Research Article

Isolation of Mitochondria and Measurement of Oxygen Consumption Rate by High-resolution Respirometry in Rat Hippocampus

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ABSTRACT

Background: Hippocampus plays an important role in memory and learning. Dysfunction of mitochondrial bioenergetics has been implicated in neurodegenerative disorders such Alzheimer disease. Oxygen consumption rate (OCR) is a useful indicator of mitochondrial function. Mitochondria isolated for OCR measurement need to have well intact function. This study aimed to describe the measurement of OCR by high resolution respirometry (HRR) in mitochondria isolated from rat hippocampus

Methods: Protocol of mitochondrial isolation was based on sucrose step density gradient centrifugation method as adapted from previous study. OCR of isolated mitochondria was measured using OROBOROS HRR Oxygraph-2k according to substrate-uncoupler-inhibitor titration (SUIT) protocol.

Results: Our results showed that OCR increased following the addition of complex I-linked substrates (glutamate, pyruvate, and malate), coupling substrate (adenosine dinucleotide) and complex II-linked substrate (succinate). OCR decreased following the addition of ATP synthase inhibitor (oligomycin), while maximal respiration was achieved following the addition of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) uncoupler. Residual oxygen consumption (ROX) was obtained following the addition of complex I inhibitor (rotenone) and complex III inhibitor (antimycin A).

Conclusions: Taken together, our findings suggested that mitochondria isolated from this protocol can be used for measurement of OCR using HRR. Measurement of OCR offers meaningful information regarding the mitochondrial bioenergetics states of a sample.

***Keywords*:** mitochondrial isolation, oxygen consumption rate, high-resolution respirometry, hippocampus

1. Introduction

The major function of hippocampus is related to learning and memory [1,2]. Hippocampus processes and retrieves 2 types of memories which are spatial and declarative memories. Spatial memory involves route and pathway, for example when locating a route during driving. On the other hand, declarative memory involves event and fact, such as when learning to memorize a speech. These short-term memories are turned into long-term memory in hippocampus and then stored in other part of the brain. Age-associated neurodegenerative diseases such as Alzheimer disease (AD) is characterized by progressive loss of memory and having difficulty to form new and long-term memories which are likely due to damages in hippocampus. Although it is a multifactorial disorder, mitochondrial dysfunction of bioenergetics appears to play an important role in the pathogenesis of AD [3,4]. Mitochondrial respiration was reported to be lower in hippocampal neurons of an AD mouse model [5] and impaired in fibroblasts of AD patients [6]. In contrast, activities of mitochondrial complexes had been shown to be increased in lymphocytes of AD patients which implied that compensatory mechanisms might exist to supply energy [7].

Cellular respiration is a metabolic pathway that converts energy from nutrients into energy of the cell: for example breaks down of glucose and production of adenosine triphosphate (ATP) which involve glycolysis, pyruvate oxidation, citric acid cycle (CAC; also known as tricarboxylic acid, TCA; or Krebs cycle), and oxidative phosphorylation (OXPHOS). The ATP can be generated by non-mitochondrial pathways such as glycolysis; and by mitochondrial pathways like OXPHOS. The primary function of mitochondria is to produce energy in the form of ATP for cellular usage through OXPHOS. OXPHOS is the formation of ATP as a result of electron transfer from nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) to oxygen (O_2) by a series of electron carriers [8]. NADH and $FADH_2$ are produced from earlier phase of cellular respiration such as glycolysis, pyruvate oxidation, and CAC; while O_2 is the final electron acceptor. Therefore, the uptake of O_2 is used as an indicator for mitochondrial bioenergetics in term of efficiency of OXPHOS. Electron transport chain (ETC) is consisted mainly of a series of large protein complexes that are span across inner membrane of mitochondria. Transfer of electrons along these complexes release energy which is used by the complexes to pump protons (H^+ ions) from matrix into intermembrane space. This creates a proton or electrochemical gradient, also known as proton-motive force, which drives the ATP synthesis by chemiosmosis. Chemiosmosis occurs when H^+ ions move down their gradient back into the matrix through ATP synthase. ATP synthase harnesses chemiosmosis to catalyze the synthesis of ATP. ETC is organized into 4 complexes, namely complex I (CI; also known as NADH:ubiquinone oxidoreductase), CII (succinate dehydrogenase), CIII (coenzyme Q:cytochrome *c*), and CIV (cytochrome *c* oxidase). CI accepts electrons from NADH, while CII is an electron acceptor for $FADH_2$. Electrons from both complexes are converged at ubiquinone which is a coenzyme that carries the electrons to CIII. The electrons are transferred from CIII to CIV via another carrier known as cytochrome *c* and eventually they are transferred to O_2 at CIV to form water [8] (Figure 1).

The function of mitochondria can be assessed by several parameters such as mitochondrial membrane potential, production of reactive oxygen species, and intactness of OXPHOS machinery based on ETC activity and protein expression. Measurement of proton flux is more informative than other intermediate parameters to assess mitochondrial function as ATP producer. The generation of proton flux from ETC provides a mean to evaluate proton flux based on rate of oxygen consumption by mitochondria [9]. Oxygen consumption rate (OCR) or respiration rate measures the uptake of O_2 by CIV to convert it into water. OCR can be extended to assess activity of other complexes in intact cell or isolated mitochondria by using appropriate mitochondrial substrates and inhibitors. OCR is useful to evaluate the effect of exogenous compounds such as natural products on mitochondrial function to identify the potential mitochondrial modulators. As the dysfunction of mitochondria is implicated in neurodegenerative diseases such as AD, it is crucial to understand how mitochondrial bioenergetics of hippocampus is associated with the pathological conditions. OCR is a meaningful indicator for measuring mitochondrial function, though it has been traditionally evaluated by a cumbersome Clarke-type oxygen electrode which requires large amount of sample. Recent techniques such as the use of O_2 specific fluorescent probe on plate-based analyzer or high-resolution respirometry (HRR) have been available to measure OCR. HRR has the advantage to continuously assess different states of respiration of the same sample in response to mitochondrial substrates, inhibitors and uncouplers. In addition, it has more stable signal, lower background noise and higher resolution than Clark-type oxygen electrode [10].

OCR can be determined from intact or permeabilized cells, and from isolated mitochondria. Due to the thickness of tissue which lack of rapid O_2 diffusion, mitochondria are usually isolated from tissue biopsy to measure OCR, though very thin muscle slices have been used for the assay [9]. Differential centrifugation is one of the most common methods to isolate mitochondria from hippocampus for measurement of OCR [11-14], though differences exist among the isolation protocols such as recipe of isolation buffer, homogenization tool, and centrifugal speed. Measurement of OCR requires the isolated mitochondria with well intact function. The appropriate protocol has to be selected to isolate mitochondria with satisfactory functionality and purity. A previous method is available to isolate mitochondria on rat brain for measurement of OCR using Clark-type oxygen electrode which is also suitable for measurement of citrate synthase activity [15,16]. It is unclear whether the method and medium are compatible for measurement of OCR using HRR; although the isolation method and medium had been modified to simultaneously measure OCR and hydrogen peroxide production using HRR [17]. Therefore, this study described the measurement of OCR in

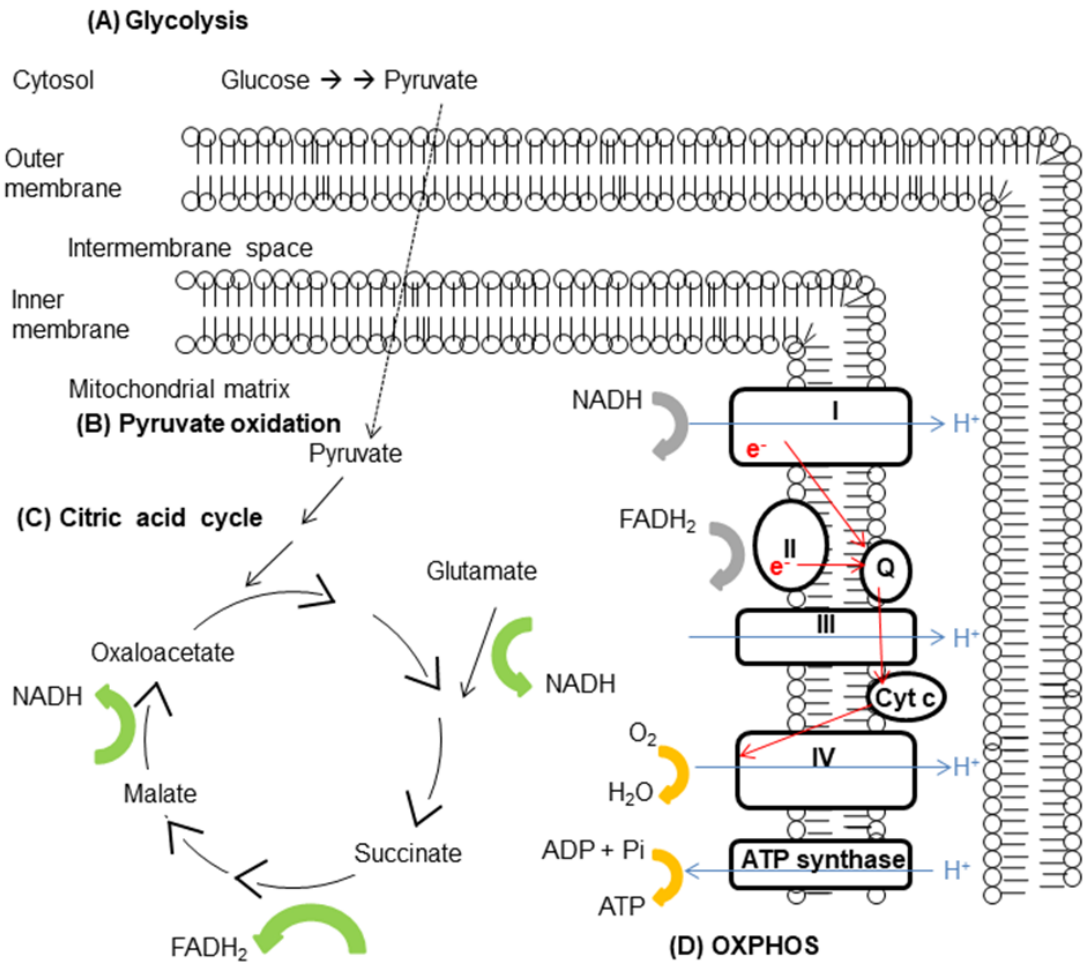

Figure 1. Pathways of cellular respiration. (A) Glucose is broken down into pyruvate through glycolysis and (B) followed by pyruvate oxidation. (C) When pyruvate, glutamate and malate are added to isolated mitochondria, NADH and $FADH_2$ are produced in the citric acid cycle. (D) NADH and $FADH_2$ donate their electrons to oxygen through a series of electron transfer using mitochondrial complexes (I to IV) to generate proton gradient. The chemiosmosis drives the synthesis of ATP by a process known as oxidative phosphorylation (OXPHOS). I: complex I; II: complex II; III: complex III; IV: complex IV; ADP: adenosine diphosphate; ATP: adenosine triphosphate; cyt *c*: cytochrome *c*; e^-: electron; $FADH_2$: flavin adenine dinucleotide; H^+: hydrogen ion; NADH: nicotinamide adenine dinucleotide; O_2: oxygen; P_i: phosphate; Q: ubiquinone; .

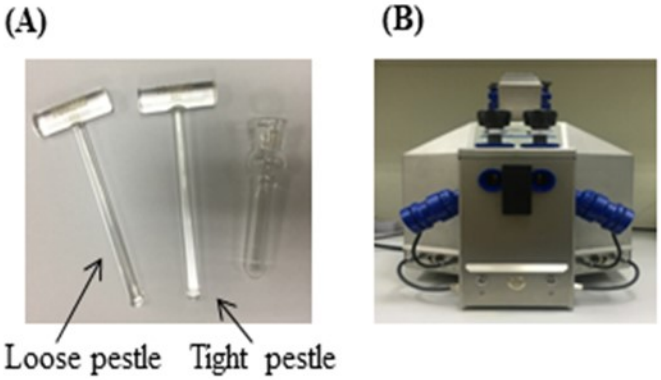

Figure 2. Tool and equipment for this study. **(A)** Dounce homogenizer with loose and tight pestles was used in the mitochondrial isolation protocol. **(B)** High resolution respirometer Oxygraph-2k was used for the measurement of oxygen consumption rate.

mitochondria of rat hippocampus using HRR. Mitochondrial isolation protocol was adapting from the previous studies [15,16] with slight modification.

2. Material and Methods

Preparation of buffers

Chemicals were purchased from Sigma-Aldrich (St. Louis, MI, USA) unless otherwise stated. Mitochondrial isolation buffer A was made up of 320 mM sucrose, 10 mM Tris pH 7.4, 1 mM Na_2EDTA (instead of K_2EDTA as in the original protocol), 2.5 g/L bovine serum albumin (BSA). Isolation buffer B was prepared as in isolation buffer A without BSA. Both buffers were aliquoted and stored at -20 ^{0}C. Reagents for respiration were prepared as described previously [18].

Isolation of mitochondria

Animal handling procedures were performed in accordance to the institutional guidelines. Male Sprague-Dawley rats of 12 weeks old were anesthetized with intraperitoneal injection of ketamine/xylazine at 0.1 mL/100 g body weight and followed by decapitation. The protocol for isolation of mitochondria was carried out using sucrose step density gradient centrifugation method as described previously with slight modifications [15,16]. Briefly, brain was dissected out from the skull and washed with 20 mL pre-cold isolation buffer A. Hippocampi from both hemispheres were separated from the whole brain and wet weight was recorded. Hippocampi were minced with scissors and transferred to a pre-cold 1 mL glass Dounce homogenizer (Wheaton, DWK Life Sciences, Millville NJ, USA) (Figure 2A). Dounce homogenizer was chosen instead of high-speed electrical-drive homogenizer in the original protocol because it is a gentle homogenizer and can accommodate small amount of sample. 1 mL of pre-cold isolation buffer A was added to the homogenizer for every 10 mg of tissue. The tissue was homogenized with loose pestle for 10 up-and-down strokes (1 round of up and down passes was considered as 1 complete stroke) in ice, followed by 10 strokes with tight pestle. The homogenate was transferred to a 1.5 mL tube and centrifuged at 1,000 x g for 10 min at 4 ^{0}C. The supernatant (contained mitochondria) was transferred to a new 1.5 mL tube and centrifuged at 6,200 x g for 10 min at 4 ^{0}C, while the pellet (contained whole cells, nuclei and cell debris) was discarded. After the medium speed centrifugation, the supernatant was discarded while the pellet (contained mitochondria) was washed with 1 mL of isolation buffer B followed by centrifugation at 6,200 x g for 10 min at 4 ^{0}C. Then, the supernatant was discarded and the pellet that contained mitochondria was resuspended with a smaller volume of isolation buffer B: 100 µL for every 10 mg of tissue wet weight which was equivalent to 0.1 mg/µL mitochondrial homogenate suspension. For measuring OCR, the mitochondrial suspension was stored in ice and used within 4 h. The remaining mitochondrial suspension was stored at -80 ^{0}C for the determination of protein concentration and citrate synthase activity.

Measurement of OCR

Mitochondrial respiration was measured using HRR Oxygraph-2k (O2k, OROBOROS Instrument, Innsbruck, Austria) (Figure 2B) at 37 ^{0}C using respiration medium: MiR05 (110 mM sucrose, 20 mM HEPES pH 7.1, 10 mM KH_2PO_4, 3 mM $MgCl_2$, 0.5 mM EGTA, 20 mM taurine, 60 mM K-lactobionate, and 0.1% fatty acid free BSA). Substrate-uncoupler-inhibitor titration (SUIT) protocol was used with modifications to determine the respiration of mitochondrial homogenate isolated from the rat hippocampus [8]. SUIT protocol is a series of titration of the exogenous mitochondrial substrates, uncouplers and inhibitors on mitochondrial homogenate to evaluate the OCR obtained using HRR. The suitable amount of homogenate used in a respiration chamber was determined by titrating the sample in 3 steps: 0.5, 1, and 2 mg of total tissue suspension (based on wet weight) per 2 mL chamber.

The ready-states of respiration in a SUIT protocol can be classified into ROUTINE respiration, LEAK respiration, OXPHOS capacity, electron transport system (ETS) capacity and residual oxygen consumption (ROX) which can be further divided into CI-, CII-, and CI&II-linked respiration [8]. The OCR was recorded to measure the ROUTINE respiration based on endogenous substrates. Then, LEAK respiration with exogenous substrates was measured following the addition of glutamate, pyruvate, and malate (final concentration: 10 mM, 10 mM, and 2 mM, respectively) which represented CI-linked LEAK (CI_L) respiration. Next, ADP (2 mM) was added to evaluate CI-linked OXPHOS (CI_P) capacity, followed by addition of succinate (10 mM) to obtain CI&II-linked OXPHOS ($CI\&II_P$) capacity. Oligomycin (1 µM) was added to assess the oligomycin-induced CI&II-linked LEAK ($CI\&II_{LOmy}$) respiration. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) uncoupler was titrated step-wise (0.5, 1.0 and 1.5 µM) to determine the CI&II-linked maximum ETS ($CI\&II_{ETS}$) capacity. Finally, rotenone (0.5 µM) was added to measure CII-linked ETS (CII_{ETS}) capacity, followed by addition of antimycin A (1 µM) to determine residual oxygen consumption (ROX). Concentrations in bracket represented the final concentration in the respiration chamber. OCR was analyzed by DatLab 7.3.0.3 software (OROBOROS Instrument).

Determination of protein concentration

Protein concentration was measured by BCA Protein Assay kit (Pierce, Thermo Fischer Scientific, Rockford, IL, USA) according manufacturer's recommendation. Briefly, mitochondrial homogenate was IL, USA) according manufacturer's recommendation. Briefly, mitochondrial homogenate was diluted at 1:20 with milliQ water. Working reagent was prepared by mixing BCA reagent A and B at a ratio of 50:1. Next, 10 µL sample or standard was added to a 96-well plate with 200 µL working reagent. The mixture was incubated at 37 0C for 30 min and cooled to room temperature before reading the absorbance at 562 nm using plate reader (EnSpire, PerkinElmer, Waltham, MA, USA).

Measurement of citrate synthase activity

Citrate synthase activity was determined as described previously with slight modifications [19,20]. Briefly, 1 uL of mitochondrial homogenate (4.9 µg of mitochondrial protein as determined using BCA kit), glutathione standards (0 to 20 nmol) or citrate synthase enzyme as positive control (3.4 mU; Sigma-Aldrich) was added to a 96-well plate with 99 µL reaction mixture consisted of 0.1 M Tris pH 8.0, 0.25% Triton-X, 0.3 mM acetyl coA, 0.5 mM oxaloacetate, and 0.1 mM DTNB. Absorbance at 412 nm was measured in a kinetic mode with 30 s interval for 40 min at 25 ^{0}C using EnSpire plate reader. The data was analyzed using EnSpire 4.13 software and Microsoft Office 2010 Excel (Redmond, WA, USA). N = 3 biological replicates with 3 technical replicates for each sample.

3. Results and Discussion

Titration of mitochondrial homogenate using tissue weight showed that the ROUTINE respiration increased in a weight dependent-manner of 0.5, 1, and 2 mg (Figure 3). A 2 mg of tissue suspension per chamber was selected for SUIT protocol in this study.

In isolated mitochondria under SUIT protocol, malate generates NADH as it oxidized to oxaloacetate as in the CAC. Malate alone is unable to support respiration of mitochondrial homogenate because oxaloacetate, which is a potent inhibitor of CII, will be accumulated in the absence of glutamate or pyruvate [8]. Therefore, the respiration can only be stimulated by a combination of CI -linked substrates such as pyruvate/malate, glutamate/ malate and pyruvate/glutamate/malate (Figure 1C). Similarly, in the SUIT protocol, $FADH_2$ is produced during the oxidation of succinate to fumarate in the CAC. $FADH_2$ donates its electrons to CII; therefore, succinate is a CII-linked substrate. Nevertheless, the addition of succinate alone to mitochondrial homogenate may lead to accumulation of oxaloacetate [8].

Coupled respiration is referred to the phenomenon that oxygen uptake of intact mitochondria is dependent on the ATP synthesis, and hence, depends on levels of ADP and inorganic phosphate because ATP is synthesized from both substrates [8]. Therefore, addition of ADP to mitochondrial homogenate increases the OCR. However, the coupling of respiration and phosphorylation will be broken in the presence of uncoupler agents such as CCCP. Uncouplers are small amphipathic molecules that increase proton permeability of phospholipid bilayers, renders the disconnection of oxygen consumption and phosphorylation. The consequence is respiration increases rapidly without any subsequent ATP synthesis.

There are 2 major types of mitochondrial inhibitors, namely respiration inhibitor and phosphorylation inhibitor. In the SUIT protocol, respiration inhibitors can block respiration after the addition of an uncoupler. While phosphorylation inhibitors can only prevent oxygen uptake if the coupling is intact. Therefore, they are added before uncouplers in a SUIT protocol. Respiration inhibitors include rotenone (CI inhibitor), malonate (CII inhibitor), antimycin A (CIII inhibitor), and cyanide (CIV inhibitor), while oligomycin is a phosphorylation that inhibits ATP synthase.

ROUTINE respiration is influenced by ATP turn over, substrates and proton leak [9]. The presence of substrates can trigger the ROUTINE respiration dramatically. Our results showed that addition of exogenous CI-linked substrates such as glutamate, pyruvate and malate increased the OCR of the mitochondrial homogenate which was represented by CI_L respiration (Figure 4). LEAK respiration is defined as consumption of oxygen without ATP synthesis. Oxygen uptake during LEAK state is to compensate for conditions such as proton leak and slip. Proton leak is a proton flux across back inner mitochondrial membrane due to proton motive force which bypasses the ATP synthase, while proton slip is the slipping back of protons to mitochondrial matrix through the CI-IV. LEAK state is a dissipative component of respiration as proton gradient is not use for coupling but producing heat. In a SUIT protocol, LEAK respiration can be measured in non-phosphorylating state which is in the absence of ADP or by inhibition of ATP synthase [8]. CI_L is the respiration of mitochondrial in the presence of exogenous CI-linked substrates such as malate, glutamates, and pyruvate.

Based on our data, OCR was increased with the addition of ADP to represent CI_P capacity which was an ADP-stimulated coupled respiration (Figure 4). OCR was further increased with the addition of succinate which was represented as $CI\&II_P$ capacity (Figure 4), a basal respiration in response to CI&II- and ATP-linked substrates (Figure 4). The basal respiration can also be tested in response to other substrates such as fatty acids to determine the preferred fuels used by a particular cell type.

Decrease of OCR after the addition of oligomycin was an indication for $CI\&II_{LOmy}$ respiration (Figure 4), a LEAK respiration induced though inhibition of ATP synthase by oligomycin as oppose to LEAK respiration in the absence of ADP (CI_L) (Figure 4). The difference difference between $CI\&II_P$ and $CI\&II_{LOmy}$ reveals an ATP-linked respiration (Figure 4) which is an indicator for coupling efficiency and ATP turnover [9,21]. Maximal respiration rate which was represented by CI&IIETS capacity was achieved following the titration of uncoupler CCCP (Figure 4). The reserve respiration capacity can be revealed by the difference between CI&IIP and CI&IIETS (Figure 4), which represents the ability of a mitochondria in response to an increase in energy demand [9,21].

OCR decreased after the addition of rotenone to show CIIETS capacity, and further decreased to minimal following addition of antimycin A to reveal the ROX (Figure 4). CIII is the oligomycin; U0.5: 0.5 µM CCCP uncoupler; U1.0: 1.0 µM CCCP; U1.5: 1.5 µM CCCP; Rot: rotenone; Ama: antimycin A. Basal respiration (orange area) is respiration based on exogenous CI&CII-linked substrates and ADP. Proton LeakOmy (red area) is LEAK respiration that was insensitive to oligomycin or

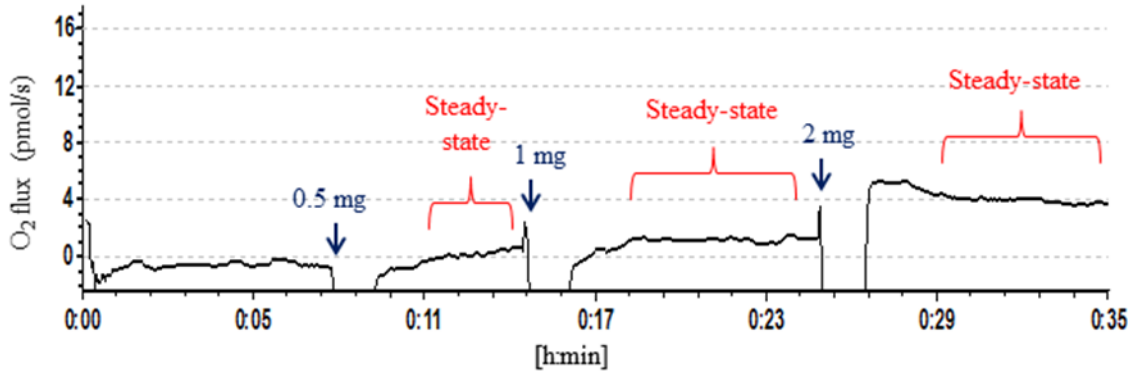

Figure 3. Oxygraph showing the titration of mitochondria homogenate based on wet weight of tissue to determine the starting amount for oxygen consumption rate (OCR) measurement. First, 5 µL mitochondrial homogenate (equivalent to 0.5 mg of tissue wet weight) was added to respiration chamber. After a steady-state of respiration was achieved, another 5 µL of homogenate was added and waited for steady-state. Lastly, 10 µL of homogenate was added to the same chamber and allowed respiration to reach steady-state. The total amount of tissue input in each step was 0.5, 1, and 2 mg of wet weight which was equivalent to 5, 10, 20 µL of mitochondrial homogenate. OCR was represented by O_2 flux (pmol/s) in the y axis.

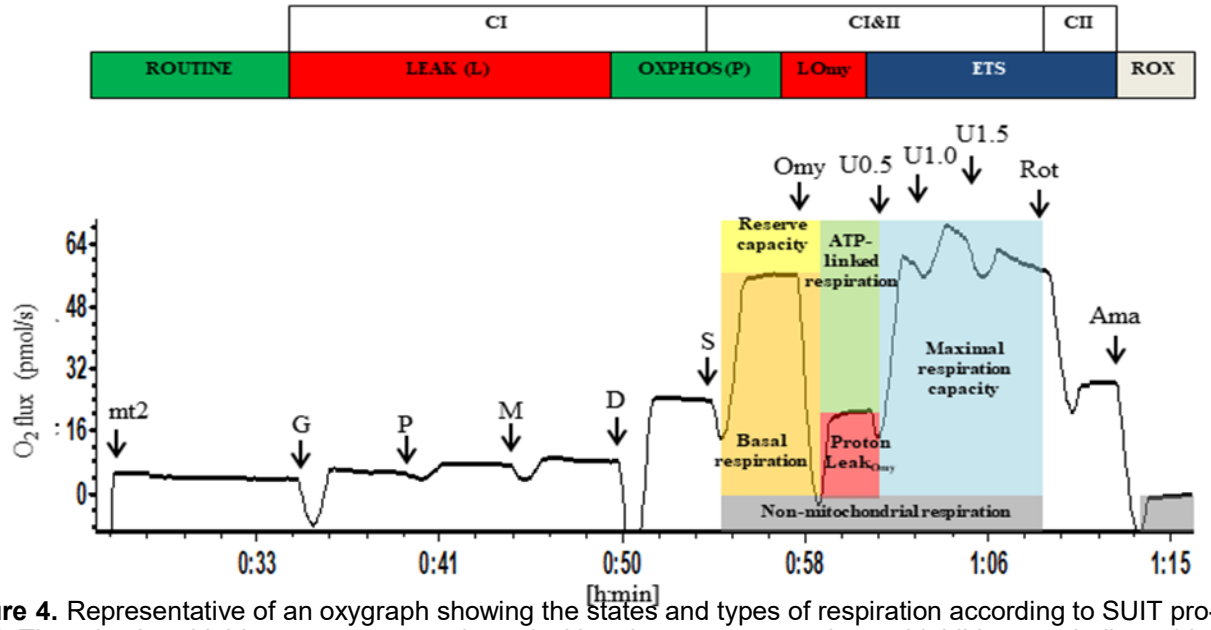

Figure 4. Representative of an oxygraph showing the states and types of respiration according to SUIT protocol. The mitochondrial homogenate was titrated with substrates, uncoupler and inhibitors as indicated by the respective arrow. N = 3 biological replicates. ROUTINE: ROUTINE respiration without exogenous substrates; CI_L: CI-linked LEAK respiration; CI_P: CI-linked OXPHOS capacity; $CI\&II_P$: CI & II-linked OXPHOS capacity; $CI\&II_{LOmy}$: Oligomycin-induced CI&II-linked LEAK resorption; $CI\&II_{ETS}$: CI&II-linked ETS capacity; CII_{ETS}: CII-linked ETS capacity; ROX: residual oxygen consumption; mt2: 2 mg tissue wet weight of mitochondrial homogenate; G: glutamate; P: pyruvate: M: malate; D: ADP; S: succinate; Omy: oligomycin; U0.5: 0.5 µM CCCP uncoupler; U1.0: 1.0 µM CCCP; U1.5: 1.5 µM CCCP; Rot: rotenone; Ama: antimycin A. Basal respiration (orange area) is respiration based on exogenous CI&CII-linked substrates and ADP. Proton $Leak_{Omy}$ (red area) is LEAK respiration that was insensitive to oligomycin or ATP turnover. ATP-linked respiration (green area) is respiration that was coupled to ATP synthesis. The maximal respiration capacity (blue area) is maximal respiration rate that could be achieved by mitochondria. Reserve capacity (yellow area) is respiration that can be achieved in response to energy demand. Non-mitochondrial respiration (gray area) which is the residual oxygen consumption (ROX) was detected as 0 in our result.

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OCR decreased after the addition of rotenone to show CII_{ETS} capacity, and further decreased to minimal following addition of antimycin A to reveal the ROX (Figure 4). CIII is the convergent junction to receive electron transfer from CI and CII. Therefore, inhibition of CIII by antimycin A prevents overall oxygen uptake. Respiration in ROX state is accounted for the non-mitochondrial respiration (Figure 4) such as activity of non-mitochondrial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [9]. Maximal respiration capacity of mitochondria can be obtained by subtracting $CI\&II_{ETS}$ from ROX (Figure 4). A low maximal respiration capacity is a strong indication of mitochondrial dysfunction [9].

Taken together, our results indicated that the mitochondrial isolation protocol can be used to assess the bioenergetics of mitochondria from rat hippocampus based on OCR. The OCR data obtained from O2k can be used to compare the bioenergetics of rats to reveal the association of cellular metabolism, more specifically mitochondrial function, with certain diseases, treatments or conditions.

Total wet weight of the both hippocampi was 10.7 mg, while protein concentration of the mitochondrial homogenate was 4.9 mg/mL. Citrate synthase activity of the mitochondrial homogenate was 0.21 U/mg protein. Citrate synthase is a mitochondrial matrix enzyme that is commonly used to normalization mitochondrial assays. In addition to normalization by tissue weight, OCR data obtained from O2k could be further normalized by total protein content or citrate synthase activity (Table 1).

The limitation of this study is that optimization of mitochondrial isolation protocol was not performed and then measured for OCR to compare which modifications produced better purity and quality of mitochondria. The parameters could be optimized based on previous studies [11-14] on isolation buffer, homogenization method,

Table 1. Values and units of OCR based on different normalization parameters using maximal respiration rate (CI&II$_{ETS}$ capacity) as an example.

Normalization parameters (unit per chamber)	CI&II$_{ETS}$ capacity	Unit
None*	68.1	pmol.s^{-1}
Tissue weight (2 mg tissue)	34.1	pmol.s^{-1}.mg^{-1} tissue
Total protein (0.098 mg protein)**	695.9	pmol.s^{-1}.mg^{-1} protein
CS activity (0.021U)***	3,243	pmol.s^{-1}.U^{-1} CS activity

*value as obtained from HRR; **Total protein per respiration chamber = 4.9 mg/mL x 20 µL mitochondrial homogenate; ***CS unit per chamber = 0.21 U/mg x 0.098 mg protein; CS: citrate synthase.

and centrifugal speed and timing. These were not performed largely due to limited amount of sample which had to be used in 4 h.

4. Conclusion

Our results showed that mitochondria isolated from rat hippocampus using this protocol could be used to measure OCR using HRR

5. Acknowledgements

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6. Conflict of Interest

Authors declare no conflict of interest.

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