Proteome Profile of Zebrafish Brain Based on Gel LC-ESI MS/MS Analysis

Sachin Kumar Singh[1], Komarla Setty Rakesh[2], Kalidoss Ramamoorthy[3], Attluri V Pardha Saradhi[4], Mohammed Mohammed Idris[5]*

[1]*Research Fellow, Department of Neurobiology, W309, CCMB, Uppal Road, Hyderabad 500007*
[2]*Senior Research fellow, Proteomics Department, CCMB, Uppal Road, Hyderabad 500007*
[3]*Research Fellow, Proteomics Department, CCMB, Uppal Road, Hyderabad 500007*
[4]*Research fellow Department of Neurobiology, W309, CCMB, Uppal Road, Hyderabad 500007*
[5]*Scientist, Department of Neurobiology, W309, CCMB, Uppal Road, Hydrabad 500007*

Abstract

The zebrafish (*Danio rerio*) is the extensively used alternate vertebrate model animal for understanding the brain function, development and evolution. Detailed brain proteome map of zebrafish is still not known inspite of its broad usage in developmental and neurological studies. We present here the large scale proteome profile of the zebrafish brain at the normal condition based on gel LC ESI MS/MS analysis. A total of 8475 different proteins details were identified based on this study with less than 1% false positive rate. All the proteins details obtained from this study were duly submitted to the database for validation and obtained accession numbers. The various proteins identified in this study were found to be involved in different biological activities, neurological functions and network pathways. With the availability of genomics information, this extensive study of proteomic profile of zebrafish brain tissue provided a complete view and details about the various proteins expressed in brain at the basal state. This study can lead to understand various new biomarkers underlying for various biological characteristics like development and neurological disease.

Abbreviations: LC: Liquid Chromatography; MS: Mass Spectrophotometer; LCMS: Liquid Chromatography Mass Spectrophotometer; ESI: Electron Spray Ionization; ACN: Acetonitrile; kDa: Kilo Dalton; SDS-PAGE: Sodium Dodecyl Poly acryl Amide Gel Electrophoresis; 1D: Single Dimension; kV: Kilo Volt; μgms: Micrograms; DTT: Dithiothreitol; CHAPS: [(3-Cholamidopropyl)dimethylammonio] - 1 propanesulfonate; ID's: Identities: NCBI: National Centre for Biotechnology Information

Background

Zebrafish (*Danio rerio*) has been recently accepted as the potential model organism towards understanding the complexity of evolution, development and function (Detrich et al., 1999; Zon, 1999). It is the most convenient vertebrate model animal for its ease of availability, short generation time, well developed human like brain and compact genome (Driever et al., 1994; Lieschke and Currier, 2007). Zebrafish have been used for understanding various neurological disorders like Alzheimer's, Parkinson's and Huntington disease (Guo, 2009; Leimer et al., 1999; Son et al., 2003; Karlovich et al., 1998) for its human like neurological system with compound brain and spinal cord. However lack of information about the brain organization, transcriptome and proteome of brain limits its use as an alternate model animal to human for understanding the normal neurological functions.

Genomic information of model animals such as mouse, rat, zebrafish, drosophila and nematode offered excellent genetic base for understanding the complexity of development and behaviour. *Danio rerio* genome sequence information (Sanger institute) and the annotation of protein-coding genes based on alignment of homologous transcript (Jekosch, 2004) have substantially facilitated zebrafish genetics inspite of the imprecise computational gene prediction. More than 21,000 zebrafish genomes were annotated (Ensemble Assembly Zv7, April 2007) based on species specific transcript data (17,000 genes), evidence and comparison of closely related species

(2500 genes) and based on computational prediction (1500 genes) (Flicek et al., 2008). Comprehending the translated product of the gene based on tandem mass spectrophotometry has always proved as a valuable alternate towards genomic annotation, as it predict the proteins profile directly resolving the gene product. Also based on proteome profile the splice forms or overlapping structure will be solved which were not possible based on cDNA annotations (De Souza et al., 2009; Jaffe et al., 2004a; Jaffe et al., 2004b; Kalume et al., 2005; Lin et al., 2009; Lucitt et al., 2009; Nasevicius et al., 2000; Savidor et al., 2006; Wang et al., 2007).

Proteome profile of brain tissue based on various proteomics approach was mostly understood among human (Fountoulakis, 2004), mouse (Gauss et al., 1999; Wang et al., 2006) and rat (Poirrier et al., 2008; Maurya et al., 2009). In the zebrafish model system, the proteome profile study has been established for understanding the proteome map of zebrafish embryo development (Link et al., 2006; Tay et al., 2006; Lin et al., 2009), cytosolic component of zebrafish liver (Wang et al., 2007), proteome profile of zebrafish gill ((De Souza et al., 2009) and brain protein level changes in zebrafish brain due to chronic ethanol administration (Damodaran et al., 2006). Understanding the zebrafish brain proteome map based on single dimension electrophoresis followed by Liquid chromatography Mass spectrophotometer (LCMS) may lead to map the all proteins expressed in the brain which are been extensively used for human disease model towards understanding the pathogenesis,

prognosis and therapeutic model for wide range of neurological disorders.

Method and Material

Animals, sample preparation and SDS PAGE

Wild zebrafish were collected from the local farmers and maintained in the standard conditions. A total of 25 adult male and female zebrafish of 6 months old were selected and anesthetized using 0.1% Tricane (Sigma, US). The total brain structure were dissected carefully, washed twice in Locke solution (0.94% NaCl, 0.0045%KCl, 004% Cacl2 (w/v) in milli-Q), pooled and homogenized in liquid nitrogen for the extraction of total brain protein using dissolving buffer (7M urea, 2 M thiourea, 4% CHAPS, 18mM Tris-HCl, 14 mM Trizmabase, 2 Tablets EDTA protease inhibitor, Triton X 0.2%, 50mM DTT). The extracted proteins were estimated using Bradford method (BioRad) and 100 μgms of total protein was electrophoresed in 7cm, 12% 1D SDSPAGE as duplicates. The total 100 μgms protein in 2X Laemmli buffer (20% Glycerol, 4% SDS, 10% 2-mercaptoethanol0.004% bromphenol blue and 0.125M Tris HCl) was electrophoresed in three lanes equally and separately. The gels were stained for overnight with CBB R250 (BioRad), destained and documented.

Enzymatic in gel digestion

After the electrophoresis each sample lane was cut into 12 sequential groups separately from the gel. The gels slices were further cut in to pieces of 1.5 mm size and washed with 100mM ammonia bicarbonate in 50% ACN for one hour and twice with water for 30 minutes followed by dehydration using 40% and 100% ACN and dried in speed vacuum. Hundred micrograms of sequencing grade α-trypsin (Promega) was solubilised in 40 mM ammonium bicarbonate, 10% ACN to a concentration of 10 ng/μl. 60 μl of trypsin solution was added to each gel pieces and incubated at 37°C for 16 hours. Following digestion, the tryptic peptides were extracted (100 μl) with 5% TFA in 50% ACN solution at room temperature for one hour. All the three sample supernatant representing respective fractions were pooled, dried by SpeedVac and reconstituted in 40 μl of 5% acetonitrile and 0.1% formic acid.

Mass spectrophotometer analysis

The extracted tryptic digested peptides were subjected to LCMS analysis using ESI-mass spectrometer with linear ion trap mass analyzer (LTQ-IT; Thermo Fischer, Waltham, MA, USA), equipped with Finnigan Surveyor MS Pump Plus. The experiment, analytical workflow and bioinformatic analysis is outlined in Figure 1. The samples are subjected to online LC-MS/MS using reverse-phase Micro LC column Bio Basic C18, (Thermo Fischer, Waltham, MA, USA). Two separate runs were performed for each tryptic digested peptides obtained from two different gel runs at a flow rate of 3 μl/min using a gradient of 0.1% formic acid in double distilled water (solvent A) and 0.1% formic acid in 95% ACN (solvent B), for 120 minutes. Chromatographically separated peptides were sprayed through a 15 cm metal needle emitter and the MS/MS spectra were acquired in data-dependent mode. The electrospray voltage was set at 4.0 kV, and capillary temperature at 200°C. The peptides were fragmented using CID with normalized collision energy of 35%. One full MS scan from 200 to 2000 m/z was acquired followed by top 7 peptide precursor ions selected for MS/MS analysis for 120 minutes LC run. Thus for each gel fraction four raw files of MS and MS/MS data were generated. Additional raw files were generated from wash cycles. All the raw files obtained from the analysis were submitted to PRIDE database for validation and obtaining PRIDE accession number (Jones et al., 2006).

Data analysis for protein identification

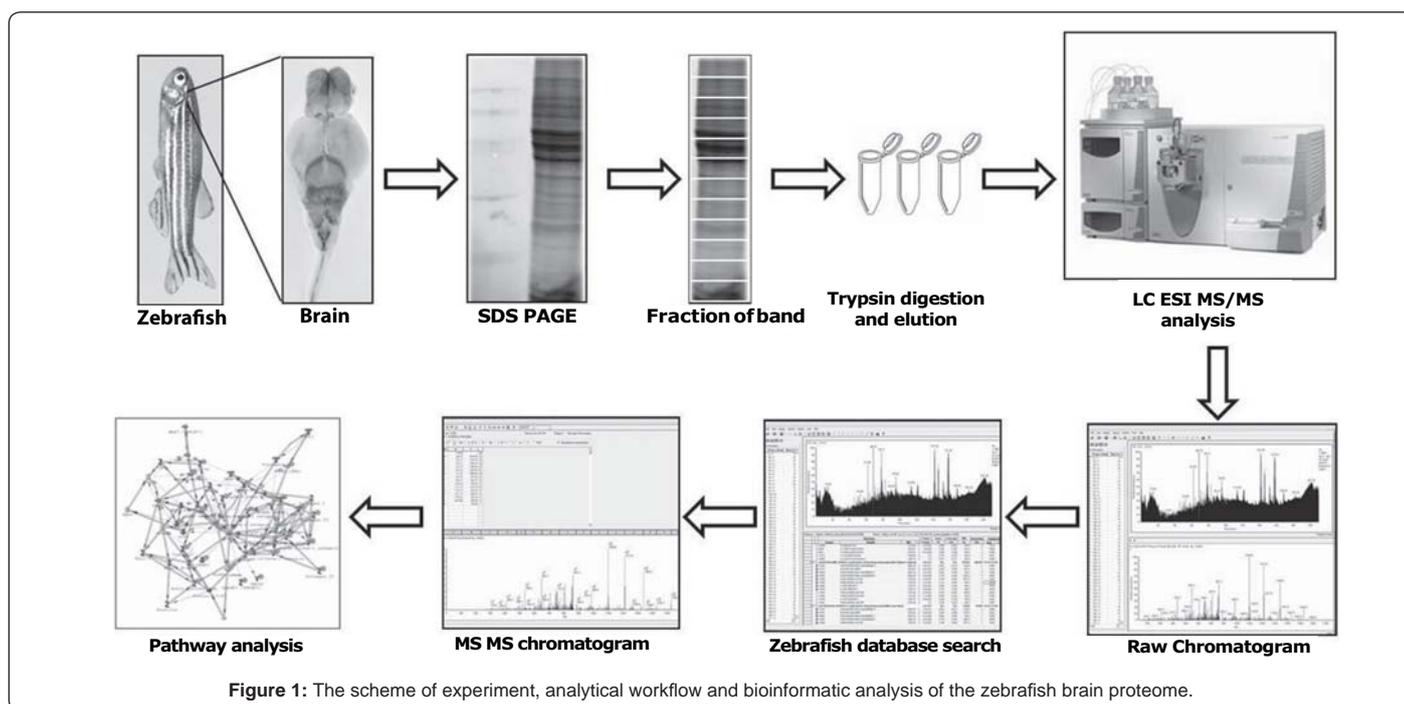The RAW files were analyzed for protein IDs using Bioworks

Figure 1: The scheme of experiment, analytical workflow and bioinformatic analysis of the zebrafish brain proteome.

version 3.2 EF2 (Thermo Fischer, Waltham, MA, USA) with an integrated SEQUEST search engine (search algorithm) and NCBI protein sequence database. Further peptide and protein redundancies were manually removed using Microsoft Excel. The obtained peptides were screened by filtering the individual spectrum of each peptide based on single scoring filter (Xcorr) (Guanghui et al., 2009) and retaining the best representation if spectral redundancy exists in the same peptide repeatedly. The protein identities were identified either by sufficient number of distinct peptides or identified by one peptide but redundantly enough to be considered reliable. Xcorr values were set to 1.9, 2.2 and 3.1 for 1+, 2+ and 3+ charged ions respectively; methionine oxidation and cysteine carbamidomethylation were set to 15.99 and 57.05, respectively. Prior to the search, the NCBI non-redundant database was modified so that the description lines from ENTREZ gene annotation were incorporated into NCBI zebrafish protein database. This helps unification of all GI numbers, incorporate ENTREZ gene names and compile multiple annotations for each protein and can be visualized under one heading. Any redundancy within the list due to multiple protein sequence accession numbers matching to same Entrez gene ID was removed by further processing. Proteins, if identified by same set of peptides, were grouped together and do not artificially inflate the number of proteins in dataset. Protein isoforms are listed in one group unless identified by one or more distinct peptides. Thus our analysis yields high quality filtered IDs from the MS datasets.

To assess the False Positive Rate (FPR) in the peptide/protein IDs, the entire dataset was searched, using same parameters, against a decoy, reverse sequence database of zebrafish proteins. The peptide sequences identified in both orientations were compared and FPR was estimated using the formula FPR= FP/ (TN+FP), wherein FP=False Positives (Peptides occurring from searches in both orientation searches assigned to proteins), TN=Peptides identified only in reverse database search. Using this criterion FPR in our analysis was found to be insignificant (< 1%).

Dataset analysis: The protein details obtained from the zebrafish brain proteome were analyzed for the function, process, location, disease and network pathway maps by GeneGo software's (www.genego.com). The identified proteins details were also validated using Data based for Annotation, Visualization and Integrated Discovery (DAVID) functional annotation tool (Dennis et al., 2003). The various significant disease maps, pathway maps and network based on GeneGo analysis were mapped and analyzed.

Results and Discussion

Based on our proteome map of zebrafish brain using high throughput LC-ESI MS/MS analysis we identified 8475 proteins expressed in the zebrafish brain unambiguously. The consensus proteins list was profiled from the 44 raw data files selected for the analysis having more than 30% peptide matches among duplicates. The identified proteome profile represented a wide range of pI and mass ranging from 3.5 to 12.4 and 4 KDa to 988 KDa respectively. The largest protein identified is the Spectrin repeat containing, nuclear envelope 1 protein (8621 aa's) and the smallest protein identified in the study is the thymosin beta (43 aa's). A total of 240,840 amino

acid characters for 15,522 peptides were obtained as dataset for the 8475 proteins identified in the study which includes 35% (2985) of the proteins with more than one peptide details (Supplementary Table 1). The maximum peptide identified is 60 for the spectrin α 2 protein and si:ch211-250g4.3 protein. All the protein identities with one peptide details (Supplementary Table 2) were also significant as per Xcorr value, which is set for higher significance and accuracy (Guanghui et al., 2009). Close to 96 % of the identified proteins were found to be classed between pI 4 to 10 with 47% being between pI 5 to 7 (Figure 2). 4697 peptides were found to be modified for methionine oxidation. 44 different accession numbers from 12033 to 12076 were obtained for all the files submitted to the PRIDE database for validation.
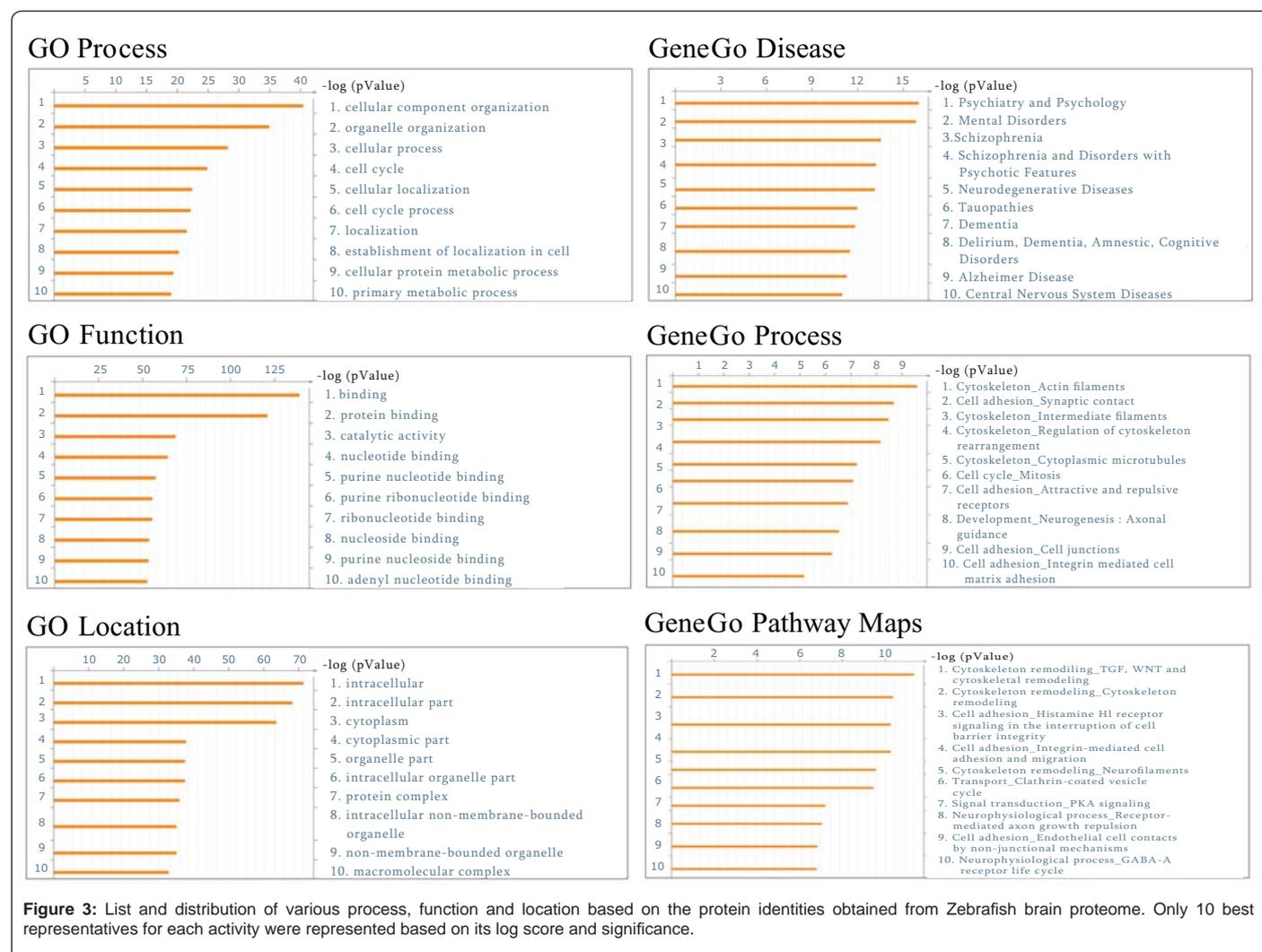
Dataset classification

The annotated protein identities of zebrafish brain based on NCBI and DAVID functional annotation tool were classified for process, function and location using GeneGo software (www.genego.com). 4479 proteins details were selected by the software as active objects for the analyses and are classified in to different categories like 739 enzymes, 200 kinases, 197 receptors, 139 proteases, 85 ligands, 62 phosphotases, 188 transcription factors and 2869 other. It is found from the analysis that the identified proteins of zebrafish brain were found to be involved in various processes such as cellular component organization, organelle organization, cellular process, cell cycle, localization and metabolic process (Figure 3i and Supplementary Table 3). Protein binding, catalytic activity and nucleotide binding, hydrolase activity, ATPase activity etc were the important functions of the identified protein (Figure 3ii and Supplementary Table 4) which were found localized in intracellular, cytoplasmic, organelle, nuclear, nucleolus, cytosol, microtubule, mitochondria, neuron projection, synapse and axon (Figure 3iii and Supplementary Table 5).

Dataset analysis

The proteins were analyzed for its involvement in various functional pathways and localization based on pathway
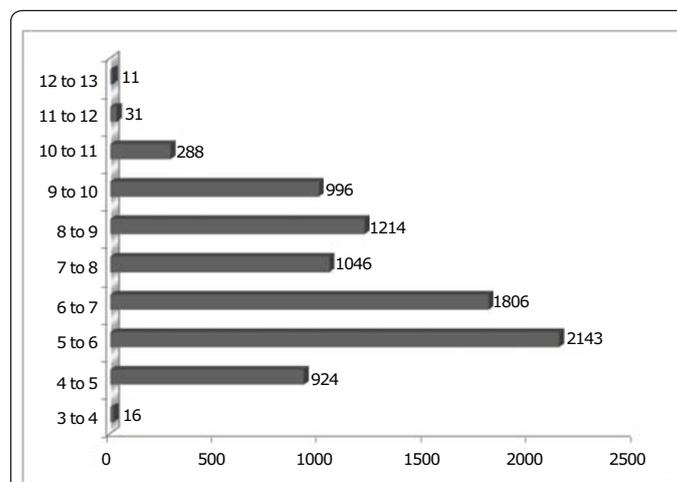
Figure 2: Histogramic distribution of all the identified proteins based on pI of the proteins. X axis represents the number of proteins in each pI range and Y-axis represents the pI range.

Figure 3: List and distribution of various process, function and location based on the protein identities obtained from Zebrafish brain proteome. Only 10 best representatives for each activity were represented based on its log score and significance.

analysis by GeneGo software (www.genego.com). The various diseases which were found to be mapped with the participation of the identified brain proteins are Psychiatry, mental disorders, Schizophrenia, Neurodegenerative disorders, tauopathies, dementia, Alzheimer and CNS disease (Figure 3iv and Supplementary Table 6). Cytoskeleton remodeling, neurofilament, cell adhesion based on Histamine H1 receptor, transport of clathrin coated vesicle cycle, signal transduction of PKA signaling, receptor mediated axon growth repulsion, neurophysiological process mediated GABA-A receptor were the different highly significant pathway map associated with the proteins (Figure 3v and Supplementary Table 7). The different process network associated with the proteins are Cytoskeleton through actin filaments, cell adhesion for synaptic contact cytoskeleton for intermediate filaments, regulation of cytoskeleton arrangements, cell cycle based on mitosis (Figure 3vi and Supplementary Table 8).

Development of neurogenesis based on axonal guidance is the most important process network identified from the dataset involving 97 of the identified proteins. The formation of the neuronal network includes many steps: neuronal migration to proper regions, neurite outgrowth, formation of polarity, guidance of axons and dendrites to proper targets, dendritic maturation and synapse formation with appropriate partners. Among them, axon guidance is one of the critical steps for the proper formation of a neural network. Axons are guided by a variety of guidance factors, such as semaphorins, ephrins, netrins. These factors and its receptors are located at periphery of network. The center of the network is occupied by kinases (such as Rho GTPases) that take part in the signal transfer from semaphorin, ephrin and other receptors to the cytosceletal and motor proteins (actin, tubulin, myosin etc) (Figure 4)

Function of proteins

Cytoskeleton remodelling, cell adhesion, transport, signal transduction, protein signalling and regulation were the important network and pathway functions associated with the 2985 proteins selected for functional analysis. The key network objects which are found participated in the various network pathways are VEGFR2, Fibronectin, GIT1, alpha2/beta1, MEKK1(MAP3K1), NCOA3, p53, SHP, LRH1, ERK2 (MAPK1), Caspase 3, 8, 6, 2, XIAP, SMAD3, Androgen receptor, E cadherin, MMP 2, TGF beta, STAT3, c Myc, STAT1, HGF receptor, Shc, c Myc, SMAD3, p53, p300, VDR, ESR2, AHR, EGFR, STAT5B, NCOA1, STAT3, ERK2, ADAM17, NCOA1, EGFR, p53, PAM, CELSR2, DA alphaMSH, c Myc, ERK5 (MAPK7), FBP3,
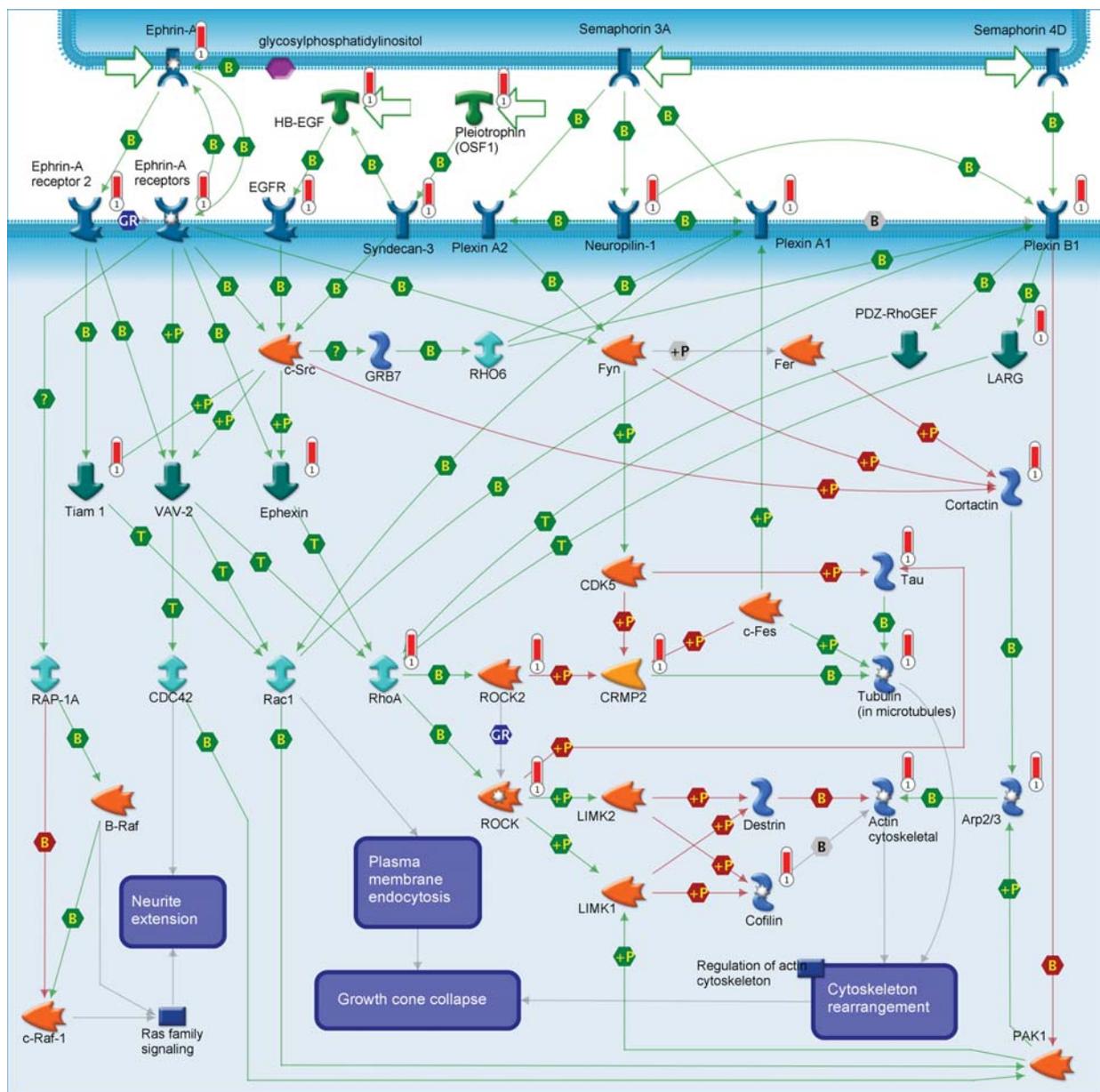
Figure 4: The process network association for the development of neurogenesis based on axonal guidance. 97 proteins were represented from the study out of 230 protein participants in the network (p value 3.235e-7).

Thrombospondin 1 and ARPC5L. These proteins were found to be involved in various pathways with high significance. The eight major pathways which are associated with identified datasets are 1. Localization of cell (cell motion), 2. cAMP biosynthetic process, 3. Induction of programmed cell death and apoptosis, 4. developmental process, 5. Protein kinase cascade, 6. Regulation of developmental process, 7. Positive regulation of cellular metabolic process, 8. Positive regulation of cellular process (Figure 5i-viii; Supplementary Table 9).

Proteome map and neurological diseases

From the proteome map and the analysis it is found that all the proteins identified in the study were justly brain derived and it is found to be involved in various housekeeping, neuro functional and neurological diseases. The various diseases for which the proteins were mapped are mostly neurological, involving both neuronal and neuronal components. The diseases which were mapped with the identified protein of the zebrafish brain are Psychiatry, Mental Disorders, Parkinson disease, Schizophrenia, Neurodegenerative Diseases, Tauopathies, Dementia, Alzheimer Disease, Central Nervous System Diseases, Nervous System Diseases, Brain Diseases, and Neuromuscular Diseases. The important proteins which were involved in neurological disorders are disc complex (disc), Contactin 2 (cntn2), Ewing sarcoma breakpoint region 1a (ewsr1a), Synucelin beta (SNCB), Cathepsin complex (CTSB), Hydroxysteroid (17-beta) dehydrogenase 10 (HSD17B10), Neuronal PAS domain protein
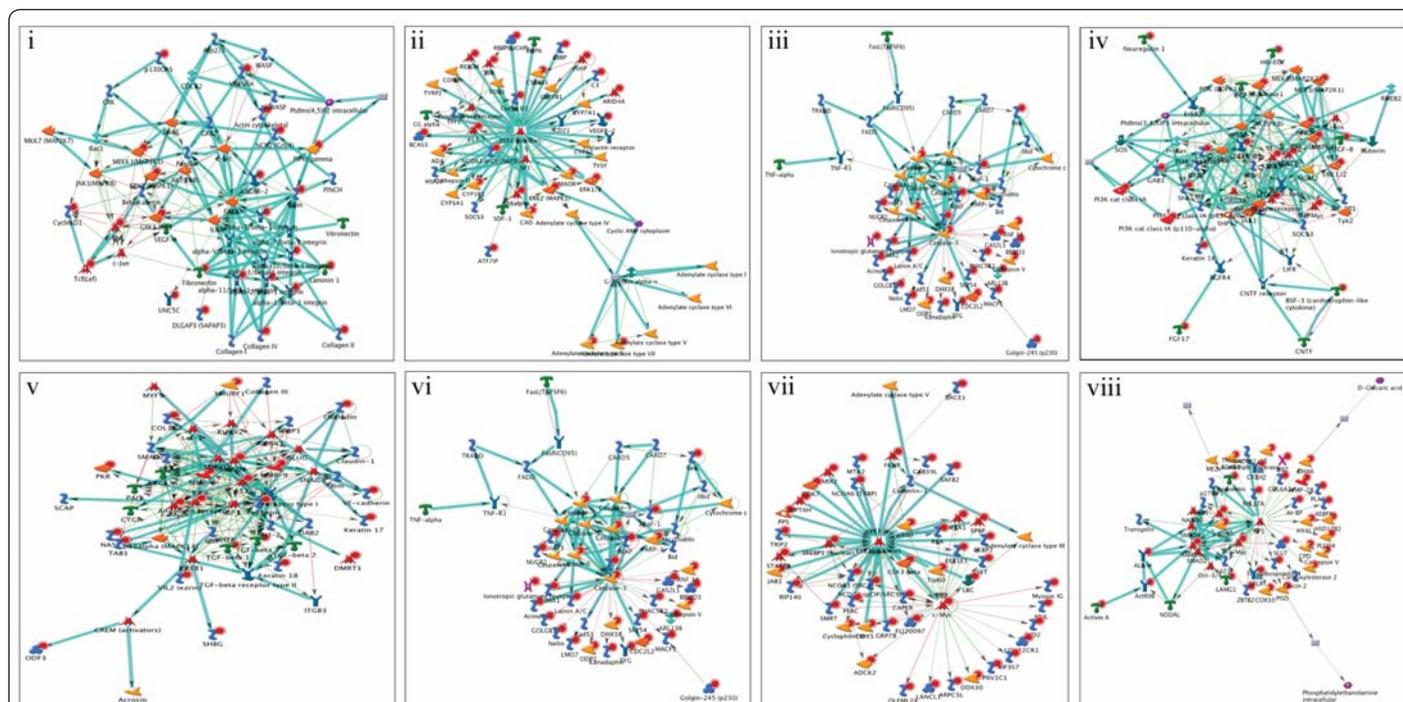
Figure 5: The various network pathways identified form the zebrafish specific brain proteins which were found to be involved in various activities. i. Localization of cell, cell motion and cell organization, ii. cAMP biosynthetic process and activation of adenylate cyclase, iii. Apoptosis and programmed cell death, iv. Protein kinase cascade, v. Development process pathway for organ development, vi. Regulation of cell proliferation, vii. Gastrulation and mesoderm development and viii. Positive regulation of transcription based on RNA polymerase II and regulation of cell cycle.

(NPSA) and parkinson disease (autosomal recessive, early onset) 7 (park7). The different inherited diseases of human associated with the identified zebrafish brain proteins ortholog are neurodegenerative disease involving ataxin 1, 2 & 7 and mental disorder like fragile X mental retardation involving fxr1.

The notable neurological disease networks which were linked to the datasets are Schizophrenia, Alzheimer's and Parkinson disease. The important proteins linked to schizophrenia from the identified dataset are SYNGRI, Chromogranin A, PCK1, NET, DNMT1, and GABA-A receptor (Figure 6i). The various proteins which are identified in the Parkinson disease are PARP-1, Caspase3, 9, MTHFR, PINK1 and Alpha synuclein (Figure 6ii). For Alzheimer's disease KLC1, APH-1A, SORL1, Neuregulin1 and Cathepsin B were found to be associated (Figure 6iii).
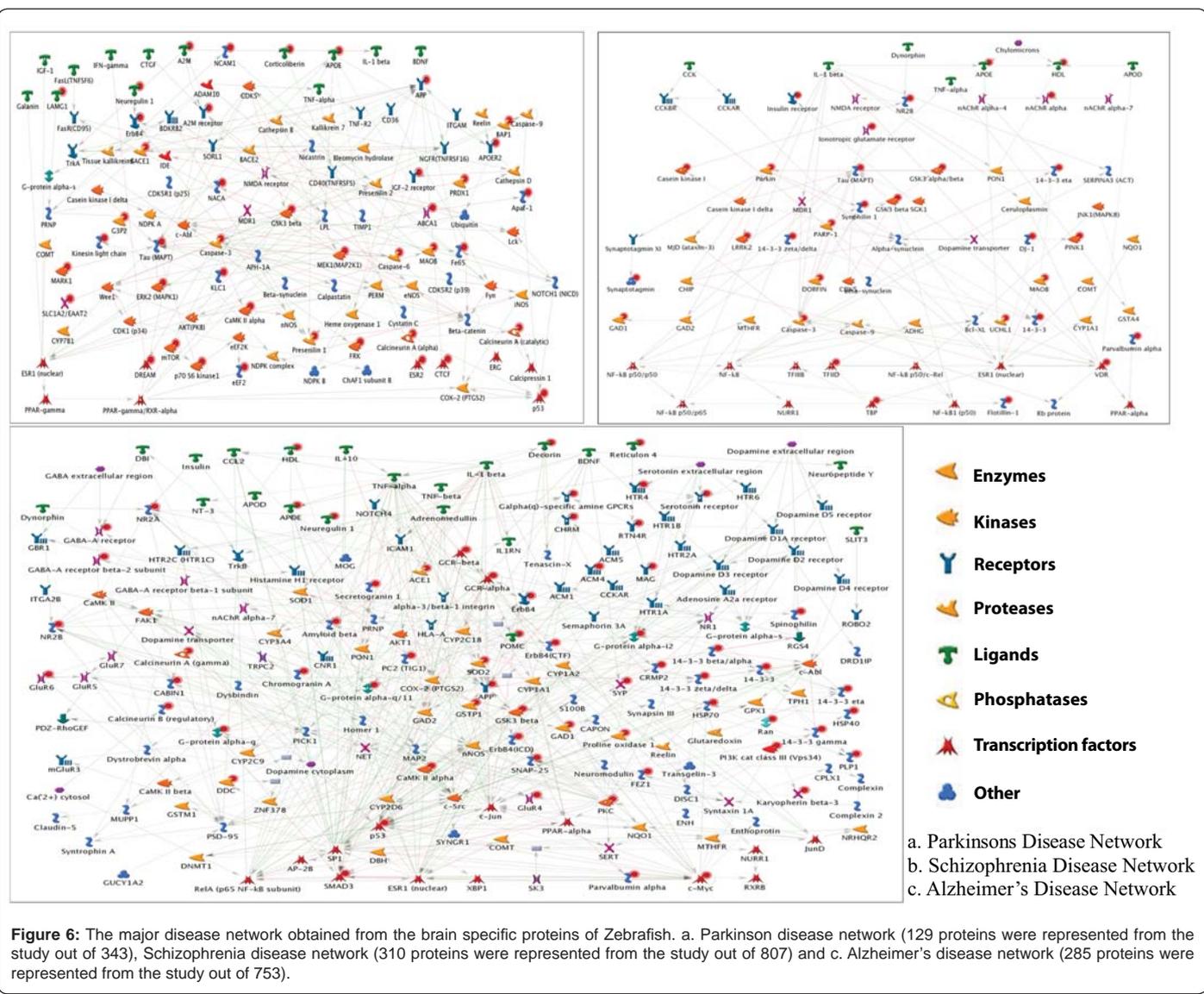
This study of understanding the proteome map of zebrafish brain based on 1D LC ESI MS/MS have identified a total of 8475 proteins expressed in the normal state. In our another study we have identified only 161 proteins based on 2-DE gel followed MALDI MS/MSMS approach, which accounts for 2 to 5 % coverage. The coverage of proteins obtained from this study is expected to be around 80 to 90% of the total proteins expressed in the zebrafish brain. The proteins identified in this study represents varied ranges of pI, mass, type and functions which were all found to be localized in both neuronal and non neuronal cells invariably in all the organelles including neuron projection, synapse of neurons and axon of the nervous system. The proteins represented various categories for its different type activities like transcription, receptor, lignands, kinases, proteases, enzymes and other types. Also our proteome map of zebrafish brain identified all those proteins identified in the zebrafish brain which was differentially expressed due to

chronic ethanol administration in zebrafish (Damodaran et al., 2006). Comparative analysis of all the proteins identified from this study against the mouse (Gauss et al., 1999; Wang et al., 2006) and human brain proteome (Fountoulakis, 2004) dataset exposed 80-90% of the proteins orthologue present in the zebrafish proteome dataset.

Through this extensive study of complete protein map of brain tissue we have identified proteins expressed in zebrafish brain and analyzed its distribution and function. The important and various functions lead by the proteins are cell locomotion, biosynthetic process, cell death, development process, metabolic process and cellular process. The various diseases of the brain associated with the proteins are Psychiatry, mental disorders, Schizophrenia, Neurodegenerative disorders, tauopathies, dementia, Alzheimer's and CNS disease. The proteins involved in various significant pathways are cytoskeleton remodeling, neurofilament development, cell adhesion, transport, signal transduction, axon growth repulsion and neurophysiological process. The study listed a huge amount proteins expressed in the brain at normal state, the list of proteins and its association is all most associated with high significance for neurological diseases like Schizophrenia, Parkinson disease, Alzheimer's disease etc.,. Understanding the various proteins which are found to be associated with various neurological diseases from this study will lead to a better understanding of the disease in the zebrafish model animal.

Conclusions and Perspectives

In summary, we described the high throughput approach of identifying all the brain specific proteins expressed in the

Enzymes
Kinases
Receptors
Proteases
Ligands
Phosphatases
Transcription factors
Other

a. Parkinsons Disease Network
b. Schizophrenia Disease Network
c. Alzheimer's Disease Network

Figure 6: The major disease network obtained from the brain specific proteins of Zebrafish. a. Parkinson disease network (129 proteins were represented from the study out of 343), Schizophrenia disease network (310 proteins were represented from the study out of 807) and c. Alzheimer's disease network (285 proteins were represented from the study out of 753).

zebrafish brain at the normal conditions. We found that the various proteins identified in this study were neuronal tissue specific and involved in various neurological functions. Additionally our work acknowledged all the various network pathways and functions associated with the identified proteins. This study of understanding all the different proteins expressed in the brain during normal conditions would not only lead to understand various biomarkers underlying for various neurological conditions but also used as a marker array tool to understand the brain involvement in various stress associated diseases and in development.

Author's Contribution

SKS carried out the 1-DE gel experiments and LCMS analysis. KSR, KR and AVSP performed the LCMS analysis. MMI was involved in the pathway analysis and drafting the paper.

Acknowledgement

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