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Proteomic Analysis of Mice Hippocampus in Simulated Microgravity Environment

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Space travel induces many deleterious effects on the flight crew due to the '0' *g* environment. The brain experiences a tremendous fluid shift, which is responsible for many of the detrimental changes in physical behavior seen in astronauts. It therefore indicates that the brain may undergo major changes in its protein levels in a '0' g environment to counteract the stress. Analysis of these global changes in proteins may explain to better understand the functioning of brain in a '0' *g* condition. Toward such an effort, we have screened proteins in the hippocampus of mice kept in simulated microgravity environment for 7 days and have observed a few changes in major proteins as compared to control mice. Essentially, the results show a major loss of proteins in the hippocampus of mice subjected to simulated microgravity. These changes occur in structural proteins such as tubulin, coupled with the loss of proteins involved in metabolism. This preliminary investigation leads to an understanding of the alteration of proteins in the hippocampus in response to the microgravity environment.

Keywords: microgravity • hippocampus • two-dimensional gel electrophoresis

1. Introduction

Microgravity induces changes in growth, cellular structure, and cell-to-cell interactions.[1] These changes are therefore considered to be a challenge to life in space. Exposure to altered microgravity during space flight has long-lasting effects on postural stability and cardiovascular function.[1] In the brain, the activity of neuronal functions has been a concern as it relates to the fluidic nature of the gray and white matter because of its enclosure within the skull. The sensitivity of the mammalian central nervous system to gravitational influences involves both direct and indirect factors. Gradual loss of cerebral circulation with increasing acceleration beyond 5 *g* has been shown to evoke changes in patterns of brain electrical activity, with epileptiform discharges triggered primarily in the hippocampus system of the temporal lobe, and spreading into other brain systems.[2,3] Results have also indicated that changes in the gravitational environment might represent a useful tool to investigate the neurobiological and behavioral responses to stressors and may provide insights into the mechanisms underlying development and plasticity of the nervous system in brain, heart, and lung tissue.[2−4]

The effect of weightlessness on the distribution of ANF-containing neurons and cardiocytes was studied in frogs that were sent into space for 9 days on the space station MIR. In control animals, the amygdala contained the most prominent group of ANF-immunoreactive cells and fibers.[5] The results supported the concept that prolonged exposure to microgravity affects biosynthesis and/or release of ANF-related peptides in discrete regions of the amphibian brain.[5] Moreover, using microarray analysis, a change in the global gene expression pattern has been reported in the skeletal muscle of space-flown rats.[6,7] These studies have reported altered expression of cytoskeletal genes during space flight that resulted in dislocation of the mitochondria in the cell and several oxidative stress-inducible genes were highly expressed in the rat muscle. Inhibition of genes regulating cell proliferation and growth factor cascades, including cell cycle genes and signal transduction proteins was shown in the skeletal muscle of rats exposed to microgravity during space flight.[7]

Recently, we have shown that in hind limb suspension model, generating simulated microgravity in mice induced oxidative stress in the brain, resulting in lipid peroxidation in various regions of the brain.[8] These data, including other observations reported on the brain during microgravity exposure, do suggest involvement of this environment in the alteration of genes and protein expression profiling.[6−10]

The hippocampus is architecturally distinct and its structures are folded into the cerebral cortex. Functionally, it has been shown to be involved in the integration of information arriving

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from different sensory organs, memory storage, and retrieval. The hippocampus contains a fairly large number of glucocorticoid receptors and, through the negative feedback mechanism, terminates glucocorticoid release during stress response.[11] Therefore, stress response signaling does occur in the hippocampus and, therefore, makes it vulnerable to changes in the environment. In addition, mice subjected to simulated microgravity undergoes stress in response to the environment and transduces stress signaling in the hippocampus.[8] In the present study, an attempt has been made to identify some of the changes in protein expression using proteomics technology in the hippocampus region of mouse brain, kept in simulated microgravity environment to better understand the effect of such stress on the proteome.

2. Materials and Methods

Exposure of Mice to Simulated Microgravity. In small mammals, the tail suspension model has been widely used as a model to simulate microgravity effects.[12,13] Studies, predominantly in rodents, have demonstrated that tail suspension has good fidelity to many of the changes that occur in larger mammals during real microgravity exposure. Many laboratories have demonstrated the feasibility of performing complex studies in the mouse and have shown substantial similarities between the mouse and the human in responses to exercise, pharmacological agents, and alterations in cytokine levels.[14] Therefore, in the present study, we have used mice as an experimental model to study the effect of simulated microgravity in hippocampus using the Tail Suspension Model that was performed as described by Wise et al., and Felix et al.[8,15] We have earlier shown in this model that simulated microgravity induced changes in cytokine and reactive oxygen species mediated nuclear transcription factor kappa B activation in brain.[8,15] Some of these changes are reported to be similar to the effect as seen in space flown mice.[10] Six- to eight-week old male BALB/c mice were obtained from Harlan Sprague Dawely (Indianapolis, IN). Mice were randomly divided into two groups, control and tail-suspended, with six mice in each group with free access to drinking water and food for 7 days. At the end of the experiment, mice were sacrificed by cervical dislocation and hippocampus was dissected and snap frozen in liquid nitrogen. The tissue was stored in −80 °C freezer for further experiments.

Sample Preparation. Hippocampus tissue was suspended in 0.5 mL of isoelectric focusing buffer containing 20 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS, 10 mM 1,4-dithioerythritol, 0.5% ampholyte (3−10) and a mixture of protease inhibitors (1 tablet Complete (Roche Diagnostics) per 10 mL of suspension buffer). The suspension was sonicated for approximately 30 s and centrifuged at 100 000 × *g* for 30 min. The protein content in the supernatant was determined using the Bradford reagent (BioRad).

Two-dimensional gel electrophoresis was performed as previously reported.[16,17] Briefly, samples were applied on immobilized pH 3−10 gradient strips (IPG 11 cm, BioRad) Focusing started at 500 V and the voltage was gradually increased to 8000 IEF unit V at 3 V/min and kept constant for a further 6 h in IPGPhore (Amersham). The focused strips were equilibrated for 15 min in 50 mM Tris-HCL, pH 8.8, 7 M urea, 2 M thiourea, 30% glycerol containing 10 mg/mL DTT followed by 25 mg/ml iodoacetamide in the same buffer for 15 min at RT. The second-dimensional separation was performed in 12.5% homogeneous SDS-polyacrylamide gels. The criterion gels (BioRad) (180 × 200 × 1.5 mm) were run at 200 V per gel, in Dodeca 2D running apparatus (Bio Rad). After protein fixation with 40% methanol, containing 10% acetic acid for 2 h, the gels were stained with Sypro Ruby for 12 h. Excess of dye was washed from the gels by distaining with 10% methanol and 7% acetic acid. Gels were scanned on ProXpress CCD scanner (Perkin-Elmer). The images were captured as 16 bit tiff files for further analysis.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF−MS). MALDI-TOF−MS analysis was essentially performed as described by Deshane et al.[17] In brief, the spots were excised and destained with 30% acetonitrile in 50 mM ammonium bicarbonate and dried in a Speedvac evaporator. Each dried gel piece was rehydrated with 10 μL of 1 mM ammonium bicarbonate, containing 50 ng trypsin (Roche Diagnostics). After 16 h at room temperature, 20 μL of 50% acetonitrile (ACN), containing 0.3% trifluoroacetic acid (TFA) were added to each gel piece and incubated for 15 min with constant shaking. This solution was purified using C18 ZipTips. The ZipTip was pre-wet by aspirating 10 μL of 50% ACN and twice with 0.3% TFA, followed by drawing 10 μL of sample. The sample was aspirated and dispensed for five times followed by washing with 0.1% TFA. The peptides were eluted in 30% ACN and 0.3% TFA, and this mixture was used for MALDI-TOF−MS analysis. The peptide mixture (1.5 μL) was mixed with the matrix solution (1 μL), consisting of 0.025% α-cyano-4-hydroxycinnamic acid (Sigma) and the standard peptides des-Arg-bradykinin (Sigma, St. Louis, MI, 904.4681 Da) and adrenocorticotropic hormone fragment 18−39 (Sigma, 2465.1989 Da) in 65% ethanol, 35% acetonitrile and 0.03% trifluoroacetic acid. The samples were spotted and analyzed in a time-of-flight mass spectrometer (Voyager DE-Pro Applied Biosystem). Peptide matching and protein searches were performed using the MASCOT search engine as described in Deshane et al., and Berndt et al.[17,18] In brief, search parameters allowed for oxidation of methionine, carbamidomethylation of cysteine, one missed trypsin cleavage, and 50 ppm mass accuracy. The peptide masses were compared with the theoretical peptide masses of all available proteins from all species. Unmatched peptides or miscleavage sites were not considered for protein identification.

Immunoblotting. Immunoblot was performed as described by Manna and Ramesh.[19] In brief, hippocampus tissue was homogenized in 2DE buffer was mixed with SDS-PAGE sample buffer to a protein concentration of 50 μg and boiled for 3 min at 95 °C in water bath. The proteins were resolved in 10% SDS-PAGE and transferred to nylon membrane. The membranes were blocked in 5% skimmed milk followed by reaction to primary antibodies specific to Pyruvate dehydrogenase or β-Synuclein. The blots were reacted to anti-rabbit horseradish-peroxidase coupled antibody and bands were visualized by incubating the blots with ECL reagent (Amersham, NJ) followed by exposing to X-ray film.

3. Results

A microgravity environment is by itself a stress as evident in the data obtained from animal studies performed in real space shuttle missions and in simulated conditions.[1−3] Ground-based models for studying microgravity include head-down bedrest and the Tail Suspension Model for animals, both cause a pooling of fluids in the upper body similar to that seen in microgravity.[12,13] These methods are stress inducible but they all mimic to a greater extent the microgravity environment.

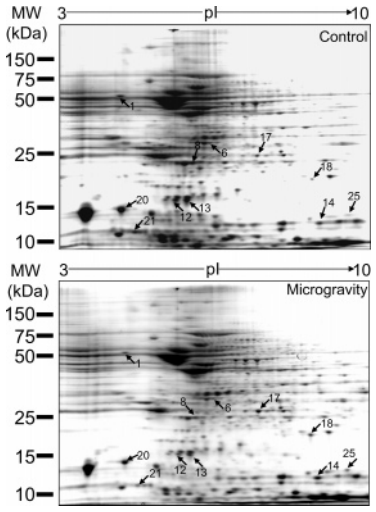

Figure 1. Representative 2-DE protein patterns from control and microgravity exposed mouse hippocampus. Control mice were maintained under normal condition in cages while other group of mice was kept for microgravity exposure by suspending them by their tail for 7 days as described in materials and methods. Hippocampus was dissected and homogenized in 2-DE extraction buffer. Approximately 150 μg of protein was used for 2-DE and stained with Sypro Ruby. Images were captured and differentially expressed proteins were identified by manual visualization. Differentially expressed proteins spots are marked on the images with arrows.

Glucocorticoid is stress-associated and needs to be corrected to delineate between the effects of simulated microgravity and stress as such generated by the model.[20] Therefore corticosterone was estimated in serum from both control and mice exposed to simulated microgravity using kit (R&D Systems Inc, MN). The results did not show any significant difference in serum corticosterone concentration between the control and the test mice. This observation therefore suggests that the stress generated is due to microgravity environment not due to tail suspension (data not shown). To investigate whether simulated microgravity affects the protein expression profile in mouse brain, we analyzed the proteome of the hippocampus region subjected to simulated microgravity. The 2-DE method we adopted did resolved and detected proteins with molecular masses between 250 kd and 10 kd and p*I*s between 3 and 10. We quantified the 2-DE protein patterns and mutually matched them by visual analysis. For comparing the protein expression differences of the microgravity and the paired control samples, we selected a total of 11 spots that were observed to be common across four sets of independent experiments performed in triplicate. Figure 1 shows a pair of 2-DE gels of the hippocampus proteins from control and microgravity groups. More numbers of proteins that had consistent difference were represented from low molecular weight proteins, which also lined up in the p*I* range of 3 to 5 as shown in Figure 1. In all, eleven protein spots were selected for MALDI-TOF−MS analysis to obtain the identity of these spots. Table 1 reports the identification of these spots and also shown are the sequence of the matched peptide with their MOWSE score. The proteins are grouped to show increased expression as compared to control against simulated microgravity and vice versa. Seven of these proteins were more represented in control, whereas four were more represented in simulated microgravity exposed hippocampus. Out of the seven spots that were more represented in control broadly included protein from family of structural proteins and proteins involved in metabolism. Thus, it seems likely that exposure of mouse brain to simulated microgravity affects the structural proteins coupled with loss of metabolic activity, and these may be linked to the adaptation to the altered environment.

Tubulin is a structural protein and this was found to be less represented in hippocampus exposed to simulated microgravity (Figure 1 Spot 1). This gene was also significantly down regulated in HepG2 cells exposed to simulated microgravity as evident in microarray analysis.[21] Loss of structural protein has been long argued in cytoskeleton and for extracellular matrix (ECM) under microgravity environment.[21] Multiple investigators have observed actin and microtubule cytoskeletal modifications in microgravity, suggesting a common root cause for the change in cell architecture.[6,22−23] The inability of the 0 g grown osteoblast to respond to sera activation suggests that there is a major alteration in anabolic signal transduction under microgravity conditions, most probably through the growth factor receptors and/or the associated kinase pathways that are connected to the cytoskeleton.[24] Therefore, loss of tubulin in the present study is at par with the hypothesis that microgravity induces a signal leading to cytoskeletal modifications. The other protein that was less represented in simulated microgravity of hippocampus was Pyruvate dehydrogenase (Figure 1 Spot 6).

Table 1. Differentially Expressed Proteins in Hippocampus from Control and Microgravity Simulated Mice[a]

spot no.	protein name	accession no.	peptide sequence	MOWSe score	predicted M.W.(Kd.)
		Control			
1	Tubulin beta polypeptide	gi|4507729	YLTVAAIFR	99	49
6	Pyruvate dehydrogenase	gi|18152793	VTGADVPMPYAK	93	38
8	Ubiquitin carboxy-terminal hydrolase L1	gi|6755929	EFTEREQGEVR	64	24
12	Phosphatidylethanolamine binding protein	gi|53236978	FKVETFR	91	20
13	Phosphatidylethanolamine binding protein	gi|53236978	FKVETFR	76	20
20	Synuclein, beta	gi|15809030	EGVLYVGSK	86	14
21	Smooth muscle and non-muscle myosin alkali light chain	gi|17986258	EAFQLFDR	71	16
		Microgravity			
14	Peptidyl-prolyl cis-trans isomerase A	gi|51761915	VSFELFADKVPK	62	18
17	Pgam1 protein	gi|12805529	HYGGLTGLNK	81	28
18	Ubiquinol-cytochrome c reductase	gi|13385168	VPDFSDYR	101	29
25	Similar to chromosome 2 open reading frame 32	gi|38091306	IQPNDGPVFFK	67	18

[a] Sequences obtained from MALDI-TOF−MS spectra are shown with their corresponding MOWSE Score. Identified proteins increased both in control and simulated microgravity of mice hippocampus are shown separately. The accession numbers indicated are from National Center for Biotechnology Information (NCBI).

Pyruvate dehydrogenase belongs to glucose metabolism, and has been shown to be involved in the metabolic abnormalities of the brain in ischemia, associated with oxidative stress.[25] Microgravity induces oxidative stress and would lead to ischemia further leading to decrease in anabolic activity. The decrease in pyruvate dehydrogenase in microgravity has been earlier reported in skeletal muscle in space flown rats.[6] Therefore, the similarity of loss of pyruvate dehydrogenase in skeletal muscle and in hippocampus provides generalized phenomena under microgravity.

Ubiquitin carboxy-terminal hydrolase L1 was down regulated in simulated microgravity as compared to the control (Figure 1 Spot 8). This protein is associated with multiple metabolic pathways in Parkinson disease model.[26] Findings from this study therefore relate microgravity associated changes that may be similar to some extent, as observed in the pathology of Parkinson disease. Phosphatidylethalomine binding protein was down regulated in microgravity as compared to control hippocampus (Figure 1, Spots 12 and 13). Phosphatidylethanolamine binding protein (PEBP) is a multifunctional protein, with proposed roles as the precursor protein of hippocampal cholinergic neurostimulating peptide (HCNP), and as the Raf kinase inhibitor protein.[26] Down-regulation of PEBP may result in lower levels of HCNP or altered coordination of signal transduction pathways that may contribute to neuronal dysfunction and pathogenesis as observed in Alzheimer disease.[27] In the hippocampus, a decrease in PEBP under simulated microgravity may therefore lead to neuronal dysfunction, mainly in relaying signal from the membrane to the cytoplasm which is one of the function of this protein predicted based on its structure.[28]

Pgam 1 protein is represented high in simulated microgravity in the hippocampus and is involved in glucose metabolism in the brain (Figure 1 Spot 17).[29] Ubiquinol-cytochrome *c* reductase levels were high in hippocampus from simulated microgravity exposed mice, and this protein is involved in the apoptosis of aging brains (Figure 1, Spot 18).[30] β-Synuclein was relatively less in hippocampus of simulated microgravity as compared to control (Figure 1, Spot 20). β-Synuclein is involved in nuclear synapse and it has been shown that both α and β-synuclein may be able to inhibit phospholipase D2 selectively.[31] Further, it is reported that β-Synuclein lead to the protection of neuronal cell lines from apoptosis through drastic reduction of p53 expression and transcriptional activity.[32,33] Thus, it may be that microgravity decreased β-Synuclein to induce apoptosis in cells. The other protein that showed significant down regulation in simulated microgravity was nonmuscle myosin alkali light chain. Therefore, our results indicate that in the hippocampus of mice exposed to modeled microgravity reduces the expression of a number of proteins as compared to induction of proteins. The proteins were focused in 11 cm IPG strips and then resolved in criterion gradient gel (BioRad) that might add to the limitation to resolve more proteins. However, most of the proteins that were differentially expressed were spread across a molecular weight ranging from 10 to 50 kD which are well resolved in our gel system.

Immunoblot Analysis of Pyruvate Dehydrogenase and *β*-Synuclein. We have further validated the expression of pyruvate dehydrogenase and β-Synuclein in the same hippocampus tissue that was used for proteomics analysis (Figure 2). The enlarged illustration shows loss of pyruvate dehydrogenase in hippocampus of mice kept on simulated microgravity (Figure 2a). The MALDI-TOF MS spectra of pyruvate dehydrogenase is shown in Figure 2b and also shown is the matching peptide

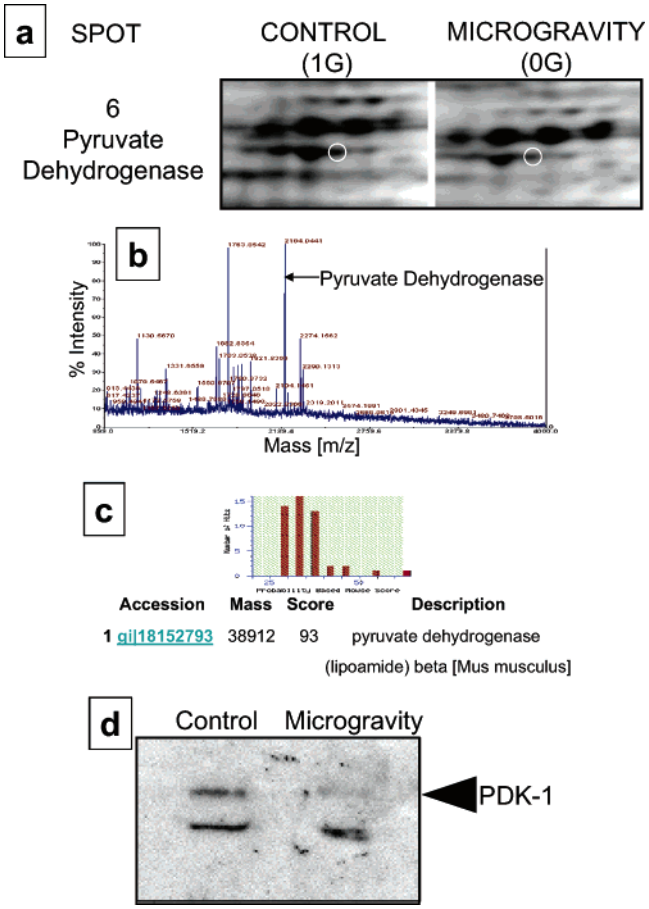

Figure 2. 2-DE profile of pyruvate dehydrogenase in control and mice kept in simulated microgravity (A−D). (A) The panel shows enlarged regions of the 2-DE protein profile representing changes in protein amount of pyruvate dehydrogenase, (B) Pyruvate dehydrogenase identification by peptide fingerprinting, (C) MOWSE score of Pyruvate dehydrogenase, and (D) Western blot analysis of Pyruvate dehydrogenase in control and in mice kept in simulated microgravity for 7 days.

as shown by MASCOT search engine Figure 2c. To further validate the protein we resolved 50 μg of hippocampus tissue and performed immunoblot analysis with pyruvate dehydrogenase specific antibody (SC-30691; Santa Cruz Biotechnology Inc. CA). This analysis showed absence of pyruvate dehydrogenase protein in hippocampus exposed to simulated microgravity (Figure 2d). The results co-related with proteomics observation and thereby suggesting that pyruvate dehydrogenase is down regulated in modeled microgravity.

In addition, we also validated β-Synuclein in hippocampus tissue that was used for proteomics analysis. The enlarged illustration shows loss of β-Synuclein in hippocampus of mice kept on simulated microgravity (Figure 3a). The MALDI-TOF−MS spectra of β-Synuclein is shown in Figure 3b and also shown is the matching peptide identification performed using MASCOT search engine (Figure 3c). For immuno blot analysis 50 μg of hippocampus tissue was resolved in 10% SDS-PAGE and probed with β-Synuclein specific antibody (SC-9565; Santa Cruz biotechnology Inc. CA) (Figure 3d). This analysis showed loss of β-Synuclein protein in hippocampus kept on simulated microgravity. Thus the analysis of the proteome of the hippocampus showing differential expression of protein was correctly portrayed.

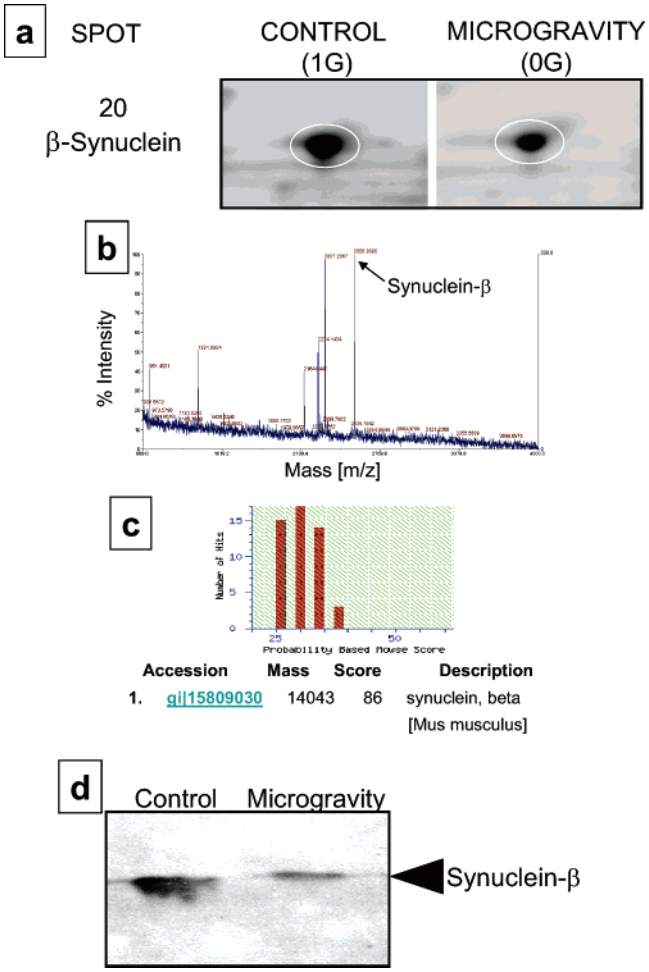

Figure 3. 2-DE profile of β-synuclein in control and mice kept in simulated microgravity (A−D). (A) The panel shows enlarged regions of the 2-DE protein profile representing changes in protein amount of β-synuclein, (B) β-synuclein identification by peptide fingerprinting, (C) MOWSE score of β-synuclein, and (D) Western blot analysis of β-synuclein in control and in mice kept in simulated microgravity for 7 days.

4. Discussion

Space travel is associated with major risk to human health and definitive markers for tissue functions are required to monitor such risks in the hypogravity environment. We have observed differential protein expression using terrestrial model for microgravity and have observed eleven proteins to be differentially expressed in simulated microgravity after extensive analysis. We have previously demonstrated that simulated microgravity induces oxidative stress that resulted in significant increase in lipid peroxidation in the hippocampus of male mice along with activation of nuclear transcription factor κB (NF-κB) was also reported.[8] Thus, the hippocampus was significantly affected by oxidative stress in mice kept on simulated microgravity. This prompted us to study the effect of simulated microgravity on the hippocampus proteome to further screen for more affected proteins. To our knowledge, this is the first report of a systematic hippocampus proteome analysis on the effect of simulated microgravity in mouse model. Most of the proteins decreased in hippocampus of simulated microgravity as compared to control thereby suggesting either they are transcriptionally deregulated or may be an adaptive response to the new environment. Preliminary analysis of 4673 human genes in a microarray analysis revealed a 2-fold change in the expression of 95 genes in HepG2 cells exposed to microgravity, whereas 10 genes were down regulated by 2-fold in these same cells.[21] Such an observation would suggest that transcriptional alterations might be a response by the cells to simulated microgravity. However, the present observations analyzed at the protein level in hippocampus show more loss of protein expression in simulated microgravity. This observation is in accordance with the previous results which show that spaceflight alters protein metabolism such that the body is unable to maintain protein synthetic rates and eventually decrease in total protein content.[34] If this phenomenon is a typical response to simulated microgravity then it was not surprising to observe loss of protein expression in simulated microgravity in the present study. This observation also signifies that the ground base model to simulate microgravity was able to induce similar effect as observed in actual microgravity experienced in space. Hippocampus by itself is an important functional part of the brain and shows its role in memory storage and retrieval, and also playing an important role in declarative memory.[11] Proteins showing changes were represented from metabolism and from the class of cytoskeleton family. Tubulin and myosin belong to the cytoskeleton group of proteins and both were significantly down-regulated in hippocampus exposed to simulated microgravity. The cytoskeleton recently attracted wide attention from cell and molecular biologists due to its crucial role in gravity sensing and transduction. The morphology of the microtubules in cardiac myocyte cytoskeleton and in MCF-7 cells became diffused under microgravity in space.[23,35−36] In a microarray based study, it was reported that the expression of cytoskeletal molecules are disturbed, including putative mitochondria-anchoring proteins, A-kinase anchoring protein, and cytoplasmic dynein in space-flown rats. The loss of the cytoskeletal proteins resulted in dislocation of the mitochondria and a postulated leakage of reactive oxygen species from the mitochondria.[23] The decrease in tubulin-beta and myosin alkali light chain in simulated microgravity in the present study further suggests that there may be loss of cellular architecture with severe consequences. One such reported consequence is the gravitational stress affected cytoskeletal dynamics, resulting in loss of the specific morphological phenotype of the cells.[37] In addition, loss of myosin heavy chain through proteolytic degradation is observed in space-flown rats similar to that observed in ground-based simulated microgravity model.[38] Together, all these observations, including the report in this communication do indicate that cytoskeletal proteins are sensitive to microgravity stress.

The mammalian pyruvate dehydrogenase is a complex within the mitochondrial matrix enzyme that catalyzes the oxidative decarboxylation of pyruvate to form acetyl CoA, nicotinamide adenine dinucleotide (the reduced form, NADH), and CO_2. This reaction constitutes the bridge between anaerobic and aerobic cerebral energy metabolism.[39] A decrease in the amount of pyruvate dehydrogenase could result from increased oxidative stress which occurs in the mouse brain in simulated microgravity.[8]

The loss of β-Synuclein in hippocampus from mice exposed to simulated microgravity might have major implications in the functional aspect of this part of the brain. β-Synuclein can prevent abnormal protein aggregations more effectively and efficiently than alpha-synuclein by acting as molecular chaperone.[40] It is likely therefore that under simulated microgravity,

there might occur more abnormal aggregations of proteins leading to more complex response in the cells.

In summary, we have shown the differential protein expression in the hippocampus of the mouse brains subjected to simulated microgravity. The proteins represented require further investigation to find out the mechanisms for their differential expression. In conclusion, the analysis warrants safety concerns regarding long duration human space flight such as planned for Mars.

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