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Analysis of regional brain mitochondrial bioenergetics and susceptibility to mitochondrial inhibition utilizing a microplate based system

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Abstract

The analysis of mitochondrial bioenergetic function typically has required 50–100 µg of protein per sample and at least 15 min per run when utilizing a Clark-type oxygen electrode. In the present work we describe a method utilizing the Seahorse Biosciences XF24 Flux Analyzer for measuring mitochondrial oxygen consumption simultaneously from multiple samples and utilizing only 5 µg of protein per sample. Utilizing this method we have investigated whether regionally based differences exist in mitochondria isolated from the cortex, striatum, hippocampus, and cerebellum. Analysis of basal mitochondrial bioenergetics revealed that minimal differences exist between the cortex, striatum, and hippocampus. However, the cerebellum exhibited significantly slower basal rates of Complex I and Complex II dependent oxygen consumption ($p < 0.05$). Mitochondrial inhibitors affected enzyme activity proportionally across all samples tested and only small differences existed in the effect of inhibitors on oxygen consumption. Investigation of the effect of rotenone administration on Complex I dependent oxygen consumption revealed that exposure to 10 pM rotenone led to a clear time dependent decrease in oxygen consumption beginning 12 min after administration ($p < 0.05$). These studies show that the utilization of this microplate based method for analysis of mitochondrial bioenergetics is effective at quantifying oxygen consumption simultaneously from multiple samples. Additionally, these studies indicate that minimal regional differences exist in mitochondria isolated from the cortex, striatum, or hippocampus. Furthermore, utilization of the mitochondrial inhibitors suggests that previous work indicating regionally specific deficits following systemic mitochondrial toxin exposure may not be the result of differences in the individual mitochondria from the affected regions.

Keywords

Mitochondria; Seahorse; XF24; Brain; Regional differences; Bioenergetics; Rotenone; Malonate

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1. Introduction

Mitochondrial bioenergetic analysis from isolated tissue historically requires samples to be run individually and prerequisite large amounts of mitochondrial protein. Traditionally, experiments utilizing a Clark-type oxygen electrode require between 50 and 100 μg of mitochondrial protein per assay (Pandya et al., 2009). Once mitochondria have been successfully purified, it is important to perform all physiological assessment within 4–5 h so that isolated mitochondria will remain well-coupled, viable, and artifact-free. Typical oxygen-electrode experiments take at least 15 min per sample which results in time becoming a limiting factor for determining sample sizes.

Recently, it has been shown that synaptosomes (Choi et al., 2009) and liver mitochondria (Gerencser et al., 2009) can be utilized on a microgram scale for mitochondrial analysis, however, this type of rapid small scale analysis has yet to be shown for purified brain mitochondria. Here we outline a procedure utilizing a Seahorse Biosciences XF24 flux analyzer for measuring mitochondrial function in isolated brain mitochondria using 10–20 $\times$ less protein than experiments utilizing an oxygen electrode and with the ability to run up to 20 individual samples simultaneously. This procedure allows for significantly less mitochondrial protein to be used for mitochondrial bioenergetic analysis negating the need to pool animals as some experiments require and/or results in having more mitochondrial protein available for subsequent experiments. Additionally, the time it takes to assay 20 samples, after the mitochondria have been purified, is less than 1 h which opens the possibilities for increasing sample size during the same experiment as compared to the traditionally utilized Clark-type oxygen electrode.

Utilizing this novel method for measuring mitochondrial bioenergetic function, we sought to rigorously assess whether regional differences exist in brain mitochondrial function and determine susceptibility to electron transport chain inhibitors. It is well understood that regional differences play an important role in mitochondrial function (for review see (Dubinsky, 2009)), however, there is limited evidence profiling basal mitochondrial function or the susceptibility to mitochondrial inhibitors across multiple brain regions within the same animal. It has been established that toxins such as 3-nitropropionic acid (3NP) or trichloroethylene (TCE) produce regionally specific mitochondrial dysfunction when given systemically (Beal et al., 1993; Gash et al., 2008) although the mechanism for this specificity has not been elucidated. Given the differences following systemic mitochondrial toxin exposure, we hypothesized that either significantly different basal mitochondrial function across brain regions or different susceptibility of the mitochondria to different inhibitors may be the underlying mechanism. The present experiments show that the utilization of the Seahorse XF24 is an effective method to analyze bioenergetic functions in isolated mitochondria and demonstrate that limited differences exist across brain regions.

2. Materials and methods

All studies were approved by the University of Kentucky Animal Care and Usage Committee. Throughout all of the experiments, 16 week old male Fischer 344 rats (Harlan) were utilized. Five to six animals per group were utilized for analysis of mitochondrial oxygen consumption. Three to five animals per group were utilized for analysis of mitochondrial enzyme activity levels.

2.1. Mitochondrial isolation

Total mitochondria were isolated using differential centrifugation, nitrogen disruption, and a Ficoll gradient as previously reported (Sullivan et al., 2003, 2004; Pandya et al., 2009). Animals were asphyxiated with CO_2 and rapidly decapitated. Following decapitation, the

brain was removed and placed in a beaker of ice-cold isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 1 mM EGTA, and 20 mM HEPES at pH 7.2) to briefly cool. Anatomical regions of interest were dissected apart and the tissue placed in ice-cold isolation buffer until homogenization. The dissection of the brain was performed by the same individual to provide consistency in the procedure. Specific brain regions were obtained using obvious anatomical markers and known structures to distinguish the different regions of interest. Samples were homogenized and then centrifuged at $1300 \times g$ for 3 min at 4 °C. The resultant supernatant was placed in a fresh tube and the pellet was resuspended in isolation buffer and centrifuged at $1300 \times g$ for 3 min at 4 °C. The supernatants were collected in separate tubes and centrifuged at $13,000 \times g$ for 10 min at 4 °C. The pellets from both tubes were combined, resuspended in 400 μ l isolation buffer and placed in a nitrogen bomb at 1200 psi for 10 min. The pressure in the nitrogen bomb was released and the sample was placed as the top layer on a Ficoll separation column which consisted of a 10% Ficoll layer and a 7.5% Ficoll layer. The Ficoll column with sample was centrifuged at $100,000 \times g$ for 30 min at 4 °C. Following the Ficoll purification, the mitochondrial pellet was resuspended in isolation buffer without EGTA and centrifuged at $10,000 \times g$ for 10 min at 4 °C in order to remove residual Ficoll from the purified mitochondrial sample. The final mitochondrial pellet was resuspended in isolation buffer without EGTA to yield a final concentration of approximately 10 mg/ml, and stored immediately on ice. Protein concentrations for each sample were determined with all the samples on the same plate using the BCA protein assay kit and measuring absorbance at 560 nm with a Biotek Synergy HT plate reader (Winooski, VT).

2.2. Preparation of mitochondrial substrates and inhibitors

Stocks of mitochondrial substrates were prepared as follows and stored at –20 °C until further usage. The concentrations for mitochondrial substrates were based upon extensive experience with the Clark-type oxygen electrode and optimized to obtain mitochondrial bioenergetic profiles which fit with previously observed findings obtained with the oxygen electrode. 500 mM pyruvate/250 mM malate was prepared by combining 550 mg pyruvate (Sigma P-2256), 335 mg malate (Sigma M-7397), and 200 μ l of 1 M HEPES in 10 ml de-ionized water (diH_2O) and the pH was adjusted to pH 7.2. A 30 mM ADP stock was prepared by combining 128.2 mg ADP (Sigma A-5285) and 200 μ l of 1 M HEPES in 10 ml diH_2O and pH was adjusted to pH 7.2. A 1 mg/ml oligomycin-A stock was prepared by combining 1 mg oligomycin-A (Biomol CM-111) with 1 ml methanol. A 1 mM FCCP stock was prepared by combining 2.542 mg FCCP (Biomol CM-120) with 10 ml 100% ethanol. A 1 M succinate stock was prepared by combining 2.36 g succinic acid (Sigma S-7501) with 400 μ l of 1 M HEPES in 20 ml in diH_2O and the pH was adjusted to pH 7.2. Rotenone was prepared by first combining 3.944 mg rotenone (Biomol CM-117) with 10 ml 100% ethanol for a final concentration of 1 mM. Rotenone was further serially diluted with 100% ethanol to obtain final concentrations of 100 μ M, 10 μ M, 1 μ M, 100 nM, and 10 nM rotenone. Malonate stock was prepared with Malonic Acid (Sigma M1296) in diH_2O and the pH was adjusted to 7.2 for a final concentration of 5 M. KOH and HCl were utilized for all pH adjustments.

2.3. Preparation and calibration of Seahorse sensor cartridge sample plate

A Seahorse Bioscience XF24 extracellular flux analyzer was used to measure mitochondrial function in intact isolated mitochondria. The XF24 creates a transient, 7 μ l chamber in specialized microplates that allows for the determination of oxygen and proton concentrations in real time. The day before the planned experiment, 1 ml of XF Calibrant solution (Seahorse Bioscience) was added to each well of a 24 well dual-analyte sensor cartridge (Seahorse Bioscience). The sensor cartridge was placed back on the 24 well calibration plate and put in a 37 °C incubator without CO_2 (Seahorse Bioscience) overnight.

The day of the experiment, the injection ports on the sensor cartridge were loaded with the appropriate mitochondrial substrates or inhibitors at 10× concentrations. Once the sensor cartridge was loaded with all of the experimental reagents it was placed into the Seahorse XF24 Flux Analyzer for automated calibration. During the sensor calibration, isolated mitochondria were then seeded in 50 µl volume of isolation buffer containing 2.5 µg or 5 µg of protein (BCA method) per well in XF24 V7 cell culture microplates. Following the centrifugation of the plates at $200 \times g$ for 4 min at 4 °C, 575 µl of respiration buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 2 mM MgCl₂, 2.5 mM KH₂PO₄ at pH 7.2) at 37 °C was gently added to each well for a final volume of 625 µl per well at the beginning to the experiment. Plates were immediately placed into the calibrated seahorse XF24 flux analyzer for mitochondrial bioenergetic analysis.

2.4. Seahorse protocol for isolated mitochondria

The following protocol was utilized for the analysis of bioenergetic function in purified mitochondria using the Seahorse Biosciences XF24 Flux Analyzer. Pyruvate plus malate plus ADP, oligomycin, FCCP, and rotenone plus succinate were injected sequentially through ports A–D, respectively, in the Seahorse Flux Pak cartridges to yield final concentrations of 5 mM (pyruvate), 2.5 mM (malate), 1 mM (ADP), 1 µg/ml (oligomycin), 1 µM (FCCP) and 100 nM (rotenone), 10 mM (succinate), respectively. FCCP is a positively charged molecule which can interact with plastic containers, such as the Seahorse cartridges utilized in these experiments, which have a negative surface charge. The interaction of FCCP with the plastic can be disrupted by the addition of the Complex II substrate succinate, which is an acid, and allow for the successful measurement of uncoupled rates of succinate driven Complex II dependent respiration even when Complex I dependent respiration can be problematic. Further optimization of the methods for using the Seahorse XF24 with isolated mitochondria indicate that a 5 µM working concentration of FCCP works better to uncouple Complex I dependent mitochondrial respiration than the 1 µM of FCCP used in the current experiments.

The following sequence was utilized for basal mitochondria respiration analysis (Table 1):

The same protocol was utilized for the log dose response experiments except that rotenone or malonate were injected through ports B–D. For the time dependent effect of rotenone administration a modified seahorse mitochondrial protocol was utilized for the repeated measure of oxygen consumption from the same well following the injection of various low concentrations of rotenone (10 fM, 10 pM, 10 nM).

2.5. Analysis of data generated by Seahorse Biosciences XF24

For optimal analysis of mitochondrial bioenergetic function, the data generated by the XF24 was further analyzed beyond the average oxygen consumption ratio generated for a given measure. The methods for analyzing the Seahorse data are based upon similar protocols for analyzing the rate of oxygen consumption using a Clark-type oxygen electrode. During a given measure, the rate of oxygen consumption varies with time as substrates bind with their targets or are consumed by the mitochondria. Using the Excel software package (Microsoft), point-by-point rates are generated using the AKOS algorithm written by AKOS Gerencser in collaboration with Seahorse Bioscience. For Complex 1 and Complex 2 function, the highest point-by-point rate is taken due to ADP being utilized over time and causing the state III rate to decrease. For the oligomycin-A rate, an average of the last three point-by-point rates is taken since the effect of oligomycin-A can take at least one minute to reach maximal effect and an average of the last 30 s of the measure provides the most consistent result.

2.6. Mitochondrial Complex I assay

Mitochondrial Complex I enzyme activity was determined by measuring the decrease in NADH absorption at 360 nm in the presence and absence of rotenone, as previously described (Gash et al., 2008; Brown et al., 2004). Frozen purified mitochondria were thawed and then diluted in 10 mM potassium phosphate (KPO₄) buffer pH 7.4 to a concentration of 1 µg/µl. Samples then underwent three cycles of 5 min freezing on dry ice followed by 5 min of thawing at 37 °C followed by 5 s of sonication. The assay was performed in a BioTek Synergy HT plate reader (BioTek, Winooski, VT) with excitation at 360 nm and emission at 460 nm. 6 µg of mitochondrial protein was added to 25 mM KPO₄ pH 7.2, 5 mM MgCl₂, 1 mM KCN, 1 mg/ml bovine serum albumin, and 150 µM NADH in a total volume of 69 µl. Either 1 µl of 1 mM rotenone or 1 µl of 25 mM KPO₄ pH 7.2 was added so that with and without rotenone samples could be compared. The reaction was started by the addition of 30 µl of coenzyme Q1 (final concentration 50 µM). Enzyme activity was calculated by subtracting the change in NADH emission in the absence of rotenone from the change in NADH emission in the presence of rotenone. IC₅₀ values were calculated from log dose response data generated during the analysis of the effect of the different concentrations of the inhibitor. Prism 4 (Graphpad Software Inc.) was utilized to calculate the IC₅₀ values using sigmoidal dose–response parameters built into the software package.

2.7. Mitochondrial Complex II assay

Mitochondrial Complex II enzyme activity was determined by measuring the change in absorbance of 2,6-dichloroindophenol sodium at 600 nm using a BioTek Synergy HT plate reader (BioTek, Winooski, VT). Frozen purified mitochondria were thawed and then diluted in 10 mM KPO₄ pH 7.4 to a concentration of 1 µg/µl. Samples then underwent three cycles of 5 min freezing on dry ice followed by 5 min of thawing at 37 °C followed by 5 s of sonication. 3 µg of mitochondria was added to 200 mM KPO₄ pH 7.0, 20 mM K-succinate, 10 µM EDTA, 0.01% Triton X-100, 0.5 µg/50 µl coenzyme Q10, ~20% DCIP for a total volume of 50 µl. The starting optical density was between 0.6 and 0.8 at 600 nm and the exact concentration of DCIP was made so that this starting density was obtained. The change in absorbance was measured at the beginning of the experiment and 2 min later. Enzyme activity was determined by subtracting the final OD from the initial OD. IC₅₀ values for the effect of malonate on mitochondrial Complex II activity were calculated in the same manner as described for Complex I IC₅₀ values.

2.8. Statistical analysis

For all experiments significance was set at $p < 0.05$. Analysis of the mitochondrial protein dependent rate of Complex I oxygen consumption was performed with a two-tailed *t*-test. Comparisons of basal mitochondrial function were conducted using a one-way analysis of variance followed by post hoc analysis to determine group differences. The log dose response experiments and the time dependent rotenone inhibitory effect were analyzed using a two-way analysis of variance followed by post hoc analysis to determine group differences.

3. Results

3.1. Calibration of Seahorse Biosciences XF24 for isolated mitochondria

A major benefit for utilizing the Seahorse Biosciences XF24 compared to previous methods is the ability to use smaller amounts of mitochondrial protein. In order to determine an optimal amount of mitochondrial protein to load per well, 2.5 µg, 5.0 µg, and 10.0 µg amounts were tested. In our initial studies it was demonstrated that 10.0 µg of mitochondrial protein/well resulted in a reduction in sensitivity (data not shown). Comparison of 2.5 µg and 5.0 µg of mitochondrial protein revealed that 5.0 µg per well had a much more robust

rate of Complex I driven, ADP dependent oxygen consumption (Fig. 1A, $p < 0.002$). An important consideration when optimizing an experiment for use in the XF24 is assurance that the rate of oxygen consumption does not exceed the ability of the apparatus to replenish the oxygen tension after a measurement is completed. Though there is a slight drop in oxygen tension once mitochondrial respiration is initiated, analysis of the concentration of oxygen in the well over time revealed no appreciable deficit in the ability for the system to replenish the oxygen tension in the wells (Fig. 1B).

3.2. Basal mitochondrial respiration across brain regions

Oxygen consumption rate was measured in real-time using a Seahorse Bioscience XF24 extracellular flux analyzer in order to determine if basal rates of oxygen consumption differed among the brain regions tested. Mitochondria isolated from different regions of the brain exhibit differences in mitochondrial bioenergetic function with the cerebellum and the striatum exhibiting significant differences. Compared with the striatum, the cerebellum exhibited a 36% slower rate of ADP dependent Complex I driven respiration (Fig. 2A, $p < 0.05$). Analysis of uncoupled rates of Complex I driven respiration revealed no significant difference between groups and had average rates of 1071.5 ± 252.5 pM/min. Analysis of maximal Complex II driven respiration revealed that the striatum was approximately 1.6 times faster than the cerebellum (Fig. 2B, $p < 0.05$). Both the striatum and the hippocampus exhibited higher rates of residual oxygen consumption in the presence of the Complex V inhibitor oligomycin-A compared to the cerebellum (Fig. 2C, $p < 0.05$). The respiratory control ratio (RCR) was calculated as the ratio of Complex I driven respiration in the presence of 1 mM ADP with and without 1 μ M oligomycin-A to determine the overall health and integrity of the mitochondria. No significant differences were observed between any of the brain regions (Fig. 2D).

3.3. Effect of mitochondrial Complex I and II inhibitors on enzymatic activity

Measurement of enzymatic activity was performed in order to determine the direct action of the inhibitors on their respective mitochondrial targets. Analysis of the effect on the Complex I specific inhibitor rotenone revealed that the concentration of rotenone required to inhibit 50% of enzyme activity, the IC₅₀, ranged from 163 to 238 pM and that no significant difference in the IC₅₀ existed across brain regions (Fig. 3C). Further analysis of the effect of rotenone revealed that Complex I enzyme activity was equally affected across brain regions as the concentration of the inhibitor was increased (Fig. 3A). Similar to the effects of rotenone, exposure to the Complex II inhibitor malonate resulted in no significant differences in the calculated IC₅₀ with values ranging from 440 to 580 μ M across brain regions (Fig. 3C). As the concentration of the inhibitor was increased, the resulting decline in Complex II enzyme activity was equal across all brain regions tested (Fig. 3B).

3.4. Effect of mitochondrial Complex I and II inhibitors on oxygen consumption

The Seahorse XF24 was utilized to assess the effects of the inhibitors on mitochondrial ADP dependent oxygen consumption. Mitochondrial enzyme activity does not always change proportionally to mitochondrial oxygen consumption following treatment with a mitochondrial enzyme inhibitor. Direct measurement of oxygen consumption was performed following inhibitor administration to determine the effect of the inhibitors their respective targets. Baseline values for the two inhibitors were calculated from mitochondrial samples in the absence of inhibitor. Acute exposure to rotenone resulted in an immediate dose dependent reduction in mitochondrial Complex I driven oxygen consumption. Significant differences were observed across brain regions as a result of exposure to Rotenone (Fig. 4A). Following exposure to 10 pM rotenone the cerebellum and hippocampus were significantly more inhibited than the striatum or the cortex (Fig. 4A, $p < 0.05$). The hippocampus remained more inhibited than the striatum following exposure to 100 pM

rotenone (Fig. 4A, $p < 0.05$). All differences between brain regions went away at concentrations of rotenone 1 nM and higher. Exposure to malonate resulted in a dose dependent decrease in mitochondrial driven oxygen consumption with no observed differences across brain regions (Fig. 4B).

3.5. Time dependent inhibition of Complex I by rotenone

Human exposure to a toxin can take place acutely or chronically over much longer timeframes than *in vitro* which are utilized to determine effective inhibitory concentrations for a particular toxin. Isolated cortical mitochondria treated with 10 nM of the Complex I inhibitor rotenone exhibited a significant reduction in mitochondrial oxygen consumption immediately following exposure. Lower doses of rotenone, 10 pM and 10 fM, did not result in any reduction in oxygen consumption up to 8 min following exposure. If exposure to rotenone at 10 pM was allowed to continue a significant reduction in mitochondrial oxygen consumption became apparent as early as 12 min following exposure. The decrease in oxygen consumption following 10 pM rotenone exposure eventually reached a 75% reduction in mitochondrial oxygen consumption.

4. Discussion

The ability to test mitochondrial bioenergetic function in a high-throughput and small scale manner will provide researchers with the ability to more rapidly conduct their experiments, allow for increased sample sizes and have tissue remaining for further experimentation. Additionally, this method requires significantly less mitochondrial protein such that different regions of the brain from the same animal can be tested individually alleviating the need to pool samples from multiple animals. In the present studies we have outlined a method which utilizes the Seahorse Biosciences XF24 flux analyzer for the profiling of brain mitochondrial bioenergetic function. We have utilized this method in order to compare differences in brain mitochondrial function and the susceptibility to mitochondrial inhibitors across brain regions. The results of these experiments demonstrate that our novel protocols for utilizing the XF24 can provide consistent results and allow for the simultaneous comparison of multiple samples.

An important factor when setting up the Seahorse Bioscience XF24 for use with isolated mitochondria was to determine the optimal amount of mitochondrial protein to use per well for a given experimental paradigm. For the present studies, determination of the optimal protein concentration was based upon determining the highest measurable rates since this would provide a larger range of oxygen consumption values to distinguish group differences. Previous work utilizing liver mitochondria at a concentration of 2.5 $\mu\text{g}/\text{well}$ has shown the ability to measure oxygen consumption with the XF24, however, no comparison to other concentrations was made nor was brain mitochondria utilized (Gerencser et al., 2009). Comparing the use of 2.5 μg or 5.0 μg of purified brain mitochondria revealed that the 5.0 μg amount provided optimal rates of oxygen consumption based upon our desired characteristics. The higher rate of Complex I dependent oxygen consumption (Fig. 1A) provides a greater measurable range for detecting group differences in future experiments. Additionally, it is important to know whether the XF24 is able to replenish the oxygen tension during an experiment so that there is not a progressive decrease in the starting oxygen tension for subsequent measurements. There was a slight drop in oxygen tension with both protein concentrations once mitochondrial respiration was started, however, comparison of 2.5 μg and 5.0 μg revealed that the XF24 was capable of replenishing the oxygen tension equally with both amounts of mitochondrial protein (Fig. 1B). Comparison of measured rates of mitochondrial oxygen consumption between samples tested with a Clark-type oxygen electrode and samples tested with the XF24 revealed that both methodologies produce similar rates. Previously published data with the Clark-type oxygen

electrode indicate that the cortex exhibits rates of oxygen consumption of approximately 300 pM O₂/min/μg of protein (Sullivan et al., 2004). The present experiments with the Seahorse XF24 indicate that the cortex has a measured rate of 328.8 ± 64.69 pM O₂/min/μg of protein which matches our previously published results. Similar to the findings in the cortex, the present studies indicate that the striatum had measured rates of oxygen consumption of 352.4 ± 64.8 pM O₂/min/μg of protein and previous studies from our group have shown rates of approximately 350 pM O₂/min/μg of protein using the Clark-type oxygen electrode (Korde et al., 2005), further indicating that the two methods can produce equivalent findings. Even though the two techniques provide similar measured rates of oxygen consumption, the Seahorse XF24 is limited by only being able to inject four different substrates during a single experiment. The initial characterization experiments reveal that 5.0 μg provides better experimental conditions since a higher starting oxygen consumption rate will provide greater experimental resolution. Additionally, since previous work has stated that RCRs greater than five indicate healthy mitochondria (Deng-Bryant et al., 2008) and studies looking at various insult models show that only the injured samples have RCRs less than five (Gash et al., 2008; Pandya et al., 2009), it was concluded that the samples utilized in these experiments were all healthy and well-coupled.

Previous studies have shown that regional differences in the activities of mitochondrial enzymes such as malate dehydrogenase and creatine kinase exist (Ryder, 1980; Gupta et al., 2000). Additionally, neuronal activity has been shown to play a direct role in the expression of mitochondrial proteins (Wong-Riley et al., 1997) and reductions in synaptic activity can lead to reduced cytochrome-*C* levels (Nie and Wong-Riley, 1996). Additionally, ischemic injury experiments have shown regionally different mitochondrial susceptibility to the injury (Sims, 1991) which could be the result of mitochondrial or cellular differences. Multiple groups have further established that in addition to brain region differences in mitochondrial function there is a significant difference between synaptic and non-synaptic mitochondria (Davey et al., 1997; Brown et al., 2006; Naga et al., 2007; Pathak and Davey, 2008). Numerous factors are likely to play role in the differential susceptibility of mitochondria such as which region of the brain the mitochondria are from (Singh et al., 2010), which portion of the CNS the mitochondria are from (Sullivan et al., 2004), or the energy demands of the cell containing the mitochondria (Zeevalk et al., 1997).

In order to further elucidate the role of the mitochondria in regionally specific toxin susceptibility, analysis of basal mitochondrial function was performed across various regions of the brain. We hypothesized that regions which appear more susceptible to Complex I or Complex II inhibition *in vivo* may have reduced basal mitochondrial function. The results indicate that the greatest differences in Complex I and Complex II dependent oxygen consumption exist between the cerebellum and the striatum (Fig. 2). The finding of reduced basal oxygen consumption in the cerebellum compared to other brain regions has not been shown before and therefore the underlying reason for this effect is currently unknown. Previous work has shown a reduced activity of Complex II enzyme activity in the cerebellum (Fagundes et al., 2007) and this could be an underlying reason for the reduced Complex II dependent oxygen consumption observed in these studies. It has recently been shown that resting aerobic glycolysis is lower in the cerebellum than other brain regions (Vaishnavi et al., 2010) and this may be the result of different ratios of neuronal and non-neuronal cells in the cerebellum (Azevedo et al., 2009). Interactions between neurons and astrocytes during *in vivo* oxygen consumption has been shown to occur (Kasischke et al., 2004) with the two cell types providing different roles during energy metabolism (for review see (Magistretti, 2006)). Given the higher percentage of neurons to astrocytes in the cerebellum, the regional differences in the mitochondrial respiration observed in the present studies may be affected by different cell type ratios.

Basal Complex I and Complex II dependent mitochondrial oxygen consumption did not differ between the cortex, striatum, or hippocampus in these experiments which indicates that mitochondria from these regions have similar bioenergetic capacities and profiles. Interestingly, the cerebellum had a significantly slower rate of oxygen consumption in the presence of the Complex-V inhibitor oligomycin-A compared to the striatum and hippocampus (Fig. 2B). The difference in oxygen consumption in the presence of oligomycin-A may be the result of lower levels of glutathione and superoxide dismutase in the striatum and hippocampus relative the cerebellum (Sanchez-Iglesias et al., 2009) which could possibly allow for increased damage to the inner mitochondrial membrane and increased proton leak across the damaged membrane. The profiling of basal mitochondrial function further indicates that mitochondrial health and integrity is not detrimentally affected by analysis with the XF24. Calculation of the mitochondrial respiratory control ratio revealed extremely high levels of coupling between the pumping of protons across the inner membrane with the formation of ATP by Complex V (Fig. 2D). These experiments indicate that not only is the XF24 capable of measuring mitochondrial function with only 5 μ g of protein but also it can be done without damaging the mitochondria.

Differences in mitochondrial spare respiratory capacity have been documented which may provide insight into the regional heterogeneity of *in vivo* mitochondrial toxin models. Previous reports utilizing the toxin trichloroethylene (Gash et al., 2008; Liu et al., 2010) or 3-nitropropionic acid (3-NP) (Beal et al., 1993) have shown that different regions of the brain do appear more susceptible than other regions. Mirandola et al., 2010 showed that following *in vivo* systemic exposure to the mitochondrial Complex II inhibitor 3-NP there are regional differences in the susceptibility to mitochondrial permeability transition in mitochondria isolated from the brain. However, in isolated mitochondria *in vitro* treatment with 3-NP affected mitochondria equally across the same brain regions. It has been suggested that one of the major reasons certain brain regions are more susceptible than others to a particular toxin is not the mitochondria itself but the environment the cells are located in (Mirandola et al., 2010). Analysis of the susceptibility of isolated mitochondria to Complex I or Complex II inhibition indicates that there are no differences between brain regions with regard to the effect these toxins have on enzyme activity (Fig. 3).

This finding is in agreement with results looking at Complex II activity and the susceptibility to 3-NP where the cortex, striatum, and cerebellum had similar basal enzyme activities and susceptibilities to inhibition (Mirandola et al., 2010). It has previously been shown that the percentage inhibition of mitochondrial Complex I activity is correlated with the percentage reduction in mitochondrial oxygen consumption (Davey et al., 1998). The current results support this previous finding since the dose dependent reduction in mitochondrial oxygen consumption in the presence of the Complex I inhibitor Rotenone (Fig. 4A) is similar to of the reduction in Complex I enzyme activity (Fig. 3A). The present study further extends this effect to Complex II dependent oxygen consumption since the effect of the inhibitor malonate had a nearly identical concentration dependent effect on oxygen consumption (Fig. 3B) as it did on enzyme activity (Fig. 3B). These results further indicate that at low concentrations of the inhibitor rotenone, differences in the percentage reduction of oxygen consumption does differ between brain regions (Fig. 4A). These results further support the ability of the XF24 to measure small changes in oxygen consumption since significant and consistent changes in the dose dependent effect of the inhibitors can be reproducibly measured.

Previous unpublished experiments from our group indicated that a time dependent effect of rotenone on Complex I dependent mitochondrial function may exist at low concentrations of the inhibitor. To test if time of exposure was a factor, repetitive measurements were taken of mitochondrial samples treated with either 0, 10 fM, 10 pM, or 10 nM rotenone. As shown in

our previous experiments (Fig. 4), the 10 nM rotenone produced an immediate significant reduction in oxygen consumption while the 10 pM rotenone did not produce a significant reduction in oxygen consumption (Fig. 5). Strikingly, continued exposure to the 10 pM rotenone produced a clear time dependent reduction in Complex I dependent oxygen consumption which became significant by 12 min post injection and reached a 75% reduction in oxygen consumption by 16 min (Fig. 5). This is an important finding to consider when attempting to understand human toxin exposure since chronic exposure to low doses of a toxin may result in a greater inhibition of mitochondrial function.

5. Conclusion

In summary, the presented experiments indicate the ability to simultaneously measure mitochondrial bioenergetic function in 20 5 µg samples utilizing the Seahorse Bioscience XF24. Furthermore, the results show that there are limited differences in basal mitochondrial bioenergetic function in samples isolated from the cortex, striatum, or hippocampus. The cerebellum did exhibit significant differences in basal mitochondrial function but the susceptibility to mitochondrial Complex I and Complex II inhibitors remained proportional to the other brain regions. Like the cerebellum, the other regions of the brain were relatively similar to mitochondrial enzyme inhibition except at low concentrations of rotenone where regional differences in susceptibility became apparent. This novel approach also shows that the effect of rotenone on Complex I function is time dependent at low concentrations. The proposed methods will allow researchers to easily and quickly measure mitochondrial function in purified samples.

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Fig. 1.

5.0 μg of protein per well is optimal for mitochondrial bioenergetic analysis. Compared with 2.5 μg of mitochondrial protein, loading 5.0 μg resulted in superior experimental characteristics since there is a larger range to measure basal or impaired mitochondrial respiration. (A) As expected, loading 5.0 μg of protein per well resulted a higher rate of Complex I dependent mitochondrial respiration. (B) There was no difference in the ability of the XF24 to replenish the amount of oxygen per well following an oxygen consumption measurement between the two protein concentrations ($n = 3-4/\text{group}$. $*p < 0.002$ with two-tailed t -test).

Fig. 2.

Regional differences exist in brain mitochondrial function. Mitochondrial bioenergetic analysis reveals regionally specific variations in mitochondrial function. (A) Compared with the striatum, the cerebellum exhibited a 36% slower rate of Complex I driven mitochondrial oxygen consumption. (B) Similar to the Complex I findings, analysis of Complex II dependent oxygen consumption revealed that the cerebellum had a significantly slower rate of oxygen consumption. (C) The striatum and the hippocampus exhibited nearly two-fold higher rates of oxygen consumption in the presence of oligomycin-A when compared to the cerebellum. (D) No significant differences exist between brain regions with respect to the mitochondrial respiratory control ratio. Mitochondria which are Ficoll purified and analyzed with the Seahorse XF24 exhibit very high RCR values indicating healthy mitochondria ($n = 6/\text{group}$). * $p < 0.05$ with one-way ANOVA and SNK-post test; # $p < 0.05$ with one-way ANOVA and protected Fischer's LSD).

Fig. 3.

Mitochondrial Complex I and II inhibitors affect enzyme activity equally across brain regions. (A) No differences were observed in the response of the different brain regions to Complex I enzyme inhibition. As the amount of rotenone was increased, all of the tested brain regions had similar percentage reductions in enzyme activity. (B) The response to Complex II inhibition was not significantly different across brain regions. Similar to the effects observed following rotenone administration, as the concentration of malonate was increased the various brain regions had similar decreases in enzyme activity. (C) No significant differences were observed in the calculated IC₅₀ values across brain regions and the reduction in enzyme activity in the presence of rotenone was comparable across all brain regions tested. The calculated IC₅₀ values for malonate did not differ significantly between the brain regions tested ($n = 3-5/\text{group}$. Two-way ANOVA)

Fig. 4.

Regional differences exist in the susceptibility to rotenone but not malonate. (A) At low concentrations of the mitochondrial Complex I inhibitor rotenone the cerebellum and hippocampus are more susceptible to inhibition of oxygen consumption than the striatum or the cortex. As the concentration of rotenone increased, the differences between brain regions eventually went away. (B) Exposure to the Complex II inhibitor malonate produced dose dependent reductions in mitochondrial oxygen consumption which did not differ across brain regions for a given concentration of inhibitor ($n = 5/\text{group}$. $*p < 0.05$ two-way ANOVA with Bonferroni post-test).

Fig. 5.

Exposure to 10 pM rotenone produces a progressive decrease in mitochondrial oxygen consumption. Samples which were exposed to 10 nM rotenone produced an immediate reduction in oxygen consumption similar to previous finding. Similarly, exposure to 10 fM rotenone resulted in no reduction in oxygen consumption. In contrast to acute measurements of rotenone dependent reductions in Complex I driven oxygen consumption, exposure to 10 pM resulted in a progressive reduction in mitochondrial oxygen consumption. For at least 8 min following exposure to 10 pM rotenone no reduction in oxygen consumption is observed. As early as 12 min following exposure to 10 pM rotenone a significant reduction in oxygen consumption occurs that continues to decrease with time. A 75% reduction in Complex I driven oxygen consumption is observed at 16 min post-exposure and this level of inhibition continues beyond this time point without further reductions ($n = 5/\text{group}$. * $p < 0.05$ two-way ANOVA with Bonferroni post-test).

Table 1

Protocol for standard mitochondrial analysis.

Step	Command
1	Calibrate probes
2	Mix 1 min
3	Time delay 1 min and 30 s
4	Mix 25 s
5	Measure 2 min
6	Mix 1 min
7	Inject port A (pyruvate/malate/ADP)
8	Mix 25 s
9	Measure 2 min
10	Mix 1 min
11	Inject port B (oligomycin)
12	Mix 25 s
13	Measure 2 min
14	Mix 1 min
15	Inject port C (FCCP)
16	Mix 25 s
17	Measure 2 min
18	Mix 1 min
19	Inject port D (rotenone/succinate)
20	Mix 25 s
21	Measure 2 min