Application Note

Bioenergetic analysis of primary neuronal deficiencies

A sensitive assay to measure mitochondrial dysfunction characteristic of Parkinson's, Huntington's and other neurodegenerative diseases

RESEARCH AREAS

Neurodegenerative Disease, Mitochondriopathies

ASSAY TYPE

Mitochondrial function: Assessing reserve respiratory capacity in primary neurons and ex vivo neuronal preparations

KEYWORDS

Oxidative Stress
Mitochondrial Dysfunction
Reserve Respiratory Capacity
Synaptosomes
Mitochondrial membrane potential

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 (synpaptosomes) from specific brain regions have enabled 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 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.

Choi *et al* exploited the ability of the Seahorse XF Analyzer to handle small sample sizes to monitor mitochondrial respiratory parameters of synaptosomes using 50-fold less protein than previously possible[2]. 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.

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.

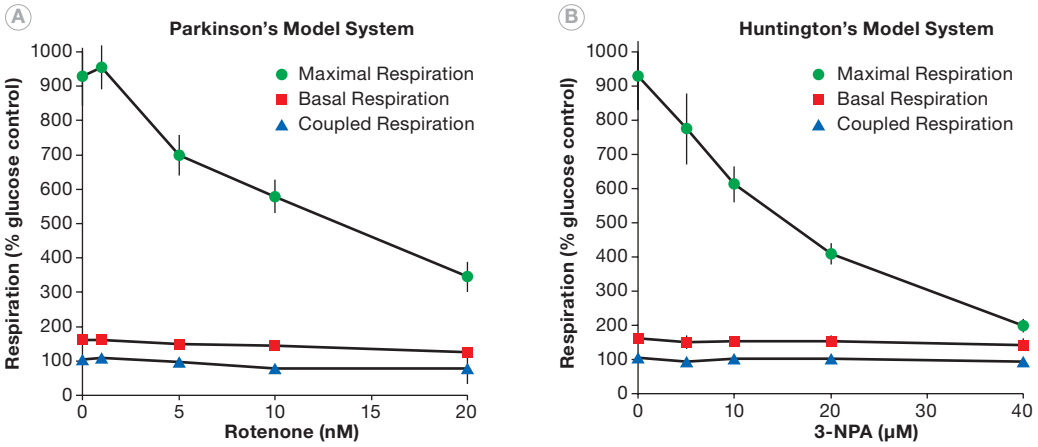

Figure 1 | Reserve Capacity of synaptosomes is sensitive to minor concentration changes of inhibitors of complexes I and II

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.

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

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 synapsotomal 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* 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 most recent 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 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]

The Seahorse XF instrument enabled a 50-fold decrease in the amount of synaptosomal protein needed for bioenergetic assays, compared with conventional respirometry.

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* 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 recently presented poster 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 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 cortices from mice between 17-21 days via 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 $MgCl_2$, 5mM $NaHCO_3$, 5mM KCl, 140mM NaCl, pH 7.4). The synaptosomes were centrifuged at 15,000g for 15 minutes to remove the Percoll, and resuspended in the ionic medium. Rotenone, oligomycin, 3NPA and FCCP were obtained from Sigma-Aldrich (St. Louis, MO, USA)

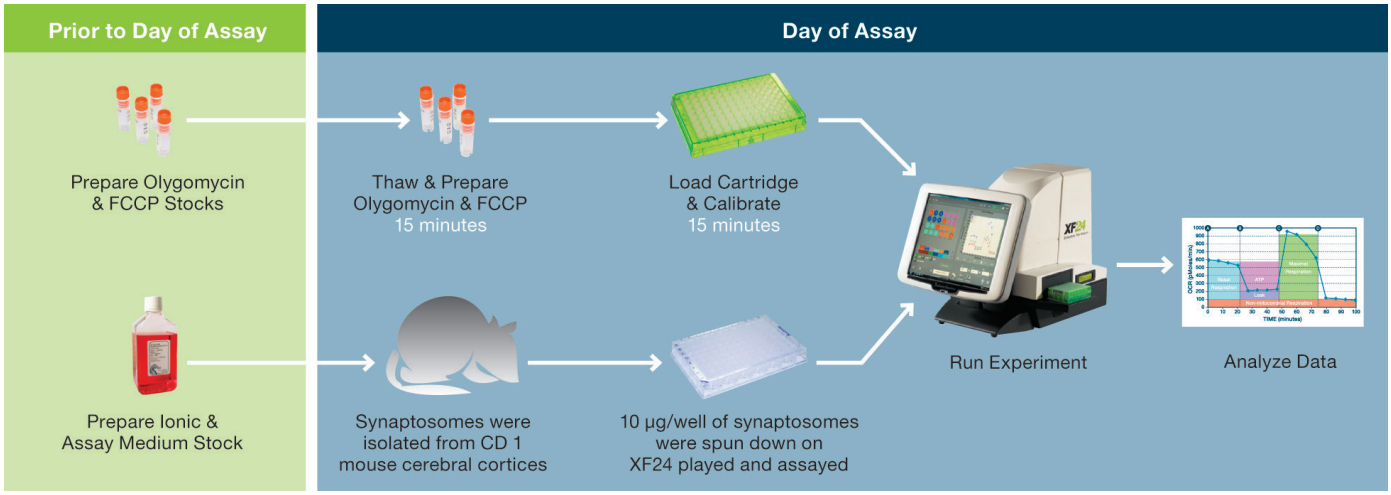
XF Bioenergetic Analysis

Bioenergetic analyses were performed in the Seahorse XF Extracellular Flux Analyzer, a fully integrated-multi well instrument that measures the uptake and excretion of metabolic end products in real-time. Bioenergetic analysis of the synaptosomes was measured using the Seahorse XF Cell Mito Stress Test Kit. This disposable assay kit contains 24 or 96 solid-state, dual-fluorescent biosensors (O_2 and pH). Each sensor is also equipped with 4 drug injection ports per well for delivering test agents into wells during an assay.

Seahorse XF24 Cell Culture Microplates were coated with polyethyleneimine (1:1500 dilution from a 50% solution) to optimize attachment. Synaptosomes (10µg protein unless otherwise shown) were aliquoted into 20 wells of a polyehtyleneimine-coated Seahorse XF24 Cell Culture Microplate and the microplate was centrifuged at 3400g 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_2PO4, 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.

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

Figure 2 | Flow Chart of XF Assay



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Additional Reading

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