Analysis of EAE Model for the development of therapies of Multiple Sclerosis through Bioinformatic tools

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ABSTRACT

Multiple Sclerosis is an autoimmune demyelinating inflammatory disease of the central nervous system (CNS) which resulted in the severe neurological defects. Mostly MS affected the adult life in their early life and it shows huge affect on family, and on professional and on daily life. The rate of multiple sclerosis according to some research in women is four times higher than in man, though the exact reason of this is still unclear but the researchers associated this with the difference in sex that linked with the brain in the MS. The disease progression of multiple sclerosis and its developments entails fundamental steps: (1). The destruction of myelin sheath and formation of lesions, (2). Inflammation. These steps are communicated together in a collaborative way, destroying the neuron tissues and causing MS. The goal of the research is to develop the EAE model for the identification of proteins involved in Multiple Sclerosis and then develop the interventions that can improve the lives of those living with MS. Synaptosomal-associated protein 25 (SNAP-25) is a 25kD protein with 206 amino acids. The presynaptic terminal of neurons is composed of a t-SNARE or target SNARE molecule. Formation of neural soluble Nethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes occurs because of SNAP -25 and has a great importance. Calcium-dependent exocytosis of synaptic vesicles, proper efficient release of neurotransmitters and propagation of action potential is done by SNARE complex. The normal levels of SNAP-25 are mandatory for neurotransmission; the changes in its expression can cause many disorders including autoimmune disorder like Multiple Sclerosis. The ultimate goal of this research is to assess the behavioral changes and pathophysiology of MS

Keywords : Inflammation, Demyelination, Multiple Sclerosis, Exocytosis, Nervous System, SNARE complex, SNAP-25

1 INTRODUCTION

Multiple sclerosis (MS) is a chronic central nervous system autoimmune demyelinating condition, which may lead to gradual neuroaxonal degeneration[1]. MS attacks the myelinated axons within the central nervous system, destroying the myelin and therefore axons to a varying degree. The symptoms of MS vary depending on the location of the plaque within the CNS. The pathological hallmarks of the disease are denoted by the loss of Myelin sheath due to the focal demyelination in the white and grey matter of the brain and spinal cord [2]. It has been reported that about 2.3 million people are being affected by MS worldwide with two times higher risk for women than men.
Currently, available MS medications will only slow down the disease's progression and do not function for all patients. Therefore, more successful therapies are needed, but a poor understanding of MS pathogenesis has disrupted their growth [3]. One of the most well-studied and well-established models to investigate neuroinflammatory pathways involved in MS is Experimental Autoimmune Encephalomyelitis (EAE). EAE can be induced in different animal models but mice are the most widely used species for this model. In the EAE model of mice, the pathophysiological reaction of the immune system against brain-specific antigens can be seen. This reaction causes inflammation and degradation of carrier structures of the antigen and produces comparable neurological and pathological features in MS patients [4].
High Mobility box 1 group (HMGB1) has emerged as a possible candidate because of its role in EAE/MS pathogenesis. HMGB1 is a proinflammatory molecule that mainly activates the toll-like receptor 4(TLR4) by combine with the receptor advanced glycation end product (RAGE)which initiates inflammatory reactions [5]. Higher HMGB1 levels in human and experimental MS enhance its function in pathogenesis, mainly through enhancement of inflammatory processes and increased neuroinflammation of demyelination [6]. HMGB1 results in the activation of DCs and T cell proliferation and in this way, it manipulates the immune system. Furthermore, Microglial activation mediated through HMGB1 shows a crucial function both in myelin injury and in the death of neurons [7]. This role of HMGB1 is pathogenically im-

portant for autoimmune disorders such as MS. The contribution of HMGB1 in MS pathogenesis has been demonstrated by the above emerging evidence [8].
The objective of this study was to determine whether HMGB1 can be a therapeutic target for EAE and characterization of HMGP1 proteins involved in the disease course of the EAE model of MS. To achieve this objective, we created an Animal model of MS by administrating the MOG35-55 peptide.

2 METHODS

Experimental work was performed in three different phases:
Phase 1 involves preparation of Animal Model and Sample Collection.
Phase 2 involves 2D Gel Electrophoresis and MS/MS analysis.
Phase 3 involves Bioinformatics Analysis.

2.1 Experimental Groups:

Three experimental groups were used. All groups were matched with respect to

- Gender
- Age
- Litter (when possible)
- Genetic Modifications (i.e., transgene, knock-in, knock-out)
- Other relevant conditions (e.g., gonadectomy, housing conditions)

Three groups were used and each group consist of four mice.

Wild type / control group: Not a single injection is given to the mice in this group so mice remain asymptomatic. All the mice in these groups are wild type with no symptoms.
Control group without MOG35–55 injection: The mice in this group were injected with all EAE reagents except MOG35–55. The mice were injected with complete Freund's adjuvant (CFA) containing Mycobacterium tuberculosis.
Mice model with MOG35–55: Mice in this group received complete EAE injections. For development of active EAE, mice **(C57BL/6)** were injected (s.c.) at flanks with $MOG^{35–55}$ peptide of 200 µg (Sigma Company) in PBS emulsified in complete Freund's adjuvant (CFA) of equal volume containing Mycobacterium tuberculosis (Sigma Company) at a final concentration of 1 mg/ml.

2.2 Materials:

2.2.1 Laboratory equipment and other material

1. Analytical Balance
2. Square anti-static weighing dishes,
3. Disposable anti-static microspatulas
4. Conical centrifuge cubes
5. Corning, orange round bottom cryogenic vial of 2.0ml
6. PrecisionGlide 27 × 1/2-gauge needle, gray
7. PrecisionGlide 25 × 5/8-gauge needle, blue
8. 1 mL syringe of Tuberculin slip.
9. 3 cc Popper & Sons Perfektum glass with proper matched number syringes. Must be cleaned and properly autoclaved.
10. Micro emulsifying needle, 20 gauge
11. Scintillation vials
12. Sterile alcohol prep pad

2.2.2 Chemicals:
Chemicals used in this study were purchased from Sigma Company and lists of chemicals used are

Table 1: List of Chemicals used

S.No	CHEMICALS	AMOUNT
1	Dulbecco's phosphate buffer saline (DPBS)	50ml
2	Lyophilized MOG35–55 peptide	200ug
3	Pertussis toxin (PTx)	400ug
4	Heat-killed Mycobacterium tuberculosis H37 RA	4mg/ml
5	Complete Freund's Adjuvant	1ml
6	Ethanol	70% (vol/vol)

1. Dulbecco's phosphate buffer saline (DPBS) were used without calcium or magnesium.
2. Lyophilized MOG35–55 peptide was used with sequence (Sequence: MEVGWYRSPFSRVVHLYRNGK; >95% purity).
3. Pertussis toxin (PTx) were lyophilized in pure water and salt-free toxin were used.

2.3 Methods

The reagents quantities were based on the mice number used in the experiment so the number of ice and quantity of reagent were first calculated. Each mouse received 1mg/ mL total of MOG35–55 -CFA through two 0.05 mL injections on both Days 0 and Day 7. MOG35–55 and M. tuberculosis were administered at 200 µg/mouse.

Critical step: Different disease profile comes from changing the $MOG35^{-55}$, M. tuberculosis, or PTx dosages. Alteration in the cytokines, immune cell population and location of lesions depends upon the concentration of dosage only when the disease course it's onsets and clinical scoring is consistent. [9].

2.3.1 Phase 1 Preparation of Animal Model:

M. tuberculosis and Complete Freund's adjuvant storage:

1. Within the BSC, M. tuberculosis H37 RA was opened carefully ampule per manufacturer's specifications.

2. The content was then transferred into a parafilm, and stored at 4°C until it is use.

3. CFA ampule was to re-suspend the M. tuberculosis. CFA ampule per manufacturer's specifications was opened within the BSC. The contents are then transferred to a parafilm, and stored at 4°C for further use.

Preparation of MOG35–55-CFA emulsion

1. Emulsifying syringes glass was attached to the emulsifying needles and then by the help of plunger it is pressed down.

2. Syringe, plunger, and needle must be clean. And both of them were correctly paired.
3. On the top there must be syringe and once it was assembled, each assembly note must unscrew easily, without any tightening both the syringes were tightly secured.
4. Plunger was then removed from the syringe and placed it on a sterile surface within the BSC.
5. 50 mL conical tube was then filled with ∼1 mL more than the calculated volume of DPBS.
6. Balance was cleaned by using Kim wipe sprayed with 70% Ethanol.
7. MOG35–55 peptide was taken from the −20°C freezer to a room temperature prior to weighing.
8. MOG35–55 peptide was then weighed out.
9. Without removing any boat from the peptides, MOG35–55 peptide was weighed from the balance and then half volume of DPBS were added, buffer might be expelled out of the boat from its edges. So, when the peptide was covered by the DBPS used on the experiment then we have transferred that into BSC after weighing properly. The DPBS were added then. The end solution was clear.
10. On the top of the emulsifying syringe used, the premeasured about of MOG35-55 were added and then all the solution was aspirated at the bottom of the string's plunger. Total MOG35–55 solution must be equally distributed.
11. CFA were vertexes to confirm the uniform suspension.
12. The predetermined amount of TB weighed out. The CFA were weighing out before transferring into the container.
13. The measured volume of solution of TB-CFA was added to the emulsifying syringe.
14. Process of emulsification continued for 25times on each side and syringes were placed at -20°C for 1 h.
15. These syringes were placed at -4°C after 1h until emulsion become softens enough to easily pass through the needle. Then in BSC chamber re-emulsified these 25 times on each side and it become total of 50 times.
16. These syringes were then stored to −20°C again for at least 1h. until this one is ready to immunize (up to 6 hrs).

Preparation of Pertussis toxin:

1. On day 0 and then on day 2 mice were administered 0.3 mL of 500ng Pertussis toxin in DPBS.
2. These things were placed in a clean BSC.
3. DPBS was aliquot into the conical tube of 50ml.
4. The needle is carefully pushed by using the rubber cap. The DPBS were automatically drained into the vial.
5. PTx vial content was then pipette into the conical tube of 50ml.
6. More than three times the DPBS with the volume of 1ml were rinsed until there will be no particles of PTx were found in the vial.
7. Each syringe was filled, must avoid bubbles. Stored at 4°C.

MOG immunization into mice (C57BL/6) for the development of EAE

1. For development of active EAE, mice **(C57BL/6)** were injected (s.c.) at flanks with $MOG^{35–55}$ peptide of 200 µg (Sigma Company) in PBS emulsified in complete Freund's adjuvant (CFA) of equal volume containing Mycobacterium tuberculosis (Sigma Company) at a final concentration of 1 mg/ml.

2. At the time of immunization and even 48 hours later each mouse received the two injections of 400ng pertussis toxin (Sigma Company). Minimum of four mice were included in each treatment group.

3. For clinical score of disease animals were properly weighed, these clinical score evaluations are very important to determine the signs of disease.

4. Mice for acute stage inflammation during EAE and their corresponding negative controls were sacrificed at 17 days post inoculation (dpi). Mice for chronic stage inflammation during EAE and their corresponding negative controls were sacrificed at 57 dpi.

5. Few of the inoculated mice had recovered and had shown no signs of inflammation at the end, so they were grouped together in "Recovered" mice group.

6. On the day of sacrifice, mice were perfused transcardialy with PBS to prevent contamination of spinal cord with blood followed by extraction of spinal cord tissue.

7. Cervical region of spinal cord was extracted and snap frozen for immunofluorescence and rest part of spinal card was extracted and snap frozen for proteomic analysis.

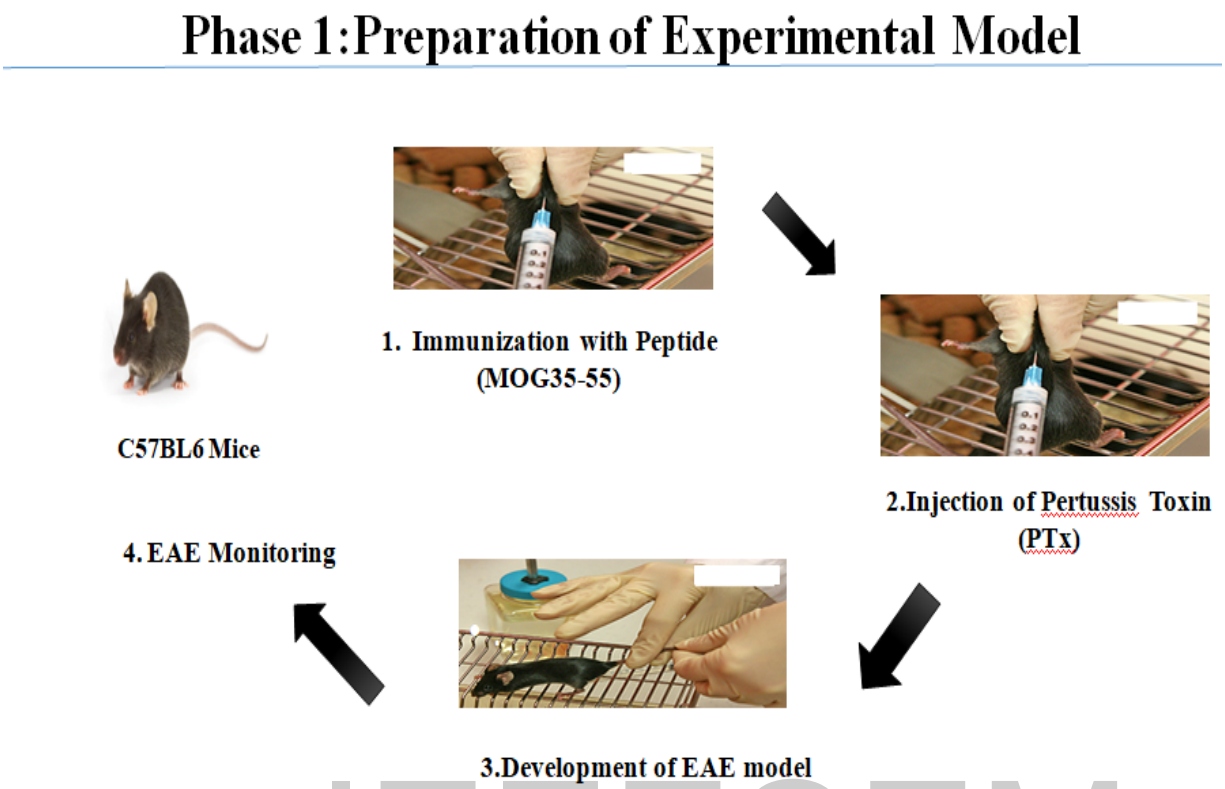


Fig 1: Development of Animal Model

The mouse used in this experiment was C57BL6 which is immunized with MOG35-55. MOG (35-55) is a myelin oligodendrocyte glycoprotein (MOG) 35-55 is a minor component of CNS myelin. Then two injections of pertussis toxin were injected intra-peritonically. Pertussis toxin increases the severity of disease in mouse model. Once the mouse model is developed then this model is further used for EAE monitoring.

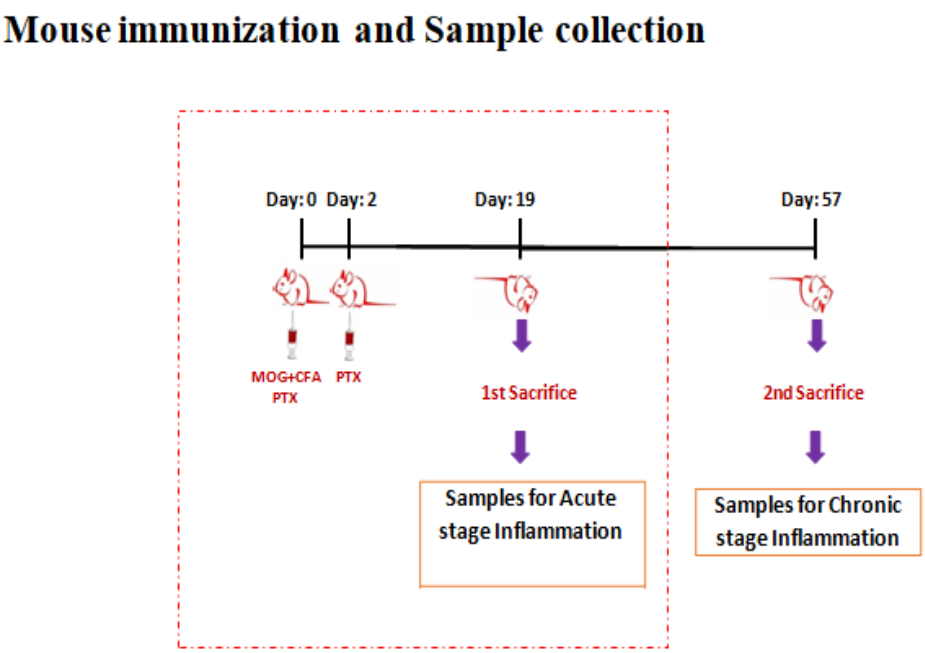


Fig 2: Mice for acute stage inflammation during EAE and their corresponding negative controls were sacrificed at 17 days post inoculation (dpi). Mice for chronic stage inflammation during EAE and their corresponding negative controls were sacrificed at 57 dpi.

Sample preparation, SDS-PAGE and Western blotting

- Sample preparation involves homogenization of tissue lysate (10% w/v) of frozen spinal cord tissue samples which were prepared in lysis buffer.
- The lysis buffer contains urea urea of 7M, Thiourea of 2M, 4 % CHAPS, 20 µl/ml Ampholytes, 10mg/ml Dithiothreitol (DTT), protease and phosphatase inhibitors.
- In the next step ultracentrifugation occur at 30,000 RPM for 30min.
- Bradford assay (Bio-Rad) is used to determine the protein concentration in tissue lysate. Boiling of samples occurred at 95°C for 5 min after mixing them with 4X Roti-Load (ROTH) as a sampling buffer.
- Cooling of sample was done, before the next step.
- It was then used for Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis (SDS-PAGE) and then finally western blotting.

2.3.2 Phase 2: 2D Gel Electrophoresis and MS/MS Analysis

Two-Dimensional Gel Electrophoresis, Visualization and Analysis of protein spots

Separation of protein in two dimensions was achieved by 2D gel electrophoresis through two-dimension process. In the first dimension isoelectric focusing (IEF) were analyzed and molecular weight with SDS-PAGE was analyzed in second dimension and the experiment was done for protein separation and different fractions of proteins were also separated from the mixture of tissue lysate through this method. Silver stain were used to visualized the protein spot after separation followed by scanning (CanoScan Scanner) for further analysis of protein spots. DECODON Delta2D software were used for the analysis of protein visually and to identify the expression of protein spots differentially between different groups which analyzed each spot intensity individually with the help and calculated by using a cut off value of 1.5-fold change and p-value <0.05 in unpaired Student's t-test.

Mass-spectrometry for the Identification of protein/ peptide sequences:

Protein spots from the silver strained 2DEgel and which show different expression were extracted and then processed for the identification of protein sequence with the Q Exactive hybrid quadrupole/orbitrap mass spectrometry. The processing involves the silver stain destaining, disulfide bonds reduction, free cysteines alkylation, digestion of trypsin, extraction of peptide and peptide sequence identification. Mass spectrometers were used to identify the protein and after the identifications, of peptide count (≥2) were used to eliminate the false positive identifiers in the proteomics data, (95%) of peptide threshold. 0.01% of minimum FDR rate which is false discovery rate were then used for the qualification of identified proteins.

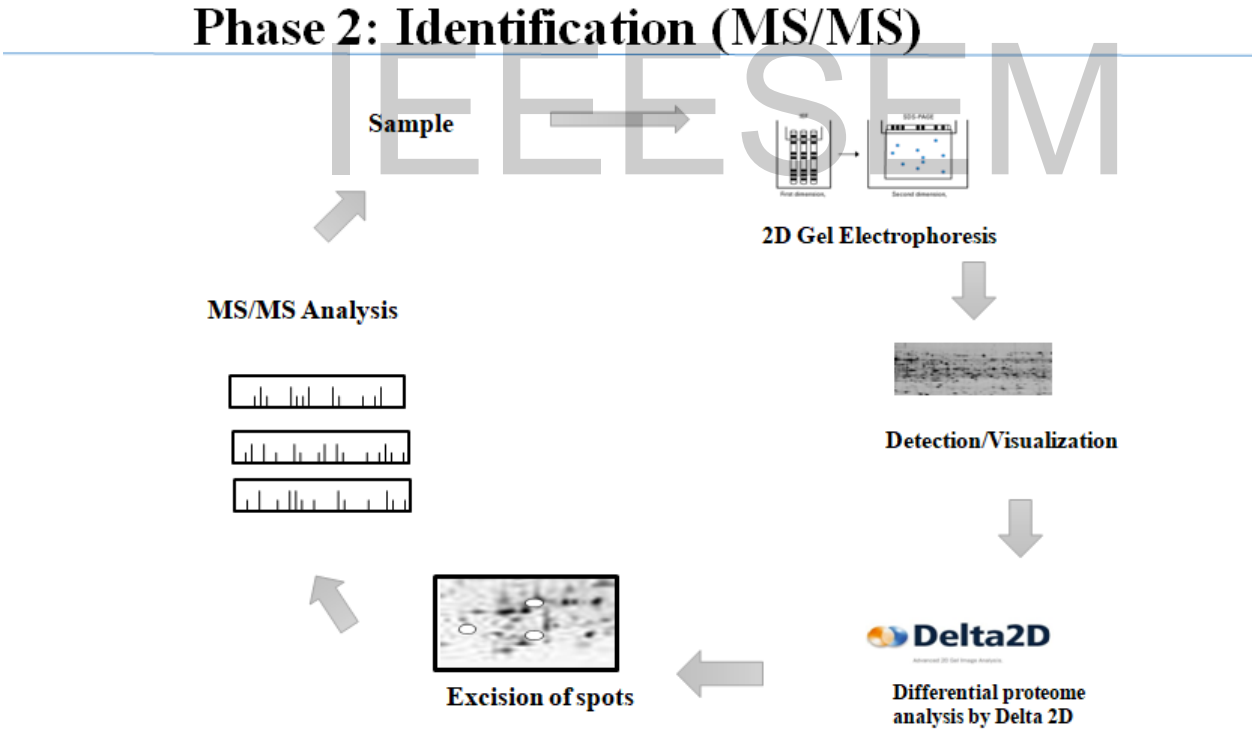


Fig 3: Proteins Separation by 2D Gel Electrophoresis and Identification of Protein by MS/MS Analysis.

2-D electrophoresis is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. It is the method available which is capable of simultaneously separating thousands of proteins. This technique separate

proteins in two steps, according to two independent properties: First-dimension is isoelectric focusing (IEF), which separates proteins according to their isoelectric points (pI); Second-dimension is SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular weights (MW). In this way, complex mixtures consisted of thousands of different proteins can be resolved and the relative amount of each protein can be determined. Each spot on the resulting two-dimensional gel potentially corresponds to a single protein species in the sample. Thousands of different proteins can be separated and information such as the protein pI, the apparent molecular weight, and the amount of each protein can be obtained. At the very beginning of the 70s, two high-performance electrophoretic separations of proteins were available: i) zone electrophoresis of proteins in the presence of SDS, as described in its almost final form by Laemmli, a technique that instantly became very popular, and still is, and ii) denaturing isoelecric focusing, as described by Gronow and Griffith. As these two techniques used completely independent separation parameters, it is not surprising that it was soon tried to couple them. Two-dimensional electrophoresis was first introduced by O'Farrell in 1975.

In 2D GE proteins are separated as per isoelectric point and protein mass. Separation of the proteins by isoelectric point is called isoelectric focusing (IEF). When a gradient of pH is applied to a gel and an electric potential is applied across the gel, making one end more positive than the other. At all pH values other than their isoelectric point, proteins will be charged. If they are positively charged, they will be pulled towards the negative end of the gel and if they are negatively charged they will be pulled to the positive end of the gel. The proteins applied in the first dimension will move along the gel and will accumulate at their isoelectric point; that is, the point at which the overall charge on the protein is 0 (a neutral charge).

In separating the proteins by mass, the gel treated with sodium dodecyl sulfate (SDS) along with other reagents (SDS-PAGE in 1-D). This denatures the proteins (that is, it unfolds them into long, straight molecules) and binds a number of SDS molecules roughly proportional to the protein's length. Because a protein's length (when unfolded) is roughly proportional to its mass, Since the SDS molecules are negatively charged, the result of this is that all of the proteins will have approximately the same mass-to-charge ratio as each other.

In addition, proteins will not migrate when they have no charge (a result of the isoelectric focusing step) therefore the coating of the protein in SDS (negatively charged) allows migration of the proteins in the second dimension. In the second dimension, an electric potential is again applied, but at a 90-degree angle from the first field. The proteins will be attracted to the more positive side of the gel (because SDS is negatively charged) proportionally to their mass-to-charge ratio. The gel therefore acts like a molecular sieve when the current is applied, separating the proteins on the basis of their molecular weight with larger proteins being retained higher in the gel and smaller proteins being able to pass through the sieve and reach lower regions of the gel.

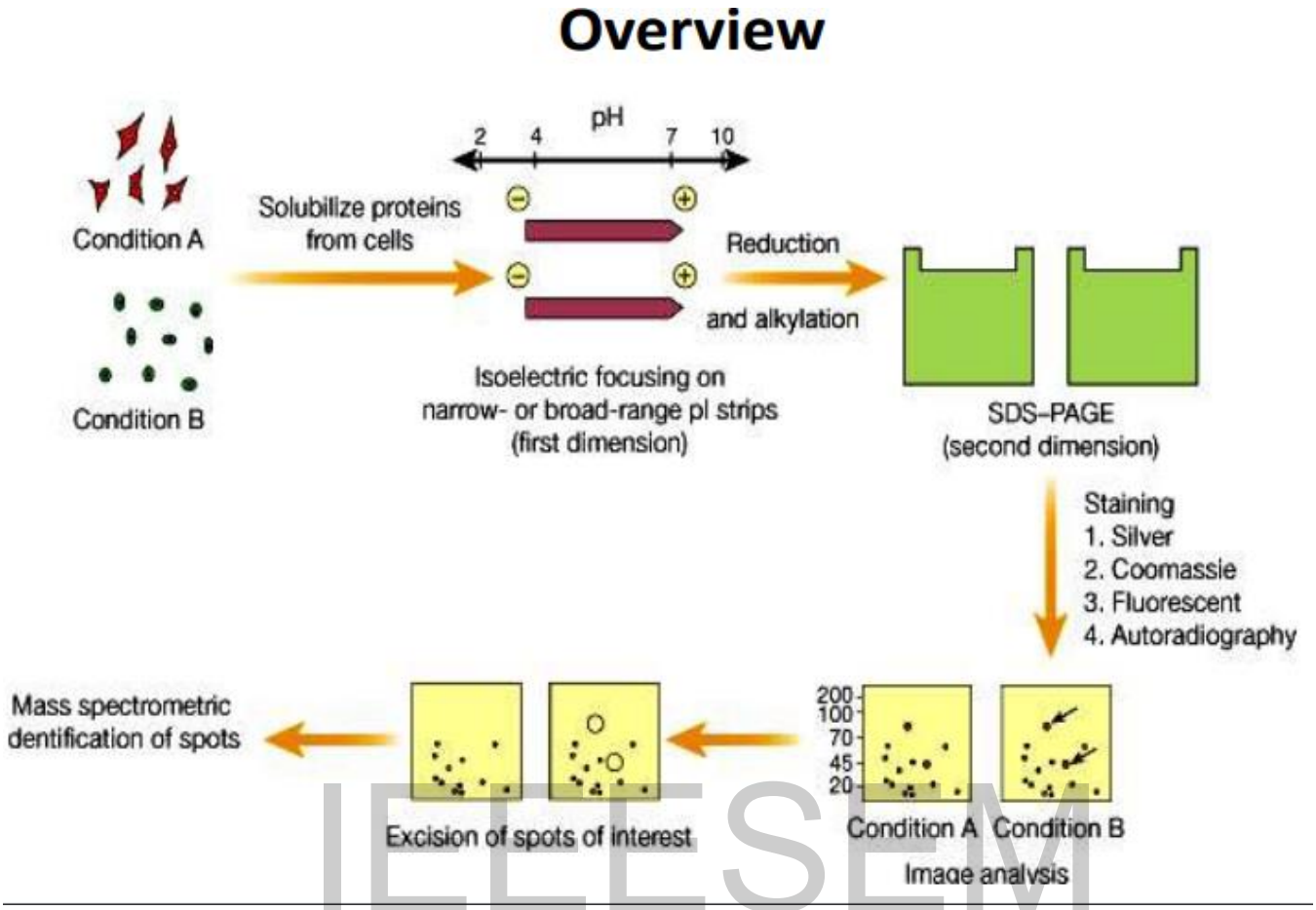


Fig 4: Overview of Separation of Protein through 2D Gel Electrophoresis.

Spinal Cord Co-Immunofluorescence:

Frozen spinal cord tissue sections (5 µm) from each of EAE (acute stage), EAE (chronic stage), CFA controls and their corresponding negative controls were fixed with methanol (9 min.) followed by acetone (1 min.). Then PBS were used to wash the sections and also it washes tissue sections of different antigenic sites. Permeabilization buffer (PBS + 0.2 % Triton X-100) exposed this section for 10 min.The non specific protein were obstructed by 5% of bovine serum albumin with RAB7 for 2h and then left it for incubation overnight (1:100) as primary antibody (pAb) then diluted in 5% serum with PBS for overnight at 4°C.Horseradish peroxidase (HRP) labeled which is anti-goat secondary antibody was used for 2h.To-Pro3 was used to stain the nuclei for 10 min. Confocal Laser Scanning was used to visualize the sections.Co-localization pattern of individual images is analyzed by using the software ImageJ(WCIF plugin) .

Sucrose density gradient formation:

Sucrose density gradient was performed in order to study the expression of oligomer formation under inflammatory stress condition during EAE. Spinal cord tissue samples were homogenized by 10% w/v in 1x PBS containing 2% w/v sarkosyl. Continuous sucrose gradient of 10-50% were made in 2mL ultracentrifugation tubes followed by layering of homogenates on the top of them. Ultracentrifugation was performed at 50,000 rpm for 73 min. at 4°C and five fractions were collected from top to bottom for each sample.

Statistical analysis:

Graph Pad Prism 5 was used to analyze the data. Experimental errors were displayed as SEM. Student´s t test and nonparametric one way ANOVA was used to calculate the statistical significance followed by Turkey's multiple comparison test. Results were considered significant when *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

2.3.3 Phase 3: Bioinformatics Analysis through LIGPLOT and GRAMM-X Protein-Protein Docking Server

(http://vakser.compbio.ku.edu/resources/gramm/grammx/)

Molecular Docking through GRAMM-X:

Molecular interactions including protein-protein, enzyme-substrate, protein-nucleic acid, drug-protein, and drug-nucleic acid play important roles in many essential biological processes, such as signal transduction, transport, cell regulation, gene expression control, enzyme inhibition, antibody–antigen recognition, and even the assembly of multi-domain proteins. These interactions very often lead to the formation of stable protein–protein or protein-ligand complexes that are essential to perform their biological functions. The tertiary structure of proteins is necessary to understand the binding mode and affinity between interacting molecules. However, it is often difficult and expensive to obtain complex structures by experimental methods, such as X-ray crystallography or NMR. Thus, docking computation is considered an important approach for understanding the protein-protein or protein-ligand interactions. (18)

Bank (PDB) and Worldwide Protein Data Bank (wwPDB) have over 88000 protein structures, many of which play vital roles in critical metabolic pathways that may be regarded as potential therapeutic targets — and specific databases containing structures of binary complexes become available, together with information about their binding affinities, such as in PDBBIND, PLD, AffinDB and BindDB molecular docking procedures improve, getting more importance than ever. (18)

Molecular docking through GRAMM-X is a widely used computer simulation procedure to predict the conformation of a receptor-ligand complex, where the receptor is usually a protein which is SNAP 25 and the ligand is RAB 32. The accurate prediction of the binding modes between the ligand and protein is of fundamental importance in moderan-based drig design, the most important application of docking software is the virtual screening, in which the most interesting and promising molecules are selected from an existing database for the further research.

- PDB file of SNAP-25 and RAB 32 were generated from PROTEIN DATA BANK
- GRMM-X was used for docking of SNAP-25 and RAB 32.
- Docking of these two proteins gave interaction between these two and also showed different interactive site with different bond lengths.
- The interation between SNAP-25 and RAB 32 provide pathway for their further role in pathogenesis of Multiple Sclerosis.

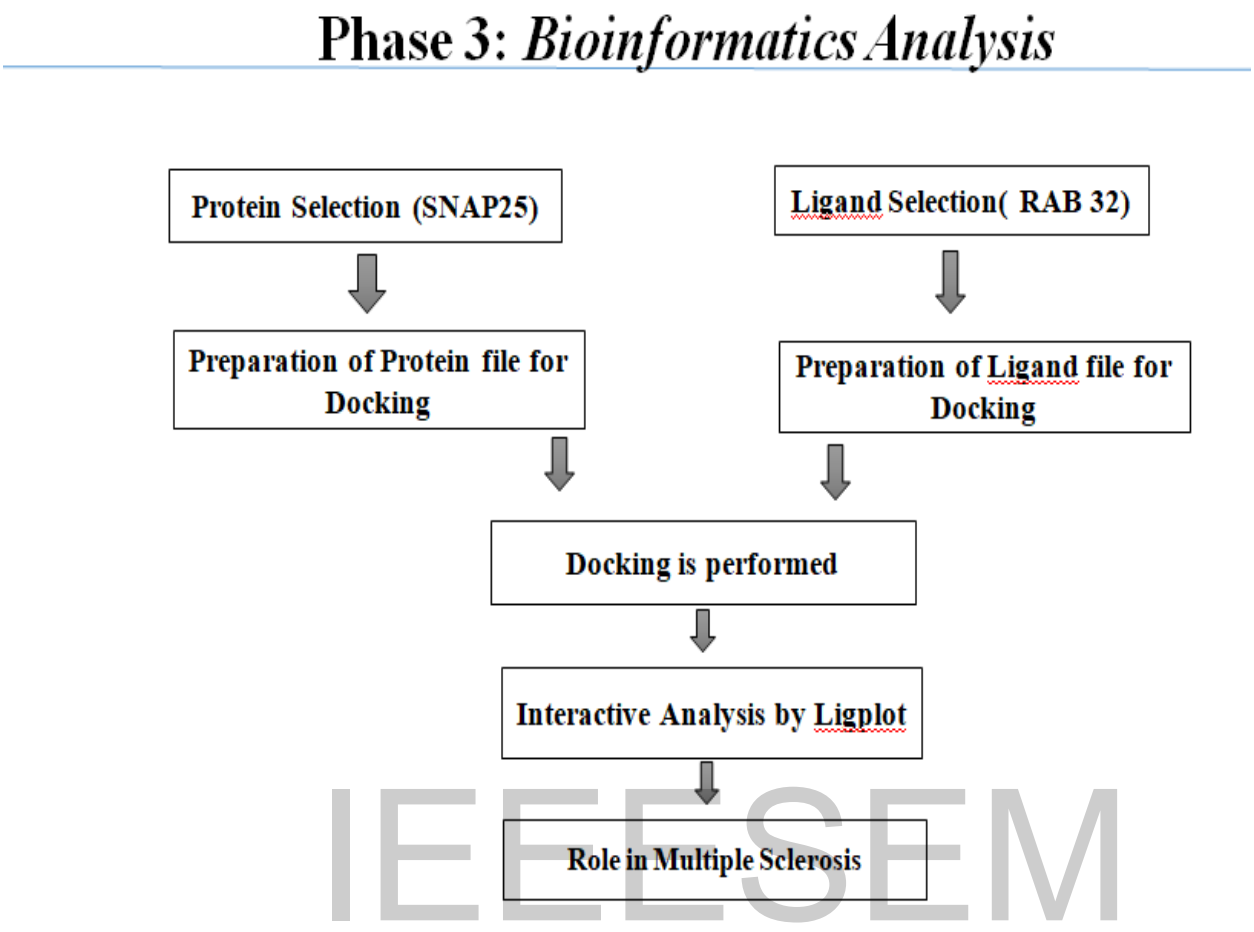


Fig 5: Docking of SNAP 25 and RAB 32

LIGPLOT Analysis:

In Bioinformatics Analysis, LIGPLOT is a computer program that generates schematic 2-D representations of protein-ligand complexes from standard Protein Data Bank file input. The LIGPLOT is used to generate images for the PDBsum resource that summarises. a). Protein-Protein Interaction, b), Protein-Structure Analysis, c). Protein-Pathway Analysis

Uniprot Database:

UniProt/SwissProt database was used for functional analysis of detected proteins from the MS/MS analysis. On the basis of annotations of UniProt/SwissProt database protein candidates from high-density fraction datasets and global proteome datasets were manually separated in modules representing a singular physiological category for proteomics. So SNAP-25 sequence and fictional information was derived from this database.

Data Input to Ligplot:

The file format for Ligplot data entry is PDB formate. The PDB file was generated from the Protein directory of Protein Data Bank and this PDF file for specific protein like SNAP -25 was used in Ligplot for determination of ligand ligand interaction and ligand protein interaction.

Determination of 3-dimentional structure of protein through Ligplot:

The main tool used in determining the structure of protein is LIGPLOT that gives information about protein structure, its interaction with ligands and other molecules and also determine their role in multiple sclerosis. The structure of a ligand in any complex with the target protein that involve in Multiple Sclerosis is considered as high source of information for understanding the ligand targets complementarity. So, the prediction of 3D structure of protein is very important in determining the function the protein whether the protein can interact with other ligands or not and how they interact with the other molecules. Gene structure is basically one-dimensional in which a sequence of nucleotides which is linear coded for a specific linear sequence of amino acids linked to each other in a head to tail rule manner (amino-carboxyl). This process is called translation in which the information present in the nucleotides is converted or transformed into amino acids using the genetic code thus it "expands" the single dimensional genetic code into a fully realized three-dimensional protein structure. 3-D structure of SNAP-25 gives information whether protein is misfolded are not. Accumulation of misfolded proteins causes the symptoms of disease. The original LIGPLOT program focused on specific type of interactions, most commonly between ligand and protein, including interactions with water molecules or with a specific residue. Other types of interactions might be also plotted, such as interaction with the dimerization surfaces and specific interactions with specific domains. The aim of research is to develop 3D coordinates of a protein and to study the ligand complex structure.

3. Results:

Diseases are usually resulting of many physiological variations at biochemical and metabolic strata. Minor differences cause major serious disorders in the broad spectrum of variability. Even changes in a single disease giving rise to its different subtypes. Likewise, differences for the progression rate amongst patients with Multiple Sclerosis, leading to the different types. Experimental autoimmune encephalomyelitis (EAE) is mostly used as an animal model for the inflammatory disease like Multiple to study the pathogenesis of the demyelination. Different types of of immunopathological and neuropathological mechanisms can be studied in EAE which further provides the pathological characteristics MS including inflammation, demyelination, axonal loss and gliosis. A complex neuropharmacology of EAE has discovered the drugs that are in use in MS, tested or validated on the basis of EAE studies. Different types of neuropharmacological. Different immunological and neuropharmacological interventions have been studied in this model so this model is considered as a great source of heterogeneity in the susceptibility to the induction.

Clinical Assessment of EAE model:

MOG-immunized C57BL/6 mice developed the signs of EAE around 15 days after immunization by displaying loss of tail tonus. The maximal clinical symptomatology, that is indicative of the acute phase, occurred at day 19 when the average clinical score reached 2. From this period on the animals slightly improved their mobility but did not completely recover from paralysis. The clinical scores that declined to an average of 1.5 did not significantly change until the 30th day that was chosen as the end point of the experiment. Variation in body weight showed an expected course characterized by a significant weight drop during the acute phase. This loss was followed by a progressive weight recovery. Animals with EAE reached weight values similar to the normal control group at the 30th day following immunization. The average clinical score for chronic EAE model is below 2.5. Above 2.5 clinical scoring is ethically prohibited.

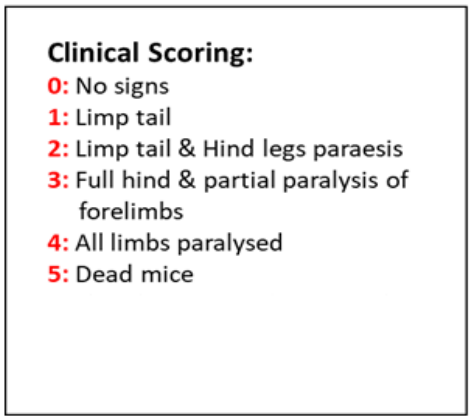


Fig a

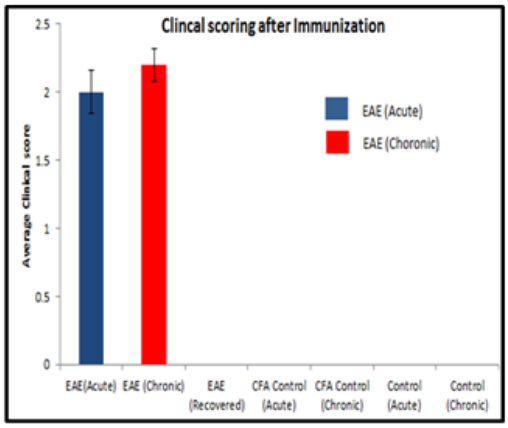


Fig b

Fig 6a: Clinical Scoring of EAE model in which 0 sets for No signs, 1 is set for limp tail, 2 is set for limp tail and hind legs paraesis, 3 is set for full hind and partial paralysis of forelimbs, 4 is set for all limbs paralyzed and 5 is set for dead mice.

Fig 6b shows the average clinical scoring of acute and chronic stage EAE model.

Identified Differential Proteome

This study involves the characterization of different proteins identified in EAE, the characterization of SNAP-25 through bioinformatics tools including Ligplot and Scaffold shows its role in the progression of multiple sclerosis. Different proteins are involved that causes the multiple Sclerosis like SNAP 25, High mobility Protein. In the current study, we aim to define the mechanistic way behind the progression rate through this software.

Table 4.1: Differential Identified Protein

Spot	Protein name	Access. #	Mole. Weight (KDa)	pI	Peptide count	Seq. Cover. (%)	Fold Change	P Value	Regu.
Identified altered proteins during the early stage of EAE									
120	Calcineurin B homologous protein 1	P61022	22.43	4.9	8	45.60	3.92	0.002	↑
161	Synaptosomal-associated protein 25	P60879	23.31	4.6	9	51.90	2.81	0.004	↑
543	cAMP-dependent protein kinase type II-beta	P31324	46.16	4.9	12	44.70	2.13	0.042	↑
552	Secernin-1	Q9CZC8	46.32	4.6	21	50.20	1.93	0.015	↑
552	Ribonuclease inhibitor	Q91VI7	49.81	4.6	15	50.40	1.93	0.015	↑
633	Junction plakoglobin	Q02257	81.80	5.7	12	18.10	3.55	0.008	↑
708	Serpin B6	Q60854	42.59	5.5	16	45.00	3.03	0.001	↑
714	UPF0160 protein MYG1 (fragment of 47-380 a.a.)	Q9JK81	37.45	5.7	11	35.50	-2.61	0.018	↓
736	Ig gamma-1 chain C region, membrane-bound form	P01869	43.38	6.0	8	26.00	1.6	0.013	↑
752	Eukaryotic translation initiation factor 3 subunit I	Q9QZD9	36.46	5.3	17	60.00	4.43	0.002	↑
1065	6-phosphogluconolactonase	Q9CQ60	27.25	5.5	12	57.20	4.10	0.004	↑
1072	Ig gamma-1 chain C region, membrane-bound form		43.38	6.0	6	24.20	3.79	0.0008	↑
1074	High mobility group protein B1	P63158	24.89	5.6	2	12.60	-2.39	0.018	↓
1202	Growth factor receptor-bound protein 2	Q60631	25.23	5.8	9	43.30	2.09	0.018	↑
1225	EF-hand domain-containing protein D2	Q9D8Y0	26.79	5.0	9	38.30	13.54	0.009	↑
1278	Myosin light chain 3	P09542	22.42	5.0	12	65.70	2.74	0.011	↑
1330	Peroxiredoxin-4 (Fragment of 41-274 a.a.)	O08807	26.47	5.8	8	39.40	2.91	0.0006	↑

Table 4.1 shows that 17 differential proteins were identified from EAE model and SNAP 25 is further validated through bioinformatics analysis .Furthermore the data was assessed by Scaffold Mass Spectroscopy to identify the unique peptide sequence of SNAP-25 which represents the level of SNAP-25 in MS.

3.1 Scaffold Results:

Identification of unique sequence through Scaffold:

The given fig shows the unique sequence of amino acid which does not appear anywhere else in the genome. The yellow color sequences are the identified unique sequences while green sequences are the modified sequence which was obtained after post translation.

SNP25_HUMAN (100%), 23,315.4 Da
Synaptosomal-associated protein 25 OS=Homo sapiens GN=SNAP25 PE=1 SV=1
8 exclusive unique peptides, 8 exclusive unique spectra, 9 total spectra, 89/206 amino acids (43% coverage)

MAEDADMRNE LEEMQRRADQ LADESLESTR RMLQLVEESK DAGIRTLVML DEQGEQLERI EEGMDQINKD
MKEAEKNLTD LGKFCGLCVC PCNKLKSSDA YKKAWGNNQD GVVASQPARV VDEREQMAIS GGFIRRVTND
ARENEMDENL EQVSGIIGNL RHMALDMGNE IDTQNRQIDR IMEKADSNKT RIDEANQRAT KMLGSG

Fig 7 shows amino acid sequence of Synaptosomal associated protein 25 with 100% probability with the size 23,315.4 Da. SNAP-25 consist of 8 exclusive unique peptide sequence, 9 total spectra out of which 8 are exclusive unique spectra with 43% coverage of aminoacids means 89/206 amino acids expand. Highlighted yellow portion in the graph shows the identified amino acids which matched with the given MS/MS spectra. Yellow highlight indicate the peptides that were confidently identified by the fdr(false discovery rate) cut off that were specified. Green highlights indicate the post translational modification.

Sequence Coverage	Protein	Accession	Category	Bio Sample	MS/MS Sa...	Prob	%Spec	#Pep	#Uni...	#Spec	%Cov	m.w.
	Synaptosom...	SNP25_HUM...	02	A_Noor_211...		97%	0.0053%	1	1	1	5.3%	23 kDa
	Synaptosom...	SNP25_HUM...	04	A_Noor_211...		100%	0.049%	8	8	9	43%	23 kDa
	Synaptosom...	SNP25_HUM...	07	A_Noor_211...		96%	0.0051%	1	1	1	5.3%	23 kDa

Fig 8: Biological replicates of SNAP 25

Valid	...	Sequence	Prob	Masc...	Masc...	Masc...
☑	1.0	(R)NELEEMQR(R)	99%	24.3	25.0	24.3
☑	1.0	(R)RADQLADESLESTR(R)	98%	21.1	25.0	21.1
☑	1.0	(R)RADQLADESLESTRR(M)	100%	30.5	25.0	30.5
☑	1.0	(R)TLVMLDEQGEQLER(I)	100%	72.1	25.0	72.1
☑	1.0	(R)IEEGMDQINK(D)	100%	34.2	25.0	24.9
☑	1.0	(K)AWGNNQDGVVASQPAR(V)	100%	106.0	25.0	106.0
☑	1.0	(R)EQMAISGGFIR(R)	100%	41.2	25.0	39.5
☑	1.0	(R)EQMAISGGFIR(R)	100%	29.0	25.0	29.0
☑	1.0	(R)HMALDMGNEIDTQNR(Q)	100%	69.5	25.0	69.5

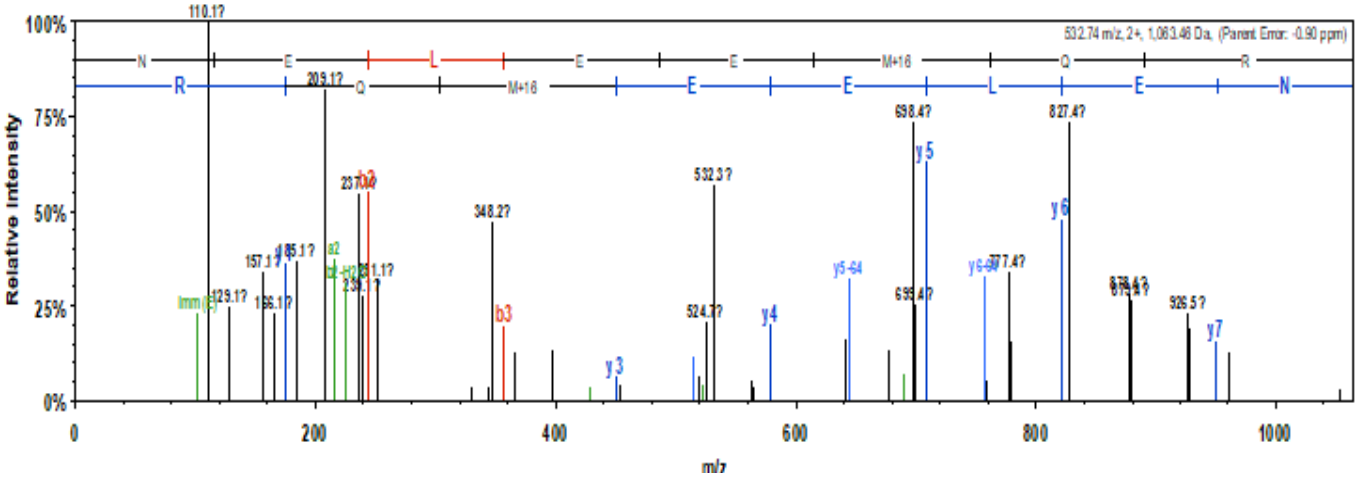
Fig 9: shows total 9 biological replicates with sequences were identified. 7 biological sequences showed 100% of probability.

Valid	...	Sequence	Prob	Masc...	Masc...	Masc...	NTT	Modifications	Observed	Actual Mass	Charge	Delta ...	Delta ...	Rete...	Intensity	TIC	Start	Stop	# Ot...	Other Prot...	Spectrum ID
☑	1.0	(R)NELEEMQR(R)	99%	24.3	25.0	24.3	2	Oxidation (+16)	532.74	1,063.46	2	-0.00096	-0.90			386100	9	16	0		Elution from: 14....
☑	1.0	(R)RADQLADESLESTR(R)	98%	21.1	25.0	21.1	2		530.93	1,589.77	3	0.0029	1.8			818500	17	30	0		Elution from: 20....
☑	1.0	(R)RADQLADESLESTRR(M)	100%	30.5	25.0	30.5	2		582.96	1,745.87	3	0.0022	1.2			964200	17	31	0		Elution from: 19....
☑	1.0	(R)TLYMLDEQGEQLER(I)	100%	72.1	25.0	72.1	2	Oxidation (+16)	838.91	1,675.81	2	-0.00084	-0.50			775600	46	59	0		Elution from: 27....
☑	1.0	(R)EEGMDQINK(D)	100%	34.2	25.0	24.9	2	Oxidation (+16)	596.78	1,191.54	2	-0.00052	-0.44			286400	60	69	0		Elution from: 15....
☑	1.0	(K)AWGNNQDGMVASQPAR(Y)	100%	106.0	25.0	106.0	2		835.41	1,668.80	2	0.00071	0.43			804300	104	119	0		Elution from: 21....
☑	1.0	(R)EQMAISGGFIR(R)	100%	41.2	25.0	39.5	2	Oxidation (+16)	612.81	1,223.60	2	-0.00073	-0.59			969000	125	135	0		Elution from: 25....
☑	1.0	(R)EQMAISGGFIR(R)	100%	29.0	25.0	29.0	2	Oxidation (+16)	612.81	1,223.60	2	-0.00073	-0.59			1169000	125	135	0		Elution from: 25....
☑	1.0	(R)HMALDMGNEIDTQNR(Q)	100%	69.5	25.0	69.5	2	Oxidation (+16),...	592.93	1,775.76	3	-0.00066	-0.37			582500	162	176	0		Elution from: 17....

Fig 10 shows the result of biological replicates of SNAP 25. According to Scaffold about 98% of probability of sequence is required for its accuracy. The Mascot score for a protein is the summed score for the individual peptides, e.g. peptide masses and peptide fragment ion masses, for all peptides matching a given protein. For positive protein identification, the mascot score has to be above the 95% confidence levels.

Determination of expression of protein though Mass spectrometry:

The graph below shows the mass spectrum of SNAP-25 protein with different peaks and top-bottom MS/MS spectra determine the expression of Synaptosomal associated 25 protein.

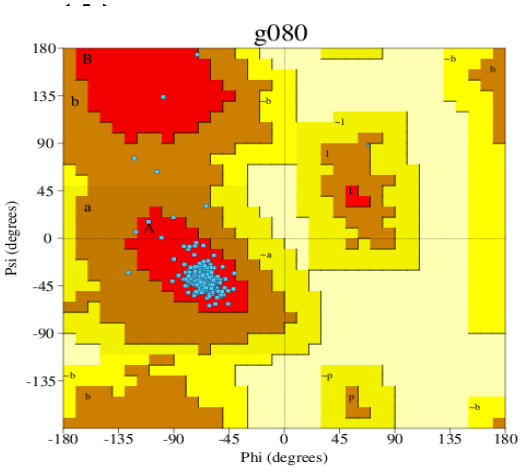


B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	115.1		98.0		N	1,064.5	532.7	1,047.4	1,046.5	8
2	244.1		227.1	226.1	E	950.4	475.7	933.4	932.4	7
3	357.2		340.2	339.2	L	821.4	411.2	804.4	803.4	6
4	486.2		469.2	468.2	E	708.3		691.3	690.3	5
5	615.3		598.2	597.3	E	579.3		562.2	561.2	4
6	762.3	381.7	745.3	744.3	M+16	450.2		433.2		3
7	890.4	445.7	873.3	872.3	Q	303.2		286.2		2
8	1,064.5	532.7	1,047.4	1,046.5	R	175.1		158.1		1

Fig 11: B ions and y ions of Synaptosomal associated 25 protein, the B ions are the charges that retained on the N-terminus and Y ions are those products when the charges are retained on the C-terminus These spectra are graphical representation which explains the difference between calculated masses as well as the masses obtained through mass spectrometry analysis. During spectrum and peptide similarity accurate matching outcomes are gained when peaks combine well with mass accuracy of employed mass spectrometer. This graphical representation of MS/MS spectra, in which red-b ions represents fragment ions whereas blue ion indicates y ion. While green represents water, grey ones are not discovered. B and y ions assigned to this sequence,. Longer peptide to have tendency to have high sequence score in sequent.Potential ions which matched the spectra are shown in red and blue lines are mention in tabular form in fragmentation table. Like wisely, the y-ion values determined by the spectrum from y2 to y8. While green represents immonium ion or neutral loss of NH_3 or water, grey ones are not discovered.That each of peaks that's labeled as a wire b corresponds to that matched the theoretical fragmentation for this peptide, we are looking for the coverage of y & b ions. We got coverage of y8 y7 but missing y6 then y5 to y1 they have pretty low abundance We have corresponding b ions that gives us a little confidence in this identification.We are looking for good signal to noise means that signal from our peak is significantly above the noise level. How many peaks that we detected are labeled or matched to the theoretical fragmentation in this case of all of them did because they are colored.

3.2- LIGPLOT Results:

Structure of protein is important to determine the function and role of protein in specific disease. LIGPLOT determine the Secondary structure of SNAP-25 protein which consists of 5 different chains of different amino acids and 66 residues



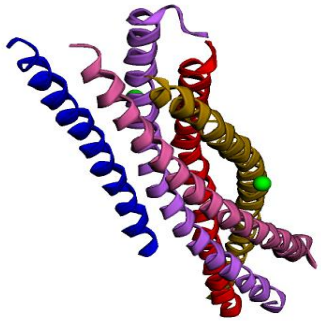


Fig. 12: Ramachandran plot calculations for SNAP-25 protein computed with the PROCHECK program. Fig 13: 3-dimensional structure of SNAP 25.

RAB 32 interaction with Culprit Synaptosomal-associated protein 25:

Protein and ligands interactions are important for all processes occurred in living organisms. Ligand binding capacity regulates the biological functions.

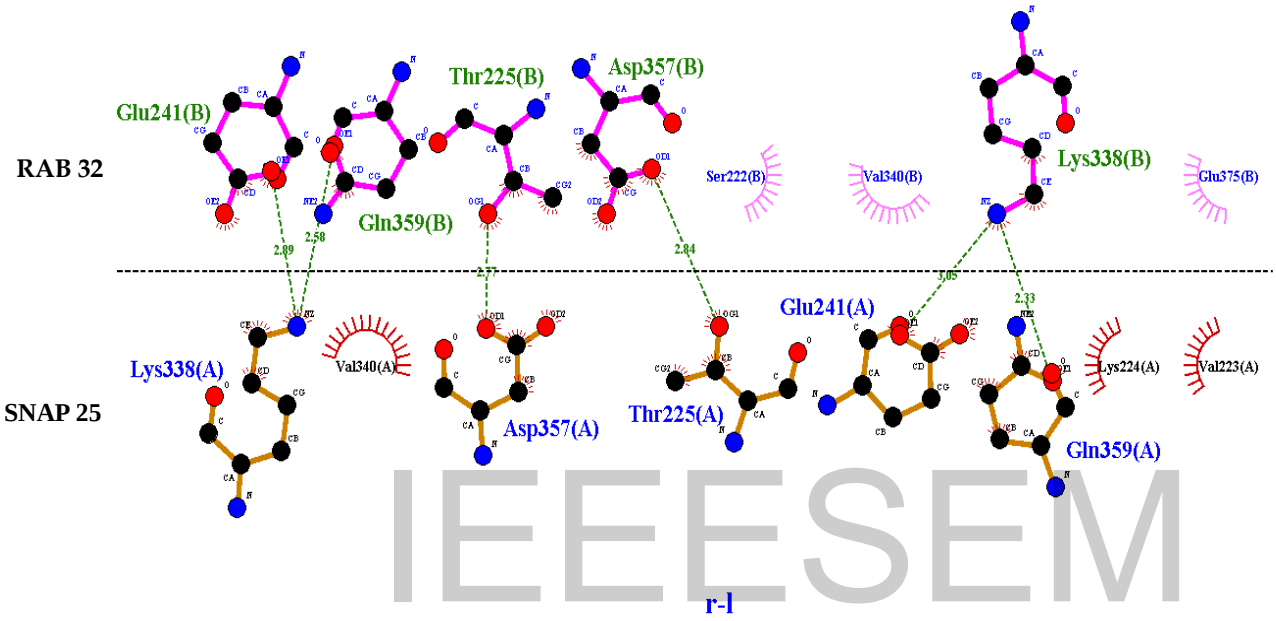


Fig 14: Interaction of SNAP 25 and RAB 32.

It shows first hydrogen bond was formed between Lys338(A) [Lysine 338 amino acid residue of chain A of SNAP 25 protein] with Glu241(B) [glutamic acid 241 amino acid residue of chain B of RAB 32 ligand] with the length of 2.89and Gln359(B)Glutamine 359 amino acid residue of china B of RAB 32 with bond length of 2.54. While second hydrogen bond of length 2.93 was formed between Asp(A) [Aspartic Acid 357 amino acid residue of chain A of SNAP 25 protein] and Thr 225(B) [Theronine 225 amino acid residue of chain B of RAB 32 ligand]. Total five interactive site of SNAP 25 shows interaction with different bond length with RAB 32.Three non-ligand amino acid residues involved in hydrophobic interactions were represented by red circular lines. The covalent/elastic bonds formed were represented as thin purple lines. DIMPLOT was applied on LIGPLOT results to study chain A and B interactions across protein-protein interfaces. The horizontal dashed black line represents the interface.

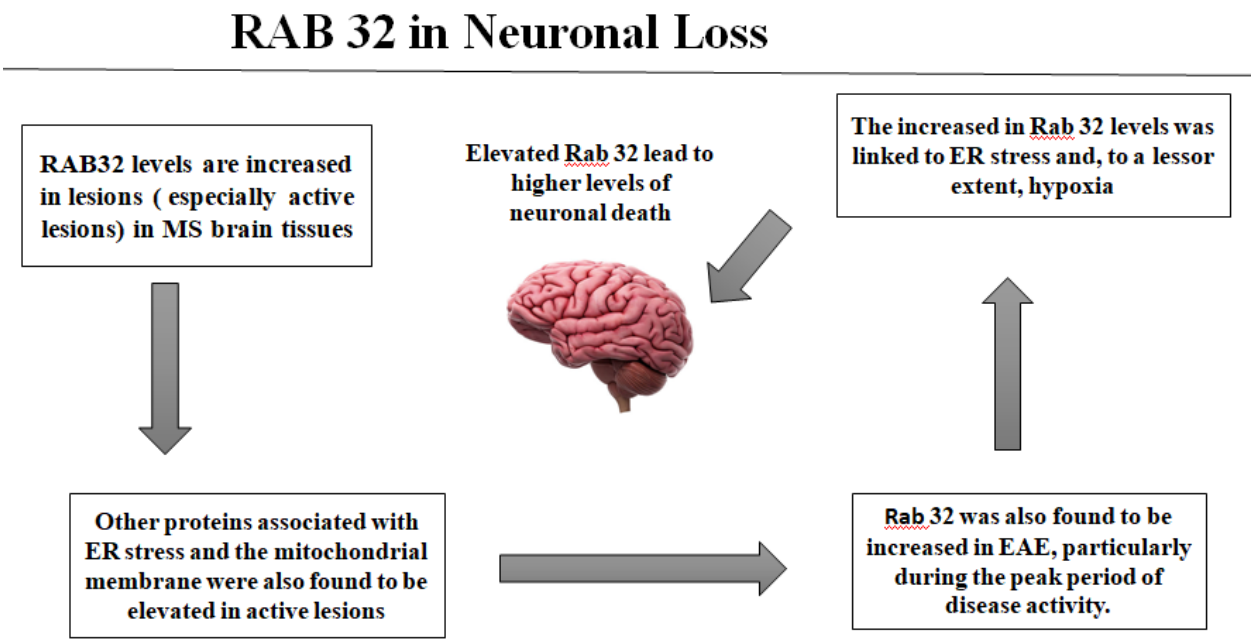


Fig 15: Diagrammatic illustration of RAB 32 role in Multiple Sclerosis.

4. DISCUSSION

Multiple sclerosis is an autoimmune and progressive demyelinating neurodegenerative disease of the central nervous system (CNS) that causes the neurological defects.MS presents in adult life and it showed a major impact on the life activites. [9]. It is considered as inflammatory disease of the central nervous system (CNS) in which there is destruction of myelin sheath results in demylenation. [10]. It was found through studies that two million people worldwide and some 100 000 people in the United Kingdom were affected with this disease. [11].

EAE is the best model for studying MS mechanisms to test or develop drugs from pathogenesis point of view. The model was producing by active immunization of protein that causes the demylenation and inflammation. The study focused on many aspects of Multiple Sclerosis in EAE model and protein study through different tools. The purpose of study is to develop animal model of MS that can easily identify the pathological mechanisms and also targeted mechanisms for therapeutic intervention. [12].

The EAE pathophysiology explains the reaction of immune cells against the specific antigens. [13]. These reactions cause the inflammation and destruction of structures carrying the antigens resulted in neurological and pathological features. Different approaches can be used for the induction of EAE model are: Actively-induced EAE (aEAE; active immunization), passively transferred EAE (pEAE; transfer of encephalitogenic cells from an immunized animal), and more recently spontaneous EAE mouse models (sEAE) which allowed the study of autoimmune mechanisms without exogenous manipulation. The induction of aEAE is easiest in mice induction that we got fast and quick results. Many researchers named it as "gold standard" of neuroimmunological animal models. [14].

Subcutaneously the animal was immunized with an emulsion of $MOG^{35–55}$ through active immunization along with complete Freund's adjuvant (CFA). On the day of immunization intraperitoneal injections of pertussis toxin were given to the mice, this pertussis toxin basically facilitates the induction of EAE model. [15]. After immunization the T-lymphocytes that are myelin specific were activated in the peripheral region and then they start migrated towards CNS across the blood brain barrirer. [16] When the T-cells entered into the CNS, these cells were then reactivated by the infiltrating antigen-presenting cells that causes the inflammatory cascades, some other cells like monocytes and macrophages are also involve in the demyelination and death of axon. [17]. The mice C57BL/6 strain was used for this immunization process and the antigen Myelin Oligodendrocytes glycoprotein (MOG) were used. After immunization the mice showed the acute and chronic progressive disease stage. The most commonly used mice strain is C57BL/6 because of presence of variety of transgenic mice and also multitude knockouts. After using the above-mentioned protocols for immunization of C57BL/6 mice with MOG_{35-55} peptide[10] the monophasic disorders appear. First symptoms in EAE occur after the recovery over the next 10-20 days. For immunization MOG_{35-55} peptide was not enough to induce this disease so the adjuvants such as CFA were used to enhance the immunogenic potential of of MOG_{35-55} peptide.

The CFA components activated the mononuclear phagocytes thus phagocytosis of these molecules was induced and also results in the secretion of cytokines. This resulted in the prolongation of the presence of antigens and also caused the efficient transfer to lymphatic system.
After immunization of mice the tissue lysate were extracted from the CSF of spinal cord and proteins were extracted from the tissue lysate for further bioinformatic analysis that defines its pathogenic role in the demyelination.
Synaptosomal-associated protein 25 (SNAP-25) is a 25kD protein with 206 amino acids. Its gene location is on chromosome 20. The presynaptic terminal of neurons is composed of a t-SNARE or target SNARE molecule. Formation of neural soluble Nethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes occurs because of SNAP -25 and has a great importance. Calcium-dependent exocytosis of synaptic vesicles, proper efficient release of neurotransmitters and propagation of action potential is done by SNARE complex. [18]. It is also important for learning, movement, memories formation and normal brain functioning. The normal levels of SNAP-25 are mandatory for neurotransmission; the changes in its expression can cause many disorders including autoimmune disorder like Multiple Sclerosis [19].
Different interactions between proteins and their cognate ligands are important for understanding of biological systems. Ligplot determines the interaction of different ligands with Synaptosomal-associated protein 25 kDa (SNAP-25). These ligands interaction is important to determine the functional and mechanistic way towards the cause of multiple sclerosis. The Structure of protein is important to describe the function and role of protein in specific disease. Ligplot determine the Secondary structure of SNAP-25 protein which consists of 5 different chains of different amino acids and 66 residues. The aim of study is finding out the changes.
This research is based on graphical interpretation of identified proteins through LIGPLOT that automatically generate numerous 2D diagrams of ligand and protein interactions from 3D coordinates. The hydrogen bonds interactions and different contacts of protein with different ligands are portrayed through Ligplot. The main-chain and side-chains determination through ligplot facilitated the series of analyzing the function of protein in pathogenic pathway. The ultimate goal of the research is to develop the EAE model for the identification of proteins like specific SNAP 25 proteins involved in Multiple Sclerosis and then develop the interventions that can improve the lives of those living with MS. Synaptosomal-associated protein 25 (SNAP-25) is a 25kD protein with 206 amino acids. **SNAP-25** not only plays a **role** in synaptogenesis and the exocytotic release of neurotransmitters. The over-expression of SNAP25 may destroy the synapse and thus stops the release of neurotransmitters.

MS is a complex multifactorial disease. Though this disease is an autoimmune disorder but many other factors are involved in the pathophysiology that causes the demyelination of neurons or neuronal death. The exact causes of this disease are still unknown. EAE model of multiple sclerosis has a vital role in the development and then validation of treatments for MS and it also helps in understanding the pathogenesis of MS. Though the pathogenesis of EAE model understanding is still unclear but results of this research helped in determination of multiple causes which results in demyelination of neuron. Studies also identifies 23 different protein in EAE model out of which SNAP 25 is further analyzed through bioinformatics tools which will give pathways towards therapeutic success. Exact mechanism behind the cause of MS is still unclear, but some studies suggest that there are some proteins which are involved in Multiple Sclerosis. Our proteomics approach endeavor a comprehensive list of known and novel interacting proteins involved in MS. This study also highlights the role of SNAP 25 protein in the progression of MS for further diagnostic strategies. EAE model of Multiple sclerosis will further investigated to detect symptoms at more early stage in the CSF of MS patients Silencing of interacting protein (SNAP 25 and RAB 32) can induced considerable changes in patients having Multiple Sclerosis. These observations could also help to explain the unknown physiological role of SNAP 25 in Multiple Sclerosis and deserve close attention in scope of neurodegenerative diseases

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