Materials and Methods
3.1 Materials

3.1.1 Animals

In the present study, male albino Wistar rats obtained from the central animal house facility of the Jawaharlal Nehru University, New Delhi, were used. Gerontological studies embodied in this thesis were done by using four different age groups of rats viz. 6, 12, 18 and 24 months. The animals were housed in cages 8”x 12”x 5” made of polypropylene with stainless steel coverings and maintained in an air conditioned room in the animal house maintained at 26 °C ± 4 °C and on a 12L: 12D (light during 0600h to 1800h) cycle. The animals were fed ad libitum with commercial rat food pellets obtained from Hindustan Lever Ltd. Each rat was checked for health status periodically during the entire course of experimentation and no evidence of the development of age-related disease symptoms were noticed.

3.1.2. Chemicals

All the chemicals used were of analytical grade and obtained from Sigma Chemical Co. USA or from standard Indian companies like BDH, SRL, Merck or Qualigens. The drug acetyl-L-carnitine was purchased from Sigma Chemical Co. and L-deprenyl from Research Biochemicals International, USA.

3.2. Experimental set up

The work embodied in this thesis was divided into four parts.

In the first part of the experimental work, rats of four age groups viz. 6, 12, 18, and 24 months were used for determining changes of various parameters during normal ageing. Age-related parameters studied are: electrophysiological parameters-electroencephalography (EEG) and multiple unit action potential activity (MUA); biochemical parameters are: determination of levels of lipid peroxidation, lipofuscin and activity of certain enzymes such as Na⁺,K⁺-ATPase, glutathione-S-transferase (GST), glutathione peroxidase (GPx); and histochemical localization of the age-pigment lipofuscin. Parameters were studied measured in the following four brain region: parietal cortex, hippocampus, striatum and thalamus.
The second part of the experimental work was aimed at determining the effect of aluminum on the brain ageing process as assessed by the above-named parameters. Rats of 6, 12, and 18 months were treated with aluminum chloride (AlCl₃) at a dose of 50mg/kg/day orally in drinking water for a period of 6 months. The dose of aluminium used was in accordance with that used in the available published data.

**The scheme of administration of aluminum chloride**

<table>
<thead>
<tr>
<th>Age at the beginning of treatment</th>
<th>Duration of treatment</th>
<th>Age at the end of treatment</th>
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<tbody>
<tr>
<td>6 months</td>
<td>6 months</td>
<td>12 months</td>
</tr>
<tr>
<td>12 months</td>
<td>6 months</td>
<td>18 months</td>
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<tr>
<td>18 months</td>
<td>6 months</td>
<td>24 months</td>
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The third part of the experiment was aimed at finding out the effects of the drugs acetyl-L-carnitine (ALC) and L-deprenyl (L-DEP) on above-mentioned parameters in the said four brain regions of 24 months old (aged) rats. ALC was administered intraperitoneally (i.p.) at a dose of 75mg/kg/day in a saline solution (Maccari et al 1990). L-DEP was also administered i.p. as a saline solution at the dose of 1 mg/kg/day (Ingram et al 1993). The doses selected are actually based on the ones used in a large number of published studies on the effects of these drugs on ageing-related parameters.

**The scheme of administration of both the drugs**

<table>
<thead>
<tr>
<th>Age at the beginning of treatment</th>
<th>Duration of treatment</th>
<th>Age at the end of treatment</th>
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<tbody>
<tr>
<td>21 months</td>
<td>3 months</td>
<td>24 months</td>
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</table>

Thus, 24 months old acetyl-carnitine- or L-deprenyl treated- rats means the animals which have been dosed by the drugs for three months by the time they became 24 months old.

The fourth part of the experimental work dealt with the effect of the above drugs (acetyl-L-carnitine and L-deprenyl) on the aluminum dosed aged (24 months old).
old) group (i.e. 18-24 month old treatment group). The aluminum treatment was started as given above and the treatment with the drugs was started after three months (i.e. at 21 months of age) of aluminum treatment and then the drug and the aluminum treatment both were continued till the animals attained 24 months of age.

The scheme of administration of the drugs to the aluminum treatment aged group is as follows

<table>
<thead>
<tr>
<th>Age at the beginning of treatment with the drugs (ALC and L-DEP)</th>
<th>Duration of treatment with drugs (ALC and L-DEP)</th>
<th>Age at the end of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 months</td>
<td>3 months</td>
<td>24 months</td>
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3.3. Details of Parameters Studied

The following parameters were studied in the normal ageing, aluminum-induced aged animals, drug treated aged animals and the aluminum + drug treated aged animals:

3.3.1 Electrophysiological Parameters

(I) EEG (electroencephalography)
(II) MUA (multiple unit action potentials)

3.3.2 Biochemical Parameters

(I) Na\(^+\),K\(^+\)-ATPase
(II) Glutathione-S-Transferase
(III) Glutathione Peroxidase
(IV) Lipid Peroxidation levels were estimated by measuring the thiobarbituric acid-reactive substances (TBA-RS)
(V) Lipofuscin (biochemically estimated by measuring 4-Hydroxynonenal)

3.3.3 Histochcmical localization of lipofuscin

The lipofuscin accumulation was done histochemically by (a) fluorescent and (b) light microscopy. The light microscopic localization was done to confirm the
pigment localization done by fluorescent microscopy by (a) Cresyl violet and (b) Nile blue sulphate staining.

3.3.4 Brain areas studied

(I) Cerebral parietal cortex
(II) Hippocampus
(III) Striatum (caudate putamen)
(IV) Thalamus

3.4. Methods

3.4.1. Electrophysiological recordings

3.4.1.1. Equipment: The equipment used to carry out the electrophysiological recordings was as follows:

I. Rat Stereotaxic Apparatus (Narishige, Japan)
II. Polygraph (Grass, Model 79 D, multichannel) (Fig. 3.1A)
III. Accessories:
(a) Electrodes: For recording the cortical and depth electroencephalographic activity (EEG) and multiple unit activity (MUA) from different regions of the brain, bipolar tungsten electrodes (Plastics One, Roanoke, Virginia, USA) were used (Fig. 3.1B).

(b) Brain Atlas: Stereotaxic coordinates for electrode implantation were according to the rat brain atlas of Paxinos and Watson (1982).

3.4.1.2. Implantation of electrodes

For implanting electrodes the rats were anaesthetized with sodium pentabarbitone (40 mg / kg) and positioned in a stereotaxic instrument (Fig. 3.1C). The burr holes were made in the calvarium at the points where electrodes were to be implanted by using a drill. The electrodes were implanted in the following regions of the brain, viz.,

(I) Parietal cortex: Stereotaxic coordinates were 2.0 mm lateral to the mid line and 2.00 mm posterior to bregma.

(II) Hippocampus: Stereotaxic coordinates were AP -4.3 mm, L 4.3 mm, V 4.5 mm. The electrodes were actually in the CA3 area of the hippocampus.
Fig. 3.1: Figure showing (A) the polygraph machine (Grass, 79 D, multichannel), (B) bipolar tungsten electrode (Plastics One) and (C) rat stereotaxic apparatus (Narishige).
(III) Striatum: Stereotaxic coordinates were AP -0.3 mm, L 3.3 mm, V 4.5 mm. The electrodes were actually in the caudate putamen.

(IV) Thalamus: Stereotaxic coordinates were AP -3.3 mm, L 2.5 mm, V 6.0 mm. The electrodes were actually in the medial ventroposterior thalamic nuclei.

All electrodes were fixed to the skull with dental acrylic cement (Roy and Singh 1988).

3.4.1.3. Electroencephalographic and Multiple Unit Activity Recordings

Cortical and depth electroencephalograms from the subcortical areas and MUA were recorded from conscious unrestrained rats of all age and treatment groups (N = five to six animals for each group and the same number for controls) using a Grass EEG / Polygraph machine (Fig. 3.1A) as described below. A reference electrode was implanted in the anterior most region of the skull. All electrode positions were histologically verified.

Animals were allowed a seven-day recovery period from surgery and were habituated to the recording environment for a period of two to three days before the commencement of recordings (Roy and Singh 1988). For recording, the animals were placed in an insulated chamber and were connected to the recording set up through flexible low noise leads, which permitted enough free movement (Nickel and Szelenyi 1989, Oliveras et al 1990). After habituation the rats generally sit quietly in their cages with rare periods of grooming and locomotion (Everett et al 1984). Overt behavior of the animal was continuously observed and noted. For MUA recordings, composite extracellular signals from the same electrodes which recorded EEGs were routed through a high impedance probe (Grass H1P 511 with FET) and amplified and filtered (300 Hz to 10 kHz) by Grass P511J or 7P511L AC preamplifiers, electronically discriminated by using window discriminators (WP1) and displayed on an oscilloscope. The standard TTL spike pulses from the window discriminator were simultaneously recorded on the polygraph. Using Grass integrator preamplifier (P10) cumulative mathematical integration of EEG traces was also recorded on one of the polygraph channels (Roy and Singh 1988, Aley et al 1990). The recordings were limited to the awake immobile state i.e. the behavioral state in which a rat sits quietly but remains awake (Vanderwolf 1969, Sirviö et al 1989). Thus, all electrophysiological activity remained uncontaminated with
movement related changes and artifacts since other waking behaviors (Vanderwolf 1969, 1982) such as walking, turning, rearing posture, grooming etc. were excluded (Vanderwolf 1969, 1982). The methods used for electrode implantation and electrophysiological recordings were essentially based on the standard procedures described in several reports from our laboratory (Roy and Singh 1988, Singh and Pathak, 1990, Sharma et al 1993, Sharma and Singh 1996, Kaur et al 1998).

3.4.2. Biochemical assays

Rats from which electrical recordings had been obtained were killed by cervical dislocation. Brains were immediately taken out, washed thoroughly with ice cold saline to remove blood smears and cooled in a deep freezer. The parietal cortex, striatum (caudate putamen), hippocampus, and thalamus were rapidly dissected out in cold on an ice plate according to the stereotaxic atlas of Paxinos and Watson (1982). Specimens dissected from the left and the right side of the brain were combined to make one sample and every assay was made on five to six samples. The different brain tissues were homogenised in 0.1 M phosphate buffer (pH 7.0) with a Potter-Elvehjem type homogenizer fitted with Teflon plunger. The ratio of the tissue to solution was 1:10. Sub-cellular fractions were prepared by differential centrifugation using the methods of Gray and Whittaker (1962). In brief, the 10% crude homogenate was initially centrifuged at 1000g for 10 min in a refrigerated centrifuge (Sorvall RC5 or RC5C). The resultant pellet (P1) consisting of nuclear and cellular material was discarded. Supernatant (S1) containing mitochondria, synaptosomes, microsomes, cytosol was further centrifuged at 12000g for 20 min to separate mitochondria, synaptosomes and myelin (P2) from microsomes and cytosol (S2). The assays for lipid peroxidation products, glutathione peroxidase and glutathione-S-transferase were done in the S2 fraction.

3.4.2.1. Estimation of Na⁺,K⁺-ATPase activity

Assay: Na⁺,K⁺-ATPase activity was measured in crude synaptosomal fraction (P2) according to the method described by Akagawa and Tsukada (1979). The fraction P2 was prepared according to the method of de Robertis et al (1963) and Gray and Whittaker (1962) in 0.32 M sucrose, 12.5 mM Tris and 1 mM EDTA (pH 7.4). The
reaction mixture contained 100 mM NaCl, 20 mM KCl, 5 mM MgCl₂, 3 mM ATP, 100 mM Ouabain, 50 mM Tris-HCl buffer (pH 7.4), enzyme preparation and the desired reagents in 1.0 ml. After pre-incubation for 5 min at 37 °C, the reaction was initiated by adding ATP followed by incubation for 20 min at the same temperature. The reaction was terminated by adding 1.0 ml of ice-cold trichloroacetic acid (TCA). After centrifugation at 1000g for 10 min, 1 ml of the supernatant was taken for determination of inorganic phosphate. Na⁺,K⁺-ATPase activity was calculated from the difference in the amounts of inorganic phosphates released in the absence and presence of ouabain and was expressed as μM inorganic phosphate produced / h / mg protein at 37 °C.

**Estimation of Inorganic phosphate:** The inorganic phosphates released were estimated according to the protocol of Fiske and Subbarow (1925). The reaction mixture comprised 1 ml of supernatant, 8.1 ml of distilled water, 0.5 ml of acid ammonium molybdate (2.5% ammonium molybdate in 10N H₂SO₄). After incubation at room temperature for 10 min, 0.4 ml of reducing agent (0.5g amino napthol sulfonic acid, 15% sodium bisulfite, 20% sodium sulfite) was added and the absorbance of the colour developed as a result was read at 660 nm in a Shimadzu UV-160A spectrophotometer.

### 3.4.2.2. Estimation of glutathione-S-transferase activity

[Systemic name: RX-glutathione-R-transferase. EC: 2.5.1.18]

The enzyme was assayed according to the method of Habig et al (1974). The enzyme glutathione-S-transferase (GST) catalyses the following reaction:

\[
\text{GST} \quad \text{GSH + CDNB} \quad \rightarrow \quad \text{GSH-CDNB}
\]

**Principle:** The enzyme activity is measured by following the increase in absorbance at 340 nm of CDNB-GSH conjugate generated as a result of GST catalysis between glutathione and 1-chloro-2,4-dinitrobenzene (CDNB).

**Assay:** The 3 ml reaction volume contained a final concentration of 0.1 M sodium phosphate buffer (pH 6.5), 1 mM CDNB in ethanol, appropriate amount of distilled water and the enzyme sample. This mixture was preincubated at 37 °C for 5 min. The
reaction was initiated by adding 1 mM reduced glutathione (GSH) and the absorbance was recorded at 340 nm for 5 min at intervals of 1 min each. The increase in absorbance at 340 nm was proportional to the activity of the enzyme. Specific activity of the enzyme was expressed as μmoles of CDNB-GSH conjugate formed/min/mg protein.

3.4.2.3. Estimation of glutathione peroxidase activity

[Systemic name: GSH: H₂O₂ oxidoreductase. EC: 1.11.1.9]

\[
\text{GPx} \\
\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + \text{H}_2\text{O} + \text{O}_2
\]

The enzyme glutathione peroxidase (GPx) was assayed according to the method of Flohe and Gunzler (1984).

**Principle:** Coupled enzyme assay with glutathione reductase (GR) was used. The oxidised glutathione (GSSG) produced as a result of GPx activity is immediately reduced by an excess of GR thereby maintaining a constant level of the glutathione (GSH) in the reaction system. The assay takes advantage of the concomitant oxidation of NADPH (reduced nicotine amide diphosphate) by GR, which is measured at 340 nm.

**Assay:** The final concentrations in 1 ml reaction volume were 50 mM sodium phosphate buffer (pH 7.0) having 0.5 mM EDTA (ethylene amide tetraacetic acid), glutathione reductase (GR), 0.3 mM reduced glutathione (GSH), 1.5 mM NADPH in 0.1% sodium bicarbonate (NaHCO₃), 1.5 mM hydrogen peroxide (H₂O₂) and appropriate amount of the homogenate. The decrease in absorbance was read at 340 nm using a Shimadzu UV-260A spectrophotometer. The non-enzymatic reaction rate was assessed by replacing the enzyme sample with buffer. The specific activity was expressed as μmoles of NADPH oxidized min⁻¹ mg⁻¹ protein.

3.4.2.4. Estimation of lipid peroxidation (thiobarbituric acid-reactive substances, TBA-RS)

Lipid peroxidation levels were estimated by measuring TBA-RS as per the protocol of Rehncrona et al (1980).
**Principle:** Oxidative damage to polyunsaturated fatty acids (PUFA) results in the formation of lipid peroxides. These are unstable and they break down to simpler aldehydes mainly malondialdehyde (MDA). Thus, MDA serves as a convenient index for the estimation of the lipid peroxide products. Heat-induced reaction of MDA with thiobarbituric acid (TBA) in an acidic medium generates a yellow compound which absorbs at 532 nm.

**Assay:** Homogenate (0.25ml) was added to 0.25 ml of 20% trichloroacetic acid and centrifuged for 4 min at 1000 rpm. 0.5ml thiobarbituric acid (0.67% in 0.026 M Tris buffer) was added to the supernatant and heated at 100°C for 15-30 min. After cooling in ice for 10 min the absorbance was read at 532 nm using Shimadzu spectrophotometer. The levels of lipid peroxides were expressed as nmoles MDA g⁻¹ tissue.

### 3.4.2.5. Estimation of lipofuscin by measuring 4-Hydroxynonenal (Fluorescent product)

Lipofuscin concentration was estimated by measuring 4-Hydroxynonenal (4-HNE) spectrofluorometrically according to the method of Esterbauer et al (1991).

**Principle:** 4-hydroxynonenal interacts with membrane phospholipids generating fluorescent chromolipids having an excitation maximum at 360 nm and emission maximum at 430 nm. This permits the fluorimetric estimation of 4-HNE.

**Assay:** For the measurement of the fluorescent lipid material formed by 4-hydroxynonenal, 0.5 ml of the homogenate was mixed with 1.875 ml of chloroform / methanol (1:2 v/v) and centrifuged. To the supernatant, 0.625 ml of chloroform and 0.625 ml of distilled water were added. 1 ml of sample from the lower chloroform layer was taken out and mixed with 0.1 ml of methanol. The fluorescence intensity of this solution was measured with Shimadzu RF-540 spectrofluorometer at an excitation wavelength of 360 nm and emission wavelength of 430 nm by using quinine sulphate (0.1μg/ml) in 0.05M H₂SO₄ as a standard.
The amount of 4-hydroxynonenal derived chromolipid products was expressed as percentage fluorescence of control (quinine sulphate-0.1 μg) from 1 ml of 10% homogenate.

3.4.2.6. Estimation of Protein

Protein estimation in the extracts was done following the protocol of Lowry et al (1951) using bovine serum albumin (BSA) as a standard.

**Principle:** Protein reacts with Folin-Ciocalteau (FC) reagent to give a colored complex. The colour so formed is due to the reaction of alkaline copper sulphate (CuSO₄) with protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The absorbance read by a Shimadzu UV160A spectrophotometer at 660 nm is a measure of the amount of protein.

**Assay:** To tissue homogenate (20 μl) was added 0.980 ml of distilled water, followed by the addition of 5 ml of alkaline reagent (solution A: 2% sodium bicarbonate; NaCO₃, 0.1N sodium hydroxide; NaOH, solution B: 1% sodium potassium tartarate, solution C: 0.5% copper sulphate, in a ratio of 100:1:1, freshly prepared). After incubation at room temperature for 15 min, Folin's reagent was added (1:1 H₂O, v/v) and the tubes vortexed. The optical density of the color so developed was read at 660 nm using a Shimadzu UV160A after 30 min.

3.4.3 Histochemical localization and distribution of Lipofuscin

3.4.3.1. Fluorescent Microscopy

**Fluorescence:** The paraffin sections were deparaffinised in xylene, passed through absolute alcohol, cleared in xylene and mounted in DPX. The slides were then observed for lipofuscin autofluorescence in a Zeiss Orthomat microscope equipped with fluorescence attachments with Plomopak Epi illuminator, H2 cube (wide band), exciter filter 390-490 nm was used. High speed Etachrome color slide film (ASA-400) was used for photography. The automatic exposure meter of the microscope was adjusted to ASA-800. Films were exposed as instructed.
3.4.3.2. Light Microscopy

Light microscopy was performed to confirm the pigment localized by fluorescence microscopy.

3.4.3.2.1. Cresyl Violet staining

The cresyl violet staining was performed according to the method of Kluver and Barrera (1953) as described by Pearse (1968). The paraffin tissue sections were deparaffinised in xylene, and passed through absolute, 95% and 70% alcohol and then dipped in distilled water. After this step, the sections were stained in cresyl violet (cresyl violet 1g, distilled water 1000ml, acetic acid 10 ml) at 45°C for 15-30 min. The sections were then dehydrated in 70%, 95% and absolute alcohol, cleared in xylene and mounted in DPX. The slides were then observed under Carl Zeiss microscope for localization of lipofuscin which was stained violet.

3.4.3.2.2. Nile blue sulphate staining

Nile blue sulphate staining was done according to the method of (Menschik, 1946) as described by Pearse (1968). The paraffin tissue sections were deparaffinised in xylene, passed through absolute, 90%, 70%, 50%, and 30% alcohol and then dipped in distilled water. The sections were then stained for 90 min at 60°C in saturated aqueous Nile blue sulphate solution (500 ml with 50 ml of 0.5% H2SO4 which had been boiled before use). After rinsing in distilled water the sections were placed for 30 min in acetone (at 50°C). The sections were then differentiated in 0.5% acetic acid for 30 min, rinsed in distilled water and again differentiated in 0.5% HCl for 3 min. The sections were finally washed in distilled water and mounted in glycerol jelly. The pigment is stained blue.

3.5. Statistical Analysis

Data are presented as mean values ± SEM. One way analysis of variance (ANOVA) was used to analyze (Downie and Heath, 1984) the data on the effect of age.
on Na\textsuperscript{+},K\textsuperscript{+}-ATPase, GST, GPx, MUA, lipid peroxidation (TBA-RS) and lipofuscin concentration (4-HNE) in the four brain areas studied.

For assessing the effect of age on Na\textsuperscript{+},K\textsuperscript{+}-ATPase, GST, GPx, MUA, lipid peroxidation (TBA-RS) and lipofuscin concentration (4-HNE), the means calculated for different age groups in all the areas studied were analysed by one way ANOVA. F-test was made to assess whether the change in the above parameters with age was significant or not. Scheffe's multiple comparison test was used to determine significance between all pairs of means. Pearson's correlations were calculated to find the correlation amongst the parameters studied. Correlations were done between

a) Na\textsuperscript{+},K\textsuperscript{+}-ATPase and MUA,

b) Na\textsuperscript{+},K\textsuperscript{+}-ATPase and lipid peroxidation,

c) Na\textsuperscript{+},K\textsuperscript{+}-ATPase and lipofuscin,

d) MUA and lipid peroxidation,

e) MUA and lipofuscin,

f) GST and Na\textsuperscript{+},K\textsuperscript{+}-ATPase,

g) GST and GPx,

h) GST and MUA,

i) GST and lipid peroxidation,

j) GST and lipofuscin,

k) GPx and Na\textsuperscript{+},K\textsuperscript{+}-ATPase,

l) GPx and MUA,

m) GPx and lipid peroxidation, and

n) GPx and lipofuscin for various age groups and as already described in our earlier reports (Sharma et al 1993).

Student's t-test was used to find out the significance between the control and the drug treated animals, between the control and aluminium treated animals and between the aluminium and aluminium + drug treated animals.