MASTER'S THESIS

Mitochondrial function and sirtuin expression in hippocampus of young and old high- and low-capacity runner rats

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ABSTRACT

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Introduction: Exercise and aerobic capacity are associated with improved learning in animals and humans. The hippocampus is a brain structure involved in learning and memory. High rates of neurogenesis have been shown to take place in the dental gyrus of hippocampus in response to physical exercise. It is not known whether mitochondrial dysfunction in the hippocampus is responsible for decline in cognitive function associated with low fitness level and aging, and whether intrinsic aerobic capacity is a risk factor for it.

Methods: Mitochondrial function was investigated in the hippocampi of young and old high-capacity runner (HCR) and low-capacity runner (LCR) rats using high-performance respirometry. Mitochondrial sirtuins and total mitochondrial respiratory chain complexes were also quantified using Western blot.

Results: Statistical analysis showed a significant difference in the LEAK respiration state ($P<0.05$) and LEAK control ratio ($P<0.01$) between young and old LCR rats (L: 8.83 ± 1.8 vs. 9.67 ± 1.92 pmol/s/mg and L/E: 0.083 ± 0.016 vs. 0.114 ± 0.025, respectively). A significant difference was found also between young HCR and old HCR rat OXPHOS (P) and ETS (E) states (P: 53.7 ± 5.6 vs. 47.0 ± 9.4 pmol/s/mg, E: 100.9 ± 17.6 vs. 83.3 ± 18.1 pmol/s/mg, respectively). No differences were found between young HCR and young LCR, or old HCR and old LCR. The correlation analysis showed a positive correlation between mitochondrial content and ETS ($r = 0.32$, $P<0.05$). Sirt4 was positively correlated with CII respiration ($r = 0.35$, $P<0.05$). ETS was positively correlated with all respiratory states, i.e. LEAK ($r = 0.44$), OXPHOS ($r = 0.71$), CI ($r = 0.67$), and CII ($r = 0.44$; $P<0.01$ for all), and negatively with the coupling control ratios L/E ($r = -0.54$) and P/E ($r = -0.52$; <0.01 for both). There was no correlation between CI and CII respiration, CII and L/E, LEAK and CII, or LEAK and P/E.

Conclusions: LCR rats show an increased LEAK state respiration as they age, which may be related to mitochondrial dysfunction. However, we did not find a difference in their mitochondrial sirtuin levels or total mitochondrial content in the hippocampus compared to HCR to explain this finding. Even HCR show a decline in their OXPHOS and ETS capacity with age, but not in LEAK state respiration.

Keywords: mitochondria, sirtuins, intrinsic aerobic capacity, aging, high-resolution respirometry

ABBREVIATIONS

CI: Complex I

CII: Complex II

CNS: Central nervous system

DG: Dentante gyrus

ETS: Electron transfer system

HCR: High-capacity runner

HRR: High-resolution respirometry

L/E: LEAK control ratio

LCR: Low-capacity runner

OXPHOS: Oxidative phosphorylation

P/E: OXPHOS control ratio

ROX: Reactive oxygen consumption

Sirt3: Sirtuin 3

Sirt4: Sirtuin 4

Sirt5: Sirtuin 5

WB: Western blot

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INTRODUCTION

Physical activity and exercise training are today known to have a significant influence not only on physical health and performance, but also on mental health and cognitive function, including learning[1,2]. Unlike many other ways to improve cognitive function, including specific cognitive training, exercise seems to work on a very broad range of domains of cognition. In humans, physical activity and exercise training has been shown to enhance cognition both acutely[3,4] and in long-term[5,6], to slow down age-related memory decline[7-9], and to prevent and enhance recovery from depression and anxiety disorders[10-13], which are associated with impaired learning abilities[14-16]. Understanding the mechanisms behind these beneficial effects of exercise on brain function is important for both future fundamental research and different applications in the fields of psychology and medicine.

1. THE RELATIONSHIP BETWEEN FITNESS AND LEARNING

Learning can be defined as any relatively lasting change in behavior resulting from experience[17]. However, the relationship between fitness and learning is complex and not yet well understood. The possible differences between the effect of acquired fitness and intrinsic fitness on cognition also remains unsolved. Is the aerobic fitness level per se, whether intrinsic or acquired, the variable associated with improved learning, or is it rather physical activity, regardless of the outcomes of the activity?

1.1 Effect of chronic exercise on learning

Several interventions with the aim of exploring short-term effects of an exercise training program on learning have been conducted especially on children and elderly people, as well as on animals[8]. In a meta-analysis published by Colcombe & Kramer in 2003, the data of 18 intervention studies were used to examine the effects of exercise training on cognitive performance in older adults (≥55 y)[18]. The results showed a positive effect of fitness training in all types of cognitive tasks, regardless of the training method or participants' characteristics. Of the examined tasks, executive-control processes showed the largest benefit of improved fitness, but also controlled processes and visuospatial processes improved.

Combined training with both strength and aerobic exercise resulted in a larger effect than aerobic training alone.

Another meta-analysis published by Smith *et al.* in 2010 studied the effects of aerobic exercise training on neurocognitive performance, analyzing 29 randomized studies with subjects aged 18 or above[19]. This meta-analysis included 12 new trials conducted after the study by Colcombe & Kramer. In this analysis, aerobic exercise training was found to be associated with slight improvements in attention and processing speed, as well as with executive function and memory. The effects of exercise on working memory were less consistent.

Heyn *et al.* (2004) analyzed the effect of exercise training programs on physical and cognitive measures in elderly people with cognitive impairment and dementia[20]. In addition to positive fitness and functional capacity outcomes, a significant positive effect was found for cognitive function and behavior.

However, in a meta-regression analysis by Etnier *et al.* in 2006, the cardiovascular hypothesis of improved cognitive function as a result of changes in aerobic fitness was tested in order to investigate whether a dose-response effect exists[21]. The fitness effect sizes and cognitive effect sizes were not found to have a significant linear or curvilinear relationship in studies with cross-sectional designs or post-test comparison, even when significant negative relationship was found between fitness and cognitive function for pre–post comparisons. Thus, even when exercise training seems to improve cognitive function, a greater increase in aerobic fitness does not necessarily predict greater improvement in cognitive performance.

Improved cardiovascular fitness is thought to be associated with changes in underlying physiological mechanisms in the brain. Exercise induces central and peripheral growth factor cascades affecting structural and functional changes in the cerebrum, while also reducing the systemic risk factors associated with the metabolic syndrome[22]. Increased neurogenesis in the hippocampus of mammals in response to aerobic exercise is well-documented[23-27]. Another factor affecting the beneficial influence of exercise on learning capabilities could be enhanced oxygen delivery to brain areas responsible for learning, as increased regional cerebral blood flow have been reported following a program of exercise training[25].

1.2 Effect of intrinsic aerobic capacity on learning

It is well known today that aerobic fitness is in great part inherited and not solely a result of lifestyle. In the HERITAGE study involving 429 individuals from 86 nuclear families, the heritability of VO_2max was estimated to be about 50 %[28], and in a recent meta-analysis of twin and sibling studies, the VO_2max heritability of children and young adults was estimated to reach 60 %[29]. The greatest heritability estimate, 71 %, was obtained in a population-based FinnTwin16 study by Mustelin *et al.* (2011), with monozygotic and dizygotic twin pairs discordant for BMI[30].

The role of intrinsic fitness for cognitive function is less clear. Not many human studies examining the question of inherited fitness and cognitive function are available in the literature, as distinguishing intrinsic from acquired exercise capacity is challenging. In the twin study by Rottensteiner *et al.* (2015), 10 adult identical twin pairs discordant for their exercise habits were compared. Independent of genetic background, the physically more active twins had improved modulation of striatum and prefrontal cortex gray matter volume[31].

Regarding animal studies, the specific question of cognitive function has been studied by Wikgren *et al.* (2012) using the rat model for intrinsic running capacity: sedentary high-capacity runners (HCR) and low-capacity runners (LCR) were tested for their ability to learn[32]. The HCR rats performed better than LCR rats in tasks requiring flexible cognition but not in a motor learning task, which suggests that inherited aerobic capacity is associated with cognitive performance. Also Sarga *et al.* (2013) studied spatial memory in trained and untrained HCR and LCR rats and showed that HCR rats had superior memory in an avoidance task even when untrained[33], and Choi *et al.* (2014) found impaired cognitive function in aged LCR rats measured by a spontaneous spatial novelty preference test in an Y maze[34].

2. ROLE OF HIPPOCAMPUS

The hippocampus is located under the cerebral cortex as a part of the limbic system (Figure 1). A region in the hippocampus critical for learning and memory is the dentate gyrus (DG). It is one of the few structures showing high rates of neurogenesis in adult mammalian brain, which is believed to play a role in learning and memory[35]. The hippocampus seems to be the structure that is, in fact, most responsive to physical exercise: a phenomenon that has been confirmed by a growing number of both animal and human studies[36]. The new neurons mature in the sub-granular zone of the dentate gyrus from neural stem cells[37].

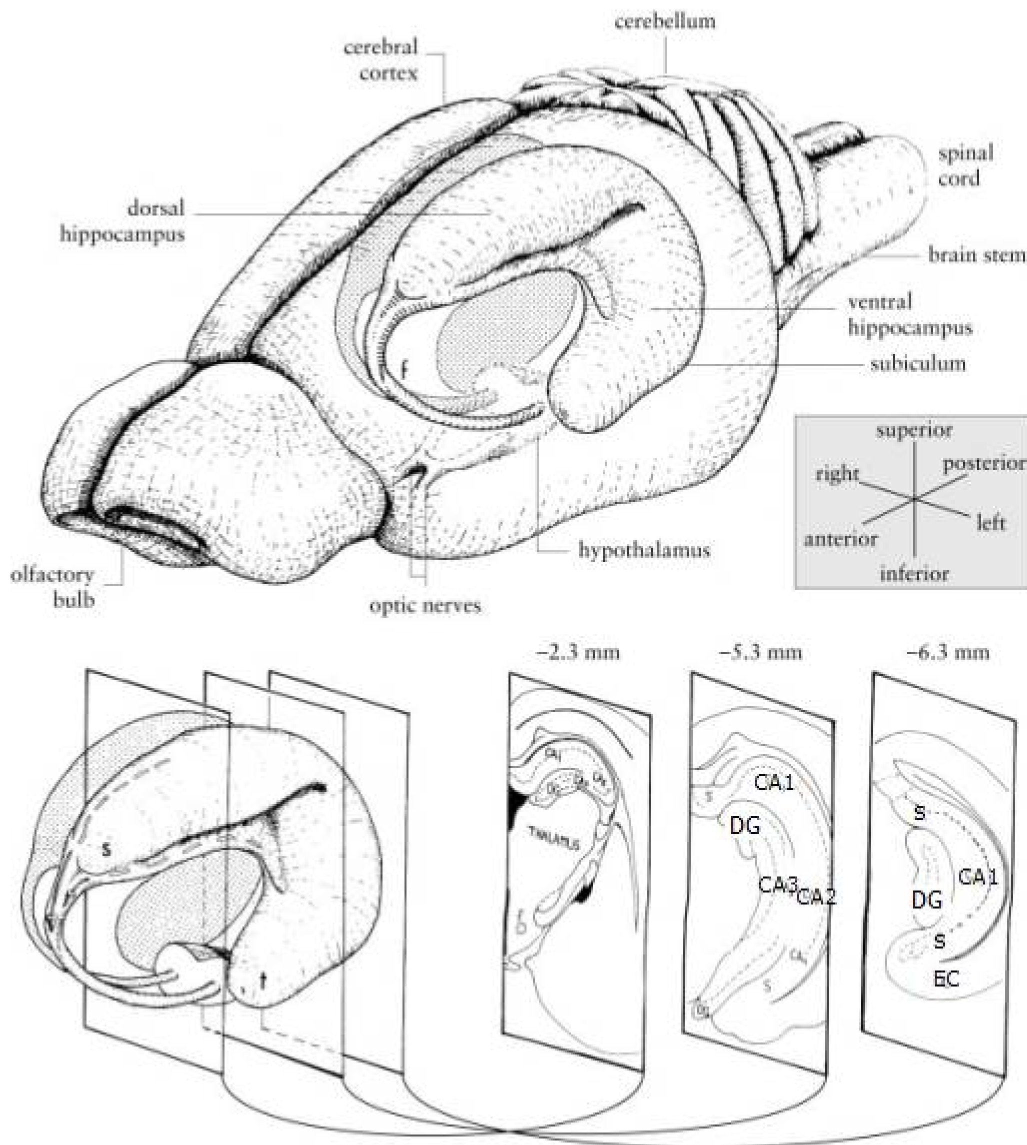

Figure 1. Rat hippocampus location in brain and its structure. CA1, CA2, CA3: cornu ammonis fields 1–3; DG: dentate gyrus; EC: entorhinal cortex; f: fornix; s: septal pole of the hippocampus; S: subiculum; t: temporal pole of the hippocampus. (Cheung & Cardinal 2005[38], Copyright Policy - open-access in OpenI)

Recently, physical exercise was shown to increase hippocampal neurogenesis and improve pattern separation in mice[39]. There is ample evidence of a beneficial effect of exercise on structural and functional plasticity of the hippocampus in rodents[40]. In humans, the volume of hippocampus has been shown to increase in previously sedentary adults as result of a 1-year aerobic training program. Hippocampal volume tends to decrease with age, which may be related to decreasing levels of BDNF (brain-derived neurotropic factor)[41].

Physical exercise also induces expression of insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF), growth factors responsible for angiogenesis in the brain[42,43] and stimulates the release of BDNF[44]. However, despite the evidence suggesting an

important role of VEGF and IGF for adult hippocampal neurogenesis, it appears that neurogenesis is not solely due to increased vascularization in the hippocampus[23,25].

2.1 Mitochondrial function

The main portion of ATP consumed by the brain is produced in mitochondria by oxidative phosphorylation. The mitochondrion is often described as the powerhouse of the cell: the ATP production occurs at the inner mitochondrial membrane, and involves electron transport through a chain of protein complexes (I-IV). These complexes transfer electrons from electron donors (NADH, $FADH_2$) to O_2. During the electron carrying steps, protons are transferred through the inner membrane against the chemiosmotic concentration gradient. The potential energy stored in this H^+ gradient is utilized to synthesize ATP from ADP and inorganic phosphate, as the protons are released through the ATP synthase.[45]

Neurons require large amounts of energy for continuously ongoing processes such as action potentials, resting membrane potential maintenance, active transport, receptor function, vesicle release, and neurotransmitter recycling[46]. Mitochondria are also the organelles mediating apoptosis, programmed cell death. Therefore, the amount and functionality of mitochondria play a significant role in neuronal proliferation and death, and have an impact on health and disease of the central nervous system[47].

Mitochondrial dysfunction is associated with pathological conditions affecting CNS, including Alzheimer's disease[48] and Parkinson's disease[49]. Even normal brain aging involves gradual alterations in memory and cognitive function. The free radical theory of aging suggests that the accumulation of mitochondrial damage produced by oxidative stress is responsible for aging[50].

One way of assessing mitochondrial function is respirometry, a method of monitoring oxygen consumption in a fresh biological sample. Any sample with functioning mitochondria can be used for respirometrical measurements: whole cells, tissue homogenates, muscle fibers, or isolated mitochondria. The inner mitochondrial membrane needs to remain intact, however, for reliable measurement of oxidative phosphorylation capacity. The substrate-uncoupler-inhibitor titration (SUIT) approach together with high-resolution respirometry allows assessing the function of different electron transferring complexes (CI and CII) in isolation while providing also the leak and the maximal electron transfer system (ETS) capacity.[51]

Figure 2. Example of a SUIT on a hippocampal homogenate. The oxygen flux at different respiration states is obtained by introducing specific substrates and inhibitors to the sample. PGM = pyruvate + glutamate + malate; Cyt c = cytochrome c; Suc = succinate; Rot = rotenone; AnA = Antimycin A.

2.2 Sirtuin expression

Sirtuins are regulatory enzymes with mainly deacylace activity involved in regulation of processes linked to energy metabolism and aging. The reversible acetylation of proteins controls their activity, and deacetylation of leads to their activation or inactivation. Mammals express seven different sirtuins, Sirt1-Sirt7. Of these, Sirt3, 4, and 5 are expressed only in mitochondria. The sirtuin-mediated deacetylation reaction couples lysine deacetylation to nicotinamide adenine dinucleotide (NAD) hydrolysis. The NAD+/NADH ratio affects sirtuin activity; at times of low energy availability, this ratio increases, leading to higher sirtuin activity and deacetylation of other proteins.

Mitochondria being the central cell organs responsible for energy production in the cell, the mitochondrial sirtuins are of particular interest when considering energy availability and metabolism. Sirt3 appears to be the predominant mitochondrial deacetylace and to play an important role in several mitochondrial pathways in all tissues[52]. The targets of Sirt3 include proteins involved for example in substrate utilization, electron transfer, and redox homeostasis[53]. In response to caloric restriction or fasting, Sirt3 deacetylates a set of mitochondrial proteins, resulting in activation, inhibition, and allosteric modification of protein functioning[52].

Unlike other sirtuins, Sirt4 does not possess deacetylace activity, but it is instead an ADP-ribosyltransferase[54]. It is activated in response to amino acids, downregulating insulin secretion by inhibiting mitochondrial glutamate dehydrogenase 1 activity[55]. Sirt4 is also involved in regulation of fatty acid oxidation and mitochondrial gene expression in liver and muscle[56].

The targets of Sirt5 are not yet well identified, although it seems like regulation of energetic flux through glycolysis is one of its main functions. The deacetylase activity of Sirt5 is weak, and its main targets are succinyl and malonyl groups[57,58]. In addition to glycolysis, it is likely involved in many other metabolic pathways, including the urea cycle, where it activates detoxification of excess ammonia that may accumulate during fasting[59].

In a recent study using a Sirt3-/- mice model relevant for neurological disease, hippocampal Sirt3 expression was found to be enhanced by running wheel exercise[60]. The striatal and hippocampal neurons of mice lacking Sirt3 showed increased vulnerability in pathological conditions. Another study showed that overexpression of Sirt3 protected against age-related hearing loss in mice via enhancing the mitochondrial glutathione antioxidant defense system, suggesting a neuroprotective role for Sirt3[61]. One recent study found a decrease in the expression of sirtuins 3-5 in the aging rat brain, although possible associations to functional or structural alterations were not investigated[62].

3. HIGH-RESOLUTION RESPIROMETRY IN MITOCHONDRIAL FUNCTION STUDIES

Respirometry is a technique used to measure metabolism of living organism indirectly by determining their oxygen (O_2) consumption and often also carbon dioxide (CO_2) production. It is based on the assumption that any consumed oxygen must be utilized by the organism in the oxidative phosphorylation – thus, the rate of energy consumption may be calculated based on O_2 consumption rate. In living whole animals including humans, different closed and open-circuit systems for ventilator gas analysis is used widely in research and fitness testing.

For a smaller scale analysis of oxygen consumption, a system with higher resolution is needed in order to detect small fluctuations in O_2 concentration. The traditional method for respirometric measurement of small biological samples is the polarographic Clark electrode, which measures oxygen on a catalytic platinum surface using the net reaction[63]:

$$O_2 + 4\,e^- + 2\,H_2O \rightarrow 4\,OH^-$$

Modern oxygen sensors are based on the same principle. The main tool in studying mitochondrial function is mitochondrial respirometry, which measures the consumption of oxygen by the mitochondria without involving an entire living animal, but a tissue sample suspended in an aqueous solution. The sample may be any of these three types: isolated mitochondria from a tissue, permeabilized cells or tissues, or a tissue homogenate[64]. By permeabilization, the cellular membrane is made permeable by the addition of detergents, leaving selectively the mitochondrial membrane intact. This allows the mitochondria to be left as functional structures that may be reached using chemicals that are normally unable to cross the cell membrane.

Mitochondrial respirometry takes place in solution: the sample is suspended in a medium in a closed chamber, and the oxygen electrode measures changes in dissolved O_2 concentration. An OROBOROS Oxygraph-2k high-resolution respirometry (HRR) instrument is presented in Figure 3. It includes two chambers for simultaneous measurement of two samples.

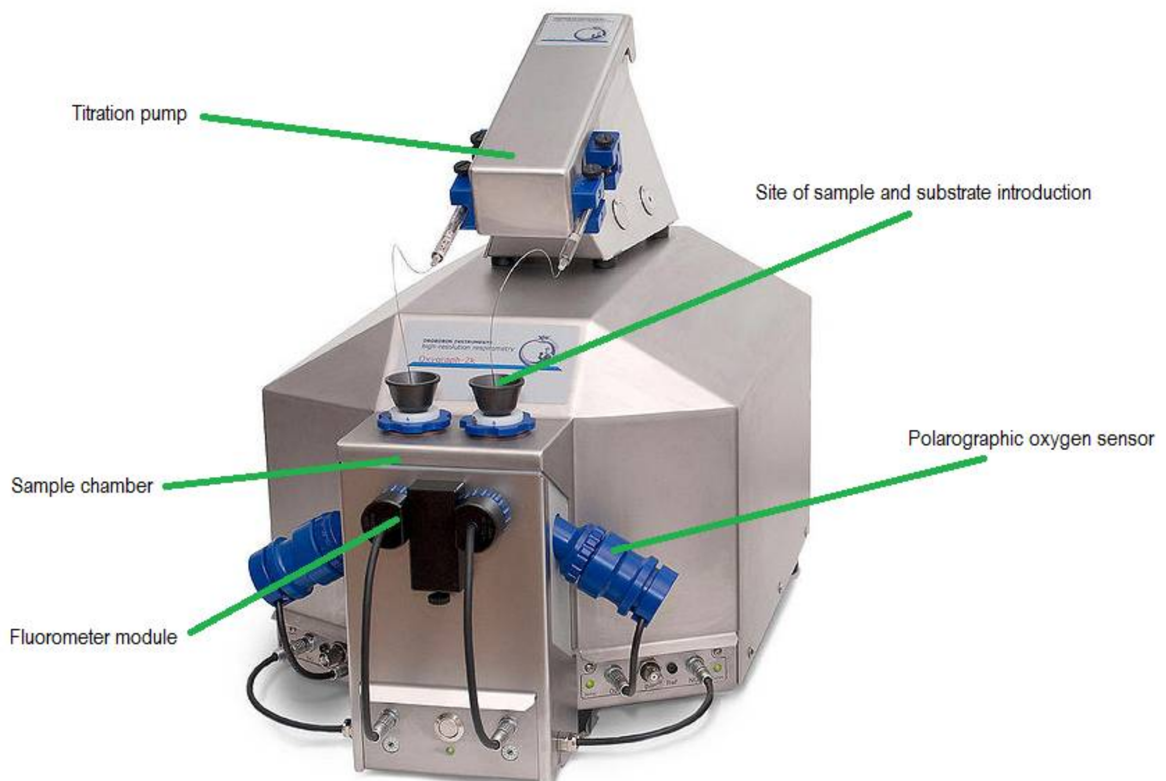

Figure 3. Overview of a OROBOROS Oxygraph-2k instrument equipped also with a O2k-Fluo LED2 fluorometer module and a titration pump. Two samples may be measured at once. The 2 ml sample chamber are made of Duran glass.

HRR is based on the selective activation and inhibition of different electron transfer system complexes and enzymes on the inner mitochondrial membrane (Figures 4 and 5). By adding

selected substrates, inhibitors, and uncouplers to the sample while monitoring its oxygen concentration, information about oxygen flux on different respiratory states is obtained.

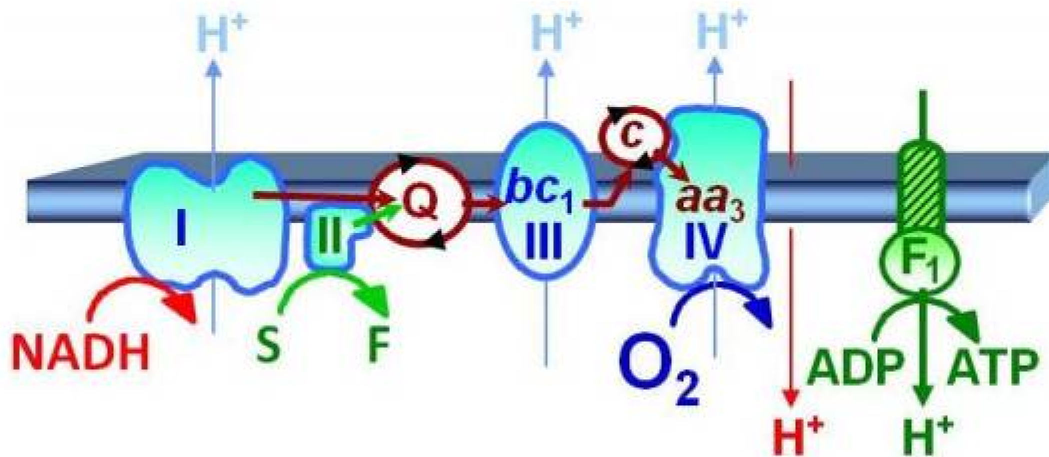

Figure 4. The simplified electron transfer system on the inner mitochondrial membrane. Complex I and II provides electrons to the ubiquinol/ubiquinone (Q-junction). NADH = Nicotinamide adenine dinucleotide, S = Succinate, F = Fumarate, c = cytochrome c. Source for image: Gnaiger (2014), open access[65]

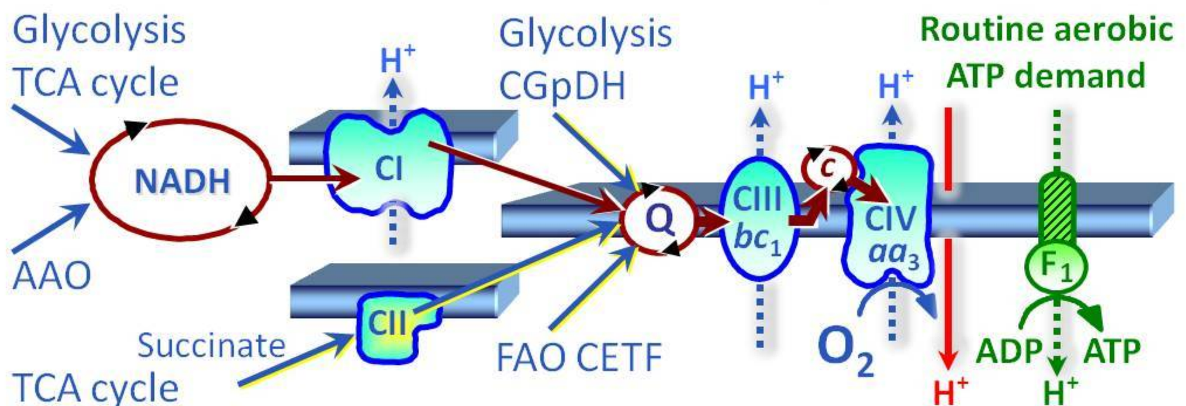

Figure 5. The ETS showing additional sources of electrons for the Q-junction and their oxidized substrates they originate from. Source for image Gnaiger (2014), open access[65]

The respiratory states used in this work are based on the ones defined by Erich Gnaiger and used in all OROBOROS protocols[65]. In mitochondrial preparations, there are three well-defined coupling states of respiration, LEAK (L), OXPHOS (P), ETS (E). LEAK is measured in presence of reducing substrates, but in absence of ADP: thus, in this state no ATP can be synthesized but O_2 is consumed and heat is produced by proton leak, proton slip, cation cycling and electron leak. OXPHOS is the respiration at saturating concentrations of ADP and inorganic phosphate: in this state, the ATP synthesis is maximized. In this text we refer to the OXPHOS state with CI+CII (complex I and complex II). The ETS state is the respiratory

electron transfer system capacity induced by adding an uncoupler to the medium: hence, the proton flow through the membrane is uncoupled from ATP synthesis and the electron carriers are working at their maximal rate. In this study the following states are used in reporting results: LEAK (L), CI (Complex I), CI+CII (OXPHOS, P), ETS (E), and CII (Complex II). Coupling control ratios are expressed as L/CI, L/P, L/E, and P/E. L/E is the LEAK control ratio, i.e. the flux ratio of LEAK respiration over ETS capacity. Its value between 0.0 and 1.0 is a measure of uncoupling or dyscoupling at constant ETS capacity, with 1.0 demonstrating 100 % uncoupling. The P/E is the OXPHOS control ratio is a measure of the OXPHOS capacity limitation by the phosphorylation system: at the upper limit of 1.0, there is no limitation of P, and the ETS capacity = OXPHOS capacity.[65]

High-resolution respirometry has proven to be a valuable tool in studying pathological effects resulting in reduced respiration: mitochondrial and metabolic diseases, apoptosis, ageing, ischemia-reperfusion injury, and oxidative stress. Minor changes in cellular respiration, small alterations in respiratory control ratios, or an altered response to inhibitors may indicate significant mitochondrial defects. These may arise from injuries of mitochondrial proteins or membranes, defects of mitochondrial DNA (mtDNA), or changes in mitochondrial signaling cascades.[66]

In the field of exercise physiology, HRR allows the measurement of human biopsies with limited amount of sample for studies regarding exercise capacity or genetic and acquired mitochondrial defects. It also enables determination of chemical oxidation rates and antioxidant capacities.[67]

4. ANIMAL MODEL FOR INTRINSIC AEROBIC CAPACITY

The overwhelming majority of existing animal research concerning the relationship between intrinsic fitness and susceptibility for disease is done using a rat model developed by Lauren G. Koch and Steven L. Britton, who in 1996 started cross-breeding rats based on speed-ramped treadmill running test by selecting the best and worst performing individuals for reproduction[68,69]. By 2011 and after 28 generations, the artificial two-way selection had produced two lines of rats which differed in their maximal running capacity about 7-fold. In one recent study, the observed difference in the covered distance was over 14-fold after 32 generations[70]. These rats are named high-capacity runners (HCR) and low-capacity runners

(LCR) and have been used as a model in a wide array of studies ranging from exercise intervention and cardiovascular and metabolic disease to neurological, cognitive, and behavioral research. Throughout the generations LCR have accumulated risk factors of cardiovascular disease. These include hypertension, endothelial dysfunction, impaired glucose tolerance and insulin resistance, visceral fat accumulation, and elevated lipids; symptoms of the metabolic syndrome[71].

Multiple genetic and environmental factors determine aerobic capacity. The genetics of HCR and LCR rats has been investigated in a few studies. Genes enriched in the HCR and LCR breeds have been identified in cardiac and skeletal muscle by Bye *et al.* in 2008. In the heart, 1540 genes related to cardiac energy substrate, growth signaling, contractility, and cellular stress, were found to be differentially expressed[72]. In a microarray analysis of the soleus muscle, of 28 000 screened transcripts, only three were differentially expressed between sedentary HCR and LCR. In contrast, in trained rats, 116 significantly differentially expressed transcripts were identified, of which many are involved in lipid/fatty acid metabolism[73]. The number of animals in this study was small, however, which may explain the discordant results with mitochondrial protein quantity differences reported by several studies.

Kivelä *et al.* (2010) also studied gene expression pattern in HCR and LCR rats and found 239 known or predicted genes being differently expressed between the lines in a genome-wide microarray analysis[74]. The analysis, which was done using four different clustering methods, revealed that the most enriched gene clusters were related to mitochondria and lipid metabolism.

Both whole body and local oxygen consumption capacity is greater in the HCR line; in the earlier phases of breeding, this seemed to be mostly due to improved O_2 utilization peripherally in the skeletal muscle[75,76]. However, with further selection, at generation 15, the continued improvement of HCR rat relatively to LCR rats was found to result from increased stroke volume[77]. Respiratory capacity of skeletal muscle in HCR rats have been found to be greater compared to LCR, which may be explained by higher oxidative enzyme activity, smaller muscle fibers, and more capillaries[78,79].

Substantial metabolic differences have been identified between the HCR and LCR lines. In addition to the mitochondrial density in skeletal muscle of HCR being higher, it seems like there is also modulation of the respiratory capacity of the mitochondria. Tweedie *et al.* (2010) observed a greater respiratory capacity per mitochondrion in the soleus muscle and a lower respiratory capacity per mitochondrion in the gastrocnemius muscle of adult HCR rats [80], and

Seifert *et al*. (2012) found direct evidence of higher intrinsic OXPHOS capacity in mitochondria isolated from skeletal muscle of HCR rats[81].

RESEARCH QUESTIONS

In this study, the biological basis for the impaired learning in rats bred for low aerobic capacity is examined. The hypothesis is that LCR rats demonstrate inferior aerobic metabolism also in their hippocampus, which contributes to their worse cognitive performance, and that the age-related decline in mitochondrial function is greater in LCR compared to HCR. The main goal is to investigate whether the mitochondrial function in the hippocampus of LCR rats differ from their high aerobic capacity counterparts. The research questions are:

- Is there a difference in oxygen flux at any of the mitochondrial respiratory states of the hippocampus between HCR and LCR, or between young and old animals?
- Is there a difference in mitochondrial sirtuin expression of the hippocampus between HCR and LCR?
- Are there correlations between the respiratory parameters and sirtuin expression?

METHODS

Tissue collection

59 male rats were used for the study (26 LCR and 33 HCR). The rats were housed in groups of two or three in an environment controlled facility at 22°C with 12/12 h light-dark cycle, without access to running wheels. They received water and standard rodent feed (R36, Labfor, Stockholm, Sweden) ad libitum. The young rats were sacrificed at 8 weeks of age (17 HCR, 12 LCR) and the old rats at 40 week of age (16 HCR, 14 LCR). The animals were first stunned in a box with rising CO_2 concentration and after that euthanized by cardiac puncture. The brain was extracted immediately and the left hippocampus was cut out. A slice (~2 mm) from the middle part of the hippocampus was excised for respirometry and stored in a tube with BIOPS medium on ice until analysis. Additional slices (~1 mm) were collected from both distally and proximally to the HRR sample for Western blot analysis and frozen instantly in liquid nitrogen.

Reagents

Antibodies where purchased from Abcam (Cambridge, MA, USA): Total OXPHOS Rodent WB Antibody Cocktail (ab110413), Anti-SIRT3 antibody (ab86671), Anti-SIRT4 antibody (ab10140), Anti-SIRT5 antibody (ab195436), Anti-GAPDH antibody, and Anti-beta tubulin antibody. All reagents were purchased from Sigma-Aldrich except for Tween20 (Fluka), and protease and phosphatase inhibitor cocktail (Thermo Scientific).

High-resolution respirometry

The high-resolution respirometry was performed using the OROBOROS Oxygraph-2k respirometer (Innsbruck, Austria). The hippocampal samples were homogenized using a shredder set provided by the manufacturer (PBI-Shredder HRR-Set). 7-9 mg of wet tissue was weighed and shredded in 0.5 ml of MiR05 medium by 10 s at level 1 and 10 s at level 2. The shredding tube was rinsed with MiR05 to a final volume of 5 ml. MiR05 was prepared according to the manufacturer's protocol: 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM D-sucrose, 1 g/l BSA (fatty acid free).

The OROBOROS was calibrated according to manufacturer's protocol every morning and allowed to stabilize in MiR05 medium for at least 30 min before introducing the sample.

The SUIT (Substrate-uncoupler-inhibitor titration) protocol was carried out in duplicates at 37 °C with 2.0 ml of tissue homogenate in each chamber. We applied the following protocol: 1) 5 mM pyruvate + 2 mM malate + 10 mM glutamate (PGM); 2) 4 mM ADP (saturating) + 2.4 mM free Mg^{2+}; 3) 10 µM cytochrome c (Cytc); 4) 10 mM succinate (Suc); 5) CCCP titration 0.5-2.5 µM; 6) 0.5 µM rotenone (Rot); 7) 2.5 µM antimycin A (AnA). The LEAK state is achieved after adding reducing substrates (PGM) but in the absence of ADP. The OXPHOS state with CI involved is reached by adding ADP, and CI+CII after addition of succinate. This is the maximal rate of OXPHOS. Adding CCCP causes uncoupling of ATP production by allowing H+ flow back through the mitochondrial membrane, skipping the ATPase but forcing maximal rate of electron transfer through the system; this is when ETS capacity is reached. Introducing the inhibitor rotenone will now inhibit complex I, leaving only complex II as a source of electrons. Antimycin A will shut down complex II, after which any remaining oxygen consumption is termed residual oxygen consumption (ROX).

Tissue homogenization for Western blot

The deep frozen hippocampal samples, stored at -80 °C, were homogenized using the Qiagen TissueLyzer II homogenizator. The pieces of tissue were weighted and added to 1.5 ml tubes with steel beads and 200 µl of homogenization buffer: 20 mM HEPES (pH 7.4), 5 mM EGTA, 1 mM EDTA, 0.2 % sodium deoxy cholate, 10 mM $MgCl_2$, 2 mM DTT, 1 % NP-40, 1 % protease phosphatase inhibitor (Thermo Scientific Halt Protease and Phosphatase Inhibitor Cocktail 100X), 1 mM Na_3VO_4, 100 mM β-glycerophosphate. The samples were lysed by 2 x 2 min, 20 Hz, transferred into clean tubes and centrifuged 10 min, 10 000 g. The supernatants were stored at -20 °C.

Western blot

Total protein concentrations were determined in the homogenates by the BCA method in the department's analytical laboratory. Of each sample, 30 µg of protein was loaded into the SDS-PAGE gel (4–20% Criterion™ TGX™ Precast Midi Protein Gel, Bio-Rad) and run at 280 V, 35-45 min. The proteins were the transferred to a nitrocellulose membrane (Amersham Protran 0.45 µm) in wet transfer for 2.5 h, 300 mA. Membranes were then stained in Ponceau S solution and imaged using the Bio-Rad ChemiDoc MP and the software Quantity One. After staining, the membranes were blocked in Odyssey blocking buffer for 1 h, RT, and then incubated O/N in the primary antibody at +4 °C in gentle rocking. The membranes were washed with TBS + 0.1 % Tween20, 4 x 5 min in shaking. The secondary Odyssey antibodies were added and the membranes were incubated for 1 h, RT, then washed again as previously. Imaging was performed using the Odyssey CLx channels 680 and 800 nm.

Data analysis

The O_2 flux signal curves from high-resolution respirometry were analyzed in OROBOROS DatLab 6: On the curve, five respiratory states were marked: leak (L), complex I (CI), complex I + II (CI+CII), electron transfer system capacity (ETS), and complex II (CII). Also the state with complex I + cytochrome C (CI+cytc), and residual oxygen consumption (ROX) were marked for mitochondrial membrane integrity and background oxygen consumption, respectively. O_2 flux values were exported to Excel. ROX levels were subtracted from respiratory states for background correction.

The WB signals were quantified in the Licor Image Studio software. Signals for sirtuins 3, 4, and 5 were normalized both to tubulin on Odyssey (channel 680 nm) and to the β-actin band from Ponceau S -dyed membranes as separate analyses. Signals for the Total OXPHOS proteins were normalized to GAPDH (channel 680 nm). Bands from all five complexes were added together for a measure of total mitochondrial content. WB signal data were exported to Excel and band intensities were normalized to their respective housekeeping protein bands.

Statistics

All HRR and WB data were exported to SPSS, and statistical analysis was performed using the non-parametric independent sample Mann-Whitney U test between groups: HCR vs. LCR, young vs. old. The P-value of 0.05 was chosen as the level of significance.

Two-tailed Pearson correlations were run for the mitochondrial content, sirtuins normalized for mitochondrial content, and respirometry parameters.

RESULTS

High-resolution respirometry

Analysis of high-resolution respirometry data revealed a significant difference in the LEAK respiration state (P<0.05) and LEAK control ratio (P<0.01) between young and old LCR rats (L: 8.83 ± 1.8 vs. 9.67 ± 1.92 pmol/s/mg and L/E: 0.083 ± 0.016 vs. 0.114 ± 0.025, respectively). Old LCR rats also showed a trend towards lower ETS capacity compared to young LCR, although not statistically significant (P<0.1). A significant difference was found between young HCR and old HCR rat OXPHOS (P) (P<0.05) and ETS (E) (P<0.01) states (P: 53.7 ± 5.6 vs. 47.0 ± 9.4 pmol/s/mg, E: 100.9 ± 17.6 vs. 83.3 ± 18.1 pmol/s/mg, respectively). No differences were found between young HCR and young LCR, or old HCR and old LCR (Figure 6, Table 1).

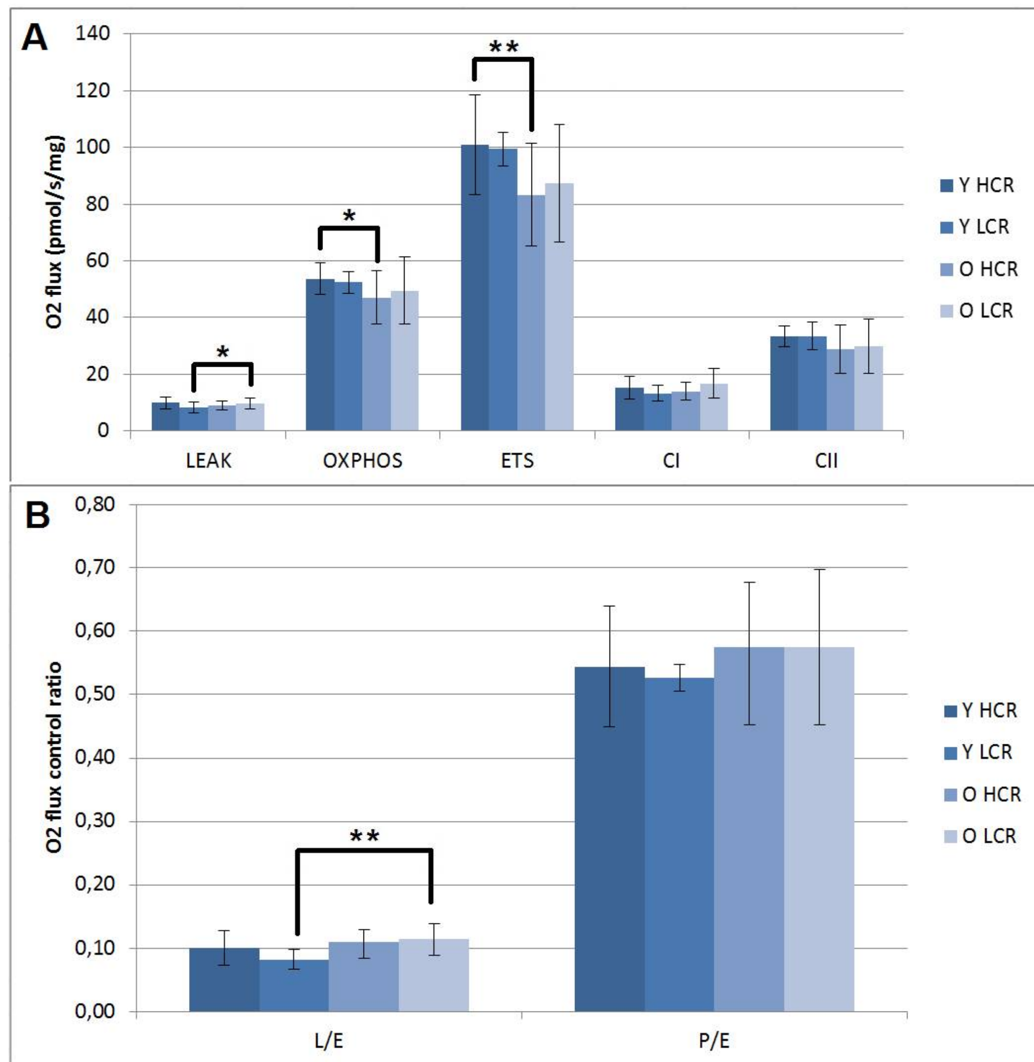

Figure 6. (A) Mean oxygen flux at the respiratory states L, OXPHOS, ETS, CI, and CII. (B) Mean respiratory control ratios L/E and P/E. The error bars represent standard deviations. *Significant at level P<0.05. **Significant at level P<0.01.

Table 1. Results from HRR. Mean O_2 flux at respiratory state LEAK, CI, OXPHOS, ETS, and CII is displayed in units pmol/s/mg. The respiratory control ratios L/E and P/E are dimensionless.

Age	Type		LEAK	CI	OXPHOS	ETS	CII	L/E	P/E
Y	HCR	Mean	9,29	15,30	48,16	85,17	29,30	0,111	0,544
		Median	9,55	14,55	47,21*	82,97**	28,22	0,107	0,515
	LCR	Mean	8,22	13,31	52,38	99,38	33,52	0,082	0,527
		Median	8,83*	14,45	53,15	100,43	33,20	0,089**	0,536
O	HCR	Mean	8,93	13,99	47,04	83,26	28,82	0,109	0,574
		Median	8,85	13,86	44,24*	81,59**	26,68	0,107	0,525
	LCR	Mean	9,70	16,79	49,44	87,35	29,85	0,114	0,576
		Median	10,50*	17,47	48,25	85,30	29,17	0,106**	0,507

* Different between young and old, $P<0.05$ ** Different between young and old, $P<0.01$

Integrity of the outer mitochondrial membrane

Cytochrome c was injected to test the integrity of the outer mitochondrial membrane. CI state respiration increased 5.1 (± 4.9) % following the injection.

Mitochondrial sirtuins

Analysis of sirtuins 3, 4, and 5 did not reveal any statistically significant differences between HCR and LCR in young or old animals, whether using the tubulin or β-actin normalization. Examples of membranes with tubulin and β-actin bands are shown in Figures 8 and 9. Both normalization methods gave similar results and therefore the sirtuin intensities were normalized to both in the final analysis (Figure 7). In addition, the sirtuin were normalized to mitochondrial content, which also did not reveal differences.

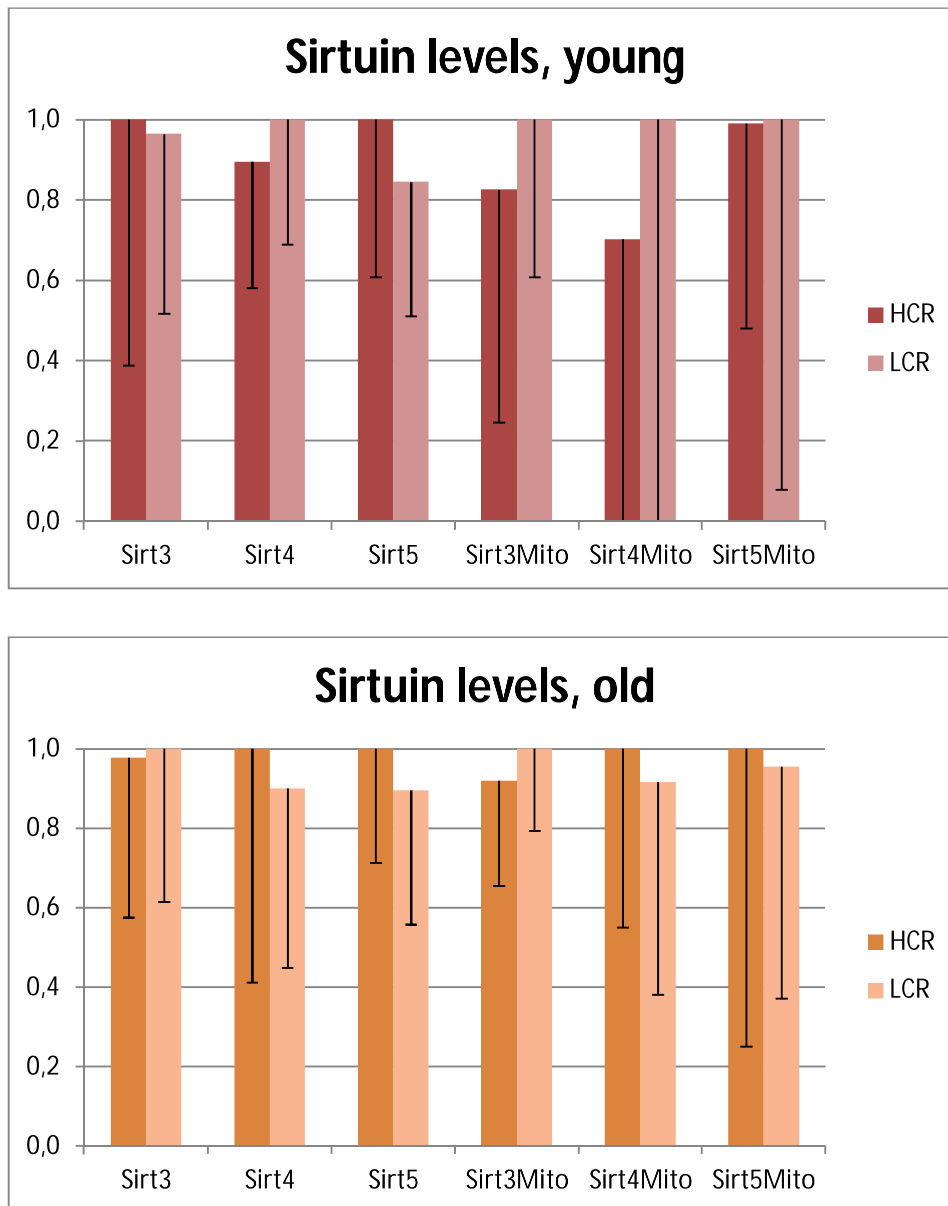

Figure 7. Mean values of WB analysis of mitochondrial sirtuin levels in hippocampal homogenates. Values are given as relative intensities (highest mean = 1.0). No differences were found between the HCR and LCR animals. Two normalization methods were used to verify the results, and the sirtuins were also normalized to mitochondrial content. Sirt3: Sirtuin 3. Sirt3Mito: Sirtuin 3 normalized to mitochondrial content. The error bars represent standard deviations

Different WB signal patterns from young and old animals were visible in the blots, with all sirtuins giving higher signals in the old vs. young. In addition, a double band was visible for sirtuins 3 and 5 for old but only a single band the young (Figure 8).

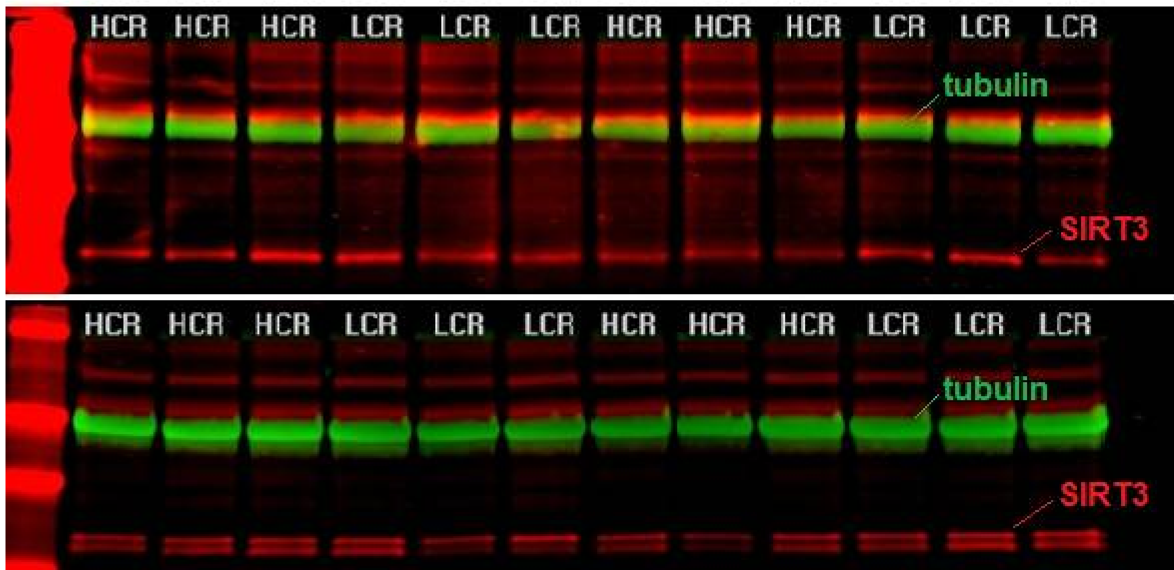

Figure 8. A higher intensity setting was needed for the analysis of membranes with young HCR and LCR rat hippocampus samples (upper image) compared to olds (lower image). In old animals, the sirtuins 3 and 5 appeared as double bands.

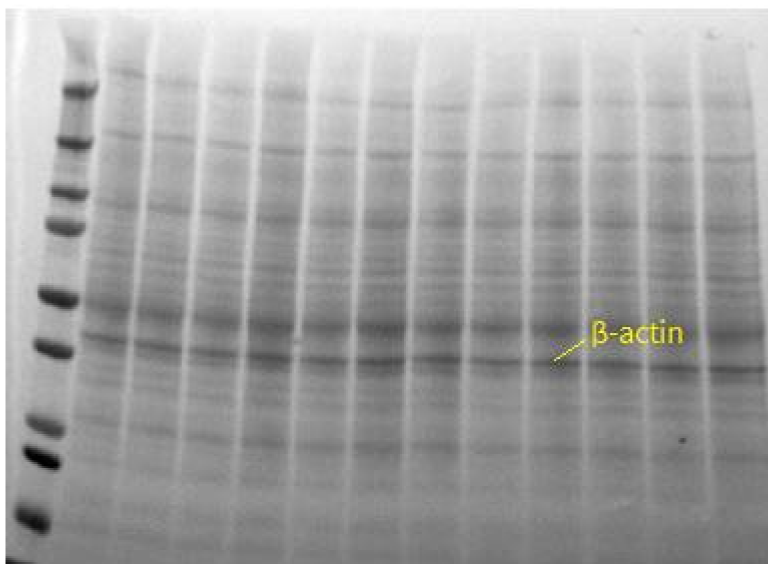

Figure 9. Example of a Ponceau S –stained membrane with a strong β-actin band.

Mitochondrial content

Analysis of total mitochondrial content based on the total WB signal of five mitochondrial complexes did not reveal any significant differences between HCR and LCR in young or old animals (Figures 10, 11).

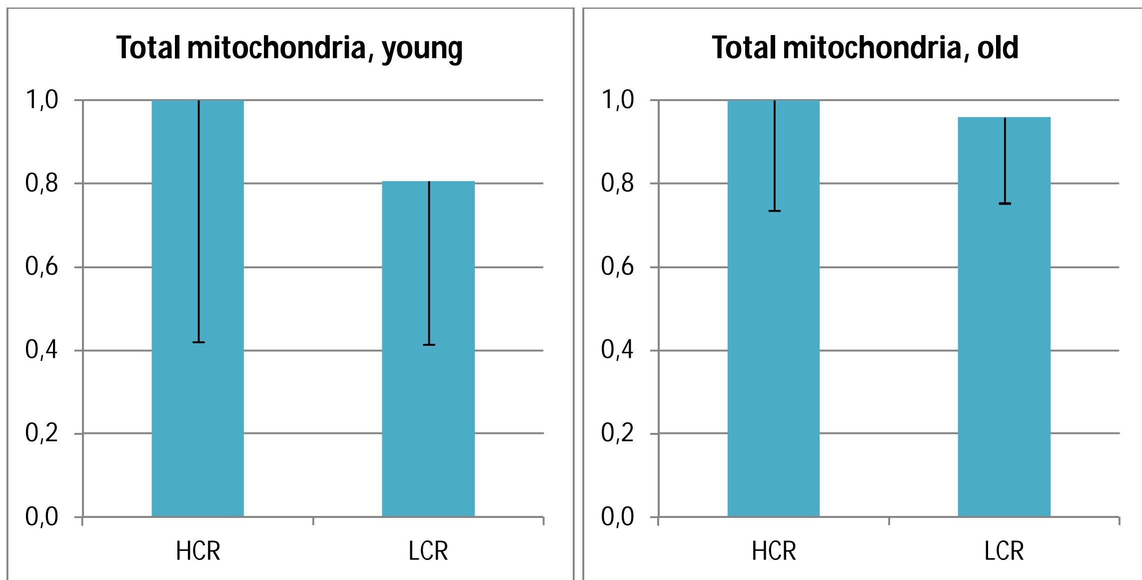

Figure 10. . Results of WB analysis of total mitochondrial content in hippocampal homogenates. Values are shown as relative intensities.

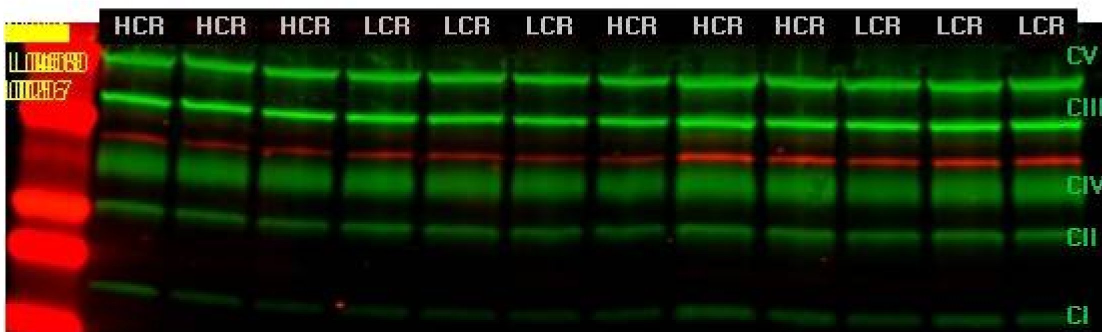

Figure 11. Image of WB using the Total OXPHOS Rodent WB Antibody Cocktail for five mitochondrial complexes on old rat hippocampal homogenates. The loading control GAPDH is visible in red. The signals from CI-CV were added together for a total estimation of mitochondrial content.

Correlations

The correlation analysis showed a positive correlation between mitochondrial content and ETS ($r = 0.32$, $P<0.05$). Sirt4 was positively correlated with CII respiration ($r = 0.35$, $P<0.05$) (Figure 10). ETS was positively correlated with all respiratory states, i.e. LEAK ($r = 0.44$), OXPHOS ($r = 0.71$), CI ($r = 0.67$), and CII ($r = 0.44$; $P<0.01$ for all), and negatively with coupling control ratios L/E ($r = -0.54$) and P/E ($r = -0.52$; <0.01 for both). There was no correlation between CI and CII respiration, CII and L/E, LEAK and CII, or LEAK and P/E (Table 2).

Table 2. Correlations displayed as Pearson Correlation factors with corresponding P-values. Significant correlations are highlighted in green.

		Mito	Sirt3/Mito	Sirt4/Mito	Sirt5/Mito	LEAK	CI	OXPHOS	ETS	CII	L/E	P/E
Mito	Pearson Correlation	1,000	-,670**	-,490**	-,744**	-0,051	0,136	0,147	,315*	0,033	-0,286	-0,214
	Sig. (2-tailed)		0,000	0,000	0,000	0,753	0,396	0,359	0,045	0,839	0,070	0,179
Sirt3/Mito	Pearson Correlation	-,670**	1,000	,484**	,893**	0,065	0,123	0,103	-0,142	0,118	0,163	0,238
	Sig. (2-tailed)	0,000		0,000	0,000	0,688	0,443	0,523	0,377	0,463	0,307	0,134
Sirt4/Mito	Pearson Correlation	-,490**	,484**	1,000	,715**	-0,185	-0,202	0,217	0,056	,349*	-0,184	0,123
	Sig. (2-tailed)	0,000	0,000		0,000	0,246	0,206	0,173	0,730	0,025	0,250	0,443
Sirt5/Mito	Pearson Correlation	-,744**	,893**	,715**	1,000	0,009	0,039	0,117	-0,070	0,151	0,066	0,165
	Sig. (2-tailed)	0,000	0,000	0,000		0,954	0,806	0,466	0,663	0,345	0,680	0,302
LEAK	Pearson Correlation	-0,051	0,065	-0,185	0,009	1,000	,709**	,447**	,442**	0,211	,494**	-0,049
	Sig. (2-tailed)	0,753	0,688	0,246	0,954		0,000	0,001	0,001	0,132	0,000	0,732
CI	Pearson Correlation	0,136	0,123	-0,202	0,039	,709**	1,000	,480**	,670**	0,116	-0,004	-,357**
	Sig. (2-tailed)	0,396	0,443	0,206	0,806	0,000		0,000	0,000	0,412	0,978	0,009
OXPHOS	Pearson Correlation	0,147	0,103	0,217	0,117	,447**	,480**	1,000	,710**	,892**	-0,234	0,218
	Sig. (2-tailed)	0,359	0,523	0,173	0,466	0,001	0,000		0,000	0,000	0,095	0,121
ETS	Pearson Correlation	,315*	-0,142	0,056	-0,070	,442**	,670**	,710**	1,000	,439**	-,544**	-,521**
	Sig. (2-tailed)	0,045	0,377	0,730	0,663	0,001	0,000	0,000		0,001	0,000	0,000
CII	Pearson Correlation	0,033	0,118	,349*	0,151	0,211	0,116	,892**	,439**	1,000	-0,183	,455**
	Sig. (2-tailed)	0,839	0,463	0,025	0,345	0,132	0,412	0,000	0,001		0,193	0,001
L/E	Pearson Correlation	-0,286	0,163	-0,184	0,066	,494**	-0,004	-0,234	-,544**	-0,183	1,000	,506**
	Sig. (2-tailed)	0,070	0,307	0,250	0,680	0,000	0,978	0,095	0,000	0,193		0,000
P/E	Pearson Correlation	-0,214	0,238	0,123	0,165	-0,049	-,357**	0,218	-,521**	,455**	,506**	1,000
	Sig. (2-tailed)	0,179	0,134	0,443	0,302	0,732	0,009	0,121	0,000	0,001	0,000	

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

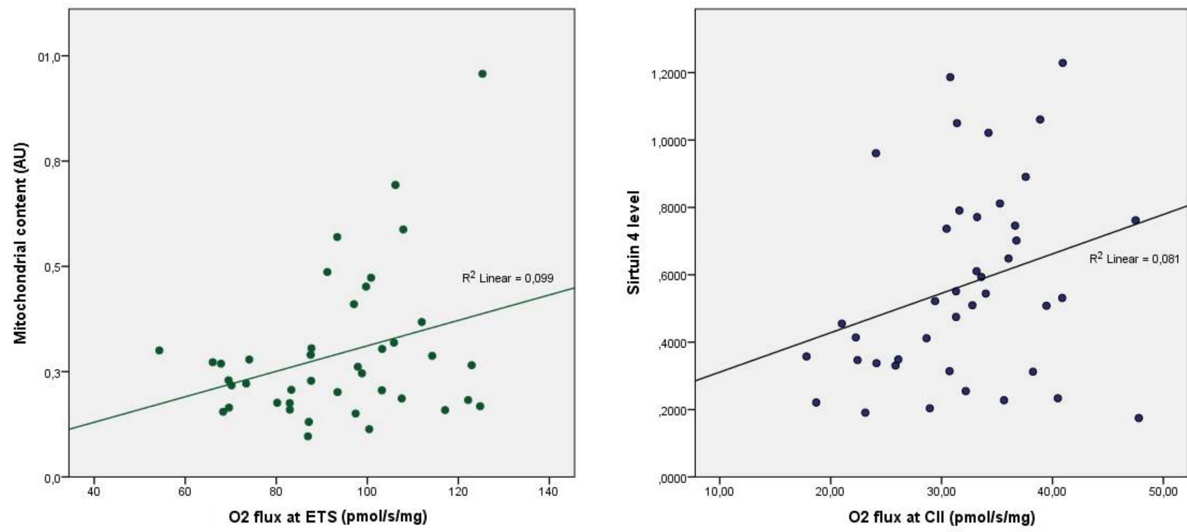

Figure 9. Scatter plot of mito vs. ETS and Sirt4 vs. CII with a linear curve fit. The correlation analysis showed a positive correlation between these varibales.

DISCUSSION

Despite evidence of impaired cognitive function in low-capacity runner rats, mechanisms at the molecular level remain largely unsolved. In this study, we manage to show that aged LCR rats have a higher LEAK respiratory state in their hippocampus than their younger brothers. This, together with a trend of lower ETS capacity, translates to a higher LEAK control ratio, which means higher uncoupling of OXPHOS. An elevated LEAK control ratio has previously been associated with mitochondrial dysfunction in diabetic rat heart[82]. In our study, HCR animals also showed a decrease in OXPHOS and ETS with age, but without an increase in uncoupling; thus, this may be a result of simply lower mitochondrial density without apparent mitochondrial dysfunction. However, protein levels of mitochondrial sirtuins or total mitochondrial respiratory chain complexes did not show statistically significant differences between HCR and LCR animals.

A surprising finding was that mitochondrial sirtuin levels did not differ between HCR and LCR animals. PGC-1α, which is previously been found at higher levels in HCR compared to LCR rats in skeletal muscle[71] as well as in the hippocampus[34], has been shown to stimulate Sirt3 gene expression[83]. On the other hand, Sirt3 may activate PGC-1α via a positive feedback mechanism[84]. As PGC-1α is known for its role in mitochondrial biogenesis, a link between Sirt3 expression and mitochondrial content could be expected. The only correlation revealed by the correlation analysis was between sirtuin 4 and complex II respiratory state, an association that is not found in previous literature and should be confirmed by more studies.

Although not quite reaching statistical significance, young HCR animals showed a trend of higher LEAK state oxygen flux than their LCR counterparts (P=0.01). Earlier research on skeletal muscle energy expenditure has revealed a lower fuel economy of activity in HCR compared to LCR[85,86], but no data from brain tissue energy economics is available. Interestingly, old LCR also showed a non-significant higher LEAK state O_2 flux compared to young LCR. It is possible that slightly different mechanisms explain a high leak in young HCR and in old LCR. Higher expression of uncoupling proteins (UCP) has been found in skeletal muscle of HCR compared to LCR[86,87], which is consistent with what we saw in young animals assuming that this is true also for brain tissue. However, in mitochondrial dysfunction associated with aging, there is also evidence of upregulation of UCP-2 in the brain as a mechanism to protect mitochondria from oxidative damage[88]. This would result in a higher leak, which may explain the trend seen in old LCR.

Despite being a sensitive method, WB has some limitations, of which sample size is one of the prominent ones. An SDS-PAGE gel can fit a limited number of samples and comparison of samples on separate gels easily leads to erroneous results. Drawing conclusions from statistics performed with a sample size of 10-20 per group should be considered carefully. Another source of uncertainty in WB is the housekeeping protein chosen for normalization. Because of the necessary overloading of the proteins in SDS-PAGE in order to get a reasonable signal from sirtuins, the tubulin band, used as the loading control, appeared very broad. Even though settings were adjusted to prevent saturation of pixels, an alternative normalization was performed using the β-actin band on the Ponceau S –dyed membranes for validation. All sirtuins normalized to tubulin were highly correlated with their β-actin normalized values, which confirms the validity of the tubulin-based normalization. Due to a long time period between the collection of the young and old rat samples resulting a prolonged storage time for the hippocampus homogenates of the young, we considered the comparison between young and old animals in WB not reliable. Therefore, results of protein levels between the different ages were not obtained.

High standard deviations were typical in the HRR measurements. As sensitive as HRR is as a method, sample preparation may cause unexpected variation in reproducibility of the measurements. A shredded sample is not an actual homogenate but contains tissue particles of various sizes, and their distribution in the two measurement chambers may vary even after careful pipeting. In addition, it was found that the initial dissolved oxygen concentration in the sample in the beginning of a SUIT titration was highly affected by the time between introducing the sample, starting mixing, and closing the chamber. An ice-cold sample will dissolve more oxygen when mixed, but it is rapidly heated in the small chamber causing a decrease in [O_2] again if not sealed. However, the [O_2] never fell below 100 µmol/l during a SUIT, which should not lead to limited respiration.

In this study, the hippocampal slice used for HRR and WB was taken from the central part of the hippocampus and not specifically from the dentate gyrus, the structure known of high rates of neurogenesis[25,35,89]. It is possible that differences in proteins related to mitochondrial biogenesis and function would be more prominent in this area.

In conclusion, this study was the first one to investigate hippocampal mitochondrial function in young and old rats bred for high and low intrinsic aerobic capacity. The results suggest that aging is indeed associated with a change in mitochondrial function in the hippocampal neurons of low-capacity runner rats, which could be related to the impaired learning seen in

an earlier study from our group[32]. However, the role of sirtuins in this decline seems complex and remains to be investigated by future studies.

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