

# Isolation of mitochondria with high respiratory control from primary cultures of neurons and astrocytes using nitrogen cavitation

Tibor Kristián<sup>a,\*</sup>, Irene B. Hopkins<sup>b</sup>, Mary C. McKenna<sup>b</sup>, Gary Fiskum<sup>a</sup>

<sup>a</sup> Department of Anesthesiology, School of Medicine, University of Maryland, 685 West Baltimore Street, MSTF 534, Baltimore, MD 21201, USA

<sup>b</sup> Department of Pediatrics, School of Medicine, University of Maryland, 685 West Baltimore Street, MSTF 534, Baltimore, MD 21201, USA

Received 18 April 2005; received in revised form 22 August 2005; accepted 30 August 2005

## Abstract

To study neurons or glia-specific mitochondria one needs to isolate these organelles from primary neuronal or astrocytic cell culture. This work provides novel method for isolation of functional and morphologically intact mitochondria from neurons and astrocytes in cell cultures. In the first step, mitochondria are released from cells by disruption of cell membranes using a nitrogen cavitation technique. This technique is based on rapid decompression of a cell suspension from within a pressure vessel. Mitochondria released from cell bodies are then separated from the rest of cell homogenate by Percoll gradient centrifugation. This is a relatively rapid technique that yields to very well coupled mitochondria that exhibited functional and morphological characteristics comparable to mitochondria isolated from brain tissue using common techniques. This technique thus will allow examination of mitochondria that are exclusively cell specific in origin.

© 2005 Elsevier B.V. All rights reserved.

**Keywords:** Primary cell culture; Neurons; Astrocytes; Mitochondria; Isolation; Nitrogen cavitation; Peripheral benzodiazepine receptor

## 1. Introduction

Mitochondria isolated from brain tissue consist of two major populations based on their presence in neurons and astrocytes, the two cell types representing the vast majority of brain mass. To date, there are no reliable techniques to separate neuronal and glial mitochondria from brain homogenates that provide mitochondrial yields with bioenergetic functional integrity similar to that observed with isolation procedures that do not separate these subpopulations. As some evidence indicates that specific metabolic enzyme activities are distinctly different in neuronal and astrocytic mitochondria (Lai and Clark, 1979, 1989; McKenna et al., 2000a,b; Sonnewald et al., 2004), additional comparisons of bioenergetic and physiologic characteristics are needed. In response to this need, attempts were made to isolate mitochondria with good functional integrity from primary cultures of pure neurons and astrocytes (Auestad et al., 1991; Almeida and Medina, 1997b). The approach used to isolate mitochondria from these cells employs mechanical disruption of cell membranes by homogenization, a technique normally

also applied to various tissues. However, while the respiratory control ratio that is commonly used as an index of bioenergetic functional integrity generally falls in the range of 5–10 for isolated brain mitochondria, the ratios reported by Almeida and Medina (1997a,b) are in the range of 3–5.

A likely explanation for the relatively low respiratory control exhibited by mitochondria isolated from primary cultures of neurons and astrocytes is the mechanical force used to homogenize the cells, which can potentially physically damage mitochondrial membranes. Typically, cell suspensions are homogenized with a Teflon pestle—glass vessel homogenizer. This process requires 20–30 strokes of the pestle up and down the vessel while rotating at 500 rpm (see Almeida and Medina, 1997b). The disadvantage of this approach is that the mitochondria released from cells disrupted early in the course of homogenization are subjected to repetitive shear stress that may result in damage. To avoid the problems associated with such mechanical homogenization of cells, we used a nitrogen cavitation technique for disrupting the plasma membrane. A similar procedure was used to isolate a crude mitochondrial fraction from cultured Jurkat cells (Krippner et al., 1996; Adachi et al., 1997, 1998; for review, see Gottlieb and Adachi, 2000). The nitrogen cavitation technique was also employed for isolation of total brain mitochondria (non-synaptic and synaptic) from brain homogenates (Brown et

\* Corresponding author. Tel.: +1 410 706 3418; fax: +1 410 706 2550.  
E-mail address: tkristian@anesthlab.umm.edu (T. Kristián).

al., 2004) and to release synaptic mitochondria from cortical synaptosomal vesicles (Sullivan et al., 2003). Here, we report a modification of this method that, when applied to primary cultures of cortical neurons and astrocytes, generates mitochondria with respiratory, and morphological characteristics superior to those exhibited by mitochondria isolated using mechanical homogenization.

## 2. Material and methods

### 2.1. Reagents

Mannitol, sucrose, EGTA, HEPES, Tris, potassium phosphate dibasic ( $K_2HPO_4$ ), magnesium chloride ( $MgCl_2$ ), malate, glutamate, oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), adenosine 5'-diphosphate (ADP), bovine serum albumin (BSA), glutaraldehyde, sodium cacodylate trihydrate, poly-L-lysine, trypsin, L-glutamine, and cytosine arabinoside were obtained from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagles's medium (DMEM), Leibovitz's L-15, Neurobasal medium (NB) with B27 supplement were purchased from GIBCO (Invitrogen Corporation, Carlsbad, CA, USA). Potassium chloride suprapure was obtained from EM Sciences (Fort Washington, PA, USA). Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT, USA). Percoll was purchased from Amersham Biosciences (Piscataway, NJ, USA). OXPHOS primary antibody was obtained from Mitosciences LLC (Eugene, OR, USA),  $\beta$ -actin antiserum was from Sigma (St. Louis, CA, USA). Antiserum against PBR was a kind gift from Dr. V. Papadopoulos. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG secondary antibodies were purchased from Amersham Biosciences.

### 2.2. Cell culture

#### 2.2.1. Cortical neurons

Primary cultures of cortical neurons were prepared from the cerebral hemispheres of 16 day gestation rat embryos using a modification of the methods of Yavin and Yavin (1980). All dissections were performed in dissecting medium (Leibovitz's L-15 with glutamine). After removing the medium, the dissected *cerebral cortexes* were minced, incubated in 0.2% trypsin at 37 °C for 21/2 min, and then the proteolytic activity stopped by addition of an equal volume of maintenance medium (neurobasal medium (NB) supplemented with 10% FBS and 2 mM L-glutamine). The cells were triturated, centrifuged at low speed, resuspended in fresh medium to 1 ml per brain, and filtered through a Falcon cell strainer (70  $\mu$ m pore size). The cells were seeded on poly-L-lysine coated (0.05 mg/ml) 150 cm<sup>2</sup> Corning plastic tissue culture flasks at a density of  $(3-7) \times 10^7$  cells per flask in a volume of 12 ml medium and incubated for 1 h at 37 °C in an atmosphere of 95% air/5% CO<sub>2</sub> (v/v) at 90% humidity. The medium was then aspirated and replaced with 20 ml maintenance medium: NB with B27 supplement and 2 mM L-glutamine. After 72 h in vitro, the cultures were treated with cytosine arabinoside (final concentration 5  $\mu$ M) for 48 h. Cultures were

re-fed with maintenance medium and were used 7 days after preparation.

#### 2.2.2. Cortical astrocytes

Astrocytes were prepared from rat brain (1-day-old pups) as described by Zielke et al. (1990) using a method based on a procedure by Booher and Sensenbrenner (1972), with some alterations. Cerebral hemispheres were removed, placed in dissecting medium (Leibovitz's L-15 medium with L-glutamine), cleaned of meninges, and trimmed to retain the *cerebral cortex*. The isolated *cerebral cortexes* were placed in a 100 mm dish containing 10 ml fresh medium. After removal of most of the medium, the tissue was then minced, resuspended in maintenance medium (DMEM/F-12 with L-glutamine plus 10% FBS at 1 ml per brain) in a 50 ml conical centrifuge tube, mechanically disrupted by vortexing (60 s) to destroy most of the neurons, and filtered through sterile nylon (Falcon 70  $\mu$ m cell strainer and Nitex screening of 10  $\mu$ m pore size) to remove blood vessels and aggregated cells. The cell suspension (15 ml/brain), enriched in astrocytes, was seeded in 185 cm<sup>2</sup> Nunc tissue culture flasks at a density of 30 ml cell suspension/flask. The cells were incubated at 37 °C in an atmosphere of 95% air/5% CO<sub>2</sub>, with 90% humidity. The culture medium was replaced after 4 days and twice weekly thereafter. All experiments were performed after 10–12 days, when cultures were confluent but had not spontaneously differentiated.

### 2.3. Isolation of mitochondria from primary cell culture

To isolate mitochondria, we used a modification of the nitrogen cavitation method described by Gottlieb and Adachi (2000). At least  $2 \times 10^8$  cells were used (about eight Nunc tissue culture flasks T-150). The cells were washed once with 6 ml of isolation medium (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.4 at 4 °C), then scraped in 3 ml of the isolation medium and collected into a pre-cooled cavitation chamber (nitrogen bomb; Parr Instrument Company, Moline, IL). The cell suspension under stirring was subjected to 1500 psi for 15 min. At the end of the 15 min period, the pressure in the chamber fell to approximately 800 psi. The cell suspension is then released through outflow tubing attached to the valve localized at the bottom of the cavitation chamber. Thus, the depressurization of the cell suspension from 800 psi to normal atmospheric pressure was instantaneous. After collecting the cell suspension from the cavitation chamber, it was centrifuged at  $1500 \times g$  for 3 min to pellet the cell debris (heavy particles, or fractions of cells). The supernatant was collected and centrifuged at  $20,000 \times g$  for 10 min. The pellet (crude mitochondrial fraction) was resuspended in 0.8 ml of 15% Percoll and layered on preformed gradient consisting of 21% (for neuronal mitochondria) or 23% (for astrocytic mitochondria) Percoll (2 ml) layered over 50% Percoll (0.8 ml) in 4 ml centrifuge tube (see Fig. 1). The purity of astrocytic mitochondrial fraction was improved when the 23% Percoll was used as compared to 21% Percoll. Following centrifugation of the gradient at  $30,700 \times g$  for 6 min, the mitochondria accumulated at the lower interface (between the 50% and 21% (or 23%) Percoll layers). The top layers were removed and the mitochon-

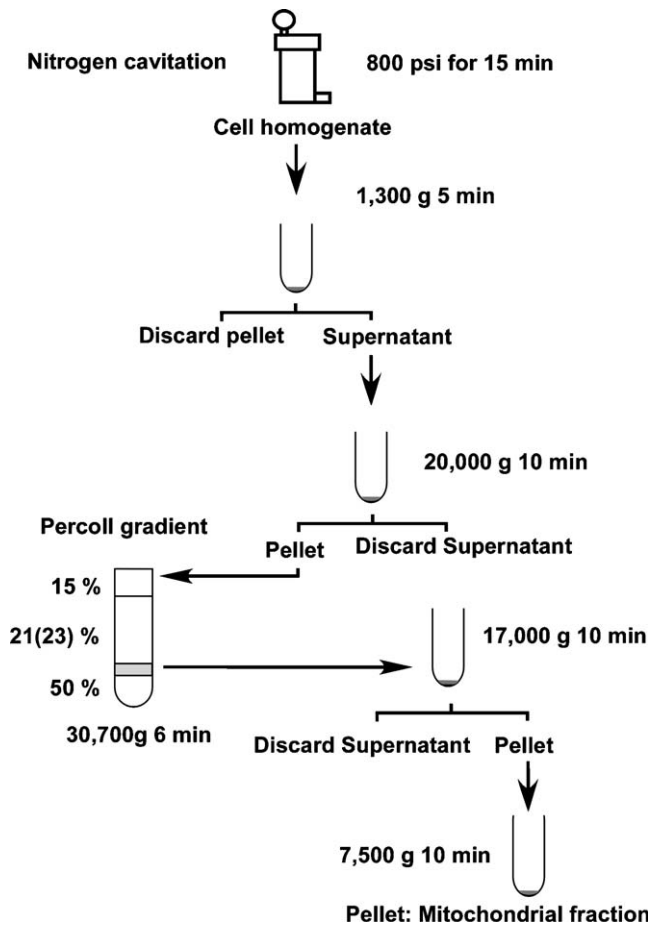


Fig. 1. Flow scheme of the isolation procedure using the nitrogen cavitation technique. The number in parenthesis represents the percentage of Percoll used to form the gradient for separation of mitochondria from astrocytes.

drial fractions collected and diluted with isolation medium (1:8). After centrifugation at  $17,000 \times g$  for 10 min, the pellet (purified mitochondria) was diluted with 1.5 ml of isolation medium and centrifuged at  $7000 \times g$  for 10 min. The final pellet was resuspended in  $30 \mu\text{l}$  of isolation medium without EGTA and used for experimental assays.

#### 2.4. Isolation of non-synaptic brain mitochondria

Non-synaptic brain mitochondria were isolated according to Sims (1990) with slight modifications (see Kristian et al., 2000, 2002). Briefly, brains were rapidly removed and homogenized in ice-cold isolation medium (225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 5 mM HEPES-Tris pH 7.4 at  $4^\circ\text{C}$ ). The brain homogenate was centrifuged at  $1300 \times g$  for 3 min. After removing the supernatant, the pellet was resuspended in the isolation medium and centrifuged again at  $1300 \times g$  for 3 min. The supernatant was then combined with the supernatant from the previous spin and centrifuged at  $17,000 \times g$  for 10 min. The resulting pellet was resuspended in 15% Percoll and layered on top of the discontinuous Percoll gradient (40, 24%). Following centrifugation of the gradient at  $30,000 \times g$  for 8 min, the mitochondria sedimented at the lower interface between 40 and 24% Percoll. The mitochondrial sample was collected and diluted in isola-

tion medium (1:5, v/v). After centrifugation at  $17,000 \times g$  for 10 min, the pellet was resuspended in isolation medium containing 10 mg/ml of defatted bovine serum albumin (BSA) and centrifuged at  $7000 \times g$  for 10 min. The final mitochondrial pellet was resuspended in approximately  $100 \mu\text{l}$  isolation medium not containing EGTA.

#### 2.5. Electron microscopy

The mitochondrial samples were fixed overnight with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at  $4^\circ\text{C}$ , post-fixed with 1% osmic acid in 0.1 M cacodylate buffer, dehydrated, and embedded in LX-112 (Ladd) epoxy resin. Thin sections were observed with a Jeol JEM-1200 EX transmission electron microscope.

#### 2.6. Respiration measurements

The respiratory functions of isolated mitochondria were measured polarimetrically with a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, England). Mitochondria were resuspended at a concentration of 0.5 mg/ml in medium consisting of 125 mM KCl, 2 mM  $\text{K}_2\text{HPO}_4$ , 1 mM  $\text{MgCl}_2$ ,  $10 \mu\text{M}$  EGTA, 5 mM malate, 5 mM glutamate, and 20 mM HEPES-Tris pH 7.0 at  $37^\circ\text{C}$ . Rates of oxygen consumption are expressed as  $\text{nmol O}_2/(\text{mg mitochondrial protein min})$ . The respiratory control ratio (RCR) was defined as the rate of ADP (0.5 mM)-stimulated oxygen consumption (State 3) divided by the rate of respiration determined in the presence of oligomycin ( $2.5 \mu\text{g/ml}$ ) (State  $4_o$ ), an inhibitor of ATP synthase. While the State  $4_o$  respiration measured in the presence of oligomycin is not equivalent to the classical State 4 rate obtained after a small bolus of ADP is almost completely converted to ATP, the use of oligomycin eliminates the contribution of ATP cycling via hydrolysis by contaminating ATPases and resynthesis by the mitochondrial ATP synthase to State 4 respiration. The oligomycin-induced State  $4_o$  rate of respiration is therefore a more specific indicator of mitochondrial proton cycling limited by passive proton leakiness of the inner membrane.

#### 2.7. Mitochondrial protein measurements

Mitochondrial protein concentrations were measured using a Lowry DC kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin used as concentration standards. Standards and the samples were measured in duplicates and the mean values were used to calculate the protein concentrations.

#### 2.8. Western blotting

Isolated mitochondria were mixed with lysis buffer containing 0.5% Nonidet p-40 (NP-40), 1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4 in 1:1 ratio (v/v) and incubated for 10 min on ice. The samples were then treated with 50 mM dithiothreitol (DDT) and NuPage LDS loading buffer (Invitrogen). After heating at  $70^\circ\text{C}$  for 10 min, the samples were separated by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis

(SDS-PAGE). Each lane was loaded with 25 µg mitochondrial protein. Following the SDS-PAGE, the proteins were transferred onto a PVDF membrane. The membranes were immediately fixed in a 0.1% glutaraldehyde in phosphate-buffered saline (PBS) with 0.05% Tween 20 buffer (PBST) for 10 min (see Connern and Halestrap, 1996). The membranes were washed in PBST and further processed according to the immunoblotting protocol recommended by the manufacturer (Mitosciences LLC). Thus, membranes were incubated at room temperature for 2 h in 5% non-fat milk in PBST. This was followed by incubation overnight at 4 °C in the primary antibody in 5% non-fat milk in PBST. The antibody cocktail consisted of monoclonal primary antibodies raised against a subunit of each of the respiratory (OXPHOS) complex, and for β-actin at 1:10,000 dilutions. Primary antibodies for peripheral benzodiazepine receptor were used at 1:5000. After overnight incubation with primary antibody, the membranes were washed with PBST three times for 10 min and incubated for 2 h in HRP-conjugated anti-mouse IgG (1:5000) or anti-rabbit IgG (1:5000) antibody. The immunoreactivity was visualized by enhanced chemiluminescence detection reagents (Amersham Biosciences).

### 2.9. Statistical analysis

The respiration data are expressed as the mean ± S.E.M. and the statistical significance was determined by one-way ANOVA followed by Scheffe's *F*-test. Differences with *P*-values of <0.05 were considered to be statistically significant. Immunoblotting experiments in which quantitative changes are detected were repeated three times to confirm reproducibility.

## 3. Results

Isolation of mitochondria from primary cell cultures employing the nitrogen cavitation technique takes approximately 1 h. The total protein yield from 10<sup>8</sup> cortical neurons or astrocytes is 0.4–0.5 mg of mitochondrial protein.

Typical examples of changes in oxygen consumption rates after ADP and oligomycin additions by astrocytic and neuronal mitochondria are shown in Fig. 2.

As shown in Table 1, the respiratory activity and coupling of isolated neuronal and astrocytic mitochondria using this tech-

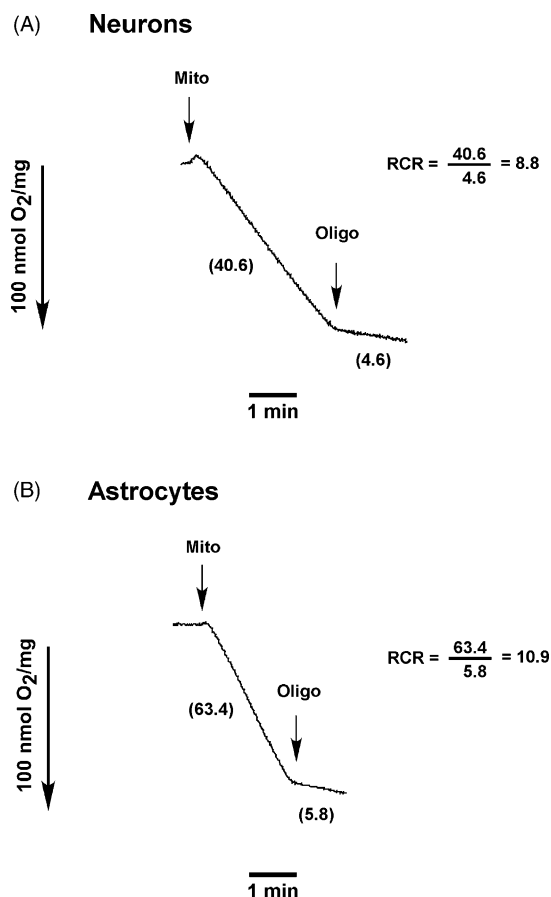


Fig. 2. Mitochondria isolated from neuronal or astrocytic culture display high respiratory control ratios. Neuronal (A) or astrocytic (B) mitochondria (0.25 mg) were added to a Clark-type oxygen electrode chamber maintained at 37 °C. The respiratory medium containing ADP (0.5 mM) stimulated the mitochondrial respiration (State 3). After about 2 min the ADP-induced oxygen consumption was stopped by addition of 1.25 µg/ml oligomycin, a mitochondrial ATP-synthase inhibitor. Rates of oxygen consumption following oligomycin addition (State 4<sub>o</sub>) represent the resting state of respiration dependent only on the unspecific leak of hydrogen ions across the inner mitochondrial membrane. The respiratory control ratio (State 3 rate divided by State 4<sub>o</sub> rate) was about 9–10 for both astrocytic and neuronal samples suggesting functionally well preserved mitochondria.

nique are very well preserved. The respiratory control ratio in the presence of complex I-linked substrates (malate and glutamate) ranges from 9 to 10 for mitochondria isolated from either astrocytes or neurons. The State 3 and State 4<sub>o</sub> respiratory rates are significantly lower for neuronal compared to astrocytic mitochondria.

The ultrastructure of mitochondria isolated by nitrogen cavitation is shown in Fig. 3. The electron micrographs indicate that mitochondria obtained by this technique have very well preserved morphological integrity. The outer and inner mitochondrial membranes are clearly distinguishable. The matrix is uniformly electron dense with no apparent damage to the inner structure. Although, the mitochondria are morphologically intact, the appearance of the infolded inner membrane (cristae) in the two types of mitochondria is different. Neuronal mitochondrial cristae exhibit a parallel alignment, whereas the cristae in astrocytic mitochondria display a more random orientation. These electron micrographs also indicate that the isolated

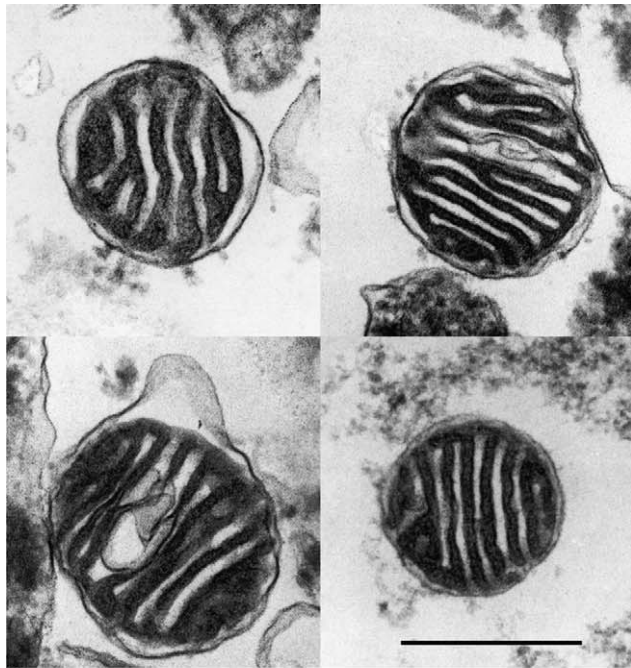
Table 1  
Mitochondria isolated from neurons and astrocytes display excellent respiratory integrity

Cell type	State 3	State 4	RCR
Astrocytes	64.1 ± 5.8	7.2 ± 0.9	9.2 ± 1.0
Neurons	31.7 ± 6.8*	3.0 ± 0.7*	10.5 ± 2.1
Non-synaptic	160.2 ± 22.5#	18 ± 2.2	9.1 ± 1.3

Respiratory parameters of mitochondria in the presence of glutamate/malate (5 mM/5 mM). The rates of respiration in State 3 and in State 4 are expressed as nmol O<sub>2</sub>/(min mg mitochondrial proteins). Results are mean ± S.E.M. of four different mitochondrial preparations isolated from different cell cultures or rat brain.

\* *P* < 0.05.

# *P* < 0.01.



(A)



(B)

Fig. 3. Electron micrograph of neuronal (A) and astrocytic (B) mitochondria. The outer and inner mitochondrial membranes are clearly identifiable. The mitochondrial matrix is electron dense with no signs of swelling or damage. Interestingly, the morphology of the cristea in neuronal mitochondria (A) have a parallel alignment, whereas in astrocytic mitochondria the cristea are randomly oriented. Both neuronal and astrocytic mitochondrial samples contain cell debris visible as diffuse dark material and small vesicles. The scale bars represent 1  $\mu$ m.

mitochondria are at least partially contaminated with other sub-cellular material.

Western immunoblots were performed with lysates of mitochondria obtained from astrocytic or neuronal primary cell culture and non-synaptic mitochondria isolated from brain homogenate to probe for possible qualitative differences in mito-

chondrial and non-mitochondrial protein levels in these preparations. Antibodies specific for specific polypeptides present in electron transport chain complexes I–III and for one sub-unit of the ATP synthase (complex V) were used and indicated that the relative distribution of different subunits of OXPHOS complexes is similar for the different mitochondrial preparations (Fig. 4). The immunoreactivity of all four mitochondrial proteins is lower in the neuronal sample compared to the astrocyte or brain mitochondria. While immunoreactivity of  $\beta$ -actin, a cytosolic marker, is undetectable in non-synaptic brain mitochondria, it is evident in astrocyte mitochondria and particularly high in neuronal mitochondria.

The cell-specific purity of our mitochondrial samples is reflected by the immunoreactivity to the peripheral benzodiazepine receptor (PBR) that is expressed preferentially in glial mitochondria (Park et al., 1996; Banati et al., 1997). As shown in Fig. 4, a strong band is evident with astrocyte mitochondria, whereas no immunoreactivity is detectable with neuronal mitochondria and only relatively weak immunoreactivity is evident with non-synaptic mitochondria.

#### 4. Discussion

We have developed a novel technique for the isolation of functionally and morphologically intact mitochondria from primary astrocytic and neuronal cell cultures. This technique combines the nitrogen cavitation cell membrane disruption approach and the purification of mitochondrial fractions from the cell homogenate using Percoll gradient centrifugation. As the dissolved gas comes out of solution, bubbles are formed, which expand and disrupt the cell membranes. The density of bubbles is proportional to the nitrogen pressure under which the gas equilibrates. At pressure greater than 1000 psi, disruption of subcellular organelles can occur (Gottlieb and Adachi, 2000). We therefore used 800 psi as the final equilibration pressure of nitrogen in the cavitation chamber.

The respiratory control ratio of mitochondria isolated from either astrocytes or neurons was approximately 9–10. A previous study using a glass homogenizer to disrupt cells reported respiratory control ratios in the range of 3.5–5.3, with the RCR of astrocyte mitochondria lower than that of neuronal mitochondria (Almeida and Medina, 1997a). The authors argued that the reason for the differences in mitochondrial coupling between the cell-specific mitochondria was not due to free fatty acid accumulation in the mitochondrial inner membrane but rather a result of different arrays of metabolic capabilities between neurons and astrocytes leading to higher exchange rates of charged metabolites in astrocytes. While a difference between the RCR of mitochondria in situ within astrocytes and neurons is possible, our results indicate that respiratory control for isolated mitochondria from astrocytes and neurons is very similar when the nitrogen cavitation procedure is used. The additional finding that the RCR values are considerably higher than those obtained with the more traditional glass vessel homogenization technique suggests that damage to mitochondrial membranes during cell homogenization is minimized by the use of nitrogen cavitation.

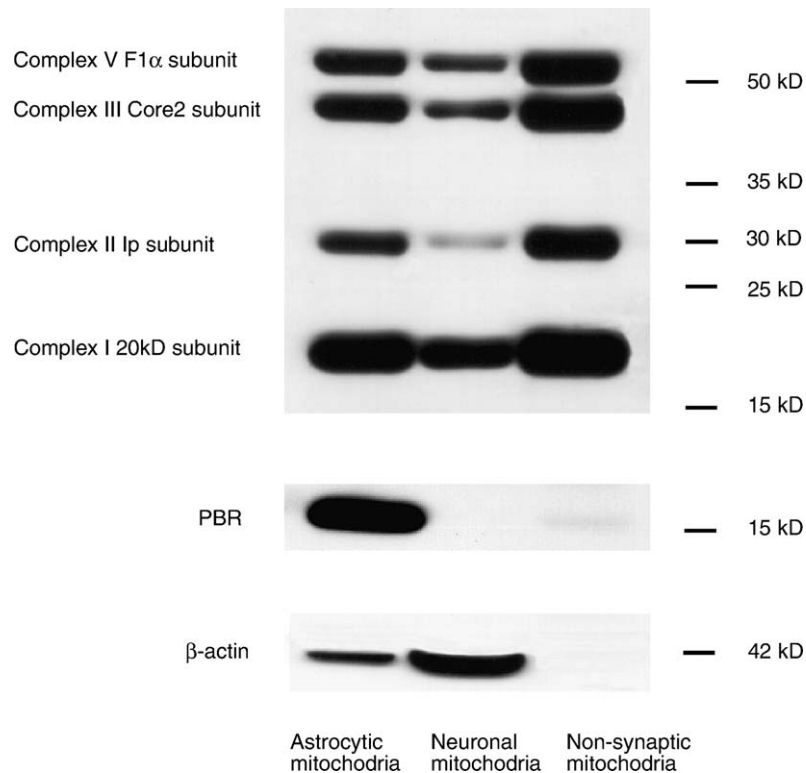


Fig. 4. Immunoblots showing the relative distribution of mitochondrial and non-mitochondrial proteins in astrocytic, neuronal, and non-synaptic mitochondria. The immunoreactivity of subunits of OXPHOS complexes was lower in neuronal mitochondria when compared to astrocytic and non-synaptic mitochondria. However, the cytosolic protein  $\beta$ -actin was not detected in non-synaptic mitochondria, while the neuronal and astrocytic mitochondria expressed some contamination by this protein. Immunoreactivity to the peripheral benzodiazepine receptor (PBR) was evident only in the astrocytic sample, completely lacking in neuronal mitochondria, and slightly detectable in the non-synaptic mitochondrial sample.

Although the respiratory control ratios are similar for isolated cortical astrocyte and neuronal mitochondria using our procedure, both State 3 and State 4 respiratory rates were lower in neuronal mitochondria when compared to astrocytic mitochondria. As Bolanos et al. (1995) reported that complex I activity is higher in astrocyte than in neuron homogenates, the lower rates in neuronal mitochondria could be also due to a greater contamination of isolated neuronal mitochondria with non-mitochondrial proteins. The presence of higher  $\beta$ -actin immunoreactivity in the isolated neuronal mitochondria supports this explanation. It has, however, been reported that actin is present within liver mitochondria (Stozharov, 1985). If this observation is valid and also applies to mitochondria within brain cells, actin should be present within mitochondria isolated from the brain. Our finding that  $\beta$ -actin is virtually undetectable in isolated adult rat forebrain non-synaptic mitochondria supports the conclusion that the actin immunoreactivity seen in the mitochondrial fraction isolated from the neurons is due to contamination by non-mitochondrial proteins. We speculate that the actin contamination is due to trapping of actin within non-mitochondrial membrane vesicles that are formed during nitrogen cavitation and that have a buoyant density so similar to mitochondria that they are present in the mitochondrial layer on the Percoll gradient.

Electron microscopic examination revealed that mitochondria isolated from both astrocytes and neurons display nor-

mal morphology, comparable to that exhibited by mitochondria isolated from tissues, e.g., the brain. In particular, the mitochondria display a condensed conformation with intact outer and inner membranes. Interestingly, the matrix cristae structures of astrocytic and neuronal mitochondria are strikingly different, assuming a parallel arrangement in neurons and a random arrangement in astrocytes. To our knowledge, this is the first report of a morphologic difference between astrocyte and neuronal mitochondria. The cause for the difference in matrix morphology and its physiologic significance remains to be elucidated.

Immunoreactivity for the peripheral benzodiazepine receptor was observed with astrocytic mitochondria and no detectable signal was obtained with isolated neuronal mitochondria. Non-synaptic brain mitochondria expressed detectable, albeit low levels of PBR. This is in agreement with reports that the PBR is localized primarily in glial cells (Park et al., 1996). However, recent studies measuring PBR ligand binding in vivo following traumatic brain injury suggest that PBR is associated primarily with activated microglia rather than reactive astrocytes (Conway et al., 1998; Raghavendra et al., 2000). Another study found that the PBR ligand PK11195 stimulates much more free radical production in cultured microglia and astrocytes than in neurons (Jayakumar et al., 2002). The presence of immunoreactive PBR in mitochondria isolated from astrocytes is therefore consistent with these observations but could also

indicate that our astrocyte cultures are partially contaminated with microglia.

The use of nitrogen cavitation has also been applied to isolation of mitochondria from brain synaptosomes. Using Percoll gradient centrifugation, non-synaptic mitochondria can be separated from synaptosomes (synaptic vesicles formed from synaptic boutons during homogenization of the brain tissue (Sims et al., 2000; Sims, 1990; Lai and Clark, 1979, 1989; Nicholls, 1978). Non-synaptic mitochondria represent somatic mitochondria from both neurons and glia. Previously, synaptosomal mitochondria were further isolated by disrupting the synaptosomal membrane either by using hypo-osmotic shock (Lai and Clark, 1979) or by applying digitonin that removes cholesterol from the plasma membranes (Elias et al., 1978; Rosenthal et al., 1987). Recently, nitrogen cavitation was employed to disrupt the synaptosomes and isolate both synaptic and non-synaptic mitochondria from brain homogenates (Brown et al., 2004). The relative ratio of glial and neuronal mitochondria in non-synaptic mitochondrial population is not known.

Since there is about the same mitochondrial to cytosolic volume ratio in neurons and glia (3–8%) (Pysh and Khan, 1972) and since there is 10–50 times more glial cells than neurons in the brain, one can assume that the majority of non-synaptic mitochondria are glial in origin. However, this assumption is correct under the condition that the average volumes of neurons and glia are the same.

In summary, currently the only method for the isolation of pure astrocyte versus neuronal mitochondria is to use pure primary cell cultures. Here, we described a technique that yields tightly coupled and morphologically intact mitochondria from rat cortical neurons and astrocytes. This technique will allow further phenotypic characterization of cell-specific mitochondria and shed more light on the physiological, metabolic and morphological differences between the two major mitochondrial populations in the brain.

## Acknowledgements

This work was supported by American Heart Association grant AHA 0256359 U, NIH grant R21NS050653 to TK and NIH grants R01NS34152 to GF and P01 HD16596 to MKM and GF.

## References

Adachi S, Cross AR, Babior BM, Gottlieb RA. Bcl-2 and the outer mitochondrial membrane in the inactivation of cytochrome c during Fas-mediated apoptosis. *J Biol Chem* 1997;272:21878–82.

Adachi S, Gottlieb RA, Babior BM. Lack of release of cytochrome C from mitochondria into cytosol early in the course of Fas-mediated apoptosis of Jurkat cells. *J Biol Chem* 1998;273:19892–4.

Almeida A, Medina JM. Different mitochondrial coupling in neurons and in astrocytes in primary culture. *Biochem Soc Trans* 1997a;25:410S.

Almeida A, Medina JM. Isolation and characterization of tightly coupled mitochondria from neurons and astrocytes in primary culture. *Brain Res* 1997b;764:167–72.

Auestad N, Korsak RA, Morrow JW, Edmond J. Fatty acid oxidation and ketogenesis by astrocytes in primary culture. *J Neurochem* 1991;56:1376–86.

Banati RB, Myers R, Kreutzberg GW. PK ('peripheral benzodiazepine')-binding sites in the CNS indicate early and discrete brain lesions: microautoradiographic detection of [3H]PK11195 binding to activated microglia. *J Neurocytol* 1997;26:77–82.

Bolanos JP, Heales SJ, Land JM, Clark JB. Effect of peroxynitrite on the mitochondrial respiratory chain: differential susceptibility of neurons and astrocytes in primary culture. *J Neurochem* 1995;64:1965–72.

Booher J, Sensenbrenner M. Growth and cultivation of dissociated neurons and glial cells from embryonic chick, rat and human brain in flask cultures. *Neurobiology* 1972;2:97–105.

Brown MR, Geddes JW, Sullivan PG. Brain region-specific, age-related, alterations in mitochondrial responses to elevated calcium. *J Bioenerg Biomembr* 2004;36:401–6.

Connern CP, Halestrap AP. Chaotropic agents and increased matrix volume enhance binding of mitochondrial cyclophilin to the inner mitochondrial membrane and sensitize the mitochondrial permeability transition to  $[Ca^{2+}]$ . *Biochemistry* 1996;35:8172–80.

Conway EL, Gundlach AL, Craven JA. Temporal changes in glial fibrillary acidic protein messenger RNA and [3H]PK11195 binding in relation to imidazoline-12-receptor and alpha 2-adrenoceptor binding in the hippocampus following transient global forebrain ischaemia in the rat. *Neuroscience* 1998;82:805–17.

Elias PM, Goerke J, Friend DS, Brown BE. Freeze-fracture identification of sterol-digitonin complexes in cell and liposome membranes. *J Cell Biol* 1978;78:577–96.

Gottlieb RA, Adachi S. Nitrogen cavitation for cell disruption to obtain mitochondria from cultured cells. *Methods Enzymol* 2000;322:213–21.

Jayakumar AR, Panickar KS, Norenberg MD. Effect on free radical generation by ligands of the peripheral benzodiazepine receptor in cultured neural cells. *J Neurochem* 2002;83:1226–34.

Krippner A, Matsuno-Yagi A, Gottlieb RA, Babior BM. Loss of function of cytochrome c in Jurkat cells undergoing fas-mediated apoptosis. *J Biol Chem* 1996;271:21629–36.

Kristian T, Gertsch J, Bates TE, Siesjo BK. Characteristics of the calcium-triggered mitochondrial permeability transition in nonsynaptic brain mitochondria: effect of cyclosporin A and ubiquinone O. *J Neurochem* 2000;74:1999–2009.

Kristian T, Weatherby TM, Bates TE, Fiskum G. Heterogeneity of the calcium-induced permeability transition in isolated non-synaptic brain mitochondria. *J Neurochem* 2002;83:1297–308.

Lai JC, Clark JB. Preparation of synaptic and nonsynaptic mitochondria from mammalian brain. *Methods Enzymol* 1979;55:51–60.

Lai JC, Clark JB. Isolation and characterization of synaptic and nonsynaptic mitochondria from mammalian brain. In: Bouiton NA, Baker GB, editors. *Carbohydrates and energy metabolism*. Clifton: Humana Press; 1989. p. 43–98.

McKenna M, O'Brien J, Kla K, Hopkins IB. Differential distribution of LDH isozemes in cortical synaptic terminals and primary cultures of cortical neurons. *J Neurochem* 2000a;74:S87.

McKenna MC, Stevenson JH, Huang X, Tildon JT, Zielke CL, Hopkins IB. Mitochondrial malic enzyme activity is much higher in mitochondria from cortical synaptic terminals compared with mitochondria from primary cultures of cortical neurons or cerebellar granule cells. *Neurochem Int* 2000b;36:451–9.

Nicholls DG. Calcium transport and porton electrochemical potential gradient in mitochondria from guinea-pig cerebral cortex and rat heart. *Biochem J* 1978;170:511–22.

Park CH, Carboni E, Wood PL, Gee KW. Characterization of peripheral benzodiazepine type sites in a cultured murine BV-2 microglial cell line. *Glia* 1996;16:65–70.

Pysh JJ, Khan T. Variations in mitochondrial structure and content of neurons and neuroglia in rat brain: an electron microscopic study. *Brain Res* 1972;36:1–18.

Raghavendra RV, Dogan A, Bowen KK, Dempsey RJ. Traumatic brain injury leads to increased expression of peripheral-type benzodiazepine receptors, neuronal death, and activation of astrocytes and microglia in rat thalamus. *Exp Neurol* 2000;161:102–14.

- Rosenthal RE, Hamud F, Fiskum G, Varghese PJ, Sharpe S. Cerebral ischemia and reperfusion: prevention of brain mitochondrial injury by lidoflazine. *J Cereb Blood Flow Metab* 1987;7:752–8.
- Sims NR. Rapid isolation of metabolically active mitochondria from rat brain and subregions using Percoll density gradient centrifugation. *J Neurochem* 1990;55:698–707.
- Sims NR, Anderson MF, Hobbs LM, Powell JA, Zaidan E. Brain mitochondrial responses to postischemic reperfusion: a role for calcium and hydrogen peroxide? *Dev Neurosci* 2000;22:366–75.
- Sonnewald U, Schousboe A, Qu H, Waagepetersen HS. Intracellular metabolic compartmentation assessed by <sup>13</sup>C magnetic resonance spectroscopy. *Neurochem Int* 2004;45:305–10.
- Stozharov A. Relationship between metabolic states of liver mitochondria and the G/F-actin ratio. *Biomed Biochim Acta* 1985;44:1287–94.
- Sullivan PG, Dube C, Dorenbos K, Steward O, Baram TZ. Mitochondrial uncoupling protein-2 protects the immature brain from excitotoxic neuronal death. *Ann Neurol* 2003;53:711–7.
- Yavin Z, Yavin E. Survival and maturation of cerebral neurons on poly(L-lysine) surfaces in the absence of serum. *Dev Biol* 1980;75:454–9.
- Zielke HR, Tildon JT, Landry ME, Max SR. Effect of 8-bromo-cAMP and dexamethasone on glutamate metabolism in rat astrocytes. *Neurochem Res* 1990;15:1115–22.