Mitochondrial viability in mouse and human postmortem brain

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Abstract

Neuronal function in the brain requires energy in the form of ATP, and mitochondria are canonically associated with ATP production in neurons. The electrochemical gradient, which underlies the mitochondrial transmembrane potential (ΔΨmem), is harnessed for ATP generation. Here we show that ΔΨmem and ATP-production can be engaged in mitochondria isolated from human brains up to 8.5 h postmortem. Also, a time course of postmortem intervals from 0 to 24 h using mitochondria isolated from mouse cortex reveals that ΔΨmem in mitochondria can be reconstituted beyond 10 h postmortem. It was found that complex I of the mitochondrial electron transport chain was affected adversely with increasing postmortem intervals. Mitochondria isolated from postmortem mouse brains maintain the ability to produce ATP, but rates of production decreased with longer postmortem intervals. Furthermore, we show that postmortem brain mitochondria retain their ΔΨmem and ATP-production capacities following cryopreservation. Our finding that ΔΨmem and ATP-generating capacity can be reinitiated in brain mitochondria hours after death indicates that human postmortem brains can be an abundant source of viable mitochondria to study metabolic processes in health and disease. It is also possible to archive these mitochondria for future studies.—Barksdale, K. A., Perez-Costas, E., Gandy, J. C., Melendez-Ferro, M., Roberts, R. C., Bijur, G. N. Mitochondrial viability in mouse and human postmortem brain. FASEB J. 24, 3590–3599 (2010). www.fasebj.org

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Unmitigated ATP supply is important in many tissues, and is especially important for the brain. The homeostatic maintenance of the ionic gradient by neuronal membranes, propagation of the neuronal action potential, and synaptic transmission require a readily available stock of ATP, in addition to energy required for the other internal functions of the neurons. Mitochondria are classically known for ATP production, and they are the major source of ATP for neurons. It is widely accepted that detrimental alterations in mitochondrial function can negatively affect neuronal function, and it has been shown that mitochondria play a key role in neuronal plasticity and death (1). Numerous studies have reported the occurrence of altered or decreased mitochondrial function in the aging brain, as well as in neurodegenerative disorders (e.g., Alzheimer’s, Parkinson’s, and Huntington’s diseases) and in psychiatric disorders such as schizophrenia and bipolar disorder (2–6), and in neurodegenerative disorders such as Leigh syndrome (7). Thus, it is clear that mitochondria play a fundamental role in normal brain function and in brain disease processes.

To understand the role of mitochondria in the central nervous system, direct assessment of mitochondrial activity is typically necessary. However, one of the vexing problems of assessing mitochondrial function is that many functional assays require abundant amounts of intact mitochondria. Animal models are commonly used for the study of mitochondrial function and dysfunction in the central nervous system because they can be manipulated genetically and pharmacologically, and because they are an abundant source of mitochondrial preparations. However, given the obvious species-specific variations in metabolic activity, mitochondria from laboratory animals cannot accurately reflect the functional or structural status of mitochondria in the human central nervous system. To assess mitochondrial activity in human brain, investigators have previously utilized frozen postmortem brain tissues to analyze mitochondrial enzymatic activities (8), mitochondrial protein levels (9), and mitochondrial DNA (10, 3). However, several other vital indices could provide insight into brain mitochondrial activity, which cannot be conducted in frozen tissue samples. These include measurements of the mitochondrial membrane potential (ΔΨmem), ATP production, calcium buffering capacity, and respiration, which together give an overall assessment of mitochondrial health and activity. For example, the ΔΨmem, which is the electrochemical

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gradient across the inner mitochondrial membrane, serves as an important overall indicator of mitochondrial activity. Also, it is a fundamental component of respiring mitochondria. The $\Delta \Psi_{\text{mem}}$ is linked to many crucial mitochondrial functions, including ATP synthesis, calcium homeostasis, mitochondrial protein import, and mitochondrial metabolite transport (11), all of which are typically analyzed in real-time measurements. However, these analyses can be conducted in structurally intact and functional mitochondria only. Furthermore, if these measurements are undertaken in isolated mitochondria, large quantities of relatively pure mitochondrial preparations are required. The need for mitochondrial purity, structural integrity, and abundance for functional studies are common barriers that can encumber brain mitochondrial research. An additional hindrance for research on human brain mitochondria is the extremely limited source of human brain tissues in general.

Postmortem human brains, when available, are potentially an abundant source of mitochondrial material. However, it is generally thought that direct measurements of mitochondrial activity, such as the $\Delta \Psi_{\text{mem}}$ and ATP production, are not possible in postmortem brain samples. This is a common belief because most organelle functions cease after death; in fact, with increasing postmortem interval (PMI), the brain tissue deteriorates rapidly. In this study, we report that postmortem mouse and human brains with relatively short PMIs are an abundant source of structurally intact and functional mitochondria, and we also report that mitochondria can be stored frozen for functional measurements at a later time.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless specified. C57/Bl6 mice were obtained commercially. All animal experiments were conducted in accordance with the University of Alabama at Birmingham Institutional Animal Care and Use Committee-approved protocols. Postmortem human tissue was obtained from the Alabama Brain Collection (ABC; Department of Psychiatry and Behavioral Neurobiology, University of Alabama at Birmingham), and human brain tissues were obtained in accordance with the University of Alabama at Birmingham Institutional Review Board. All tissue donations were obtained through the Alabama Organ Center in compliance with the Alabama uniform anatomical gift act.

Postmortem human brain samples were obtained from the ABC in collaboration with the Alabama Organ Center. Consent was obtained from the next of kin. Serology was performed for hepatitis A, B, or C; HIV; cytomegalovirus; and syphilis. One case was positive for cytomegalovirus (case S2). Brain tissue from the prefrontal cortex (Brodmann Area 10, BA10) was obtained from 3 different cases with postmortem intervals below 10 h (Table 1). For each case, ~5 cm³ of fresh tissue was placed in isolation buffer (215 mM mannitol; Acros Organics, Morris Plains, NJ, USA), 75 mM sucrose (Roche Diagnostics, Indianapolis, IN, USA), 0.1% fatty acid-free bovine serum albumin (FAF-BSA; Sigma-Aldrich), 20 mM HEPES (Sigma-Aldrich), and 1 mM ethylene glycol tetraacetic acid (EGTA; adjusted to pH 7.2 with 5 M KOH) immediately after dissection for further processing.

### Isolation of mitochondrial populations from neuronal processes and cell bodies

Methods for this isolation were adapted from Brown et al. (4). Tissues collected from either mouse or human were dissociated by Dounce homogenization in ice-cold isolation buffer. The same amount of ice-cold 30% Percoll (GE Healthcare Life Sciences, Uppsala, Sweden) in isolation buffer was added (~2% vol) for a final 15% Percoll solution in the sample. The Percoll solutions were prepared in isolation buffer and filtered to remove contaminants. Centrifuge tubes were layered from bottom to top, first with 40% Percoll, followed by 24% Percoll, and finally the 15% Percoll mitochondrial sample. Samples were centrifuged in a Sorvall RC-5C Plus centrifuge (Thermo Scientific, Asheville, NC, USA) using a fixed-angle SS34 rotor at 34,500 g for 30 min at 4°C. After centrifugation, three hazy bands were formed in the Percoll. The second and third bands from the top (synaptosomes and cell bodies, respectively) were removed from the Percoll medium and placed in separate tubes, and 10 ml of isolation buffer was added to wash the Percoll from the samples. Samples were centrifuged at 16,700 g for 15 min. The loose pellets obtained were then resuspended in isolation buffer and placed in a nitrogen cavitation bomb (Parr Instrument Company, Moline, IL, USA). The samples were subjected to nitrogen cavitation for further homogenization (800 p.s.i. for 10 min for mouse samples and 1900 p.s.i. for 10 min for human samples). The mitochondria were then separated into two general populations: mitochondria from cellular processes, designated as pMito, are mitochondria that exist predominantly in the axons, neurites, and the synapses. Mitochondria

### Table 1. Demographic data of the human cases used in the study

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Gender</th>
<th>Race</th>
<th>PMI (h)</th>
<th>pH</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>32</td>
<td>Female</td>
<td>Caucasian</td>
<td>7.5</td>
<td>6.3</td>
<td>Cardiac arrest</td>
</tr>
<tr>
<td>S2</td>
<td>77</td>
<td>Female</td>
<td>Caucasian</td>
<td>8.25</td>
<td>6.4</td>
<td>Lymphoma</td>
</tr>
<tr>
<td>S3</td>
<td>70</td>
<td>Male</td>
<td>Caucasian</td>
<td>8.5</td>
<td>6.4</td>
<td>Myocardial infarction</td>
</tr>
</tbody>
</table>

Note that PMI < 10 h for all cases used; pH is provided as a quality control for the tissue.
in the cell soma, designated as cMito, are mitochondria that are mostly resident in the perinuclear area and in the cell bodies.

To purify the pMito fraction, the homogenates were mixed with an equal volume of 10% Percoll in isolation buffer. The samples were layered over a 20% and a 12% Percoll cushions and centrifuged at 34,500 g for 30 min at 4°C. Following centrifugation, the bottom band containing pMito was collected. Isolation buffer (10 ml) was added, and the sample was centrifuged at 16,700 g for 15 min. The loose pellet was collected, washed once with isolation buffer, and centrifuged for 10 min at 17,600 g in an Eppendorf 5417R tabletop centrifuge (Eppendorf, Hamburg, Germany) at 4°C. This additional Percoll purification step was necessary for the removal of synaptic material from the pMito fraction. To purify the cMito following nitrogen cavitation, samples were centrifuged for 10 min at 750 g in an Eppendorf 5417R tabletop centrifuge at 4°C. The supernatant was retained and centrifuged for 15 min at 17,600 g, then the pellet was collected and washed once with isolation buffer and centrifuged for 10 min at 17,600 g. Mitochondria of both types (pMito and cMito) were kept pelleted on ice for a maximum of 40 min until used for the assays.

Mitochondrial cryopreservation

Mitochondria isolated from mouse cortices at 0 and 10 h postmortem were used. Previous reports have shown that mitochondria isolated from brain tissue can maintain structural and functional integrity after being frozen in a buffer containing 20% dimethyl sulfoxide (DMSO) and 1% FAF-BSA (1). pMito and cMito were resuspended in isolation buffer containing sterile 20% DMSO (Research Organics, Cleveland, OH, USA) and 1% FAF-BSA in Nalgene Cryoware cryogenic vials (Nalgene Nunc International, Rochester, NY, USA). Vials were then placed in a foam-insulated thermofreezing container with an approximate cooling rate of 1°C/min (Aladdin Industries Inc., Nashville, TN, USA), and the container was placed at −80°C for 7 d. Mitochondrial suspensions were then thawed at 4°C, washed with isolation buffer, and prepared for functional studies (ΔΨmem and ATP production) and the assessment of mitochondrial integrity and morphology by transmission electron microscopy.

Mitochondria functional assays

Rhodamine 123 assay for analysis of ΔΨmem

Mitochondria were washed with isolation buffer containing no BSA and then assayed for protein concentration using the bicinchoninic acid assay (Pierce, Rockford, IL, USA). During the protein assay incubation, mitochondria were pelleted (2 min centrifugation at 17,600 g) in isolation buffer to avoid exposure to air. Both cMito and pMito were tested for ΔΨmem. Mitochondria (60 μg) were resuspended in 200 μl isolation buffer containing 0.1% BSA and added to a 96-well clear-bottom plate. Rhodamine 123 (Rh123; 300 nM; Axonora, San Diego, CA, USA) was added to each sample just before reading. Samples were read in a Biotek Synergy 2 spectrofluorometer (Biotek, Winooski, VT, USA) at 25°C. Readings were taken every 45 s at 485 ± 20 nm excitation and 528 ± 20 nm emission wavelengths. Baseline readings were measured for 5 min before the addition of any substrates or treatments. Malate (7 mM), glutamate (7 mM), and ADP (100 μM) were added, and fluorescence was read for 10 min. The mitochondrial complex I inhibitor rotenone (5 μM) was added, and samples were monitored for 10 min, followed by the addition of succinate (7 mM) and ADP (100 μM) and another 10 min of fluorescence readings. Finally, carbonyl cyanide m-chlorophenylhydrazine (CCCP; 50 μM) was added, and fluorescence was monitored for the last 10 min. Water and DMSO were used as vehicle controls.

ATP production assay

pMito were assessed for ATP production. After determining protein concentration, 250 μg aliquots of pMito were pelleted and placed on ice until ready for use. For the ATP production assay, pellets were resuspended in 185 μl respiration buffer (20 mM HEPES-KOH pH 7.4; 80 mM potassium acetate; 5 mM magnesium acetate; 250 mM sucrose; 1 mM glutamate; and 1 mM malate). Buffer B (0.5 M Tris-acetate, pH 7.75; 0.8 mM D-tubulin; and 20 μg/ml luciferase) (10 μl) was immediately added to the reaction, and sample luminescence was read in standard mode on a Turner 20/20 luminometer (Promega, Sunnyvale, CA, USA) for 4 s with no delay. ADP (0.1 mM) was then added to the sample, and the luminescence was read every 4 s for a total of 60 s. Buffer background measurements were taken using buffers containing all substrates, and these measurements were subtracted from the mitochondrial readings. ATP standards were read using the same conditions and used to extrapolate ATP generation rates for each sample.

Transmission electron microscopy

Isolated mitochondria were pelleted and resuspended in cold fixative [4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffer, pH 7.4 (PB)]. Mitochondria were pelleted using centrifugation (17,600 g for 5 min), and fresh fixative was added to the pellet. The mitochondrial pellets were maintained in fixative for 12 h at 4°C. Pellets were then washed thoroughly with filtered PB, postfixed for 1 h with 1% osmium tetroxide in PB, rinsed with PB, and dehydrated in 50% and 70% ethanol baths. A 2% uranyl acetate in 70% ethanol solution was applied for contrast (1 h at 25°C in the dark) followed by 2 rinses in 70% ethanol (5 min each). Dehydration in ethanol was completed with two steps of 5 min in 95% and 100% ethanol baths. Then, 100% propylene oxide (Electron Microscope Sciences, Fort Washington, PA, USA) was applied to the samples (2 baths of 10 min each). Samples were then placed gradually in a 1:1 mixture of propylene oxide and Epon resin (25.9% Embed 812+15.5% Araldite 502+55.9% DDSA+2.7% BDMA; all resin components from Electron Microscopy Sciences) for 1 h at 25°C, a 1:2 mixture of propylene oxide-Epon resin (1 h at 25°C), and finally transferred to pure Epon resin and kept overnight at 25°C. The following day, the samples were placed in freshly prepared resin for an additional hour at 25°C and finally allowed to polymerize in an oven at 70°C for a minimum of 4 d. Ultrathin sections (90 nm thick) were obtained using a Leica EM UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany), mounted on formvar-coated copper grids and observed and photographed using a Hitachi transmission electron microscope, model H7650 (Hitachi, Japan), equipped with a Hamamatsu ORCA-HR digital camera (Bridgewater, NJ, USA). Figure plates were prepared using Corel PhotoPaint 12 and CorelDraw 12 (Corel, Ottawa, ON, Canada).

RESULTS

Presence of a robust ΔΨmem in postmortem mouse cortex

This study aimed to examine whether mitochondria maintain their integrity and activity after the death of...
an individual. To test whether functional mitochondria could be isolated from postmortem brain, adult male C57/Bl6 mice were euthanized by cervical dislocation and the intact bodies were placed at 4°C for increasing lengths of time (0, 10, 18, and 24 h) to test the possible time-dependent degradation of brain mitochondria. These time points were used because the typical PMI for human postmortem tissues is beyond 8 h, although on very rare occasions human brain tissues with shorter PMIs are available. Furthermore, this mode of death and body storage was used to mimic a generalized scenario of human death and subsequent body handling. Mitochondria from the postmortem mice were compared to mitochondria from 0 h animals in which the mice were euthanized and cortical mitochondria were isolated immediately after death. In the initial time course study, cell body mitochondria (cMito) were isolated from mouse cortex. The potentiometric dye Rh123 used to measure $\Delta \Psi_{\text{mem}}$. Rh123 is transported electrophoretically into intact mitochondria and sequestered within the organelle due to the charge difference across the mitochondrial inner membrane (12, 13). Nonrespiring mitochondria and mitochondria lacking structural integrity cannot accumulate Rh123 (13). Thus, when the mitochondrial inner membrane is intact and polarized, Rh123 is sequestered within mitochondria and Rh123 fluorescence decreases (13). On depolarization, the sequestered Rh123 is released from the mitochondria into the surrounding buffer, resulting in increased fluorescence (13). A PMI time course was established to examine how increasing PMI would affect $\Delta \Psi_{\text{mem}}$. Treatment with the respiratory substrates malate, glutamate, and ADP caused efficient sequestration of Rh123, resulting in decreased fluorescence in all the time points tested (0 to 24 h PMI), and all samples of mitochondria responded equivalently to the addition of respiratory substrates (Fig. 1A–D). These results indicate that isolated mouse brain mitochondria are intact and can be induced to hyperpolarize, with respect to baseline readings, for $\geq$24 h after death.

Manipulation of the $\Delta \Psi_{\text{mem}}$ with respiratory toxins provides an additional assessment of mitochondrial integrity and viability. The addition of the complex I inhibitor rotenone resulted in the rapid depolarization of mitochondria, indicated by the upward shift in Rh123 fluorescence across all time points (Fig. 1A–D), but depolarization of the 10, 18, and 24 h PMI mitochondria was clearly more robust than the 0 h mitochondria. Bypass of complex I by the addition of succinate (which activates complex II) resulted in complete restoration of the $\Delta \Psi_{\text{mem}}$ at all the PMI time points (Fig. 1A–D). The addition of the protonophore CCCP equivalently dissipated the $\Delta \Psi_{\text{mem}}$ for all the
PMI time points (Fig. 1A–D). At PMI > 10 h, the brain architecture and the tissues overall were substantially degraded, and an ~70% reduction in mitochondrial yield occurred. Therefore, for the remainder of the studies, the 0 and 10 h PMI were tested.

The next goal was to test whether any statistical variation occurred between 0 and 10 h PMI. The only variation between the two time points was in response to rotenone treatments, whereby rotenone-induced membrane depolarization at the 10 h PMI increased significantly (Fig. 1E, F). Thus, complex I may have acquired more sensitivity to rotenone with increasing PMIs, but the downstream complex II was unaffected. Dimethyl sulfoxide (DMSO) was used as a vehicle control for rotenone and CCCP, but no effect of DMSO on $\Delta \Psi_{\text{mem}}$ was evident, indicated by open squares (Fig. 1E, F). The fluorescence of the hyperpolarized mitochondria from the 10 h PMI remained stable and constant throughout the 45-min timeframe (Fig. 1F, open squares). Thus, no leakage of Rh123 from the mitochondria occurred, indicating that the mitochondrial membranes remained fully intact. This result indicates that postmortem mouse brain cortical mitochondria can be induced to engage their $\Delta \Psi_{\text{mem}}$ 24 h postmortem.

To get a visual confirmation of mitochondrial structural integrity, mitochondrial pellets from the 0 and 10 h PMI were visualized by electron microscopy. In Fig. 1G, I, it is evident that the mitochondrial fraction is mostly free from nonmitochondrial debris, which could occlude accurate measurements of mitochondrial function. Higher magnification images (Fig. 1H, J) illustrate that most of the mitochondria in the 0 and 10 h PMI samples appear to be intact and maintain their orthodox configuration, which has been shown to be indicative of mitochondria with normal respiration, energy production, and cell survival signaling (14–16). These data together indicate that mitochondria isolated from mouse postmortem brains are structurally intact, as well as functionally viable.

Mitochondrial $\Delta \Psi_{\text{mem}}$ and ATP production in postmortem human cortex

The next objective was to extend these observations to the human brain and to examine whether $\Delta \Psi_{\text{mem}}$ in mitochondria isolated from postmortem human cortex also responded to respiratory substrates and toxins. Human cortical tissue was extracted from BA10 from three deceased individuals with no documented history of neurological or psychiatric disorders (Table 1). In all three cases, the PMI was <10 h and the pH was between 6.3 and 6.4. Mitochondria from cell bodies (cMito) and neuronal processes (pMito) were isolated as described in Materials and Methods and tested for $\Delta \Psi_{\text{mem}}$. In subject 1 (S1), the pMito and cMito hyperpolarized in response to the respiratory substrates (Fig. 2A, B), and the pMito robustly depolarized in response to treatments with rotenone and CCCP (Fig. 2A). By comparison, on the addition of rotenone, the cMito from S1 maximally depolarized, and were then refractory to further succinate and CCCP treatments (Fig. 2B), which suggests that complex II in this cMito sample was impaired. The pMito from subject 2 (S2) also displayed the characteristic responses to substrates and toxins (Fig. 2C), although visibly less than pMito from S1. Also, the cMito from S2 showed a noticeably mitigated response to the stimuli (Fig. 2D) compared to the matched pMito. In contrast, pMito and cMito isolated from subject 3 (S3) were neither responsive nor could correlated responses be found in their $\Delta \Psi_{\text{mem}}$ to malate/glutamate, rotenone, and succinate (Fig. 2E, F). However, membrane polarity was not dissipated in these mitochondria because the $\Delta \Psi_{\text{mem}}$ was still amenable to total depolarization by treatment with the uncoupler CCCP. Since the mitochondrial sample from S3 displayed an aberrant $\Delta \Psi_{\text{mem}}$ profile, we wanted to test further whether these mitochondria were metabolically active. ATP production was measured in comparison to S2 mitochondria, which displayed the more characteristic $\Delta \Psi_{\text{mem}}$ profile in our studies and were isolated from an equivalent age (77 yr for S2 and 70 yr for S3) and PMI brain sample (8.25 h for S2 and 8.5 h for S3) (see Table 1). Since the mitochondria from S3 did not hyperpolarize in response to glutamate/malate, it was interesting to find ample ATP production capacity in response to these substrates and only 20% less ATP production than those from S2 (Fig. 2G). Furthermore, ATP production in S3 mitochondria was inhibited by 87.5% by the addition of CCCP (Fig. 2G). This finding suggests that the mitochondria from S3 retain their ATP-generating capability despite the lack of substrate-induced changes in the $\Delta \Psi_{\text{mem}}$. Mitochondrial integrity from all the human samples was also verified visually. The electron micrographs (Fig. 3) show that the isolated human postmortem brain mitochondria are predominantly intact and retain much of their orthodox internal morphology. Overall, these data show for the first time that mitochondria in postmortem human brain remain intact and metabolically active for at least 8.5 h following the death of the individual.

Cryogenic preservation of postmortem mitochondria minimally affects $\Delta \Psi_{\text{mem}}$ and bolsters ATP production

Cryogenic storage of freshly isolated mitochondria has been reported previously (1, 17, 18). The purpose here was to test whether mitochondria from postmortem brains could also withstand cryogenic storage conditions. This finding would be an important assessment for instances when comparative studies of mitochondrial functional indices must be measured simultaneously under standardized conditions. Given the sporadic nature of human tissue procurement, these types of studies in fresh samples would be impossible.

For these studies, cMito and pMito isolated from mouse cortex 0 and 10 h after death were stored as described in the Materials and Methods. After 7 d of...
storage, mitochondrial preparations were thawed on ice and tested for \( \Delta \Psi_{\text{mem}} \) and ATP production, and the stored mitochondria were examined for structural changes. Cryogenic storage did not appear to cause any alterations in the \( \Delta \Psi_{\text{mem}} \) profile of the cMito and pMito in response to respiratory substrates and toxins (Fig. 4A–D). Furthermore, the \( \Delta \Psi_{\text{mem}} \) profile of the frozen cMito was not altered significantly when compared to freshly isolated cMito (Fig. 4E), although a slight, but consistent potentiation in the responses of the frozen mitochondria to the respiratory substrates and to rotenone was found. For extended storage, future studies will be necessary to determine the maximum length of time that cryogenic storage is feasible without loss of mitochondrial integrity. However, the present findings show that cryogenic storage of postmortem brain mitochondria for at least 7 d does not cause overt damage to mitochondrial membrane integrity (Fig. 4F). A direct comparison of \( \Delta \Psi_{\text{mem}} \) profiles from same-sample freshly isolated 0 h mitochondria and 7 d cryopreserved 0 h mitochondria (Fig. 5) also confirms that proper cryopreservation does not result in major detrimental effects on \( \Delta \Psi_{\text{mem}} \).

To assess whether the postmortem interval combined with cryogenic storage can affect metabolic output, ATP production rate was also measured in

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**Figure 2.** Mitochondrial \( \Delta \Psi_{\text{mem}} \) in human postmortem brain. Mitochondria isolated from human postmortem brains are intact and responsive to substrates and toxins. Mitochondria from neuronal processes (pMito) and cell bodies (cMito) were isolated as described in Materials and Methods. \( \Delta \Psi_{\text{mem}} \) results are shown as percentage of initial reading. A–F) pMito and cMito were isolated from BA10 of 3 different individuals with different PMIs ranging from 7.5 to 8.5 h (A, B: S1; C, D: S2; E, F: S3). pMito and cMito suspensions were treated sequentially with malate/glutamate/ADP (MGA), rotenone (ROT), succinate/ADP (S/A), and carbonyl cyanide \( m \)-chlorophenylhydrazone (CCCP). G) Comparison of ATP production by isolated mitochondria from S2 and S3 after the addition of mitochondrial substrates malate, glutamate, and ADP. As a control for the assay, the addition of CCCP to the sample was used to verify that ATP production could be inhibited.

**Figure 3.** Structurally intact mitochondria can be obtained from postmortem human brain. A–D) Low-magnification (A, C) and high-magnification images (B, D) of isolated mitochondria from case S1. E–H) Low-magnification (E, G) and high-magnification images (F, H) of isolated mitochondria from case S2. Note that differences in age do not affect obtaining viable mitochondria from human brain tissue (see Table 1 for demographic information). Scale bars = 2 \( \mu \text{m} \) (A, E, G); 1 \( \mu \text{m} \) (C); 0.5 \( \mu \text{m} \) (B, D, F, H).
The rate of ATP production was decreased significantly, by 56%, in the freshly isolated 10 h PMI mitochondria, compared to the 0 h freshly isolated mitochondria (Table 2), indicating that the postmortem interval does cause a decrement in mitochondrial ATP output. It was interesting to find that the cryogenic storage of mitochondria augmented ATP production, with and without CCCP treatment, in both 0 and 10 h PMI mitochondria. ATP production was also bolstered in the same-sample 0 h PMI cryopreserved mitochondria compared to the freshly isolated mitochondria, indicating that the cryopreservation process affects mitochondrial ATP production. However, the stored 10 h PMI mitochondria had a proportional, 57.9% reduction in the rate of ATP production compared to the cryopreserved 0 h mitochondria (Table 2), as was found in the freshly isolated samples. This finding indicates that cryogenic conditions did not alter the percent reduction in ATP output from the 10 h PMI mitochondria. These results highlight the fact that isolated mitochondria can be cryogenically archived and then reanimated at a later time. However, studies of comparative mitochondrial ATP measurements between different postmortem mitochondrial samples must be matched for PMI and storage conditions.

DISCUSSION

Moments after death, the brain is placed in a condition of asphyxia, and tissues are deprived of oxygen, which is required as the final electron acceptor at the terminus of the electron transport chain. As a consequence, mitochondrial ATP generation ceases. Among the early ramifications due to the loss of ATP production is the destruction of cellular membranes (19), because phospholipids in the membranes become rapidly oxidized and are not replaced by the ATP-dependent processes of membrane repair and renewal. The loss of cell membrane integrity disgorges normally sequestered hydrolytic enzymes, and the process of cell and or-

Figure 4. Cryopreservation for 1 wk does not affect ΔΨmem. Mitochondria isolated from 0 and 10 h PMI mouse cortex were cryopreserved (20% DMSO, −80°C) for 1 wk. Responsiveness of the ΔΨmem to substrates and toxins was tested in cryopreserved cMito and pMito isolated from 0 h (A, B), and 10 h PMI (C, D) mouse cortex. Error bars = SEM. E) Comparison of ΔΨmem responses to mitochondrial substrates and toxins between freshly isolated (Fr) and cryopreserved (CP) mitochondria from 10 h PMI mouse cortex. No statistical difference was found in the responsiveness of ΔΨmem to substrates and toxins between the freshly isolated and the cryopreserved mitochondria. (Student’s t test; P>0.05; n=3). F) Electron micrographs of cMito cryopreserved in 20% DMSO for 1 wk after 0 and 10 h PMI. Scale bars = 2 μm.

Figure 5. Comparison of freshly isolated and cryopreserved mitochondria obtained from the same sample. Mitochondria were isolated from 0 h PMI mouse brain (n=3 animals) and separated into two aliquots. One mitochondrial aliquot was tested immediately for function in the Rh123 assay, while the other aliquot was cryopreserved for 1 wk prior to testing. A) Averaged membrane potential data obtained from fresh (gray diamonds) and cryopreserved pMito (open squares). B) Representative experiment indicating membrane potential in fresh (gray diamonds) and cryopreserved cMito (open squares).
Mitochondria are not a homogenous population in the brain, and mitochondria in neuronal processes (pMito) and mitochondria in the cell soma (cMito) have been shown to have functionally distinct characteristics (22, 4, 23). However, the findings here suggest that PMI and cryopreservation have little or no differential effects on ΔΨmem between cMito and pMito.

In contrast to the standardized mouse husbandry and controlled storage conditions of mouse tissues, which yields mitochondria of consistent quality, the condition of mitochondria from human postmortem brain tissues is far more variable. This is because the premortem state of the individual, including age, drug use, and possible brain infections, as well as cause of death, storage condition of the body, tissue pH, and PMI, altogether affect the overall yield and quality of human brain mitochondria. The human brain tissues used in this study were all <10 h PMI; however, we found marked interindividual and intermitochondrial (cMito vs. pMito) variations, as each of the different mitochondrial samples displayed uniquely distinct ΔΨmem profiles, which was not evident in the mouse brain mitochondrial samples. The small increase in PMIs in samples S2 and S3, compared to S1, may be one factor that imparts differences in the ΔΨmem profiles. In addition, the age of the individuals could also be another factor contributing to ΔΨmem differences, which would be worthwhile to assess in more detail in future studies. The mitochondrial sample from S1, a 32-year-old individual, responded most robustly to the different treatments, while the other two samples (from S2 and S3, both of whom were in their 70s) produced variable results by comparison.

The S3 mitochondria sample was particularly intriguing because the ΔΨmem was unresponsive to the addition of oxidizable substrates and to rotenone. Although it is impossible to draw firm conclusions from one individual, these results nevertheless suggest that complex I and complex II in these mitochondria may have been impaired but not completely inactive, since CCCP treatment still resulted in robust depolarization, indicating that some level of membrane polarity was still present. Ostensibly, this is sufficient to drive ATP generation. This finding reflects the inherent complexity of working with heterogeneous human samples, a problem that can be overcome by the inclusion of greater sample numbers. However, the findings presented here with three human samples clearly demonstrate the feasibility of using human postmortem brains as an abundant source of metabolically active mitochondria for research purposes.

The remarkable resiliency of the mitochondrion is likely due to the combination of its enclosed asymmetric double membrane barrier, the dissimilar composition of the mitochondrial membranes, and the intricate crisscrossing cristae, which together scaffold the mitochondrial structure and protect it from the normal tissue degradation processes. The resistance of mitochondria to proteolytic degradation is illustrated further by findings from mitochondria isolated from cultured cells (24, 25). When these mitochondria were incubated in a 30°C water bath in the presence of the proteolytic enzymes trypsin or proteinase K, the proteins that were exposed on the cytosolic face of the outer mitochondrial membrane were digested rapidly, but the proteins encapsulated within the mitochondrial membranes were unaffected (24, 25). This finding

### TABLE 2. Comparison of ATP production at 0 and 10 h PMI in freshly isolated and in cryopreserved pMito from mouse cortex

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mal/Glut</th>
<th>Mal/Glut/CCCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h PMI</td>
<td>53.28 ± 10.34</td>
<td>6.86 ± 1.83</td>
</tr>
<tr>
<td>10 h PMI</td>
<td>23.37 ± 7.16</td>
<td>2.76 ± 0.76</td>
</tr>
<tr>
<td>0 h PMI CP</td>
<td>161.98 ± 0.57*</td>
<td>72.4 ± 0.09</td>
</tr>
<tr>
<td>10 h PMI CP</td>
<td>68.2 ± 9.37#</td>
<td>14.9 ± 1.66</td>
</tr>
</tbody>
</table>

Comparison of fresh and cryopreserved pMito from same sample

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mal/Glut</th>
<th>Mal/Glut/CCCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h PMI</td>
<td>41.29 ± 3.11</td>
<td>7.42 ± 2.90</td>
</tr>
<tr>
<td>0 h PMI CP</td>
<td>130.20 ± 14.25</td>
<td>15.05 ± 7.67</td>
</tr>
</tbody>
</table>

Values are mean ± s.e rates of ATP production (nmol/min/μg mitochondrial protein). CP, cryopreserved. *P < 0.05 vs. 0 h PMI, #P < 0.05 vs. 10 h PMI; Student’s t test.
suggests that the double mitochondrial membrane is mostly impervious to proteolytic enzymes, thereby insulating the internal contents of the mitochondria from the digestive milieu. Therefore, in the dying brain tissues, mitochondria could possibly withstand the early degradation processes that typically destroy other cellular structures.

One of the beneficial consequences of utilizing post-mortem brains is the abundant yield of mitochondria. The storage of mitochondria for archival purposes and subsequent analysis liberates the researcher from the long, tedious process of analyzing mitochondrial functions one sample at a time. Instead, with appropriate storage, it could be possible to assess mitochondrial activity, for example $\Delta \Psi_{\text{mem}}$ and ATP production, in a rapid and standardized process. In the present study, cryogenic storage of mouse brain mitochondria for at least one week clearly did not affect their $\Delta \Psi_{\text{mem}}$ profile. Cryogenic storage was not attempted for the human brain mitochondria because it would be difficult with such a small number of human samples to determine whether any alterations were due to the cryogenic process or inherent differences in the individuals’ mitochondria. Although, the mouse data do suggest feasibility of such an undertaking, one must be made aware that the same cryopreservation protocol may yield different results with human samples. The concept of cryogenic storage of mitochondria dates back to 1961 (26, 27). More recent studies have confirmed that mitochondria within rat brain synaptosomes and isolated rat brain mitochondria can be reanimated following cryopreservation (28, 1). The most important consideration of mitochondrial cryopreservation is the addition of a cryoprotective agent, because freezing in its absence destroys mitochondrial structure and function (1). Numerous cryoprotectants are available, but the most commonly used are DMSO and glycerol. A comparative study using these two cryoprotectants revealed that DMSO is far superior in preserving mitochondrial integrity and function on freeze-thawing than glycerol (17). In our study, it was interesting to observe that $\Delta \Psi_{\text{mem}}$ not only was unaffected in the DMSO cryopreserved mitochondrial samples but, curiously, we observed a substantial enhancement of ATP production for pMito cryogenically stored, both after 0 and 10 h PMI. To our knowledge no one has reported ATP production measurements from cryopreserved brain mitochondria; however, respiration measurements have revealed decreased respiratory control ratios in cryopreserved rat brain mitochondria (1). The reason for the enhancement of mitochondrial ATP production following cryopreservation is unclear, although some evidence suggests that DMSO can affect the affinity of the F1 subunit of ATP synthase for P, and influence ATP formation (29). Although cryopreservation accentuated mitochondrial ATP output in the 0 and 10 h PMI mitochondria, it did not seem to affect the relative difference in the rate of ATP production, as in, both the freshly isolated samples and the cryopreserved samples the 10 h PMI had 56.2% and 57.9% reduction, respectively, in ATP production compared to the 0 h PMI mitochondria. Thus, although $\Delta \Psi_{\text{mem}}$ measurements could be unaffected by cryopreservation, ATP production from cryopreserved mitochondrial samples must be matched to other cryopreserved samples.

In conclusion, the common perception is that once an individual is deceased all metabolic activity is irreversibly arrested. Our findings show for the first time that, in actuality, brain mitochondrial $\Delta \Psi_{\text{mem}}$ can be resurrected, and hence mitochondrial metabolic activity can be effectively reinitiated within at least 10 h after death in mice and 7.5 h in human postmortem tissue. The ability to access a source of fully functional human brain mitochondria allows for the first time the assessment of indices of mitochondrial function that could not be accomplished previously in human brain tissues. Although the therapeutic implications are yet to be discovered, these results provide mitochondrial researchers a novel and additional resource to directly investigate mitochondrial structure and function in the normal and diseased human brain.

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REFERENCES


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