mesulam 1987) which is formed mostly from protein and peroxidized lipid material autophagocytosed from various cellular constituents and fragmented within the lysosomes (Dowson et al. 1992,1993, Brunk et al.1992,Winterbourne & Johnson 1994). Increasing oxidative stress is a causal factor both in lipofuscinogenesis and the ageing process (Sohal et al. 1989, Sohal & Allen 1990).

Age-related rise in lipofuscin may be correlated with age-related loss of neurons suggesting that neurons may die of increasing oxidative stress (Sohal et al.1989, 1990).Lipofuscin accumulation has also been correlated with age related decline in cognitive functions (Nandy 1978, 1983,Brizzee & Ordy 1979, Hayes 1974). Therefore,quantitative infrmation regarding the number of neurons accumulating lipofuscin in a brain during ageing will also be an important ageing parameter besides the gross lipofuscin contents in a particular brain region (Sharma et

al. 1993) or the amount of lipofuscin per neuron (Dowson et al. 1992, Drach et al.1994). In the present study, therefore, we report the age-related changes in the number of neurons containing dispersed, clumped, PAS-positive, NBS-positive and no lipofuscin in the parietal cerebral cortex of the rat. Correlations between various relevant parameters were determined to have an insight into the statistical evidence showing possible causal factors underlying the observed changes.

The drug centrophenoxine (CPH) reverses several age-dependent deteriorative changes and has antilipofuscin and antioxidative effects (Sharma et al.1993). In the present work the effect of this drug has been studied mainly to find out quantitatively the effect of the drug in terms of neuronal numbers and whether the drug exertsits effects against both young PAS-positive(scattered) and senile NBS-positive (aggregated) lipofuscins (Dowson 1985).

Materials and Methods

Male albino Wistar rats of 4, 8, 16 and 24 months of age, maintained on the standard commercial feed obtained from Hindustan Lever Ltd. Bombay, were used. Another group of rats (n=30,24-month-old) selected randomly from the pool was used for centrophenoxine treatment. Daily a dose of 80 mg/kg was administered (i.p) for 10, 20 or 30 days. Controls were given saline (0.9% NaCl). Animals after proper anesthetisation with chloroform were perfused via the left ventricle with saline in order to remove the blood and to prevent the tissue from cellular damage. The parietal cortex from each brain was separated and left in 10% formalin for 48 hr. Localisation of lipofuscin was done in paraffin sections by Periodic acid-schiff (PAS),Sudan black B (SBB),Nile blue sulphate (NBS), Ferric-ferrocyanide (Pearse 1972) and fluorescence microscopy methods. Neurons were counted by the method of Few and Getty (1967). Twenty sections from each animal and the population of cells appearing in a minimum of ten micro-scopic fields in each section under the 100x magnification (oil immersion) focused in different areas were taken into account. In each microscopic field, the number of nonpigmented (without lipofuscin) neurons, and of those containing clumped, diffused, NBS or PAS positive lipofuscin were counted. In the rats of each age group as well as in centrophenoxine-treated old rats, the percentage of various types of neurons was determined. To assess the relationship among them Pearsons correlative test was used.

Statistics: One way analysis of variance (ANOVA) was used to analyse the effect of age and centrophenoxine on various types of neurons.Scheffes multiple comparison test was used to make post hoc comparisons. Pearsons correlations were done between all the parameters for various age groups as in our recent study (Sharma et al.1993).

Results

Age-related Changes in the Number of Pigmented (Lipofuscin-containing) and Non-pigmented Neurons

Figure 1A shows an age-related decline in the number of nonpigmented neurons. The one way ANOVA indicated a significant effect of age on non - pigmented neurons: $F(3,16) = 440.87$; $p < 0.01$. At 24 months of age the parietal cortex had only 22% neurons without lipofuscin. Rest 78% neurons had acquired lipofuscin deposits. Figures 1B-D show an increase in the number of igmented neurons with age as discerned by fluorescence microscopy. Based on the stainability characterstics of lipofuscin two cell types were distinguishable: PAS-positive and NBS-positive. In figure 2 are shown samples of PAS positive (scattered distribution) and NBS- positive (aggregated or clumped distribution) neurons from young (4 month) and old (24 month) animals. It will be observed that NBS-positivity increases with age while PAS-positivity

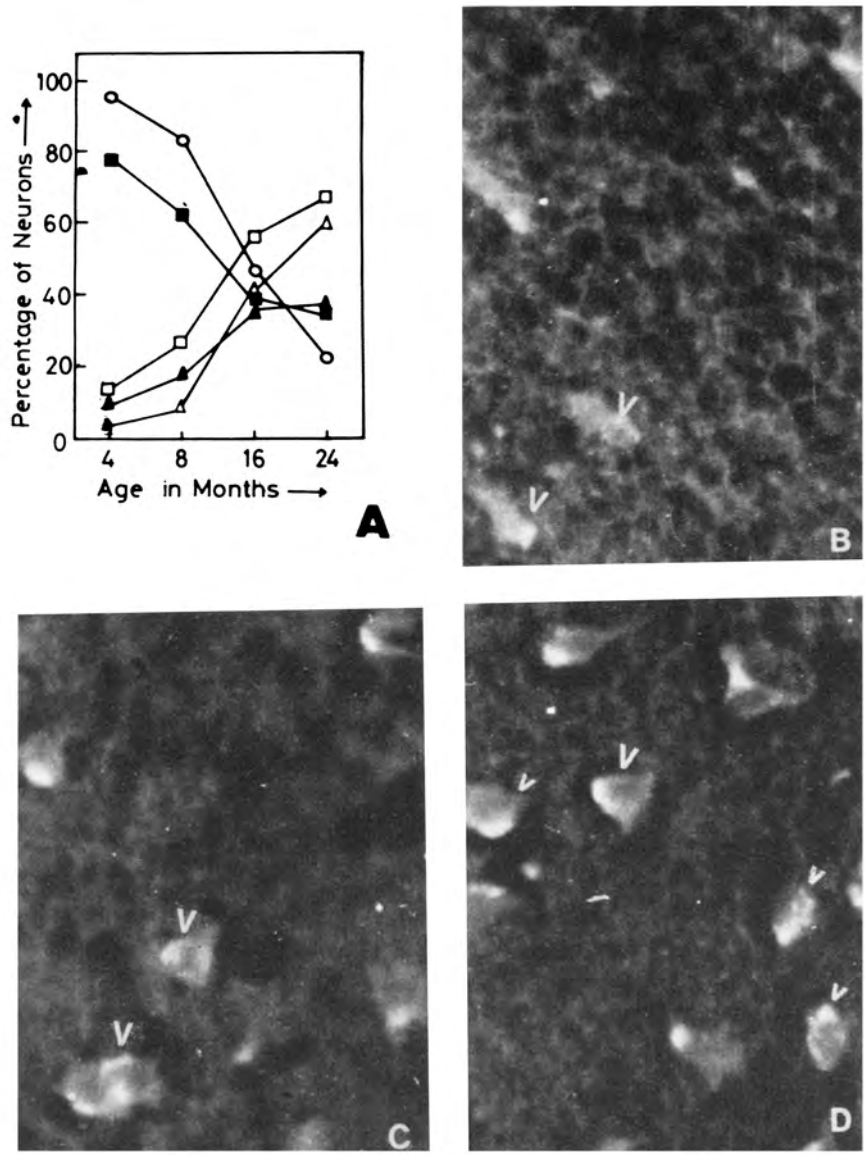

Figure 1 A-D A, Age changes in the number of neurons with no lipofuscin o—o, neurons containing diffused ^—^, aggregated ^—^, PAS positive [ ]—[ ],and NBS positive lipofuscin [ ]-—[ ]of the rat parietal cortex; **B,C & D,** Fluorescent photomicrographs show increase in the number of lipofuscin neurons with ageing: 4-,8- and 24-months-old rats respectively

declines. According to the morphological nature of lipofuscin two cell types were discerned:neurons containing pigment randomly dispersed throughout the cell body and neurons containing dense aggregation of lipofuscin in the form of one or more large clumps (figure 3).

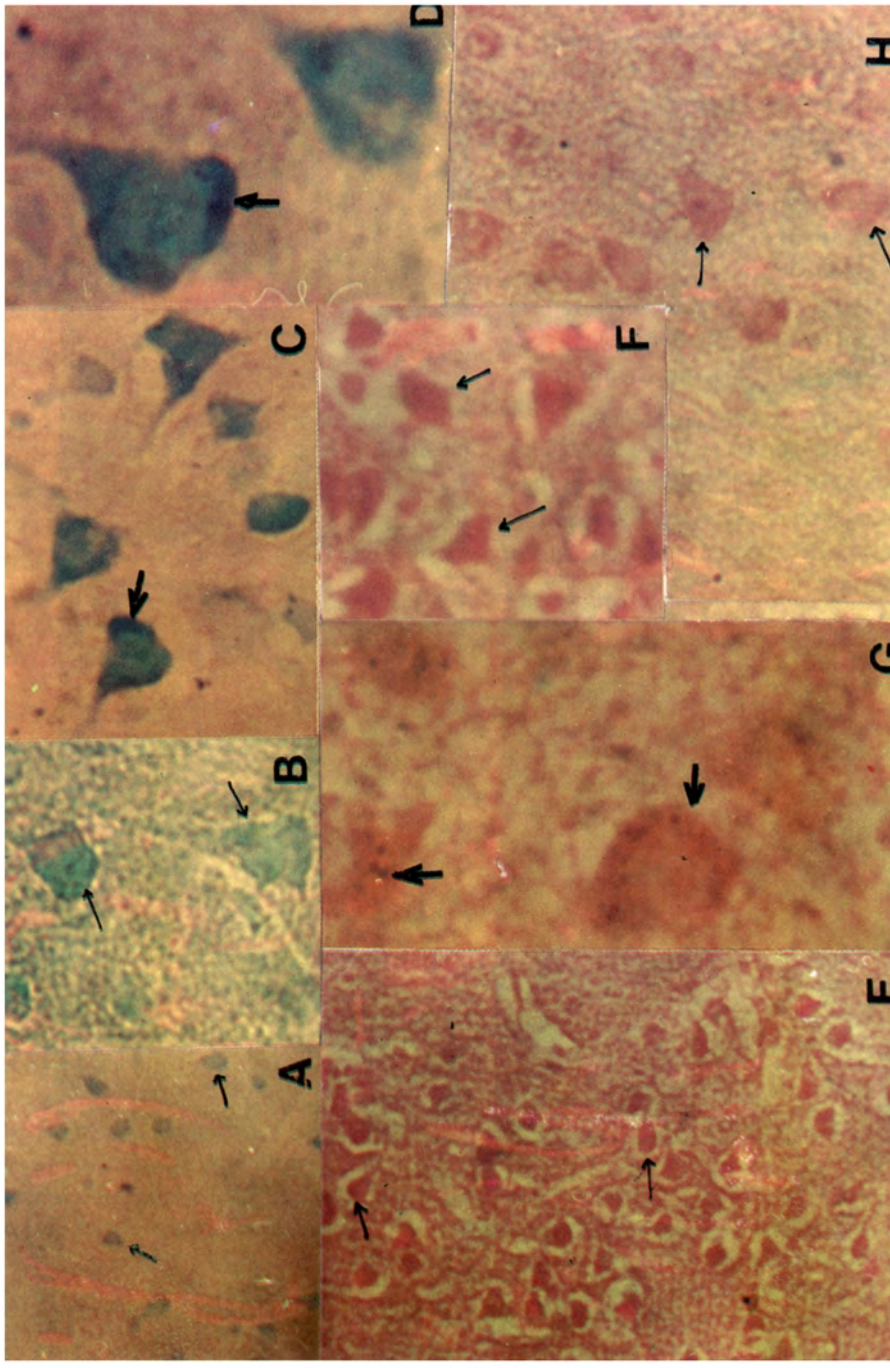

Figure 2 A-H Nile blue sulphate(**A -D**), and PAS stained (**E-H**) sections of the brain parietal cortex from 4 - and 24-month-old rats; **A & B** (4 -month - old rats); **C&D** (24- month- old rats); show age-related increase in NBS- positivity of lipofuscin staining & aggregatedness; **E,F & G** (4 -month-old rats); **H,** (24-month-old rats) show decrease in staining

While the number of PAS-positive neurons was decreased with age, that of NBS-positive neurons increased (figure 1A). The one way ANOVA indicated a significant effect of age on both, the PAS positive ($F\ 3, 16 = 124.42; p < 0.01$) and the NBS- positive ($F\ 3, 16 = 307.01; p < 0.01$) neurons. Together with this the number of neurons containing both diffused and aggregated lipofuscin was increased with age. The one way ANOVA indicated a significant effect of age on both types of neurons i.e. those containing diffused ($F\ 3,16 = 125.27; p < 0.01$ and those containing aggregated ($F\ 3,16 = 381.67; p < 0.01$) lipofuscin.It would thus appear that the number of lipofuscin -containing neurons relative to that of neurons not containing it was increased with age. This generally indicates that more and more neurons accumulate lipofuscin with age. An age-related decline in PAS-positivity together with an age-related increase in NBS-positivity (figure 1A) would indicate that with advancing age lipofuscin loses PAS positivity and becomes NBS-positive.

Statistical Correlation between Age-related Changes in the Number of Various Cell Types

The age-related increase in the number of neurons containing scattered lipofuscin correlated strongly positively with the age-related increase in those containing aggregated lipofuscin (figure 4A, $r=0.95$, $p < 0.01$). This correlation suggests that with advancing age scattered lipofuscin tends to aggregate.

There was a strong positive correlation between the age-related increase in the number of neurons containing aggregated lipofuscin and that of the neurons containing NBS-positive lipofuscin (figure 4B, $r=0.98$, $p < .001$). This indicates that with advancing age the aggregated lipofuscin acquires NBS-positivity but loses PAS-positivity. A strong negative correlation between the age-related decline in the number of PAS-positive neurons and the age related increase in that of NBS-positive neurons (figure 4F, $r=-0.98$; $p < .001$) together with a positive correlation of the number of NBS-positive neurons with that of scattered-lipofuscin neurons

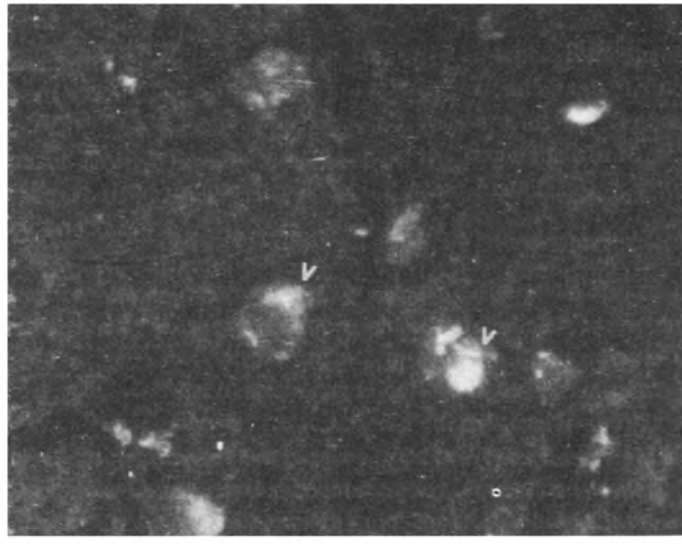

Figure 3 Fluorescence photomicrographs showing scattered (diffused) and aggregated (clumped) lipofuscin

(figure 4E ,r=0.98; p < .001) would further support the conclusion that with advancing age NBS-positivity replaces PAS-positivity. Furthermore, the age-related changes in both aggregatedness and diffuseness of lipofuscin were negatively correlated with PAS-positivity changes with age (figure 4 C & D, r=-0.98&r=-0.95; p < 0.001). This further suggests that with advancing age lipofuscin loses its PAS-positive character and acquires NBS-positivity. A comparison of the figures 3A and 3E would show that scatteredness of lipofuscin was positively correlated with both aggregatedness and NBS-positivity. This reinforces the conclusion that with age the scattered lipofuscin becomes aggregated and acquires NBS-positivity. A strong negative correlation of PAS-positivity with age-related increase in the number of neurons containing scattered lipofuscin (figure.4C r=-0.98, p < .001) would also suggest loss of PAS-positivity with age.

Effect of Centrophenoxine (CPH) on Changes in the Numbers of Lipofuscin Containing Neurons

In rats treated with centrophinoxine for 10,20 or 30 days , the numbers of neurons devoid of lipofuscin were significantly higher when compared with controls (figure 5A). The one way ANOVA indicated a significant effect of CPH treatment on the non-pigmented neurons (F 3,16 = 176.90; p < 0.01). Figures 5 B-D show the neuronal number changes following the drug treatment as seen by the fluorescence microscopy. The numbers of neurons containing NBS-positive, aggregated and diffused lipofuscin were less than those found in controls (Figure.5A). The one way ANOVA indicated that there was a significant effect of the drug treatment on neurons containing predominently NBS-positive (F 3,16 = 102.53; p <0.01): aggregated (F 3,16 = 204.80; p < 0.01) and diffused lipofuscin (F 3,16 = 134.93; p < 0.01). The increase in the number of cells deviod of lipofuscin and the decrease in that

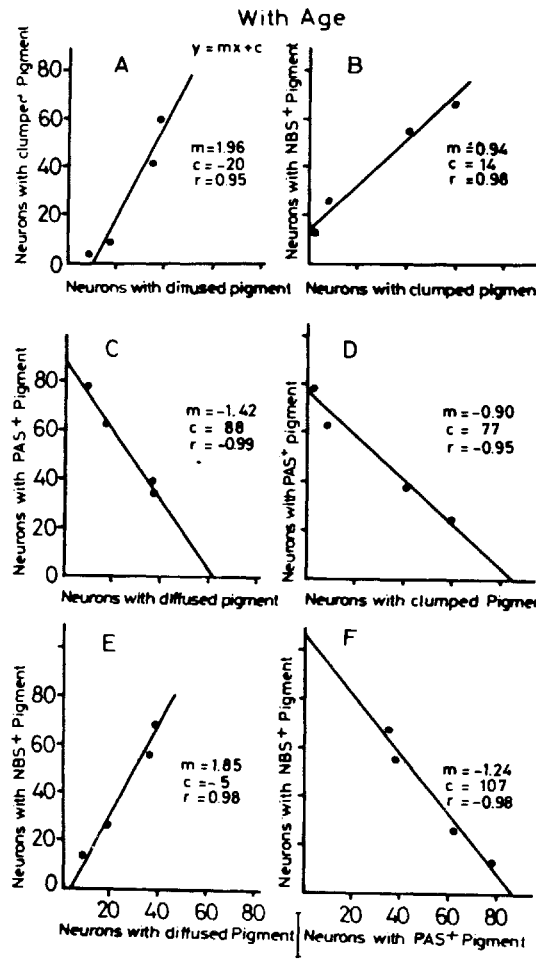

Figure 4 A-F, showings correlations between lipofuscin neurons of various types for the four age groups (See legend for figure 1 text)

of cells containing lipofuscin was related to the length of the duration of the drug treatment; the longer treatment durations produced greater changes. The neurons with PAS-positive lipofuscin were however, practically unaffected by 10 & 20 days of drug treatment. After 30 days treatment, slight increase in the PAS-positive neurons was observed.

Statistical Correlations between Neuronal Number Changes following Centrophenoxine Treatment

Correlational analysis of cell number changes in centrophenoxine-treated animals showed that the drug-induced decrease in neurons containing clumped lipofuscin was positively correlated with the drug induced decrease in cells containing NBS-positive lipofuscin (figure 6D). This correlation is consistent with the finding that the drug treatment decreased the number of both NBS-positive and aggregated lipofuscin neurons. This may also reflect the NBS-positive nature of the clumped lipofuscin. The drug-induced decline in the number of neurons containing clumped lipofuscin was negatively correlated with the drug-induced rise in neurons with PAS-positive lipofuscin (figure 6 A & B). A negative correla- ation was also evident between the drug- induced elevation in PAS-positive neurons and the drug induced decline in NBS-positive neurons (figure 4F). These correlations are consistent with the finding that while PAS-positive neurons increase after CPH-administration, the numbers of neurons containing diffused and clumped lipofuscin decrease. A positive correlation between the drug-induced decrease in neurons with clumped and diffused lipofuscin (figure 6A) is reflective of the drug induced alteration in the neuronal numbers (figure 5A). Correlations seen in figures 6C and 6F are consistent with the corresponding changes in cell numbers observed after CPH administration (figure 5A).

Discussion

Our data provide a quantitative statistical evidence of a general age-related increase in the number of lipofuscin loaded neurons and a decrease in that of neurons without lipofuscin. The cells containing PAS-positive, and NBS-positive lipofuscin are distinguishable. Although the precise basis for the difference in staining is not known, PAS-positivity is believed to be due to auto-oxidized unsaturated lipids, hydroxy keto groups etc (Pearse 1972), and NBS-positivity due to the presence of carbonyl, 1-3 , diketones and enol groups (Lillie 1965). The conversion of PAS-positive lipofuscin into NBS-positive lipofuscin is believed to involve complex chemical changes which are not known. Chemically, right from the beginning of its formation lipofuscin undergoes substantial chemical changes during ageing till its maturity in senility (Sharma & James 1991, Wolf 1993). Our finding of a tight correlation between the age-related increase in the NBS-positive neurons and the decrease in the PAS- positive neurons is a statistical evidence indicative of age changes in the chemical nature of lipofuscin. Concerning the relationship between age and morphological form of lipofuscin, a tight correlation observed between the age-related changes in the number of neurons containing scattered and clumped lipofuscin is a significant evidence supporting the view of the transformation of the scattered lipofuscin into the clumped one with ageing (Braak 1984). A strong positive correlation between the numbers of neurons containing clumped and NBS-positive lipouscin would support the notion that the aggregated lipofuscin is NBS-positive.

Our centrophenoxine experiments clearly showed the drug-induced changes in the chemical characterstics of the intraneuronal lipofuscin. The drug treatment also depleted a considerable number of neurons of their intraneuronal lipofuscin deposits. These drug effects have been known from several descriptive histochemical studies (Nandy 1968, Riga & Riga 1974, Roy et al.1983 ,1984, Sharma et al.1991) Our data permits a quantitation of these effects. Another important finding from our work is that the drug has a greater effect on the NBS-positive/clumped lipofuscin than on the PAS-positive/ scattered lipofuscin. In the case of PAS-positive neurons, the number seemed to increase after 30 days of the drug treatment. This would seem to be largely

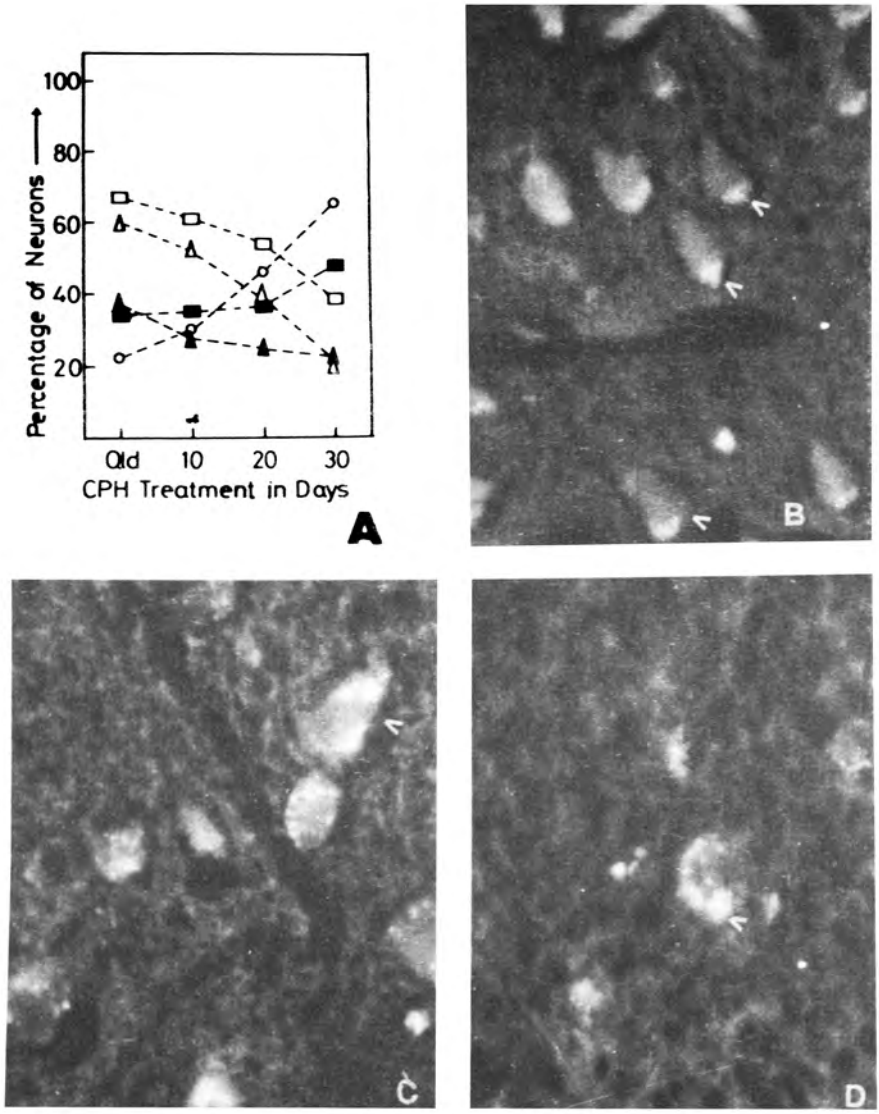

Figure 5 A, Effect of centrophenoxine on the number of non-lipofuscin neurons.o—o, neurons with non-aggregated ^—^,aggregated or clumped ^—^,PAS-positive []—[], and with NBS-positive lipofuscin []—[] **B,** Fluorescent photomicrographs show decreased number of pigmented neurons after 10-,20- and 30 days of centrophenoxine treatment

due to drug induced alterations in the stainability of lipofuscin. The drug alters the NBS-positive lipofuscin and makes it PAS-positive and hence an increase in the number of PAS-positive neu-

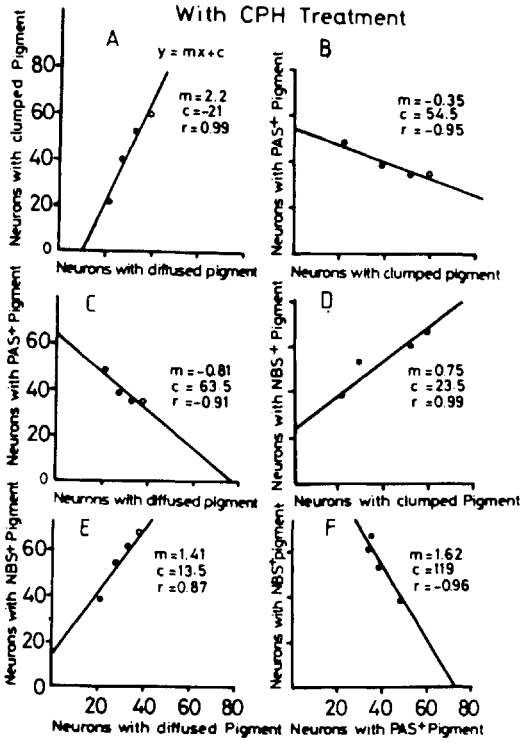

Figure 6 A-F, showing correlations between centrophenoxine induced changes in the number of lipofuscin neurons (discribed in figure .5A) of the parietal cerebral cortex of 24-month-old rat. Data point at filled circles represent vales of control rats while open circles represent values obtained after 10-, 20- and 30 day centrophenoxine treatment

rons is likely. Statistical correlations between the neuronal number changes following centrophenoxine treatment are consistent with the suggestion that the drug alters the stainability of lipofuscin. Based on the microscopic histochemical data it has been often suggested that centrophenoxine treatment makes the senile lipofuscin somewhat similar to that found in the neurons of younger animals (Nandy 1971). Our statistical data are some what consistent with this suggestion as the number of PAS-positive neurons increases following the drug treatment. The negative correlation between the drug-induced increase in PAS-positive neurons and the decrease in neurons with diffused lipofuscin would however, indicate that lipofuscin that becomes PAS-positive following the drug treatment might not become clearly scattered.

The positive correlation between the drug-induced decrease in NBS-positive neurons and the drug induced decrease in neurons containing clumped as well as those with scattered lipofuscin suggests drugs effectiveness against the clumped as well as scattered lipofuscin. Similarly, the positive correlation between the drug induced changes in neurons containing clumped and neurons containing scattered lipofuscin would show that the drug induced changes are related with each other and that the drug causes alterations in the chemical features of lipofuscin.

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References

Braak H 1984 Architectonics as seen by lipofuscin stains; in *Cerebral Cortex* **Vol 1** pp 59-104 ed. A Peters and E G Jones (New York: Plenum Press)

Brizzee K R and Ordy J M 1979 Age pigments, cell loss and hippocampal functions; *Mech. Ageing Dev.* **9** 143-162

Brunk U T, Jones C B and Sohal R S 1992 A novel hypothesis of lipofuscinogenesis and cellular aging based on interactions between oxidative stress and autophagocytosis; *Mutation Res.* **275** 395- 403

Drach L M, Bohl J and Goebel J 1994 The lipofuscin cntent of nerve cells of the inferior olivary nucleus in Alzheimers disease; *Dementia* **5** 234-239

Dowson J H 1985 Quantitative studies on the effect of aging, Meclophenoxate,and Dihydroergotoxine on intraneuronal lipopigment accumulation in the rat; *Exp.Gerontol.* **20** 333-340

——, Fattoretti P, Cairns M, James N T, Cox W and Bertoni-Freddari C 1992 The effect of aging and vitamin E-deficient diet on the lipopigment content of rat

hippocampal and Purkinje neurons; *Arch.Gerontol.Geriatr.* **14** 239-251

——, Wilton-Cox H and Cairns M R 1993 Effects of chronic chlorpromazine or lithium administration on ageing-related lipopigment in rat Purkinje neurons *J. Psychopharmacol* **7** 195-202

Few A and Getty R 1967 Occurrence of lipofuscin as related to aging in the canine and porcine nervous system; *J. Geront.* **22** 357-368

Hayes K C 1974 Pathophysiology of vitamin E deficiency in monkeys; *Amer. J. Clin. Nutr.* **27** 1130-1134

Lillie R D 1965 *Histopathologic Technique and Practical H istochemistry* (New York:Mc Graw Hill)

Mesulam M M 1987 Involutional and developmental implications of age-related neuronal changes : in search of an engram for wisdom; *Neurobiol Aging* **8** 581-583

Nandy K 1968 Further studies on the effect of centro phenoxine on the lipofuscin pigment in the neurons of senile guinea pigs; *J. Gerontol.* **23** 82-92

——, 1971 Properties of neuronal lipofuscin pigment in mice; *Acta Neuropath* **19** 25-32

—— 1978 Centrophenoxine: Effect on aging mammalian brain; *J.Am.Geriatr. Soc.* **26** 74-81

—— 1983 Aging neurons and pharmacological agents; *Aging* **21** 401-413

Pearse A G E 1972 *Histochemistry Theoretical & Applied* Vols I & II (Balltimore: Williams & Wilkins)

Riga S and Riga D 1974 Effect of centrophenoxine on the lipofuscin pigment in the nervous system of old rats; *Brain Res.* **72** 265-275

Roy D, Pathak D N and Singh R 1983 Effect of centro phenoxine on the antioxidative enzymes in various regions of the aging rat brain; ***Exp. Geront.*** **18** 185-197

——, ——, —— 1984 Effect of chlorpromazine on the activities of antioxidant enzymes and lipid peroxidation in the various regions of aging rat brain; *J. Neurochem.* **42** 628-633

Sharma D, Maurya A K and Singh R 1993 Age-related decline in multiple unit action otentials of CA3 region of hippocampus: correlation with lipid peroxidation and lipofuscin concentration and the effect of centrophenoxine; *Neurobiol. Aging* **14** 319-330

——, Singh R and Maurya A K 1991 Response of age related intraneuronal cytomorphological impairments to centrophenoxinein pyramidal neurons of lamina pyramidalis (layer V) in cerebral cortex in aged rats; *Proc.Nat. Acad. Sci.*, India **B61** 59-63

Sharma S P and James T J 1991 Existence of bluish-white fluorescing age-pigment- pre-lipofuscin"; *Free Rad. Biol. Med.* **10** 443-444

Sohal R S, Marazabadi M R, Galaris D, and Brunk U T 1989 Effect of ambient oxygen concentration on lipofuscin accumulation in cultured rat heart myocytes- A novel *in vitro* model of lipofuscnogenesis; *Free Rad. Biol. Med.* **6** 23-30

—— and Allen R G 1990 Oxidative stress as a causal factor in differentiation and aging:a unifying hypothesis; *Exp. Gerontol.* **25** 499-522

Winterbourne D J and Johnson J W 1994 Purines induce lipofuscin formation in colon carcinoma cell line; *Biochem.J.* **301** 373-377

Wolf G 1993 Lipofuscin, the age pigment; *Nutrit. Reviews* **51** 205-206