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Age and Sex-Related Changes in Rat Brain Mitochondrial Function

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Key Words

Aging • Sex differences • Mitochondrial function • Brain

Abstract

Aging is responsible for the decline in the function of mitochondria and their increase in size and number - adaptive mechanism to restore mitochondrial function. Estrogens increase mitochondrial function, especially in female rats. The aim of this study was to determine the age-related changes in rat brain mitochondrial function focusing on sex differences. Cellular and mitochondrial protein and DNA content, mitochondrial oxidative and phosphorylative function in male and female rat brain from four different age groups (6, 12, 18 and 24 months old) were analyzed. Mitochondria protein/DNA content decreased with aging shifting toward lesser mitochondrial functional capacity and the mitochondria number increased. A sex dimorphism was determined, with female rat brain showing mitochondria with greater functional capacity than males. These sex differences gradually increased during aging.

Introduction

Aging is characterized by a general decline of physiological function, determined by cellular dysfunction and mitochondria impairment, with a more marked effect in the functions that depend on the central nervous system [1, 2]. Many studies have reported a decline in mitochondrial function upon aging consisting of a decrease in electron transfer activity and ATP production [2, 3] whereas other authors have described an increase of mtDNA copy number in senescent tissues [4] and age-dependent increased mitochondrial size [5], supporting the idea of an adaptive mechanism designed to restore mitochondrial function.

Brain has a very high metabolic rate, as it consumes about 20% of the oxygen inspired at rest, while accounting for only 0.5-2% of the body weight [6]. This high metabolic demand is because neurons are highly differentiated cells needing large amounts of ATP for maintenance of ionic gradients across the cell membranes and for neurotransmission. Since most neuronal ATP is generated by oxidative metabolism, neurons critically depend on mitochondrial function and oxygen supply [7, 8].

Sex hormones, specifically estrogens, have profound neuro-protective effects, having been reported to increase

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mitochondrial respiration efficiency and mitochondrial functions [9]. Several studies have reported that female rats show more highly differentiated mitochondria than males in liver [10, 11], brown adipose tissue [12-14], cardiac [15] and skeletal muscle [16], and aged brain [17], meaning greater mitochondrial machinery and, as a result, higher capacity and efficiency.

Taking all these precedents into account, the aim of this study was to determine the age-related changes in rat brain mitochondrial function focusing on sex differences. To tackle this aim we analyzed both cellular and mitochondrial protein and DNA content, and mitochondrial oxidative and phosphorylative function in male and female rat brain from four different age groups: 6, 12, 18 and 24 months old.

Materials and Methods

Chemicals

Routine chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA), Roche Diagnostics (Basel, Switzerland), Bio-Rad (Hercules, CA, USA) and Panreac (Barcelona, Spain). Antibodies against COX IV subunit and COX II subunit were obtained from MitoSciences (Eugene, OR, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA) respectively.

Animals, sacrifice and mitochondrial isolation

Animals were treated in accordance with the University Bioethical Committee guidelines for animal care and European Union regulations (2003/65/CE).

Wistar rats, supplied by Charles River (Barcelona, Spain), were housed individually at 22 °C with a 12-h light/dark cycle and fed *ad libitum* with pelleted standard diet (A04 Panlab, Barcelona, Spain). Eight groups were made: males and females of four different ages, 6 months old (youngest rats), 12 months old (adult rats), 18 months old (old rats) and 24 months old (senescent rats), including 6-8 animals in each group.

All animals were weighed and sacrificed by decapitation at the start of the light cycle. After sacrifice animals underwent a visual internal inspection in order to exclude old animals with any pathological signal. Brain was quickly removed and weighed. Homogenization and mitochondrial isolation were performed according to previously described procedure [18]. Briefly, brain was homogenized in 35 mL of cold isolation medium (0.25 M sucrose, 10 mM tris-HCl and 0.5 mM EDTA-K^+, pH 7.4) in a manual homogenizer with a glass pestle, total clearance 0.1 mm. An aliquot of the homogenate was stored at -20° C for posterior analyses and the remainder was used to perform biochemical assays and for mitochondrial isolation. Nuclei and cell debris were twice sedimented by differential centrifugation at 2000xg for 3 min and the pellets were discarded. Supernatant was subjected to a further centrifugation at 12500xg for 8 min and the crude mitochondrial pellet obtained was resuspended with 0.8 mL of isolation medium. Protein concentration was determined in both homogenate and mitochondrial fraction by Bradford's method [19]. Total DNA content was tested in homogenate as previously described [20].

Quantification of mitochondrial DNA by real-time PCR

Mitochondrial DNA (mtDNA) extraction and semiquantification were carried out as previously described [21]. Briefly, real-time PCR was performed to amplify a 162-nt region of the mitochondrial NADH dehydrogenase subunit 4 gene, which is exclusive to mtDNA. Amplification was carried out in a LightCycler rapid thermal cycler system (Roche) using a total volume of 10 µl containing each primer at 0.375 µM, 3 mM $MgCl_2$, 1 µl LightCycler FastStart DNA Master SYBR Green I, and 2.5 µl of pretreated mitochondrial sample. The PCRs were cycled 35 times after initial denaturation (95°C, 10 min). The primer sequences were 5'-TAC ACG ATG AGG CAA CCA AA-3' and 5'-GGT AGG GGG TGT GTT GTG AG3'.

Enzymatic activities

Citrate synthase (CS, EC 2.3.3.1) [22] and ATP synthase (ATPase, EC 3.6.1.3) [23] activities were determined in resuspended mitochondrial pellet. Ferrocytochrome-c:oxygen oxidoreductase (COX, EC 1.9.3.1) [24] activity was measured in brain homogenate.

Mitochondrial oxygen consumption

Oxygen consumption of brain mitochondria was measured by a thermostatically controlled Clark-type O_2 electrode (Oxygraph, Hansatech, UK). Reaction conditions were 1 mg of mitochondrial protein in up to 0.5 mL of respiration buffer (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA and 0.1% BSA fatty acid free, pH 7.4) at 37º C. The substrate used was pyruvate/malate (5 mM and 2.5 mM respectively) in the presence (State 3, phosphorylating) and absence (State 4, resting) of 500 µM ADP. Mitochondrial viability was checked by the respiratory control rate (RCR = state 3/ state 4).

Western blot of COX IV subunit and COX II subunit

10 µg of total protein in the case of COX IV and 40 µg in the case of COX II were electrophoresed on 15% v/v polyacrilamide SDS-PAGE gels. Proteins were electrotransferred onto a nitrocellulose membrane and Ponceau S staining was performed to check the correct loading and electrophoretic transfer. Membranes were incubated with the corresponding antibodies against COX IV subunit and COX II subunit; immunoreactive bands were developed by chemiluminescence, detected by a ChemiDoc XRS system (BIO-Rad, CA, USA) and quantified by the image analysis program Quantity One© (BIO-Rad, CA, USA). Apparent molecular weights of proteins were estimated using a protein molecular-mass standard.

Statistics

All statistical analyses were performed with the Statistical Program for Social Sciences software SPSS version 17.0 for Windows (Chicago, IL, USA). Data are expressed as mean ±

Table 1. Biometric parameters and brain protein and DNA content in male and female rats during aging process. Values are mean ± SEM; n = 6 - 8 animals per group. ANOVA ($p<0.05$). A: age effect; S: sex effect; AxS: age and sex interaction; NS: no significant differences. Student's t-test post hoc analysis ($p<0.05$). a 12-mo old vs. 6-mo old; b 18-mo old vs. 6-mo old; c 24-mo old vs. 6-mo old; d 18-mo old vs. 12-mo old; e 24-mo old vs. 12-mo old; f 24-mo old vs. 18-mo old. * females vs. males. M: males; F: females.

		6 months	12 months	18 months	24 months	ANOVA
Body weight (g)	M	484 ± 13	601 ± 15[a]	579 ± 17[b]	707 ± 32[cef]	A, S, AxS
	F	264 ± 6*	310 ± 9*[a]	353 ± 14*[bd]	396 ± 16*[cef]	
Brain weight (g)	M	2.00 ± 0.04	2.15 ± 0.01	2.11 ± 0.03	2.20 ± 0.05	A_{abc}, S
	F	1.82 ± 0.02*	1.95 ± 0.02*	2.02 ± 0.02*	2.01 ± 0.04*	
Brain weight to body weight (%)	M	0.408± 0.002	0.359± 0.009[a]	0.366± 0.013[b]	0.314 ± 0.013[cef]	A, S, AxS
	F	0.691± 0.013*	0.633± 0.014*[a]	0.578± 0.028*[bd]	0.510 ± 0.016*[cef]	
Brain total protein (mg/g tissue)	M	51.8 ± 1.1	52.4 ± 0.3	55.4 ± 0.9	53.3 ± 3.0	A_{bcef}, AxS
	F	52.0 ± 0.6	52.8 ± 1.4	54.2 ± 1.2	61.2 ± 2.6*	
Brain total DNA (mg/g tissue)	M	1.00 ± 0.03	1.09 ± 0.05	1.13 ± 0.05	0.69 ± 0.04	A_{cef}, AxS
	F	1.15 ± 0.05*	1.08 ± 0.04	1.03 ± 0.04	0.61 ± 0.01	
Total protein/total DNA (mg/mg)	M	51.9 ± 1.2	48.6 ± 2.2	49.4 ± 2.2	78.4 ± 7.2[cef]	A, S, AxS
	F	46.7 ± 1.0	49.2 ± 2.6	53.2 ± 2.1[b]	100.8 ± 3.0*[cef]	

Table 2. Mitochondrial DNA, protein and citrate synthase in male and female rat brain during aging process. Values are mean ± SEM; n = 6-8 animals per group. ANOVA ($p<0.05$): A: age effect; S: gender effect; AxS: age and gender interaction; NS: no significant differences. Post hoc analysis (LSD test, $p<0.05$): a 6-mo old vs. 12-mo old; b 6-mo old vs. 18-mo old; c 6-mo old vs. 24-mo old; d 12-mo old vs. 18-mo old; e 12-mo old vs. 24-mo old; f 18-mo old vs. 24-mo old. Post hoc analysis (Student's t-test, $p<0.05$): * females vs. males. M: males; F: females. a.u., arbitrary units; levels found in 6-month-old male rats were set as 100%. mt DNA: mitochondrial DNA; mt protein: mitochondrial protein.

		6 months	12 months	18 months	24 months	ANOVA
mtDNA (a.u./g tissue)	M	100 ± 6	149 ± 9	188 ± 23	247 ± 17	A_{abcdef}, S
	F	65 ± 8*	132 ± 3	143 ± 23	219 ± 16	
mtDNA/totalDNA (a.u./ug)	M	12.9 ± 0.9	17.4 ± 0.8[a]	21.6 ± 2.9[bd]	46.1 ± 4.4[e]	A_{abcef}
	F	7.4 ± 0.9*	15.7 ± 0.8	18.0 ± 3.1[bd]	46.1 ± 3.2[ce]	
mt protein (mg/g tissue)	M	28.7 ± 1.6	34.4 ± 1.3	39.2 ± 1.3	42.1 ± 3.0	A_{abcdef}, S
	F	29.5 ± 1.2	40.7 ± 1.7*	42.6 ± 2.9	49.2 ± 0.9*	
mt protein/mtDNA (mg/a.u.)	M	2.32 ± 0.19	1.74 ± 0.13	1.82 ± 0.19	1.23 ± 0.06	A_{abcef}, S
	F	3.88 ± 0.43*	2.59 ± 0.21*	2.55 ± 0.48	1.71 ± 0.20*	
Citrate synthase activity (mIU/g tissue)	M	63.8 ± 3.1	58.2 ± 2.2	84.6 ± 3.7[bd]	94.1 ± 8.6[ce]	A, S, AxS
	F	59.4 ± 2.6	69.7 ± 2.7*[a]	86.3 ± 5.0[bd]	113.0 ± 6.2[cef]	

SEM. Differences between groups were assessed by two-way analysis of variance (ANOVA) to determine the effect of age and sex, and Student's t-test for post-hoc comparisons. Statistical significance was set at $p<0.05$.

Results and Discussion

Body and brain weight and brain DNA and protein content

Table 1 shows biometric parameters and protein and DNA content in male and female rat brain during aging process (6, 12, 18 and 24-mo old rats). Body and brain weights gradually increased with age, but were always higher in male than in female rats. A slight increase with age until 18-mo was observed in brain total protein content in both genders and a marked sex dimorphism, with an increase in females and no change in males between 18-mo to 24-mo, was detected. Total DNA content in rat brain presented no changes until 18-mo old and a decrease at 24-mo, similar to previously published results [25]. This decrease was more evident in female rats than in males (-47% females and -31% in males); suggesting that the decrease in brain cell number was accompanied by a slight, gradual increase in protein content from 6-mo to 18-mo old rats in both sexes, and showing a marked increase in 24-mo only in females. The total protein/total DNA ratio reflected this pattern and showed a sex dimorphism, with a greater increase in brain cell protein density in females compared to males at senescence (+116% females vs. +51% males).

Mitochondrial DNA, protein and citrate synthase

mtDNA content (Table 2), a parameter that could reflect the mitochondria number [26, 27], showed a marked, yet gradual increase from 6-mo to 24-mo of age, which was higher in females than males (+237% females vs. +147% males). An increase in the amount of mtDNA with age has previously been described in other tissues such us liver [28], brown adipose tissue [29], heart [4], spleen and kidney [30]. The mtDNA/total DNA ratio, which reflects the mitochondrial content per cell, showed a sharp increase, more marked from old age to senescence. This pattern is more evident in females than in male rats (6.3 vs. 3.6 fold respectively, when 6-mo to 24-mo ages were compared). Mitochondrial protein, indica-

Table 3. Mitochondrial oxidative and phosphorylative function in male and female rat brain during aging process. Values are mean ± SEM; n = 6-8 animals per Group. ANOVA ($p<0.05$): A: age effect; S: gender effect; AxS: age and gender interaction; NS: no significant differences. Post hoc analysis (LSD test, $p<0.05$): a 6-mo old vs. 12-mo old; b 6-mo old vs. 18-mo old; c 6-mo old vs. 24-mo old; d 12-mo old vs. 18-mo old; e 12-mo old vs. 24-mo old; f 18-mo old vs. 24-mo old. Post hoc analysis (Student's t-test, $p<0.05$): * females vs. males. M: males; F: females. a.u., arbitrary units; levels found in 6-month-old male rats were set as 100%. COX: ferrocytochrome-c:oxygen oxidoreductase; ATPase: ATP synthase. S3: respiration state 3; S4: respiration state 4.

		6 months	12 months	18 months	24 months	ANOVA
COX activity (a.u./g tissue)	M	100 ± 8	100 ± 1	97 ± 2	77 ± 17	S
	F	143 ± 8*	122 ± 7*	118 ± 2*	131± 12*	
ATPase activity (IU/g tissue)	M	7.78± 0.44	7.44± 0.53	10.07 ± 0.53	13.26 ± 0.97	A_{bcdef}, S
	F	7.70± 0.32	9.50± 0.53*	10.85 ± 0.98	16.30 ± 0.73*	
O2 consumption S3 (nmol/min·g tissue)	M	201 ± 16	241 ± 16	272 ± 25	360 ± 40	A_{abcef}
	F	213 ± 13	271 ± 15	257 ± 8	385 ± 22	
O2 consumption S4 (nmol/min·g tissue)	M	32.7± 1.4	39.3± 4.0	42.9 ± 3.2	53.4 ± 4.7	A_{abcef}
	F	34.6± 2.1	43.2± 4.0	43.0 ± 0.6	57.3 ± 6.6	
RCR	M	5.83± 0.17	6.00± 0.56	6.63 ± 0.38	6.41 ± 0.21	NS
	F	5.84± 0.18	6.20± 0.61	5.89 ± 0.20	5.92 ± 0.02	

tor of mitochondrial functionality, underwent a progressive increase with age in both sexes and this increase was again greater in females than in males, reaching sex differences at 24-mo old. These results highlight the fact that the increase in brain mitochondrial protein content rose at a lower rate than the mtDNA content did, thus, the mitochondrial protein/mtDNA ratio showed that protein content per mitochondrion decreased with age in both sexes, with females showing higher protein content per mitochondrion at any age, indicative of a sex dimorphism. Likewise, citrate synthase – a Krebs cycle key enzyme - activity was also increased during aging with a similar profile to mitochondrial protein, again showing a sex dimorphism, with a greater increase in females with respect to males (+90% females vs. +48% males). Considering mitochondrial differentiation as an increase in the functional capabilities and integrity of pre-existing mitochondria in order to acquire the ultrastructure and functional features of mature mitochondria [27, 31], the mitochondrial protein/mtDNA ratio, which reflects the mitochondrial machinery per mitochondria [32, 33], could be taken as a good mitochondrial differentiation marker. Our results show that rat brain mitochondria lost functional capacity with age in both sexes, especially from old age; however, females maintained mitochondrial functionality with a greater degree of differentiation than males at any age.

Mitochondrial oxidative and phosphorylative function

Indicative parameters of mitochondrial oxidative and phosphorylative function in both sexes during aging are shown in Table 3. These data are expressed per gram of tissue to determine the total functional capacity of the studied animal brain. COX activity was measured to assess the oxidative mitochondrial capacity, since it catalyzes a rate-controlling step of mitochondrial respiration. Rat brain COX activity was maintained with age in both male and female rats; however, female COX activity was higher than that of males at any analysed age. Other authors report a decline of COX activity in old rats but only in males [34]. ATPase activity was increased with age in both sexes, showing the same sex-dependent difference as the other mitochondrial parameters analyzed, that is, the increase was again more marked in female rat brain than in males, reaching higher levels at 24-mo of age. Oxygen consumption using pyruvate/malate as substrate, both in the presence (State 3) and the absence (State 4) of ADP, showed a gradual increase during aging, with similar levels between sexes.

These mitochondrial function results are in agreement with the aforementioned increase in mitochondrial protein with age in both sexes, showing only a clear sex dimorphism in ATPase activity of the oldest rats. On the other hand, COX activity showed a different pattern, maintaining its levels during aging; nevertheless, the sex dimorphism was present at all ages.

COX content

Because of the different pattern followed by COX activity compared with the other mitochondrial features and taking into account that enzymatic activity can be related to enzymatic content, we decided to quantify the protein levels of the nuclear DNA (nDNA)-encoded COX IV and the mtDNA-encoded COX II subunits by western blot (Fig. 1). COX IV subunit content remained practically unchanged with age in both sexes, whereas COX II subunit content underwent a pronounced increase with age, greater in females than in males, leading to a marked difference between sexes at the end of the studied period. In a given cell or tissue, the total mitochondrial function, depending on mitochondrial biogenesis, is the result

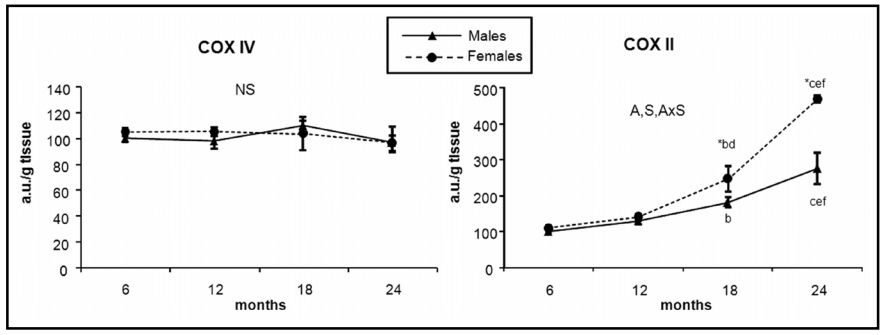

Fig. 1. Protein content of COX IV subunit and COX II subunit in male and female rat brain during aging process. Values are mean ± SEM; n = 6-8 animals per group. ANOVA ($p<0.05$): A: age effect; S: gender effect; AxS: age and gender interaction; NS: no significant differences. Post hoc analysis (LSD test, $p<0.05$): a 6-mo old vs. 12-mo old; b 6-mo old vs. 18-mo old; c 6-mo old vs. 24-mo old; d 12-mo old vs. 18-mo old; e 12-mo old vs. 24-mo old; f 18-mo old vs. 24-mo old. Post hoc analysis (Student's t-test, $p<0.05$): * females vs. males. a.u., arbitrary units; levels found in 6-month-old male rats were set as 100%.

of both the proliferation process, which defines mitochondrial population, and the differentiation process, which defines mitochondrial functional capabilities [10, 35]. Mitochondrial protein content is the result of the coordinated expression of both mtDNA and nDNA genes [36]. In this context, our results could indicate that although the mtDNA-encoded COX II subunit content increased with age in parallel to the increase in mtDNA, the nDNA-encoded COX IV subunit content remained unchanged with age, which agrees with the unchanged profile of COX activity.

However, the sex dimorphism in COX activity, with higher levels in females with respect to males could not be explained by COX IV subunit content since they were very similar in both sexes. This fact could suggest a COX activity up-regulation in female rat brain, which could be related to the different hormonal milieu as has been described in other tissues [11, 16]. In this sense, 17β-estradiol and progesterone have been proved to be involved in the enhancement of both the function [37] and expression of nuclear and mitochondrial genes encoding proteins from the mitochondrial respiratory chain [38]. Although sex hormone levels, which show a marked sex dimorphism, have been described extensively as declining toward the end of life, males and females still had higher levels of corresponding sex-hormones than the other sex at senescence [39]. This could explain, at least in part, the sex-dependent differences found in some mitochondrial features and function.

In summary, this study has demonstrated that rat brain mitochondria decreased the protein/DNA content per mitochondrion during aging, especially between old age and senescence, indicating a shift toward mitochondria with lower functional capacity linked with age. As in other tissues, a sex dimorphism in mitochondrial function has been determined, with female rat brain showing more highly differentiated or maturated mitochondria than male ones. These sex differences gradually increased during aging, which could lead to lesser mitochondrial dysfunction induced by aging of female rat brain.

Abbreviations

mtDNA (Mitochondrial DNA); nDNA (Nuclear DNA); CS (Citrate synthase); COX (Ferrocytochrome-c:oxygen oxidoreductase); ATPase (ATP synthase); state 3 (Active respiration); State 4 (Resting respiration).

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