Hippocampal Proteomics Profiling along with Protein-Protein Interaction Analysis Elucidates Alzheimer’s Disease Pathways and Genes

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ABSTRACT

Alzheimer’s disease (AD), according to the amyloid cascade hypothesis, is caused by the accumulation of amyloid beta peptide (Aβ), derived from the plasma membrane bound amyloid precursor protein (APP), inside the neurons called as neurofibrillary tangles or outside the neurons called as plaques. The present study has been designed to understand the expression, interaction and dynamics of the hippocampal proteins’ molecular networks that accompany the progression of AD in the rat models compared with those of the controls. Compared with those of the controls, the AD rats had been found altered expression and network of memory and learning related protein (both interacting and functional pathways, as revealed by the STRING and IPA analysis, respectively). Observed findings might be attributed to the infused Aβ in the model animals.

Keywords: Alzheimer’s disease, Hippocampal proteins, Amyloid beta peptide, Rat models

INTRODUCTION

Alzheimer’s disease (AD), a neurodegenerative disorder and the most common form of dementia, poses grave threat towards the ever-increasing lifespan of the humanity. There are more than 40 million people worldwide suffering from AD [1]. Although AD is an age-onset physical complication, manifested usually after the age of 60, its initiation and progression occurs during early stages of life. According to the amyloid cascade hypothesis, AD is caused by the accumulation of amyloid beta peptide (Aβ), derived from the plasma membrane bound amyloid precursor protein (APP), inside the neurons called as neurofibrillary tangles or outside the neurons called as plaques [2]. Aβ aggregates cause neuronal cell toxicity, cell function loss and/or cell death leading to AD complications including memory loss and behavioral alteration. As a consequence the affected person becomes unable to perform daily normal activities, becomes confused about time and space, faces problem in planning and executing even errands [3]. Advanced stage, the AD patients suffer from difficulties in speaking and writing, sleeping and awakening and even cannot recall their own names [3]. They become irritated and suspicious even about their caregivers though they become dependent on their caregivers and family members.

Prevalence of AD is highest in the Western Europe, followed very closely by the USA while lowest in the sub-Saharan Africa. AD is the prime cause of disability in later age of life. In 2016, global cost of AD is estimated to be 605 billion US dollars. Prevalence of AD increases up to 15 times during the age range of 65-80 years. AD prevalence is higher in the developed countries than those of the developing and least developed [4]. This might be due to the increased life span of the people living in the developed countries. As the developing countries are also harboring increased number of aged people, AD prevalence trend is also upward in those countries [5]. AD is posing threat to the global economic policy as it impacts world economy negatively. By 2050, the global figure of the centenarians (people aged 100 years) is expected to reach up to 2 million [1]. During their lifetime, one in every eight males and one in every four females has been predicted to develop AD [1]. Although AD is going to plague the aged humanity worldwide, till today, there is no specific drug for the treatment of AD; the available medico-strategies just delay the worsening of the symptoms. Therefore, to aid the ever-increasing global aged and AD prone populace, finding out

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of safe, less expensive and easy to achieve therapeutic agents has emerged as an urgent need. In this regard, identifying the novel mechanistic approaches governing AD pathogenesis seems apt for strategizing future therapeutic approaches against AD.

Proteomics approach would aid much in high-throughput screening of the etiology and therapeutic approaches for AD. Cellular proteomics studies might shed light on AD biomarker development and elucidate the AD pathomechanism. Mechanistically, functions of proteins are mediated through protein-protein interaction (PPI) and thus, identification of the PPI strategy remains elusive in AD brain proteomics [6]. Therefore, the present study has been designed to understand the expression, interaction and dynamics of the hippocampal proteins’ molecular networks that accompany the progression of AD in the rat models compared with those of the controls.

MATERIALS AND METHODS

Animals

Wistar male rats (120 ± 5 g) were divided into two groups: Control (C) and AD (A), each group containing 15 rats. AD model rats were prepared by infusing Aβ1-42 (ab120959, abcam, USA) into the cerebral ventricles following the method of Fang et al. (2013) [7]. All the experimental protocols had been approved by the ethical permission committee, University of Malaya Institutional Animal Care and Use Committee (UMIACUC) [Ethics reference no. ISB/25/04/2013/NA (R)].

Brain sample preparation and protein quantification

Rats were anaesthetized with intra-peritoneal injection of sodium pentobarbital (35 mg/kgbw), sacrificed, head removed followed by collection of brain on ice bath. Brains were frozen in liquid nitrogen and stored at -80°C. Protein extraction from the brain samples was performed following homogenization of the brain sample (50 mg) with lysis buffer (1 ml) using a homogenizer (Polytron PT 1200, Kinematica). To avoid protein degradation, we added 10 μL of protease inhibitor cocktail during homogenization followed by centrifugation at 10000× g at 4°C for 10 min. The supernatant was collected and preceded towards protein separation through SDS-PAGE and protein quantification through LC-chip MS/MS Q-TOF was performed.

Protein separation through SDS-PAGE

The mini-PROTEAN tetra cell (165-8000, BIO-RAD, USA) was used according to the manufacturer’s instructions for running SDS-PAGE in the current study. Coomassie brilliant blue (0.1%) was used for staining the proteins with shaking for about 20 min. For destaining, 10% acetic acid solution (aqueous) was used. The gel immersed into the destaining solution contained in a covered box underwent occasional stirring until the entire gel was fully destained. Then, individual bands were cut and if any gel plug still contained stain, repeated shaking of the gel plugs in 50 μL of 50% acetonitrile (ACN) in 50 mM ammonium bicarbonate was continued.

In-gel tryptic digestion

For disrupting the tertiary structures of the solubilized proteins, reduction and alkylation are applied to them so that the disulfide linkages are broken and cannot be re-formed. For reduction, the gel plugs were incubated in 150 μL of 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate buffer at 60°C for 30 min. After cooling at room temperature, the gel plugs were alkylated by incubating in 150 μL of 55 mM iodoacetamide (IAA) in 100 mM ammonium bicarbonate for 20 min in the dark chamber. The gel plugs were washed in triplicate with 500 μL of 50% ACN in 100 mM ammonium bicarbonate for 20 min. For dehydration of the gel plugs, shaking in 50 μL of 100% ACN for 15 min was performed followed by drying the gel plugs in speed vacuum for 30 min at 4°C. For enzymatic digestion, the gel plugs were incubated with 25 μL of 6 ng/μL trypsin in 50 mM ammonium bicarbonate at 37°C overnight.

Extraction

After overnight digestion, the digested products were spun down by vortexing and transferred liquid to the fresh tubes. Adding 50 μL 50% ACN to the tubes, shaking was continued for 15 min. Then, the gel plugs were incubated with 50 μL of 100% ACN and shook for another 15 min. Transferring the liquid to the previous tubes, the digested samples were completely dried using the speed vacuum at 1000 rpm. The dried tubes were stored at -80°C and later on de-salting and zip tipping performed.

LC-Chip-MS/MS Q-TOF quantification

All the MS/MS instruments and software used in the present section of the study were from Agilent (Agilent, Santa Clara, CA, USA). Eluted sample obtained from zip tip procedure was dried and 10 μL of the lyophilized samples was reconstituted in the first LC mobile phase (0.1% formic acid) in triplicate. The peptides with a Nano-LC 1260 linked directly with an Accurate Mass Q-TOF 6550 containing a Chip-Cube interface Nano-ESI ion source. Polaris High Performance Chip was utilized and enriched the peptides using 360 nl enrichment column followed by their separation using the separation column (C18 reverse phase, 150mm × 75Åμm, 5 μm) with solvent A (0.2% formic acid in water) and a 5-80% gradient of solvent B (0.1% formic acid in acetonitrile) for 34 min with a flow rate of 0.35 μL/min. Mass data acquisition was undertaken at 8 spectra/second in the range of 100-200 m/z and subsequent collision induced dissociation (CID) of the twenty most intense ions. Setting the mass-tolerance of precursor and product ions at 20, MS/MS data acquisition was performed in the range of 200-3000 m/z. In order to identify the proteins, the acquired
MS/MS data was compared against the UniProtKB/SwissProt rat (Rattus norvegicus) database using the Spectrum Mill and X! Tandem. The differentially expressed proteins in the different groups were identified using their canonical sequence and proteins having fold change of at least 1.5 times were considered as the deregulated proteins. For validation of the identified proteins, the data was exported to the Scaffold database (version 4.5.1, Portland, USA). Proteins were grouped together if they would share at least two peptides and maintained their threshold level at 95.0% and <1% false discovery rate (FDR) by the Peptide Prophet algorithm with Scaffold delta-mass correction for the matched peptide-spectra. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Proteins were annotated with GO terms from NCBI.

Statistical analysis
In the present experiment, label-free relative quantification was performed depending on the regulation of the peptides. For statistical analysis, the data was exported to the Mass Profiler Professional (MPP) software that analyzed depending on the MPP entities, the intensity of the total spectra of the proteins. Setting the baseline of the spectra to the median of the samples, frequencies of the entities were filtered minimally at all the replicates of each treatment. To overcome the complications of false discovery associated with multiple test analyses, ANOVA (P<0.05) was performed.

Bioinformatics and analysis of protein-protein interaction (PPI)
Most of the proteins do not work singly rather they participate in complex network or scaffold and interact with others. Thus, analysis of the relevant protein-protein networks provides important information in deciphering any bio-molecular system. We identified the functional interaction networks of the proteins using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (version 10.0; http://string-db.org/). STRING displays protein-protein interactions in a large network of connectivity and protein hubs. Active prediction methods that we used were experiments, neighborhood, databases, gene fusions, co-expression, co-occurrence and text mining, using high confidence (0.7).

For further identifying over-representing pathways and biological functions, we used the ingenuity pathway analysis (IPA), build version: 389077M, content version: 27821452, (Release date: 2016-06-14) (https://www.ingenuity.com/wp-content/themes/ingenuity-qigen). We uploaded the datasets (AD versus C, AD versus AE and C versus AE) of the proteins significantly expressed (p<0.05) and having log fold change of 1.5 and higher. Our analysis setup was as given in Table 1.

### Table 1. Analysis setup.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Set up</th>
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<tr>
<td>Reference set</td>
<td>Ingenuity knowledge base (genes only)</td>
</tr>
<tr>
<td>Relationship to include</td>
<td>Direct and indirect. Includes endogenous chemicals.</td>
</tr>
<tr>
<td>Optional analyses</td>
<td>My pathways my list</td>
</tr>
<tr>
<td>Filter summary</td>
<td>Consider only molecules and/or relationships where (species=human) and (data sources=ClinicalTrials.gov or ClinVar or Ingenuity Expert Findings or Ingenuity ExpertAssist Findings)</td>
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RESULTS AND DISCUSSION

Quantitative analysis of the identified proteins

Total 822 proteins with protein threshold at 95.0%, minimum peptide of 2 and peptide threshold at 0.1% FDR were identified in the present study. Number of commonly expressed proteins was 361. Among all the identified proteins (822), 329 were differentially expressed with statistical significance (P<0.05). Among the significantly regulated (P<0.05) 329 proteins, 289 met the criteria of fold change (LogFC of 1.5) cut off value. Number of proteins linked with AD was 59. The highest amount of proteins differentially expressed in the AD rats were those involved in metabolic processes (26% increase in the rats) followed by those involved in anti-oxidant activities.

Functional classification of the significantly regulated proteins

A. Proteins involved in neuronal structure and function: We observed differential expression of the
neurotransmission, synaptic plasticity, neurogenesis, memory and learning related proteins such as neurochondrin, synaptophysin, synapsin-1, synapsin-2, synaptogyrin 3 (Syngr3), 4-aminobutyrate aminotransferase and 14-3-3 protein gamma in the different rat groups. Associative learning and long-term memory related proteins glutathione-S-transferase 3 and tenasin R were up regulated. Synaptic plasticity promoting Ras related protein Rab 5a and nerve growth factor (NGF) signaling Rap-1A were also among the significantly up-regulated group. Similar was the case for the heat shock proteins (HSP) involved in the regulation of neuronal migration (HSP 90-alpha) and apoptosis (Hsp 60). Up-regulation was also observed for the proteins involved in post-synaptic excitatory potential (serine/threonine-protein phosphatase, syntaxin 1B), pre- and post-synaptic density (isoform 2 of clathrin coat assembly protein AP180) and tyrosine phosphorylation (hemopexin) in the AD versus C group.

Proteins involved in synaptic organization (neurofascin) and synaptic vesicle budding (ADP-ribosylation factor 1), vesicle mediated transport (syntaxin 1A), neuronal differentiation and development (Dihydopyrimidinase-related protein 1 and 2), axonogenesis (2", 3"-cyclic-nucleotide 3"-phosphodiesterase), axonal choice point recognition (neuromodulin) and axonal transport (neurofilament light polypeptide) were also differentially expressed in the hippocampi of the three rat groups. Beta-soluble NSF attachment protein (SNAP-β) involved in the regulation of glutamatergic synaptic transmission, disassembly of SNARE complex and synaptic vesicle priming was also up regulated. In addition to these proteins, we observed differentially up regulated expression of glial fibrillar acidic protein (GFAP). GFAP is involved in long-term synaptic potentiation, neurotransmitter uptake, neurogenesis, glial and Schwann cell proliferation.

We observed down-regulated expression of memory and learning related proteins such as clusterin (stimulator of Aβ and NFT), neuromodulin, neurofascin, NCAM 1 and proteins involved in dopamine decarboxylation in the AD versus control group. Following are the AD related other proteins differentially expressed in the present study:

**Syntaxin-1A:** Syntaxin-1A regulates vesicular trafficking during exocytosis and trans-membranal protein insertion. Decreased expression of syntaxin-1 A in the AD rats might have affected synaptic functions [8].

**Synaptogyrin-1:** Synaptogyrin-1 is involved in maintaining short and long-term synaptic plasticity. Level of hippocampal syntaxin-1 A and synaptogyrin-1 had been found to be reduced in line with AD progression [9].

**Neuromodulin (GAP-3):** Neuromodulin is a neuronal growth and neurite forming protein whose level decreases in AD brains [10].

**Neural cell adhesion molecule (NCAM):** NCAM plays important role in brain development and increased level of NCAM 1 in transgenic AD mouse model (Tg2576) and of NCAM 2 in human AD patients have been reported [8].

**Endophilin A1:** Endophilin A1 is a membrane bending protein involved in CNS development, apoptosis, signal transduction and microtubule based movement. AD rats’ hippocampi showed decreased expression while the control rats experienced increased expression of endophilin A1 in the present study. In the temporal neocortex of the AD patients, decreased level of endophilin A1 has been observed [11].

**Clathrin:** Clathrin group of proteins are involved in neuronal secretory functions and synaptic maintenance [12]. AD pathogenesis involves altered clathrin-associated membrane trafficking resulting in neurodegeneration [12]. Between the light and the heavy chains of clathrin, impaired distribution of the former has been linked with the AD pathogenesis [12]. We observed similar findings in the AD group of rats compared with others in the present study.

**Septin:** Septins are GTP-binding proteins found to be co-localized with the NFT in the AD brains [11]. We observed increased expression of septins (septin-2, septin-3), NAD-dependent protein deacetylase sirtuin-2 in different rat groups. Our findings are compatible with those of [11].

**UCH L1:** Ubiquitin carboxy-terminal hydrolase L1 (UCH L1) is an important enzyme for maintenance of cognitive and synaptic [13]. Conflicting information regarding its expression has been documented in different AD cases.

**Soluble NSF-attachment protein beta (SNAP-β):** N-ethylmaleimide sensitive fusion proteins (NSF) are the part of APP and overexpressed in AD [14]. Soluble NSF-attachment proteins are involved in intracellular membrane fusion and vesicular trafficking. Among α-, β- and γ-SNAPS, α- and γ-SNAPs are expressed in different tissues while the β-SNAP is brain specific. In AD brain, differential expression and oxidized form of SNAP-β had been detected through redox proteomics.

**Neuropolyptide h3:** Neuropolyptide h3 is a cholinergic neuro-stimulating peptide that falls in the phosphatidylethanolamine binding protein group and is also known as Raf-kinase inhibitor protein (RKIP) and/or hippocampal cholinergic neurostimulating peptide (HCNP) [15]. Our finding of down regulated neuropolyptide h3 is in agreement with other studies [8]. Oxidatively modified loss of function of neuropolyptide h3 impairs phospholipid asymmetry that might be involved in extrusion of phosphatidyl serine to the outer membrane of neuron and signal for apoptosis and cause neuronal death [16]. Also, neuropolyptide h3 mediated stimulation of acetylcholine esterase (AchE) becomes compromised and this effect is heightened when HNE interacts with AchE in presence of Aβ (1-42) in synaptosome [17]. Thus, in AD brains,
neuropolypeptide h3 is linked with cholinergic abnormalities and altered lipid metabolism that are the early events in AD pathogenesis [14].

Annexin: AD rats showed increased expression of annexin in the hippocampi. Previous studies have linked increased plasma annexin5 with increased AD risk [18].

Glycogen synthase kinase 3 β (GSK3β): Glycogen synthase kinase 3 β (GSK3β) is a serine/threonine kinase having diversified regulatory functions ranging from glycogen metabolism to gene transcription. Over activity of GSK-3β has been linked with elevated Aβ production, tau hyper phosphorylation and impaired memory and learning activities and “mitochondrial traffic jam”[19].

Serine/Threonine protein phosphatase: Serine/Threonine protein phosphatase negatively regulates memory and learning abilities by impairing synaptic plasticity and LTP [20]. Up regulation of serine/threonine protein phosphatase in the AD rats might contribute towards impaired memory and learning performance in the present study.

Serine protease inhibitors (serpins): Serine protease inhibitors (serpins) regulate proteolytic processing of proteins. Previous studies indicated their increased level in plasma and CSF of AD patients [20]. We also observed increased expression of serpins (α1-antitrypsin) in the AD rats’ hippocampi. Alpha 1-antitrypsin (A1AT) has been reported to be co-localizing with Aβ plaques and NFTs [21].

B. Proteins involved in Ca²⁺transportation, homeostasis and signaling

Dysregulation of Ca²⁺ metabolism and signaling has been linked with neurodegeneration and AD pathogenesis (Brawek and Garaschuk, 2014) [22]. We observed differential expression of calmodulin, CamK2a and CamK2b involved in Ca²⁺transportation, homeostasis and signaling.

a. Calmodulin: AD rats showed up regulated expression of calmodulin. Calmodulin is a biomarker of AD whose increased expression and its binding proteins are associated with AD pathogenesis [23,24].

b. CamK2a: Ca²⁺/calmodulin-dependent serine/threonine protein kinase (CamK2a) is highly important for maintenance of the glutametargic synaptic plasticity [25]. Its role in spatial learning becomes also evident from its supporting role towards NMDAR-dependent LTP in the hippocampus [26]. Through Ca²⁺/calmodulin kinase II signaling, CamK2a also regulates neurotrophin-3 and BDNF secretion from the hippocampal post-synaptic neurons [26,27]. In the present study, the CamK2a showed down regulated pattern compared with their control and mushroom-treated counterparts. Our findings are in line with those [28,24]. Its deficiency has been shown to hamper neuronal development and dentate gyrus formation as well as behavioral alteration [28]. On the other hand, up-regulated expression of CamK2a has been linked with improved cognitive performance of the SMP8 mice [23,28].

c. Annexins (Annexin-1, -5 and -6)

Our findings of increased annexin expression in the AD rats’ hippocampi are compatible with those of [18,25,29]. Annexins are intracellular Ca²⁺-respondents capable of binding with membrane phospholipids and participate in membrane trafficking, endo- and exo-cytosis [25].

Aβ-induced Ca²⁺ dysregulation (increased intracellular level) hyper activates c-Jun N-terminal kinase (JNK), cyclin-dependent kinase 5 (CDK5), tau phosphorylation and disrupts microtubule network [19]. It leads towards mitochondrial trafficking defects that impair the normal movement of mitochondria across the microtubules and thus cause “mitochondrial traffic jam” in the AD neurons and affects neuronal functions [20]. Thus, intracellular Ca²⁺ dyshomoeostasis might be among different mechanisms involved in disrupted neuronal activity and corresponding impaired memory of the AD rats in the present study.

C. Proteins involved in signal transduction

a. 14-3-3 proteins (Ywhag): We found up regulated expression of the 14-3-3 proteins (ζ/δ, θ, η, γ, β/α) in the AD rat brains. They constitute about 1% of total soluble proteins of the normal brain. 14-3-3 proteins participate in signaling through binding with the phospho-serine containing proteins and can regulate the activities of the kinases, phosphatases and trans-membrane proteins [29]. Thus, they mediate diversified activities involving neuronal plasticity, neurotransmission, neurite outgrowth generation and neurogenesis [30]. In AD brains, they have been found to be closely associated with tau and aid in the formation of the NFTs [29]. Differential expression of 14-3-3 proteins β/α, ζ/δ and ε had been observed in the temporal neocortex and other parts of the brains of the AD patients [11]. In the AD hippocampi, both intra- and extra-cellular expression of the 14-3-3 proteins have been detected. Among different isoforms, the highest immunoreactivity towards NFT has been observed for the 14-3-3ζ [31]. 14-3-3ζ-mediated tau phosphorylation involves protein kinases such as glycogen synthase kinase-3 beta (GSK3β). GSK-3β hyperactivity impairs mitochondrial intra-neuronal anterograde movement and causes “mitochondrial traffic jam” and disrupts neuronal activities [19]. 14-3-3ζ also binds with δ-catenin and disrupts the formation of the adherens junction complex that compromises the neural structure and cognitive performance [19]. Also, interaction of δ-catenin with pre-senilin 1 is an important stimulator of the wnt signaling and thus of AD pathogenesis and neuronal under-development [24].

b. VDAC (voltage-dependent anion selective channel 1): Voltage-dependent anion selective channels (VDAC1 and VDAC2) are mitochondrial porins involved in transportation of ATP and Ca²⁺ and in apoptotic signaling [32]. Its altered expression has been noticed in AD and other
neurodegenerative diseases and in mitochondrial dysfunctions.

c. **SLC12A5**: SLC12A5 are neuronal K+/Cl- symporter involved in maintaining intra-neuronal low Cl- concentration [33]. It mediates neuronal excitotoxicity and synaptic inhibition [34]. Its increased expression is linked with the glutamate transporter Slc17a7 that actively participates in GABAergic neurotransmission [33].

D. **Proteins involved in apoptosis**

Apoptosis is an important feature of neuronal and synaptic cell losses. Decreased levels of the apoptosis regulatory enzymes (peptidyl prolyl cis-trans isomerase, protein phosphatase) found in the present study are distinct hallmarks of AD pathogenesis [35].

E. **Proteins involved in neuronal cytoskeleton maintenance**

Derangement of neuronal cytoskeleton through microtubule disassembly is an important feature of neurodegeneration [36]. STRING analysis revealed strong networks among microtubule assembling the cytoskeletal proteins such as tubulin, β-actin and keratin.

a. **Tubulin**: AD neurons suffer from disrupted microtubule structure and functioning [37]. Tubulin is the main component of microtubule and consists of dimers imparted by the alpha and beta chains. Differential expression of tubulin α-1c, -4a, β-2a, -2B, -3 and -5 chains were observed in the present study. Molecular function based sub-network analysis showed different tubulin chains to be clustered together and deranged in AD. Both animal and human studies have linked decreased level of α and β tubulin with human AD [38,39]. In AD brain, β tubulin becomes abnormally hyper phosphorylated and modified tubulin fails to assemble microtubules. Consequently, microtubule disassembles leads towards cytoskeletal vulnerability. Recently, micro tubular disassembly has been implicated in causing “mitochondrial traffic jam” in the AD neurons as mitochondrial shifting across the “rail-road of microtubule” becomes impeded in the AD brain [19].

b. **β-Actin**: Normally, β-actin is involved in maintenance of cytoskeleton, internal cell motility, neuronal network integrity and aids in memory and learning performances. Its altered expression and oxidized form had been linked with AD pathogenesis [15]. Impaired expression of actin is in agreement with the synaptic dysregulation associated with AD and age-related altered cytoskeletal structure, axonal dystrophy, reduced dendritic spines and impaired transport across membranes [40]. Enhanced accumulation of actin enhances tau-governed neurotoxicity [41].

c. **Dihydropyrimidinase-related protein 2 (DRP-2)**: Dihydropyrimidinase related protein 2 (DRP2) is involved in regulation of axonal outgrowth and becomes hyper phosphorylated in NFT and its increased level had been observed in AD model animals [42]. Compared with the normal neurons, AD neurons possess shortened dendrites that are a characteristic of their lowered communication with neighboring neurons [15]. Oxidative modification of DRP2 might have caused reduced length of the dendrites and communication leading to lowered cognitive performance of the AD rats in the present study [15].

d. **Glial fibrillary acidic protein (GFAP)**: GFAP provides structural support to the astrocytes and its elevated level in AD model animals and in human subjects [42]. Our findings are conforming to those [42].

e. **RhoA proteins**: Ras homolog gene family, member A (RhoA) proteins are involved in neuronal cytoskeleton regulatory processes such as dendrite development, axonal extension and protrusion [43]. They also stabilize the Aβ-disrupted microtubules [43]. Aβ increases RhoA-GTPases and decreases neuronal spine production and neural connection both in the cell lines and also in the brains of the transgenic AD models [14].

f. **Septin**: Septins are microtubule associated, filament-forming and GTP-binding proteins that participate in dendritic spine formation and in neurotransmitter release [44]. Like that of we found increased expression of septin-2 and -3 in the AD rats’ hippocampi. Its increased expression might be involved in disrupting micro tubular filament formation and associated cytoskeletal derangement in the AD rats.

g. **Cofilin**: Brain cofilin activity reduces with age and in the AD subjects, it goes down aberrantly [45]. As cofilin is a regulator of actin, decreased cofilin expression in AD rats points towards decreased actin turnover and lowered depolymerization of actin filament.

h. **Dynamin**: Dynamin is a neuronal GTPase capable of free entry into and release from the synaptic vesicles [45]. Our finding of its decreased expression in the AD rats is consistent with [12,48] Aβ-induced depleted dynamin1 level had been found to impair memory in the AD model rats [47].

i. **Gelsolin**: Gelsolin is a member of the actin-binding proteins having anti-oxidative, Aβ binding and fibrillation inhibitory potentiality [48]. Its overproduction and/or administration showed Aβ lowering effect and thus, gelsolin has been regarded as an AD therapeutic agent [47]. We found decreased level of gelsolin expression in the AD rats’ hippocampi that is in par with those of [49].

**Protein-protein interaction (PPI) findings**

In addition to functional, modular and pathway-related insights, PPI maps provide disease specific information. As identification of the target protein in any disease pathogenesis is an important aspect, PPI analyses shed light towards understanding the complex connectivity and

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identifying the protein of interest for further evaluation and management [50].

Based on the analysis of the PPI networks and pathways of the differentially expressed proteins, it is obvious that AD causes a disturbed protein expression affecting the global protein-protein interactive networks and the relevant biological pathways. We categorized them into functional framework of metabolic process, intracellular signaling cascade, signal transduction, oxidation reduction, cell communication, molecular transport, regulation of biological processes, regulation of cellular processes and apoptosis.

**PPI among the up-regulated proteins**

**PPI among up-regulated proteins of AD vs. control group:** PPI interaction among the up-regulated proteins in the AD versus control group can be divided into several network clusters (Figure 1). Among them, the first two consists mainly of the cytoskeletal proteins such as 14-3-3 proteins along with tubulin, actin, Ras-related proteins, coflin, sirtuin, actin, myosin and the Ras-like proteins. The heat shock proteins (HSP) of different molecular weight and function formed another important network cluster (Figure 1). Calmodulin, Ca2+/calmodulin-dependent protein kinases, serine/threonine-protein phosphatase and synapsin-1 formed another functional interaction (Figure 1). Enzymatic proteins involved in metabolism and energy generation formed functional interactions among themselves (Figure 1). There was strong interaction among the proteins (at high confidence score of 0.700). As some of the proteins had been involved in different functions, functional overlap of the proteins led them towards extended integration and interaction beyond any single class (Figure 1).

![Figure 1. PPI network of the up-regulated proteins of the AD vs. Control group.](image-url)
Legends (applicable for all the PPIs of all the groups)

Red color is indicator of up-regulation and green color of down-regulation. Nodes and edges are indicators as follows:

<table>
<thead>
<tr>
<th>Node</th>
<th>Node size</th>
<th>Node color</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Small</td>
<td>Large</td>
</tr>
<tr>
<td></td>
<td>Colored</td>
<td>White</td>
</tr>
</tbody>
</table>

Network nodes represent proteins

<table>
<thead>
<tr>
<th>Protein of unknown 3D structure</th>
<th>Some 3D structure is known or predicted</th>
<th>Query proteins and first shell of interactors</th>
<th>Second shell of interactors</th>
</tr>
</thead>
</table>

Legends of the nodes and edges used in IPA

<table>
<thead>
<tr>
<th>Edges</th>
<th>Known interactions</th>
<th>Predicted interactions</th>
<th>Others</th>
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<td>From curate d data bases</td>
<td>Experiment ally determined</td>
<td>Gene neighbor hood</td>
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Legends of functional networks in IPA

- Complex
- Enzyme
- Group/Complex/Other
- Ion Channel
- Phosphatase
- Transcription Regulator
- Transporter
- Direct Relationship
- Indirect Relationship

a. Pathway analysis of the up regulated proteins in the AD vs. control group

Through KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis, we found 65 pathways to be significantly enriched (P<0.05) in the AD versus control group. Among them the most notable were the AD pathway (pathway ID 05010) involving the genes Camk2a, Camk2b, Ndufs1, Ndufv2 and Atp5o (FDR 0.0176); the LTP pathway (pathway ID 04720) involving the genes Calm1, Camk2a, Camk2b and Rap1a (FDR 0.0037) and the neurotrophin signaling pathway (pathway ID 04722, FDR 0.0246) involving the genes Calm1, Camk2b, Rap1a and Ywhae. The P13K-Akt signaling pathway (pathway ID 04151, FDR 0.0161) involving the genes Hsp90aa1, Ywhab, Ywhae, Ywhag, Ywhh and Ywhaq; Ca\textsuperscript{2+} signaling pathway (ID 04020, FDR 0.0176) with the genes Calm1, Camk2a, Camk2b, Ppp3ca, Slc25a4 and Slc25a5; cGMP-PKG signaling pathway (ID 04022, FDR 0.0032) involving the genes Camk2a, Camk2b, Atp1a1, Atp1b1 and Slc25a5 were also among the interacted signaling pathways. PFAM and INTERPRO protein domain analyses identified 14-3-3 (PF0024) as the most significantly enriched protein in the AD versus control group.

PPI among the down-regulated proteins

PPI among the down-regulated proteins of the AD vs. control group: Among the down regulated AD versus control group, the most notable interaction had been observed among the cytoskeletal proteins such as keratin isoforms (Krt1, Krt2, Neflh, Krt5, Krt8, Krt10, Krt13, Krt15,
Krt14, GFAP, Krt17, Krt42, Krt73), junction plakoglobin (Jup), neurofilament heavy chain (Neflh) and glial fibrillary acidic protein (GFAP) (Figure 2). Down regulated anti-oxidant proteins peroxiredoxin 5, superoxide dismutase 1, protein disulfide isomerases (Prdx5, Sod1, P4hb, Hspe1 and Cct3), oxoglutarate/malate carrier protein and alcohol dehydrogenase also formed a sub-network (Figure 2). Besides, metabolic and ATP (Atp5c1, Atp6v1d and Atp6v1e1) generating proteins were also interacted with each other (Figure 2).

Figure 2. PPI network among the down-regulated proteins of the AD vs. C group.

Legends (applicable for all the PPIs of all the groups)

<table>
<thead>
<tr>
<th>Node</th>
<th>Node size</th>
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<tr>
<td></td>
<td>Small</td>
<td>Large</td>
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<tr>
<td>Network nodes represent</td>
<td>Protein of unknown 3D</td>
<td>Some 3D structure is</td>
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Identification of functional network interaction through integrated pathway analysis (IPA)

Based on IPA Knowledge Base (IPAKB), genes are transformed into relevant networks. In the network, relationships among the genes are expressed as the “edges” and genes become connected with each other only if there is any path among them in the global network. In this case, molecules from the dataset that are uploaded are called the “focus molecules”. We performed core analysis through IPA so that we could interpret our datasets in the form of their functional networks. In the IPA Knowledge Base (IPAKB), corresponding objects were mapped with the protein identifiers [51]. Depending on the physical interaction (direct relationship) among the eligible proteins, IPAKB generated the networks and the score (probability value) of the networks [51]. Higher the network connectivity greater is the representation of significant biological functions of the relevant genes [51]. Statistical justification of the network connectivity is performed through measuring “p scores (-log10 p value, Fisher’s exact test)” and “network score”. Network score is also measured through Fisher's exact test that is based on the focus protein and biological functions and thus shows the relevancy of the analysis.

Functional networks in AD vs C

IPA of the AD vs C identified 18 networks of which the top-most one had score of 90 having 93 focus molecules among 140 total selected molecules (Figure 3). The top-most network has been associated with cell death and survival, neurological diseases and psychological disorders.
Figure 3. Top-scoring gene networks differentially expressed between AD and C group (top-most network 1).
Legends of functional networks in IPA

The second network had score of 54 and focus molecules 64 among 140 total selected molecules (Figure 4). The relevant physiological system development and functions included molecular transport, cell-cell signaling and interaction, nervous system development and functions. Nervous system development and functions involved 40 molecules (p value 4.99E-02 - 3.48E-05). The other networks had score of 1 and focus molecule 1 and most were related to metabolism.

Figure 4. Top-scoring gene networks differentially expressed between AD and C group (top-most network 2).
The top upstream regulators with corresponding p value of overlapping were PPARG (6.50E-05), NFE2L2 (3.34E-04), STAT3 (1.36E-03), CEBPA (5.25E-03), GATA4 (5.25E-03). Peroxisome proliferator activated receptors (PPARs) are the groups of nuclear hormone receptors that regulate lipid metabolism, energy production, metabolic balance between lipid and carbohydrates by acting as the lipid sensors [52]. AD ameliorating effect of the non-steroidal anti-inflammatory drugs (NSAIDs) has been associated with PPAR stimulating effect [53].

Neurological diseases were among the top diseases and biofunctions with 14 molecules and p value of 4.74E-02 - 5.26E-04. Among the top toxicity lists were OS (22.2%, 7.95E-11), mitochondrial dysfunction (12.7%, p 2.30E-12), LXR/RXR activation (14.4%, 5.73E-11), positive acute phase response proteins (34.6%, 2.43E-10) and FXR/RXR activation (13.0%, 9.72E-10).

The top-most up-regulated molecules with their log ratios were (THY1 18.403), ATP5F1 (18.175), GSTP1 (17.998), VAMP2 (17.922), IgH-6 (17.867), SLC1A3 (17.808), SOD2 (17.784), HSPA9 (17.354), TPPP (17.347) and RAB11B (17.254).

The top-most down-regulated molecules with their log ratios were DDT (-18.544), LOC259246 (-18.299), CYB5A (-18.203), SOD1 (-17.609), HIST1H3E (-17.581), DSG1 (-17.409), FABP1 (-17.253), HSPE1 (-17.230), LOC100911847 (-16.892) and JUP (-16.852).

Top canonical pathways with their corresponding overlap and p values were phagosome maturation (21.5%, 2.98E-19), 14-3-3-mediated signaling (16.1%, 2.37E-14), remodeling of epithelial adherens junctions (24.6%, 6.49E-14), epithelial adherens junction signaling (14.0%, 1.55E-12) and mitochondrial dysfunction (12.9%, 1.80E-12). 14-3-3-mediated signaling and the network associated affected proteins have been depicted in Figure 5. Besides, pathways associated with glucose metabolism (glycolysis and gluconeogenesis), TCA cycle, oxidative phosphorylation, unfolded protein response, HIPPO signaling and xenobiotic metabolism signaling were also among the notable functionally interacted ones. The HIPPO signaling entails the protein kinase “Hippo (HPO)” and is evolutionarily conserved for developing the mammalian nervous system. Its emerging role in AD pathogenesis has intrigued the current AD research field [54].

![Figure 5. 14-3-3 protein-mediated signaling and associated proteins in the AD vs. C group.](image-url)
Consistent with our quantitative proteomics findings and interaction analysis by STRING, the proteins altered in their expression are also functionally related with each other. The most prominent proteins in this regard are those involved in nervous system development and function (YWHAE; NCAM1, NEFH, SIRT2, VSNL1; NCAM1, NEFH, SIRT2, VSNL1; NME2; UGT1A1; SEPT1; CAMK2A; 2, SEPT5, SNCA, SOD1; KG1, TUB1A1). Proteins involved in anti-oxidative defense through free radical scavenging (GPX1, GPX3, PRDX1, PRDX2, PRDX6, SNCA, SOD1 and SOD2) also were functionally related. Similar trend was observed for the proteins involved in synaptic functions, cytoskeletal arrangements, microtubule-associated proteins, cellular stress response (especially the HSPs) and calcium binding. Our findings closely resemble those of [25].

Besides, proteins involved in mitochondrial structure and functions were functionally closed. Deranged expression pattern of metabolism, energy generation and mitochondria-associated proteins had been observed. The IPA analysis tally with those and indicate that the AD mitochondria suffer from disrupted expression and functionality of the proteins involved in metabolism, energy generation, OS regulation and Ca2+ homeostasis [51]. Another important aspect of neuronal mitochondria is maintenance of cellular dynamics so mitochondrial “traffic jam” is overcome through proper translocation of mitochondria through the neuronal cytoskeleton [19]. Present findings of the affected proteins linked together in the AD subjects are “red signal” across the neuronal cytoskeletal “cross-roads”. In line with the mitochondrial derangement, the functional interaction among the affected cytoskeleton and microtubule-associated proteins (especially tubulins) reinforce the observed jumble of the AD hippocampal proteins.

Proteins involved in metabolism of almost all the biomolecules have been affected in the AD groups. The most notable alteration was those metabolizing glucose and lipids (APOA1, HSD11B1, PHB, SERPINA1, SIRT2; Akr1c14, COMT, HSD11B1, Sult1a1; ACSL1, ALB, FABP1; ABAT, ACSL1, ALB, APOA4, APOE, CS, DLAT, DLD, F2, FABP1, KNG1, MDH1, MDH2, PDHB, RAC1, RGN, STX1A, SUCLA2). Top-ranking proteins involved in protein metabolism and interacted together were ACADM, ALDOC, ANXA5, CRP, EEF1A1, Gmnt/LC100911564, HBA1/HBA2, HBB, HSD17B10, IVD, JUP, MAT1A, NEFL, NME2, SOD2, VCP and YWHAB. Metabolism-related proteins were linked in the peripheral nodes of the IPA.

CONCLUSION

Compared with those of the controls, the AD rats had been found altered expression and network of memory and learning related protein (both interacting and functional pathways, as revealed by the STRING and IPA analysis, respectively). Observed findings might be attributed to the infused Aβ in the model animals. The altered proteins, functional networks and pathways might be targeted as an AD withstanding stratagety. Thus, the present study paves a new vista in the realm of AD therapeutics.

HIGHLIGHTS

• Control and Alzheimer’s disease (ad) model rats’ hippocampal proteomics has been compared.

• Ad model rats showed altered expression of proteins and protein-protein interaction networks and functional pathways.

Proteins and networks involved in neuronal structure maintenance and regulation had been found to be mostly affected in the ad rats’ hippocampi.

REFERENCES


