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Proteomic analysis of mitochondria from embryonic and postnatal rat brains reveals response to developmental changes in energy demands

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Abstract

Many biological processes converge on the mitochondria. In such systems, where many pathways converge, manipulation of the components can produce varied and far-reaching effects. Due to the centrality of the mitochondria in many cellular pathways, we decided to investigate the brain mitochondrial proteome during early development. Using a SWATH mass spectrometry-based technique, we were able to identify vast proteomic alterations between whole brain mitochondria from rats at embryonic day 18 compared to postnatal day 7. These findings include statistically significant alterations in proteins involved in glycolysis and mitochondrial trafficking/dynamics. Additionally, bioinformatic analysis enabled the identification of HIF1A and XBP1 as upstream transcriptional regulators of many of the differentially expressed proteins. These data suggest that the cell is rearranging mitochondria to accommodate special energy demands and that cytosolic proteins exert mitochondrial effects through dynamic interactions with mitochondria.

Keywords

Mitochondria; Neurodegeneration; Oxidative stress; Bioenergetics/electron transfer complex; Development

Introduction

Mitochondria are self-replicating, double-membrane organelles that support cellular functions by producing energy via oxidative respiration through the electron transport chain

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Author Contributions

All authors contributed to the conception and design of the study, LMV acquired and analyzed the data, all authors contributed to the interpretation of data, LMV wrote the first draft of the article, all authors participated in its editing/revision and gave final approval of the submitted version.

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(ETC) and also play a crucial role in many other essential pathways such as apoptosis [1], calcium homeostasis [2], iron homeostasis [3] and reactive oxygen species (ROS) signaling [4]. These organelles are regulated not only by the nuclear genome but also the mitochondrial genome providing two sources of genetic regulation. Proteins encoded by the nuclear genome are translated in the cytoplasm and must access the mitochondrial import machinery to enter a mitochondrion. Although the vast majority of mitochondrial proteins are nuclear encoded, the mitochondrial genome encodes several critical components of the ETC as well as a complete set of transfer RNAs and ribosomal RNAs. In addition, because the mitochondrial genome is in close proximity to the source of ROS, the ETC, alterations in mitochondrial efficiency resulting in elevated ROS levels can damage mitochondrial DNA in addition to proteins and lipids resulting in mitochondrial functional changes. The interplay of transcription, translation, ROS production and import machinery in conjunction with protein degradation mechanisms modulates protein levels, which result in a vast array of mitochondrial metabolic states and activity levels.

Previous research has identified tissue-specific mitochondrial proteomic differences in both mice and rats conducted at a single time point [5, 6], establishing the heterogeneity of mitochondria throughout the body. Other work has examined the mitochondrial proteome during postnatal brain development, revealing a dynamic proteome early in postnatal life [7]. Together, these previous studies demonstrate that changes in the mitochondrial proteome are important for modulating cellular responses.

Protein regulatory mechanisms within the cell contribute to the mitochondrial proteomic diversity as many cytosolic proteins interact dynamically with the mitochondria [8]. These dynamic interactions not only drive the mitochondria towards certain functions, but also allow for further regulation of mitochondria through protein recruitment. The regulation of mitochondrial protein levels can be altered by the ubiquitin-proteasome system (UPS) as well as through autophagic degradation of mitochondria (mitophagy). The UPS and mitophagy can work separately or synergistically to either target whole mitochondria (all mitochondrial proteins) or select proteins for degradation.

Mitochondrial disorders are associated with devastating genetic diseases in children that are often linked to neuronal degeneration. Additionally, genetic and sporadic neurodegenerative disorders that arise in adults are frequently associated with mitochondrial dysfunction [9]. Yet, the means by which mitochondrial changes occur during development, between tissues, and during degenerative disorders remain largely elusive. Understanding changes occurring in the mitochondrial proteome may help elucidate the mitochondrial alterations responsible for neurodegeneration.

In order to assess potential mechanisms of mitochondrial alterations in neurons, we utilized a developmental system to model a time of distinct change in the brain which is predominantly neuronal. While oxygen levels are limited in the fetus, following birth increased oxygen is available and neuronal development and function makes demands on energy production; furthermore brain mitochondrial activity increases and higher levels of ATP are present [10]. In order to examine mitochondria at the protein level and potential changes between these stages in brain development, we investigated the brain mitochondrial

proteome of embryonic day 18 (E18) and postnatal day 7 (P7) rats. Using a combination of a mass spectrometry technique and bioinformatics approach, we identified marked alterations in mitochondrial trafficking, mitochondrial dynamics and association of glycolytic proteins.

Materials and Methods

Animals

All animal experiments were conducted with Sprague-Dawley rats obtained from Charles River (Wilmington, MA). Four animals each were used in the E18 and P7 groups. All protocols were conducted within NIH-approved guidelines with the approval and oversight of the University of Nebraska Medical Center IACUC.

Cell line mitochondria isolation for SWATH mass spectrometry (SWATH-MS) library

The rat cell lines B35, H19-7/IGF-IR, RN33B and PC12 were obtained from the ATCC (Manassas, VA). Cells were grown in DMEM-F12 containing 10% FBS and 1% penicillin/streptomycin. RN33B and H19-7/IGF-IR cells were grown at 33° C, while B35 and PC12 cells were grown at 37° C. Cells were harvested and mitochondria were isolated by sequential differential centrifugation (Mitosciences, Eugene, OR) followed by an immunomagnetic (anti-TOM22) affinity isolation (Miltenyi Biotech, Auburn, CA) [11]. Mitochondria were lysed in 4% sodium dodecyl sulfate (SDS) and protein concentration was quantified using a Pierce 660 assay with bovine serum albumin standards (Thermo Fisher Scientific, Rockford, IL).

Cell line mass spectrometry analysis

Mass spectrometry for initial analysis of the cell line mitochondrial proteome was conducted using a LTQ Orbitrap XL nano-LC system (Thermo Fischer Scientific) featuring two alternating peptide traps and a PicoFrit C18 column emitter (New Objective, Woburn, MA). Samples were resuspended in 1% formic acid in water. Peptides were injected with an autosampler and eluted with a linear gradient of acetonitrile from 0–60% over the course of 60 mins. The machine was calibrated before samples were analyzed using the manufacturers' standards. Peptides were identified in a data-dependent acquisition mode. One precursor scan in the Orbitrap identified the 5 most abundant peptide peaks for fragmentation and detection in the LTQ. System variables were set to values as previously described [12]. Briefly, precursor peaks were scanned from 300 to 2000 m/z with a resolution of 60,000 and dynamically excluded after two selections for 60 s. Background peaks were included in a mass rejection list. Collision energy was set to 35 using an isolation width of 2 and an activation Q of 0.250.

Data obtained from the LTQ-Orbitrap was analyzed with MaxQuant (version 1.2.2.2) to generate a peak list. Using the Andromeda algorithm, the peak lists were compared against the Uniprot rat database. Spectral counts were assessed for each protein. The search parameters were set as a maximum of two missed cleavages, carbamidomethyl (C) as fixed modification, N-acetyl (protein) and oxidation (M) as variable modifications, top 6 MS/MS peaks per 100 Da; and MS/MS mass tolerance of 0.5 Da. Exclusion criteria to remove proteins from analysis were as follows: FDR of 0.05 for both peptides and proteins, peptides

must contain at least 6 amino acids, contaminants as identified through the database search and proteins identified as being in the reverse database. All data analysis was performed on data normalized by total spectral count.

Construction of the SWATH-MS spectral library

Mitochondrial lysates from B35, H19-7/IGF-IR, PC12 and RN33B cell lines were mixed in equal amounts then digested with trypsin, quantified, and fractionated by isoelectric focusing. Isoelectric focusing was performed using an Agilent 3100 OffGEL Fractionator into 12 fractions from pH 3–10 according to manufacturer supplied protocols (Agilent Technologies, Santa Clara, CA). Peptides from each fraction were prepared for mass spectrometry with Pierce C-18 PepClean Spin Columns (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. Samples were dehydrated with a Savant ISS 110 SpeedVac Concentrator (Thermo Fisher Scientific) and resuspended in 6 μ L of 0.1% formic acid for LC-MS/MS analysis. The mitochondrial isolation, protein and peptide processing was performed twice independently.

The resulting 24 fractions of peptides were analyzed by nano-LC-MS/MS in SWATH-MS mode on the 5600 TripleTOF instrument. The SWATH-MS acquisition was performed using the published protocol [13]. The acquisition method was in data-dependent mode with one precursor scan followed by fragmentation of the 50 most abundant peaks. Precursor peaks with a minimum signal count of 100 were dynamically excluded after two selections for 6 seconds within a range ± 25 mDa. Charge states other than 2–5 were rejected. Rolling collision energy was used. DDA files were searched in Protein Pilot. Combined results yielded a library of spectra representing 2,004 proteins identified with high confidence (FDR 5%).

Isolation of whole brain mitochondria

Whole brains were isolated from E18 or P7 Sprague-Dawley rats (Charles River). After extraction, brains were immediately rinsed with ice-cold PBS to remove blood. The meninges and cerebellum were removed. Tissue was chopped and homogenized using a Dounce homogenizer. Brain mitochondria were isolated and protein was quantified as stated above for the cell lines.

Culture of cortical neurons and isolation of mitochondria

E18 fetal rat pups were removed and brains were extracted. The cerebellum and meninges were removed. Brains were treated with trypsin and titrated. Cells were pelleted and trypsin removed. Cells were plated at a density of 6×10^6 cells/6cm poly-D-lysine coated dish. Cells were grown for 7 days in neural basal media supplemented with B27, penicillin/streptomycin, and L-glutamine (Sigma). Cells were scraped off the plates at day 7 and collected and pelleted. Mitochondria were isolated and protein was quantified as described for cell lines.

Sample preparation for Mass Spectrometry

Protein from the isolated mitochondria was digested with trypsin (Promega, Madison, WI) using the filter aided proteome preparation technique [14] with a 20- μ m filter (Pall

Corporation, Ann Arbor, MI). The resultant peptides were cleaned with an Oasis mixed-mode weak cation exchange cartridge (Waters, Milford, MA). Peptides were quantified using a Nanodrop (Thermo Fisher Scientific) in conjunction with Scopes method for protein quantitation [15].

Data-independent SWATH-MS analysis

Unfractionated samples of peptides from E18 and P7 rat brain mitochondrial lysates were analyzed in quadruplicate (four biological replicates per age group) using SWATH data-independent analysis (DIA). All of the fragment ion chromatograms were extracted and automatically integrated with PeakView (v. 1.1.0.0). The raw peak areas as reported by PeakView were used for all the quantification calculations with no data processing (neither denoising nor smoothing) of any kind applied to the extracted ion chromatograms. To calibrate retention times, synthetic peptides (BiognoSYS; Zurich, Switzerland) were spiked-in the samples in accordance with the manufacturer's protocol. In accordance with previously published work [13], we selected 5 peptides and 5 transitions option for quantitative analysis and targeted data extraction for each peptide was performed. Samples were normalized to the area counts of the synthetic peptides. Briefly, for each peptide the fragment ion chromatograms were extracted using the SWATH isolation window set to a width of 10 min and 50 ppm accuracy for quantification purposes in accordance with previously established protocols [13]. All mass spectrometry data were log₂ transformed for statistical analysis. T-tests were performed between experimental groups with a Benjamini-Hochberg correction for multiple testing, using a false discovery rate of $\alpha < 0.05$.

Bioinformatic analysis

Lists of proteins were generated from databases for mitochondrial proteins (MitoMiner, <http://mitominer.mrc-mbu.cam.ac.uk/release-3.1/begin.do>) [16], autophagy proteins (Autophagy Database; <http://www.tanpaku.org/autophagy>) [17], lysosomal proteins [18], mt-UPR proteins [19], and synaptic proteins (SynDB; <http://syndb.cbi.pku.edu.cn>) [20]. Heap maps of protein expression were built with Multi Experiment Viewer (Version 4.8.1, <http://www.tm4.org/mev.html>) [21]. Gene Ontology enrichment was determined using the Database for the Annotation, Visualization and Integrated Discovery (DAVID, <http://david.abcc.ncifcrf.gov>) [22]. Analysis of upstream regulators was performed using Ingenuity Pathways Analysis (IPA; <http://www.ingenuity.com/products/ipa>) [23]. For this analysis, all proteins that were found to be significantly different between groups were uploaded into the software. After uploading proteins exhibiting changes from E18 to P7 brain mitochondria, a list of probable upstream regulators was generated. Bias-correction was applied to the data and graphical representations of interactions were generated. Graphics depicting these interactions were generated through IPA.

Results

We chose to quantify mitochondrial proteins from rat brains at two distinct stages during early brain development, at prenatal embryonic time point (E18) in which immature neurons are present and at postnatal time point (P7) when neuronal maturation has occurred. One caveat to studies on the brain is the heterogeneity of the cell types present. However at P7

the rat brain is still approximately 90% neurons [24], thus we can assess neuronal mitochondria from two stages in which documented changes in mitochondrial function occur [10].

Cell line analysis for SWATH-MS library

SWATH-based mass spectrometry (SWATH-MS) is a data independent proteomics approach that relies on the compilation of a library of peptide elution times [13]. These elution times are computed experimentally based upon a 'library' of peptides. With the knowledge of peptide elution times, the computer is able to assign scan peaks to certain peptides without the normally required precursor scan. In doing so, this method increases the number of peptides quantified. A corollary of this methodology though is for a peptide to be identified, its corresponding elution time must be present in the library. Therefore, to be identified in the 'final' experiment, a peptide must have a good signal-to-noise ratio and confidence in a precursor scan so as to be identified for the library.

To build our library, we relied on previous finding which demonstrated that a specific mixture of cell lines can be used to provide a varied yet accurate representation of a particular proteome [11]. Our goal with the cell line mix was to provide a diverse representation of the mitochondrial proteome for the SWATH-MS analysis, thereby increasing the number of high confidence peptides in the library.

Our cell line mix was derived from readily available rat cell lines of neurological phenotype: we chose cell lines derived from a neuroblastoma (B35) and a pheochromocytoma (PC12), as well as cell lines derived from hippocampal (H19-7/IGF-IR) and medullary raphe nucleus (RN33B) neurons. To assess whether their mitochondrial proteomes are complementary and determine if they provide an accurate view of the mitochondrial proteome for a SWATH-MS library, we used mass spectrometry to analyze the mitochondrial proteome of each of the cell lines by label-free proteomics following a one dimensional reverse phase liquid chromatography (Figure 1A).

In total, 1174 proteins were identified from the four cell lines (Figure 1B). The mitochondrial proteome varied based upon the cell line, with 377 proteins (32% of the total proteins identified) being identified in only a single cell line (Figure 1B). Since only one dimensional separation was performed we cannot truly ascertain which proteins may be cell line specific, but nevertheless this illustrates differences between the cell lines. To determine the variability in the expression levels of the common 391 proteins found in all cell lines, hierarchical clustering (Figure 2) was performed utilizing normalized spectral counts for quantification. Indeed the analyses confirmed that the mitochondrial proteomes of the investigated cell lines differed and were complementary.

SWATH-MS Analysis

Recently new methodology, SWATH-MS, in which targeted data extraction is performed from fragment ion spectral libraries has been found to have accuracy similar to that of selected reaction monitoring while allowing for quantification of large numbers of proteins over a wide dynamic range [13]. For our experiment, we built a library using mitochondria isolated from the B35, H19-7/IGF-IR, PC12, and RN33B cell lines. In order to expand

peptide and protein identification, we utilized an additional dimension of separation, isoelectric focusing of the trypsin digested peptides, and performed mass spectrometry on two independent replicates. The resulting library contained 2,004 proteins available for quantification by SWATH-MS was used to extract quantitative levels of mitochondrial proteins from brains harvested from E18 and P7 rats (Figure 3).

Brain mitochondria were isolated, protein purified, quantified, and used for shotgun proteomics, with four independent biological replicates for each age (Figure 3). A total of 1096 proteins were identified in all samples from each experimental group (Supplementary Table 1). Of these proteins, 556 proteins were annotated by MitoMiner as being mitochondrial (Supplementary Table 1). In addition bioinformatic analysis using DAVID identified significant enrichment of mitochondrial proteins based upon gene ontology cellular component analysis (Table 1) [22]. Furthermore, we calculated the coefficient of variation between subjects for each individual protein. We found that using SWATH-MS, the variation between biological replicates was small (Supplemental Figure 1).

These techniques demonstrated the ability to quantify mitochondrial proteins from cells in the brain. While our primary interest was in neurons, which dominate at the time periods examined, the brain still contains a variety of cell types. To examine whether our proteomic approach indeed identifies and quantifies neuronal mitochondrial proteins, E18 and P7 brain mitochondria proteomes were compared against a mitochondrial proteome derived from primary cultured cortical neurons. Using our SWATH library, every protein was identified in each sample. This experiment demonstrated the protein levels observed in the E18 and P7 samples were similar to the protein levels in cortical neurons (Figure 4). The Pearson's correlation coefficient, r , between the mitochondrial proteomes of the cortical neurons and the E18 and P7 brains was 0.92 and 0.94 respectively. Combined with the predominance of neurons at these stages, the observed proteomic alterations found here are most likely related to neuronal mitochondrial proteomic alterations.

Due to the metabolic demands occurring in P7 as compared to E18 rat brains, our initial investigation probed the differences in the proteins involved in oxidative phosphorylation. The subunits identified included both catalytic and non-catalytic subunits. A total of 33 subunits of the electron transport chain (ETC) were identified. Complex II, III, and V subunits showed little change between E18 and P7 rat brains (Figure 5). Most of the subunits were unchanged between E18 and P7 animals, but COX5A of complex IV showed a 1.42-fold increase in the P7 animals ($p=0.021$) while AIFM of complex I was decreased 1.75-fold in P7 animals ($p=0.018$). These results suggest the composition and relative amounts of the ETC components are not altered between the E18 and P7 stage.

Despite an apparent dearth of changes in the ETC subunits, analysis of the glycolytic and citric acid cycle proteins revealed an almost ubiquitous increase in P7 rat brains compared to E18 (Figure 6A). We identified 28 proteins from these processes in our mass spectrometry experiment with 13 proteins significantly increased in the P7 rats. No glycolytic proteins identified in our experiment were decreased (Figure 6A). Thus although the ETC components show relatively few changes, the flux into the ETC may be increasing.

Due to the dynamic nature of the mitochondria, we also investigated known mitochondrial trafficking/dynamics proteins (Figure 6B). The majority of mitochondrial trafficking/dynamics proteins were unchanged between groups, but both OPA1 and DRP1 displayed increases of 1.52 and 2.77 fold ($p = 0.02$ and 0.017 respectively). Furthermore, the motor proteins DNMT1 and trafficking-associated adaptor proteins SH3GLB1 and SH3GLB2 were significantly increased in P7 mitochondrial with fold changes of 6.00, 1.84, and 3.15 ($p = 0.001$, 0.037 and 0.0008 respectively). Changes in trafficking and fission/fusion proteins may mean mitochondria are reorienting in the cell to spatially match energy demand with energy production.

Surprisingly, our analysis identified many proteins involved in organelle acidification (Figure 6C). Three of the V-type ATPases (ATP6V0A1, ATP6V1B2 and ATP6V1E1) were increased in P7 brain mitochondria 3.87, 3.46, and 4.32 fold ($p = 0.0005$, 0.008 , and 0.0008 respectively). The presence of such proteins in our analysis may be an indication of increased mitochondrial turnover through mitophagy as V-type ATPases associate with mitochondrial during lysosomal acidification [25]. Alternatively, V-type ATPases at the synapse are involved in synaptic vesicle loading [26] and synaptic vesicle fusion [27, 28], and possibly, the mitochondrial may become associated with this process at the synapse in order to efficiently provide energy for these processes.

Further examination led to support for both possibilities. We were able to demonstrate increasing mitochondrial association of both lysosomal (Figure 6D) and synaptic proteins (Figure 6E). Of the lysosomal proteins, we identified 12 proteins with four significantly increased (CTSD, DPP7, LAMP2, and SCARB2) and one significantly decreased (ABCA2) at P7 versus E18. Although ABCA2 was significantly decreased, it has been demonstrated present in neural progenitor cells but only a subset of differentiated neurons as well as oligodendrocytes [29]. With this in mind, the decrease in ABCA2 may be indicative of the neuronal differentiation process. Therefore, our data is consistent with an increasing association of lysosomal proteins which would be expected if there was increase in mitophagy. Still, in addition, we identified 17 synaptic proteins in our analysis (Figure 6E). Of these proteins, 10 were found to be significantly increased in the P7 animal brains. The increasing association of synaptic proteins and mitochondria again is consistent with neuronal differentiation, and indicate there is increased mitochondrial trafficking in the brain of P7 animals.

Given the overall increase in lysosomal proteins, our next step was to investigate mitophagy-related proteins. We identified nine proteins involved in mitophagy (Figure 6F). The majority of the proteins were increased with four proteins significantly increased. These proteins included GABARAPL2, MAP1LC3A, MTOR and TM9SF4. Each of these proteins displayed increases of two-fold or greater and p -values of less than 0.05. The increased expression of these proteins, and particularly two of the mammalian homologs of yeast autophagy-related gene 8 (Atg8), GABARAPL and MAP1LC3A, indicated an increase in mitophagy.

Because of the increase in mitophagy proteins, we investigated proteins commonly associated with the mitochondrial unfolded protein response (mt-UPR; Figure 6G). The

mitochondrial unfolded proteins response is increased in response to stress in order to activate nuclear transcription of stress relation genes, and mitochondrial homeostasis is compromised in the presence of an inadequate response [30]. We found that the components of the mt-UPR were ubiquitously decreased in the P7 animals. Of the six proteins identified, four were significantly decreased. Of particular note is the CLPX protein (1.84-fold decrease; $p=0.04$) which has been demonstrated to be integral to mounting the mt-UPR. These results suggest that the stress in the mitochondria at the P7 stage is minimal.

After we determined all the proteins that were significantly altered in the P7 rat mitochondria (Supplementary Table 1), we used IPA to predict which transcription factors were activated and inhibited to determine transcriptional changes that may be upstream of the observed proteomic changes. From this analysis, we identified HIF1A as the top activated transcription factor with a bias-corrected activation score of 1.888 and XBP1 was the most inhibited transcription factor with a bias-corrected activation score of -3.887 (Supplementary Table 2). Analysis of the proteins downstream of these regulators demonstrated that HIF1A (Figure 7) and XBP1 (Figure 8) control the expression of proteins involved in various pathways including those demonstrated to increase in the P7 rat brain mitochondria in our proteomic analysis. HIF1A was found to regulate many proteins involved in glycolysis including ALDOC, GAPDH, ENO1, PPIA, PGK1, ALDOA, and LDHA. Furthermore, it transcriptionally regulates the V-type ATPase ATP2A2. Interestingly, XBP1 activity is a key control mechanism for the endoplasmic reticulum (ER)-UPR [31], its inhibition indicates that the ER-UPR activity at the P7 stage is likely minimal.

Discussion

Our results indicate that mitochondria undergo a proteomic transformation during development from the E18 to P7 stage. Based upon the SWATH-MS data, we were able to characterize the mitochondrial proteomic alterations between E18 and P7 rat brains. We identified significant increases in proteins associated with mitochondrial trafficking, mitochondrial degradation, and glycolysis. These results suggest that mitochondria are affected in terms of neuronal location as well as turnover by the ongoing neuronal differentiation, as well as providing the needed ATP at P7 via an increased flux through the citric acid cycle and ETC due to increased mitochondrial association of glycolytic proteins.

Although commonly assigned a cytoplasmic localization, many of the glycolytic enzymes have been found to be partially localized to the outer mitochondrial membrane. Certain glycolytic enzymes are completely localized to the mitochondria and include hexokinase I, hexokinase II, and hexokinase IV [32, 33]. Previous research has documented that many of these glycolytic enzymes have a dynamic association with the outer mitochondrial membrane, are functional, and act to increase substrate for the ETC generated by glycolysis [8, 34]. Furthermore, aerobic glycolysis in the brain has been shown to correlate with synaptic formation and growth [35]. From E18 to P7, the brain is remodeled through the processes of neurogenesis, neural migration, and synaptogenesis [36–39] with low levels of gliogenesis [38, 39]. The development of neuronal functional properties, specializations, and interactions coincide with the shift from placental oxygen exchange to pulmonary oxygen

exchange [12]. By increasing the association of glycolytic proteins to mitochondria, mitochondria are able to increasingly shuttle precursors of the ETC into the mitochondria.

Despite the increase in substrates for the ETC as predicted by increased mitochondrially-associated glycolytic enzymes, the components of the ETC were largely unchanged. Interestingly, a key component of the ETC, AIFM, was demonstrated to be decreased in P7 brains. AIFM-deficiency leads to complex I impairment in the brain [40]. While AIFM has an antioxidant role in mitochondria [41], AIFM can also be released from mitochondria in response to stress and translocate to the nucleus to stimulate non-canonical apoptosis [42]. A decreased expression could be indicative of two situations: (1) AIFM was already released in response to stress or (2) AIFM protein was decreased as a result of the mitochondria establishing new sensitivities to stimuli.

The mitochondrial fission/trafficking pathways are demonstrated to be differentially regulated between E18 and P7 rat brains. From our data the outer mitochondrial membrane fission protein DRP1 was found to be increased in P7 animals. OPA1, an inner membrane fusion protein, was increased also but the action of OPA1 is dependent on increases in MFN1 [43] which is unchanged in our experiment suggesting fusion dynamics are unchanged in our system. The increases in fission as denoted by DRP1 could be indicative of two processes: (1) increased mitophagy [44] and/or (2) increased mitochondrial trafficking to synapses [45].

Two pieces of our findings can be interpreted as supporting the aforementioned finding in relation to the increase in DRP1. The presence of increased DNM1 in P7 animals suggests there is increasing amount of mitophagy as previous work has demonstrated the centrality of DNM1 to mitophagy [46]. Conversely, it may signal an increase in mitochondrial trafficking to the presynaptic membrane as DNM1 has presynaptic functions and localization [47]. Along these lines, we identified increased mitochondrial association with synaptic proteins such as MARCKSL1, STX1A, STX1B, VAMP2 and VAMP3 (Figure 6E). It is likely our findings indicate both processes are increasingly occurring. As stated previously, the brain is undergoing synaptic remodeling requiring additional energy at the synapse [4, 11, 12, 36, 38], and additionally, previous research has demonstrated mitochondrial fission as necessary to proper mitochondrial synaptic distribution [48–50].

Although there is evidence supporting increased mitochondrial trafficking to synapses, protein expression levels of mitophagy proteins and V-ATPases suggest increasing degradation of mitochondria, perhaps turnover linked to the differentiation and remodeling of the developing brain structure. Of the proteins commonly associated with mitophagy, four proteins were significantly increased, including two Atg8 homologues, key in recognizing autophagic receptors on mitochondria [51]. The V-type ATPases (lysosomal proteins commonly associated with lysosomal acidification) were increased in P7 brains suggesting that this pathway may be important for mitochondria turnover. Previous work has demonstrated the V-type ATPases are also associated with neurotransmitter loading in synaptic vesicle [52, 53]. Due to the P7 stage of development to be highly associated with axonal and dendritic proliferation [54] with dendritic formation and synaptic strength dependent on signaling [55–60], the increase in the V-type ATPases observed is likely also

associated with increased synaptic signaling. These results suggest there is increased mitochondrial trafficking to the synapses and increased mitophagy occurring at the P7 stage of brain development.

Although our results indicate increased mitophagy, this function could be in response to stress or be a homeostatic mechanism during development and differentiation. To elucidate this point, we identified proteins involved in the mt-UPR. We found these proteins to be universally decreased in our model at the P7 stage. The absence of an increase mt-UPR, as well as low XBP1 activity indicating low ER-UPR, indicates the brain is not receiving increased stress. The lack of increased stress indicates the mitophagy occurring is likely a homeostatic mechanism, and indeed, mitophagy is neuroprotective when damaged mitochondria are present [61].

The top predicted transcriptional regulator activity changes were for HIF1A and XBP1. The prediction of XBP1 activity as decreased in P7 animals was consistent with many of our findings in which the proteins regulated by XBP1 were also increased (Figure 8). The presence of HIF1A as the most increased transcription factor activity may again seem counterintuitive because HIF1A expression is commonly associated with decreased oxygen tension. However, though the mechanisms are unclear, HIF1A expression is intimately linked to the glycolytic pathway which we found to be increased [62], and HIF1A can contribute to proliferation, which would be expected at P7 [63].

When interrogating the mitochondrial proteomic alterations, it became clear many proteins including the glycolytic proteins were attached to the outer mitochondrial membrane. These results are interesting because the majority of protein studies focus on inner mitochondrial membrane proteins such as the ETC complex subunits. Previous research has demonstrated the feasibility of analyzing mitochondrial proteomes [64–66], and by incorporating SWATH-MS, we were able to interrogate the mitochondrial proteome more completely and with higher confidence. Interestingly, previous experiments have demonstrated that alterations of post translational modifications could alter the mitochondrial interactome and metabolic regulation [67–69]. Alterations of post-translational modifications offer a mechanism for the observed changes, but future research is necessary to clarify the issue.

Undoubtedly, a mass spectrometry experiment always raises concerns about reproducibility of the data. We attempted to minimize this issue by performing our experiment in quadruplicate on a SWATH-MS platform. Using our protocol, the data were highly reproducible between biological replicates (Supplemental Figure 1), furthering the argument for mass spectrometry being a principal mode of analysis [70]. Combined with the relative ease of running replicates on the experimental samples once a library is built, this protocol can become a model of future mass spectrometry experiments.

When performing these experiments, we focused on brain proteomic alterations. Therefore, proteomic alterations in any brain neuronal or non-neuronal cell type could theoretically contribute to the proteomic alterations observed. To address this issue, we compared the mitochondrial proteomic results from whole brain samples against those from primary culture cortical neurons. The whole brain samples (both E18 and P7) were similar to the

cortical neuron sample as denoted by the high correlation coefficient (Figure 4). These results are unsurprising because previous work has demonstrated non-neuronal cells do not contribute substantially to brain growth until the second and third postnatal weeks, after the P7 stage [24]. While we cannot discount non-neuronal cell contribution to the proteomic alterations observed, these data suggest the majority of proteomic alterations occurring are in neuronal mitochondria.

It should be noted that while we performed mitochondrial isolation by two sequential purification techniques before proteomics, many of the proteins identified in our experiments were not annotated as mitochondria. However in the literature many of these proteins have indeed been demonstrated to interact with mitochondria and new data concerning mitochondrial localization continue to arise. Furthermore many proteins can associate functionally with mitochondria depending on the physiological condition. In our study we cannot distinguish between integral mitochondrial proteins and those that are also located in the cytoplasm and dynamically interacting with mitochondria. The increased or decreased interaction of such dynamically-interacting mitochondrial proteins could signify alterations in the mitochondrial properties of these cells and adds another layer of regulation to the various cellular pathways that converge on the mitochondria.

Understanding the early functional mitochondrial alterations of neurons provides further insight into such long-lasting, early-acting disorders. In this paper, we have demonstrated, using a subcellular organelle SWATH-MS study, alterations in the glycolytic pathway, the fission pathway, mitochondrial degradation proteins as well as other changes. These proteins alterations, we believe, can largely be traced to the massive remodeling the brain is experiencing which demonstrates that the mitochondria are sensitive to the environment and take an active role in the developmental processes. These proteomic differences undoubtedly produce effects in all biological pathways converging on the mitochondria and may be one of the reasons why mitochondrial disorders are early acting and generally affect the brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Although mitochondria play critical roles in many cellular pathways, our understanding of how these organelles change over time is limited. The changes occurring in mitochondria at early time points are especially important as many mitochondrial disorders produce neurological dysfunction early in life. Herein, we utilize a SWATH mass spectrometry approach to quantify proteomic alterations of rat brain mitochondria between embryonic and postnatal stages. We found this method to be highly reproducible, enabling the identification of alterations in many biochemical pathways and mitochondrial properties. This insight into the distinct changes in these biological pathways to maintain homeostasis under divergent conditions will help elucidate the pathological changes occurring in disease states.

Highlights

- SWATH-MS provides a highly effective method to characterize proteomic differences in subcellular organelles.
- Mitochondria utilize distinct mechanisms to adapt to environmental/developmental changes
- Coordinated changes in the mitochondrial proteome map to distinct transcriptional regulators

Figure 1. Schematic depicting workflow for analysis of cell lines for SWATH mass spectrometry experiment (A) and a Venn diagram of the results from the analysis showing in which samples a protein could be identified (B).

Figure 2. Hierarchical clustering of the 391 proteins identified in all cell lines. Expression values are displayed as Log₂ ratios of the spectral counts.

Figure 3.
Flow chart depicting the process for performing a SWATH-MS experiment.

Figure 4.

Scatterplot of the mitochondrial protein expression levels from cortical neurons vs. E18 brain (Blue) and P7 brain (Red). Protein expression values were $\log_2$ transformed prior to graphing.

Figure 5.

Heat map displaying the change in protein expression of mitochondrial electron transport chain (ETC) subunits between E18 and P7 brain mitochondria as observed through SWATH-MS. The values displayed are on a log₂ scale. n=4 biological replicates. Protein names in red are significantly changed (p<0.05).

Figure 6.

Heat map displaying protein expression changes in the glycolytic pathway (A), mitochondrial trafficking/dynamics pathway (B), V-type ATPases (C), autophagy (D), mt-UPR (E), lysosomal proteins (F) and synaptic proteins (G). The values displayed are on a $\log_2$ scale. $n=4$ biological replicates. Protein names in red are significantly changed ($p<0.05$).

Figure 7.
Graphical representation of the top predicted activated upstream transcriptional regulator using IPA to assess the significantly changed proteins.

Figure 8.
Graphical representation of the top predicted deactivated upstream transcriptional regulator using IPA to assess the significantly changed proteins.

Gene Ontology (GO) annotation for cellular component enrichment obtained from DAVID, assessing the SWATH-MS identified proteins. The top two groupings are shown, both of which have overall enrichment scores greater than 50. Count and percent indicate the number and percent of the identified proteins in a given GO term, with the fold indicating the fold enrichment relative to what is encoded in the genome. The p-value is based on a modified Fisher's exact test, and the values for Bonferroni's and Benjamini's multiple testing correction are indicated.

Table 1

Annotation Cluster 1						
Enrichment Score: 55.22						
Term	Count	%	Fold	P-Value	Bonferroni	Benjamini
GO:0044429-mitochondrial part	256	13.70	3.18	4.91E-77	3.26E-74	1.63E-74
GO:0031980-mitochondrial lumen	110	5.89	4.02	2.13E-45	1.42E-42	1.77E-43
GO:0005759-mitochondrial matrix	110	5.89	4.02	2.13E-45	1.42E-42	1.77E-43

Annotation Cluster 2						
Enrichment Score: 51.61						
Term	Count	%	Fold	P-Value	Bonferroni	Benjamini
GO:0044429-mitochondrial part	256	13.70	3.18	4.91E-77	3.26E-74	1.63E-74
GO:0031090-organelle membrane	333	17.82	2.27	1.39E-55	9.25E-53	3.08E-53
GO:0031967-organelle envelope	235	12.57	2.75	1.85E-55	1.23E-52	3.08E-53
GO:0031975-envelope	235	12.57	2.73	9.27E-55	6.15E-52	1.23E-52
GO:0005740-mitochondrial envelope	183	9.79	3.03	3.31E-50	2.20E-47	3.67E-48
GO:0031966-mitochondrial membrane	172	9.20	3.04	1.77E-47	1.18E-44	1.68E-45
GO:0019866-organelle inner membrane	143	7.65	3.04	2.38E-39	1.58E-36	1.76E-37
GO:0005743-mitochondrial inner membrane	137	7.33	3.10	8.11E-39	5.38E-36	5.38E-37