Bioenergetic Analysis of Primary Neuronal Deficiencies

A Sensitive Assay to Measure Mitochondrial Dysfunction Characteristic of Parkinson's, Huntington's, and Other Neurodegenerative Diseases

Application Brief

Introduction

Mitochondria play central roles in meeting the demands of neuronal synapses for energy (ATP). Mitochondrial dysfunction results in impaired neuroplasticity, neuronal degeneration, and cell death, and is now recognized as a key element in neurodegenerative diseases including Alzheimer's (AD) and Huntington's (HD) diseases, dementia with Lewy bodies (DLB), and Parkinson's disease (PD)[1].

The development of model assay systems such as primary neurons, isolated brain mitochondria, and pre-synaptic nerve terminals (synaptosomes) from specific brain regions have enabled the identification of mitochondrial defects associated with neurodegenerative diseases. Synaptosomes are a particularly relevant model system as they preserve mitochondrial bioenergetic function, membrane excitability, and receptors, as well as structures relevant for neurotransmitter exocytosis and re-uptake, and they can be made from experimental animals of any age. However, the need for relatively large quantities of synaptic protein for conventional assays has limited synaptic bioenergetic analyses.

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Choi; *et al.*[2] exploited the ability of the Agilent Seahorse XF Analyzer to handle small sample sizes to monitor mitochondrial respiratory parameters of synaptosomes using 50-fold less protein than previously possible. The results demonstrated a decrease in mitochondrial spare respiratory capacity in bioenergetically compromised synaptosomes that may model deficits characteristic of PD and HD. Figure 1 shows that maximal respiration, as opposed to basal or coupled respiration, is extremely sensitive to very minor concentrations of Rotenone and 3-NPA, inhibitors of complexes I and II, which were used to model mitochondrial dysfunction found in Parkinson's and Huntington's, respectively.

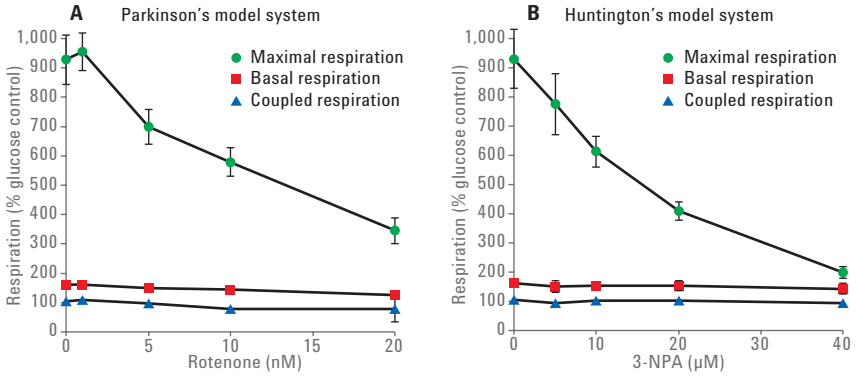

Figure 1. Reserve capacity of synaptosomes is sensitive to minor concentration changes of inhibitors of complexes I and II. Titration of basal, coupled (oligomycin sensitive) and maximal (FCCP-stimulated) respiration with (A) rotenone and (B) 3-NPA. Synaptosomes were incubated in the presence of 15 mM glucose + 10 mM pyruvate and the indicated concentrations of inhibitors were added to model mitochondrial dysfunction found in Parkinson's and Huntington's, respectively. Rates are expressed relative to basal respiration in medium containing glucose but not pyruvate.

Choi; *et al.* went on to demonstrate a heterogeneous response in synaptosomes subjected to increased energy demand, together with a time-dependent decrease in membrane potential. These findings indicated that distinct synaptosome sub-populations are differentially susceptible to bioenergetic failure under conditions of increased energy demand.

The experimental results support the use of the Agilent Seahorse XF Analyzer, combined with membrane potential measurements, to study synaptosomes isolated from specific brain regions of animal models of human neurodegenerative diseases.

Results and Discussion

Several human neurodegenerative diseases have been associated with mitochondrial dysfunction, including mitochondrial depolarization, enlargement, ultrastructural changes, and mitochondrial bioenergetics deficits. Development of model systems, ranging from synaptosomal preparations to cultured primary neurons that reproduce these deficits could potentially allow the identification of novel therapeutics aimed at specific bioenergetic targets.

Gohil; *et al.*[3] recently published a study implicating meclizine as neuroprotective in models of Huntington's disease. In a previous paper, they demonstrated the ability of meclizine to shift energy metabolism towards glycolysis and away from aerobic respiration. In this study, meclizine's protective effect on murine striatal cells expressing polyQ-expanded Huntington's was shown to be due to its ability to attenuate mitochondrial respiration. Using the Agilent Seahorse XF Analyzer, they were able to show that as little as a 10 % decrease in oxygen consumption rate (OCR) leads to an almost 60 % increase in viability of STH$dh^{Q111/111}$ cells[3].

Given that meclizine is well tolerated and can be dosed to achieve subtle inhibition of respiration, it may be possible to establish neuroprotection *in vivo*.

In another study, Yao; *et al.*[4] compared mitochondrial function in female triple transgenic Alzheimer mice (3xTg-D) to normal age-matched controls using mitochondrial preparations isolated from whole brain. This transgenic mouse model bears mutations in three genes linked to AD and frontotemporal dementia, and exhibits an age-related neuropathological phenotype, including amyloid *beta* deposition and *tau* hyperphosporylation. Brain mitochondrial dysfunction was evidenced by decreased mitochondrial respiration and decreased pyruvate dehydrogenase protein levels as early as 3 months of age. The transgenic mouse mitochondria also showed increased oxidative stress as indicated by increased hydrogen production and lipid peroxidation[4].

To determine the cellular contribution to mitochondrial deficits in these mice, basal cellular respiration and glycolysis were assessed in primary hippocampal neuronal cultures from transgenic (TG) and nonTG mice. Both basal and maximal respiration were significantly lower in hippocampal neurons from transgenic mice, suggesting an impairment of the reserve respiratory capacity in these neurons that would potentiate mitochondrial dysfunction in the setting of increasing metabolic demand. Similarly, primary neurons from AD mice showed lower basal respiration and maximal respiratory capacity than those from non-TG mice. The investigators showed that mitochondrial dysfunction, especially that leading to compromised energy production, precedes plaque formation, and showed that female TRG mouse brain recapitulates multiple indicators

of mitochondrial dysfunction found in human AD patients, including decreased bioenergetics, increased oxidative stress, and increased amyloid load in AD mouse models.

A poster presented by L. H. Sanders[5] at the 2010 Neuroscience meeting explored the hypothesis that DNA damage is an early event in dopaminergic cell loss in the substantia nigra using a Rotenone rat model of Parkinson's disease. They performed a series of XF assays showing that sublethal amounts of Rotenone inhibited respiration equally in primary mesencephalic and cortical neurons. This sublethal treatment with Rotenone induced selective nuclear and mitochondrial DNA damage in substantia nigra *in vivo*. The results are consistent with a model that would lead to a PD phenotype based on complex I dysfunction.

These studies establish the utility of the Agilent Seahorse XF Analyzer in characterizing mitochondrial biochemical defects in models of neurodegenerative disease.

Materials and Methods

Synaptosomes and compounds

Synaptosomes were isolated from CD1 mouse cerebral cortexes from mice between 17–21 days using Dounce homogenization, then Percoll gradient centrifugation[2]. For bioenergetic studies, the synaptosomal band was diluted into Ionic Medium (20 mM HEPES, 10 mM D-glucose, 1.2 mM Na_2HPO_4, 1 mM $MgCl_2$, 5 mM $NaHCO_3$, 5 mM KCl, 140 mM NaCl, pH 7.4). The synaptosomes were centrifuged at 15,000 g for 15 minutes to remove the Percoll, and resuspended in the ionic medium. Rotenone, oligomycin, 3-NPA, and FCCP were obtained from Sigma-Aldrich (St. Louis, MO, USA).

XF Bioenergetic analysis

Bioenergetic analyses were performed in the Agilent Seahorse XF Analyzer, a fully integrated multiwell instrument that measures the uptake and excretion of metabolic end products in real time. Bioenergetic analysis of the synaptosomes was measured using the Agilent Seahorse XF FluxPak. This disposable assay kit contains 24 or 96 solid-state, dual-fluorescent biosensors (O_2 and pH). Each sensor is also equipped with four drug injection ports per well for delivering test agents into wells during an assay.

Agilent Seahorse XF24 Cell Culture Microplates were coated with polyethylenimine (1:1,500 dilution from a 50 % solution) to optimize attachment. Synaptosomes (10 µg protein unless otherwise shown) were aliquoted into 20 wells of a polyethylenimine-coated Agilent Seahorse XF24 Cell Culture Microplate, and the microplate was centrifuged at 3,400 g for 1 hour to allow attachment of synaptosomal aggregates that were sufficiently robust to withstand machine-mixing protocols. Ionic Medium was replaced by Incubation Medium (3.5 mM KCl, 120 mM NaCl, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 1.2 mM Na_2SO_4, 2 mM $MgSO_4$, 15 mM D-glucose, 4 mg/mL bovine serum albumin, 37 °C), and plates were used immediately or stored on ice for not more than 3 hours.

OCR and extracellular acidification rate (ECAR) data were then determined. OCR is reported in pmoles/minute and ECAR in mpH/minute.

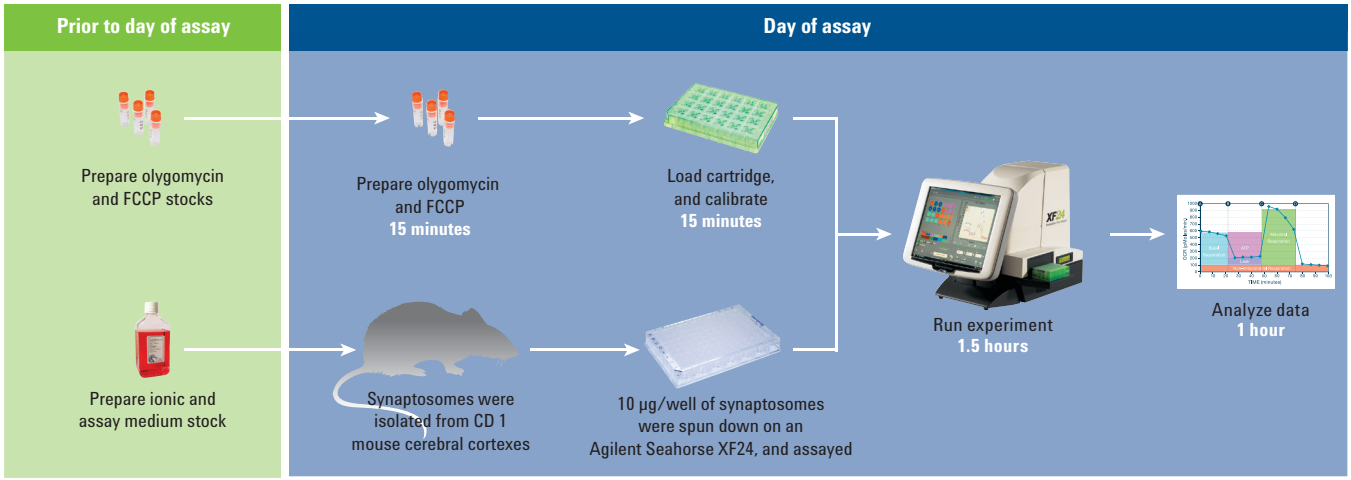

Figure 2. Flow chart of the XF assay.

References

1. Swerdlow, R. H. Mitochondrial Medicine and the Neurodegenerative Mitochondriopathies. *Pharmaceuticals* **2009**, *2*, 150-167.

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3. Gohil, V. M.; *et al.* Meclizine is neuroprotective in models of Huntington's disease. *HMG Advance Access* published October 25, **2010**.

4. Yao, J.; *et al.* Mitochondrial bioenergetic deficit precedes Alzheimer's pathology in female mouse model of Alzheimer's disease. *PNAS*, *106(34)*, 14670-5. Epub **2009** August 10.

5. Sanders, L. H.; *et al.* DNA Damage Precedes Cell Death In Rotenone Rat Model Of Parkinson's Disease. *Neuroscience* **2010**, Poster 252, November 14, 2010, San Diego CA.

Additional Reading

Bartesaghi, S.; *et al.* Loss of Thymidine kinase 2 alters neuronal bioenergetics and leads to neurodegeneration. *Hum. Mol. Genet*. **2010**, *19(9)*, 1669-77. Epub 2010 Feb 1.

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Published in the USA, December 1, 2016
5991-7127EN

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