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Age-Related Variations of the Brain Mitochondrial Permeability Transition Pore and Complex I in Response to Hypoxia

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ABSTRACT

The opening of the mitochondrial permeability transition pore (mPTP) is thought to be one of the processes leading to cell death during ischemia, while the regulation of opening of the mPTP during development and aging remain obscure. In this study, we compared the effects of *in vitro* 90 min hypoxia on mPTP sensitivity to Ca^{2+} mitochondrial respiration, and infarct zone size in 7 day old, 2-3, 8-10 and 24-26 months old rat brains with a particular focus on mitochondrial respiratory chain complex I. Expression of the mitochondrial complex I subunit NDUFS2 (NADH dehydrogenase [ubiquinone] iron-sulfur protein 2) was only lower in 24-26 months rats, whereas mitochondrial complex I activity was the lowest in 7 days old animals and increased with age. While, after hypoxia, complex I activity decreased in all age groups except the 7 days old rats. In comparison to other age groups, the cerebellar mitochondria of 2-3 months old rats were more sensitive to Ca^{2+} induced mPTP, but hypoxia had no effect on it. Mitochondria isolated from cerebellar of 8-10 and 24-26 months and cortex of all rats age groups demonstrated an increase in mPTP sensitivity to Ca^{2+} after hypoxia. Though, the content of cyclophilin D (CypD) in brain mitochondria remains consistent throughout the rat's lifespan. In all age groups of animals, hypoxia decreased cortex and cerebellum mitochondrial respiration and led to necrosis. These data indicate that despite age-related variations in mPTP sensitivity to Ca^{2+}, hypoxia-induced acute mitochondrial dysfunction and brain necrosis are not likely mediated by mPTP opening.

Keywords: Brain Ischemia; Aging; Mitochondrial Permeability Transition; Complex I

Abbreviations: mPTP: Mitochondrial Permeability Transition Pore; CypD: Cyclophilin D; NDUFS2: Structural Subunit of Complex I - Nadh Dehydrogenase [Ubiquinone] Iron-Sulfur Protein 2; VDAC1: Voltage-Dependent Anion Channel 1; CRC: Mitochondrial Calcium Retention Capacity; LEAK: Mitochondrial Proton Leak Respiration; OXPHOS: Mitochondrial Oxidative Phosphorylation Respiration; TTC: 2,3,5- Triphenyl Tetrazolium Chloride

Introduction

Aging is a major risk factor for ischemic diseases, including ischemic stroke [1]. Nevertheless, understanding of the molecular basis of age-associated differences in the pathogenesis of this disease is still limited. There are evidences suggesting that mitochondrial permeability transition pore (mPTP) is one of the factors involved in the progress of ischemic brain injury [2,3], though many aspects of mPTP regulation, and even structure are still under debate [4]. Blockage of blood supply to the brain results inhibition of mitochondrial respiration and ATP depletion leading to disruption of ion homeostasis and induces excessive release of glutamate. The release of glutamate stimulates specific receptors, and this results in an excessive influx of Ca^{2+} into cells that may lead to the so called excitotoxic neuronal death (for the review see [5]). Excessive intracellular Ca^{2+} concentration is a trigger for the opening of mPTP [6,7]. Prolonged opening of mPTP has been suggested to contribute to cell death after ischemia as it causes mitochondrial swelling which leads to rupture of the outer membrane and release of cytochrome c and other proapoptotic factors into the cytosol [8-12]. In addition, opening of the mPTP may

abolish mitochondrial membrane potential, leading to the cessation of ATP synthesis and eventually necrotic cell death [13]. The structure of mPTP is still under debate, with the only one experimentally confirmed component being cyclophilin D (CypD), which is located in the mitochondrial matrix [4,14].

The role of other possible components of mPTP, such as adenine nucleotide translocator (ANT) [4,15-17], phosphate carrier [18,19] and ATP-synthase [4,20-22], are still controversial. It is generally agreed that cyclophilin D (encoded by the Ppif gene) is one of the main regulators of mPTP opening [23], and cyclosporin A (CsA), which increases the amount of Ca^{2+} required to open mPTP by interacting with CypD [24], is the most prominent pharmacological inhibitor of mPTP opening. Various *in vitro* and *in vivo* animal studies have shown that CsA or genetic depletion of CypD desensitizes mitochondria to Ca^{2+} and protects against ischemic brain damage [25-27]. Nevertheless, administration of CsA in clinical trials with stroke patients proved to be ineffective [28,29]. It must be emphasized that in preclinical studies, the effects of CsA were investigated in young adult animals [30-33], while stroke most commonly occurs in advanced-age patients. On the other hand, increasing experimental evidence suggests that regulation of mPTP is more complex and may also involve mitochondrial respiratory complex I in addition to CypD [34,35]. It has been shown that some ubiquinone derivatives [4], as well as rotenone (a selective inhibitor of complex I), increase the amount of Ca^{2+} needed to open mPTP, thus acting as inhibitors of mPTP [35]. Furthermore, some biguanides (metformin, phenformin) that inhibit complex I have been shown to be able to reduce ischemic brain injury in rats [36-39].

It was also suggested that the involvement of complex I and CypD in mPTP regulation may differ among tissues [34] and may depend on the content of these proteins in mitochondria [40,41]. The interaction between complex I and mPTP has also been proposed to mediate brain injury in hypoxia/hyperoxia-reperfusion experiments using 10-day old mice [42,43]. Multiple studies have shown that aging affects mitochondrial metabolism and Ca^{2+} retention capacity [44-46], which may alter the response of mitochondrial enzymes to hypoxia. However, most of the research on hypoxia-induced brain damage is performed on young animals, prenatal or neonatal primary cell cultures, or age-unrelated cell lines. Despite the fact that stroke and other brain ischemia-associated pathologies usually affect aged individuals, there is very little information about the effects of aging on mPTP regulation and involvement in hypoxia/ischemia-induced damage. In this context, it is important to note that the content of components of the respiratory complexes and other proteins in mitochondria changes during development and aging [47]. Studies show that aging has an impact on mitochondrial energy production [46,48], management of intracellular calcium homeostasis [49], and sensitivity of mPTP to Ca^{2+} [50]. It has also been proposed that changes in the assembly and functional activity of mitochondrial complex I contribute to aging [51,52]. There is very little information on whether such age-related differences in mitochondrial content of complex I components and/or CypD affect the sensitivity of mPTP to Ca^{2+} and hypoxia.

These insights prompted us to investigate the structural subunit of complex I - NADH dehydrogenase [ubiquinone] iron-sulfur protein 2 (NDUFS2), which participates in the binding of ubiquinones [53] and rotenone [54], both of which have been shown to inhibit opening of mPTP [35,55]. In this study, we performed a comparative analysis of the effects of hypoxia on brain infarct size, mitochondrial calcium retention capacity, respiration, and complex I activity in the brains of newborn (7 days), young (2–3 months), adult (8–10 months) and aged (24–26 months) Wistar rats. We also investigated levels of CypD and NDUFS2 proteins in the brains of the aforementioned animal age groups.

Materials and Methods

Experimental Design

Experimental procedures were carried out according to the EU Directive 2010/63/EU (86/609/EEC) for animal experiments and the Law of the Republic of Lithuania on the care, keeping, and use of experimental animals. In experiments, Wistar rats of different age were used: newborn (7 days), young (2–3 months), adults (8–10 months), and aged (24–26 months) rats. In experiments where expression of proteins and activity of mitochondrial complex I were measured, an additional group of 18 months rats were included. To study the effect of ischemia on mitochondrial functions and cell death, we used *in vitro* hypoxia model which is considered as an efficient and suitable method for investigations of basic biochemical mechanisms [56]. Hypoxic group brain tissue was placed into a Petri dish together with 1 ml of isolation medium (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.4) and inserted into a hermetic glass camera with a continuous supply of gas mixture (93% N2, 5%O2, 2% CO2) and maintained at 37°C, with a constant flow of gas mixture for 90 min. After hypoxia the brains were used for mitochondrial isolation or sectioned for staining with TTC. Isolated mitochondria were used in the measurements of mitochondrial respiration rates, calcium retention capacity, mitochondrial complex I activity evaluation, and analysis of protein levels by Western blot.

Mitochondrial Complex I Activity Assay

In these experiments, mitochondria isolated from the whole brain were used. The enzymatic activity of mitochondrial complex I was expressed as the rate of NADH oxidation, measured spectrophotometrically at 340 nm [57]. For disruption of mitochondrial membranes, samples were suspended in hypotonic buffer (25 mM KH2PO4; pH 7.2) followed by three freeze-thaw cycles. For measurements, 0.2 mg of mitochondrial suspension was added to the cuvette and incubated in 800 ml of distilled water for 1 minute at 37°C. After incubation, the cuvette was supplemented with 200 µl of 50 mM Tris buffer (pH 8.0) containing 0.8 mM NADH, 500 mM sodium azide, 25 µM anti-

mycin A, and 5 mg/ml bovine serum albumin (BSA). Measurements were started by adding 100 μM of coenzyme Q1 which was the final electron acceptor in this system. The decrease in absorbance was recorded for 3 minutes, then additional 3 minutes were recorded in the presence of 5 μM rotenone. The activity of complex I is expressed as rotenone-sensitive NADH oxidation rate/min/mg protein.

Western Blotting

Mitochondria isolated from whole brains were lysed in RIPA buffer (Sigma) on ice for 60 min. The lysate was centrifuged at 20000 xg for 15 min at 4°C and the supernatant was collected. The samples were stored at -80°C until use. The amount of total proteins in lysates was quantified using the Bradford reagent (Sigma) according to the manufacturer's instructions. An equal amount of protein (15μg) was loaded into each well and separated in 10% SDS-PAGE gels. After separation, the proteins were transferred onto PVDF membranes using the Novex Semi-Dry Blotter (Life technologies). Membranes were blocked in 5% BSA solution in Tris-buffered saline with 0.1% Tween (TBST) for 1 hour at room temperature with gentle shaking. After blocking, membranes were probed with primary antibodies overnight at 4°C. The primary antibodies were: NDUFS2 antibody (NBP2-30413, Novus biologicals) diluted 1:1000 in blocking buffer (5% BSA in TBST); Cyclophilin-F Antibody (JM71-39, Novus biologicals) diluted 1:2000 in blocking buffer; and VDAC1 Antibody (SA93-03, Novus biologicals) diluted 1:2000 in blocking buffer. The following day, membranes were washed in TBST and incubated with the secondary antibody (goat anti- Rabbit, Human IgG (H+L) Secondary Antibody [HRP], NBP2-30348H, Novus biologicals) diluted 1:2000 in blocking buffer for 1 hour at room temperature with gentle shaking. After incubation with secondary antibody, membranes were washed in TBST and antibody binding was detected with Pierce ECL western blotting substrate (Thermo Scientific).

The blots were imaged on the BioSpectrum 600 imaging system (UVP, Cambridge, UK). To determine the molecular weight of the protein bands, PageRuler plus prestained protein ladder, 10 to 250 kDa (Thermo Scientific) was used. Densitometric analysis was performed using Image J software and the expression of NDUFS2 and CypD were calculated relative to the expression of VDAC1 (porin).

Mitochondrial Isolation

After 90 min. *in vitro* hypoxia mitochondria were isolated from either the whole brain, brain cortex or cerebellum. For this we used medium containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.4 at 4°C. Homogenates were prepared using a glass-teflon homogenizer and were centrifugated at 1000xg for 5 min at 4°C. Then the supernatant was collected and centrifuged again at 10000xg for 10 min at 4°C. Mitochondrial pellets were resuspended in isolation medium. Total protein content was determined by the modified Biuret method [58].

Mitochondrial Respiration

Mitochondrial respiration was measured using Oroboros high-resolution respirometry in the medium containing 110 mM KCl, 2.24 mM MgCl2, 5 mM KH2PO4, 10 mM Tris-HCl, pH 7.2 at 37°C. Proton leak respiration assay was started with the addition of mitochondria (0.25 mg/ml) and complex I substrates pyruvate (1 mM) and malate (1 mM). Oxidative phosphorylation was achieved by the addition of 0.4 mM ADP. Succinate (5 mM) and rotenone (50 nM) were used to measure complex II-dependent oxidative phosphorylation. The addition of atractyloside (100 nM) inhibited oxidative phosphorylation in mitochondria by preventing ADP transport into the matrix and represented succinate-dependent leak respiration [58].

Mitochondrial Calcium Retention Capacity

Mitochondrial calcium retention capacity was measured fluorometrically (Perkin Elmer LS-55 spectrofluorimeter; excitation 507 nm, emission 538 nm) using the fluorescent dye Calcium Green 5N. For measurements, 0.17 mg of mitochondria were incubated in the medium consisting of 200 mM sucrose, 10 mM Tris-HCl, 1 mM KH-2PO4, 0.01mM EGTA pH 7.4 supplemented with 150 nM Calcium Green 5N, 1 mM pyruvate and 1 mM malate. Pulses of 1.6μM CaCl2 were added every 2 minutes until a large increase in fluorescence signal was recorded and considered as the opening of mPTP [59].

Staining of Brain Slices with TTC

The cerebral hemispheres were separated from the cerebellum, the meninges were removed and placed into hypoxic camera. Then brains were immobilized caudal end down and sectioned with the vibratome (Vibratome 1000, Technical Products International Inc., Saint Louis, MO, United States) in PBS and glucose-filled cooling bath at 1°C. The brains were coronally sliced with low profile microtome blade (Leica Biosystems) into six 2 mm thick slices with an amplitude of 2 mm and minimal advancing speed. All acquired slices were stained with 60 mM TTC (2,3,5- triphenyl tetrazolium chloride) in saline at 37°C for 15 min and fixed with 4% paraformaldehyde. For estimation of the infarct area, pictures of all brain slices were captured and analyzed using Image J software. The results of infarct area were expressed as a percent of the total area of the tissue slice. The figures show the percentage of infarct zone area compared to the corresponding age control group.

Statistical Analysis

SPSS Statistics 20 software was used for statistical analysis. Data were expressed as means ± S.E from at least 3 experiments on separate animals. Differences in means were analyzed using ANOVA followed by Tukey's or LSD multiple comparison test. A value of $p<0.05$ was considered a statistically significant result.

Results

Age-associated Differences in Expression levels of Cyclophilin D, NDUFS2 and Activity of Complex I

The protein expression levels of CypD and NDUFS2 (Figure 1), as well as complex I enzymatic activity (Figure 2), were measured in mitochondria isolated from the whole brains of animals at the age of 7days, 2-3 months, 8-10 months, 18 months, and 24-26 months. Protein expression was analyzed by Western blots and the relative levels of the proteins were normalized to the level of voltage-dependent anion channel 1 (VDAC1), the expression of which did not differ among the investigated age groups of rats. Comparison of the density of CypD protein bands in the blots (Figure 1A) showed that there were no significant age-dependent changes in the expression of this protein. Meanwhile, we detected differences in NDUFS2 expression when comparing different age groups: the expression level of NDUFS2 was similar in age groups from 7 days up to 18 months; however, its expression level was significantly decreased in aged (24–26 months) animals (Figure 1B). As shown in (Figure 2), the activity of complex I in mitochondria isolated from control brains of 7 days animals was the lowest and increased with age. We also tested whether 90 min of hypoxia has an effect on complex I activity in brain mitochondria from various age animals. As demonstrated in (Figure 2), hypoxia had no effect on complex I activity in brain mitochondria of the newborn group, though in all other groups the activity was reduced by 12-35% under hypoxic conditions (Figure 2). Altogether, these data indicate that aging causes a decrease in NDUFS2 content in brain mitochondria; however, there was no correlation between expression levels of this component and activity of complex I or its sensitivity to hypoxia.

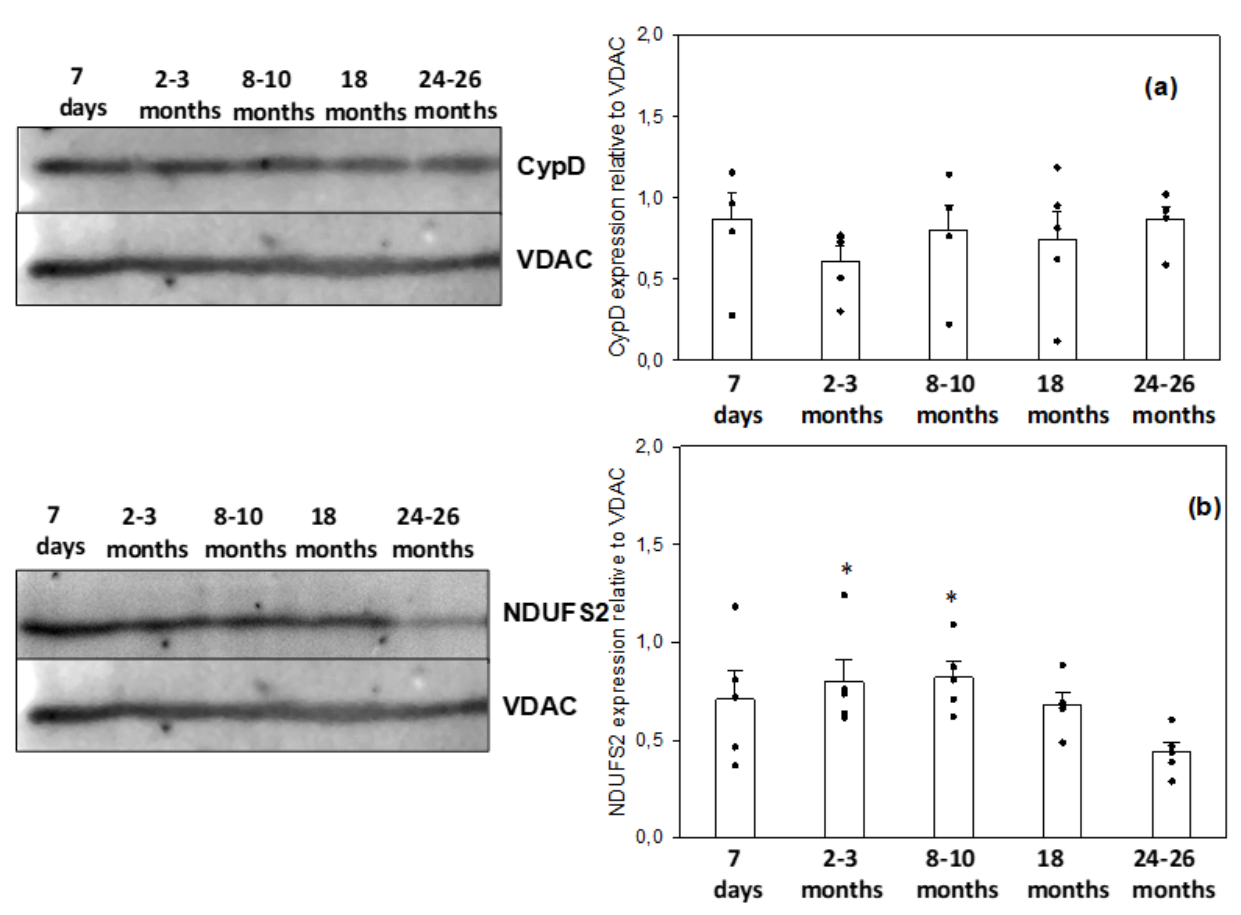

Figure 1: Protein levels of CypD and NDUFS2 relative to VDAC1.
(a) Representative blots and quantification of relative CypD levels (n=4).
(b) Representative blots and quantification of relative NDUFS2 levels (n=5). *- statistically significantly different compared to 24–26 months group.

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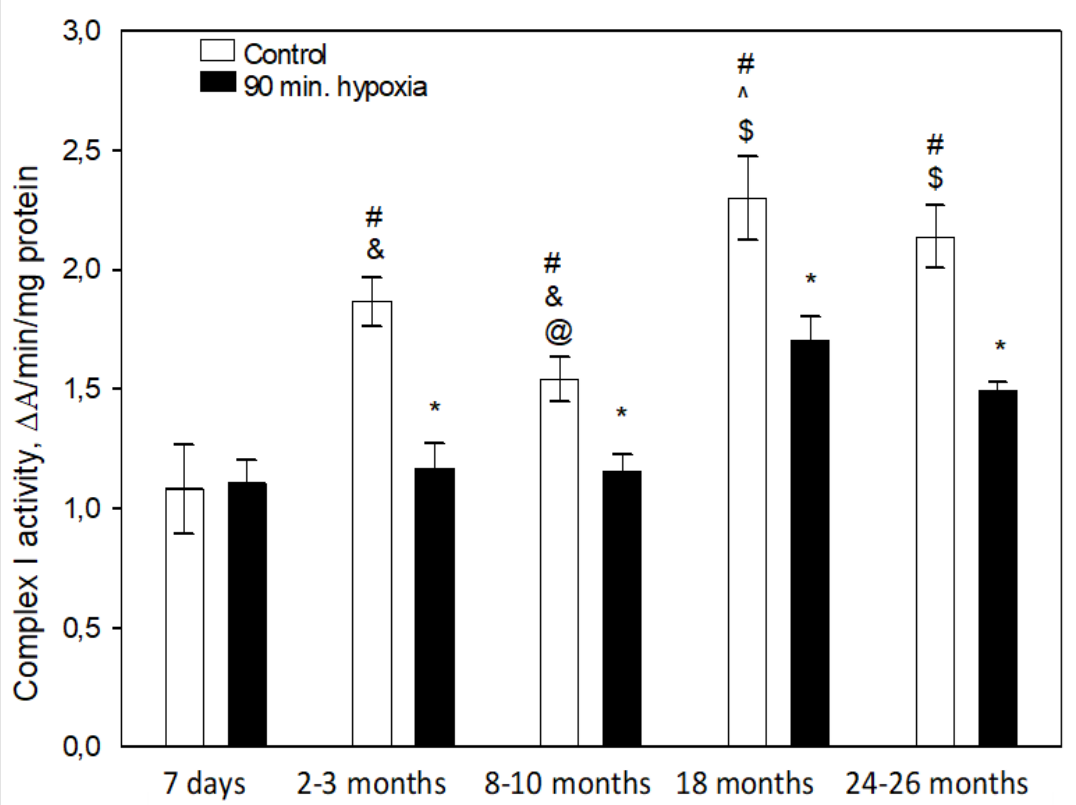

Figure 2: Complex I activity in normal and 90 min hypoxia-affected whole brain mitochondria (n=5-6). *- statistically significant effect of hypoxia compared to the respective control group. # - statistically significantly different compared to 7 days control group. ^- statistically significantly different compared to 2–3 months control group. $ - statistically significantly different compared to 8 - 10 months control group. & - statistically significantly different compared to 18 months control group. @ - statistically significantly different compared to 24 – 26 months control group.

Effects of Age and Hypoxia on mPTP Opening

In the next series of experiments, we evaluated the effects of 90 min hypoxia on the sensitivity of mPTP to Ca^{2+} in animals of 4 age groups: 7 days, 2-3 months, 8-10 months, and 24-26 months rats. As most ischemic pathologies are related to cortical injuries, we compared the effects of hypoxia on mitochondria isolated from histologically distinct regions: cortex (rich in glial cells) and cerebellum, where neuronal cells are predominant [60]. Opening of the mPTP was evaluated as the mitochondrial Calcium Retention Capacity (CRC). As shown in (Figure 3A), CRC of cortex mitochondria was similar in all age groups, though a tendency for higher CRC was observed in the 7 days old group compared to other groups. In cerebellar mitochondria, the lowest CRC was observed in the 2-3 months group compared to 7 days and 24-26 months old groups (Figure 3B). 90 min hypoxia sensitized cortical mitochondria to Ca^{2+} in all age groups: CRC was decreased by 40% in the 7 days group, 48% in 2-3 months, 20% in 8-10 months, and 22% in 24-26 months groups compared to the corresponding controls (Figure 3A). Hypoxia had no effect on CRC of cerebellar mitochondria of newborn and young rats, while CRC of mitochondria isolated from 8-10 months old rats cerebella after hypoxia decreased by 41%. In the 24-26 months old animal group, hypoxia caused a 35% decrease in CRC of cerebellar mitochondrial.

These data indicate that age has no significant effect on CRC of cortical mitochondria, whereas hypoxia sensitizes cortical mPTP to calcium in all age groups. In the cerebellum, the effect of hypoxia on mPTP opening is detectable in adult and aged animals.

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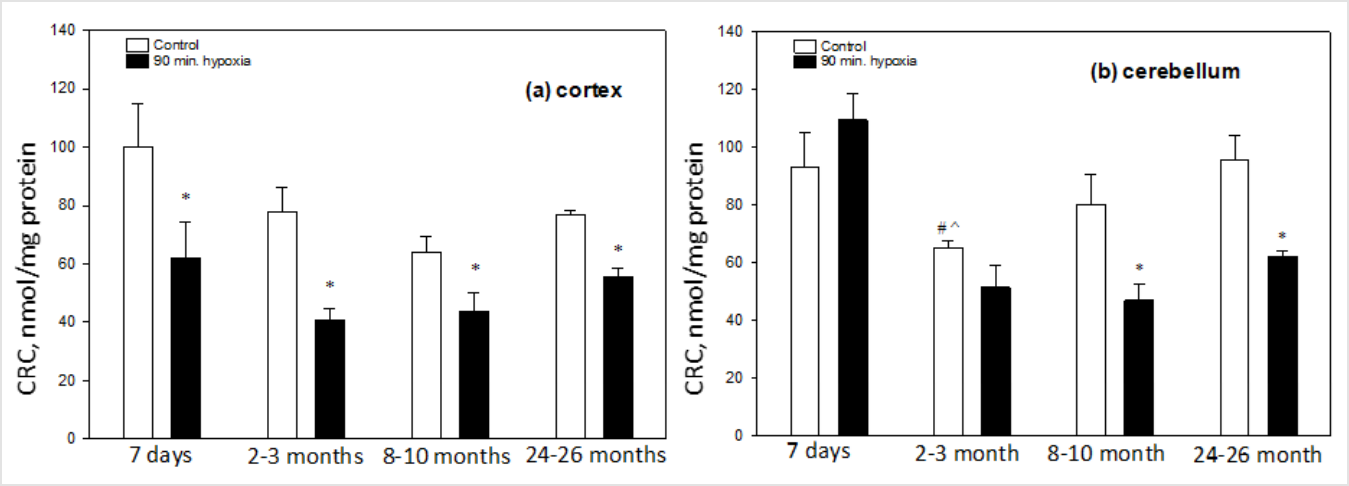

Figure 3: Effect of 90 min hypoxia on mPTP opening
(a) Effect of 90 min hypoxia on calcium retention capacity on cortical mitochondria
(b) Effect of 90 min hypoxia on calcium retention capacity on cerebellar mitochondria. CRC was measured as the amount of Ca2+ added to open the mPTP. *- statistically significantly different compared to the corresponding control group. # - statistically significantly different compared to 7 days control group. ^- statistically significantly different compared to 24-26 months control group (n=4-7).

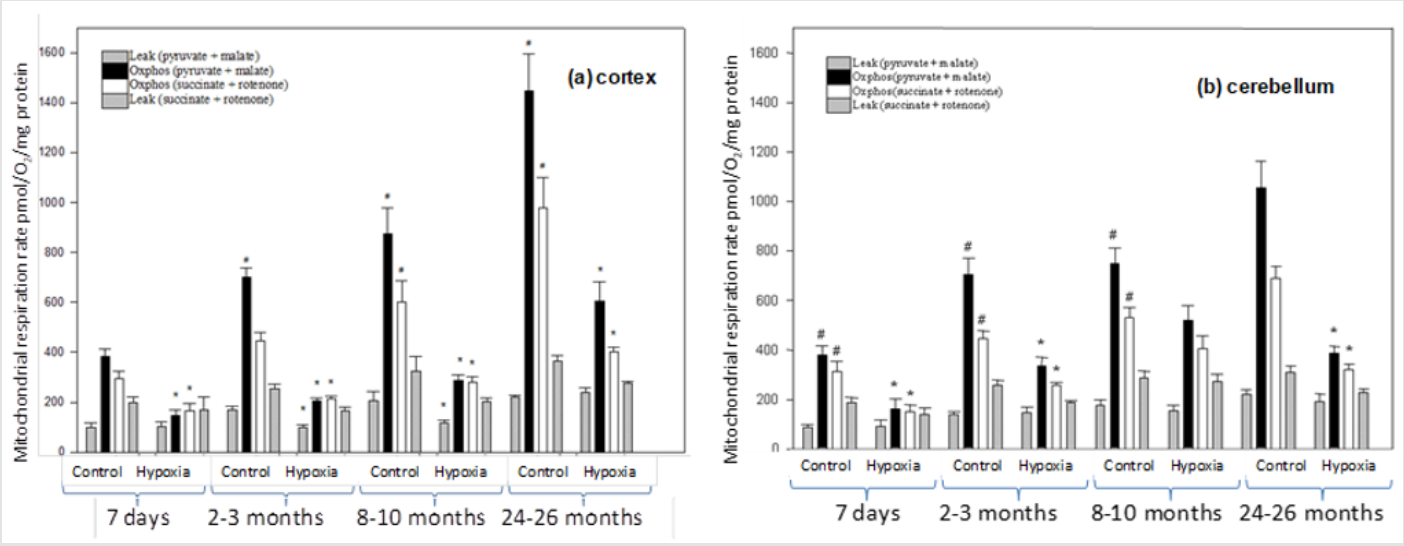

Figure 4: Effect of Age and Hypoxia on Mitochondrial Respiration
(a)Effect of 90 min hypoxia on respiration of cortex mitochondria.
(b)Effect of 90 min hypoxia on respiration of cerebellum mitochondria. Leak (pyruvate + malate) – mitochondrial LEAK respiration was measured by adding mitochondria (0.25mg/ml) and 1 mM pyruvate and 1 mM malate. OXPHOS – mitochondrial oxidative phosphorylation was achieved by addition of 0.4 mM ADP and indicated substrate (pyruvate + malate or succinate + rotenone) Addition of 100 nM atractyloside was used to inhibit phosphorylating respiration in the presence of 5 mM succinate and 50 nM rotenone. *- statistically significantly different compared to the respective control, # - statistically significantly different compared to 24-26 months control OXPHOS (n=4-7).

Effects of Age and Hypoxia on Mitochondrial Respiration

We measured the respiration rates of mitochondria isolated from cortices and cerebella from various age animals using complex I and complex II-dependent substrates in LEAK and OXPHOS states (see 5.5. Mitochondrial respiration for details). As shown in (Figure 4A) OXPHOS respiration rates of cortical mitochondria with pyruvate were lowest in 7 days old animals (383 ± 29 pmolO2/min/mg) and gradually increased with age reaching 1054 ± 109 pmolO2/min/mg in 24-26 months old animals. A similar tendency was observed with succinate as substrate: OXPHOS respiration rates were similar in 7

days old (295.4 ± 28.2 pmolO2/min/mg) and 2-3 months old cortex mitochondria (447 ± 32 pmolO2/min/mg) and increased gradually in 8-10 months old and 24-26 months old animals up to 670 ± 47 pmolO2/min/mg. LEAK respiration of cortical mitochondria was similar in all age groups with both substrates (Figure 4A). 90 min hypoxia significantly inhibited cortex mitochondrial OXPHOS respiration rate with pyruvate in all age groups: by 60% in the 7 days group; 70% in the 2-3 months group; 66% in the 8-10 months group, and 65% in the 24-26 months group [61] compared to the respective control group (Figure 4A). Similarly, hypoxia suppressed OXPHOS with succinate as substrate: in 7 days group respiration decreased by 31%, while in other groups respiration rates decreased by 52% compared with the respective controls (Figure 4A). There was no significant effect of hypoxia on LEAK respiration regardless of the animal age or the substrates used (Figure 4A).

OXPHOS respiration rate of cerebellar mitochondria with pyruvate as well as with succinate was also lowest in 7 days old animals (Figure 4B). OXPHOS respiration of cerebellar mitochondria from 24-26 months old rats was the highest and significantly different from other age groups of animals (Figure 4B). And again, there were no significant differences in LEAK respiration with both substrates (Figure 4B). Hypoxia inhibited cerebellar mitochondrial OXPHOS respiration rate with pyruvate as substrate by 64% in 7 days old animals; 52% in 2-3 months old animals; 21% in 8-10 months old animals, and 62% in 24-26 months old animals. Similarly, hypoxia reduced OXPHOS respiration with succinate as substrate in the presence of rotenone in 7 days group by 58%, in 2-3 months group by 43%, and in 24-26-months old group by 56%. In 8-10 months old animal group, cerebellar mitochondrial respiration after hypoxia was not statistically significantly different compared to the respective control, though a tendency for lower respiratory rates was observed here as well (Figure 4B). These data suggest that 90 min hypoxia inhibited mitochondrial phosphorylating respiration rates in cortical and cerebellar mitochondria from animals of all age group to a similar extent.

Evaluation Of Infarct Size in Brain Slices After 90 Min Hypoxia

Finally, we compared 90 min hypoxia-induced infarct sizes in brain hemispheres and cerebella from rats of various ages. As demonstrated in (Figure 5), after 90 min hypoxia infarcted area in all age groups was in the range of 41–53% of the hemispheres. In cerebella, hypoxia caused a similar tissue infarction (53–65%) in all age groups. According to these results, all animals, regardless of age, had similar brain tissue damage after 90 minutes of hypoxia.

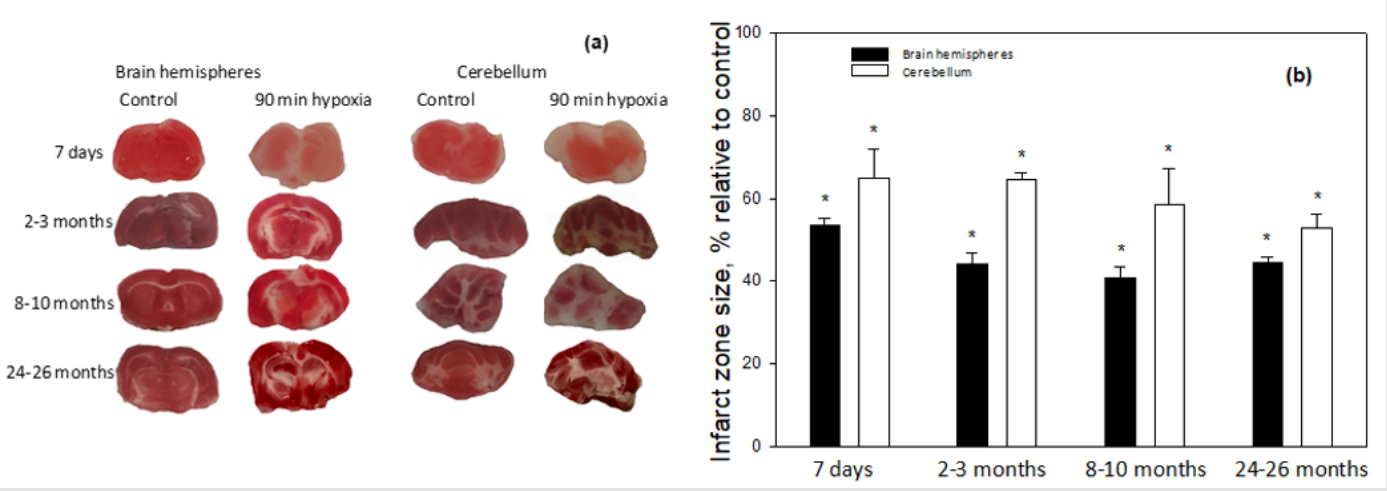

Figure 5: Evaluation of 90 min hypoxia-induced infarct size in brain slices.
(a) Representative images of TTC staining of brain slices. Representative images were form similar rat brain zone (Bregma -4mm -6 mm) [61] and one of 2 cerebellar slices per brain are shown for each age group Images of one middle slice of 6 slices per hemisphere and one of 2 cerebellar slices per brain are shown for each age group.
(b) Quantitative expression of effects of 90 min hypoxia on the infarcted area in brain hemispheres (cortex) and cerebella of various age rats (n=4-7). *- statistically significantly different compared to the corresponding control group.

Discussion

The present comparative study was designed to get more insights on mPTP activation and sensitivity to hypoxia, as well as complex I activity and structural composition in the brains of newborn (7 days), young (2-3 months), adult (8-10 months), and aged (24-26 months) rats. We compared expression levels of CypD in brain mitochondria throughout the lifespan and demonstrated that the content of this structural and regulatory component of mPTP does not change with age. Furthermore, this study is the first which examined age-associated changes in expression levels of NDUFS2 – a component of complex

I of the mitochondrial electron transfer system, and found that the content of this protein in brain mitochondria decreases in aged animals (24-26 months old). Our findings of constant CypD expression in brain mitochondria over the lifespan are consistent with previously reported similar CypD content in 6 and 24 months old rat brain and liver mitochondria [55]. In contrast, other investigators have reported enhanced levels of CypD in the brains of aged mice [62]. A study by Elissev, et al. [63] has found that the expression of CypD at transcriptional and protein levels was highest during neuronal development at early neonatal stages, while in adulthood, reduced expression of CypD was detected along with increased resistance of mPTP to Ca^{2+} [63]. The differences between the results of various studies most likely are related to species specificity. Complex I of the mitochondrial electron transfer system has been suggested to be involved in the regulation of mPTP [64,65].

It has been previously reported that inhibitors of complex I increase the concentration of Ca^{2+} required for mPTP opening [38,66] and act synergistically with CypD inhibitor cyclosporine A [67]. Therefore, we measured the levels of NDUFS2—a core subunit of complex I which contains the inhibitor-binding site [53,68]. Our results demonstrate that NDUFS2 expression decreased only in the 24-26 months old animal group, whereas in all other age groups of animals NDUFS2 content in brain mitochondria was similar. It seems that there is no direct correlation between the content of this protein and complex I activity, which was found to be lowest in newborn brain mitochondria but increased with age and was the highest in the 24-26 months old group. However, we cannot exclude the possibility that a decrease in NDUFS2 content affects the capacity of complex I to generate ROS at the ubiquinone-binding site [69]. The decrease in the expression of complex I components might be a sign of "healthy aging" as the increased expression of proteins of this complex has been shown to lead to increased ROS production and loss of enzymatic activity [52,70]. In our study, no significant age-related differences in mPTP sensitivity to Ca^{2+} were found for normal cortex mitochondria. This correlates well with the absence of age-related differences in the content of mPTP regulatory protein CypD in brain mitochondria. Interestingly, we found that mPTP sensitivity to calcium was enhanced in normal cerebellar mitochondria of young (2–3 months) rats, and this is not related to CypD content in mitochondria, which was not changed. The causes of this are not clear and need to be further investigated. An interesting idea has been suggested by a study on 8, 16, and 24 months old mice, that showed the age-dependent increase in interactions between Cyp D and OSCP subunit of ATP synthase leading to ATP synthase dysfunction and subsequently lowered ATP production, increased ROS production, and sensitization of mPTP [58]. There is accumulating evidence that ATP synthase may be involved in the structural composition of mPTP [4,20-22]. However, the influence of aging on ATP synthase and its role in mPTP functioning and/or regulation has not been investigated yet. Studies with mice [71] and rats [55] show that the abundance of most of the ATP synthase subunits stays constant during aging. However, other factors such as oligomerization and post-translational modifications of subunits of ATP synthase may change with age and alter the activity of this complex [71,72]. It has been suggested that age-associated glycation of ATP synthase constituents as well as reduced dimerization and oligomerization of ATP synthase increase the susceptibility of mPTP to Ca^{2+} [71]. In addition, other post-translational modifications of ATP synthase such as phosphorylation [73,74] and nitration [75] have been described during aging, but their role in mPTP functioning or regulation is still unclear [76].

Several studies provide experimental evidence that mPTP is formed by the c ring of the F0 domain of ATP synthase [77-79]. However, studies concerning expression and modifications of the c subunits of ATP synthase during aging are still lacking and need further investigation. The results of our study also revealed regional and age-associated differences in mPTP sensitivity to hypoxia demonstrating that mPTP in the cerebellar mitochondria of newborn animals is most resistant to hypoxia. The sensitivity of mPTP to Ca^{2+} was found to be increased by hypoxia in the cortex of all age animals and the cerebellum of adult (8-10 months) and aged (24-26 months) animals. In these groups, hypoxia caused the opening of mPTP to occur at significantly lower Ca^{2+} concentrations compared to the respective controls. This is consistent with previously reported findings demonstrating enhanced susceptibility of mPTP opening to Ca^{2+} in non-synaptic mitochondria from aged (> 18 months) rats compared to young (3 months) rats [80], or in whole brain mitochondria from aged (> 26 months) versus young (> 4 months) rats [81]. The described regional variations in sensitivity to hypoxia may be due to different ratios of neuronal and glial cells in each of the brain regions, with the cortex being rich in glia and cerebellum - in neuronal cells [82]. Another study on post-mortem human brain samples showed that the ratio of neurons and glial cells in these regions changes throughout the lifespan [82].

Interestingly, when we compared the respiratory functions of mitochondria isolated from cortices and cerebella of various age rats, we found that the rate of phosphorylating respiration was lowest in newborn rat brains. In young rats, this respiratory function, particularly with complex I substrate pyruvate, was significantly increased, reaching the highest activity in aged rats. This was in correlation with age-dependent changes in complex I activity, which were also found to be lowest in newborn and highest in aged rat brains. Notably, 90 min hypoxia caused a substantial reduction in the respiratory function of cortical as well as cerebellar mitochondria in all age groups. Similarly, *ex vivo* hypoxia-induced infarct size was similar in both brain regions in all age groups. This suggests that hypoxia-induced acute necrosis in the brain is most likely to be related to the impairment of the oxidative phosphorylation system independently of mPTP opening. On the other hand, hypoxia-induced sensitization of mPTP to Ca^{2+} in cortex mitochondria of rats of all age groups or aged cerebellar mitochon-

dria may be important in mediating delayed neuronal death, which occurs in penumbra days and weeks after ischemic insult. [5]. It is important to point out that young adult rats (1-6 months old) are widely used in experimental studies on ischemic brain damage and neuroprotective treatments. According to the findings of our study, age-dependent as well as brain regional differences, must be considered in such studies in order to identify specific and more effective targets for neuroprotection.

Highlight

- In groups of rats aged 7 days, 2-3, 8-10, and 24-26 months, *in vitro* 90 min hypoxia inhibits the brain cortex and cerebellum mitochondrial respiration.
- *In vitro* hypoxia sensitized cortical mitochondria to mitochondrial permeability transition pore opening in all age groups, but cerebellar mitochondrial permeability transition pore opening after hypoxia is detected in the 8-10 and 24-26 months rat groups.
- The expression of the mitochondrial complex I subunit NDUFS2 decreases in the group of rats aged 24-26 months, while the enzymatic activity of mitochondrial complex I increases with age.

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Author Contributions

Vilmante Borutaite: Conceptualization, Supervision, Writing - Review & Editing, Funding acquisition. Danielius Umbrasas: Methodology, Investigation, Visualization, Formal analysis, Writing - Original Draft, Writing - Review & Editing. Odeta Arandarcikaite: Project administration, Methodology, Investigation, Visualization, Formal analysis, Writing - Original Draft, Writing - Review & Editing. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The data generated and presented in this study are available on request from the corresponding author.

Conflicts of Interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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