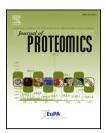


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## Proteomic analysis of the mitochondria from embryonic and postnatal rat brains reveals response to developmental changes in energy demands



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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 the mitochondria to accommodate special energy demands and that cytosolic proteins exert mitochondrial effects through dynamic interactions with the mitochondria.

#### Biological 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 the 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.

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#### 1. Introduction

The mitochondria are self-replicating, double-membrane organelles that support cellular functions by producing energy

via oxidative respiration through the electron transport chain (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

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[4]. These organelles are regulated not only by the nuclear genome but also by 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 the 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 toward certain functions, but also allow for further regulation of the 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 the 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 make demands on energy production; furthermore brain mitochondrial activity increases and higher levels of ATP are present [10]. In order to examine the 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 bioinformatic approach, we identified marked alterations in mitochondrial trafficking, mitochondrial dynamics and association of glycolytic proteins.

#### 2. Materials and methods

#### 2.1. 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.

## 2.2. 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 the mitochondria were isolated by sequential differential centrifugation (Mitosciences, Eugene, OR) followed by an immunomagnetic (anti-TOM22) affinity isolation (Miltenyi Biotech, Auburn, CA) [11]. The mitochondria were lysed in 4% sodium dodecyl sulfate (SDS) and protein concentration was quantified using a using a Pierce 660 assay with bovine serum albumin standards (Thermo Fisher Scientific, Rockford, IL).

#### 2.3. 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 to 60% over the course of 60 min. The machine was calibrated before samples were analyzed using the manufacturer's 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

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