CHAPTER 3
CHAPTER 3
EXPERIMENTAL PROCEDURES

1.1 Induction and Selection of Animal Model for Human M.S

This study was conducted to understand the outcomes of the active immunization of two different CNS antigens viz. myelin oligodendrocyte glycoprotein (MOG, peptide 35-55) and myelin basic protein (MBP) in Wistar rats (Rattus Norvegicus). These two antigens were selected because of their wide usage and established neuroinflammatory properties. The target of the study was to select a model which is reliable, easily reproducible and mimics the human RR type M.S in most of its characteristics.

1.1.1 Animal Preparation

Young age matched female Wistar rats (150±10 gm) were obtained from Jamia Hamdard central animal house facility. All the procedures done were approved by ethical committee regulating use of laboratory animals for experimentation in Jamia Hamdard (IAEC) vide proposal no-572/2009. Animals were housed (n=2/cage) under a 12:12h light- dark cycle (temp. 25 ± 2°C starting at 08.00h in the morning for initial acclimatization for about 1 week, and permitted food and water ad libitum. Care was taken to avoid the infections as they can challenge the animals before immunization.

1.1.2 Preparation of Inoculums for Induction

Myelin antigens viz. MOG (peptide 35-55) and MBP and Freud's complete adjuvant (CFA) were removed from the cold storage and allowed to stand at room temperature for about 30min. The antigens were dissolved in 0.98% saline separately in microtubes. After complete dissolution, equal volumes of the antigen solution and CFA (containing 1mg/mL of Mycobacterium strain HR 37a) were mixed together on vortex shaker for about 10min. Following mixing, the mixture was passed between two glass syringes connected together through a stopper. With this procedure, a thick white emulsion (Fig. 3.1) was formed. The
homogeneity of emulsion was revealed by its stability in water. Inoculums were always prepared freshly.

1.1.3 Immunization

Randomly, each animal was assigned to 3 groups with 20 animals (Wistar rats) per group. After anesthesia (Choral hydrate 400mg/Kg, i.p), rats were laid on a bench. Gently, an 18 gauge needle containing the inoculum or vehicle only was inserted into the subcutaneous aspects near auxiliary surface so that the injection is close to the auxiliary lymph nodes and 100-200μL (50μg antigen) (Weissert et al., 1998) of inoculum was injected on multiple sites on the back of the animals. Following immunization, animals were returned to their well ventilated cages with easy access to food and water. Following is the group wise description of the immunization schedule

❖ Group-I, 100-200μL of innoculum containing 50μg MOG in saline emulsified with Freud’s complete adjuvant (CFA) (1:1) containing heat killed Mycobacterium strain HR 37 a subcutaneously (1mg/mL).
❖ Group-II, 100-200μL of innoculum containing 50μg MBP (myelin basic protein) with saline emulsified in Freud’s complete adjuvant (CFA) (1:1) containing heat killed Mycobacterium strain HR 37 a subcutaneously (1mg/mL).
❖ Group-III, 100-200μL CFA containing heat killed Mycobacterium strain HR 37 a subcutaneously (1mg/mL), subcutaneously only as vehicle control.

1.1.4 Assessment of Neurological Deficits and Weight Changes

The course of the disease was followed until the 31 days post immunization (p.i) for neurological signs (0, healthy; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, scale 3 plus forelimb weakness; 5, moribund or dead) (Eugster et al., 2001).

Along with the neurological changes, the body weight was monitored before the immunization, 20 days p.i and 31 days p.i and record of the weight changes was maintained. The animals were weighed on a balance which was corrected to “0” everyday before taking any record.
1.1.5 Criteria for Selection of Animal Model

The selection of animal model after successful immunization was based on several inclusion and exclusion criteria. These criteria were selected keeping in view the need for approaching the most common type of multiple sclerosis in humans viz. the remitting relapsing type (RRMS). The criteria for selection of the animal model are described in Table 3.1.

**Table 3.1 Inclusion and Exclusion Criteria**

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Exclusion Criteria</th>
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<tbody>
<tr>
<td>1. Remitting Relapsing type Disease</td>
<td>1. Mono-phasic Disease</td>
</tr>
<tr>
<td>2. Defined Phases</td>
<td>2. Non-defined Phases</td>
</tr>
<tr>
<td>4. Target organ specific Autoimmunity</td>
<td>4. Non-specific Inflammation</td>
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2 Characterization of the Selected Animal Model.

2.1 Animal Grouping

Wistar rats were immunized with 50μg myelin oligodendrocyte glycoprotein (35-55) as described in section 1.1.3 making three groups. Following immunization, rats were sacrificed after the first acute phase and at the start of the first relapse.

- **Group I** (n=20): 100-200μL of CFA (Scarificed 20 & 31 days p.i)
- **Group II** (n=10): 100-200μL of MOG(50μg) emulsified with CFA (Sacrificed 20 days p.i)
- **Group III** (n=10): 100-200μL of MOG(50μg) emulsified with CFA (Sacrificed 31 days p.i)

2.2 Study of Cerebrospinal Fluid (CSF)

2.2.1 Collection of CSF

CSF was collected as per previously published method for rodents (Consiglio & Lucion, 2000) at the end of the first acute phase i.e. 20 days post induction and beginning of the first relapse i.e. 31 days post induction. Briefly, animals were anesthetized with chloral hydrate (400mg/Kg). 20-25min. later, an incision was made from the top of the skull to the dorsal aspects of the rats. Muscles between the first vertebra and base of the skull were carefully...
removed to expose the meninges overlying the cisterna magna. After careful removal of tissues above the cisterna magna and cleaning of the surrounding area with an ethanol (70%) dipped cotton swab, the arachnoid membrane covering the cisterna magna was punctured with a needle. CSF (50-70µL/rat) that oozed out of the needle hole was collected for about a minute using a glass capillary. Immediately after the procedure, the tissues were sutured and the wound was sprinkled with neomycin antibiotic powder and normal saline (1mL) was injected i.p as to rehydrate the animal. CSF collected from rats was centrifuged successively for 5min. at 500xg to remove the blood impurities. Purified unconcentrated CSF was transferred to sterilized microtubes and appropriately sealed with a parafilm and stored at -80°C till further analysis.

2.2.2 Determination of Total Protein in CSF

CSF samples were recovered from storage at -80°C. Immediately before the analysis, samples were thawed and allowed to reach the room temperature. A 1µL sample (finely vortex mixed) was added to 19µL of D. H₂O in 96-well plate and mixed well. Finally a 200 µL working solution of Bradford reagent was added to the mixture and allowed to stand for 5-10 min. at room temperature. Absorbance (Ab) of the samples was recorded at 595nm in a microplate reader (Bio-Rad, USA). The concentration of protein present in the sample was determined using a standard curve generated from standard BSA (bovine serum albumin) and expressed as mg/mL.

2.2.3 Determination of Oligoclonal Bands in CSF by SDS-PAGE.

Oligoclonal bands are immunoglobulin molecules present in multiple sclerotic patients resulting from B-cell or plasma cell infiltration. Some models of human M.S are demonstrated to show these bands after their induction. Since we used MOG model for our studies, we wanted to check whether, MOG immunization can lead to the formation of oligoclonal bands in the cerebrospinal fluid of Wistar rats at after the first acute phase and the beginning of the first relapse.

The procedure was performed according to an already described method with some modifications (Abdolmohammad et al, 1982). Unconcentrated CSF (≈ 20µg protein) was
mixed with sample loading buffer (containing glycerol, SDS, 2-ME and bromophenol blue) and allowed to stand for 5-10 min. at room temperature. Afterwards, denaturation was done at 80-95°C in a water bath. Denatured samples were separated using 7.5% SDS PAGE without stacking since it can lead to the entrapment of IgM molecules. The voltage condition were 50V (9mA) for first one hour and 145 (15mA) volt after that until complete run. Gels were washed with 50% ethanol and fixed in fixing solution (50% ethanol, 10% acetic acid and 50μL of formaldehyde) for 15 min. Comassie blue staining(0.25% Commasie blueR-250 in methanol (50%), distilled water (40%) and acetic acid (10%) was done for 30 min. and with two washes of destain solution. Finally gels were transferred to 10% ethanol for storage and photographs were recorded for documentation.

2.3 Study of Blood Serum following EAE
Target organ specific autoimmunity generates auto-antibodies against the target proteins and is the important and can be used as an indirect proof for the antigen specific autoimmune responses.

2.3.1 Collection of Blood and Separation of Sera
Rats from both the groups viz. adjuvant injected controls and EAE were anesthetized with ether and blood (1-3mL) was collected from the orbital sinus (Retro Orbital) (Herck et al., 1998) using a glass capillary in sterilized tubes without anticoagulant and sealed immediately. The tubes were allowed to stand at room temperature overnight with appropriate disinfection. Next day, the clear upper layer was isolated by centrifugation at 1500xg for 10 min. The sera so collected after centrifugation were aliquoted and transferred into microtubes and frozen at -80°C for further analysis.

2.3.2 Determination of anti-MBP, anti-MOG Antibodies in Blood Serum
For the estimation of anti-MOG and anti-MBP-specific antibodies, in the blood serum of the adjuvant control and EAE rats at the end of the first acute phase and start of the first relapse, a standard ELISA technique (Abdelhadi et al, 1995) was applied. Briefly, ELISA plates were coated overnight with 10μg/mL of purified MOG (Alexis Axxora, USA) and MBP (Sigma Aldrich, USA) at 4°C. Next day, after saturation with 5% milk powder for 90
min., the plates were washed using PBS (pH 7.4) and serum samples (appropriately diluted) were added to the plates. After a 2h incubation period, plates were extensively washed with PBS (pH 7.4) and MBP and MOG specific antibodies were detected by addition of horseradish peroxidase-conjugated rabbit anti-rat IgG diluted in 10% normal goat serum (Invitrogen, Carlsbad, USA). Sapphire substrate solution was added and color development was assayed after adding the stop solution (2M H$_2$SO$_4$) in an ELISA plate reader. Each serum sample was tested in duplicate. Results were expressed as % change of controls determined from absorbance at 450nm.

2.4 Evaluation Blood Brain Barrier (BBB) Integrity

To estimate BBB integrity, rats from both the groups were intraperitoneally injected, the macromolecular albumin-binding dye Evans blue (5 mL of 2% solution in 0.9% NaCl, Sigma, St. Louis, MO) (Ernst Seiffert et al, 2004). The rats after injection were immediately returned to the cages. One or two hours later, the dye reaches almost all of the organs and can be seen with naked eye as the color of the rats changes to bluish after injection. After confirmation, the rats were anesthetized (Chloral hydrate 400mg/Kg) and were transcardially perfused with 150mL of chilled normal saline to exclude the interference of vascular dye moiety and to estimate the concentration of dye only in CNS parenchyma. Finally, the brains were quickly removed. Evans blue-albumin dye complex in the brain parenchyma as a proof of the penetration of macromolecular dye across BBB was quantified by two approaches.

2.4.1 Colorimetric Analysis

EAE or control brain slices were dissected out, dissolved in 0.1 M phosphate buffer solution (10μL/mg tissue) containing 1% SDS (SD Fine, India), homogenized, and centrifuged (12000xg; 5 min) with an Remi (India, New Delhi) centrifuge (C30BL) to precipitate confounding cell debris. Albumin–Evans blue complex concentrations were spectrophotometrically measured in the supernatant at 595nm wavelength using a microplate reader (BioRad U.S.A).
Experimental Procedures

2.4.2 Microscopic Analysis
Coronal sections (approx. 0.5mm-thick) were cut and incubated in 4% paraformaldehyde solution for 24h, followed by incubation in 30% sucrose (in 0.1 M phosphate buffer) for another 24h. 50μm sections were then cut on a microtome and mounted on glass slides for microscopy. A tissue concentration of the fluorescent albumin–Evans blue complex was visualized by recording the intensity in digitized low power-field imaging at 580nm in a fluorescent microscope.

2.5 Characterization of Demyelination after EAE

2.5.1 Assay for 2', 3'-Cyclic Nucleotide 3'-Phosphodiesterase activity (CNPase)
Activity of CNPase in rat brains was carried out according to previously reported method (Prohaska, et al, 1973). Tissues were homogenized in 9 volumes of 0.32M sucrose. Aliquots (15μL) of homogenate was mixed with 0.5% (wt/vol) sodium deoxycholate (SD Fine, India) in 0.2M Tris-HCl (Hi-Media) (pH 7.5) (0.3mL) and incubated for 10min. at 4°C. The homogenates were diluted in three volumes of distilled water and the protein concentration measured by Bradford method. Homogenates (20μg protein) were incubated with 10.5mM 2'-3' cAMP in 50mM Trismaleate pH 6.2 for 10min. at 30°C. The reaction was terminated by placing tubes in boiling water for 30s. The solutions were returned to 30°C and 27μL of 0.3M Tris (pH 9.0) containing 21mM MgCl₂ and 0.72 units of alkaline phosphatase (Fermentas) were added and the mixture incubated at 30°C for 20min. A mixture of isobutanol and toluene (1:1) (32.5μL) was then added together with 1.2mL of 1.5% (wt/vol) (NH₄)₆Mo7O 24•4H₂O in 0.5 M H₂SO₄. After 20 min. of shaking, the solutions were centrifuged 1000xg for 4min. and the absorbance of the yellow layer was read spectrophotometrically at 410nm. Activity was expressed μmoles phosphate release per mg of protein per min.

2.5.2 Immunohistochemistry of Myelin Basic Protein (MBP).
Rats (n=3) from both the groups were anesthetized with chloral hydrate (400mg/Kg). After complete anesthesia, rats were laid on a bench and transcardially perfused with chilled normal saline solution (150mL) followed by infusion of 4% paraformaldehyde by same procedure (Agrawal et al., 2004). The brains were quickly dissected from the cranium and stored overnight in 4% paraformaldehyde. Next day, brains were transferred to the 10%,
Experimental Procedures

20% and 30% sucrose solution for 3 consecutive days until they sank which marked their dehydration. Next the brains were embedded in molten wax and blocks were prepared from each group. Serial coronal sections (5-7µm) were cut through the coronal plane of both the groups and mounted on the poly L-lysine coated glass slides. MBP expression was evaluated by immunohistochemical localization using monoclonal antibody (Merkler et al, 2006). The serial sections were deparaffinised by two washes of xylene (2min. each) and then serially rehydrated in a descending series of ethyl alcohol. After, rehydration, the sections were thoroughly washed with PBS (pH-7.4) for three times. Thereafter, the sections were incubated at 85°C in a solution containing 0.1M Tris-HcL (pH-10) for the retrieval of the antigens because paraformaldehyde can lead to the crosslinking of the antigens and hence poor immunoreactivity. After antigen retrieval, sections were appropriately cooled and immediately immersed in 0.3% H₂O₂ to quench the activity of endogenous peroxides. The sections were washed again and blocked in blocking buffer (0.5% BSA, 1% NGS and 0.1% Triton X-100 in PBS). Afterwards, the sections were incubated with monoclonal rabbit anti-MBP antibody diluted in blocking buffer (1:100) at 4°C overnight. Next day, the sections were washed and immediately incubated with mouse anti rabbit secondary-HRP conjugated antibody in blocking buffer (1:200) for one hour at room temperature. After extensive washing, peroxides complex was visualized with DAB (Sigma Aldrich, USA) and sections were counterstained, dehydrated in ethanol and coverslipped in mounting medium.

2.6 Pathological Studies (Hematoxylin & Eosin and Toluidine Blue Staining Procedures)

2.6.1 Fixation, dehydration, infiltration and block preparation
The tissues were dehydrated by passing through graded series of ethyl alcohol (50%, 70%, 90% and 100%) for one hour in each giving two changes followed by cleaning in xylene (two changes of one hour each). The cleared tissues were placed for 5 min. in xylene containing molten paraffin wax at 50-60°C for infiltration. After giving two changes of molten paraffin (1h each) the tissues were embedded in fresh paraffin wax for block preparation. Embedded tissue was sectioned (5-6µm) on a grading type microtome and spread on gelatin coated glass slides and finally dried at 35-40°C.

2.6.2 Staining
Sections were deparaffinized by dipping in xylene (2 changes). These slides were then passed through graded concentration of ethyl alcohol (100%, 90%, 70%, 50% and 30%) with two changes of 2 min. each, and then stained with hematoxylin for 1 minute (Okuno et al., 2001) and again washed in running water thoroughly. Slides were then passed through 50% and 70% ethyl alcohol and subsequently put into eosin stain (prepared in 70% ethyl alcohol) and allowed to stand in Scott's tap water substitute for 10-30 min. These were then passed through graded series of ethyl alcohol [70%, 90%, 100%, 100% + xylene (1:1)] and finally given two changes of xylene.

For toulidine blue staining, slides after rehydration were stained with toulidine blue in ethyl alcohol for 1 min followed by incubation in Scott's tap water substitute for about 20 min. The slides were then mounted in D.P.X covered with glass cover slips and kept for drying at room temperature.

3 Studies of Cell death in EAE

3.1 Animal Grouping

Female Wistar rats were immunized with 50 μg myelin oligodendrocyte protein (peptide 35-55) as described in section making two groups. Following immunization, rats were sacrificed after the first acute phase, 20 days p.i.

- Group I(n=20) - 100-200 μL of CFA (Adjuvant Injected Controls)
- Group II(n=20) - 100-200 μL of MOG (50 μg) emulsified with CFA (EAE)

3.2 ELISA for Th-1 Cytokines (TNF-α and IFN-γ)

3.2.1 Preparation of cellular lysates

Single cell suspensions were prepared from the required brain parts. Small blocks of the periventricular tissue after dissection were put in Petri-dishes on ice and were chopped using crossed scalpels. Warm (37°C) trypsin was added to the tissues for about 2-6 min. and its activity was quenched by adding DMEM. The enzyme digested tissues were then passed through a 10 mL syringe several times to obtain a homogenous single cell suspension. The cell suspension from all the groups was centrifuged at 800 x g for 5 min. to settle all the cells in pellet. Thereafter, pellet was washed 2-3 times in PBS (pH 7.4). The pellet from the
Experimental Procedures

respective groups was incubated in cell lysis buffer containing (20mM Tris-HCl, 150mM NaCl, 1mM EDTA and 0.1% Triton-X-100) for 30min. with occasional stirring (Tatebayashi et al., 1999). After 30min., the cell extracts were sonicated for 5-15s and contents were centrifuged at 10000xg for 10min. and resulting supernatant was used for cytokine analysis.

3.2.2 ELISA
Th-1 type cytokines from EAE and adjuvant injected control groups were assayed using commercial ELISA kits (eBiosciences, USA). Briefly, sterilized flat well 96-well plates were coated with detection antibody (separate for TNF-α and IFN-γ respectively) in assay diluents (1X) at 4°C overnight (for maximal sensitivity) with care taken to adjust the plates in a horizontal direction. Next day, the wells were washed three times using 250μL/well wash buffer (included in the kit). A 100μL sample from both the groups was added in the appropriate wells and allowed to incubate for at least 2h after which the wells were immediately washed two times with the wash buffer with 1min. gap between the successive washes. Finally the plates were dried on the paper towel to drain off the residual buffer. Now, detection antibody (separate for TNF-α and IFN-γ respectively) was added which was prediluted in assay diluent (1X) for 1h at room temperature. The wells were aspirated and washed 5 times with 1min. gap between successive washes. A 100μL of Avidin-HRP conjugate (in assay diluent) was added to each well and plates incubated for 30min. at room temperature. Next the wells were washed 7 times and plates were inverted on a paper towel. A 100μL of the substrate was added per well and plates were incubated for 15min. at room temperature until the color development. Immediately, 50μL of the stop solution (2M H₂SO₄) was added per well and absorbance recorded at 450nm in a microplate reader (Bio-Rad, USA). Results were expressed as percentage change of adjuvant injected controls determined from absorbance.

3.3 Studies Related to Reactive Oxygen Species (ROS) Generation
3.3.1 Preparation Post Mitochondrial Supernatant (PMS)
In order to assess free radical mediated effects in the periventricular tissue of the selected animal model, estimations of peroxidation of lipids (LPO) and reduced glutathione (GSH) were carried out with other enzymatic antioxidants (total superoxide dismutase and catalase).
Rats (n=6) were sacrificed by cervical dislocation followed by decapitation and brains were dissected quickly on ice packs. Further, equal amounts of the tissues were dissected carefully and weighed. The post mitochondrial supernatant was used as the source for the different estimation and for lipid peroxidation, lipid fraction was isolated.

The cellular lysates as obtained from section 3.2.1 were centrifuged at 3000xg to obtain the lipid fraction which was immediately stored at -20°C. The supernatant was again centrifuged at 10,000xg for 10min. and the resulting supernatant (cell lysates) was considered as PMS which was stored at -80°C till further analysis.

3.3.2 Malondialdehyde formation

LPO in brain tissues was measured by estimating malondialdehyde (MDA) release as described already (Bohme et al, 1977) with minor modifications, adapted to microtiter plates by bringing the final volume to 150 µL. In brief, 30µL of the lipid fraction was incubated for 0°C and 37°C for 1hr. Subsequently, 60 µL of 28% w/v TCA was added and the volume was made up to 150µL by adding 60µL of d.H2O. Following centrifugation at 3000xg for 10min., 125 µL of the supernatant was taken and color was developed by addition of 25µL of 1% w/v TBA dissolved in 0.05N NaOH and kept in boiling water bath for 15 min. until the appearance of pink color. The absorbance was read at 532 nm in a plate reader (Bio-Rad U.S.A). The results were expressed as µmoles MDA formed/min.

3.3.3 Reduced Glutathione (GSH)

GSH was measured in the groups following the method reported earlier (Sedlak and Linsay et al 1968). Briefly, PMS was deproteinized by adding an equal volume of 10% TCA and was allowed to stand at 4°C for 2hrs. The contents were centrifuged at 2000xg for 15min. 50µL supernatant was added to 200µL of 0.4 M Tris buffer (pH 8.9) containing 0.02 M EDTA (pH 8.9) followed by the addition 20µL of 0.01M DTNB. The absorbance was read in a microplate reader at 412nm and results are expressed as µg GSH/mg protein.

3.3.4 Total Superoxide Dismutases (SOD) activity
Total SODs were measured in the brain tissues of the animals by an already described method (Beauchamp and Fridovich, 1971) adapted to microtiter plates by bringing the final volume to 100μL. In brief, reaction mixture consisted of 0.05M phosphate buffer (pH 7.4), 1mM Xanthine and 57μM NBT. After incubation at room temperature for 15min., reaction was initiated by addition of 50μU Xanthine oxidase mixture. The SOD activity is expressed in Units /mg protein.

3.3.5 Catalase activity
Catalase activity was assayed according to earlier reported method (Sinha, 1972) using H₂O₂ as substrate. The reaction was adjusted to multiwell flat bottom plates by reducing the final volume to 200μL. Briefly, reaction mixture of 75μL in an eppendorf tube consisted of 50 μL phosphate buffer (0.01 M, pH7.0), 20μL distilled water and 5μL of PMS. Reaction was started by adding 25μL H₂O₂ (15%), incubated at 37°C for 1min. and reaction was stopped by addition of 100μL of dichromate: acetic acid reagent (1:3). The tubes were immediately kept in a boiling water bath for 15min. and centrifuged for 10min. (1500xg). The colour developed during the reaction was read at 570nm in a microplate reader. The enzyme activity was expressed as μmol H₂O₂ consumed/min/mg protein.

3.4 Nitric Oxide Analysis
3.4.1 Determination of Nitrite in Serum and Brain Tissues
The sera separated as described in the section 2.3.1 were used for the estimation of the nitrite in serum and their concentration in brain by Griess reaction system. For the estimation of nitrite inside periventricular tissues of brain, animals were sacrificed and the brains were washed with PBS and placed on ice. Brain slices from pooled periventricular tissue were chopped with scalpels and forceps and quickly weighed and placed in borosilicate glass tubes containing chilled PBS, volume 10 times the tissue weight. Tissues were then homogenized on ice using a tissue homogenizer (Remi India). The homogenate was centrifuged at 10000xg for 20 min. The supernatant was collected and centrifuged again at 75000xg for 15min. 500μL of supernatant was filtered through a micron filter (0.2μm) and filtrate collected and immediately assayed as the refrigeration can degrade the NO metabolites.
A nitrite content of the samples (100μL each) was determined by reading absorbance at 540nm after adding equal amount of Griess reagent (Sigma Aldrich, USA). Each experiment included a standard curve for nitrite. Standard curves were used to determine nitrite concentration (μmol), which was divided by wet tissue weight and expressed as μmol/mg wet tissue weight. In case of serum, nitrite concentration was expressed as μmol/mL.

3.5 Assay for Myeloperoxidase activity (MPO)

MPO activity was determined by the method as described earlier (Bradley et al., 1982). Rats were perfused with 0.98% saline and brains were quickly removed. Small blocks of periventricular tissue were finely minced at 4°C and homogenized with a homogenizer (Remi India) for 30s in 10mM KH$_2$PO$_4$ (pH 6.0) with 0.5% hexadecyltrimethylammonium bromide in an approximate concentration of 50mg of tissue/1mL. This homogenate was sonicated for 30s, and an aliquot (1.5mL) was then frozen and thawed twice for the full release of MPO polymorphonuclear neutrophils. After centrifugation at 15000xg for 15min. at 4°C, an aliquot (5-100μL) of the final supernatant was mixed with 3mL of 10mM potassium phosphate buffer (pH 6.0) containing 0.07% of 20mM aqueous guaiacol and 20μL of 3% hydrogen peroxide. Absorbance at 470nm was read with a spectrophotometer. One unit of MPO activity was defined as the quantity catalyzing the decomposition of 1μmol of hydrogen peroxide to water per minute at 37°C. Protein content was measured by Bradford method. MPO activity was expressed in units per mg protein.

3.6 Determination of Peroxynitrite formation

Peroxynitrite accumulation in the brain tissues was estimated by an already described method (Ischiropoulos et al., 1995). This method utilizes the peroxynitrite-dependent oxidation of dihydrorhodamine 123 to rhodamine 123. Briefly, a homogenous single cell suspension of the brains tissues from the EAE and adjuvant injected control rats were obtained after serial crossed scalpel chopping followed by trypsin digestion as described in section 3.2.1. Cell suspensions so obtained were washed (2-3 times) in PBS (p.H-7.4). Cells were resuspended in medium (DMEM-serum free) containing 5μM dihydrorhodamine 123 in PBS and incubated for 1h at room temperature. After incubation, cells were centrifuged at 800xg and
Experimental Procedures

the pellet was resuspended in PBS(1X) and fluorescence intensity of rhodamine 123 was measured using Perkin Elmer fluorometer (LS50-B) at an excitation wavelength of 490 nm and an emission wavelength of 520 nm (slit widths, 2.5 and 3.0 nm, respectively) and results were expressed as fluorescence intensity.

3.7 Study of Cellular Apoptosis
3.7.1 Immunohistochemistry of BAX
Rats brains (n=3) were processed for the immunohistochemical procedure as described in section 2.5.2. After antigen retrieval, sections were appropriately cooled and immediately immersed in 0.3% H2O2 to quench the activity of endogenous peroxides. The sections were washed again and blocked in blocking buffer (0.5% BSA, 1% NGS and 0.1% Triton X-100 in PBS). Afterwards, the sections were incubated with monoclonal mouse anti-BAX antibody diluted in blocking buffer (1:300) at 4°C overnight. Next day, the sections were washed and peroxides complex was visualized using a staining kit (Bio-Sb, USA) as per the manufacturer instructions containing DAB as substrate and the sections were dehydrated in ethanol and coverslipped.

3.7.2 Assay for Caspase-3 activity
Caspase-3 activity was performed using a commercially available kit (CPP32, Biovision-USA) as per manufacturer's protocol. Briefly, cell was obtained by trypsin digestion as described in section 3.1.1. The cells so obtained were spun and the pellets were lysed with Tris lysis buffer, pH 7.4 (50 mM Tris HCl, 1 mM EDTA, and 10 mM EGTA) containing 10 μM digitonin for 20min. at 37°C. Lysates were centrifuged at 900x g for 3 min, and the resulting supernatants were assayed for the protein concentration. Approximately, 50-200μg protein was diluted to 50μL of cell lysis buffer from each sample. Afterwards, the samples 50μL were mixed with the reaction buffer (containing 10Mm DTI). All the samples were incubated for 1-2 hr at 37°C after adding 5μL of 4mM DEVD-pNA (200μM). The absorbance was read at 405nm in microplate reader (Bio-Rad U.S.A) and the absorbance of cell lysates and buffers was subtracted to calculate the caspase-3 activity. Results were expressed as μM pNA hydrolysed per min. per mL.
3.7.3 Assay for PARP- activity
3.7.3.1 Preparation of Cell Lysates
Tissues from both EAE and adjuvant injected control rats were removed and placed in cold PBS in a 50 mL conical tube. Tissues were washed with PBS to remove blood clots and other debris. Tissues were transferred to a Petri dish on ice and minced to small pieces with surgical scissors and then put in a clean stainless steel sieve. Sieve with the tissue pieces was put in a Petri dish which contained about 20 mL of cold 1X PBS. A single cell suspension of the tissue was prepared using a pestle or a round bottom tube to grind the tissue pieces thoroughly until the bulk of the tissue passed through the sieve. The PBS containing the single cell suspension was then transferred to a 50 mL conical tube with PBS and mixed by inverting the tube several times. Tube was made to stand on ice for 1 min. to allow large aggregates of tissue to settle out of solution and carefully the supernatant was transferred to a clean 50 mL conical centrifuge tube. The suspension was centrifuged at 400 × g for 10 min. at 4°C and the supernatant discarded. The cell pellet was suspended in 1 mL of ice-cold 1X PBS and transferred to a prechilled 1.5 mL microtube and centrifuged at 10,000 × g for 12 sec at 4°C and supernatant discarded.

The cell pellet obtained above was mixed in 5-10 pellet volumes of cold 1X PARP Buffer containing 0.4 mM PMSF, other protease inhibitors, 0.4 M NaCl, and 1% Triton X-100 or 1% NP-40 nonionic detergent and incubated on ice, with periodic vortexing, for 30 min. The disrupted cell suspension was centrifuged at 10,000 × g for 10 min. at 4°C to remove insoluble material. The supernatant was recovered to a fresh tube in prechilled on ice. Protein concentration of the cleared cell lysates was determined and the cleared cell extracts were snap frozen in small aliquots and stored at -80°C.

3.7.3.2 PARP assay
PARP activity was assayed using a commercial kit (Cat# 4671-096-K, Trivigen-USA) as the manufacturer recommendations. Histone coated flat 96-well plates were washed 4 times with 1X PBS + 0.1% Triton X-100 (200 μL/well) and enough care was taken to ensure that all the liquid is removed following each wash by tapping plate onto paper towels. Plates were blocked by adding 100 μL of 1X Strep-Diluent to every well and allowed to stand at room temperature for 1 h. Following blocking, plates were washed 4 times with 1X PBS + 0.1%
Triton X-100 (200μL/well) and excess liquid was removed following each wash by tapping plate onto paper towels. 10μL 1X PARP buffer was mixed with 8-15μL (20 μg) cell extract in a well. Finally, 25μL of PARP Cocktail was added in each well and mixed good and plates were incubated at room temperature for 30-60min. Subsequently, plates were washed 4 times with 1X PBS + 0.1% Triton X-100 (200μL/well) and 50μL per well of diluted Strep-HRP were added and again incubate at room temperature for 20 min. Plates were again 4 times with 1X PBS + 0.1% Triton X-100 (200μL/well) and 50μL per well of TACS-Sapphire as the colorimetric substrate was added and left in the dark, for 10-30 min. Color development was monitored for observable blue color in samples reaction was stopped by adding 50μL per well of 0.2 M HCl or 5% Phosphoric Acid and the absorbance read at 450 nm. Results expressed were as % change from adjuvant injected controls.

3.8 Choline-O-acetyl Transferase Immunohistochemistry (ChAT)

Rats (n=3) were processed for the immunohistochemical procedure as described in section 2.5.2. After the antigen retrieval, sections were appropriately cooled and immediately immersed in 0.3% H2O2 to quench the activity of endogenous peroxides. The sections were washed again and blocked in blocking buffer (0.5% BSA, 1% NGS and 0.1% Triton X-100 in PBS). Afterwards, the sections were incubated with monoclonal mouse anti-ChAT antibody diluted in blocking buffer (1:500) at 4°C overnight. Next day, the sections were washed and peroxides complex was visualized using a staining kit (Bio-Sb, USA) as per the manufacturer instructions containing DAB as substrate and the sections were dehydrated in ethanol and covered slipped.
4. Studies of the Cell Cycle and Migration of Proliferating Progenitors

4.1 Animal Grouping
For the studies of progenitor proliferation and migration, rats were divided into groups as per the need of the experiment described in forthcoming sections.

4.1.1 Identification of Proliferative Population
For the location of proliferation, rats were divided into following groups

❖ Group I (n=5) - Adjuvant injected rats, 2 i.p injections of BrdU(50mg/Kg) per day, spaced 2h for 4 days and perfused 20 days p.i.
❖ Group II (n=5) - EAE rats, 2 i.p injections of BrdU(50mg/Kg) per day, spaced 2h for 4 days and perfused 20 days p.i.

4.1.2 Cumulative BrdU Labeling
For this procedure, 4 EAE rats after 20 days p.i were used per group and ten different groups were made according to the injection schedules described in Table 3.2.

4.1.3 BrdU+ cell Migration
For the BrdU+ cell migration, rats were divided into following groups

❖ Group I (n=3) - Adjuvant injected rats, 7 i.p injections of BrdU (50mg/Kg) spaced at 2h intervals a day before immunization.
❖ Group II (n=3) - EAE rats, 7 i.p injections of BrdU (50mg/Kg) spaced at 2h intervals a day before immunization.

4.2 Preparation of BrdU
BrdU (a thymidine analog) (Sigma, St Louis, USA) prepared in phosphate buffered saline (pH 7.4) containing 0.007N NaOH.
4.2.1 Identification of Proliferative Population

For determination whether the EAE in Wistar rats leads to the proliferation of the germinative zones, BrdU was injected into the animals i.p two times a day for a period of 4 days since it takes almost 4 days to label all the proliferating population in rodents. Rats were sacrificed 20 days p.i and brains so obtained processed for immunohistochemistry.

4.2.2 Cumulative BrdU labeling

To estimate the length of cell cycle (TC), the length of S phase (TS) and the actively proliferating population (GF) of SVZ and DG, a cumulative BrdU labeling scheme established by Nowakowski et al., 1989 and Takahashi et al., 1993 was used. This protocol requires that the serial BrdU injections (50mg/Kg) be spaced so that the length of non labeled intervals between injections is less than the length of S phase such that all nuclei passing through S phase will be labeled. Briefly, adult rats were intraperitoneally injected with BrdU (50 mg/kg, Sigma) at 2h intervals over a total period of 18.5h, beginning at 08.00h (Table 3.2). At 30min. after each of the BrdU injections, selected rats were perfused. Three to four animals per time point were killed with a total of 10 time points. Adjuvant injected rats were used as controls and underwent a similar tracing protocol.

4.2.3 SVZ Cell Migration

To study SVZ cell migration, adjuvant injected controls and EAE rats received seven i.p. injections of BrdU at 2h intervals the day before EAE induction. Rats were euthanized 20 days p.i. using this tracing protocol, labeled cells are restricted to the SVZ in adjuvant controls (Nait-Oumesmar et al., 1999; Litzenburger et al., 2000). Therefore, detection of labeled cells after EAE induction, in structures other than the SVZ, is assumed to originate from the SVZ.
Table 3.2- Cumulative BrdU labeling protocol for the estimation of total cell cycle length (TC) and total time in synthesis phase (TS) of proliferating progenitors in SVZ (subventricular zone) and DG (dentate gyrus) of EAE and control rats.

<table>
<thead>
<tr>
<th>Rat Group(RG) Serial No.</th>
<th>BrdU Pulse time</th>
<th>Time Schedule of BrdU Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>RG 1</td>
<td>0.5 hr</td>
<td>7.30 A.M</td>
</tr>
<tr>
<td>RG 2</td>
<td>2.5 hr</td>
<td>8.00+ 9.30</td>
</tr>
<tr>
<td>RG 3</td>
<td>4.5 hr</td>
<td>8.00+10.00+11.30</td>
</tr>
<tr>
<td>RG 4</td>
<td>6.5 hr</td>
<td>8.00+10.00+12.00+ 1.30</td>
</tr>
<tr>
<td>RG 5</td>
<td>8.5 hr</td>
<td>8.00+10.00+12.00+ 2.00+ 3.30</td>
</tr>
<tr>
<td>RG 6</td>
<td>10.5 hr</td>
<td>8.00+10.00+12.00+ 2.00+ 4.00+ 5.30</td>
</tr>
<tr>
<td>RG 7</td>
<td>12.5 hr</td>
<td>8.00+10.00+12.00+ 2.00+ 4.00+ 6.00+ 7.30</td>
</tr>
<tr>
<td>RG 8</td>
<td>14.5 hr</td>
<td>8.00+10.00+12.00+ 2.00+ 4.00+ 6.00+ 8.00+ 9.30</td>
</tr>
<tr>
<td>RG 9</td>
<td>16.5 hr</td>
<td>8.00+10.00+12.00+ 2.00+ 4.00+ 6.00+ 8.00+ 10.00+11.30</td>
</tr>
<tr>
<td>RG 10</td>
<td>18.5 hr</td>
<td>8.00+10.00+12.00+ 2.00+ 4.00+ 6.00+ 8.00+ 10.00+12.00+ 1.30 A.M</td>
</tr>
</tbody>
</table>

Figure 3.2- Schematic representation of the SVZ and DG of Wistar rats at AP +10.6 & AP +9.2
4.2.4 Immunohistochemistry of BrdU

An already described method was employed with modifications. Rats after different treatment protocols were anesthetised by ether. Following anaesthesia, rats were mounted on an operation table with fitted in water supply nozzles and two flow rate controlled drips with 21-gauge needles, one filled with freshly prepared 4% paraformaldehyde and another with chilled P.B.S (p.H 7.4) (Agrawal et al, 2004). Then the anterior aspects of rat abdomen were opened by incision up till thorax which allowed access to the heart. Perfusion was done by injecting 200mL of prechilled P.B.S (p.H 7.4) by transcardial perfusion for about 1.5h. In order to fix the tissues after blood removal; 150mL of 4% paraformaldehyde was infused through the same procedure. Later on, the overlying skin of the head was removed and skull was opened and brains were dissected out while taking care to preserve the anatomical structures from physical damage. The brains so obtained were transferred to the sterile Petri dishes (Qiagen-India) in P.B.S (p.H 7.4) and lids were closed. Brains after washing were taken out and were put in 4% paraformaldehyde overnight. From next day, brains were dehydrated in 10%, 20% and 30% sucrose successively up till 3 days. The post dehydration fixation was done primary with different fixatives but afterwards, the formaldehyde fixative (40% w/v) was selected since it caused least damage to the epitopes that was evident during initial experimentation. Fixation was done until 1 month before immunochemical staining. For immunochemical detection, brains were dissected as per need of the experiment and were mounted for paraffin embedding preparing blocks. 5-8μm thick sections of paraffin embedded blocks were cut through the coronal plane using grading type microtome under freezing conditions.

Before starting the antigen detection, sections were processed for antigen retrieval. Since, the BrdU resides in nucleus after its uptake by replicated DNA, it is necessary that the nuclear membrane is dissolved to localize it. Firstly, the sections were incubated in warm trypsin (37°C) for about 2min and repeatedly washed in P.B.S (p.H 7.4) to remove excess trypsin. Afterwards, sections were incubated in 2Ν HCl for 10 min and washed 3 times with P.B.S (p.H 7.4) to remove HCl. After antigen retrieval, sections were incubated shortly in 0.3% H2O2 in methanol to block activity of endogenous peroxides present in the tissue which decreases noise and gives differentiated signal. After this, sections were incubated in
blocking buffer (for blocking non specific binding) containing 0.1M PBS, 0.04% Triton X-100 and 10% NGS. Sections were then incubated in monoclonal anti BrdU antibody (1:100) (AbD Serotec, USA)) at 4°C overnight in a humid chamber. After rinsing in buffer, sections were processed using a peroxidase staining kit from BioSB (U.S.A) using a tertiary antibody conjugated with HRP (horse raddish peroxidase). The peroxides complex was visualized with 3, 3-diaminobenzidine. Lastly the slides were counterstained hematoxylin in for about 20s. Slides were then cleaned in xylene, gradually dehydrated with ethanol and cover slipped.

4.2.5 Analysis of BrdU immunostaining

4.2.5.1 Analysis of Cumulative Labeled BrdU

For analysis of BrdU-labeled cells, BrdU immunostained coronal sections at the level of AP + 10.6mm and AP + 9.2mm (Paxinos and Watson, 1986) (Fig.3.2) were used. 5-Bromo-2-deoxyuridine- labeled and unlabeled cells along the SVZ of the ventricular wall and DG were counted in only one focal plane to avoid oversampling. 5-Bromo-2-deoxyuridine-labeled and unlabeled cells in each SVZ & DG coronal section are presented as the number of the labeled cells /section. Density for the four sections per rat was averaged to obtain a mean density value for each brain according to published methods (Kuhn et al., 1996; Zhang et al., 2001). Using this method, the number of BrdU cells in the SVZ and the dentate gyrus were counted. The results are comparable to the number obtained from the stereology method (Kuhn et al., 1996; Zhang et al., 2001).

4.2.5.2 Analysis of Cell Migration.

The number of BrdU (+) cells was estimated in three sections in a rat and total 3 rats were studied per group. Cell counting was done in three of the anatomically defined compartments viz. SVZ, corpus callosum (CC) and cortex (CTX). Since presence of the BrdU immunopositive cells in the structures other than SVZ was considered as migration and respective numbers in each structure allowed estimating the extent of migration.

4.2.5.3 Calculation and interpretation of Results from Cumulative Labeling.

For each brain, an average labeling index (LI), that is, the ratio of labeled cells to total cells, was determined by averaging the LIs of three nonadjacent sections at each time point, and
was plotted as a function of time after the initial injection (Nowakowski et al., 1989; Takahashi et al., 1993). The GF (growth fraction), that is, the ratio of proliferating cells to the total cells in the population, and the parameters of TC and TS were calculated by using least-squares (LS) line fit to all considered data points. Total cell cycle length (TC) and total time in synthesis phase (TS) were calculated from the graphs based on two relationships: (1) the time required to label the GF, that is, the inflection point of the curve, is equal to TS/TC; and (2) the y-intercept of the curve is equal to (TC/TS) x GF (eq.1) (Nowakowski et al., 1989; Takahashi et al., 1993).

4.3 Immunohistochemistry of CSPG3 (neurocan)
Rats (n=3) from both the groups were processed for immunohistochemical procedure described in section 2.5.2. After antigen retrieval, sections were appropriately cooled and immediately immersed in 0.3% H2O2 to quench the activity of endogenous peroxides. The sections were washed again and blocked in blocking buffer (0.5% BSA, 1% NGS and 0.1% Triton X-100 in PBS). Afterwards, the sections were incubated with monoclonal mouse anti-CSPG3 antibody diluted in blocking buffer (1:150) at 4°C overnight. Next day, the sections were washed and peroxides complex was visualized using a staining kit (Bio-Sb, USA) as per the manufacturer instructions containing DAB as substrate and the sections were dehydrated in ethanol and coverslipped.

4.4 SVZ Explants Migration in vitro
To test whether EAE regulates neuronal cell migration in vitro (Lois et al., 1996), EAE and non EAE rat tissue explants from the SVZ were harvested and plated in 24 wells (lower surface of the wells scratched to enhance adhesion) with 1000μL of Neuralbasal-A medium containing 2% B27 supplement (Invitrogen) (Leventhal et al., 1999). The average linear distance and the total area of cell migration from the explant culture edge were captured using a 10x objective via the computer imaging analysis system and measured at day 3 & 5 post plating using the Motic 2.0 software. The longest migration distances and the area encompassing migration were assessed in each explant culture and measured.
Protein content

The content of the protein where ever need in these experimental procedures was estimated as per Bradford, 1976.

Statistical Analysis

All the presented data are Mean ± SEM.

Chapter 5: Data was analyzed using ANOVA with post hoc analysis done by Tuckey’s multiple comparisons. Any variations with P<0.05 were considered significant.

Chapter 6, 8: Data was analyzed using non-parametric one tailed t-test. Any variations with P<0.05 were considered significant.

Chapter 7: Data from BrdU labeling was analyzed using Least Squares Fit.