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Rat hippocampal proteomic alterations following intrahippocampal injection of amyloid beta peptide (1–40)

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ABSTRACT

Amyloid beta peptide 1–40 ($A\beta_{1-40}$) is closely associated with the progressive neuronal loss and cognitive decline observed in Alzheimer's disease (AD). This study aimed to establish a proteomic strategy for the profiling of AD tissues for disease-specific changes in protein abundance. Intrahippocampal injection of $A\beta_{1-40}$ induced spatial memory and learning decline in rats. Proteomic analysis revealed the changes in protein expression in the rat hippocampus treated with $A\beta_{1-40}$. Four proteins of interest which was in abundance was significantly altered in $A\beta_{1-40}$-treated rats were identified by peptide mass fingerprint (PMF). These proteins corresponded to synapsin Ib, protein disulfide-isomerase A3 precursor, tubulin β chain and ATP synthase β subunit. Our results provide new insights into the relationship between Aβ and the pathogenesis of AD, and suggest potential targets for the therapy of AD.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, and represents the most common form of dementia in elderly people [15]. AD is characterized by the deterioration of cognitive and mental functions including learning and memory skills [6]. Extensive studies have indicated that the amyloid β peptide (Aβ) deposited as plaques in AD contributes to the neuronal cell loss and pathogenesis of AD [13,20]. However, prevention or removal of Aβ deposits improves cognitive performance in animals [2,26]. $A\beta_{1-40}$ is the prone-to-aggregation product of amyloid precursor protein (APP) proteolytic cleavage, and is the major constituent of senile plaques observed in the brain of AD [22]. Recently, Passos and colleagues described a mouse model of acute inflammation induced by $A\beta_{1-40}$ intracerebroventricular injection that appears to mimic the early phase of AD progression [16,18]. Studies indicated that specific proteins associated with $A\beta_{1-40}$ in AD exert a significant effect on multiple neurosignaling pathways [1]. However, the precise role of this protein in the memory loss and neuronal degeneration associated with AD are not well understood.

In this study, a rat model with a spatial memory retention lesion, assessed by step-down passive avoidance test and Morris water maze (MWM) test, was established using bilateral injection of $A\beta_{1-40}$ into hippocampus. To observe the protein changes responding to $A\beta_{1-40}$ exposure and screen for potential markers associated with the spatial learning and memory decline in the rats hippocampus at the initial stage of AD development, we used a proteomics approach that combined two-dimensional gel electrophoresis (2-DE) with MALDI-TOF mass spectrometry (MS).

Eight-week-old male Sprague-Dawley rats (SCXK 2003-0002, $n = 14$) weighing 200–220 g were obtained from Shanghai Super B&K Laboratory Animal Co. Ltd. The rats were housed in plastic cages with food and water supplied *ad libitum*, and were maintained on a 12-h light/dark cycle at room temperature (21–23 °C). All experimental protocols and animal handling procedures were approved in advance by the Animal Care and Use Committee of Dalian Medical University, and were consistent with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

$A\beta_{1-40}$ (Sigma) solution was dissolved in Milli-Q water at a concentration of 5 μg/μl and incubated at 37 °C for 7 days prior to use

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[8]. Rats were anesthetized with sodium pentobarbital (45 mg/kg, i.p.) and placed in a stereotaxic instrument (Model 900, David Kopf Instruments, CA, USA). An incision was then made into the scalp and the scalp was retracted. Holes were then drilled into the skull using a dental burr. Stereotaxic coordinates were, anterior–posterior (AP) = −3.5 mm, medial-lateral l (ML) = 2.0 mm from the bregma and dorsal–ventral (DV) = 2.7 mm from the dura. 14 rats were randomly divided into two groups: (1) Milli-Q water-injected rats served as the control group and (2) fibrillar neurotoxic $A\beta_{1-40}$-injected rats served as the $A\beta_{1-40}$ group. Fibrillar $A\beta_{1-40}$ or Milli-Q water (2 μl) was injected at a flow rate of 0.5 μl/min. Following injection, the needle was maintained in place for 5 min prior to its slow extraction. A second injection was also administered at the same coordinate in the opposite hemisphere [25].

The behavioral performances of animals were assessed by the step-down passive avoidance test and Morris water maze test as previously described [9]. The details are available in Supplementary Material. At the end of behavioral testing, rats were deeply anesthetized with 2% sodium pentobarbital (60 mg/kg), and the hippocampus was rapidly removed and stored at −80 °C.

The freezing hippocampus sections were analyzed for Aβ immunostaining by immunohistochemistry. The details are described in the methods of Supplementary Material.

Hippocampus was homogenized mechanically in 1 ml of ice-cold lysis buffer containing 1 M HEPES (pH 7.4), 10 mM NaCl, 0.2 M Na_3VO_4, 0.5 M EGTA, and 10% Triton X-100. The homogenate was then centrifuged at 13,000 rpm for 5 min and the supernatant was collected. Protein concentration was measured using the 2D Quant protein assay kit (GE Healthcare).

Protein samples were then subjected to 2-DE analysis. Total protein (100 μg) from each sample was diluted in 2× rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG buffer and 20 mM DTT. Then, 13-cm 3-10 NL IPG strips were subjected to electrophoresis using an Ettan IPGphor II system (GE Healthcare). Focusing was carried out using the following conditions: (I) 30 V, 12 h, step, (II) 500 V, 1 h, step, (III) 1000 V, 1 h, step, (IV) 1000–8000 V, 13,500 Vh, gradient, and (V) 8000 V, 56,000 Vh. Prior to the second dimension, the IPG strips were equilibrated twice with solutions containing 100 mM Tris–HCl (pH 8.0), 6 M urea, 30% glycerol, 2% SDS, and a trace amount of bromophenol blue. DTT (1%) and IAM (4.5%) were added to the solutions during the first and second equilibration steps, respectively. IPG strips were then placed on top of 12.5% polyacrylamide gels precast with low-fluorescence glass plates. SDS-PAGE was carried out at 30 mA for 3 h at 20 °C, or until the bromophenol blue dye-front reached the bottom of the gel. The separated proteins were then visualized using silver staining as described by Yu [29].

For 2-DE gel analysis, 0.5 mg of total protein was loaded onto the gel and the protein spots visualized using a modified silver-staining method compatible with MS analysis [28]. The silver-stained 2-DE gels were acquired using a GS-710 imaging densitometer and the digitized images were analyzed with imagemaster software (GE Healthcare). The protein expression levels of differentially spots were analyzed using a Student's t-test. The spots of interest with an average ratio more than 1.5 or less than −1.5 ($p < 0.05$) between controls and $A\beta_{1-40}$-treated rats were selected for further analysis.

Gel plugs were digested in-gel as described previously [23]. Briefly, gel plugs were destained in 30 mM potassium ferricyanide/100 mM sodium thiosulfate (1:1, v/v) for 20 min, and equilibrated and dehydrated in two 10-min changes in 25 mM ammonium bicarbonate and 100% ACN, respectively. The dehydrated gel plugs were then incubated in 10 mM DTT at 56 °C for 45 min, cooled to room temperature and incubated in 55 mM IAM. Protein within the gel was then digested with porcine modified trypsin (Promega) in 25 mM ammonium bicarbonate for 16 h at 37 °C. Tryptic peptides were then extracted from the gel in two washes of 50% ACN and 1% trifluoroacetic acid (TFA). The combined extract solution was then concentrated using a speed vacuum.

All mass spectra data was acquired using an AutoFlex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). A 1-μL aliquot of the peptide extract was then pre-mixed with 1 μL of matrix containing 10 mg/mL CHCA in 35% ACN and 0.1% TFA, and spotted onto a MALDI target plate. The spectrometer was operated in the delayed extraction and positive-ion linear mode with the following parameters: 20-kV acceleration voltage, 95% grid voltage, 100-ns delay time and 500-m/z low-mass gate. For the acquisition of a mass spectrometric peptide map, measurements were externally calibrated using a standard peptide mixture of angiotensin II, and internally recalibrated with peptide fragments arising from autoproteolysis of trypsin. The protein mass fingerprint (PMF) data was combined using Flex Analysis and the combined data set was submitted to MASCOT for protein identification by searching against the National Center for Biotechnology non-redundant (NCBInr) database. Peptides were identified by matching with sequences originating from *Rattus* taxonomy. All cysteine residues were searched as carboxylamidomethyl-cysteine (+57.0215 Da), and methionine residues were allowed to be oxidized (+15.9949 Da). The peptide mass tolerance was initially set at 100 ppm and the fragment mass tolerance was set at ±0.5 Da. Only significant hits as defined by the MASCOT probability analysis ($p < 0.05$) were accepted.

Proteins identified as tubulin β chain, synapsin Ib and ATP synthase β subunit by MS were further investigated by western blotting. The details are described in Supplementary Material.

All data were presented as mean ± standard deviation (S.D.). Mean daily measures of MWM were subjected to repeated measures ANOVA. Mauchley's Test was used to evaluate the sphericity of within-subject effects and when necessary, Greenhouse-Geisser was applied to adjust the degrees of freedom. Differences of single day between groups in WMW and other data were analyzed by Student's t-test. $p < 0.05$ was considered significant. All analyses were performed using SPSS v 13 (Chicago, IL, USA).

For the step-down passive avoidance test, longer step-down latencies and greater number of errors were found in $A\beta_{1-40}$-treated rats than the control. During the memory performance test, the rats treated with $A\beta_{1-40}$ were observed shorter step-down latencies and more errors than the vehicle-treated rats [9]. In MWM test, rats showed significant difference in escape latency between training days ($p = 6.07 \times 10^{-5}$) and groups ($p = 0.002$), but no interaction between the factors of day and groups ($p = 0.152$). The $A\beta_{1-40}$-treated rats required longer to find the platform hidden under the water [9] (Fig. 1), which was consistent with the findings previously reported [17]. In the visible platform test, the latency for each group was similar (20.56 ± 11.31 s in control and 14.68 ± 9.97 s in $A\beta_{1-40}$ group, $p = 0.304$), indicating that the animals did not present sensorimotor deficits. These data suggested that the rats had impaired learning and memory function in $A\beta_{1-40}$ group.

The morphological features of Aβ immunostaining in rat hippocampus infused with or without $A\beta_{1-40}$ peptides are shown in Supplementary Material Fig. 1. Stronger Aβ immunostaining on the image of hippocampus combined with the worse behavioral performance proved that the $A\beta_{1-40}$-treated model was constructed successfully.

Approximately 2000 spots were detected on the gel image and more than 1500 ones were matched in all gels. 13 differentially expressed protein spots were found by the 2-DE analysis using Student's t-test. Among these spots, 6 ones were significantly up-regulated and 7 ones significantly down-regulated in $A\beta_{1-40}$ group comparing with the control (Supplementary Material Table 1 and Fig. 2).

Table 1
Identification of the differentially expressed protein spots by mass spectrometry.

Spots no.	Protein name	Accession no. (GI)	Matched peptides	Sequence coverage (%)	Mw (kDa)/pI	Score	Function
376	Synapsin Ib	206933	8	17	70.0/9.84	73	Neuronal phosphoprotein
594	Protein disulfide-isomerase A3 precursor	1352384	11	22	57.0/5.88	121	Isomerase
670	Tubulin beta chain	4507729	13	26	50.3/4.78	75	Microtubule constituent
755	ATP synthase beta subunit	1374715	15	44	51.2/4.92	146	Protein metabolism

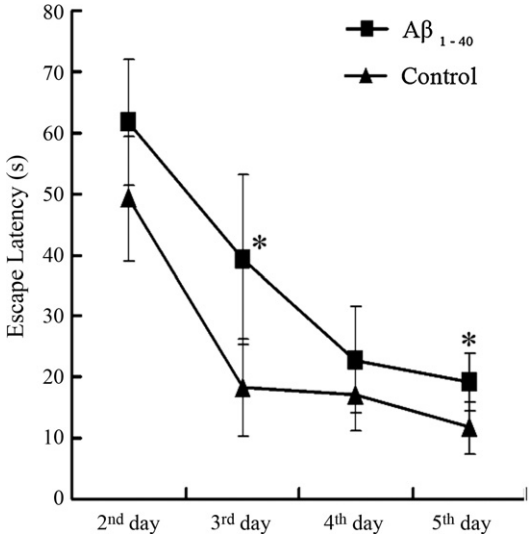

Fig. 1. Escape latency for the Morris water maze test. Results are presented as the mean ± S.D. $^{*}p < 0.05$ when $A\beta_{1-40}$ group was compared to the control.

Four proteins were successfully identified by MALDI-TOF/TOF MS (Table 1). The identity of spots 376, 594, 670 and 755 corresponded to synapsin Ib, protein disulfide-isomerase A3 precursor, tubulin β chain and ATP synthase β subunit, respectively (Fig. 2). In the up-regulated protein spots, spots 1699, 1596, 374 and 529 were analyzed by MS but did not reach the standard of successfully identified.

The levels of differentially expressed proteins tubulin β chain, synapsin Ib and ATP synthase β subunit were further tested via Western blot analysis. Tubulin β chain was significantly down-regulated in $A\beta_{1-40}$ group ($p = 0.014$) when compared with control (Fig. 3A and B). Similar changes were also observed for ATP synthase β subunit ($p = 0.010$) and synapsin Ib between $A\beta_{1-40}$ and control groups (Fig. 3A, C and D), but not significantly for synapsin Ib ($p = 0.295$). The results of western blot were consistent with the proteomic analysis, confirming the reliability of the proteomics methodology.

Aβ plays a critical role in the neurotoxicity and subsequent development of amyloid plaque and contributes to the pathology of AD [7]. The neurotoxic effects of $A\beta_{1-40}$ have been confirmed in primary cultures of rat cortical neurons and hippocampal slice *in vitro*, revealing that toxicity may rely on the aggregation of $A\beta_{1-40}$ [14]. Furthermore, *in vivo* neuroanatomical and neurochemical studies have indicated that the neurotoxicity of $A\beta_{1-40}$ may also be directly observed in the rat brain [10,11]. The results of our current work indicated that exogenous accumulation of $A\beta_{1-40}$ in the rat hippocampus may promote spatial memory and learning decline in rats during the initial stages of AD development.

The proteins identified here have been reported to be potentially associated with AD and AD-like disease. However, to the best of our knowledge, the current work describes for the first time that these proteins are closely associated with the spatial memory decline induced by $A\beta_{1-40}$ injection into the rat hippocampus *in vivo*.

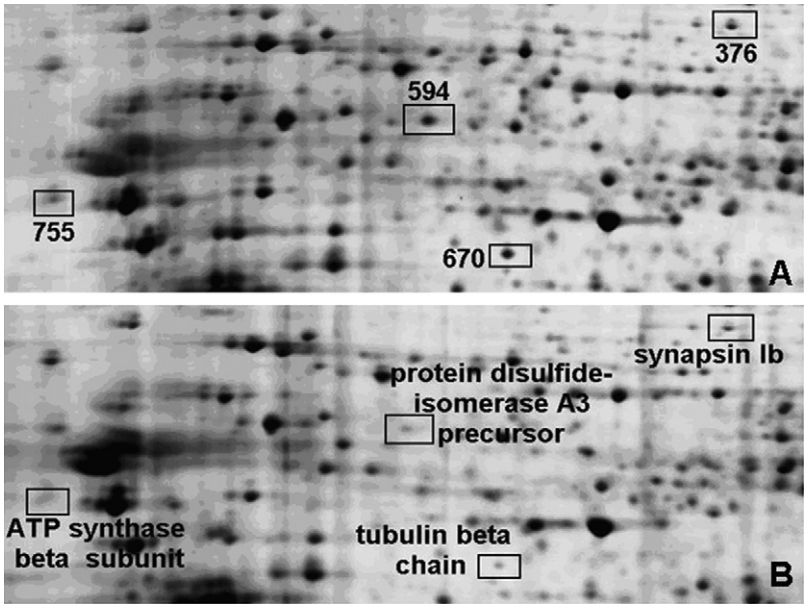

Fig. 2. The location of spots successfully identified by MS on the 2-DE gel. (A) The spots are marked with their spot numbers on the control group gel. (B) The spots are marked with the name of the matched proteins identified by MS on the $A\beta_{1-40}$ group gel.

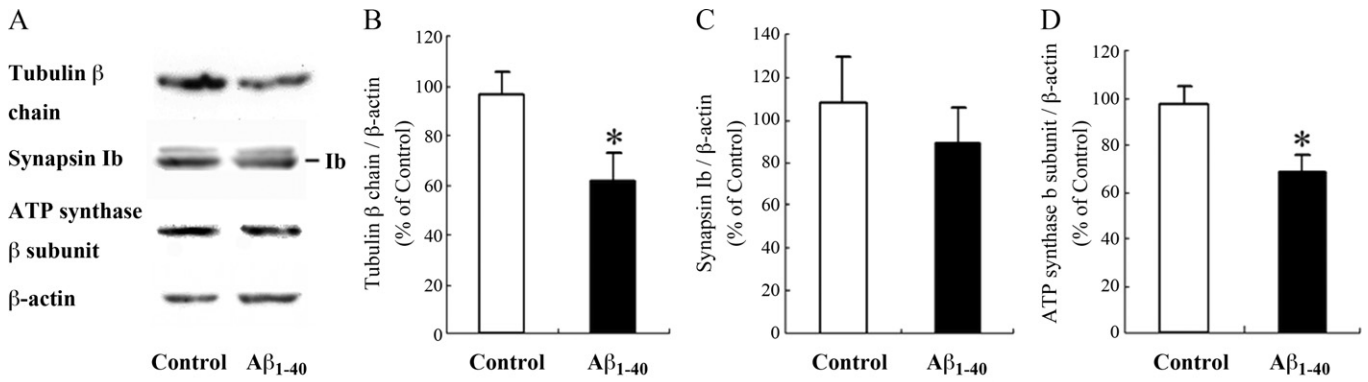

Fig. 3. Western blot analysis of three differentially expressed proteins in the hippocampus (A), corresponding to tubulin β chain (B), synapsin Ib (C) and ATP synthase β subunit (D), respectively. Blots were digitized and the bands were quantified using an image analysis system. Bars represent the means ± S.D. from 3 independent experiments. $^{*}p < 0.05$ when $A\beta_{1-40}$ group was compared to the control.

As a core protein of microtubules and cytoskeleton, tubulin facilitates the transportation of membrane-bound organelles, and the extension and maintenance of neurites. The oxidation of tubulin makes its function idle, compromises cellular structure and results in neuronal connection and communication deficiencies. As a consequence, neurodegeneration may occur [3]. Interestingly, our results indicate that the tubulin β chain is down-regulated in the hippocampus of $A\beta_{1-40}$-treated rats, which may provide a novel insight into neurodegenerative disease research.

Synapsin I is a member of the family of neuron-specific phosphoproteins and is involved in synapse formation, synthesis of synaptic vesicle proteins and the release of neurotransmitters [12,19]. Synapsin I is thought to be associated with axonogenesis and synaptogenesis via regulation of nerve terminal function in mature synapses. The cognitive alterations observed in AD patients are most likely associated with synaptic loss and neurofibrillary pathology in the limbic system and neocortex [5]. Thus, our proteomic results indicate that the decreased expression of synapsin Ib in the rat hippocampus may induce the decline in spatial learning and memory ability. Although not significantly, the western blotting analysis indicated the decline trend of synapsin Ib in the $A\beta_{1-40}$-treated hippocampus, which also supported the proteomic results.

ATP synthase is a key enzyme required for mitochondrial function. Previous studies have indicated that the failure of mitochondrial electron transporting chain enzymes may generate reactive oxygen species (ROS) that cause neuronal death. The ATP synthase subunit β was found to be significantly down-regulated (50–60%) at an mRNA level in the temporal cortex of AD patients [4]. Abnormal ATP synthase expression has also been observed in protein samples extracted from the hippocampus of AD patients [21]. Our proteomic results demonstrated that the ATP synthase β subunit is decreased in the hippocampus of rats exhibiting spatial memory deficits, implicating the ATP synthase β subunit as a possible novel marker for AD.

Protein disulfide-isomerase (PDI) catalyzes the thio-disulfide interchange processes required for the folding of normal proteins in the endoplasmic reticulum (ER) [27]. PDI prevents neurotoxicity caused by ER stress and protein misfolding. The up-regulation of PDI is thought to be an adaptive response to protect neuronal cells [24]. It has been reported that the NO-mediated S-nitrosylation of PDI may inhibit its function, which leads to dysregulated protein folding in the ER, and consequently results in ER stress that promotes neuronal cell death [24]. Consistent with these findings, our current study indicates that PDI is down-regulated in rat hippocampus exposed to $A\beta_{1-40}$, implicating the importance of PDI in neurodegenerative disease.

In the current study, a rat model with lesions in spatial memory retention due to the injection of $A\beta_{1-40}$ into hippocampus has been proven to be an ideal animal model for the study of neurodegenerative diseases in their initial stages *in vivo*. The proteomics approach combining 2-DE with MALDI-TOF MS was successfully established and applied to obtain the differentially expressed proteins in response to $A\beta_{1-40}$ injection in the rat hippocampus. Data suggest that the behavioral deficits observed in these animals at the initial stage were associated with changes in four proteins, including synapsin Ib, protein disulfide-isomerase A3 precursor, tubulin β chain and ATP synthase β subunit in the hippocampus. These proteins may also serve as potential protein indicators. In addition, this work suggests that the protein expression maps of the rat hippocampus can be used as a reference source for future investigation into the molecular mechanisms underlying the brain pathogenesis during the initial stages of neurodegenerative disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2011.06.009.

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