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Isolation and Functional Assessment of Mitochondria from Small Amounts of Mouse Brain Tissue

Christos Chinopoulos, Steven F. Zhang, Bobby Thomas, Vadim Ten, and Anatoly A. Starkov

Abstract

Recent discoveries have brought mitochondria functions in focus of the neuroscience research community and greatly stimulated the demand for approaches to study mitochondria dysfunction in neurodegenerative diseases. Many mouse disease models have been generated, but studying mitochondria isolated from individual mouse brain regions is a challenge because of small amount of the available brain tissue. Conventional techniques for isolation and purification of mitochondria from mouse brain subregions, such as ventral midbrain, hippocampus, or striatum, require pooling brain tissue from six to nine animals for a single mitochondrial preparation. Working with pooled tissue significantly decreases the quality of data because of the time required to dissect several brains. It also greatly increases the labor intensity and the cost of experiments as several animals are required per single data point.

We describe a method for isolation of brain mitochondria from mouse striata or other 7–12 mg brain samples. The method utilizes a refrigerated table-top microtube centrifuge, and produces research grade quality mitochondria in amounts sufficient for performing multiple enzymatic and functional assays, thereby eliminating the necessity for pooling mouse brain tissue. We also include a method of measuring ADP-ATP exchange rate as a function of mitochondrial membrane potential ($\Delta\Psi_m$) in small amounts of isolated mitochondria, adapted to a plate reader format.

Keywords

Adenine nucleotide translocase; Adenine nucleotide carrier; Systems biology

1. Introduction

The availability of a multitude of genetical mouse models of neurodegenerative diseases greatly stimulated the demand for in vivo and in vitro approaches to study mouse brain mitochondria functions. Although the in vivo methodology to study mitochondrial functions is not yet well developed, a wealth of in vitro approaches is readily available for in-depth studies with isolated mitochondria. However, such studies still represent a challenge because of small amount of the available brain tissue, especially when a particular brain region is of interest. For example, dissected mouse striata weight only about ~12 mg (wet weight). If using conventional techniques for isolation, purification and functional assessment of striatal mitochondria, one has to pool brain tissue from six to nine animals for a single mitochondrial preparation. Tissue pooling has long been a routine practice in the field (e.g., where striata from ten mice had to be pooled per single experiment) (1). A disadvantage of this experimental approach is that *working with pooled tissue significantly decreases the quality of the data* because of the time required to dissect several brains; it also greatly increases the labor intensity and the cost of experiments as several animals are required per

single data point. To overcome these limitations, we developed a method for isolation of brain mitochondria that allows us to isolate a workable amount of mitochondria from 7 to 12 mg brain samples, which eliminates the necessity for pooling mouse brain tissue.

One of most useful and frequently assessed mitochondria functional characteristics is their oxidative phosphorylation. Classically, it is evaluated by respirometry as the ratio of the amount of phosphorylated ADP to the amount of the oxygen consumed (ADP:O ratio), which characterizes the efficiency of oxidative phosphorylation and a degree of intactness of mitochondria. The amount of produced ATP is not measured in these experiments; however, it can be evaluated separately by enzymatic assays. Recently, we have developed a kinetic assay to measure the efflux of ATP from mitochondria (2). This method allows one to estimate ADP-ATP exchange rate mediated by the ANT in mitochondria, which is influenced by many parameters (3), most steeply by mitochondrial membrane potential ($\Delta\Psi_m$) (2, 4–7). We describe this methodology adapted to a small volume assay and using commonly available instrumentation, such as a plate reader.

2. Materials

2.1. Materials and Equipment

1. *Blunt-blunt tip straight Student Surgical Scissors* (1, Fig. 1).
2. *Sharp-sharp tip straight Extra Fine Bonn Scissors* (2, Fig. 1).
3. Razor blade (3, Fig. 1).
4. *Curved tip Dumont #7 Medical Forceps – Inox Standard Tip* (4, Fig. 1).
5. Straight tip Dumont #5 Mirror Finish Forceps (5, Fig. 1).
6. *Curved serrated tip Standard Pattern Forceps* (6, Fig. 1).
7. Kontes glass homogenizer, small clearance pestle “B” (7, Fig. 1).
8. Bucket filled with ice.
9. Glass or plastic dissection support surface (e.g., a Petri dish) and a piece of Whatman paper.
10. Wash bottle (~50 ml) filled with MSEGTA buffer (see Subheading 2.2, item 1).
11. A refrigerated microtube centrifuge (see Note 1).
12. A vacuum aspirator (see Note 2).
13. 10–12 1.7 ml Eppendorf® tubes (see Note 3).
14. 1 ml pipettor and tips.
15. Plate reader capable of measuring multiwavelength fluorescence and absorbance (see Note 4).
16. White opaque 96-well plates with flat-bottom wells and general lab equipment (e.g., pipettors, tips, etc.).

¹We use a “Microfuge 22R centrifuge” (Beckman-Coulter, USA) refrigerated table top centrifuge for the procedure described above.

²The vacuum aspirator is optional though preferable, but a pipettor can be used instead.

³Eppendorf® tubes can be substituted with any other brand of small polypropylene tubes of similar volume, with a conical bottom.

⁴These instructions assume the use of the monochromator-based Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA 94089, USA). If a monochromator-based plate reader is not available and a filter-based plate reader is to be used instead, a 505/535 nm excitation/emission filter is required for MgG, and a 495/585 nm excitation/emission filter for safranin O.

2.2. Reagents

1. MSEGTA buffer: 225 mM mannitol, 75 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM EGTA, dissolved in water. Store at +0°C to +4°C (see Note 5).
2. MSEGTA-BSA: MSEGTA buffer supplemented with 0.2 mg/ml bovine serum albumin (BSA) essentially fatty acid-free. Store at +0°C to +4°C.
3. 100% Percoll™-MSEGTA buffer: 225 mM mannitol, 75 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM EGTA, dissolved in 100% Percoll™. Store at +0°C to +4°C.
4. Experimental buffer: 8 mM KCl, 110 mM potassium gluconate, 10 mM NaCl, 10 mM Hepes (acid), 10 mM KH₂PO₄, 0.005 mM EGTA, 10 mM mannitol, 1.5 mM MgCl₂, 0.5 mg/ml BSA essentially fatty acid-free, pH 7.25 (adjusted with KOH). Store at +4°C (see Note 6).
5. 50 mM diadenosine pentaphosphate, (Ap5A), dissolved in water. Store at –20°C (see Note 7).
6. 2 mM magnesium green (MgG) 5 K⁺ salt (Invitrogen) dissolved in water. Store in 30 µl aliquots at –20°C.
7. 1 M K⁺ glutamate, pH 7.0 and 0.5 M K⁺ malate dissolved in water, pH 7.0 (adjusted with KOH). Store at –20°C in 1 ml aliquots (see Note 8).
8. 200 mM K⁺ ADP, pH 6.3–6.9. Store at –20°C (or preferably at –80°C) in 0.2 ml aliquots (see Note 9).
9. 200 mM K⁺ ATP, pH 6.3–6.9. Store at –20°C (or preferably at –80°C) in 0.2 ml aliquots (see Note 9).
10. Solution of 0.5 M EDTA.
11. Solution of 1 M MgCl₂.

⁵All water solutions and reagents are prepared using distilled (>17.4 MΩ resistance) water, such as e.g., Milli-Q.

⁶If an alternative buffer is used, K_d values of ADP and ATP for Mg²⁺ must be determined as described in (2). K_d values of ADP and ATP for Mg²⁺ are temperature-dependent, so all experiments must be performed at the same temperature. Also, it is important to use no less than 1.5 mM MgCl₂ if 2 mM ADP is to be subsequently added because $[Mg^{2+}]_f$ must be sufficiently high to fall within the dynamic range of MgG used in the plate reader. It is encouraged to calculate own “empirical correction” values, pertaining to the experimental conditions. During the conversion of MgG fluorescence to $[Mg^{2+}]_f$, an additional point emerges that serves as a quality-control for the validity of the experiments: assuming that the total amount of MgCl₂ present in the buffer is 1.5 mM, and the K_d value of ADP for Mg²⁺ for the experimental conditions is 0.906 mM, addition of 2 mM ADP into the medium will decrease $[Mg^{2+}]_f$ from 1.5 mM to 0.658 mM. This value was calculated by the following standard binding equation:

$$[Mg^{2+}]_f = 0.5 \cdot \left([Mg^{2+}]_t - K_d - [L]_t + \sqrt{([Mg^{2+}]_t - K_d - [L]_t)^2 + 4K_d[Mg^{2+}]_t} \right) \quad (2)$$

where $[Mg^{2+}]_t$ is the total $[Mg^{2+}]$, $[L]_t$ is the total concentration of added ADP, and K_d is the affinity constant of ADP for Mg²⁺ under these experimental conditions, i.e., 0.906 mM. If after conversion of MgG fluorescence to $[Mg^{2+}]_f$ a value different than 0.658 mM is recorded, that probably means that either the K_d of ADP for Mg²⁺ has not been correctly estimated, or the ADP stock has not been properly made/stored.

⁷Diadenosine pentaphosphate (Ap5A) stock, the inhibitor for adenylate kinase, does not require pH adjustment and can withstand at least ten freeze-thaw cycles. It is generally a good practice to supplement a 10 ml aliquot of the buffer with Ap5A, the dye (MgG or safranin O cannot be simultaneously added due to absorption overlap), and the mitochondrial substrates on the day of the experiment, and add only the mitochondria and the adenine nucleotides to the well, during the experiment. The purpose of this is to keep the increase of the experimental volume in the well due to additions of the materials to less than 5%.

⁸The frozen stocks of the mitochondrial substrates are stable for at least 1 year. It is difficult to prepare K⁺ malate stocks at a concentration higher than 0.5 M.

⁹ADP and ATP stocks must be carefully made from the highest purity available materials, and the exact concentrations known, otherwise results obtained from Eq. 1 are invalid. During their preparation, ADP and ATP unbuffered stocks are highly acidic, and require pH adjustment to a pH range 6.3–6.9.

12. 1 mM safranin O dissolved in water. Make at least 20 ml and store at room temperature in the dark for at least 5 days before the experiment. Stable for a year if stored as described (see Note 10).
13. 1 mM SF 6847 (“Tyrphostin 9,” 3,5-di-tert-butyl-4-hydroxy-benzylidenemalononitrile) in ethanol. Store at -20°C .

3. Methods

3.1. Dissection of Mouse Striata for Mitochondrial Preparation (see Fig. 2)

1. Sacrifice the mouse by a method approved at your Institution.
2. Decapitate with (1) *Blunt-blunt tip straight Student Surgical Scissors*.
3. Open the skull on the top with (2) *Sharp-sharp tip straight Extra Fine Bonn Scissors*, and carefully remove the brain from the skull. Take care not to damage the brain structure.
4. Place excised brain on the ice-chilled glass or plastic surface (e.g., a Petri dish) covered with wet Whatman paper; wash out the blood out of the brain surface (use mitochondria isolation buffer to wash and to wet the paper).
5. Using a razor blade (3), cut out the forebrain along the line *a* in Fig. 1, and cut it in two halves as shown in Fig. 2.
6. Flip the cut piece of forebrain on its side (see Fig. 3). Hold the block with (6) *Curved serrated tip Standard Pattern Forceps* and gently work out the space between hippocampus and striatum lengthwise using (4) *Curved tip Dumont #7 Medical Forceps – Inox Standard Tip*.
7. Flip out tissue flap as shown in Fig. 4.
8. Using tips of (4) *Curved tip Dumont #7 Medical Forceps – Inox Standard Tip*, gently separate the striatum from surrounding tissue and lift (“scoop”) it out.
9. Immediately place it in 1 ml of ice-cold MSEGTA-BSA buffer.

3.2. Isolation of Mitochondria

1. Homogenize brain tissue obtained in step 9 in Subheading 3.1 above in 1 ml MSEGTA-BSA with 2 ml Kontes™ Dounce homogenizer, tight pestle (7, see Fig. 1), with 30–35 strokes, transfer into two 1.7 ml Eppendorf® tubes;
2. Add MSEGTA-BSA to the tubes up to the groove at the top (~1.7 ml, total volume), mix by pipetting or inversion;
3. Centrifuge at $\sim 500 \times g \times 5 \text{ min}$;
4. Transfer the supernatant into clean 1.7 ml Eppendorf® tubes, centrifuge at $14,000 \times g \times 10 \text{ min}$;
5. While centrifuging at step 4, fill 1.7 ml Eppendorf® tube with 1 ml of 24% Percoll™-(see Note 11) MSEGTA, and prepare 12% Percoll™-MSEGTA, 0.2 ml;
 - a. Take 0.1 ml of 24% Percoll™- MSEGTA and dilute with 0.1 ml MSEGTA;

¹⁰Upon long-term storage, the safranin O solution tends to form a dark precipitate. Do not shake the safranin O containing bottles, and always take from the top of the solution. If stored in the dark, a 1 mM safranin O stock solution is stable for at least 1 year.

¹¹24% Percoll™-MSEGTA is prepared from 100% Percoll™-MSEGTA by diluting it with MSEGTA. Can be stored for 2 weeks at $+4^{\circ}\text{C}$ or frozen for longer storage.

- b. Keep both 24% and 12% Percoll™-MSEGTA-filled tubes in ice;
6. Aspirate off the supernatant, resuspend both pellets (combine them) in 0.2 ml of 12% Percoll™-MSEGTA;
7. Carefully layer the suspension over the 24% Percoll™-MSEGTA solution, by holding the tube at about 30 degrees, placing the tip of the pipettor into the tube's groove and slowly releasing the suspension. Close the tube, turn it upright and place in ice before proceeding to the next step. Fill another 1.7 ml Eppendorf® tube with 1.2 ml of MSEGTA to use as a counterbalance in the centrifuge.
8. Centrifuge the prepared sample from step 7 at $18,000 \times g \times 15$ min. After the centrifugation is completed, check the appearance of the sample. There should be almost transparent band approximately in the middle of the tube, with cloudy top and bottom portions of the sample.
9. Aspirate off 0.7 ml of the top portion of the sample.
10. Add 1.2 ml MSEGTA, mix by inversion, and centrifuge at $18,000 \times g \times 5$ min;
11. Aspirate 1.5 ml of the supernatant, resuspend the pellet in the remaining 0.2 ml of the isolation buffer, add 1.5 ml MSEGTA, mix by inversion, centrifuge at $14,000 \times g \times 5$ min.
12. If the pellet is fuzzy, repeat previous step 11;
13. If the pellet appears solid with well-defined shape, aspirate off the supernatant completely (place 20 μ l tip over the vacuum aspirator nozzle) and resuspend the pellet in 0.1 ml MSEGTA. This is purified mitochondrial fraction.

3.3. $[Mg^{2+}]_f$ Determination from Magnesium Green Fluorescence in the Extramitochondrial Volume of Isolated Mitochondria

1. (See Note 4) Set the acquisition rate of plate reader at 0.33 Hz (one acquisition every 2 s plus 1 s for mixing in between each acquisition) and set 505 excitation and 535 nm emission wavelengths. Turn on the incubator and set it to 37°C.
2. Perform all experiments at 37°C. Prewarm the plate and the experimental buffer to this temperature.
3. Use white opaque 96-well plates with flat-bottom wells.
4. Add 0.2 ml of the experimental buffer minus the volume of the mitochondrial suspension that is intended to be added, 2 μ M of magnesium green 5 K⁺ salt, 5 mM glutamate, 5 mM malate and 50 μ M Ap5A to a well (see Notes 7 and 12).
5. Add 20–40 μ g of mitochondria (see Note 13).
6. Record baseline until steady (usually a couple of minutes).
7. Add 2 mM of K⁺ ADP. Record the first abrupt and second progressive drop in magnesium green fluorescence for at least 2 min. (If de-energized mitochondria are

¹²The experimental buffer contains BSA, and therefore it tends to foam. While pipetting materials in the well, care must be taken to avoid extra foaming, that may affect the fluorescence signal.

¹³For mouse brain, 20 μ g of energized mitochondria in 0.2 ml volume exhibit sufficiently high ADP-ATP exchange rates to produce changes in $[Mg^{2+}]_f$ with an adequate signal-to-noise ratio. If, however, mitochondria are partially de-energized, i.e., uncoupled for any reason, or alternative type of mitochondria are used with low ADP-ATP exchange rates, it is advisable to increase the amount of mitochondria added in the well in order to increase the signal-to-noise ratio of the MgG fluorescence measurements. For the mitochondria protein determination, bicinchoninic acid (BCA) method was used (BCA kit is currently available from Thermo Scientific company).

to be used, i.e., in the absence of substrates, use 2 mM K⁺ ATP in lieu of ADP. For the remaining section, it will be assumed that mitochondria are energized by substrates).

8. At the end of the experiment, record minimum fluorescence ($F_{\min}$) by adding 10 mM EDTA, followed by recording of maximum fluorescence ($F_{\max}$) elicited by addition of 20 mM MgCl₂.
9. Free Mg²⁺ concentration, (Mg^{2+}_f), is calculated from the equation: $Mg^{2+}_f = (K_d(F - F_{\min})/(F_{\max} - F)) - 0.068$ mM, assuming a K_d of 0.9 mM for the MgG–Mg²⁺ complex (7), (see Fig. 4a) (see Note 6). The correction term –0.068 mM is empirical, and possibly reflects chelation of other ions by EDTA that have an affinity for MgG, and alter its fluorescence.

3.4. Conversion of [Mg²⁺]_f to ADP-ATP Exchange Rate

ADP-ATP exchange rate is estimated using the recently described method (2) based on a concept developed previously (8, 9), exploiting the differential affinity of ADP and ATP to Mg²⁺. The rate of ATP appearing in the medium following addition of ADP to energized mitochondria (or vice versa in case of de-energized mitochondria), is calculated from the measured rate of change in free extramitochondrial [Mg²⁺] using the following equation:

$$[ATP]_t = \left(\frac{[Mg^{2+}]_t}{[Mg^{2+}]_f} - 1 - \frac{[ADP]_t(t=0) + [ATP]_t(t=0)}{K_{ADP} + [Mg^{2+}]_f} \right) / \left(\frac{1}{K_{ATP}[Mg^{2+}]_f} - \frac{1}{K_{ADP} + [Mg^{2+}]_f} \right) \quad (1)$$

Here, [ADP]_t and [ATP]_t are the total concentrations of ADP and ATP, respectively, in the medium, and [ADP]_t($t=0$) and [ATP]_t($t=0$) are [ADP]_t and [ATP]_t in the medium at time zero. For the calculation of [ATP] or [ADP] from free [Mg²⁺], the apparent K_d values are identical to those in (2) due to identical experimental conditions ($K_{ADP} = 0.906 \pm 0.023$ mM, and $K_{ATP} = 0.114 \pm 0.005$ mM), (see Note 6). As a comparison, the program Winmaxc (<http://www.stanford.edu/~cpatton/maxc.html>), using constants from (10) predicts apparent K_d values of $K_{ADP} = 0.739$ mM, and $K_{ATP} = 0.060$ mM – in reasonable agreement with our measured values. Equation 1 is available for download as an executable file at: <http://tinyurl.com/ANT-calculator>. ADP-ATP exchange rate can be calculated by performing a linear regression on the values shown in Fig. 4b.

3.5. Mitochondrial Membrane Potential Determination

1. (See Note 4) Set the acquisition rate at 0.33 Hz (one acquisition every 2 s plus 1 s for mixing in between each acquisition); set 495 excitation and 585 nm emission wavelengths. Turn on the incubator and set it to 37°C.
2. Perform all experiments at 37°C. Prewarm the plate and the experimental buffer to this temperature.
3. Use white opaque 96-well plates with flat-bottom wells.
4. Add 0.2 ml of the experimental buffer minus the volume of the mitochondrial suspension that is intended to be added, 5 μM of safranin O, 5 mM glutamate, 5 mM malate, and 50 μM Ap5A to a well (see Notes 7 and 12).
5. Add 20–40 μg of mitochondria.
6. Record baseline until steady (usually a couple of minutes).
7. Add 2 mM of K⁺ ADP. Record for at least 2 min (see Fig. 4c).

8. At the end of the experiment, record maximum fluorescence elicited by addition of 1 μ M SF 6847.

3.6. Conversion of Safranin O Fluorescence to mV

Safranin O fluorescence can be “converted” to approximate values of $\Delta\Psi_m$ expressed in mV using the following three assumptions: (1) well-coupled mitochondria respiring on glutamate plus malate, at pH 7.25, exhibit $\Delta\Psi_m$ equal to -180 mV (2, 3) (see Note 14); (2) in the same conditions, addition of ADP clamps $\Delta\Psi_m$ to -145 mV for as long as there is ADP in the well (2, 3); and (3) addition of 1 μ M SF 6847 collapses $\Delta\Psi_m$ to 0 mV. From these three points, the power function calibration can be constructed: $f = y_0 + a \times x^b$, in which safranin O fluorescence can be converted to mV. The “ y_0 ” constant of the power function represents the residual safranin O fluorescence after mitochondria are fully charged; “ x ” is for safranin O raw fluorescence, “ a ” and “ b ” are constants and “ f ” represents calculated $\Delta\Psi_m$ in mV (see Fig. 4d).

3.7. Results

To assess the quality of mitochondria, we measured the respiration rates of the isolated striatal brain mitochondria (bSm). The oxygen consumption was measured with Hansatech oxytherm respirometry system (“Hansatech,” UK) in 0.25 ml of incubation buffer (125 mM KCl, 2 mM KH_2PO_4 , 20 mM HEPES (pH 7.2), 1 mM Mg^{2+} , 0.2 mg/ml BSA essentially fatty acid-free, 0.2 mM EGTA; 5 mM α -ketoglutarate, 1 mM Na^+ -glutamate, 5 mM Na^+ -pyruvate, 1 mM Na^+ -malate). Mitochondria were added at 0.1 mg/ml concentration. We found that mitochondria were well coupled and exhibited excellent phosphorylation efficiency (Table 1. Note that ADP:O ratios >3 is due to the choice of oxidative substrates that activate the so-called substrate phosphorylation carried out by the succinyl thiokinase in the matrix of mitochondria, e.g., see (11)).

Electron microphotography revealed normal appearance of isolated striatal mitochondria and relatively low level of contaminating nonmitochondrial membranes (see Fig. 3a). The purity of the mitochondrial preparation was further assessed by immunoblotting (see Fig. 3b). The contamination by cytosol and unbroken nerve terminals (creatine kinase, cytosolic isoform BB “BBCK,” marker) and that by endoplasmic reticulum (calnexin, marker) was very low and comparable with that in a conventional whole brain mitochondria preparation obtained by isopycnic centrifugation according to (12).

3.8. Conclusions

Overall, the method produces mitochondria of research grade quality in amounts sufficient for performing multiple enzymatic and functional assays, thereby eliminating the necessity for pooling mouse brain tissue.

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¹⁴If the mitochondria are uncoupled, either because of poor preparation, or for whatever reason they were impaired to begin with (experimental tissue damage, genetic/pharmacological manipulation, etc.), they will exhibit a lower membrane potential. In this case, the assumption of a resting membrane potential of -180 mV must be abandoned, and a two-point (ADP effect and SF 6847 effect) calibration of safranin O to mV conversion is advised. A two-point calibration is not as accurate as the three-point calibration elaborated above.

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Fig. 1.
Tools. (1) Blunt-blunt tip straight student surgical scissors; (2) Sharp-sharp tip straight Extra Fine Bonn Scissors; (3) Razor blade; (4) Curved tip Dumont #7 Medical Forceps – Inox Standard Tip; (5) Curved serrated tip standard pattern forceps; (6) Dounce homogenizer.

Fig. 2.
Dissection procedure. see text for details.

Fig. 3. Electron microphotograph (a) and immunoblot (b) of isolated mouse striatal mitochondria. Abbreviations: *H* whole brain homogenate, *M* whole brain mitochondria isolated by a conventional procedure described in (12), *sM* striatal mitochondria isolated as described in this manuscript, *mCK* mitochondrial isoform of creatine kinase, *BBCK* cytosolic isoform of creatine kinase, *clnx* calnexin, *MnSOD* mitochondrial superoxide dismutase, *bAct* beta-actin.

Fig. 4.

Estimation of ATP appearing in the medium and $\Delta\Psi_m$ upon ADP addition in isolated mitochondria. **(a)** Time course of $[Mg^{2+}]_{fr}$. **(b)** Time course of $[ATP]_{calc}$ appearing in the medium, calculated from $[Mg^{2+}]_{fr}$. **(c)** Reconstructed time course of safranin O fluorescence. **(d)** Reconstructed time course of $\Delta\Psi_m$ in mV.

Table 1

The yield of the striatal (bSm) mitochondria and their respiratory characteristics

Parameter	bSm	Units
Tissue weight	12.5 ± 0.3	mg
Protein yield	110 ± 14	μg
State 4	32 ± 4	nmol O ₂ /min/mg
State 3	193 ± 15	nmol O ₂ /min/mg
R.C.I.	6.1 ± 0.5	State 3/State 4 ratio
A.C.I.	12 ± 1.1	State 3/V(cAtr) ratio
ADP:O	3.19 ± 0.07	ADP(nmol)/O(natoms) ratio

State 4 is the resting respiration rate when no active oxidative phosphorylation occurs; State 3 is the active respiration rate when mitochondria phosphorylate added ADP; RCI is the respiratory control index which reflect the quality of the mitochondrial preparation; ACI is the acceptor control index which under our experimental conditions characterizes the permeability of the inner membrane to protons, thereby reflecting the “true coupling” of the mitochondrial preparations; the V(cAtr) is the rate of respiration in the presence of carboxyatractylate, a specific inhibitor of ADP/ATP exchange across the mitochondrial membrane. ($n = 6$)