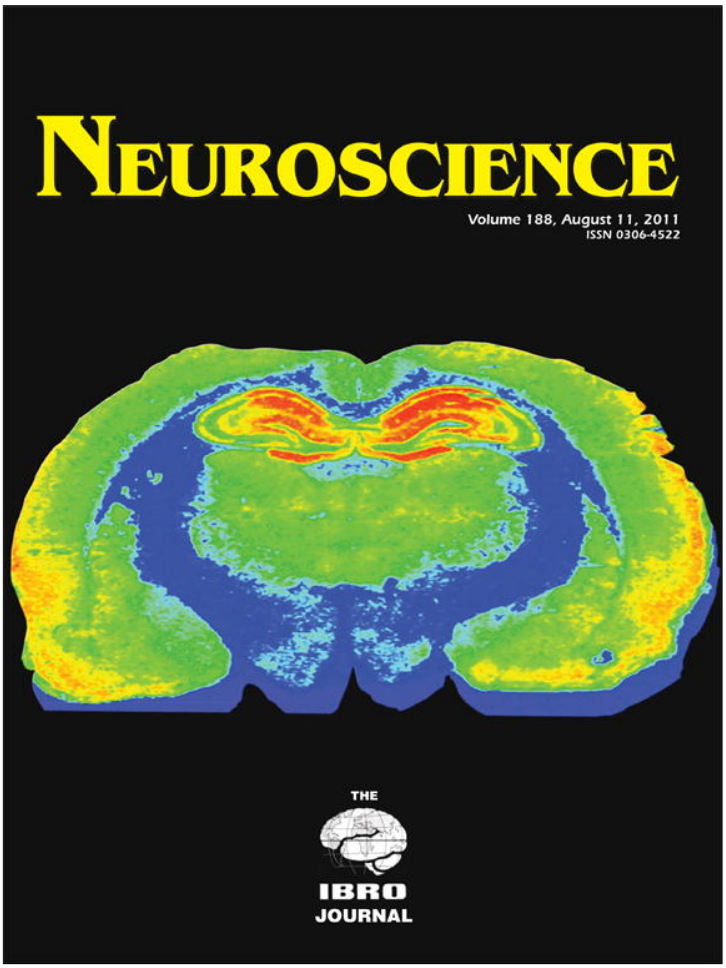

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AGE-RELATED ALTERATIONS IN MITOCHONDRIAL PHYSIOLOGICAL PARAMETERS AND NITRIC OXIDE PRODUCTION IN SYNAPTIC AND NON-SYNAPTIC BRAIN CORTEX MITOCHONDRIA

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Abstract—Brain aging has been associated with mitochondrial dysfunction and changes in nitric oxide levels. The aim of this study was to evaluate the susceptibility of synaptic and non-synaptic mitochondria to aging-dependent dysfunction. State 3 respiratory rate and respiratory control were 43% and 33% decreased, respectively in brain cortex synaptosomes from 14-month-old animals, as compared with synaptosomes from 3-month-old mice. Respiratory rates were not significantly affected by aging in non-synaptic mitochondrial fractions. Mitochondrial dysfunction was associated with increases of 84% and 38% in H_2O_2 production rates in brain cortex synaptosomes and non-synaptic mitochondria, respectively, from 14-month-old mice, as compared with young animals. Synaptic mitochondria seem to be more susceptible to calcium insult in 14-month-old mice, as compared with non-synaptic mitochondria, as measured by response of both types of fractions to calcium-induced depolarization. With aging, nitric oxide (NO) production was 44% and 27% decreased both in synaptosomal and non-synaptic mitochondrial fractions, respectively. The results of this study suggest that with aging, mitochondrial function at the nerve terminals would be more susceptible to suffer alterations by the constant calcium changes occurring as a consequence of synaptic activity. Non-synaptic mitochondria would be more resistant to age-related dysfunction and oxidative damage.

Key words: synaptosomes, non-synaptic mitochondria, aging, cerebral cortex, mitochondrial respiration, nitric oxide synthase.

High neuronal activity requires increased metabolism to fuel pumps, the maintenance of membrane gradients and enough ATP levels to support synaptic activity (Dubinsky, 2009). Therefore, mitochondria in nerve terminals are subjected to extensive calcium fluxes and high energy demands, both at the pre-synapsis due to neurotransmitter exocytosis and release and at the post-synapsis where Ca^{2+} and Na^{+} fluxes activate ATPases (Parihar and Brewer, 2007).

Mitochondria in the presence of phosphate take up Ca^{2+} to a fixed capacity, in a membrane-potential–dependent fashion. When mitochondria become overloaded with Ca^{2+}, they undergo the mitochondrial permeability transition (MPT) via formation of a non-selective pore that allows solutes <1500 Daltons to pass through the usually impermeable inner mitochondrial membrane with a resultant rupture of the outer mitochondrial membrane due to osmotic swelling (Bernardi, 1999).

Recent studies by Brown et al. (2006) have shown that non-synaptic mitochondria isolated from rat cortex can accumulate significantly more Ca^{2+} before undergoing MPT than the synaptic mitochondria. Synaptic mitochondria are synthesized in the cell body of neurons and transported down the axon or dendrite. Within the CNS, mitochondria have an apparent half-life of approximately 1 month. As a result of transport, synaptic mitochondria may be "older" than mitochondria in the soma of neurons and glial cells, and may exhibit greater cumulative damage from oxidative stress (Brown et al., 2006).

The aging process involves changes in mitochondria, including a reduction in bioenergetic capacity, increased oxygen free radical generation and perturbation of calcium homeostasis. Mitochondria are considered the main intracellular source of reactive oxygen species (ROS) and also the main target of oxy-radical–mediated damage. Cumulative free radical damage leads to significant changes in brain mitochondrial function with aging (Sastre et al., 2000). There is increasing evidence to suggest that in aging and in Alzheimer disease, damaged synaptic mitochondria might not satisfy the high energy demands required at the synapses, which might lead to impaired neurotransmission and, ultimately, to cognitive failure (Reddy and Beal, 2008).

The aim of this study was to evaluate potential differences in the functioning of synaptic and non-synaptic mitochondria during the aging process. As the decline in mitochondrial function may begin before the onset of old age, we decided to study two different age groups: 3 and 14 months. For the evaluation of mitochondrial function of mitochondria at nerve terminals, synaptosomes were isolated from mouse brain cortex. Parameters of mitochondrial function such as oxygen uptake, hydrogen peroxide (H_2O_2) generation and mitochondrial membrane potential were determined in intact brain cortex synaptosomes and in non-synaptic mitochondria. Enzyme activity of nitric oxide synthase (NOS), monoamine oxidase (MAO) and respiratory complexes activity were measured in brain cortex synaptosomal and submitochondrial membranes from 3- or 14-month-old animals. Acetylcholinesterase activity, an enzyme normally affected during the aging process, was

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Abbreviations: MAO, monoamine oxidase; MPT, mitochondrial permeability transition; NO, nitric oxide; NOS, nitric oxide synthase.

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also determined in brain cortex synaptosomal samples from the two groups of age.

EXPERIMENTAL PROCEDURES

Animals and sample preparation

Male Swiss mice of 3 or 14 months of age were used. All efforts were made to minimize animal discomfort and to reduce the number of animals used.

Animals were killed by decapitation and the brains were immediately excised. Brains were weighed, cerebral cortex was dissected and homogenized in a medium consisting of 0.23 M mannitol, 0.07 M sucrose, 10 mM Tris–HCl and 1 mM EDTA (MSTE), pH 7.4, and homogenized at a ratio of 1 g brain/5 ml homogenization medium. Homogenates were centrifuged at 600 g for 10 min to discard nuclei and cell debris and the supernatant was centrifuged at 8000 g for 10 min. The resulting pellet was washed and resuspended in the same buffer.

Further mitochondrial purification and synaptosomal fraction separation were performed by Ficoll gradient (Clark and Nicklas, 1970), with modifications. The crude mitochondrial fraction was resuspended in MSTE buffer and layered on Ficoll gradients containing steps of 13%, 8% and 3% Ficoll (Rodríguez de Lores Arnaiz and Girardi, 1977). The gradients were centrifuged at 11,500 g for 30 min. Three layers and a pellet were obtained. The fraction occurring at 8% was used to study the synaptosomal fraction. All the procedure was carried out at 0–2 °C. Submitochondrial membranes were obtained from mitochondria by twice freezing, thawing and homogenizing by passing the suspension through a 15/10 hypodermic needle (Lores-Arnaiz et al., 2004a). Protein content was assayed by using the Folin phenol reagent and bovine serum albumin as standard (Lowry et al., 1951).

Mitochondrial protein content was estimated in our preparations, resulting between 3.5 and 5 mg/g tissue for non-synaptic mitochondria and between 4 and 6.5 mg/g tissue for synaptosomes. Yield of mitochondrial protein after isolation of a crude mitochondrial fraction was previously estimated between 100 and 140 mg/50 g tissue (Basford, 1967); preparation of synaptosomes by a rapid Percoll gradient procedure resulted in a yield of synaptosomes between 2.5 and 4 mg of synaptosomal protein per gram rat brain (Dunkley et al., 2008). Measurement of a mitochondrial marker enzyme (MAO) activity in total homogenates, in non-synaptic mitochondria and in synaptosomal fractions indicated that our isolation procedure resulted in a 2.3-fold enrichment of the mitochondrial pool. Procedures incorporating density gradient centrifugation usually produce highly enriched fractions of mitochondria but typically result in yields of 10% or less of the original organelles (Anderson and Sims, 2000). In our study, mitochondrial yields were estimated for synaptosomal and non-synaptic mitochondrial fractions, based on measurements of MAO activity in each fraction as compared with total MAO activity determined in cerebral cortex homogenates. Our isolation procedure resulted in yields of mitochondrial recovery of 9.3% for synaptosomes and 5.9% for non-synaptic mitochondria. Non-synaptic mitochondria were less than 5% contaminated with synaptosomal components, according to acetylcholinesterase activity determinations.

Mitochondrial respiration

A two-channel respirometer for high-resolution respirometry (Oroboros Oxygraph, Paar KG, Graz, Austria) was used. Mitochondrial respiratory rates were measured in cerebral cortex synaptosomes and non-synaptic mitochondria from 3- or 14-month-old animals, in a reaction medium containing 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 4 mM $MgCl_2$, 5 mM phosphate and 0.2% bovine serum albumin at 30 °C. Malate 6 mM and glutamate 6 mM were used as substrates to measure state 4 respiration and 1 mM ADP was added to measure state 3 respiration. The respiratory control ratio (state 3 respiration/state 4 respiration) was determined (Estabrook, 1967).

In order to verify if malate, glutamate and ADP could penetrate through the plasma membrane and get to the mitochondria, a simple assay was carried out to compare respiratory rates in intact synaptosomes and in a synaptic mitochondrial fraction obtained by permeabilization of synaptosomes. For this assay, synaptosomes were diluted in the isolation buffer with the addition of 0.01% digitonin, mixed gently and rapidly centrifuged for 10 min at 11,500 g. After immediate removal of digitonin, the pellet was resuspended in the same isolation buffer.

Hydrogen peroxide production

Hydrogen peroxide generation was determined in intact cerebral cortex synaptosomes or non-synaptic mitochondria (0.1–0.3 mg protein/ml) by the scopoletin-HRP method, following the decrease in fluorescence intensity at 365–450 nm ($\lambda exc - \lambda em$) at 37 °C (Boveris, 1984).

The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), 0.8 μM HRP, 1 μM scopoletin, 6 mM malate, 6 mM glutamate and 0.3 μM SOD. A calibration curve was made using H_2O_2 (0.05–0.35 μM) as standard to express the fluorescence changes as nmol H_2O_2/min mg protein.

Mitochondrial membrane potential

Mitochondrial population with specific side scattering (SSC) and forward scattering (FSC) properties were analyzed; 20,000 events per sample were collected. Brain cortex synaptosomes or non-synaptic mitochondria from 3- or 14-month-old mice were loaded with 30 nM of the potentiometric probe $DiOC_6$ for 20 min at 37 °C and immediately acquired by a FAC-SCAN flow cytometer equipped with a 488 nm argon laser and a 615 nm red diode laser. Mitochondrial autofluorescence (no probe) was also measured. Data from the experiments were collected in a Becton-Dickinson FACSCalibur and analyzed using the WinMDI 2.6 version software.

Nitric oxide synthase activity

Both synaptosomes and non-synaptic mitochondria were ruptured by freezing and thawing to measure NOS activity. Nitric oxide (NO) production was determined in synaptosomal fractions and in non-synaptic submitochondrial membranes from 3- or 14-month-old animals, by following spectrophotometrically the oxidation of oxyhemoglobin to methemoglobin at 37 °C, in a reaction medium containing 50 mM phosphate buffer (pH 5.8 for non-synaptic mitochondria and 7.4 for synaptosomal membranes), 1 mM $CaCl_2$, 50 μM L-arginine, 100 μM NADPH, 10 μM dithiothreitol, 4 μM Cu-Zn SOD, 0.1 μM catalase, 0.5–1.0 mg submitochondrial protein/ml and 25 μM oxyhemoglobin (expressed per heme group). The kinetics was followed at 577–591 nm (ε=11.2 mM^{-1} cm^{-1}) in a double-beam double-wavelength spectrophotometer (Boveris et al., 2002).

MAO activity

MAO activity was measured in synaptosomal fractions and in non-synaptic submitochondrial membranes by following spectrophotometrically the oxidation of kynuramine at 30 °C, in a reaction medium containing 50 mM phosphate buffer (pH 7.4). Kinetics were followed at 360 nm (ε =4.28 mM^{-1} cm^{-1}) (Weissbach et al., 1960).

Evaluation of respiratory complexes activity

NADH cytochrome c reductase activity (complex I+III) was measured in synaptosomal fractions and in non-synaptic submitochon-

drial membranes by following spectrophotometrically the reduction of cytochrome c at 30 °C at 550 nm (ε=19.6 mM^{-1} cm^{-1}) in a reaction medium containing 100 mM phosphate buffer (pH 7.4), 0.2 mM NADH, 0.1 mM cytochrome c and 0.5 mM KCN. Enzyme activity was expressed in nmoles cytochrome c reduced per min per mg of protein. Succinate cytochrome c reductase activity (complex II+III) was similarly determined and expressed, except that NADH was substituted by 20 mM succinate. Cytochrome oxidase activity (complex IV) was assayed spectrophotometrically at 550 nm by following the rate of oxidation of 50 μM ferrocytochrome c (Yonetani, 1967). The activity was expressed as nmoles ferrocytochrome c oxidized per min per mg of protein.

Acetylcholinesterase activity

Acetylcholinesterase activity was measured spectrophotometrically by using 1 mM acetylthiocholine, 0.2 mM 5,5′-dithiobis-(2-nitrobenzoic acid) in potassium phosphate buffer (0.1 M, pH 8.0), following absorbance change at 412 nm for 2 min. Enzyme activity was expressed as nmol acetylthiocholine hydrolyzed/min mg protein (Ellman et al., 1961).

Drugs and chemicals

ADP, L-arginine, catalase, dithiothreitol, EDTA, glutamic acid, malic acid, mannitol, NADPH, N_ω-nitro-L-arginine, haemoglobin, scopoletin, horseradish peroxidase, succinate, sucrose, superoxide dismutase, cytochrome c, Trizma base, cyclosporin A, digitonine, kynuramine, acetylthiocholine and 5,5′-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents were of analytical grade.

Statistics

Results are expressed as mean values±SEM. Student's *t*-test was used to analyze the significance of differences between paired groups.

RESULTS

Mitochondrial respiration

Malate-glutamate–dependent oxygen consumption rate was measured in state 4 (resting or controled respiration) and in state 3 (active respiration, the maximal physiological rate of O_2 uptake and ATP synthesis) and the respiratory control ratios were calculated.

Experimentally the synaptosome is accessible to many of the techniques employed for isolated mitochondria, including the oxygen electrode, mitochondrial membrane potential and studies of Ca^{2+} transport and ATP generation (Nicholls, 2003). The use of synaptosomal preparations for measurements of oxygen uptake has been reported by several authors (Rodríguez de Lores Arnaiz and Girardi, 1977; Kilbride et al., 2008; Flynn et al., 2011). When using the synaptosomal fraction to measure oxygen uptake, it could be hypothesized that sucrose present in the buffer was metabolized through glycolysis to generate a respiration rate. However, additional assays carried out in the presence of sucrose (without glutamate/malate) showed that oxygen uptake was almost negligible (data not shown).

A simple assay was carried out to test if mitochondria present in our synaptosomal fraction were accessible to the respiratory substrates in the oxygen chamber. Synaptosomes from 3-month-old mice were disrupted by treatment with 0.01% digitonin, centrifuged and resuspended to obtain a synaptic mitochondrial fraction; special attention was followed during the permeabilization procedure, optimizing the digitonin time and concentration effects. We observed that in a particular condition, digitonin-permeabilized synaptosomes present higher respiratory rates than intact synaptosomes, thus suggesting that malate, glutamate and ADP could be more rapidly incorporated to the mitochondria when synaptosomes were previously permeabilized (data not shown). However, considering that our study requires structurally intact mitochondria, we decided to avoid the risk of partial disruption of the outer mitochondrial membrane due to permeabilization procedures.

Synaptosomal and non-synaptic mitochondrial preparations from cerebral cortex were highly metabolically active as indicated by the high rates of oxygen uptake in the presence of ADP. They exhibited high respiratory control ratios (5–7) indicating the presence of a well-preserved inner mitochondrial membrane (Table 1).

State 3 respiratory rate was decreased by 43% in cerebral cortex synaptosomes from 14-month-old mice, as compared with young animals, but was not significantly affected by aging in non-synaptic mitochondria (Table 1). No changes were observed in state 4 respiratory rates in synaptosomes or in non-synaptic mitochondria (Table 1). Respiratory control was significantly decreased in cerebral cortex synaptosomes from 14-month-old mice as compared with young animals (Table 1).

Mitochondrial membrane potential

In this study, the response to calcium-induced MPT was evaluated by mitochondrial membrane potential determinations.

In 14-month-old animals, calcium addition did not disturb $\Delta\Psi$ in non-synaptic mitochondria, but induced a 14% decrease in $DiOC_6$ fluorescence signal of synaptosomal samples (Figs. 1 and 2B). No changes in mitochondrial membrane potential were observed in brain cortex non-synaptic mitochondria or in synaptosomes from 3-month-old mice after 100 μM calcium incubation (Fig. 2A).

The mitochondrial uncoupler FCCP was used as positive control for mitochondrial depolarization. Both in synaptosomes and non-synaptic mitochondria of cerebral cortex from 3- or 14-month-old animals, significant mitochondrial depolarization (45–55%) was observed after FCCP treatment (Fig. 2A, B).

Table 1. Oxygen consumption by cortical non-synaptic (NS) mitochondria or synaptosomes from 3- or 14-mon-old animals

Oxygen uptake (natgO/min mg protein)	NS mitochondria		Synaptosomes	
	3 mon	14 mon	3 mon	14 mon
State 4	5.5±0.1	5.0±0.4	6.5±0.9	5.5±1.2
State 3	42±1	35±3	35±2	20±3*
RCR	7.6±0.8	7.0±0.9	5.4±0.6	3.6±0.5*

Values represent the mean±SEM.
* $P<0.05$.

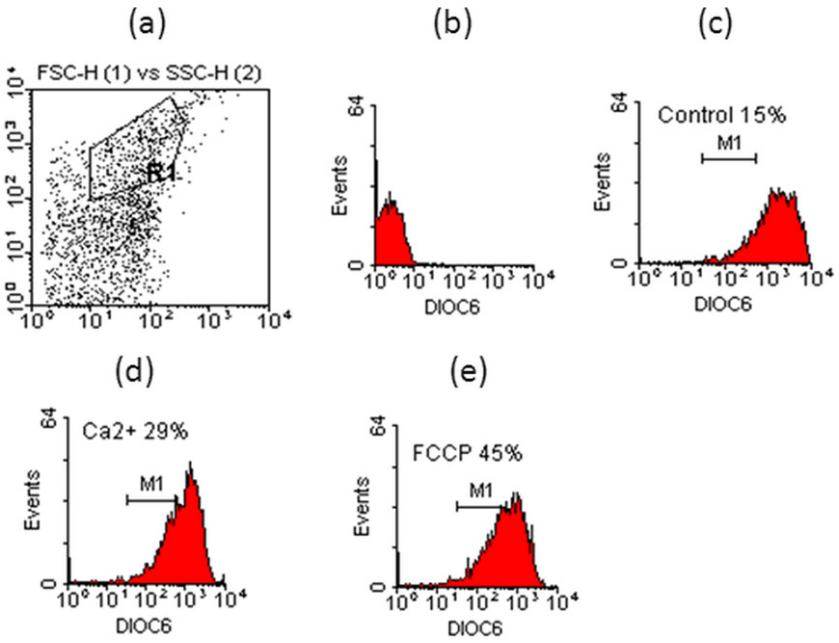

Fig. 1. Histograms of gated events (R1) versus relative $DiOC_6$ fluorescence intensity (FL-1) which indicate mitochondrial membrane potential of cerebral cortex synaptosomes from 14-mon-old animals. (a) Dot blot of FSC-H vs. SSC-H indicating a gated mitochondrial population, (b) unloaded (autofluorescence), (c) control synaptosomes, (d) synaptosomes loaded with 200 nmol Ca^{2+}/mg protein, (e) 0.5 μM FCCP. Each histogram represents a typical experiment, which was performed in triplicate.

H_2O_2 production rate

H_2O_2 production was measured in brain cortex synaptosomes or in non-synaptic mitochondria from 3- or 14-month-old animals, using malate-glutamate as mitochondrial substrates. Control H_2O_2 production rate was approximately 0.19±0.02 nmol/min mg protein for synaptosomes and 0.37±0.03 nmol/min mg protein (Fig. 3) for non-synaptic mitochondria.

Increases of 84% and 38% in H_2O_2 production rates were observed in brain cortex synaptosomes and non-synaptic mitochondria from 14-month-old mice, as compared with young animals (Fig. 3).

Monoamine oxidase activity

MAO activity was determined both in synaptosomal fractions or in non-synaptic submitochondrial membranes from 3- or 14-month-old animals. MAO activity was found unchanged in cerebral cortex from 3- or 14-month-old mice both when measured in synaptosomal samples (Table 2) and in non-synaptic submitochondrial membranes (data not shown).

Acetylcholinesterase activity

Acetylcholinesterase activity was determined in brain cortex synaptosomal fractions from 3- or 14-month-old mice. Enzymatic activity was 159±10 nmol/min mg protein for brain cortex synaptosomal fractions from young animals and was 37% increased in 14-month-old mice (Table 2).

NO production

Control NOS activity associated to cerebral cortex synaptosomal fractions and non-synaptic submitochondrial membranes was 0.9±0.1 nmol/min mg and 0.66±0.05 nmol/min mg, respectively. NOS activity was decreased by 27% and 44% in synaptosomal fractions and non-synaptic submitochondrial membranes from 14-month-old mice, as compared with young animals (Fig. 4).

Respiratory complexes activity

Activity of complex I–III was not affected in non-synaptic submitochondrial membranes from 14-month-old animals, but was significantly increased by 40% in synaptosomal fractions from 14-month-old animals, as compared with 3-month-old mice (Table 3). Activity of complex II–III was 34% and 27% decreased in non-synaptic submitochondrial membranes and in synaptosomal fractions, respectively, in cerebral cortex from 14-month-old animals (Table 3). Complex IV activity was decreased by 25% in cerebral cortex synaptosomes from 14-month-old animals, as compared with young mice, while was unaffected in non-synaptic submitochondrial membranes in the different groups of age.

DISCUSSION

The bioenergetics demands in the pre-synaptic terminal are high, with ATP being required for endo- and exocytosis in addition to maintenance of ion homeostasis. Pre-synaptic mitochondria are constantly exposed to high Ca^{2+} transients associated with neurotransmitter release.

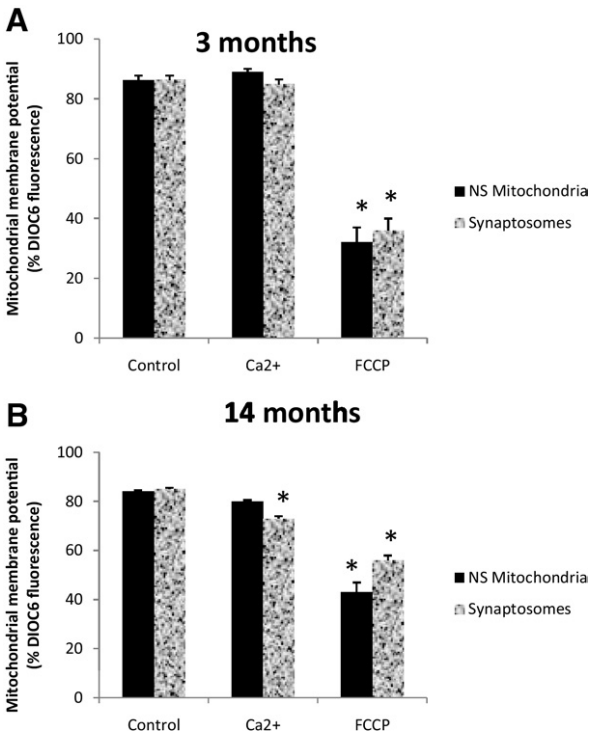

Fig. 2. Bars scheme quantification of mitochondrial membrane potential ($\Delta\Psi_m$) of cerebral cortex synaptosomes and nonsynaptic mitochondria from 3-mon-old animals (A) or 14-mon-old animals (B) in three different experimental conditions: unloaded, loaded with 200 nmol Ca^{2+}/mg protein or with 0.5 μM FCCP. Bars represent mean values±SEM, from three independent experiments. * $P<0.05$.

Similar respiration rates were observed in the non-synaptic and synaptic mitochondria in the different classical states of respiration or in the RCR (Brown et al., 2006). In our study, both populations of mitochondria from 3-month-old animals were well-coupled and bioenergetically active following the isolation procedure. Respiratory controls were higher for non-synaptic mitochondria (7.6±0.8) as compared with synaptosomes (5.4±0.6) from young mice. Malate, glutamate and ADP seem to be more

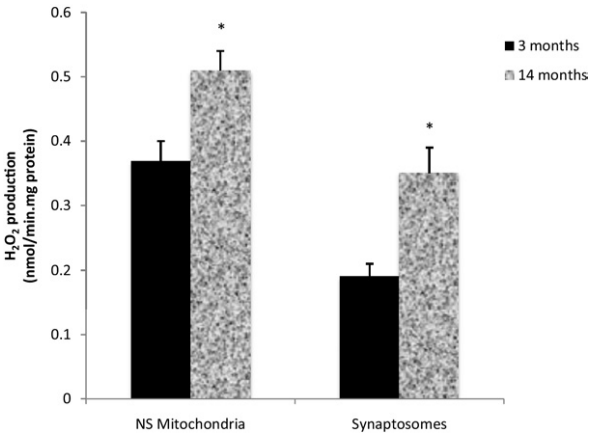

Fig. 3. Bars scheme of H_2O_2 production rate of cerebral cortex synaptosomes and nonsynaptic mitochondria from 3- or 14-mon-old animals. Bars represent mean values±SEM of four to six mice. * $P<0.05$.

Table 2. Enzymatic activity of MAO and acetylcholinesterase (AChE) in cortical synaptosomal membranes from 3- or 14-mon-old animals

Enzyme activity (nmol/min mg protein)	Synaptosomes	
	3 mon	14 mon
MAO	10±2	9±1
AchE	159±10	218±18*

Values represent the mean±SEM.
* $P<0.05$.

rapidly incorporated to the mitochondria when synaptosomes are previously permeabilized with digitonin, as digitonin-permeabilized synaptosomes present higher respiratory rates than intact synaptosomes. However, digitonin permeabilization still represents a high risk of partial disruption of the outer mitochondrial membrane (Anderson and Sims, 2000). Therefore, considering that our study requires structurally intact mitochondria, we decided to avoid permeabilization and report only data from intact synaptosomes.

When comparing respiratory rates for mouse cortical synaptosomes between 3-month-old mice and 14-month-old animals, a significant decrease in state 3 oxygen consumption, with a concomitant decrease in respiratory control was observed, suggesting synaptic dysfunction in the respiratory transport chain with age. Non-synaptic mitochondria, however, seem to be more resistant to age-related impairment, as no significant changes were found in respiratory rates of this mitochondrial population when comparing 3- or 14-month-old animals. It could be speculated that mitochondria present in the terminals require synapse integrity to be capable of exerting its optimal function. The different response of synaptic and non-synaptic mitochondria could be attributed to the fact that, being evaluated in the synaptic bouton, mitochondrial dysfunction inside synaptosomes could probably be the result of synapse degeneration by aging.

High respiratory activity depends on the accurate functioning of mitochondrial respiratory complexes. Our results

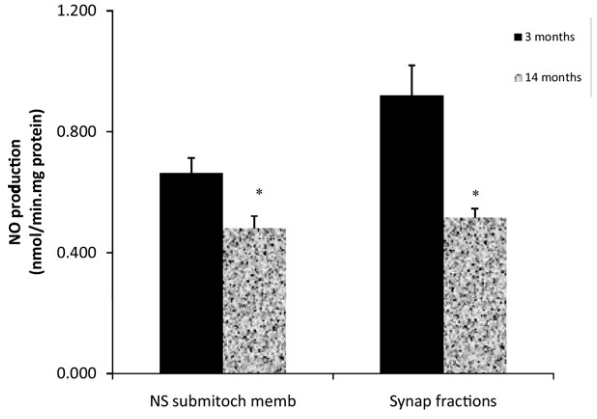

Fig. 4. NO production by nonsynaptic (NS) submitochondrial membranes and synaptosomal fractions of cerebral cortex from 3- or 14-mon-old animals. Bars represent mean values±SEM of four to six mice. * $P<0.05$.

Table 3. Activity of enzyme complexes of mitochondrial respiratory chain in non-synaptic (NS) mitochondria and in synaptosomal preparations from 3- or 14-mon-old animals

	Enzymatic activity (nmol/min mg protein)		
	Complex I–III	Complex II–III	Complex IV
NS mitochondria			
3 mon	132±10	58±1	79±8
14 mon	138±12	38±4*	76±6
Synaptosomes			
3 mon	89±4	26±1	59±4
14 mon	125±8*	19±2*	44±2*

Values represent the mean±SEM.
* $P<0.05$.

show decreased activity of mitochondrial respiratory complexes II–III and IV in synaptosomes from 14-month-old animals, as compared with young animals. This result is in agreement with data from Kilbride et al. showing compromised function of these electron transport chain components in brain synaptosomes from 18-month-old animals, compared to those from 6-month-old rats (Kilbride et al., 2008). Inhibition of complexes II–III and complex IV activities in synaptic mitochondria has been found to reduce oxygen consumption and ATP synthesis by up to 15% in synaptosomal mitochondria (Davey et al., 1998). Studies by Davey et al. (1997) have shown that complex IV activity can be 50–60% reduced before respiration and ATP synthesis are severely compromised in synaptosomal and non-synaptic mitochondria. Non-synaptic mitochondria have similar complex I thresholds between 50% and 60%, but in synaptosomal mitochondria, respiration rates and ATP synthesis start to decrease rapidly when only 25% of complex I activity is inhibited (Davey et al., 1997).

Activity of mitochondrial respiratory complexes I–III was found increased in cerebral cortex synaptosomes from 14-month-old animals as compared with young mice. Accordingly, Manczak et al. (2005) have published evidence that mRNA expression of complex I subunits is increased in middle-aged mice (12 months old). However, other studies have suggested that the aging-associated energy decline could be attributed to somatic mutations of mitochondrial DNA induced by oxygen radicals, being complex I mostly affected and strongly rate limiting for electron transfer (Lenaz et al., 2000). A progressive decrease in enzyme activities of NADH-dehydrogenase and cytochrome oxidase has been reported during aging in whole brain mitochondria (Navarro and Boveris, 2007). Other laboratories have shown a decreased NADH oxidation in brain cortex non-synaptic mitochondrial particles from old rats, while no major changes in NADH oxidation rate were found in synaptic mitochondria from brain cortex (Genova et al., 1997).

Studies by Brown et al. have shown that mitochondria isolated from rat cortical synaptosomes have an increased propensity to undergo MPT in response to added Ca^{2+} as compared to mitochondria isolated from a non-synaptosomal fraction. This difference does not seem to result of differences in mitochondrial bioenergetics or initial Ca^{2+} load, but may reflect the largely neuronal origin of synaptic mitochondria versus the mixed cellular origin of non-synaptic mitochondria, or the different functions of synaptic versus non-synaptic mitochondria (Brown et al., 2006). High levels of cyclophilin D in neurons and synaptic mitochondria and low levels in astrocytes and non-synaptic mitochondria influence both mitochondrial Ca^{2+} buffering capacity and levels of CsA required to inhibit MPT (Brown et al., 2006).

In our study, no significant differences were observed in mitochondrial membrane potential of synaptosomal or non-synaptic mitochondrial preparations, or in the response to calcium overload in young animals. However, in 14-month-old animals, calcium addition induced a decrease in $\Delta\Psi$ of synaptosomal samples. The higher susceptibility of synaptosomes from old animals to undergo mitochondrial depolarization, possibly by MPT induction, could be due to the presence of a higher cyclophilin D content of synaptic mitochondria than in non-synaptic mitochondria (Naga et al., 2007). Differences in $\Delta\Psi$ with aging have been previously reported in different tissues. For instance, Kokoszka et al. (2001) monitored $\Delta\Psi$ in mouse liver mitochondria preparations and reported a 10 mV decrease in mitochondrial membrane potential in the preparation from old mice relative to young and middle-aged animals.

Synaptic mitochondria seem to be more susceptible than non-synaptic mitochondria to suffer age-decline in respiratory function and oxidative damage. At young ages, there are no differences in respiratory functioning between synaptic and non-synaptic mitochondria, indicating that the electron transport chain was well coupled to oxidative phosphorylation allowing the maintenance of normal mitochondrial membrane potential in both populations of mitochondria. However, at middle-age, synaptic mitochondria may handle calcium differently than mitochondria in other regions of neurons and other cell types.

Given the brain's high energy requirements, any decline in brain respiratory function could have a significant impact on brain function (Beal, 1998). The aging process involves increased reactive oxygen species production together with alterations in Ca^{2+} homeostasis and mitochondrial dysfunction. In our study, rates of H_2O_2 production were found increased in non-synaptic mitochondria and synaptosomes from cerebral cortex of 14-month-old animals, as compared with young mice, being synaptosomes more prone to generate active oxygen species with increasing age. MAO activity was found unchanged in both fractions of the two groups of age, suggesting that the H_2O_2 increases came from a mitochondrial origin and are not a consequence of an increased MAO activity. In agreement, Kilbride et al. (2008) have shown increased H_2O_2 production in synaptosomes from 18-month-old animals compared to those from 6-month-old rats, in association with decreased $\Delta\Psi$ and oxygen consumption. Mitochondrial dysfunction during aging was also reported by Petrosillo et al. (2008) showing an age-related decrease in complex I activity which was attributed to ROS-induced cardiolipin oxidation.

Acetylcholinesterase is modulated by the aging process. According to our data, an increased acetylcholinesterase activity was observed in cerebral cortex synaptosomal fractions from 14-month-old mice, as compared with young animals. While it has been established that acetylcholinesterase activity was severely decreased in brain samples from Alzheimer disease patients (Perry et al., 1977), the enzyme was actually increased in the hippocampus and frontal cortex from patients with mild cognitive impairment (DeKosky et al., 2002).

Changes in nitric oxide synthase activity and protein expression have been reported in association to the aging process. Studies of the ultrastructural distribution of NADPH-diaphorase in synaptic structures of hippocampus have shown that the NOS enzyme was predominately attached to membranes of the endoplasmic reticulum, and in synaptic regions mainly the pre-synaptic sides (Faber-Zuschratter et al., 1996). Also, Batista et al. (2001), have reported the subcellular localization of nNOS in the rat superior colliculus, showing nNOS immunoreactivity on the external membrane of mitochondria, endoplasmic reticulum, in pre- and post-synaptic profiles and also diffusely distributed in the cytosol. These data are in agreement with studies by Lacza et al. (2006), suggesting that NOS is not contained within the mitochondrial matrix but attached to the outer membrane, thus solving part of the controversy of its subcellular localization. In this study, decreased generation of NO has been observed both in non-synaptic submitochondrial membranes and in synaptosomal fractions from 14-month-old mice as compared with young animals. The decrease was more marked for synaptosomal samples than for non-synaptic submitochondrial membranes, again giving support to the concept that synaptic mitochondria are more susceptible to suffer aging-associated damage. Nitric oxide is an important messenger in the CNS, playing an important role in synaptic activity, neuronal plasticity and memory formation (Lores-Arnaiz and Bustamante, 2008). A decreased NO generation would lead to impairment of synaptic plasticity and cognition alterations (Lores-Arnaiz et al., 2004b, 2006).

CONCLUSION

According to our results, brain cortex mitochondria from 3-month-old mice are capable of responding to ATP requirements, producing low levels of H_2O_2, having an efficient calcium buffering capacity and generating physiological NO levels. On the contrary, synaptic mitochondria from 14-month-old animals seem to be partially dysfunctional, probably leading to ATP deficiency, increased H_2O_2 generation, alterations of calcium homoestasis and decreased NO levels. Partial energy deficiency could be compensated by an increased complex I–III activity in synaptosomal fractions from 14-month-old animals. Non-synaptic mitochondria would be more resistant to age-related dysfunction and oxidative damage.

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REFERENCES

Anderson MF, Sims NR (2000) Improved recovery of highly enriched mitochondrial fractions from small brain tissue samples. Brain Res Brain Res Protoc 5:95–101.

Basford RE (1967) Preparation and properties of brain mitochondria. Methods Enzymol 10:96–101.

Batista CM, de Paula KC, Cavalcante LA, Mendez-Otero R (2001) Subcellular localization of neuronal nitric oxide synthase in the superficial gray layer of the rat superior colliculus. Neurosci Res 41:67–70.

Beal MF (1998) Mitochondrial dysfunction in neurodegenerative diseases. Biochim Biophys Acta 1366:211–223.

Bernardi P (1999) Mitochondrial transport of cations: channels, exchangers, and permeability transition. Physiol Rev 79:1127–1155.

Boveris A (1984) Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. Methods Enzymol 105:429–435.

Boveris A, Lores-Arnaiz S, Bustamante J, Alvarez S, Valdez L, Boveris AD, Navarro A (2002) Pharmacological regulation of mitochondrial nitric oxide synthase. Methods Enzymol 359:328–339.

Brown MR, Sullivan PG, Geddes JW (2006) Synaptic mitochondria are more susceptible to Ca^{2+}overload than nonsynaptic mitochondria. J Biol Chem 281:11658–11668.

Clark JB, Nicklas WJ (1970) The metabolism of rat brain mitochondria. Preparation and characterization. J Biol Chem 245:4724–4731.

Davey GP, Canevari L, Clark JB (1997) Threshold effects in synaptosomal and nonsynaptic mitochondria from hippocampal CA1 and paramedian neocortex brain regions. J Neurochem 69:2564–2570.

Davey GP, Peuchen S, Clark JB (1998) Energy thresholds in brain mitochondria. Potential involvement in neurodegeneration. J Biol Chem 273:12753–12757.

DeKosky ST, Ikonomovic MD, Styren SD, Beckett L, Wisniewski S, Bennett DA, Cochran EJ, Kordower JH, Mufson EJ (2002) Upregulation of choline acetyltransferase activity in hippocampus and frontal cortex of elderly subjects with mild cognitive impairment. Ann Neurol 51:145–155.

Dubinsky JM (2009) Heterogeneity of nervous system mitochondria: location, location, location! Exp Neurol 218:293–307.

Dunkley PR, Jarvie PE, Robinson PJ (2008) A rapid Percoll gradient procedure for preparation of synaptosomes. Nat Protoc 3:1718–1728.

Ellman GL, Courtney KD, Andres V Jr, Feather-Stone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95.

Estabrook R (1967) Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. Methods Enzymol 10: 41–47.

Faber-Zuschratter H, Seidenbecher T, Reymann K, Wolf G (1996) Ultrastructural distribution of NADPH-diaphorase in the normal hippocampus and after long-term potentiation. J Neural Transm 103:807–817.

Flynn JM, Choi SW, Day NU, Gerencser AA, Hubbard A, Melov S (2011) Impaired spare respiratory capacity in cortical synaptosomes from Sod2 null mice. Free Radic Biol Med 50:866–873.

Genova ML, Bovina C, Marchetti M, Pallotti F, Tietz C, Biagini G, Pugnaloni A, Viticchi C, Gorini A, Villa RF, Lenaz G (1997) Decrease of rotenone inhibition is a sensitive parameter of complex I damage in brain non-synaptic mitochondria of aged rats. FEBS Lett 410:467–469.

Kilbride SM, Telford JE, Davey GP (2008) Age-related changes in H_2O_2 production and bioenergetics in rat brain synaptosomes. Biochim Biophys Acta 1777:783–788.

Kokoszka JE, Coskun P, Esposito LA, Wallace DC (2001) Increased mitochondrial oxidative stress in the Sod2 (+/−) mouse results in the age-related decline of mitochondrial function culminating in increased apoptosis. Proc Natl Acad Sci U S A 98:2278–2283.

Lacza Z, Pankotai E, Csordás A, Gero D, Kiss L, Horváth EM, Kollai M, Busija DW, Szabó C (2006) Mitochondrial NO and reactive nitrogen species production: does mtNOS exist? Nitric Oxide 14: 162–168.

Lenaz G, D'Aurelio M, Merlo Pich M, Genova ML, Ventura B, Bovina C, Formiggini G, ParentiCastelli G (2000) Mitochondrial bioenergetics in aging. Biochim Biophys Acta 1459:397–404.

Lores-Arnaiz S, D'Amico G, Czerniczyniec A, Bustamante J, Boveris A (2004a) Brain mitochondrial nitric oxide synthase: *in vitro* and *in vivo* inhibition by chlorpromazine. Arch Biochem Biophys 430: 170–177.

Lores-Arnaiz S, D'Amico G, Paglia N, Arismendi M, Basso N, Arnaiz L (2004b) Enriched environment, nitric oxide production and synaptic plasticity prevent the aging-dependent impairment of spatial cognition. Molec Aspects Med 25:91–101.

Lores-Arnaiz S, Bustamante J, Arismendi M, Vilas S, Paglia N, Basso N, Capani F, Coirini H, López Costa JJ, LoresArnaiz MR (2006) Extensive enriched environments prevent old rats from the aging dependent impairment of spatial cognition, synaptic plasticity and nitric oxide production. Behav Brain Res 169:294–302.

Lores-Arnaiz S, Bustamante J (2008) Nitric oxide and mitochondrial function in neuronal aging and plasticity. In: Free radical pathophysiology, Section: free radicals in neurodegeneration (Alvarez S, Evelson P, Boveris A, eds), pp 329–343. Trivandrum, India: Research Signpost.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Manczak M, Jung Y, Park BS, Partovi D, Reddy PH (2005) Time-course of mitochondrial gene expressions in mice brains: implications for mitochondrial dysfunction, oxidative damage, and cytochrome c in aging. J Neurochem 92:494–504.

Naga KK, Sullivan PG, Geddes JW (2007) High cyclophilin D content of synaptic mitochondria results in increased vulnerability to permeability transition. J Neurosci 27:7469–7475.

Navarro A, Boveris A (2007) Brain mitochondrial dysfunction in aging: conditions that improve survival, neurological performance and mitochondrial function. Front Biosci 12:1154–1163.

Nicholls DG (2003) Bioenergetics and transmitter release in the isolated nerve terminal. Neurochem Res 28:1433–1441.

Parihar MS, Brewer GJ (2007) Mitoenergetic failure in Alzheimer disease. Am J Physiol Cell Physiol 292:8–23.

Perry EK, Gibson PH, Blessed G, Perry RH, Tomlinson BE (1977) Neurotransmitter enzyme abnormalities in senile dementia. Choline acetyltransferase and glutamic acid decarboxylase activities in necropsy brain tissue. J Neurol Sci 34:247–265.

Petrosillo G, Matera M, Casanova G, Ruggiero FM, Paradies G (2008) Mitochondrial dysfunction in rat brain with aging Involvement of complex I, reactive oxygen species and cardiolipin. Neurochem Int 53:126–131.

Reddy PH, Beal MF (2008) Amyloid beta, mitochondrial dysfunction and synaptic damage: implications for cognitive decline in aging and Alzheimer's disease. Trends Mol Med 14:45–53.

Rodríguez de Lores Arnaiz G, Girardi E (1977) The increase in respiratory capacity of brain subcellular fractions after the administration of the convulsant 3-mercaptopropionic acid. Life Sci 21:637–646.

Sastre J, Pallardó FV, García de la Asunción J, Viña J (2000) Mitochondria, oxidative stress and aging. Free Radic Res 32:189–198.

Weissbach H, Smith TE, Daly JW, Witkop B, Udenfriend S (1960) A rapid spectrophotometric assay of mono-amine oxidase based on the rate of disappearance of kynuramine. J Biol Chem 235: 1160–1163.

Yonetani T (1967) Cytochrome oxidase: beef heart. Methods Enzymol 10:332–335.

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