Proteomic analysis of mouse brain subjected to spaceflight

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Abstract

There is evidence that spaceflight poses acute and late risks on the central nervous system. To explore possible mechanisms, the proteomic changes following spaceflight in mouse brain were characterized. Space Shuttle Atlantis (STS-135) was launched at the Kennedy Space Center on a 13-day mission. Within 3-5 hours after landing, brain tissue was collected to evaluate protein expression profiles using quantitative proteomic analysis. Our results showed that there were 26 proteins that were significantly altered after spaceflight in the grey and/or white matter. While there was no overlap between the white and grey matter in terms of individual proteins, there was overlap in terms of function, synaptic plasticity, vesical activity, protein/organelle transport, and metabolism. Our data demonstrate that exposure to the spaceflight environment induces significant changes in protein expression related to neuronal structure and metabolic function. This might lead to a significant impact on brain structural and functional integrity that could affect the outcome of space missions.

Introduction

Long-term deep space missions expose astronauts to an environment that is characterized mainly by ultraviolet and ionizing radiation, microgravity, and physiological/psychological stressors. These conditions present a significant hazard to spaceflight crews during and after the course of mission activities. The hazards posed to normal tissues, such as the central nervous system (CNS), are not fully understood. The health risk of spaceflight-induced neuronal damage and potential neurodegenerative effects has long been a concern. Brain damage and degeneration can be promoted by many factors including aging, ischemia, fluctuation in oxygen tension, oxidative stress, and increased intraocular pressure.

There is some evidence that low-dose, space-relevant radiation induces changes in neuronal functions [1]. Microgravity induces intraocular pressure and vascular changes [2,3] and promotes apoptosis of astrocytes [4]. Spaceflight also induces cognitive and perceptual motor performance deterioration under stress [5]. Studies have shown that exposure to spaceflights has a strong impact on metabolic and stress response [6]. Collective evidence indicates that the exposure to stressful spaceflight environment might be able to induce changes in brain neuronal structure and function. However, the pathophysiological consequences and cellular mechanisms of stress stimuli, especially due to the spaceflight environment, in facilitating brain damage and neurodegeneration is less studied and remains unclear.

Grey matter consists of neurons (i.e. it contains the cell bodies, dendrites and axon terminals of neurons), nerve fibers, astrocytes, microglia, and capillaries. Grey matter is closely associated

with the functional domains of performance, locomotion, learning, memory and coordination. On the other hand, white matter consists mostly of oligodendroglial cells, myelinated axons and capillaries. White matter allows communication to and from grey matter areas, and between grey matter and the other parts of the body. It functions by transmitting the information from the different parts of the body towards the cerebral cortex. It modulates the distribution of action potentials, acting as a relay and coordinating communication between different brain regions [7]. Changes in grey matter are known to be primarily associated with Alzheimer's disease and other neurodegenerative diseases, with secondary effects on the white matter [8]. The deficits range from language ability to delayed memory and visuospatial construction. Disrupted white matter organization has been linked to poorer motor performance [9]. Studies have shown altered expression of a number of genes and proteins involving a wide spectrum of biological functions following exposure to space environment. These alterations induced distinct changes specific to the regions of the brain [10]. Regional difference in stress response was also documented following simulated microgravity in human brain grey matter and white matter [11].

The purpose of the present investigation was to study spaceflight condition-induced changes in protein expression profiles in mouse gray matter and to compare these changes in white matter regions. Our unique data might provide new insights and improve risk assessment for future long-term space travel.

Materials and Methods

STS-135 Flight Mice and Control Conditions

Space Shuttle Atlantis, i.e., Space Transportation System 135 (STS-135), was launched at the Kennedy Space Center (KSC) on a 13-day mission in July of 2011. Female C57BL/6 mice (Charles River Laboratories, Inc., Hollister, CA) were flown in STS-135 using NASA's animal enclosure modules (AEMs). A set of ground controls (Ground AEMs) was housed at the Space Life Science Laboratory (SLSL) at KSC. Ground AEM control mice were placed into the same hardware used in flight and environmental parameters such as temperature and carbon dioxide (CO_2) levels were matched as closely as possible based on 48-hour delayed telemetry data.

All mice were under ambient temperature of 26-28°C with a 12-hour day/night cycle during the flight. The mid deck CO_2 levels that the mice were exposed to averaged 2,150 parts per million (ppm) and ranged from a few hundred ppm while on the ground before installation in the shuttle to a maximum level of 3,480 ppm in the shuttle during the mission. AEM controls were fed a special NASA food bar diet same as space-flown mice. All mice received the same access to food and water *ad libitum*.

The Loma Linda University (LLU) Institutional Animal Care and Use Committee (IACUC) was consulted, but no protocol was required since only tissues were obtained after euthanasia. However, it should be noted that all NASA research with vertebrate animals is done in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (NIH).

Upon return to Earth, animals were removed from the AEM nursing facility and assessed for survival and health. It was reported that all the mice survived the 13-day space mission. All animals were in good condition as described by the inspecting personnel.

Dissecting and Preservation of the Mouse Brains Post Flight

Within 3-5 hours after landing, the mice were euthanized and brains were removed (4-8 mice/group). As part of the primary science, all mice underwent dual energy X-ray absorptiometry (DEXA) densitometry (Piximus, Inc., Fitchburg, WI) immediately prior to anesthesia and euthanization. Mice were anesthetized with 3-5% isoflurane and euthanized with 100% CO_2 and exsanguination via cardiac puncture. The whole brain hemispheres were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 24 hours and then rinsed with PBS, infiltrated overnight with 30% sucrose in PBS at 4°C. Fixed samples were sent to LLU via courier for analysis. Fixed midbrain samples were then embedded in optimal cutting temperature (OCT) compound (Sakura, Torrance, CA) and frozen at -80°C for further assays.

Brain Sectioning for Proteomic Analysis

Brains were gross sectioned coronally at a thickness of 30 microns. After mounting and de-paraffinizing, six 3 mm diameter circular punches of tissue were obtained to provide 3 replicate samples each of white matter and gray matter. Each tissue punch (disk) was transferred to a vial for individual processing (work up) by a modification of the methodology of Craven et al. [12]. The preparation of tissue samples for proteomic mass spectrometry (MS) analysis in this paper is described by the steps below.

Protein Extraction from Tissue

We used boil-proof 1.7 mL Polypropylene Low Retention Snap-top Vials to process tissues. Lysis buffer was made by combining 50 µL each of stock solutions A and B, plus 10 µL of freshly prepared 1.1 M dithiothreitol (DTT)???. Solution A consists of 300 mM Tris-Cl pH 8.2. Solution B was made by combining five liquid components: 20% sodium dodecyl sulfate (SDS), glycerol, trifluorethanol, thiodiethanol, and water in volumetric ratio of 3:2:1:1:3. Protein extraction was initiated by addition of 110 µL lysis buffer to each vial containing a tissue punch (disk), followed by incubation for 30 minutes at 105°C in a fume hood because of trifluorethanol toxicity. After cooling the vials were stored at -80°C indefinitely.

Trypsin Digestion

This was done using Amicon Ultra-0.5 mL 30k Centrifugal Filter Devices (http://www.emdmillipore.com). Urea-ammonium bicarbonate buffer (UA) that is 50 mM with respect to ammonium bicarbonate (ABC) and 8 M with respect to ultrapure urea (https://www.thermofisher.com) was prepared fresh on the day of use. Use of the Amicon Ultra Centrifugal Filter Device is outlined as follows: a) To each vial after protein extraction (see above), add 300 µL UA, vortex, transfer sample components to an Amicon Ultra, centrifuge at 10,000 x G for 10 minutes and discard the filtrate. b) To complete the transfer initiated above, and to continue replacement of reagents from Step 1 with UA constituents, repeat the process above this one twice using 400 µL UA each time and discard both filtrates. c) Combine 5 µL each of 5 mM Tris (2-carboxyethyl) phosphine (TCEP) and 10 mM acrylamide with 200 µL UA and transfer

entire volume to Amicon Ultra, incubate 30 minutes at ambient temperature, centrifuge and discard filtrate. d) Wash out reagents by adding 400 µL UA, centrifuging, and discarding filtrate. e) Wash out urea by adding 400 µL 50 mM ABC, centrifuging, and discarding filtrate. Repeat this process two more times. f) Add 200 µL 50 mM ABC and perform protein assay (Nanodrop, http://www.nanodrop.com, or Micro BCA, http://www.thermofisher.com) on an aliquot. g) Prepare a trypsin digestion solution for addition to protein remaining in Amicon Ultra. To 100 µL 250 mM ABC add 2.5 µL of 1% Promega PMax (http://promega.com) to give an 0.025% working solution plus sufficient sequencer-grade trypsin to result in an enzyme to protein ratio in the range of 1:25-50. h) Incubate overnight at 37°C to convert proteins to peptides. i) Next morning add 150 µL water, centrifuge, and collect the filtrate containing peptides. Repeat using another 150 µL water and combine filtrates. j) Add 20 µL 10% TFA to the combined filtrates to destroy the PMax. k) Concentrate filtrate in a vacuum centrifuge to a 100 µL volume and store at -80°C. l) Just prior to MS analysis, a 2-5 µg portion of each protein/peptide sample was purified with a Zip-tip C18 P10 (http://www.emdmillipore.com). The sample preparations from each set of 3 replicate "punches" were pooled, resulting in a total of 20 samples for MS analysis.

MS Analyses and Data Processing

An Easy-nLC system with an autosampler was attached to an LTQ-Orbitrap Velos Pro mass spectrometer (http://thermoscientificbio.com). This was used for all MS analyses utilizing 2 to 2.5 µg peptide loadings after Zip-tip purification. After injection, the 5 µL peptide samples in 0.25% TFA, were passed through a 2 cm x 100 µm C18, 5 µm particle size, precolumn (http://thermoscientificbio.com) in series with a 10 cm x 75 µm in-house-prepared capillary

column packed with Microm Magic C18, 5 μ particle size (http://www.michrom.com/) for separation and elution.

A two-hour gradient was used (Solvent A 0.1% FA in water, Solvent B 0.1% FA in ACN, from 5 to 30% Solvent B). Collision-induced disassociation was used to fragment the top 10 most abundant ions and the MS/MS spectra were collected between 250 and 1500 m/z, following the parent full scan mass spectrum collected at 60,000 resolution. The raw data files from these analyses were processed first through Proteome Discoverer (http://thermoscientificbio.com) for Precursor Intensity with the following search parameters using the mouse Uniprot/Swissprot database: 2 missed trypsin cleavages allowed, dynamic oxidation on methionine, deamidation on asparagine and glutamine, and static proprionamide attachment on cysteine.

All MSF files were then combined in Scaffold version 4.4.6 (http://proteomesoftware.com) for further processing (20 categories: 6 ground control white matter, 6 ground control grey matter, 4 flight white matter, and 4 flight grey matter). Protein and peptide thresholds, as well as minimum peptide number, were adjusted to give decoy false discovery rates (FDRs) <1% for 679 proteins. Flight and AEM ground controls were first processed for significant P values using TOTAL SPECTRA as the quantitative method (P </= 0.1, minimum value 0.0, normalization on, NORMALIZED TOTAL SPECTRA as the display option). These were selected and re-processed for both P values and fold changes (minimum value for fold change at 10,000), with AVERAGE PRECURSOR INTENSITY as the quantitative method with NORMALIZED TOTAL SPECTRA as the display option. Both (P values and Fold Changes) were combined in an Excel spread sheet.

All spread sheets were then exported into Ingenuity Pathway Analysis (IPA; http://www.ingenuity.com/products/ipa). In IPA, only direct relationships were allowed in general settings and genes only. Endogenous chemical interactions and causal networks were both included. All items were selected under Data Sources, Confidence, Species and Mutation.

Results

Comparison between White and Grey Matter Protein Levels

There were 9 and 17 proteins that were significantly altered after spaceflight in the white (Table 1) and grey (Table 2) matter, respectively (P<0.05, Log Fold Change > 1.0 or < -1.0). In general, proteins that were significantly altered were upregulated in both areas of the brain. However, there was no overlap between the brain area data sets. If Log Fold Change constraints were reduced to > 0.5, the numbers of proteins increased to 16 and 25 for white and grey matter, respectively.

There were no significant changes in canonical pathways or upstream regulators in the pathway analysis for either the white or grey matter proteins. However, there were strong trends for changes in functionally related proteins in both brain regions (Table 3). In the white matter, there was a strong trend for a downregulation in functions related to the formation of cellular protrusions (Z = -1.98). In the grey matter, there was a significant downregulation in functions related to overall organismal death and organ degeneration (Z < -2.0). There were also strong

trends for a downregulation in cellular and neural degenerative functions (Z = -1.98). Interestingly, there was a significant upregulation in functions related to viral infection (Z > 2.0).

Discussion

While there do not appear to be enough proteins that are significantly different in either white or grey matter in this analysis to appear as a significantly activated canonical pathway, there still appear to be common functional themes. The first involves synaptic plasticity and vesicle activity.

In the white matter, there were three proteins related to synaptic plasticity that were upregulated. This is consistent with the upregulation in proteins related to the "formation of cellular protrusions" found in the IPA analysis (Table 3). **Calcium voltage-gated channel auxiliary subunit α2δ1 (CACNA2D1)** is intimately involved in calcium channel trafficking [13], and regulates excitatory synapse formation during development or after injury [14]. **PTPRF interacting protein alpha 3 (PPFIA3, aka Liprin-alpha-3)** is typically found to be co-expressed with a variety of pre-synaptic proteins in neurons, but has also been found in astrocytes [15,16]. It is thought to be involved in presynaptic plasticity and synaptic vesicle release, particularly in excitatory synapses [16,17]. **Myosin VA (MYO5A)** is an F-actin-based motor protein that is also important in the generation and movement of synaptic vesicles. It has been found in dendritic spines, synaptic vesicles and appears to be critical for synaptic plasticity and organelle transport (reviewed in [18]).

Several proteins involved with synaptic function are upregulated in the grey matter after flight involved vesicle formation, exocytosis and endocytosis. **Syntaxin 1A (STX1A)** is soluble N-

ethylmaleimide-sensitive fusion attachment protein receptor (SNARE) protein that is expressed in most neurons [19] and is a critical component of synaptic vesicle formation and exocytosis [20,21]. **DNAJ heat shock protein family (Hsp40) member C5 (DNAJC5)** is a pre-synaptic DNAJ C-class Hsp40 co-chaperone that is primarily expressed in the brain and retina (reviewed in [22]). It is part of a complex of proteins that resides on synaptic vesicles and chaperones pre-synaptic SNARE proteins, making it critical during repeated synaptic vesicle cycles [23]. Indeed, DNAJC5 knockout mice have progressive, age-dependent sensorimotor deficits and the protein appears to be critical to preventing pre-synaptic degeneration via deficits in endocytosis [22,24].

Upregulated within grey matter, **dynamin 3 (DNM3)** is expressed in the dendritic spines and is primarily associated with regulating synaptic vesicle endocytosis and recycling [25-27]. There are three isoforms of dynamin that share about 80% overall homology and have mostly redundant roles in clathrin-mediated endocytosis and membrane fission [25,28]. **SH3 domain containing GRB2 like 2, endophilin A1 (SH3GL2, aka endophilin-1)** is a potential tumor suppressor gene [29] that is highly expressed in the brain, particularly in presynaptic ganglion [30]. However, the primary function of this protein is to regulate clathrin-mediated endocytosis (reviewed in [31]). Finally, while not directly related to neuronal communication, **ATPase H+ transporting V0 subunit a1 (ATP6V0A1)** involves a form of endocytosis in microglia. This protein that was upregulated in grey matter, is involved with the merging of lysosomes and phagosomes during phagocytosis in the brain [32]. Interestingly, clathrin-medicated endocytosis was significantly and highly upregulated in the liver of these mice as well [33], suggesting a systemic response.

In addition to vesicle formation, several of the proteins upregulated in grey matter after spaceflight have been implicated in neurite and dendritic spine formation. **Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon (YWHAE)** is believed to play a critical role during neuronal development and migration [34] and neurite formation during cortical development [35]. Over expression of this gene disrupts neurite formation through the microtubule binding protein, doublecortin [35]. **Enolase 2 (ENO2)** has also been shown to have neurotrophic activity [36] and is involved in cytoskeletal remodeling and neurite regeneration [37].

Similarly, both **dynamin 3 (DNM3)** [38] and **SH3 domain containing GRB2 like 2, endophilin A1 (SH3GL2)** [39] that were upregulated in grey matter are involved with dendritic spine morphogenesis and stability. Another upregulated protein in the grey matter is **actinin alpha 1 (ACTN1).** Expressed in the dendritic spines of the post-synaptic density (PSD) [40,41], this protein is an actin-crosslinking protein [42] that is involved with synaptic plasticity [40]. This is interesting because changes in dendrite activity might be related to synaptic plasticity [43,44].

Consistent with the upregulation of proteins related to neurite and dendrite growth is the downregulation of **SEC22B (SEC22 homolog B, vesicle trafficking protein) in grey matter.** Knocking down this protein using siRNA reduced neurite length, but had no impact on neuronal migration [45].

Another broad category impacted by spaceflight involved intracellular communication. Specifically, 1) axonal signaling that is "insulated" via myelin and 2) protein and organelle transport. Downregulated in the murine white matter, **myelin basic protein (MBP)** has an important role in the process of myelination of axons, particularly in the adhesion of myelin layers between cytosolic surfaces [46,47]. MBP is implicated in auto-immune responses within the human CNS, and is thought to be a target for T cell activity in multiple sclerosis and other demyelinating or degenerative disorders. Its reduction over an extended period is usually associated with glial inflammation activation and proliferation, leading to reactive astrocytosis [48,49].

Interestingly, three important factors found in oligodendrocytes and involved in myelin formation were upregulated in the grey matter: **acyl-coenzyme A: cholesterol acyltransferase 1 (ACAT1)** [50], **2',3'-cyclic nucleotide 3' phosphodiesterase (CNP)** [51,52], and **neurofilament light (NEFL)** [53,54].

Two components of protein/organelle transport were upregulated in the white matter. As stated previously, **myosin VA (MYO5A)** is important for organelle transport (reviewed in [18]). Similarly, **dynein light chain LC8-type 2 (DYNLL2, aka DLC2)**, originally identified as part of the microtubule-based motor protein, dynein [55], is involved with transporting mitochondria along the axons of neurons in response to local energy and metabolic requirements [56]. However, DYNLL2 has also been shown to have a variety of other targets including nNOS, post synaptic scaffolding proteins, and pro-apoptotic proteins [55].

However, there were also two factors involved in protein transport that were downregulated in white matter. **Vacuolar protein sorting 35 ortholog (VPS35)** is a component of the "cargo recognition complex" of the retromer complex responsible for retrograde transport of proteins from endosomes to the trans-Golgi network or the plasma membrane [57,58]. Already mentioned above as an important component of myelin, **MBP** also interacts with the cytoskeleton and/or tight junctions, making it critical for communicating extracellular signaling to the inside of the cell [47]. Decreases in MBP have been associated with glial activation [59].

Consistent with our previous results in the liver and skin from these same mice where we found that spaceflight had a major impact on metabolism [33,60-62], the next broad category involved to be impacted by spaceflight in the brain include glycolysis and metabolism.

Two proteins involved in glycolysis or metabolism were altered by spaceflight in the white matter. This first one was upregulated. **Phosphatidylinositol transfer protein alpha (PITPNA)** is involved with coordinating lipid metabolism and signaling [63], transferring phospholipids out of the endoplasmic reticulum into other membranes [64]. Interestingly, an increase in oxidative stress has been shown to cause a decrease in this protein, particularly in the brains of aged or Parkinson's disease models [65], and its lack has been associated with neurodegenerative disease that has been linked to changes in glucose homeostasis [66]. **Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)** is an abundant enzyme in brain tissue that is critical to energy metabolism and glycolysis [67]. In conditions of oxidative stress, GAPDH activity is impaired,

leading to cellular aging and apoptosis [67]. This enzyme can undergo sulfhydration, S-nitrosylation and oxidation, which, in turn, can lead to the memory loss, apoptotic cell death and neurodegeneration noted in ischemia and Alzheimer's disease models [68-70].

In the grey matter, **enolase 2 (ENO2)** was upregulated after flight, also suggesting glycolysis may be impacted. **ENO2** is a glycolytic enzyme [71] found in neurons, neuroendocrine cells and microglia [71,72]. However, one of the few proteins that were downregulated in the grey matter, **SEC22B (SEC22 homolog B, vesicle trafficking protein),** also complexes with SNARE proteins in the endoplasmic reticulum (ER) [45,73]. However, knocking down this protein using siRNA had no impact on exocytosis [45]. Instead, SEC22B interacts with lipid transfer proteins and inhibiting this factor has been shown to result in changes in lipid metabolism and transfer [45].

Simultaneously, four proteins critical to mitochondrial function were also upregulated in the grey matter. The mitochondrial localized enzyme, **acetyl-CoA acetyltransferase 1** (**ACAT1),** has been linked to cholesterol homeostasis and metabolism [74,75] and can be found in axons of the cerebral cortex and hippocampus [50]. **Dynamin 1 like (DNM1L)** is critical to mitochondrial fission [76]. This protein drives mitochondrial division by self-assembling into filaments that constrict around the organelle [77]. **2',3'-Cyclic nucleotide 3' phosphodiesterase (CNP)** can also be found in the inner membranes of mitochondria [78], and is important for Ca^{2+} transport [79]. Interestingly CNP levels decreased in non-synaptic mitochondria in the brains of old rats [79]. An increase in CNP release suggests mitochondria may be in a calcium-overloaded condition [80]. **Inner membrane mitochondrial protein (IMMT)**, also known as MIC60 & mitofilin, is important

for protein translocation across the mitochondrial membrane, regulating both morphology and protein biogenesis [81].

The changes noted in metabolism are likely related to increases in proteins involved with oxidative stress and inflammation in the white matter. **Arginase 1 (ARG1)**, which was highly upregulated, is an important enzyme of the urea cycle that is generally found in the liver and is critical to removing ammonia from the body [82]. However, ARG1+ is also commonly expressed by alternatively-activated macrophages and microglia [83], which tend to be anti-inflammatory. In the brain, ARG1+ microglia have been implicated in amyloid beta plaque removal [84]. Furthermore, it is important to nitric oxide (NO)-mediated vasodilation in microvascular endothelial cells [85]. In activated macrophages, ARG1+ competes with NO synthase (NOS) for their common substrate, L-arginine, leading to a reduction in NO production [86].

Not surprisingly, many of the mitochondrial proteins that were upregulated in the grey matter are also involved in the oxidative stress response. **ACAT1** expression has been shown to be elevated during conditions of oxidative stress [50]. Mutations in **DNM1L** often result in death associated with oxidative stress-induced neurodegeneration [77,87]. In vitro, deleting DNML1 from Purkinje cells resulted in increased oxidative damage via the peroxidation of proteins and lipids [77]. Decreases in **IMMT** appears to result in increases in ROS levels [88] and seems to be an anti-apoptotic protein important for the regulation of cytochrome c release [88-90]. Interestingly, oxidative damage due to lipid peroxidation found in the interfibrillar mitochondria of diabetic heart tissue was reduced by overexpressing IMMT [91,92].

Increases in the above oxidative stress factors is consistent with the decreases noted in another mitochondrial protein, **UQCRB (ubiquinol-cytochrome c reductase binding protein),** a subunit of complex III in the mitochondrial respiratory chain [93] in the grey matter. UQCRB is important in mediating mitochondrial-derived reactive oxygen species (ROS) that is both independent of NADPH oxidase and important for angiogenesis [94]. Drugs which inhibit the activity of UQCRB reduce ROS produced by the mitochondria [93-95].

Although not found in mitochondria, **Quinonoid dihydropteridine reductase (QDPR)** is also upregulated in the grey matter. **QDPR** is primarily associated with the regeneration of tetrahydrobiopterin (BH4) from quinonoid dihydrobiopterin (qBH2). This is important because BH4 is a critical co-factor in the generation of all three NO synthases, iNOS, nNOS and eNOS [96]. In QDPR$^{-/-}$ knockout mice, biomarkers of folate-dependent oxidative stress such as ophthalamate, spermine and gamma-Glu-Cys all appear to be elevated [96].

Given the changes in markers indicative of oxidative stress, it should not be surprising that there are also changes in proteins related to cell damage and death. In the grey matter, at least four upregulated proteins dealing with intracellular damage and/or cell death appear to be involved. This is consistent with the IPA analysis that found changes in proteins related to "organismal death," degeneration of cells" and "neurodegeneration." **ATP6V0A1** is critically important in mediating autophagosome-lysosome fusion [97]. **DNAJC5** appears to have some influence on protein folding and endosomal autophagy, depending on the presence of SGT and Hsc70 [98].

ACAT1 is instrumental in induction of necroptosis through lipid droplet formation that has been demonstrated to be the initial key event in cell death [99]. Finally, **CNP** appears to play a role with caspase-independent apoptosis [100].

Finally, there were several changes in the white matter involved in sympathetic activity & catecholamine production. As mentioned previously, **CACNA2D1** was upregulated in the white matter. Although we did not specifically look at areas within the brain, this protein is active in the periventricular nucleus (PVN) and is involved with sympathetic outflow [14]. This is interesting because two proteins involved with sympathetic responses were also upregulated in the grey matter. **Quinonoid dihydropteridine reductase (QDPR)** is primarily associated with the regeneration of tetrahydrobiopterin (BH4) from quinonoid dihydrobiopterin (qBH2). BH4 is a critical co-factor in the biosynthesis of the neurotransmitters dopamine and serotonin [96]. **Syntaxin 1A (STX1A)** is a expressed in endocrine cells [19] and STX1A knockout mice have decreased circulating levels of the stress hormones, CRH and ACTH, as well as serotonergic precursors [19].

Conclusions

In summary, this study is the first to identify spaceflight-induced proteomic significance and biomarkers in the grey and white matter of the murine brain. These unique "proteomic signatures" of brain tissue may provide new mechanistic insight into the complex biological response to space environment. We propose that spaceflight condition induces changes in neuronal structure, cellular function, oxidative response, mitochondrial function and

metabolism, which, in turn might lead to tissue injury and late neurodegeneration. Diverse

changes in protein expression profiles in white and grey matter in response to spaceflight

condition warrants further investigation. Further studies are also necessary to elucidate the

possible tissue and functional impact responsible for our findings and to identify effective

countermeasures.

Acknowledgements

Table 1. Spaceflight alters the proteomic profile in the white matter.

Symbol	Entrez Gene Name	Expr p-value	Expr Log Ratio	Location	Type(s)
ARG1	arginase 1	0.037	3.742	Cytoplasm	enzyme
CACNA2D1	calcium voltage-gated channel auxiliary subunit alpha2delta 1	0.024	2.244	Plasma Membrane	ion channel
PPFIA3	PTPRF interacting protein alpha 3	0.025	2.189	Plasma Membrane	phosphatase
PITPNA	phosphatidylinositol transfer protein alpha	0.010	1.328	Cytoplasm	transporter
MYO5A	myosin VA	0.034	1.304	Cytoplasm	enzyme
DYNLL2	dynein light chain LC8-type 2	0.040	1.152	Cytoplasm	other
Ank2	ankyrin 2, brain	0.012	0.837	Plasma Membrane	other
MYH9	myosin heavy chain 9	0.004	0.686	Cytoplasm	enzyme
DLAT	dihydrolipoamide S-acetyltransferase	0.006	-0.572	Cytoplasm	enzyme
PALM	paralemmin	0.014	-0.615	Plasma Membrane	other
ALB	albumin	0.033	-0.803	Extracellular Space	transporter
ATP1A1	ATPase Na+/K+ transporting subunit alpha 1	0.003	-0.823	Plasma Membrane	transporter
OPA1	OPA1, mitochondrial dynamin like GTPase	0.041	-0.905	Cytoplasm	enzyme
VPS35	VPS35, retromer complex component	0.046	-1.164	Cytoplasm	transporter
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	0.015	-1.478	Cytoplasm	enzyme
MBP	myelin basic protein	0.003	-2.536	Extracellular Space	other

Table 2. Spaceflight alters the proteomic profile in the grey matter.

Symbol	Entrez Gene Name	Expr p-value	Expr Log Ratio	Location	Type(s)
QDPR	quinoid dihydropteridine reductase	0.0229	2.458	Cytoplasm	enzyme
DNM3	dynamin 3	0.00186	1.781	Cytoplasm	enzyme
ACAT1	acetyl-CoA acetyltransferase 1	0.0000214	1.637	Cytoplasm	enzyme
DNAJC5	DnaJ heat shock protein family (Hsp40) member C5	0.00702	1.501	Plasma Membrane	other
SH3GL2	SH3 domain containing GRB2 like 2, endophilin A1	0.0136	1.377	Plasma Membrane	enzyme
RAP1GDS1	Rap1 GTPase-GDP dissociation stimulator 1	0.0284	1.363	Cytoplasm	other
DNM1L	dynamin 1 like	0.0069	1.284	Cytoplasm	enzyme
CNP	2',3'-cyclic nucleotide 3' phosphodiesterase	0.000816	1.261	Cytoplasm	enzyme
YWHAE	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon	0.00989	1.261	Cytoplasm	other
ACTN1	actinin alpha 1	0.0308	1.254	Cytoplasm	transcription regulator
ATP6V0A1	ATPase H+ transporting V0 subunit a1	0.00028	1.191	Cytoplasm	transporter
IMMT	inner membrane mitochondrial protein	0.00436	1.154	Cytoplasm	other
NEFL	neurofilament light	0.00557	1.153	Cytoplasm	other
ENO2	enolase 2	0.0281	1.123	Cytoplasm	enzyme

STX1A	syntaxin 1A	0.00859	1.074	Cytoplasm	transporter
NDUFA10	NADH:ubiquinone oxidoreductase subunit A10	0.0297	0.938	Cytoplasm	transporter
GNAI2	G protein subunit alpha i2	0.0403	0.891	Plasma Membrane	enzyme
ACO2	aconitase 2	0.00635	0.888	Cytoplasm	enzyme
MDH2	malate dehydrogenase 2	0.0323	0.764	Cytoplasm	enzyme
CA2	carbonic anhydrase 2	0.00641	0.733	Cytoplasm	enzyme
PFKM	phosphofructokinase, muscle	0.0466	0.719	Cytoplasm	kinase
TUBB4A	tubulin beta 4A class IVa	0.0326	0.688	Cytoplasm	other
DYNC1H1	dynein cytoplasmic 1 heavy chain 1	0.000217	0.681	Cytoplasm	peptidase
UQCRB	ubiquinol-cytochrome c reductase binding protein	0.0447	-1.059	Cytoplasm	enzyme
SEC22B	SEC22 homolog B, vesicle trafficking protein (gene/pseudogene)	0.00601	-2.709	Cytoplasm	other

Table 3. Spaceflight causes changes in white and grey matter proteins indicative of functional deficits.

Region	Categories	Diseases or Functions Annotation	p-Value	Activation z-score
White Matter	Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance	formation of cellular protrusions	1.69E-03	-1.982
	Organismal Survival	organismal death	3.76E-02	-1.156
	Cellular Assembly and Organization, Cellular Function and Maintenance	organization of cytoplasm	6.40E-05	-1.154
	Cellular Assembly and Organization, Cellular Function and Maintenance	microtubule dynamics	9.47E-05	-1.154
	Tissue Morphology	quantity of cells	3.44E-02	-0.44
	Cell Death and Survival	apoptosis	4.26E-02	0.003
	Cell Death and Survival	necrosis	1.41E-02	0.014
	Cell Death and Survival	cell death of tumor cell lines	4.40E-03	0.028
	Cell Death and Survival	cell death	1.41E-02	0.166
	Cell Death and Survival	apoptosis of tumor cell lines	3.86E-02	0.176
	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	concentration of lipid	7.47E-03	0.333
	Categories	**Diseases or Functions Annotation**	**p-Value**	**Activation z-score**
Grey Matter	Organismal Survival	organismal death	1.22E-03	-3.257

Organismal Injury and Abnormalities	Organ Degeneration	1.46E-05	-2.186
Cellular Compromise	degeneration of cells	1.36E-04	-1.982
Developmental Disorder	Growth Failure	1.45E-02	-1.982
Neurological Disease	Neurodegeneration	8.36E-05	-1.981
Cell Death and Survival	necrosis	1.84E-05	-1.6
Cell Death and Survival	apoptosis	5.78E-03	-1.404
Cell Death and Survival	neuronal cell death	2.96E-03	-0.958
Molecular Transport	transport of molecule	9.80E-03	-0.722
Cell Death and Survival	cell viability	1.48E-02	0.555
Infectious Diseases	infection by HIV-1	2.45E-03	1.98
Infectious Diseases	Viral Infection	1.12E-02	2.2

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