Ganoderma Lucidum Modulates Neuronal Structure and Function Regulatory Protein Expression and Protein-protein Interaction in Alzheimer’s Disease Model Animals

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Research

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Abstract

Background

Alzheimer's disease (AD) is the leading neurodegenerative disorder affecting memory, learning and behavior. Altered expression of proteins involved in neuronal structure and function is a recent observation of AD pathogenesis. Modulation of altered protein expression seems promising in AD therapeutics. In the present experiment, AD ameliorating effect of medicinal mushroom Ganoderma lucidum had been evaluated through its effect on neuronal structure and function related protein expression pattern in AD model rats.

Methods

Wistar male rats (120 ± 5 gm) were divided into three groups: control (C), AD (A) and G. lucidum hot water extract (HWE) fed AD (AE), each group containing 15 rats. AD model rats were prepared by infusing Aβ1-42 (ab120959, abcam, USA) into the cerebral ventricles. Protein extraction from the brain samples was performed following homogenization of the hippocampus (50 mg) with lysis buffer (1ml) using a homogenizer (Polytron PT 1200, Kinematica). Protein separation through SDS-PAGE and protein quantification through LC-chip MS/MS Q-TOF had been performed for label-free relative quantification. For statistical analyses, the data were exported to the Mass Profiler Professional (MPP) software and ANOVA (P<0.05) had been performed to overcome the complications of false discovery associated with multiple test analyses. Functional interaction networks of the proteins were identified using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (version 10.0; http://string-db.org/). For further identifying over-representing pathways and biological functions, the ingenuity pathway analysis (IPA), build version: 389077M, content version: 27821452, (Release date: 2016-06-14) was used (https://www.ingenuity.com/wp-content/themes/ingenuity-qiagen). Datasets of the proteins significantly expressed (P<0.05) and having log fold change of 1.5 and higher were uploaded (AD versus C, AD versus AE and C versus AE).

Results

Among 2,212 proteins identified in the present study, 819 had been found to be differentially expressed. Of the differentially expressed ones, 40 proteins linked with neuronal structure and function regulation had been observed in the present study. AD rats manifested altered expression of proteins associated with neuronal structure and function. G. lucidum hot water treatment ameliorated the altered expression of those proteins.

Conclusions

Altered expression of hippocampal proteins is a hallmark of AD. Neuroproteomics regulatory approach towards AD amelioration seems promising. Inclusion of G. lucidum for proteomics based AD therapeutics in regulation of the proteins involved in neuronal structure and function seem apt. Thus, G. lucidum could be considered as an AD therapeutic agent.

1. Introduction

According to United Nations Education Scientific and Cultural Organization (UNESCO), mushroom had included in the Ben Cao Gang Mu ([ Retrieves, the first pharmacopoeia in China, compiled during Ming dynasty (1590 AD]) as an aesthetic, spiritual and therapeutic agent [1–2]. Medicinal mushroom, Ling zhi (Ganoderma lucidum) has 2000 years-old reputation of usage in the Chinese medicine [3]. The State Pharmacopoeia of the People’s Republic of China (2000) has incorporated G. lucidum as the potent replenisher of Qi (life force), salient mind soothing and cough relieving as well as anti-asthmatic agent [2]. Modern medicinal sector also acknowledge its inclusion as a therapeutic agent against numerous physiological disorders including, but not limited to, antioxidant, anti-inflammatory, antitumor, anticancer, antimicrobial, immunomodulatory and hepatoprotective agent [3–7]. Content of more than 400 gano-components had accredited Ganoderma as the “fungal biofactory”, “panacea” and the “elixir of life” [3–7]. Gano-components conferring medicinal values range from polysaccharides to triterpenes, sterols, proteins, peptides, fatty acids and vitamins [3–7]. Recently, utilization of G. lucidum in ameliorating neurodegenerative diseases like Alzheimer’s disease (AD), have received epoch-making attention [8]. We have also observed promising effect of G. lucidum as an AD therapeutic agent [9–15].

AD is a neurodegenerative disorder affecting mostly the elderly people is posing threat to the ever increasing aged humanity of the world. Currently, more than 46 million people over the world have been suffering from AD and this number had been projected to double by 2050 [16–17]. AD patients suffer from progressive loss of memory and learning abilities, behavioral abnormalities, disorientation about time and space, inability to smell and taste, difficulty in performing errands [17]. At severe stage, they become solely dependent on their family members and care-givers that lays extra economic burden on national and global health-care budget. Though the number of AD patients is skyrocketing, the world still awaits successful medication against this age-onset malady. AD occurs due to loss of neurons and synapses associated with
memory, learning and behavioral performances [17]. Since its identification in 1901, different hypotheses relating AD pathogenesis had been put forward. Among them, genetic predisposition, formation of amyloid beta (Aβ) plaques, neurofibrillary tangles (NFT) and mutation had received attention [14]. Recently, proteomics approaches towards understanding the mechanism and modulation of AD pathogenesis has received high attention [10, 18–21]. Differential expression of proteins between AD and normal subjects would aid in formulating therapeutic strategies against AD. Though different approaches of AD therapeutics had been linked with G. lucidum, there is scarcity of reports describing AD neuroproteomics modulating effect of G. lucidum. Thus, the present study had been designed to evaluate the AD neuroproteomics modulatory effect of G. lucidum, especially the expression pattern of proteins regulating neuronal structure and function.

2. Materials And Methods

a. Animals

Wistar male rats (120 ± 5 gm) were divided into three groups: control (C), AD (A) and G. lucidum hot water extract (HWE) fed AD (AE), each group containing 15 rats. AD model rats were prepared by infusing Aβ1−42 (ab120959, abcam, USA) into the cerebral ventricles following an established method [22]. 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)].

b. Brain sample preparation and protein quantification

Following our previously established method [10], rat brain samples had been prepared and protein quantified in the hippocampi of C, AD and AE rats. Briefly, protein extraction from the hippocampi had been performed by homogenization (brain sample 50 mg; lysis buffer 1 ml; homogenizer Polytron PT 1200, Kinematica); protein separation had been performed through SDS-PAGE [mini-PROTEAN tetra cell (165–8000, BIO-RAD, USA)] and protein quantification through LC-chip MS/MS Q-TOF [10]. To identify the proteins, the acquired MS/MS data were compared against the UniProtKB/Swiss Prot 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 were 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.

c. Statistical analysis

Data had been exported to the Mass Profiler Professional (MPP) software that analyzed depending on the MPP entities, the intensity of the total spectra of the proteins. Analysis of variance (ANOVA) had been performed to overcome the complications of false discovery associated with multiple test analyses.

d. 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. Functional interaction networks of the proteins were identified 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, coexpression, cooccurrence and text mining, using high confidence (0.7). For further identifying over-representing pathways and biological functions, the ingenuity pathway analysis (IPA), build version: 389077M, content version: 27821452, (Release date: 2016-06-14) was used (https://www.ingenuity.com/wp-content/themes/ingenuity-qiagen). Datasets of the proteins significantly expressed (P < 0.05) and having log fold change of 1.5 and higher were uploaded (AD versus C, AD versus AE and C versus AE).

3. Results

Liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) had identified 2,212 proteins had been identified with protein threshold at 95.0%, minimum peptide of 2 and peptide threshold at 0.1% FDR. Among them, 819 proteins were differentially expressed with statistical significance (P < 0.05). Among the significantly regulated (P < 0.05) 819 proteins, 498 met the criteria of fold change (LogFC of 1.5) cut off value. Number of proteins linked with AD was 40 (Table 1). Number of commonly expressed proteins was 761. The highest amount of proteins differentially expressed in the AD rats were those involved in maintaining neuronal structure and function (Table 1).
| No. | Protein                          | Swiss-Prot ID | M. wt. (KDa) | p value | AD vs. C Regulation | Log FC | AD vs. AE Regulation | Log FC | AE vs. C Regulation | Log FC |
|-----|---------------------------------|---------------|--------------|---------|---------------------|--------|----------------------|--------|----------------------|--------|
| 1   | Syntaxin 1A                     | Q9QXG3        | 33.38        | 1.09E-08 | down                | -15.97 | up                   | 16.57  | up                   | 16.31  |
| 2   | Syntaxin-1B                     | P61265        | 33.47        | 3.15E-13 | down                | -16.21 | up                   | 16.56  | up                   | 16.31  |
| 3   | Synaptogyrin-1                  | Q62876        | 25.90        | 0.038714 | down                | -15.72 | down                 | -5.45  | down                 | -6.06  |
| 4   | Neuromodulin                    | P07936        | 23.72        | 0.042242 | down                | 9.61   | up                   | 9.84   | up                   | 15.23  |
| 5   | Neural cell adhesion molecule 1 | P13596        | 95.46        | 0.036474 | down                | -15.72 | down                 | -5.45  | down                 | -6.06  |
| 6   | Synapsin-1                      | P09951        | 74.16        | 5.39E-07 | up                  | 16.92  | up                   | 16.92  | up                   | 16.92  |
| 7   | Synapsin-2                      | Q63537        | 63.74        | 7.18E-05 | up                  | 2.20   | down                 | -14.80 | down                 | -14.80 |
| 8   | Annexin                         | Q66HH8        | 35.83        | 0.02747  | down                | -15.78 | down                 | -6.36  | down                 | -6.36  |
| 9   | Synaptophysin                   | P07825        | 33.60        | 1.22E-12 | up                  | 16.92  | up                   | 16.92  | up                   | 16.92  |
| 10  | Synaptotagmin-1                 | P21707        | 47.74        | 1.15E-04 | up                  | 1.58   | down                 | -17.09 | down                 | -17.09 |
| 11  | Neuromodulin                    | P07936        | 23.72        | 0.042242 | down                | 9.61   | up                   | 9.84   | up                   | 15.23  |
| 12  | Neurochondrin                   | O35095        | 80.41        | 2.09E-09 | up                  | 17.00  | up                   | 16.92  | up                   | 16.92  |
| 13  | Septin-2                        | Q91Y81        | 41.76        | 7.80E-10 | down                | -16.09 | down                 | -16.09 | down                 | -16.09 |
| 14  | Isoform 2 of Septin-5           | Q9JJM9-2      | 44.38        | 1.09E-11 | down                | -17.09 | down                 | -17.09 | down                 | -17.09 |
| 15  | Neurofascin                     | R9PY05        | 138.87       | 0.024378 | down                | -9.67  | up                   | 4.01   | up                   | 13.68  |
| 16  | Serpina3n-like protein          | E0A3N4        | 48.21        | 7.49E-04 | up                  | 2.41   | down                 | -17.09 | down                 | -17.09 |
| 17  | Clathrin heavy chain            | F1M779        | 193.32       | 8.61E-04 | up                  | 1.63   | down                 | -17.09 | down                 | -17.09 |
| 18  | Annexin                         | Q66HH8        | 35.83        | 0.02747  | down                | -15.78 | down                 | -6.36  | down                 | -6.36  |
| 19  | Neurofilament heavy polypeptide | P16884        | 115.66       | 0.006005 | down                | -1.90  | up                   | 2.89   | down                 | -6.06  |
| 20  | Neurofilament light polypeptide | P19527        | 61.39        | 0.004508 | down                | -9.67  | up                   | 4.01   | up                   | 2.68   |
| 21  | Beta-soluble NSF attachment protein | F8WFM2     | 33.90        | 3.83E-08 | up                  | 15.83  | up                   | 15.28  | up                   | 15.28  |
| 22  | Heat shock protein HSP 90-alpha | P82995        | 85.21        | 2.71E-04 | up                  | 1.64   | up                   | 1.64   | up                   | 1.64   |
| 23  | Heat shock protein HSP 90-beta  | P34058        | 83.62        | 1.84E-04 | up                  | 1.71   | up                   | 1.71   | up                   | 1.71   |
| 24  | Heat shock 70 kDa protein 12A (Predicted), isoform CRA_a | D3ZC55      | 75.17        | 9.72E-08 | up                  | 16.07  | down                 | -14.60 | down                 | -14.60 |
| 25  | 14-3-3 protein zeta/delta       | P63102        | 27.94        | 2.73E-04 | up                  | 2.19   | down                 | -1.83  | down                 | -1.83  |
| 26  | 14-3-3 protein theta            | P68255        | 28.06        | 0.0126   | up                  | 1.91   | down                 | -1.83  | down                 | -1.83  |
| 27  | 14-3-3 protein eta              | P68511        | 28.38        | 0.00353  | up                  | 2.03   | down                 | -1.83  | down                 | -1.83  |
| 28  | 14-3-3 protein gamma            | P61983        | 28.47        | 0.019343 | up                  | 1.69   | down                 | -1.83  | down                 | -1.83  |

Here, C: control rats; AD: Alzheimer's disease model rats; AE: G. lucidum hot water extract fed AD rats; FC: fold change; KDa: kilo Dalton; M. wt: molecular weight.
| No. | Protein                          | Swiss-Prot ID | M. wt. (KDa) | p value     | AD vs. C Regulation Log FC | AD vs. AE Regulation Log FC | AE vs. C Regulation Log FC |
|-----|---------------------------------|---------------|--------------|-------------|----------------------------|----------------------------|----------------------------|
| 29  | 14-3-3 protein beta/alpha       | P35213        | 28.17        | 0.008179    | up                         | 1.86                       |                            |
| 207 | Ras-related protein Rap-1A     | P62836        | 21.33        | 0.04164     | up                         | 5.30                       | down -10.44                |
| 30  | Ras-related protein Ral-A      | P63322        | 23.72        | 1.62E-12    | down                       | -15.55                     | down -15.55               |
| 31  | Ras-related protein Rab-2A     | P05712        | 23.71        | 2.27E-09    | up                         | 17.04                      | down -15.85               |
| 32  | Ras-related protein Rab-7a     | P09527        | 23.79        | 1.85E-05    | up                         | 17.05                      | up 3.55                   |
| 33  | Ras-related protein Rab-11B    | O35509        | 24.60        | 3.01E-11    | up                         | 17.25                      | up 2.30                   |
| 34  | Ras-related C3 botulinum toxin substrate 1 | Q6RUUV5 | 21.85          | 5.65E-10     | down                       | -16.32                     | down -16.32               |
| 35  | Ras-related protein Rab-5A     | M0RC99        | 23.85        | 0.047559    | up                         | 10.75                      | down -4.90                |
| 36  | Ras-related protein Rab-18     | Q5EB77        | 23.26        | 6.58E-09    | down                       | -14.47                     | down -14.47               |
| 37  | Isoform Syn2 of Alpha-synuclein | P37377-2     | 15.87        | 1.05E-11    | down                       | -16.01                     | down -16.01               |
| 38  | Isoform 2 of Clathrin coat assembly protein AP180 | Q05140-2 | 91.66        | 0.003282    | up                         | 3.08                       | up 1.99                   |
| 39  | 4-nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog 1 (C. elegans), isoform CRA_b | G3V728 | 33.63          | 1.22E-12     | down                       | -15.22                     | down -15.22               |
| 40  | Excitatory amino acid transporter 1 | P24942 | 59.87        | 5.51E-10    | up                         | 17.81                      | up 3.44                   |

Here, C: control rats; AD: Alzheimer’s disease model rats; AE: *G. lucidum* hot water extract fed AD rats; FC: fold change; KDa: kilo Dalton; M.wt: molecular weight.

4. Discussion

3.1 Differential expression of the significantly regulated proteins

Differential expression of the proteins involved in 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 were observed in the different rat groups (C, A and AE) (Table 1). Associative learning and long-term memory related proteins glutathione-S-transferase 3 and tenascin 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 compared to the mushroom-treated (AE) group.

Proteins involved in synaptic organization (neurofascin) and synaptic vesicle budding (ADP-ribosylation factor 1), vesicle mediated transport (syntaxin 1A), neuronal differentiation and development (Dihydropyrimidinase-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 hippocampus 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, differentially up regulated expression of glial fibrillary acidic protein (GFAP) was also observed. GFAP is involved in long-term synaptic potentiation, neurotransmitter uptake, neurogenesis, glial and Schwan cell proliferation.

In the present study, down-regulated expression of memory and learning related proteins in the AD group was observed when compared with both C and AE group. However, there was inter-group variation in the extent of fold change in case of the down-regulated proteins. In the AD versus control group, down regulated expression of the proteins involved in dopamine decarboxylation, clusterin (stimulator of Aβ and NFT), neuromodulin, neurofascin and NCAM 1 was observed. In the AD versus AE groups, α-synuclein, synaptogyrin 1 and transgelin-3 were among the most important down regulated proteins. In addition to these, neuromodulin, excitatory amino acid transporter and park 7 were the mostly up regulated proteins in the AE versus C groups (Table 1).

Following are the AD related proteins differentially expressed in the present study

**Syntaxin-1A**

Syntaxin-1A regulates vesicular trafficking during exocytosis and trans-membranal protein insertion [22]. Decreased expression of syntaxin-1 A in the AD rats might have affected synaptic functions [23]. Mushroom treatment might have synaptic function improving effect as up-regulated expression of this protein has been observed in the mushroom-treated group.

**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 [24]. Its lowered expression in the AD and increased level in the AE rats reveals the ameliorating effect of *G. lucidum*.

**Neuromodulin (GAP-3)**

Neuromodulin is a neuronal growth and neurite forming protein whose level decreases in AD brains. As an CSF biomarker, its lowered level has been found in other studies also [25–27]. However, in the present study, mushroom treatment (AE) has been found to increase the abundancy of this protein as is evidenced by the up-regulated expression.

**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 [28–29]. Current findings were compatible with the previous ones as *G. lucidum* treatment helped increase the abundancy of NCAM.

**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 and AE 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 [30].

**Clathrin**

Clathrin group of proteins are involved in neuronal secretory functions and synaptic maintenance [31]. AD pathogenesis involves altered clathrin-associated membrane trafficking resulting in neurodegeneration [31]. Between the light and the heavy chains of clathrin, impaired distribution of the former has been linked with the AD pathogenesis [31–32]. Similar pattern was observed for the AD rats in the current experiment and an increasing trend following *G. lucidum* treatment (AE).

**Septin**

Septins are GTP-binding proteins found to be co-localized with the NFT in the AD brains [33]. Differential expressions of septins have been observed in the present study. Contrary to the findings of Musunury *et al.* (2014) [30] and Shin *et al.* (2004) [34], down-regulated expression of septin-2 had been observed in the AD versus AE group of the present study. As septin-2 is involved in synaptic plasticity, its down-regulation in the AD versus AE group bears supports to the synaptic dysfunction associated with the AD pathogenesis. However, its down-regulated expression in the CE versus AE group is of intriguing. Interestingly, the isoform-2 of septin-5 had also been found down-regulated in both the AD versus AE and AE versus C groups which is compatible with the findings of Musunury *et al.* (2014) [30], who found similar expression status in the temporal brain neo-cortex of the AD patients. Thus, differential expression even of the different isoforms of the same protein might be
implicated in the AD pathogenesis and corresponding modulation demands differential therapeutic strategy. Current observation of the G. lucidum HWE upon differential expression of different isoforms of septin is a unique finding that demands further studies.

**UCH L1**

Ubiquitin carboxyl-terminal hydrolase L1 (UCH L1) is an important enzyme for maintenance of cognitive and synaptic functions [35]. Conflicting information regarding its expression has been documented in different AD cases. Though most of the researchers have noticed decreased and oxidatively modified form of UCH L1 in AD subjects [36–38], Sultana et al (2007), reported its 1.31-fold increase in the AD brain hippocampi [39]. Increased expression of UCH L1 was observed in the G. lucidum-treated (AE) group.

**Soluble NSF-attachment protein beta (SNAP-β)**

N-ethylmaleimide sensitive fusion proteins (NSF) are the part of APP and overexpressed in AD [40]. 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 [41].

**Neuropolypeptide h3**

Neuropolypeptide h3 is a cholinergic neuro-stimulating peptide that falls in the phosphatidyloethanolamine binding protein group and is also known as Raf-kinase inhibitor protein (RKIP) and/or hippocampal cholinergic neurostimulating peptide (HCNP) [36]. Our finding of downregulated neuropolypeptide h3 is in agreement with those of Butterfield (2004) [42]. Oxidatively modified loss of function of neuropolypeptide 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 [43]. Also, neuropolypeptide 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 [44–45]. Thus, in AD brains, neuropolypeptide h3 is linked with cholinergic abnormalities and altered lipid metabolism that are the early events in AD pathogenesis [46].

**Annexin**

AD rats showed increased expression of annexin in the hippocampi. Previous studies have linked increased plasma annexin5 with increased AD risk [47]. Transgenic AD mice (Tg2576) also expresses increased annexin in the brain cortex [48].

**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. Overactivity of GSK-3β has been linked with elevated Aβ production, tau hyperphosphorylation and impaired memory and learning activities [49]. Memory affecting mechanism of GSK-3β involves interruption of intra-neuronal anterograde mitochondrial transportation and causation of “mitochondrial traffic jam” [50–51].

**Serine/Threonine protein phosphatase**

Serine/Threonine protein phosphatase negatively regulate memory and learning abilities by impairing synaptic plasticity and LTP [52]. Upregulation 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 [53]. 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 [54].

### 4.2 PPI network of the upregulated protein clusters in the AD vs AE group

In the AD versus AE group, the most notable protein networks included those involved in the biological processes such as redox mechanisms, neurogenesis, neurotransmission, neuronal development and metabolism (Fig. 1). Interacted protein networks involved in molecular functions such as SNARE binding, syntaxin binding, syntaxin-1 binding were also enriched (Fig. 1).

The main network of the upregulated proteins can be divided into several subsets with respect to their gene content such as sub-set 1 consisting of synaptogamin-1, syntaxin 1A, syntaxin 1B, beta soluble NSF attachment protein (Syt1, Stx1a, Stx1b and Napb), sub-set 2...
including AP2 forming proteins and clathrin heavy chain (Ap2a2, Ap2a1, Cltc), sub-set 3 consisting of metabolic enzymes NADH dehydrogenase, dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, pyruvate dehydrogenase E1 component subunit beta and isocitrate dehydrogenase (Ndufv2, Ndufa9, Dlat, Pdhb and Idh3a) and those involved in signal transduction, membrane trafficking and cytoskeletal maintenance (Ubb, Vcp, C3, Rab7a, Rab11b, Gpi and HK1) (Fig. 1). Besides the main network, two other protein-protein interaction networks involved immune-regulatory proteins (Igga-2a, Igga-1a, IgkC and Crp) and proteins associated with Ca\(^{2+}\)-mediated signaling and metabolism (Calm1, Camk2a, Camk2b and Ppp3ca) (Fig. 1).

### 3.3 Identification of functional pathway interaction through IPA

Based on ingenuity pathway analysis 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”. Core analysis of IPA was performed to interpret the datasets in the form of their functional networks. In the IPAKB, corresponding objects were mapped with the protein identifiers [55]. Depending on the physical interaction (direct relationship) among the eligible proteins, IPAKB generated the networks and the score (probability value) of the networks [55]. Higher the network connectivity, greater is the representation of significant biological functions of the relevant genes [55]. Statistical justification of the network connectivity has been performed through measuring "p scores (\(-\log_{10} p\) value, Fisher's exact test)" and “network score”. Network score has also been measured through Fisher's exact test that is based on the focus protein and biological functions and thus shows the relevancy of the analysis.

#### 3.3.1 Functional network in AD vs AE

IPA of the AD vs AE identified 20 networks of which the top-most one had score of 75 and focus molecules 83 among 140 total selected molecules (Fig. 2). The top-most network functions include cell death and survival, cell morphology and organ morphology. The second one had score of 56 and focus molecules 73 among 140 total selected molecules (Fig. 2). The second-top network related functions include molecular transport, cell signaling, nervous system development and function categories. The third network had score of 8 and 25 focus molecules among 135 total selected molecules involved in CVD and functions, cellular and organismal development (Fig. 2).

Up to present day, about 96 proteins have been reported to be linked with AD [30]. Most of the differentially expressed proteins identified in the present study fall within that category. The reason for not detecting direct expression of APP or A\(\beta\) in the AD rats of the present study might be that A\(\beta\) was infused intra-ventricularly and this does not fall under the category of expression or repression. But its effect is revealed through up- and/or – down regulation of multiple proteins in the AD subjects. Provided the same amount of protein being processed for quantification, variability in the output may be considered due to reduced accessibility of the AD proteins towards trypsin digestion as the proteins in AD subjects are much aggregated and less soluble compared to the controls. Importantly, inability to detect proteins of target does not imply their absence in the sample; rather it is an indication of their differential expression. As the AD rats had been found to experience impaired memory and learning activities and altered expression and networks (both interacting and functional pathways, as revealed by the STRING and IPA analysis, respectively) of the memory and learning related proteins, differential expression of the proteins might be attributed to the infused A\(\beta\) in the respective model animals [8–12]. On the other hand, proteins up- and down-regulated in the control and G. lucidum treated animals might have been involved in improving those derangements and thus ameliorating the AD consequences as observed through memory related tests described elsewhere [8–12]. Another important feature of the present study is that some of the proteins differentially expressed in the AD model rats are highly interacted with each other and formed pathway-based functional networks. This inter-relationship sets a novel ground for AD therapeutics. Thus, findings of the current research suggest a novel target for AD pathogenesis management and incorporation of G. lucidum as an AD ameliorating agent seems pertinent. Differential expression of the proteins in the mushroom-fed AD rats might either be an adaptive response or protective strategy against A\(\beta\)-mediated stresses. Admittedly, the regulated proteins, identified in the current experiment but not previously been studied, warrants extensive exploration for much conclusive remarks. In this endeavor, western blot analysis of the mostly regulated proteins for validation of the current findings is the immediate future aspect of the present study.

### 5. Conclusion

Present study indicates the Alzheimer's disease modulatory effect of medicinal mushroom G. lucidum through differential expression of neuronal structure and function related proteins, restoration of disrupted protein-protein interaction network and maintenance of integrated pathways. Thus, utilization of G. lucidum in AD therapeutics seems promising. However, further studies should be carried out to determine therapeutic dosage, toxicity and safety.

### Abbreviations

A
Alzheimer's disease model rat; AE: *Ganoderma lucidum* hot water extract fed Alzheimer's disease model rat; C: Control rat; AD: Alzheimer's disease; GL: *Ganoderma lucidum*; HWE: Hot water extract; FC: fold change of protein; KDa: Killo Dalton; M.wt: Molecular weight; PPI: Protein-protein interaction; IPA: Ingenuity pathway analysis; STRING: Search Tool for the Retrieval of Interacting Genes/Proteins.

**Declarations**

**Ethics approval and consent to participate:** 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)].

**Consent for publication:** All authors consented to publish.

**Availability of data and material:** Not applicable.

**Competing interests:** Authors declare no competing interest.

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**Authors’ contributions:** Mohammad Azizur Rahman designed and conducted the experiments, interpreted outcomes, composed manuscripts. Other authors supervised the experiments.

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Figures

Figure 1

Protein-protein interaction (PPI) of AD versus AE rat groups.
Figure 2

Integrated pathway analysis (IPA) of AD versus AE rat groups.