

Proteins Expressed in the Hippocampi of C57BL/6J Mice During Spatial Learning and Memory

¹Seyedeh Zeinab Taheri Mirani, ¹Perumal Ramasamy and ²Than Myint

¹Department of Biomedical Sciences and Therapeutic, School of Medicine,
Universiti Malaysia Sabah, 88450, Kota Kinabalu, Sabah, Malaysia

²Department of Community & Family, School of Medicine,
Universiti Malaysia Sabah, 88450, Kota Kinabalu, Sabah, Malaysia

Abstract: Studies have indicated that protein synthesis is required for the initiation of the molecular process for memory consolidation and storage. The hippocampus has been shown to play a critical role in this process. The Morris water maze (MWM) was used to study the effect of training on hippocampus-dependent learning. Using two-dimensional gel electrophoresis and MALDI TOF/MS mass spectrometry, we determined new proteins expressed in the hippocampal tissue of C57BL/6J mice during long-term memory consolidation. A total of 18 male mice were divided into 3 groups; 6 trained to find a hidden platform following cues on the wall of the pool, 6 swim controls that underwent training without the platform and 6 resting controls that were kept in home cages without exploring the MWM. Over nine days, 4 trials of 150 s each, were conducted daily. In each case mice were sacrificed at the end of the experimental procedure, the proteins were extracted and subjected to iso-electric focusing in one direction and polyacrylamide gel electrophoresis in the second direction. The statistical analysis of protein spots on 2D gel showed significant intensity in 9 spots. The proteins identified are known to be involved in energy metabolism, signal transduction, chaperones, cytoskeletal system and transporter function.

Key words: Hippocampus • Spatial memory • Consolidation • Protein synthesis

INTRODUCTION

Learning is a long process. The Morris water maze is a tool that is extensively used to investigate molecular and cellular changes in spatial learning and memory. This task has both spatial learning as well as swimming stress [1]. The mouse is used mostly as an experimental animal to elucidate the function of genes and proteins in the molecular mechanism underlying physiological and pathological conditions [2]. Proteomics and mass spectrometry can be used together to investigate the role of protein expression in learning and consolidation of memory in the mouse hippocampus, a critical region of the brain. Memories are formed and stored temporarily in the hippocampus. For permanent memory storage, they are transferred to distal cortical sites in the consolidation system [3, 4]. The objective of the present study was to compare protein expressions during acquisition and

control performances in mice. Memory formation is associated with the activation of many synaptic plasticity-related proteins, such as N-Methyl-D-aspartic acid (NMDA) receptor, Ca^{2+} /Calmodulin-dependent protein kinases (CaMKK, CaMKII, CaMKIV), mitogen-activated protein kinases (MAPKs), the cAMP-response element binding proteins (CREB) and dopamine receptors [5, 6]. Biochemical cascades, such as the influx of Ca^{2+} , persuade the binding of calcium/calmodulin to CaMKIV and initiate the phosphorylation of CREB ser¹³³ which is the crucial molecule for long-term potentiation. Up-regulation of these proteins occurs at different times during learning. These proteins achieve the final modification of learning in a synaptic system within a neuronal network [7].

Besides of proteins which are involved in cell molecular pathway; there are proteins such as neurofilaments (NF) and nerve growth factor (NGF)

Corresponding Author: Seyedeh Zeinab Taheri Mirani, Department of Biomedical Sciences and Therapeutic, School of Medicine, Universiti Malaysia Sabah, 88450, Kota Kinabalu, Sabah, Malaysia.
Tel: +60-178190522.

families which are caused of axonal growth have a crucial role in neuronal plasticity learning cognition and lack of this protein has been observed in degenerative disease like Alzheimer disease [8-11].

MATERIALS AND METHODS

Animals: Eighteen 2 month old male C57Bl/6J mice from BioLasco Taiwan were used. They were housed in sanitized polycarbonate cages (25×47.5×20cm) in a room with controlled temperature (22±1°C) under alternate 12 h light/ dark cycle with access to food and water ad libitum. All experimental procedures were performed in accordance with the ethical committee of School of Medicine University Malaysia Sabah.

Behavioral Study: All mice were tested at the same time, 9 am, before starting training. All mice were placed in the training room for 30 min every day.

Morris Water Maze: Behavioral testing was done using a Morris water maze (MWM) to evaluate spatial learning. The apparatus consisted of a circular tank (182 cm diameter and 90 cm height) placed 10 cm above the floor. The pool was filled with water rendered opaque with nontoxic white pastel color and an escape platform was hidden 0.5 cm below the water surface in one of the quadrants of the pool. The water temperature was kept at 23±1°C during the training. The pool had visual cues fixed on the walls to help the animal to spatially learn the location of the hidden platform. Each trial was recorded automatically by plus 2020 video tracking system software [12].

Behavioral Procedure: The experimental mice consisted of three different groups: quiet controls, swim and training. The Morris Water Maze was divided into four quadrants (N, S, W and E). In the training phase the mouse was allowed to swim to locate the escape platform within 150 s. Each animal received this training for nine days, four sessions per day with 15 min inertial interval. The mice released from one of the four start locations had to search for a hidden platform under the water surface. The platform was located in the same position during the training days. In free swim group (n=6), the mice had to swim 150 sec without the platform. The quiet control group did not receive any training and the mice were left in their cages until tissue processing. The escape latencies, total distance swum and time spent in each quadrant were monitored and analyzed by the video tracking system (Plus 2020, United Kingdom).

Tissue Preparation: Animals from the trained, swim and quiet control groups were sacrificed, at the end of their respective sessions, by cervical dislocation and the hippocampus was dissected out. The tissue was homogenized and suspended in lysis buffer [6M Urea (Sigma, USA), 2M Thiourea (Fulka, USA), 30mM Tris pH 8.8 (Sigma), 4% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1propanesulfonate) (Sigma)] and left 30 minutes in ice. The homogenate was centrifuged at 4°C, at 13,000g for 15 min. The supernatant was collected [13]. The protein content of the supernatant was determined using the 2D Quant kit (Amersham, U.K.) and the absorbance was measured at 480 nm.

Two-Dimensional Gel Electrophoresis: 2-D gel electrophoresis was run on proteins isolated from hippocampi prepared from the mice. 40 µg of protein were applied on immobilized pH 3-10 gradient strips. Focusing was started at 200 V and the voltage gradually increased to 8000 V. After the first dimensional electro focusing, the strips (13 cm) were equilibrated for 15 min in the buffer pH 8.8, containing 6M urea, 50mM Tris, 30% glycerol, 2% SDS, 1% DTT and then suspended for 15 min in the same buffer containing 2.5% iodo-acetamide, instead of DTT. After equilibration, the IPG strips were loaded onto 12.5% sodium dodecyl sulfate polyacrylamide gels for the second-dimensional separation. Standard protein markers (Biorad, USA) were used for molecular mass determination (10-250kDa and pI 3-10). Immediately after the run, the gels were fixed in 50% ethanol and 12% acetic acid overnight. Then all gels were stained with silver nitrate. The gels were then scanned using Image Scanner Software (Amersham, USA). Based on the protein spots configurations on the gels, they were quantified using 2D-platinum software (Sweden).

Gel Image Analysis: All the gels were scanned using Image Master Scanner with the Lab Scan software (Amersham Bioscience, Sweden). Analysis of the gel maps to compare protein spots between the control and trained groups was carried out using 2D-platinum software (GE Healthcare, Sweden).

Analysis of Peptides with Matrix-assisted Laser Desorption Mass Spectrometry: A total of 18 (2 x 9) most significant spots were selected by the software for protein identification. All spots were manually eluted out and washed with 10 mM ammonium bicarbonate and 50 % acetonitrile in 10 mM ammonium bicarbonate. The spots were shrunk with additional acetonitrile and then dried. The dried samples were digested in 40 ng/µl

trypsin in digestion buffer, including 5mM octyl β -D-glucopyramidinase (OGP) and 10 mM ammonium bicarbonate and incubated overnight at 37°C. Peptide extraction was performed in 20 μ l of 1 % TFA and 5 mM OGP for 30 minutes and subsequently in 0.1 % TFA and 4% acetonitrile for 30 min. The extracted peptides were used for MS and MS/MS Spectra analysis. These were obtained using ABI 4800 Proteomics Analyzer MALDI-TOF/TOF Mass Spectrometer (Applied Bio systems) operating in a result-dependent acquisition mode. For MS analysis, 1000 shots were accumulated for each well of sample. Six external standards (mass standard kit for the 4700 Proteomics Analyzer calibration mixture, Part Number 4333504, Applied Bio systems) were used to calibrate each spectrum to a mass accuracy within 50ppm. The ten most intense ions from each sample excluding trypsin autolysis and keratin tryptic peptides were selected for MS/MS. The MS/MS analyses were performed using air, at collision energy of 2kV and a collision gas pressure of $\sim 1 \times 10^{-6}$ Torr. Stop conditions were implemented so that 2000 to 3000 shots were accumulated depending on the quality of the spectra. GPSExplorer™ software Version 3.6 (Applied Biosystems) was used to create and search files with MASCOT search engine (version 2.1; Matrix Science) for peptide and protein identification. International Protein Index (IPI) Mouse database Version 3.75. was used for the search and was restricted to tryptic peptides. N-terminal acetylation, Cysteine carbamido methylation and methionine oxidation were selected as variable modifications. One missing cleavage was allowed. Peptide mass tolerance and fragment mass tolerance were set to 150 ppm and 0.4 Da respectively. Maximum peptide rank was set to 2 and the minimum ion score C.I. % (peptide) was set to 50.

Statistical Analysis: One way repeated measurement ANOVA statistical analyses were performed using R statistic software version 2.8.0. Behavioral data, mean escape latencies and distances of each day were compared between the platforms areas.

RESULTS

Behavioral Study: The escape latencies and the distance of mice movement decrease clearly across the days during training [$F_{(8,32)}=4.74$, $p=0.004$; blue column and $F_{(8,32)}=6.754$, $p=0.0005$; red column, Figure 1. Both escape latencies and distance bring the same conclusion that the mice learnt where the platform was located during the training session and long-term memory was established.

Protein Identification: Spots with statistically significant intensities were identified by mass spectrometric analysis. Such proteins were identified in C57Bl/6J mice as belonging to metabolic pathway related to amino acid metabolism (glutamate dehydrogenase I), carbohydrate metabolism (fructose biphosphatealdolase C, triosephosphateisomerase), chaperone (heat-shock 60 kDa protein), transport (voltage-dependent ion channel), cytoskeletal related to neurofilament family (α -internexin) and signal transduction (dihydropyrimidinase related protein 2, adp-ribosilation factor 2 and calmodulin).

Representative two-dimensional maps of overall protein expression from the hippocampus of control and trained mice are shown in Figure 2 and Table 2.

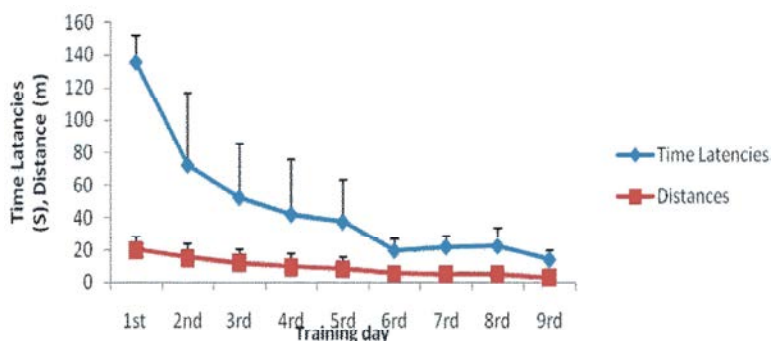


Fig. 1: The escape latencies and distance of mice movement to find the hidden platform during learning progress. This graph and schematic pathway indicator the escape latencies and distance of movement has decreased within ninth days of training

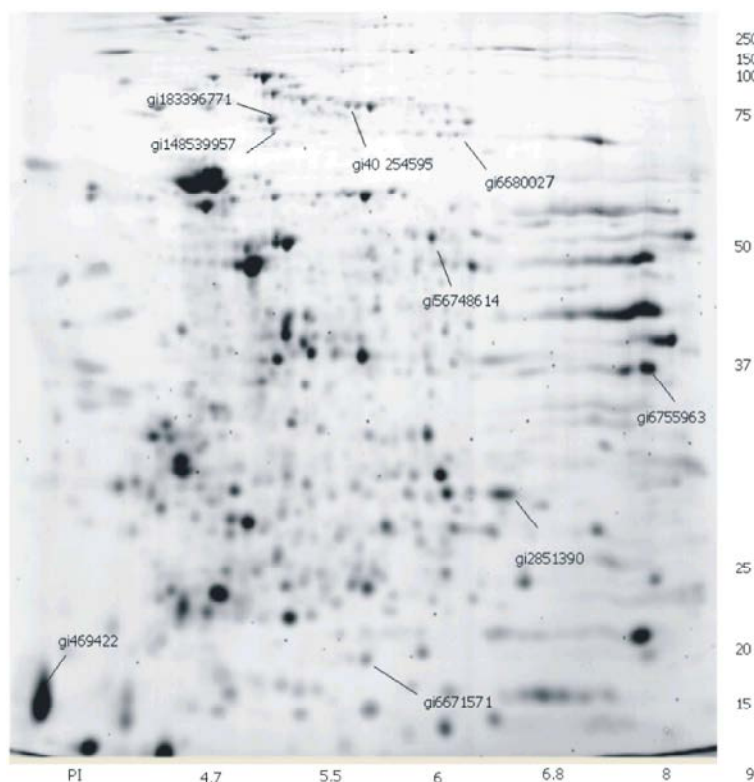


Fig. 2: Two-dimensional gel electrophoresis of mouse hippocampal proteins.

The proteins were extracted and 40µg applied on an immobilized pH 3-10 gradient strip, followed by 12.5 % polyacrylamide gel. Gels were stained with silver nitrate; spots were identified by MALDI-TOF/MS and proteins assigned using MASCOT software. Accession numbers for protein identification were obtained from database (<http://www.matrixscience.com>).

Table 1: Functional proteins showing significant intensities in the C57Bl/6J mouse hippocampus

Functional Class	Protein Name	Accession No.	MS Score	Match. pept.	Spot No.	Sequences coverage%	Molecular Weight	PI	Significance
Signal Transduction	Dihydropyrimidinase related protein 2	gi/40254595	49	7	261	10	62	5.95	0.0026
	ADP-ribosylation factor 2	gi/6671571	57	7	26	24	20.7	6.2	0.013
	Calmodulin kinase IV	gi/469422	136	14	10	38	17	4.1	8.11E-04
Energy Metabolism	Fructose biphosphatealdolase C	gi/56748614	57	10	197	14	39.2	6.79	0.025
	Glutamate dehydrogenase 1	gi/6680027	153	7	247	7	61	6.7	0.012
	Triosephosphate isomerase	gi/2851390	289	19	108	39	26.7	6.09	0.003
Chaperon	Heat shock 60 kDa protein	gi/183396771	91	9	253	17	60	5.35	5.39E-04
Transport	voltage-dependent anion channel	gi/6755963	186	12	155	26	32	8.55	0.0022
Cytoskeletal	α -internexin	gi/148539957	221	26	244	27	55	5.25	0.0099

Spot numbers and significance values were obtained using the 2D platinum 7.0 Software. Accession No. is the primary accession number obtained from SWISS/Prot databases. Functional classes were determined by searching the Human Protein Reference Database (<http://www.hprd.org/moleculeClass>).

DISCUSSION

The results of monitoring mice performance in the Morris Water Maze by looking at the time latencies and distance showed that they decreased during training progress and this showed that mice had learned where the platform was located [14, 15]. The three cues on different parts of the wall guided the mice to find the platform. During the training the mice tried to swim around the pool for the first three days.

They found the platform by chance during the session. In the rest of the training days the mice had learned where the platform was and reached the platform within a few seconds by the end of training. This showed the mice learning behaviour and developed memory for this task which they retained over long term retention. Moreover, Alikhani *et al.* [11] reported that learning performance can be affected by cognitive and motivational photos via different performance as it has shown in this study [16-18].

Protein Synthesis: Adaptation of the neuronal cell to long-lasting changes in structure and function is caused by alternation in the synaptic strengths called long-term potential (LTP). This process which is critical for long-term memory and learning is initiated in the hippocampus by the synthesis of many proteins that contribute to strengthening the neural network. Influx of Ca^{2+} through the synapses, as well as ATPase synthases and cytoskeletal proteins have important roles in creating long lasting memory [19]. In addition some studies have shown that many molecular processes occur during training that support cognitive and memory function. However, it is not well known how training regulates the action of various factors at the molecular level, particularly those which are related to energy metabolism and synaptic plasticity. Proteomics provides us with a useful tool to identify protein expression and modification. After analysing many protein spots of relatively high abundance from hippocampus on two-dimensional electrophoresis gels, it was particularly obvious that proteins differentially expressed by training are associated with energy metabolism and neuronal plasticity.

Protein Involved in the Signal Transduction: Dihydropyrimidinase-related protein 2 (DRP-2) was identified as one of the proteins which is expressed in learning. DRP-2 is involved in the process of axonal outgrowth regulated by extracellular signals [20]. The expression of DRP-2 in adult brains is assumed to be important as a mechanism of repair and regeneration of neurons. Changes in DRP-2 expression are considered to play an important role in Down syndrome. Changes in DRP-2 levels are probably disease-related. According to Kimball and colleagues [20] this protein is necessary for repairing synapses and neuron communication. They found that DRP-2 exists at basal level all the time [21]. It was shown by Sultana [22] that DRP-2 expression is decreased in Alzheimer disease. Therefore DRP-2 contributes to memory and cognitive loss [22, 23]. Another protein that is categorized in signal transduction based upon molecular function is calmodulin. Calmodulin IV is a 17 kDa acidic modulator protein that has been located in the nucleus. Calmodulin has four sites to bind Ca^{2+} and when all calmodulin sites bind to Ca^{2+} it causes conformational change. It has been assumed that this conformational change leads to calmodulin's ability to activate or inactivate enzymes. These enzymes include adenylyl cyclase, Ca^{2+} dependent protein kinase, glycogen synthase which are regulated by Ca^{2+} and probably via

calmodulin. Calmodulin-dependent kinase can activate cAMP responsive element binding protein (CREB) by phosphorylating on ser¹³³ [24,25].

Protein Involved in Transporter: Mitochondrion and glycolysis are used for vesicle mobilization necessary for synaptic transmission. Glutamate dehydrogenase can reuse glutamate in the Kerbs cycle to continue ATP production. Glutamate dehydrogenase is an enzyme in mitochondrial membrane [26]. Voltage-dependent anion channel (VDAC 1) is located in the outer membrane of the mitochondrion and has a central role in energy metabolism of neurons by maintaining cellular levels of ATP and modulating calcium buffering [27, 28]. The most important function of mitochondria is oxidative phosphorylation, oxidation of a variety of respiratory substrates to produce ATP from ADP. VDAC expression is associated with ATP production. ATP synthesis is decreased by reduction of VDAC expression [29]. Pumping of protons creates an electrochemical gradient used by complex V (ATP synthase) to synthesize ATP. Protons are provided by respiratory Complexes I, III and IV (NADH-ubiquinone oxidoreductase, ubiquinol-cytochrome C oxidoreductase and cytochrome c oxidase. All metabolites can enter and leave mitochondria via VDAC. The VDAC has the ability to close and inhibit the energy of metabolites [30].

Chaperone Proteins: Molecular chaperones are multifunctional proteins that protect other proteins from unfolding. These proteins increase in response to stress to protect the cells [31, 32]. Most of the molecular chaperones are heat shock proteins (HSP). Chaperones are associated with ATPase activity which is critical to their effect on protein folding. Their function is further extended to anti-apoptosis and synaptic plasticity. The chaperone found to be increased by training, is heat shock 60 kDa protein (hsp 60). The heat shock 60kDa protein is located in mitochondria. Its function is to protect mitochondria from oxidative stress by assisting in the correct assembly of mitochondrial proteins [21, 33]. Evidence indicated that heat shock protein expression is decreased in Alzheimer's disease [27]. Heat shock 60kDa proteins are expressed in neural cells like microglia, oligodendrocytes, astrocytes and neuron cells [35].

Cytoskeletal Proteins: Studies have shown that training influences neurogenesis in adult brains. In support of this, our results indicate that training increases the expression of proteins related to cytoskeletal function and development. The protein that we found increased was

α -internexin which is one of five major intermediate neurofilament families (NF-L; neurofilaments). Cytoskeletal protein changes may assist in changing neuronal network involved in the cellular mechanism of learning [36, 37]. α -internexin is involved in the stabilization of neurons and their processes. It produces a structure for the assembly of the other neurofilament proteins which are quickly assembled into filament structures and transferred into the axons. As was shown by Yuan, [36], α -internexin in the absence of other neurofilaments was reduced in axons of parts of the brain including the corpus callosum, hippocampus, frontal cortex, cerebellum and spinal cord. α -internexin is required for axonal transport [36].

Proteins Involved in Energy Metabolism: Changes in energy metabolism enzymes are assumed to be critical in learning and memory. Energy metabolism involves four stages: glucose metabolism, ATP synthesis, transferring of energy and glutamate turnover. Fructose-bisphosphatealdolase C is one of the enzymes involved in glycolysis. Aldolase C catalyses fructose-1,6bisphosphate to glyceraldehyde3-phosphate and glycerol phosphate. While fructose bisphosphates are broken into phosphoglycerate, kinase 1(PGK-1) participates in the second phase of glycolysis to change 1,3-diphosphoglycerate into 3-phosphoglycerate. Once this change has occurred one molecule of ATP is formed. Likewise, glutamate dehydrogenase catalyses glutamate oxidative deamination into α ketoglutarate and glutamate to feed into the tricarboxylic acid cycle and also provides an alternative to Krebs's cycle [38].

In this present study we found that a few enzymes related to glycolysis and synaptic plasticity and transport were affected by training. Some of the enzymes are fructose-bisphosphatealdolase C, triosephosphate isomerase and glutamate dehydrogenase related to energy metabolism. These proteins are related to ATP synthesis which is required for proper function of cells. ATP has the key role for operating pumps, adjusting pH and maintaining cell potential gradients. Altered energy metabolism appears to be important in neurodegenerative disease as presented in Alzheimer disease. Dihydropyrimidinase and ADP-ribosylation are factors in protein synthesis reported by Ergen and colleagues [39] to be involved in axonal outgrowth transmission and modulation of extracellular signals [40, 41].

Heat shock 60 kDa protein and α -internexin are involved in synaptic plasticity and calmodulin in protein kinase [42] activity.

Low expression of DRP-2 protein has been reported in many diseases such as Alzheimer Disease and Down syndrome [23]. α -internexin protein is one of the neurofilaments (NFs) families which are axonal proteins. These proteins form axonal structures and assemble to form long macromolecular filaments. The level of NF-L was also reported to be decreased in AD brains.

In summary, studies such as this in combination with 2D dimensional gel electrophoresis and mass spectrometry or time of flight provide a powerful tool for studying protein expression in the process of learning and memory.

CONCLUSION

This study demonstrated that training affects a diverse network of proteins related to energy metabolism and synaptic plasticity in the hippocampus, the central region for cognitive function. We have identified a number of proteins that appear to be expressed in learning and memory. This study also revealed protein changes related to the cytoskeleton, signal transduction, energy metabolism, transport and chaperones or protein folding. Different proteins are expressed in different cellular pathways that might affect learning and memory.

ACKNOWLEDGEMENT

Many thanks for Ministry of Science Technology and Innovation e-Science for funding (SCF 0023-BIO-1/2007) project. The research was conducted at the Department of Biomedical Science and Therapeutic, School of Medicine, Universiti Malaysia Sabah (UMS).

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