Proc Indian Natn Sci Acad **75** No.4 pp. 173-181 (2009)

Lipofuscin, Lipid Peroxidation and Antioxidant Status in Discrete Regions of the Aged Rat Brain

MAHDI HASAN[1*], SANDEEP TRIPATHI[2], ABBAS ALI MAHDI[2], KALYAN MITRA[3] and MAHENDRA PAL SINGH NEGI[4]
[1]*INSA Honorary Scientist, Department of Anatomy, CSM Medical University, Lucknow-226003, India*
[2]*Department of Biochemistry, CSM Medical University, Lucknow-226003, India*
[3]*Electron Microscopy Division, Central Drug Research Institute, Lucknow-226001, India*
[4]*Biometry and Statistics Division, Central Drug Research Institute, Lucknow-226001, India*

(Received on 15 October 2009; Accepted 11 February 2010[†])

The present study was designed to assess whether a correlation exists between the degree of lipofuscin accumulation in various regions of the rat brain and the rate of lipid peroxidation/antioxidant status. Twenty four male Wistar rats were divided into two age groups (6m: n=12; 24m: n=12). For electron microscopy, transcardiac perfusion fixation was done and pieces of prefrontal cortex and hippocampus dissected and processed by standard electron microscopy techniques. For biochemical assays (lipid peroxidation and antioxidant status), brain regions viz. hippocampus hypothalamus, rest of cerebrum, cerebellum and brain stem were dissected after sacrificing rats by an overdose of sodium pentobarbitone. Protein content, protein carbonyl, lipid peroxide, conjugated dienes, SOD, catalase, glutathione peroxidase and glutathione reductase were estimated by standard methods. Statistical analysis of the results was accomplished by Student's 't' test, Pearson correlation and hierarchical clustering. Oxidative stress markers (LPO, CD, LOOH, LIF and PC) in 5 different brain regions were significantly increased while the activities of antioxidant enzymes (SOD, CAT, GPx and GR) in the same brain regions were significantly depleted. Reduced and oxidized glutathione ratio showed remarkable depletion in the old group in comparison with the same brain regions of young rats. Our results suggest that increment of the rate of lipid peroxidation is well correlated with the decline in the antioxidant status of the various brain regions. The morphological indicator of lipid peroxidation, neuronal lipofuscin, formed consequent to mitochondrial residues remaining after lysosomal degradation, also exhibits regional heterogeneity and linear increment with age.

Key Words: Lipofuscin; Lipid Peroxidation; Antioxidants; Rat Brain; Regional Heterogeneity

1. Introduction

Accumulation of intracellular granules of the pigment, lipofuscin, with the passage of time has been well-documented in postmitotic cells [1]. Ledda *et al.* [2] have ultrastructurally quantitated the age-dependent deposition of lipofuscin in the rabbit spinal ganglia and demonstrated that strong linear correlation seems to exist between the degree of lipofuscin accumulation and the chronological age. On the other hand, Sheehy *et al.* [3] showed that the rate of lipofuscin accumulation correlates negatively with longevity. Various premature ageing disorders, viz. neuronal ceroid lipofuscinosis, progeria and Werner's syndrome are characterized by accelerated accumulation of lipofuscin [4].

Chemical analysis of isolated lipofuscin granules indicates the presence of compounds of protein and lipid origin and some metal ions, especially Fe, Cu, Zn, Al, in variable quantities [5, 6]. An important property of lipofuscin is its broad fluorescence. Because of the difficulties inherent in the analysis of lipofuscin fluorophores, its nature and composition has not yet been fully elucidated. Nevertheless, *'in vitro'* experiments suggest that reactions between carbonyl (mainly aldehyde, resulting from lipid peroxidation reaction) and amino compounds produce Schiff bases, that display autofluorescent properties. It is well-known that mitochondria are the main sites of reactive oxygen species (ROS) formation and also the main target of their attack [7]. Oxidatively damaged mitochondria may contain some already peroxidized undergradable macromolecules.

The main objectives of the present study are to find out whether age-dependent correlation exists between the degree of lipofuscin accumulation and the rate of lipid peroxidation in discrete regions of the rat brain. Also, a comparative evaluation of the relative values of the oxidative stress markers in the hippocampus, hypothalamus, cerebrum, cerebellum and the brain-stem of the young and aged rats will be undertaken and correlated with the antioxidant status of the

** Author for Correspondence: INSA Hony. Scientist, Department of Anatomy, C.S.M. Medical University, Lucknow-226003, India*
E-mail: dramahdihasan@rediffmail.com, Phone +91-9415011603, Fax: +91-522-2253030

[†] *The December 2009 Issue of the Journal had got delayed. To avoid delay in publication, this article has been included in the present Issue even though it was accepted after December 2009.*

aforementioned brain regions and a hierarchical clustering of these disparate brain regions will be accomplished.

2. Material and Methods

2.1. ***Animals:*** Twenty-four male, *Rattus norvegicus*, Wistar strain rats of two age groups (n = 12), young (6 month, weight 220 ± 10 g) and old (24 month, weight 450 ± 10 g), were purchased from Industrial Toxicology Research Institute, Lucknow, (UP) India. The animals were separately housed in polypropylene cages at a room temperature of 22 ± 2 ^{0}C, relative humidity of 50 ± 10 % and 12h light dark cycles. They were fed a commercial pellet diet (Dayal Industries, Barabanki, UP, India) and allowed access to water *ad libitum*. The ICMR 'Guide for care and use of animals' was followed and the Institutional Animal Ethics Committee approved this study prior to the initiation of the experiment and also approved all experimental protocols.

2.2. *Preparation of Tissue Homogenate*

Rats were sacrificed by anesthetic overdose of sodium pentabarbitone (100 mg/kg). The whole brain was removed within one minute and dissected into discrete parts i.e., cerebrum, cerebellum, brain stem, hippocampus and hypothalamus. The brain parts were homogenized in 10% w/v cold potassium phosphate buffer (50 mM: pH 7.4). The whole homogenate was divided into two portions; the first portion was centrifuged at 700 x g fir 20 min in order to determine lipid peroxide level, conjugated diene, lipid hydroperoxide and GSH level. The second portion of the homogenate was centrifuged at 20,000 Xg for 30 min for the determination of the activity of antioxidant enzyme.

2.3. *Biochemical Assay*

2.3.1. Protein Content

The protein content of the samples was determined by the method of Lowry *et al*. [8] using bovine serum albumin (BSA) as standard and was represented as mg/g protein.

Protein Carbonyl Content

Protein carbonyl content was measured by the method of Liu *et al.* [9]. Protein was precipitated with 20% trichloroacetic acid (TCA). After centrifuging at 11,000 xg and 4^{o}C for 15 min, the supernatant was removed. The pellet was resuspended in 0.5 ml of 10mM 2, 4-dinitrophenylhydrazine (DNPH)/2M HCl (50^{0}C). Samples were kept in a dark place and vortexed every 10 minutes for one hour. The samples were precipitated with 0.5 ml of 20% TCA, and centrifuged at 11,000 xg and 4^{0}C for 3 min. The same procedure was repeated with 10% TCA for three times. Precipitate was dissolved in 2ml of NaOH at 37^{0}C. Absorbance was recorded at 360 nm. Protein carbonyl levels were expressed as nmol carbonyl/mg protein using ε max =22,000M^{-1} cm^{-1}.

Lipid Peroxidation

Lipid peroxide was estimated in the brain regions using thiobarbituric acid (TBA) test described by Ohkawa *et al.* [10]. 0.1 ml of homogenate of brain regions was added to the test tube containing 0.2 ml of 8% of SDS, 1.5 ml of 20% acetic acid solution (pH 3.5) and 1.5 ml of 0.8% TBA solution. The mixture was diluted to 4 ml with distilled water and heated at 95^{0}C for 60 minutes. After cooling on ice, the samples were extracted with 4 ml mixture of n butanol and pyridine (15: 1 v/v). The organic phase was collected and the absorbance measured at a wavelength of 532 nm. The concentration of TBA was determined using the extinction coefficient of 1.56 x 105 M^{-1} cm^{-1}.

Lipid Hydroperoxide

Lipid hydroperoxide level was measured by the method of Haldebrandt and Roots [11]. In this method, ferrousthiocynate was measured on peroxidation of Fe^{2+} to Fe^{3+} by H_2O_2 and resulted in intense pale colour, which was read at 480 nm. Standard cumine hydroperoxide was used and results were expressed as nmole of cumin hydroperoxide per gram/tissue.

Conjugated Dienes

Conjugated dienes was measured by the method of Racknell and Ghosal [12]. The cytosolic fraction was extracted from long chain fatty acids by chloroform: methanol (2:1 v/v) and read at the absorbance of 220 nm. Result was expressed as µmole dienes / 100g lipid using the molar extinction coefficient of 2.1 x 2.1x10^3.

Lipofuscin

Lipofuscin (LIF) was measured using 2:1 chloroform-methanol extraction mixture [13]. The concentration was measured with a fluoro-spectrophotometer at an excitation maximum of 360 nm and emission maximum of 420 nm. The content of the fluorescence was determined using quinine fluorescence as a standard. Data were presented as U/g tissue. One unit (U) of lipofuscin is defined as fluorescence of 0.01 g/ml quinine sulfate.

Superoxide Dismutase

The superoxide dismutase (SOD EC 1: 15.1.1) activity was determined from its ability to inhibit the reduction of NBT in presence of PMS [14]. The reaction was monitored spectrophotometrically at 560 nm. The SOD activity was expressed as U/mg protein (1 unit is the

amount of enzyme that inhibits the reduction of NBT by one half in above reaction mixture).

Catalase

Catalase (CAT, EC 1.11.1.6) activity was assayed using hydrogen peroxide as substrate; the decomposition of H_2O_2 was followed at 240nm on spectrophotometer. The CAT activity was expressed as U/mg protein [15].

Glutathione Peroxidase

The glutathione peroxidase (GSHPx, EC 1.11.1.0) was assayed using GSH, NADPH and H_2O_2 as reactants. The oxidation of GSH into GSSG was measured in terms of oxidation of NADPH to $NADP^+$ and assayed as decrease in the absorbance of reaction mixture at 340 nm on spectrophotometer [16]. The activity of GSHPx was expressed as n moles of NADPH oxidized/min/mg protein.

Glutathione Reductase

Glutathione reductase (EC.1.6.4.2, GR) activity was assayed by the method of Hazelton and Lang [17]. Activity of GR was expressed as nmoles of NADPH oxidised/min/mg protein of cell extract.

Measurement of Reduced and Oxidized Glutathione

Reduced glutathione was measured in deproteinized supernatant of the brain regions. Tissue homogenate was deproteinated with tetrachloroacetic acid, centrifuged and supernatant was used for the estimation of reduced glutathione (GSH) with the help of Ellman reagent (5, 5' dithiobis (2-nitro benzoic acid). The optical density of the pale colour was measured on the spectrophotometer on 412 nm. An appropriate standard (pure GSH) was run simultaneously. The level of GSH was expressed as µg / g tissue [18]. The oxidized glutathione (GSSG) was estimated by the decrement of GSSG in the presence of NADPH and glutathione reductase and determined the decrement of NADPH absorbance at 340 nm. The result was expressed as mg/g tissue [19].

Electron Microscopy Study

Two rats of the "young" and "old" group were perfusion fixed through the heart with Karnovsky's, 0.1 M paraformaldehyde and glutaraldehyde solution (pH 7.3) after anaesthetising with nembutol (sodium pentobarbitol) solution, 50mg/ kg body weight. Brain was quickly dissected within 1 minute and placed on ices and the frontal cortex and hippocampus were dissected out. Small pieces of 2-3 mm size were immersed in the same fixative for two hours, then washed with cacodylate buffer (pH 7.3) and transported in buffer solution to the Electron Microscope lab of CDRI–Lucknow where further processing was undertaken. Post-fixation with OsO^4 and dehydration in graded alcohol, with two changes in absolute alcohol followed. Embedding was done in an Epon-araldite mixture and the silver, gray section cut with glass-knife on LKB ultra microtone. On grid staining with urenyl acetate and lead citrate was done and the section examined with a Phillips (FEI Tecnai 12 twin) Electron Microscope at CDRI, Lucknow.

Statistical Analysis

The data in 6 replicates are summarized as Mean ± SEM. The significance of mean difference between two groups was evaluated by Student's 't' test. The association between variables was done by Pearson correlation. The similarity between brain regions and variables was done by cluster analysis (Hierarchical clustering; Single linkage and Euclidean distances) after standardizing the data i.e. each variable has a mean of 0 and a standard deviation of 1. The two tailed ($á$=2) probability $p<0.05$ was considered to be statistically significant.

3. Results

Oxidative Stress Parameters

The level of oxidative stress markers (LPO, CD, LOOH, LIF, and PC) in 5 different brain regions (hippocampus, hypothalamus, cerebrum, cerebellum and brain stem) of young rats and old rats are summarized in Table 1. The mean levels of all oxidative stress markers in brain regions of old rat were higher than the corresponding regions of young rat. On comparing, the mean level of all oxidative stress markers in all brain regions of the old group was found to be significantly different and higher ($p<0.05$ or $p<0.01$) than the young rat, except LOOH in the cerebellum. As compared to young, the mean level of CD in cerebrum of old showed the maximum increase (86.35%) while PC in cerebellum, the minimum (20.0%).

Antioxidant Enzymes

The level of antioxidant enzymes (SOD, CAT, GPx and GR) in 5 different brain regions of young rats and old rats were summarized in Table 2. The mean levels of all antioxidants in all brain regions of young rat were higher than the old rat. On comparing, the mean level of all antioxidants in all brain regions of young rats was found to be significantly ($p<0.01$) different and higher than the old rats except GPx in hypothalamus, cerebrum, and cerebellum, and GR in cerebellum and brain stem. As compared to old, the mean level of CAT in hippocampus of young showed the maximum increase (63.4%) while GPx in hypothalamus the minimum (12.8%).

Reduced and Oxidized Glutathione

The level of reduced and oxidized glutathione (GSH and GSSG) and their ratio (GSH/GSSG) in 5 different brain regions of young rats and old rats are summarized in Table 3. The mean level of GSH in all brain regions of young was higher than the old while the level of GSSG was higher in old than the young. On comparing, the mean level of both GSH and GSSG in all brain regions of the two rat groups was found to be significantly ($p<0.05$ or $p<0.01$) different except GSSG in cerebrum and cerebellum. In contrast, the mean level of GSH/GSSG ratio in all brain regions of the young group was found to be significantly (*$p<0.05$ or $p<0.01$*) different and higher than the old. As compared to the aged, the mean level of GSH in hippocampus of young showed the maximum increase (56.8%) while its level in cerebrum showed the minimum (35.4%). Similarly, as compared to young, the mean level of GSSG in hypothalamus of old showed the maximum increase (1.9 fold or 48.2%) while its level in Cerebrum showed the minimum (1.3 fold or 22.4%). Further, in comparison to the aged, the mean level of GSH/GSSG ratio in hippocampus of young showed the maximum and exceptionally high increment (76.4%) while its level in cerebrum showed the minimum (49.8%).

Correlation

The association between variables was done by correlating the relative levels of all assessed variables in all brain regions of all rats (young and old) and summarized in Table 4. Most of the variables showed significant ($p<0.05$ or $p<0.01$) direct (positive) and inverse (negative) correlation with each other. The LPO, CD, LOOH, LIF, PC and GSSG showed significant

Table 1: Baseline levels of oxidative stress markers in different brain regions of young and old rats

Variables	Rats	Hippocampus	Hypothalamus	Cerebrum	Cerebellum	Brain stem
LPO	Young	318.25 ± 17.55	364.80 ± 16.04	301.47 ± 13.97	367.77 ± 15.66	231.98 ± 16.60
	Old	545.18 ± 15.77**	606.00 ± 13.22**	437.92 ± 20.03**	475.43 ± 18.33**	375.57 ± 22.91**
CD	Young	24.72 ± 1.59	31.25 ± 1.33	22.26 ± 1.36	26.38 ± 1.85	19.30 ± 2.39
	Old	86.17 ± 4.61**	154.60 ± 4.90**	162.48 ± 6.04**	93.30 ± 6.05**	91.95 ± 6.96**
LOOH	Young	21.78 ± 2.52	20.03 ± 2.61	19.65 ± 2.04	25.32 ± 2.75	18.85 ± 1.73
	Old	38.22 ± 4.42**	31.73 ± 3.69*	33.73 ± 2.10**	32.48 ± 2.05^{ns}	26.97 ± 2.35*
LIF	Young	6.52 ± 0.93	7.02 ± 0.81	5.58 ± 0.93	4.22 ± 0.93	4.77 ± 0.93
	Old	32.38 ± 3.72**	29.27 ± 2.89**	26.68 ± 2.71**	30.68 ± 2.72**	22.55 ± 2.03**
PC	Young	14.38 ± 0.94	16.91 ± 1.15	15.62 ± 0.67	18.23 ± 0.66	15.38 ± 1.07
	Old	27.68 ± 1.81**	31.65 ± 1.65**	19.97 ± 1.15**	22.79 ± 1.33*	21.18 ± 1.07**

Units used in this table are as follows: lipid peroxide concentration (LPO; nmole MDA /g tissue), lipid hydroperoxide (LOOH; nmole of cumine hydroperoxide / g tissue), conjugated dienes (CD; µmole diene / 100g lipids), lipofuscin (LF; unit / g tissue) and Protein carbonyl content (PC; nmole carbonyl content/mg protein). The results are expressed as Mean ± SEM in six rats of each groups. Superscripts relate to significance (ns- $p>0.05$, *- $p<0.05$ and **- $p<0.01$) in comparison with young and old rats.

Table 2: Baseline levels of antioxidant enzymes in different brain regions of young and old rats

Variables	Rats	Hippocampus	Hypothalamus	Cerebrum	Cerebellum	Brain stem
SOD	Young	6.88 ± 0.17	7.73 ± 0.18	5.77 ± 0.20	5.86 ± 0.17	5.14 ± 0.16
	Old	4.12 ± 0.20**	4.89 ± 0.25**	3.59 ± 0.25**	3.62 ± 0.21**	3.69 ± 0.17**
CAT	Young	2.46 ± 0.16	2.66 ± 0.26	2.40 ± 0.28	1.86 ± 0.10	2.02 ± 0.31
	Old	0.90 ± 0.09**	0.98 ± 0.08**	1.03 ± 0.08**	0.80 ± 0.08**	0.88 ± 0.16**
GPx	Young	89.24 ± 5.29	94.76 ± 4.78	58.18 ± 6.18	80.94 ± 5.67	85.00 ± 8.86
	Old	54.04 ± 5.11**	82.67 ± 6.60^{ns}	46.88 ± 6.00^{ns}	67.29 ± 6.31^{ns}	58.02 ± 4.91*
GR	Young	29.30 ± 1.37	29.40 ± 1.15	17.97 ± 1.34	21.20 ± 2.28	17.92 ± 2.32
	Old	20.57 ± 2.17**	21.55 ± 1.59**	11.25 ± 1.11**	15.67 ± 2.87^{ns}	14.65 ± 1.58^{ns}

The activity of superoxide dismutase (SOD; U/mg protein), catalase (CAT; U/mg protein), glutathione peroxidase (GPx; NADPH oxidised/min/mg protein) and glutathione reductase (GR; NADPH oxidised/min/mg protein) are expressed as Mean ± SEM in six rats of each group. Superscripts relate to significance (ns- $p>0.05$, *- $p<0.05$ and **- $p<0.01$) in comparison to young and old.

Table 3: Baseline levels of reduced and oxidized glutathione in different brain regions of young and old rats

Variables	Rats	Hippocampus	Hypothalamus	Cerebrum	Cerebellum	Brain stem
GSH	Young	7.18 ± 0.51	7.21 ± 0.48	5.02 ± 0.17	5.49 ± 0.26	5.68 ± 0.61
	Old	3.10 ± 0.34**	3.93 ± 0.26**	3.24 ± 0.35**	3.48 ± 0.46**	3.16 ± 0.31**
GSSG	Young	1.75 ± 0.28	1.50 ± 0.18	2.50 ± 0.36	2.90 ± 0.36	2.55 ± 0.22
	Old	2.81 ± 0.21*	2.90 ± 0.16**	3.23 ± 0.37^{ns}	4.05 ± 0.38^{ns}	4.01 ± 0.45*
GSH/GSSG	Young	4.84 ± 1.02	5.32 ± 0.93	2.20 ± 0.29	2.05 ± 0.28	2.31 ± 0.30
	Old	1.14 ± 0.17**	1.36 ± 0.06**	1.11 ± 0.21*	0.98 ± 0.26*	0.83 ± 0.10**

The concentrations of reduced glutathione (GSH; µg/ g tissue) and oxidized glutathione (GSSG; µg/ g tissue) are expressed as mean ± SEM for 6 animals in each group. The superscripts relate to significance (ns- $p>0.05$, *- $p<0.05$ and **- $p<0.01$) comparison between young and old.

Table 4: Correlation (n=60) among baseline values of different variables in all brain regions of all young and old rats

Variables	Age	LPO	CD	LOOH	LIF	PC	SOD	CAT	GPx	GR	GSH	GSSG	GSH/GSSG
Age	1.00												
LPO	0.74**	1.00											
CD	0.87**	0.72**	1.00										
LOOH	0.64**	0.60**	0.55**	1.00									
LIF	0.90**	0.75**	0.80**	0.67**	1.00								
PC	0.71**	0.77**	0.66**	0.55**	0.66**	1.00							
SOD	-0.80**	-0.42**	-0.64**	-0.53**	-0.68**	-0.41**	1.00						
CAT	-0.83**	-0.61**	-0.70**	-0.51**	-0.76**	-0.59**	0.78**	1.00					
GPx	-0.47**	-0.22ns	-0.37**	-0.43**	-0.39**	-0.22ns	0.58**	0.48**	1.00				
GR	-0.46**	-0.03ns	-0.42**	-0.22ns	-0.43**	-0.07ns	0.63**	0.51**	0.41**	1.00			
GSH	-0.77**	-0.48**	-0.64**	-0.47**	-0.73**	-0.54**	0.79**	0.70**	0.48**	0.62**	1.00		
GSSG	0.55**	0.27*	0.43**	0.32*	0.46**	0.29*	-0.67**	-0.56**	-0.30*	-0.57**	-0.61**	1.00	
GSH/GSSG	-0.61**	-0.40**	-0.50**	-0.35**	-0.53**	-0.41**	0.76**	0.59**	0.44**	0.58**	0.78**	-0.76**	1.00

The correlation between age and different variables e.g., lipid peroxide levels (LPO), lipid hydroperoxide (LOOH), conjugated dienes (CD) lipofuscin (LF), Protein carbonyl content (PC), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), reduced glutathione (GSH), oxidized glutathione (GSSG) and GSH: GSSG ratio. Superscripts relate to significance (ns- $p>0.05$, *- $p<0.05$ and **- $p<0.01$) in comparison with young and old rats.

($p<0.01$) and positive correlation with the age while SOD, CAT, GPx, GR, GSH and GSH/GSSG ratio showed the negative. The correlation of GPx and GR with LPO and PC, and GR with LOOH were found to be insignificant ($p>0.05$).

Hierarchical Clustering

The natural similarity of brain regions on the basis of baseline values of different variables and of variables on the basis of brain regions of rats (young and old) are shown graphically by Fig. 1. The five brain regions clustered in 2 distinct clusters. The hippocampus and hypothalamus formed the similar group of first cluster with nearest neighbours while cerebrum, brain stem, and cerebellum the second of which cerebellum showed the farthest neighbour (Fig. 1a). Similarly, variables formed two distinct clusters. The LPO, PC, CD, LIF, LOOH, and GSSG formed a similar group of first cluster with CD and LIF the nearest neighbours and LOOH the farthest while SOD, GSH, GSH/GSSG, CAT, GR, and GPx formed the second cluster with SOD and GSH the nearest and GPx the farthest.

4. Discussion

Brain is a heterogeneous conglomeration of many discrete “little organs”, rather than one large organ. Cerebral cortex itself exhibits around 52 distinct Brodmann’s areas! Hence, instead of evaluating neurochemical parameters in the whole brain or its 3 major components (cerebrum, cerebellum and brain stem), as commonly observed in the literature, we have dissected out hippocampus, hypothalamus, rest of cerebrum, cerebellum and brain stem for the estimation of lipid peroxidation and antioxidant parameters.

In the present study, age-related changes in the brain lipids, caused by reactive oxygen species (ROS), were evaluated by estimating lipid peroxide, lipid hydroperoxide and conjugated dienes. Their mean levels in the various brain regions of the old rats were significantly higher in comparison with the young ones. However, when mean levels of all oxidative stress markers in all regions of the old rats were compared with the values of young rats, they were found to be significantly different and higher except lipid hydroperoxide level in the cerebellum. The endogenous antioxidant, glutathione (GSH), is the most abundant antioxidant in the body and is responsible for neutralizing reactive oxygen species (ROS) produced in the mitochondria [20]. It scavenges superoxide radicals and hydrogen peroxide resulting in the formation of disulfide glutathione (GSSG), which is subsequently reduced by GSSG reductase.

With the passage of time, either generation of ROS is increased, or the ability to detoxify ROS is compromised. Although decreases in the levels or activity of the antioxidant enzymes, superoxide dismutase (SOD) and catalase, may be a contributing factor in increased ROS and subsequent cellular damage, compromised mitochondrial function, membrane permeability and/or increased metal content are additional factors that may potentially lead to increased generation of ROS. In this regard, we have already reported [21] that levels of both iron and aluminum (Al) increase in the brain with age. Increased iron content, in particular, causes increased generation of highly reactive OH radical from H_2O_2 via the Fenton reaction and suppresses enzyme activity contributing to diminished neutralized of ROS [22]. The ratio of GSH/GSSG determines the relative amount of reduced or working glutathione (GSH) compared to the disulfide, or bound, glutathione (GSSG). A larger ratio reflects a more efficient glutathione redox system because GSH is normally maintained in a highly reduced state via NADPH-dependent enzymes, specifically GSSG

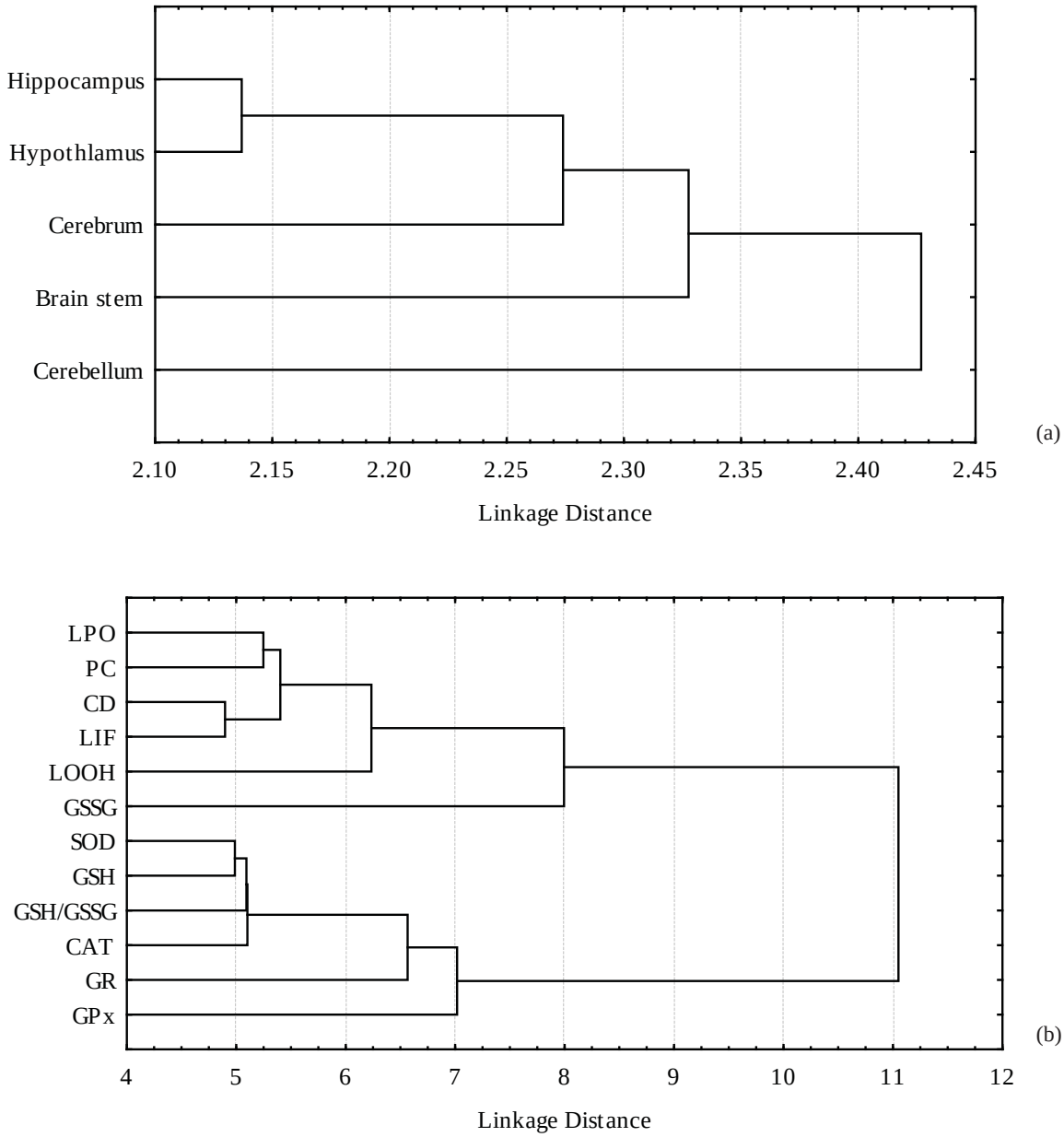

Fig. 1: Cluster diagram for 5 brain regions (a) on the basis of baseline values of 12 different variables (n=144) and of 12 variables (b) on the basis of their accumulation in all brain regions (n=60) of young and old rats.

reductase (GR). A larger ratio found by us in young rats reflects a more efficient glutathione redox system which deteriorates with the passage of time in the old rats. The mean level of GSH in the hippocampus of the old rats showed the maximum depletion of 56.8%. Similarly, the GSH/GSSG ratio in hippocampus of aged animals showed exceptionally remarkable decline (76.4%) Superoxide dismutase and catalase too exhibited similar, though less remarkable depletion in the aged rats. Our data are generally in concordance with those of Siqueira *et al.* [23] in so far as the age-related increase in lipid peroxidation in hippocampus and cerebral cortex is concerned. However, our finding of significant depletion of superoxide dismutase activity in the cerebellum is at variance with Siqueira *et al.* [23] who reported its high activity in this brain region. Both SOD and catalase play an important role in the free radical detoxification of the brain [24]. The age-related decrease in the activity of these enzymes in the specific regions of the rat brain observed by us may predispose these regions to increased free radical damage.

Our electron microscopic investigation has revealed age-related increment of variegated lipofuscin granules in the neuronal perikarya of hippocampus and frontal cortex neurons: Recently, Brunk and Terman [25] have considerably strengthened our previous reports [1, 26] that accumulation of lipofuscin is a recognized hallmark of ageing. Ledda *et al.* [2], on the other hand, have reported that lipofuscin profiles were found scattered singly in young adults but were often clustered in old

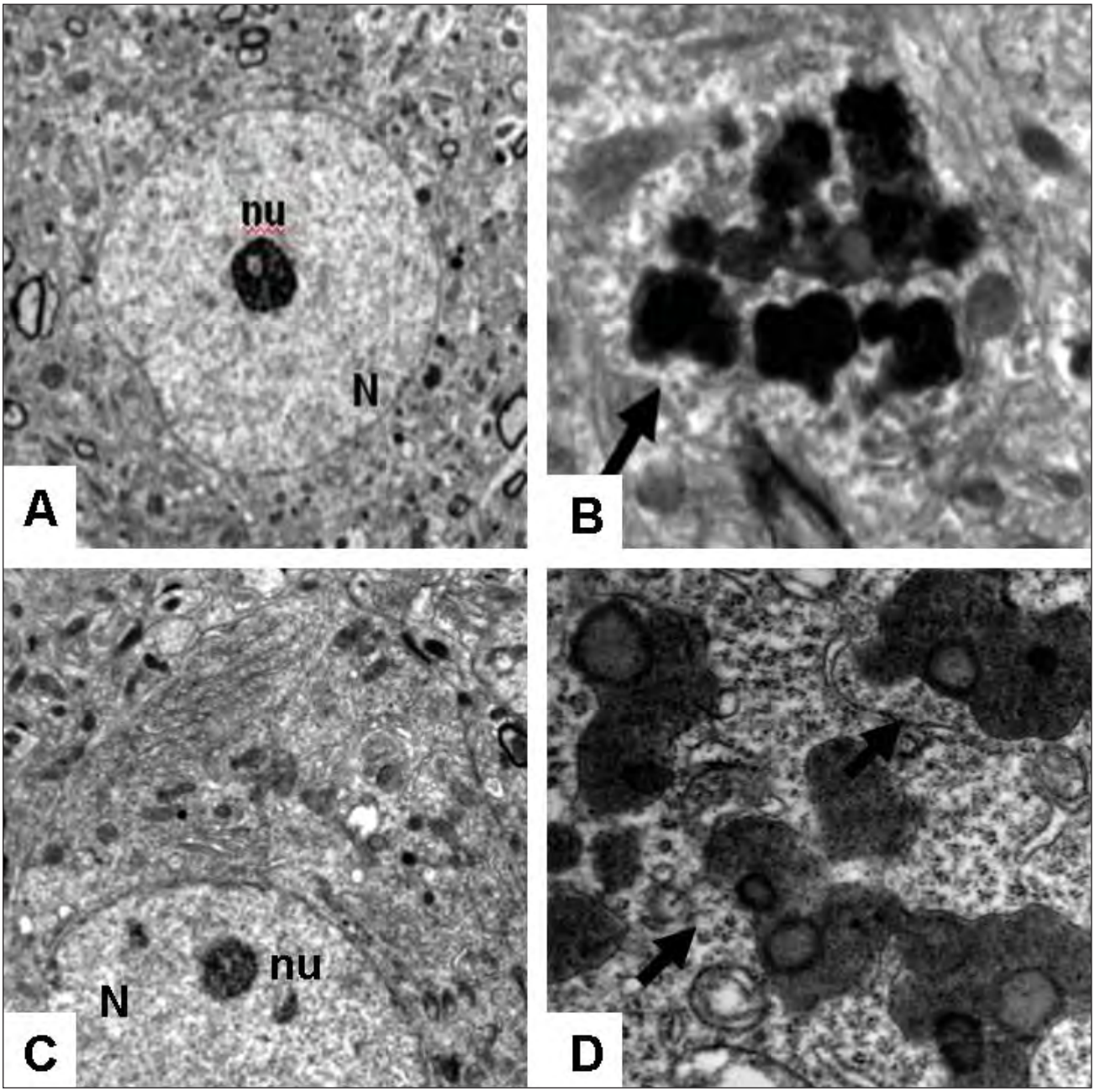

Fig.1 (A) Electron micrograph (EM) of part of a neuron of frontal cortex of young rat (prominent nucleus (N) and nucleolus, perikaryon exhibited well–preserved organelles but little lipofuscin X 20,000), (B) part of perikaryon of neuron of frontal cortex of old rat. A cluster of lipofuscin granules, 8 variegated profiles seen X 30,000), (C) EM of part of hippocampal neuron of a young rat (6m) depicting a segment of nuclear profile (N), in the bottom with a nucleolus (nu). The perikaryon shows a few lysosomes and mitochondria but no lipofuscin granules. (D) EM of part of hippocampal neuron of 24m old rat showing a cluster of 7 variegated lipofuscin granules with membrane bound electron-lucent vacuoles in 5 profiles.

animals. Whereas lipofuscin occupied an average of 0.36% of neuronal perikaryal volume in the young, it increased to 2.55% in the aged rabbits (more than 7 folds increment).

The topographic pattern of lipofuscin accumulation in the brain is not uniform, but displays a particular predilection for certain areas. It appears prominent in areas of brain involved in learning and memory (e.g. hippocampus) and also in regions involved in initiating, monitoring and controlling movements. Chemically, lipofuscin is a complex mixture of oxidized protein and lipid degradation residues along with some metals. Iron is predominant among metals in lipofuscin [6]. The genesis of lipofuscin has since long been controversial. However, it is now generally accepted that ageing is to a large extent related to macromolecular damage by mitochondrially produced ROS, mostly affecting long-lived cells, such as neurons and cardiac myocytes [27]. The inherent inability of cellular degradation mechanisms to remove damaged structures completely results in progressive accumulation of garbage, including cytosolic protein aggregates, defective mitochondria and

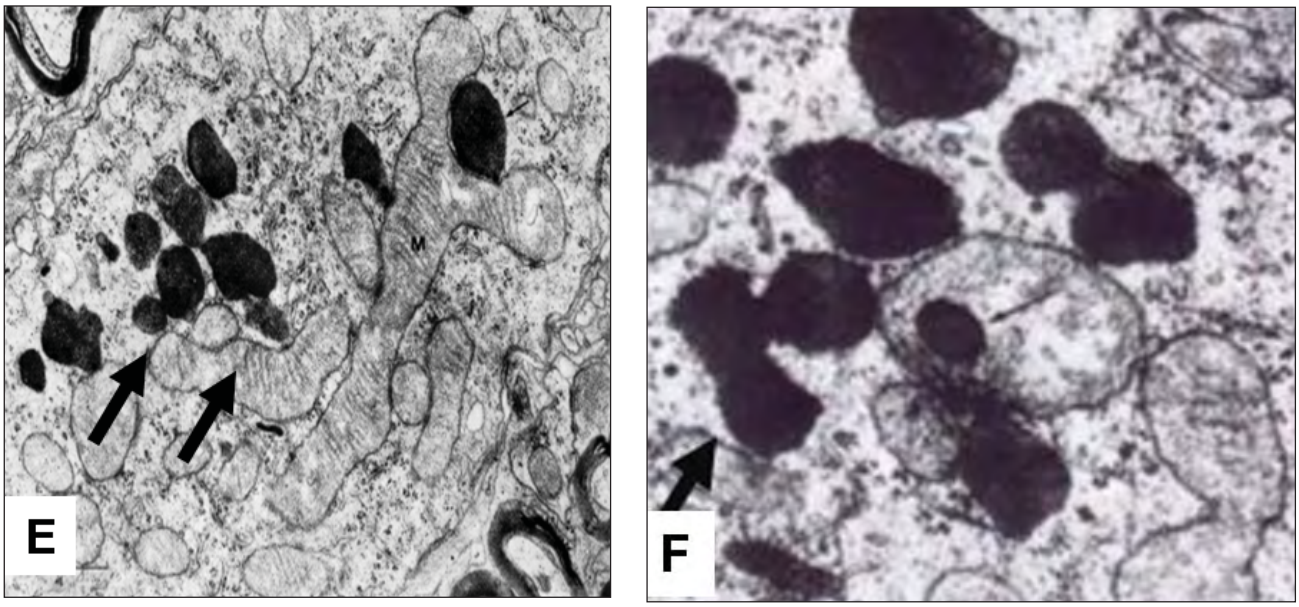

Fig 2. (E) EM of part of the perikaryon of aged rat (24m) showing elongated mitochondrial profiles, one (m) appears to split in a y-shaped manner to surround a lipofuscin granule. Two arrows show a cluster of intermingled lipofuscin and mitochondrial profiles. (F) EM of part of hippocampal neuron of an aged rat (24m) exhibiting 8 round to elongated lipofuscin granules, one within a mitochondrion (thin arrow). Thick arrow shows fusion of an elongated and round lipofuscin-granule with mitochondrial membrane.
Note: Figs. E and F are reproduced from the book "Neuronal lipofuscin in aging and disease" by Glees and Hasan (1976) with the permission of authors.

lipofuscin, which is an intralysosomal indigestible material. Terman *et al.* [28] have once again stressed the importance of the cross-talk between mitochondria and lysosomes in the genesis of lipofuscin. The decline in antioxidant system during ageing augments the oxy-free radical damage to mitochondria [29]. We have demonstrated frequent occurrence of intra-mitochondrial electron dense bodies resembling lipofuscin granules (Fig.1). Robust support to our [1, 26] hypothesis of mitochondrial genesis of lipofuscin stems from the recent landmark study of Brunk and Terman [25], coupled with the most recent observations of Monniera *et al.* [30], Nakanishi and Wu [31] and Terman *et al*. [28] that neurons progressively accumulate lipofuscin as a result of insufficient digestion of oxidatively damaged macromolecules and organelles by autophagy [32].

The authors can safely conclude that there is regional heterogeneity in the rate of lipid peroxidation, lipofuscin accumulation and antioxidant status of the rat brain. The increment of lipid peroxidation significantly correlates with the decline in the activity of antioxidant enzymes in the various brain regions. However, their correlation with the decline, if any, in functional status of various brain regions, particularly possible age-related deterioration of cognitive functions, remains to be elucidated. Further behavioural studies are underway in our laboratory, in which a number of antioxidants and memory enhancers for the aged, including herbal preparations, are to be evaluated.

Acknowledgements

The authors are grateful to the Indian National Science Academy, New Delhi for sanctioning Honorary Scientist Scheme, to one of the authors (MH).

References

1. Glees P and Hasan M Lipofuscin in neuronal ageing and diseases. Stuttgart, Georg Thieme Publishers (1976).
2. Ledda M, Barni L, Altieri L and Pannese E Amount and distribution of lipofuscin in nerve and satellite cells from spinal ganglia of young and aged rabbits. *J Submicrosc Cytol Pathol* **31** (1999) 237-246.
3. Sheehy MR, Greenwood, JG and Fielder DR Lipofuscin as a record of "rate of living" in an aquatic poikilotherm. *J Gerontol* **50** (1995) 327-336.
4. Haustein J, Pawlas U andCervos-Navarro J The Werner syndrome: a case study. *Clin Neuropathol* **8** (1989)147-51.
5. Jolly RD, Douglas BV, Davey PM, Roir JE Lipofuscin in bovine muscle and brain: a model for studying age pigment. *Gerontology* **41** (1995) 283-295.
6. Tokutake S Accumulation of aluminum and silicon in lipofuscin granules suggests retardation of blood-brain barrier function by aging. *Ann NY Acad Sci* **826** (1997) 510-512.
7. Wong YT, Ruan R and Tay FE Relationship between levels of oxidative DNA damage, lipid peroxidation and mitochondrial membrane potential in young and old F344 rats. *Free Radic Res* **40** (2006) 393-402.
8. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ Protein measurement with the Folin phenol reagent. *J Biol Chem* **193** (1951) 265-268.
9. Liu R, Liu IY, Thompson RF, Doctrow SR, Malfroy B and Baudry M Reversal of age related learning and brain oxidative stress in mice with superoxide dismutase/catalase mimetics. *Proc Natl Acad Sci USA* **100** (2003) 8526–8531.

10. Ohkawa H, Ohishi N and Yagi K Assay for lipid peroxides in animal tissue by thio-barbituric acid reaction. *Anal Biochem* **95** (1979) 351-358.
11. Haldebrandt AG and Roots I Reduced nicotinamide adenine dinucleotide phosphate (NADPH) dependent formation breakdown of hydrogen peroxide during mixed function oxidation reaction in liver microsome. *Arch Biochem Biophysics* **171** (1975) 385-397.
12. Racknagel RD and Ghosal AK Quantitative estimation of peroxidative degeneration of rat liver micro somal and mitochondrial lipids alters carbon tetrachloride poisoning. *Exp Mol Pathol* **5** (1966) 413-426.
13. Sohal RS and Donato HJ Effect of experimental prolongation of life span on lipofuscin content and lysosomal enzyme activity in the brain of the housefly, *Musca domestica J Gerontol* **34** (1979) 489-496.
14. McCord JM and Fridovich I SOD enzyme function for erythrocuprein. *J Biol Chem* **224** (1969) 6049-6055.
15. Aebi H, Catalase. In: Bergmeyer HU (ed). Methods of Enzymatic Analysis. NY Acad Press Inc. **2** (1974) 673–684.
16. Paglia DE and Valentine WN Studies on the qualitative and quantitative characterization of erythrocyte GPx. *J Lab Clin Med* **20** (1967)150-168.
17. Hazelton GA and Lang CA GSH content of tissue in aging mouse. *Biochem J* **188** (1985) 25-30.
18. Ellman GL Tissue sulfydryl groups. *Arch Biochem Biophysics* **82** (1959) 70.
19. Folbergrova J, Rehncron S and Siesj BK Oxidized and reduced glutathione in the rat brain under normoxic and hypoxic conditions. *J Neurochem* **32** (1979)1621-1627.
20. Bush IA Metals and neuroscience. *Curr Poin Chem Biol* **4** (2000) 184-191.
21. Tripathi S, Mahdi AA, Nawab A, Chander R, Hasan M, Siddique MS, Mahdi F, Mitra K and Bajpai VK Influence of age on aluminium induced lipid peroxidation and neurolipofuscin in frontal cortex of rat brain: A behavioural, biochemical and ultrastructural study. *Brain Res* 1253 (2009) 107-116.
22. Deloncle R, Guillard O, Bind JL, Delaval J, Fleury N, Mauco G and Lesage G Free radical generation of protease-resistant prion after substitution of manganese for copper in bovine brain homogenate. *Neurotoxicol* **27** (2006) 437-444.
23. Siqueira IR, Fochesatto C, de Andrade A, Santos M, Hagen M, Bello-Klein A and Netto CA Total antioxidant capacity is impaired in different structures from aged rat brain. *Int J Dev Neurosci* **23** (2005) 663-671.
24. Alper G, Sozman EY, Kanit L, Mentes G, Ersoz B, Kutay FZ Age related alteration in superoxide dismutase and catalase activities in rat brain. *Tr J of Medical Sciences* **28** (1998) 491-494.
25. Brunk UT and Terman A Lipofuscin: mechanism of age–related accumulation and influence on cell function. Free radical *Biol Med* **33** (2002) 611-619.
26. Glees P, Hasan M and Spoerri PE (1974) Mitochondrial genesis of neuronal lipofuscin: evidence based on electron microscopic studies of the brain, heart and neural tissue culture. *J Physiol (Lond.)* **239** 16.
27. Kurz T, Eaton J and Brunk U Redox Activity within the Lysosomal Compartment: Implications for Aging and Apoptosis. *Antioxid Redox Signal* 2009.
28. Terman A, Kurz T, Navratil M, Arriaga EA and Brunk U Mitochondrial turnover and aging of long-lived postmitotic cells: the mitochondrial-lysosomal axis theory of aging. *Antioxid Redox Signal* **12** (2010) 503-535.
29. Srividhya R, Zarkovic K, Stroser M, Waeg G, Zarkovic N and Kalaiselvi P Mitochondrial alterations in aging rat brain: effective role of (-)-epigallo catechin gallate. *Int J Dev Neurosci* **27** (2009) 223-231.
30. Moreira PI, Carvalho C, Zhu X, Smith MA and Perry G Mitochondrial dysfunction is a trigger of Alzheimer's disease pathophysiology. *Biochim Biophys Acta* **1802** (2010) 2-10.
31. Nakanishi H and Wu Z Microglia-aging: roles of microglial lysosome- and mitochondria-derived reactive oxygen species in brain aging. *Behav Brain Res* 201 (2009) 1-7.
32. Sulzer D, Mosharov E, Talloczy Z, Zucca FA, Simon JD and Zecca L Neuronal pigmented autophagic vacuoles: lipofuscin, neuromelanin, and ceroid as macroautophagic responses during aging and disease. *J Neurochem* **106** (2008) 24-36.