

The role of resveratrol treatment and regular
physical activity on sirtuins in brain of rats
artificially selected for intrinsic aerobic running
capacity

PhD thesis

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Abbreviations

8-oxoG	8-oxoguanine
A β	Amyloid beta
AcOGG1	AcetylatedOGG1
ADP	Adenosine diphosphate
AMPK	5' AMP-activated protein kinase
APE1	AP Endonuclease1
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
β -AKT	Beta-Actin
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CH	Control high capacity of running
CL	Control low capacity of running
CREB	cAMP response element-binding protein
ERK1-2	Extracellular-signal-regulated kinases
FOXO1-4	Forkhead box protein O1-4
GPx	Glutathione peroxidase
HCR	High capacity of running
HDACI-II	Histone deacetylases I-II
LCR	Low capacity of running
MAPK	Mitogen-activated protein kinases
MnSOD	Manganese superoxide dismutase
mTOR	Mammalian target of rapamycin
NAD ⁺ /NADH	Nicotinamide adenine dinucleotide
NAM	Nicotinamide
NAMPT	Nicotinamide phosphoribosyltransferase
NeuN	Neuronal Nuclei
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells

OGG1-2	8-oxoguanine glycosylase 1-2
p300	Histone acetyltransferase
p53	Protein 53
p-AMPK	phospho-5' AMP-activated protein kinase
PAR	Poly ADP-ribose
PARP1	Poly [ADP-ribose] polymerase 1
PBEF	pre-B-cell colony-enhancing factor 1
PGC1- α	Peroxisome proliferator-activated receptor gamma coactivator 1- α
PPAR α	Peroxisome proliferator-activated receptor α
PPAR γ	Peroxisome proliferator-activated receptor γ
ROS	Reactive oxygen species
RsvH	Resveratrol treated high capacity of running
RsvL	Resveratrol treated low capacity of running
sir2/sirtuin	Silent mating-type information regulation 2
siRNA	Small interfering RNA
SIRT1-7	Human sir2 homologues
SOD-1	Superoxide dismutase 1
TrH	Training high capacity of running
TrL	Training low capacity of running
TrRsvH	Training+Resveratrol treated high capacity of running
TrRsvL	Training+Resveratrol treated low capacity of running
VO ₂ max	Maximal aerobic capacity

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1. Introduction

1.1 Sirtuins – A new promise of a longer life

One of the biggest wishes of mankind is that of eternal life. Although it is not possible, humanity is still seeking new ways to elongate life. Great milestones show the way that science has already walked on this path. For example we know more and more about cell differentiation, cell proliferation, apoptosis ...etc. every day. These processes are built up like a symphony of molecular mechanisms. In the orchestra one of the most important members is DNA (Deoxyribonucleic acid), surrounded by histones. Among many functions histones protect the genetic information by making it untouchable for harmful molecules. In these reactions the key regulators are the histone deacetylases. There are four classes of deacetylases in mammals, among them, enzymes in class III are unique ones. They are referred to as silent information regulator two proteins (sirtuins), named after their yeast homologue (sir2). (Afshar & Murnane, 1999) Since their first debut this family of molecules has been quoted many times as promises of a longer life.

1.1.1 General characterization of sirtuins

Sirtuins are NAD⁺-dependent (Nicotinamide adenine dinucleotide-dependent) enzymes that are phylogenetically conserved from archeobacteria to humans. Sequence alignment studies on the sirtuin proteins show that they contain a conserved catalytic core consisting of approximately 275 amino acid residues and variable N- and C- terminal chains. (Sanders, Jackson, & Marmorstein, 2010) The catalytic core exists in an elongated shape and consists of a large Rossmann fold domain (which is a hallmark of NAD⁺/NADH-binding proteins), a structurally more diverse but smaller zinc-binding domain and many loops connecting these domains (as shown on Figure 1.).

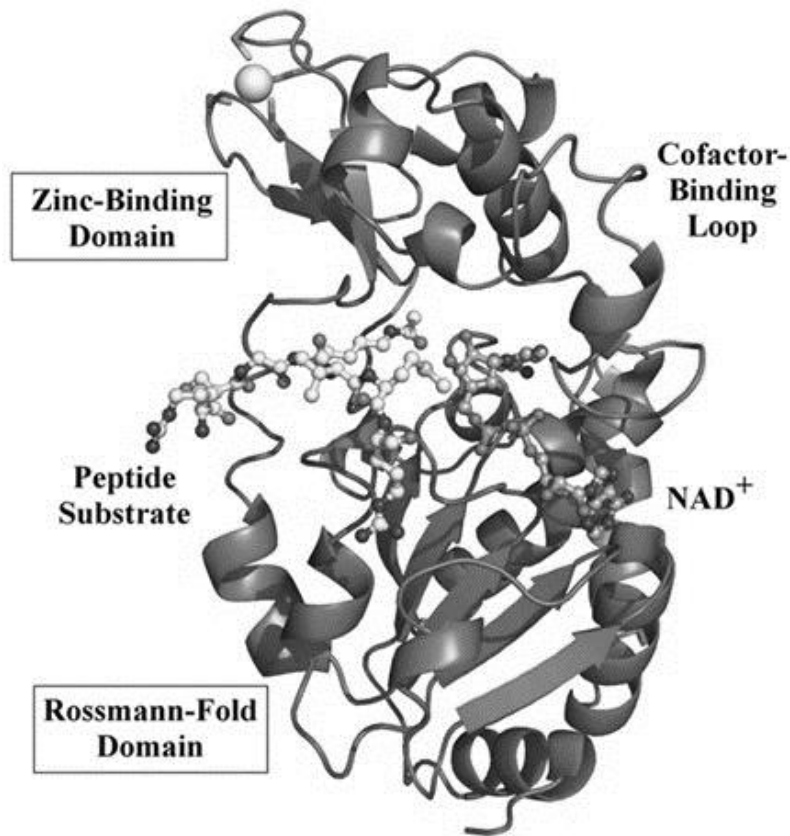


Figure 1: Structure of sirtuins

The catalytic core consists of a large Rossmann fold domain, a smaller zinc-binding domain and many loops connecting these domains. (Moniot, Weyand, & Steegborn, 2012)

The Rossmann fold is the large domain of the catalytic core exhibiting the classical α/β Rossmann fold structure. The Rossmann fold is a protein structural motif found in proteins that bind nucleotids, especially the cofactor NAD^+ . It has all the characteristics of a NAD^+ -binding site including a Gly-X-Gly sequence for phosphate binding, a pocket for NAD^+ and charged residues for the ribose moiety. An unusual characteristic of this family of enzymes is that the NAD^+ binds in an inverted direction relative to most NAD^+ dehydrogenases. (Finnin, Donigian, & Pavletich, 2001) The Zn-binding domain is a small domain that results from two insertions in the Rossmann fold represents the most diverse region among sirtuin family members. Two structural modules have been suggested for the domain: a three-stranded antiparallel β -sheet and a

variable α -helical region depending on the type of the sirtuin. The structural diversity observed in the small domain may potentially have a role in substrate specificity and protein-protein interactions, thus finally affecting the enzyme function. (Zhao, Harshaw, Chai, & Marmorstein, 2001) At the acetyl-lysine binding site the peptide substrate binds to the cleft between the small and large domains with the acetyl-lysine side chain sliding into a hydrophobic tunnel within the cleft. The peptide backbone of the acetyl-lysine chain forms β -sheet-like interactions flanked by two strands in the enzyme to form a three-stranded antiparallel motif called a β -staple. (Cosgrove, et al., 2006) It has been suggested that NAD^+ and peptide may bind co-operatively, considering that the region around the peptide binding site adopts a more closed conformation in the presence and not in the absence of bound NAD^+ . (Avalos, et al., 2002) The crux of peptide binding to sirtuins is said to be mainly controlled by the insertion of the acetyl-lysine into the conserved hydrophobic tunnel and formation of the β -staple as discussed previously. The substrate specificity of sirtuins is more or less thought to lie outside the highly conserved catalytic core of the enzyme. However, it was observed that some sirtuins could distinguish between substrates that have multiple acetylated lysine-residues, suggesting that the catalytic core could be sufficient for substrate specificity. (Garske & Denu, 2006) (Borra, Langer, Slama, & Denu, 2004) Until this question is answered theoretically every protein with acetyl-lysine binding site can be substrate of sirtuins.

Sirtuins have either deacetylase or mono-ADP-ribosyl transferase activity or both as shown on Figure 2. Both reactions are strictly dependent on NAD^+ . After NAD^+ and the substrate peptide attached to a sirtuin it will cleave the acetyl group and pass it to the NAD^+ . NAD^+ meanwhile will hydrolyse into nicotinamide and 2'-O-acetyl-ADP-ribose. The dependence of sirtuins on NAD^+ links their enzymatic activity directly to the energy status of the cell via the cellular NAD^+ : NADH ratio, the absolute levels of NAD^+ , NADH or nicotinamide or a combination of these variables.

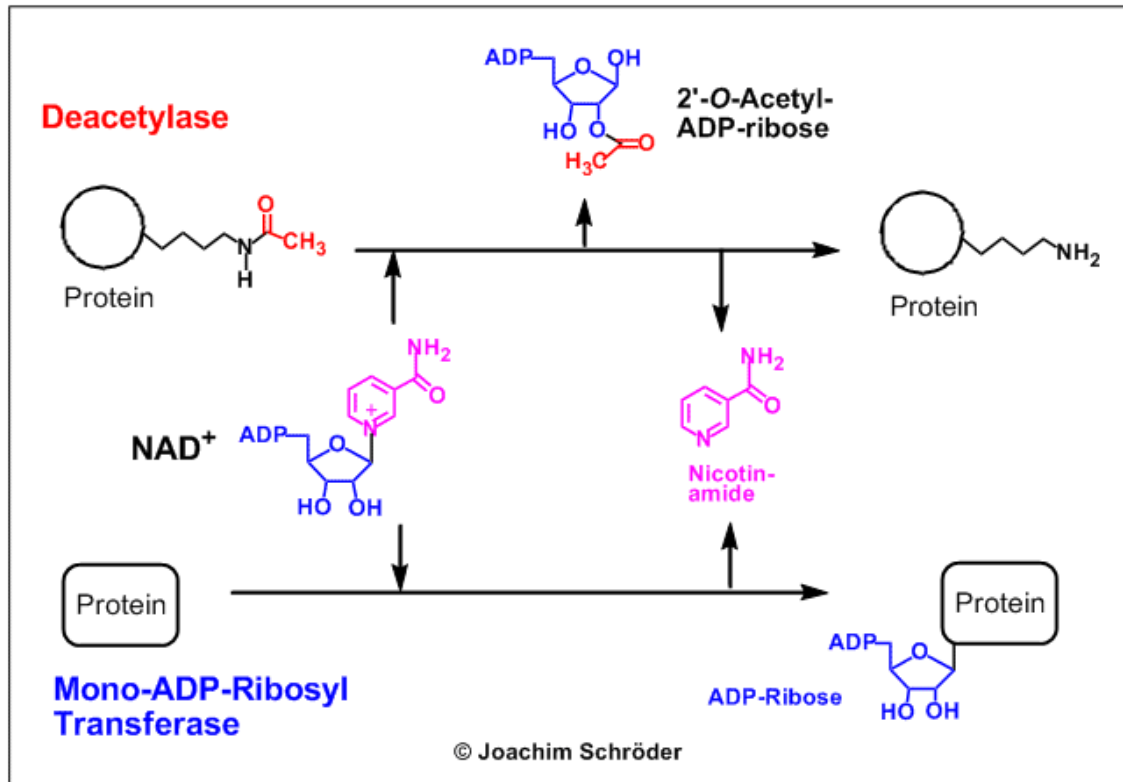


Figure 2: The main enzymatic activities of sirtuins

Sirtuins have either deacetylase or mono-ADP-ribose transferase activities or both. (Michan & Sinclair, 2007)

Mammals possess seven types of sirtuins. Here I collect the main data in a nutshell about each member, particularly about their function in the nervous system.

1.1.2 SIRT1

This is the best known member among the sirtuin family. It was originally documented to localize in the nucleus, but later studies confirmed that SIRT1 (Sirtuin 1) is able to shift between the nucleus and the cytoplasm. There are target molecules on both sides which can be deacetylated by SIRT1. This was the first sirtuin in which the deacetylation function was proved at first on histone targets, later on several protein targets in the cell. SIRT1 is expressed in every cell of the body in a tissue-specific manner of course. During mouse embryogenesis, SIRT1 is highly expressed in the brain, spinal cord, and dorsal root ganglion, with the peak expression at E4.5. (Salminen & Kaarniranta, 2012) SIRT1 is also expressed in the adult brain, at high levels in the

cortex, hippocampus, cerebellum, and hypothalamus, and at low level in white matter. Among the various cell types of brain, SIRT1 is predominantly, if not exclusively, expressed in neurons. (Adler, et al., 2007) The only exception is that SIRT1 is found in microglia when co-cultured with neurons. (Schmitz, Mattioli, Buss, & Kracht, 2004) SIRT1 is abundantly expressed in several regions in the hypothalamus of mice, especially in the arcuate, paraventricular, ventro- and dorsomedial nuclei; so calorie restriction increases SIRT1 levels in the hypothalamus, which increases body temperature, food intake, and physical activity. (Ramadori, et al., 2008) (Dietrich, et al., 2010)

SIRT1 is involved in several different mechanisms during the cells' life, so it has to be precisely regulated on all levels:

- Transcriptional upregulation: The basal level of SIRT1 is regulated by the transcription factor E2F transcription factor 1 (E2F1), through binding to the SIRT1 promoter at a consensus site. Cellular stresses increase the transcriptional activity of E2F1 and upregulate the level of SIRT1. (Wang, et al., 2006) FOXO1 (Forkhead box protein O1) binds to several consensus sites within the SIRT1 promoter and enables its transcription. (Xiong, Salazar, Patrushev, & Alexander, 2011) FOXO3a is another SIRT1 regulator. Starvation in mammal cells activates FOXO3a and consequently augments SIRT1 expression. (Nemoto, Fergusson, & Finkel, 2004)
- Transcriptional downregulation: Hypermethylated in cancer 1 (HIC1) binds SIRT1, forming a transcriptional repression complex through its N-terminus. This complex directly binds to the SIRT1 promoter, and thereby inhibits SIRT1. (Chen, et al., 2005) A recent study showed that PPAR γ (Peroxisome proliferator-activated receptor γ) binds the promoter of SIRT1 and inhibits its expression. (Han, et al., 2010)
- Post-transcriptional regulation: MicroRNAs are a group of short RNAs with an average length of 22 nucleotides. They cause gene silencing by binding to complementary sequences on their target mRNAs, leading to the degradation of mRNAs. Several microRNAs have been identified that reduce SIRT1 expression, including miR-9, miR-34a, miR-132, miR-181 and miR-199, miR-217. (Saunders, et al., 2010) (Lee & Kemper, 2010) (Menghini, et al., 2009)

Also an RNA-binding protein, HuR associates with the 3' untranslated region of the Sirt1 mRNA. This interaction leads to increased stability of Sirt1 mRNA, promoting the translation of SIRT1. (Abdelmohsen, K; Pullmann, R Jr, et al., 2007)

- Regulation by other proteins: An active regulator of SIRT1 (AROS) is a recently identified nuclear protein that increases the deacetylating activity of SIRT1. (Kim, Kho, Kang, & Um, 2007) In contrast, deleted in breast cancer-1 (DBC1), another nuclear protein, functions as a negative regulator. (Chini, Escande, Nin, & Chini, 2010)
- Post-translational regulation: Sumoylation and desumoylation of SIRT1 can function as a molecular switch to regulate SIRT1 activity in response to cellular stresses. (Zschoernig & Mahlknecht, 2008) It is reported that c-jun N-terminal kinase 1 (JNK1) phosphorylates two serine residues plus Thr530 of SIRT1. (Nasrin, et al., 2009) The phosphorylation of SIRT1 occurs under oxidative stress and increases the nuclear translocation and enzymatic activity of SIRT1. The cell cycle checkpoint kinases (CHKs) and dual specificity tyrosine phosphorylation-regulated kinases (DYRKs) are also groups of kinases that phosphorylate SIRT1 to increase its activity. On the other hand a recent study showed that SIRT1 is phosphorylated by mammalian sterile 20-like kinase 1 (MST1) after induced DNA damage, leading to reduced activity of SIRT1. (Yuan, et al., 2011)
- Metabolic regulation: According to the deacetylation mechanism of SIRT1, it is a reasonable prediction that substrates of SIRT1 will activate it, and products of the reaction will inhibit it. Therefore NAD^+ can increase the enzymatic activity of SIRT1, whereas nicotinamide and 2'-O-acetyl-ADP-ribose may inhibit it. (Neugebauer, Sippl, & Jung, 2008) (Tong & Denu, 2010)
- Pharmacological regulation: Splitomicin and sirtinol, the frequently used SIRT1 inhibitors, are effective both in vitro and in vivo. On the contrary, SIRT1 can be activated by several small-molecule compounds. Most of these activators are polyphenols including resveratrol, quercetin, curcumin, and catechins.

Initially SIRT1 was documented as a histone deacetylase. In nuclei, core histones, including H2, H3 and H4, form ball-like structures for the DNA to wrap around,

keeping DNA in a resting status. When acetylated on the lysines, however, histones lose their positive charges and release DNA, allowing DNA unwinding and subsequent gene transcription. In contrast, deacetylation polarizes histones and promotes their binding to DNA, leading to genome-wide but non-specific transcription silencing. (Imai, Armstrong, Kaeberlein, & Guarente, 2000) Since that time it has been demonstrated that SIRT1 has several protein targets both in nucleus and cytoplasm. It is complicated to summarize these interactions, therefore I chose to do it briefly with a help of a table in alphabetical order (Table 1.).

Table 1: Summary of SIRT1 deacetylation's known targets

Target	Target's function	SIRT1's Effect	Literature
APE1 (AP Endonuclease1)	base excision repair enzyme	↑	(Yamamori, et al., 2010)
eNOS (endothelial Nitric oxide synthase)	vasodilatator which generates endothelial nitric oxide	↑	(Mattagajasingh, et al., 2007)
FOXO1 (Forkhead box protein O1)	transcription factor that plays important roles in regulation of gluconeogenesis and glycogenolysis	↓	(Yang, Hou, Haller, Nicosia, & Bai, 2005)
FOXO3 (Forkhead box protein O3)	upregulates pro-apoptotic factors, but antioxidants as well	↑	(Wang, Nguyen, Qin, & Tong, 2007)
FOXO4 (Forkhead box protein O4)	promotes survival against oxidative stress	↑	(van der Horst, et al., 2004)
HIFs (Hypoxia-inducible factors)	hypoxia-inducible factors	↑	(Dioum, et al., 2009) (Lim, et al., 2010)
Ku70	double-stranded DNA break repair	↑	(Jeong, et al., 2007)
LKB-1 (Liver kinase B1)	activates AMPK	↑	(Zu, et al., 2010)

NF- κ B (Nuclear factor kappa-light-chain-enhancer of activated B cells)	upregulates pro-inflammatory mediators	↓	(Wang, et al., 2009)
NRF2 (Nuclear factor erythroid 2-related factor 2)	cytoprotective during oxidative stress, carcinogenic when overactivated	↓	(Kawai, Garduño, Theodore, Yang, & Arinze, 2011)
p300 (Histone acetyltransferase)	histone acetyltransferase, promoting the transcription of other transcription factors	↓	(Bouras, et al., 2005)
p53 (Protein 53)	upregulates pro-apoptotic molecules (Bcl-2 family)	↓	(Luo, et al., 2001)
PARP-1 (Poly [ADP-ribose] polymerase 1)	single-stranded DNA break repair	↓	(Rajamohan, et al., 2009)
PGC-1 α (Peroxisome proliferator-activated receptor gamma coactivator 1- α)	increase mitochondrial energy metabolism	↑	(Nemoto, Fergusson, & Finkel, 2005)
PPAR- α (Peroxisome proliferator-activated receptor α)	increases anti-inflammatory mechanisms	↑	(Deplanque, et al., 2003)
PPAR- γ (Peroxisome proliferator-activated receptor γ)	downregulates inflammatory cytokines	↓	(Shie, Nivison, Hsu, & Montine, 2009)

RAR- β (Retinoic acid receptor β)	increases the transcription of α -secretase	↑	(Donmez G. , Wang, Cohen, & Guarente, 2010)
Tau	microtubule-binding protein, promotes the assembly of microtubules	↓	(Min, et al., 2010)
XPA (Xeroderma pigmentosum, complementation group A)	nucleotide excision repair enzyme	↑	(Fan & Luo, 2010)
α -secretase	process soluble APP which helps avoid Alzheimer's disease	↑	(Donmez G. , Wang, Cohen, & Guarente, 2010)

1.1.3 SIRT2

This protein is mostly found in the cytoplasm and has both deacetylase and mono-ADP-ribosyl transferase activity. (North, Marshall, Borra, Denu, & Verdin, 2003) New data uncovered a novel role for SIRT2 opening new perspectives for therapeutic intervention in neuroinflammatory disorders. Reduction of SIRT2 in microglia dramatically increased the expression of inflammatory markers, the production of free radicals, and neurotoxicity. Consistent with increased NF- κ B-dependent transcription of inflammatory genes, NF- κ B was found hyperacetylated in the absence of SIRT2. (Pais, et al., 2013) So SIRT2 has been related to synaptic plasticity, learning and memory that are also in turn related to neuronal motility and migration. Kireev and colleagues showed significantly decreased SIRT2 expressions in the dentate gyrus of old male rats. Interestingly melatonin or growth hormone treatments have been shown to modulate the pro-antiapoptotic ratio and increase SIRT2's expression restoring the situation found in young animals. (Kireev, Vara, & Tresguerres, 2013)

1.1.4 SIRT3

Among the known sirtuins SIRT3 was identified as a stress responsive deacetylase recently shown to play a role in protecting cells under stress conditions. It has been proven that SIRT3 is a mitochondrial protein with a robust deacetylase activity. Mitochondria are the major site for the generation of the reactive oxygen radical superoxide, and also where the superoxide is dismutated by mitochondrial MnSOD (Manganese superoxide dismutase). Recent reports show that SIRT3 deacetylates MnSOD at Lys122 and increases its activity, reducing oxidative and radiation stress in mice. Overexpression of SIRT3 protects HEK293 from oxidative stress and prevents age-related cochlear cell death in mice. (Someya, et al., 2010) Overall these observations suggested anti-oxidative and neuroprotective roles of SIRT3. Furthermore, it was found that mitochondrial SIRT3 is increased following PARP-1 mediated NAD⁺ depletion in neurons, which can be reversed by either inhibition of PARP-1 or exogenous NAD⁺. (Kim, Lu, & Alano, 2011) The massive amount of ROS produced under this NAD⁺ depleted condition mediates the increase in mitochondrial SIRT3. By transfecting primary neurons with a SIRT3 overexpressing plasmid or SIRT3 siRNA, it was shown that SIRT3 is required for neuroprotection against excitotoxicity. The ability of calorie restriction to induce SIRT3 expression has been well documented. (Kincaid & Bossy-Wetzel, 2013) Additional stimulators of the sirtuins have also been proposed, such as resveratrol, a polyphenol found in red wine. Treatment with resveratrol did not affect SIRT3 expression levels. However, a recent report found that a resveratrol derivative, trans-(-)- ϵ -viniferin, is able to increase SIRT3 expression and provide protection in cell models of Huntington's disease (HD). Specifically, viniferin treatment of striatal precursor cells overexpressing mutant huntingtin resulted in increased SIRT3 expression, increased the NAD⁺/NADH ratio, reduced intracellular ROS accumulation, and decreased acetylated MnSOD levels. Thus, SIRT3 is required for viniferin-mediated neuroprotection in HD models. (Fu, et al., 2013)

1.1.5 SIRT4

This enzyme also can be found in mitochondria but shows only mono-ADP-ribosyltransferase activity in a NAD^+ -dependent manner. There are some publications regarding SIRT4's role in metabolic homeostasis but none of these focus on neural tissues. It has recently been reported that congenital hyperinsulinism/hyperammonemia (HI/HA) syndrome is caused by an activation mutation of glutamate dehydrogenase 1 (GDH1), a mitochondrial enzyme responsible for the reversible interconversion between glutamate and α -ketoglutarate. (Komlos, et al., 2013) GDH1 is allosterically regulated by many factors, and has been shown to be inhibited by SIRT4. SIRT4 is highly expressed in glial cells, specifically astrocytes, in the postnatal brain and in radial glia during embryogenesis. The authors also found that SIRT4 and GDH1 overexpression play antagonistic roles in regulating gliogenesis.

1.1.6 SIRT5

SIRT5 is a mitochondrial sirtuin which can demalonylate and desuccinylate proteins (Peng, et al., 2011) in particular the urea cycle enzyme carbamoyl phosphate synthetase I (CPS I). (Du, et al., 2011) Glorioso and colleagues investigated the transcriptome changes during "normal" human brain aging by microarray analysis in two cohorts and four brain areas, focusing on the overlap of aging and disease pathways, and then tested whether subject molecular brain aging rates were associated with several candidate longevity gene polymorphisms. In support of a genetic modulation or control of this molecular aging-by-disease risk model, they showed that the cross-sectional trajectory of a large component of molecular aging was differentially affected in subjects carrying a common polymorphism in the SIRT5 putative longevity gene (SIRT5prom2), which they also show correlated with reduced SIRT5 expression. Based on these results, they predict that SIRT5-risk allele (C/C) carriers may be at increased risk for mitochondrial-dysfunction related disorders, including Parkinson's and Huntington's diseases. (Glorioso, Oh, Douillard, & Sibille, 2011)

1.1.7 SIRT6

According to a study (Schwer, et al., 2010) the highest level of Sirt6 mRNA was detected in the brain, heart and liver and the lowest expression level was observed in skeletal muscle. They also proved that SIRT6 protein is localized in the nucleus of cells. They could not show any deacetylase activity but they could detect a great mono-ADP-ribosyltransferase activity. Later researchers could provide evidence that SIRT6 deacetylates histone H3K9 and H3K56. (Yang, Zwaans, Eckersdorff, & Lombard, 2009) (Michishita, et al., 2009) Until recently, there was no possibility to study Sirt6 knockout mice, because this deletion was found to be lethal.

In 2010 a group of scientists was able to generate a neural-specific Sirt6 knockout mouse to study the roles of SIRT6 in the central nervous system. (Schwer, et al., 2010) KOSirt6 mice appeared normal at birth, but at 4 weeks of age they were significantly smaller. They also demonstrated that neural-specific Sirt6 deletion, likely through reduced GH and hypothalamic neuropeptide levels – Proopiomelanocortin (POMC), Single-minded homolog 1 (SIM1), and Brain-derived neurotrophic factor (BDNF) -, promotes adult-onset obesity in mice.

1.1.8 SIRT7

This sirtuin is located in the nucleoli, but there were no clue for a long time what kind of enzymatic activity it has. In 2013 Tsai and co-workers evidenced that SIRT7 regulates rDNA transcription and that reduced SIRT7 levels inhibit tumor growth. (Tsai, Greco, & Cristea, 2013) A key feature of cancer cells is uncontrolled proliferation that ultimately overcomes the intrinsic limit of mitotic cycles. However, tumor cells must achieve a critical cell mass before committing to another round of cell division to increase the tumor cell population. Ribosome synthesis is a key process necessary to fulfill the required cell mass. This group of scientists presents the first experimental evidence that SIRT7 interacts with proteins involved in ribosome biogenesis (DNA Polymerase I,III through mammalian target of rapamycin (mTOR)), and that its levels are critical for regulating protein synthesis.

More than 2000 publications are dedicated to the therapeutic potential of sirtuins. A main trend is to activate sirtuins via a natural, dietary way. To start a diet like this, it is suggested to consume several types of fruits, vegetables and nuts. These foods contain high amounts of antioxidants. A huge section of antioxidants are polyphenols.

1.2 Resveratrol – All good things come from fruits

Polyphenols are naturally occurring phytochemicals which are present within fruits, vegetables and natural products. These phytoalexins found in the tissues of a widespread range of plants, they are characterized by the presence of multiple hydroxyl groups on aromatic rings. They can be divided into two main categories - flavonoids and non flavonoids. There are several subcategories based on the chemical structures as it is nicely demonstrated on the figure of David Vauzour (Figure 3.):

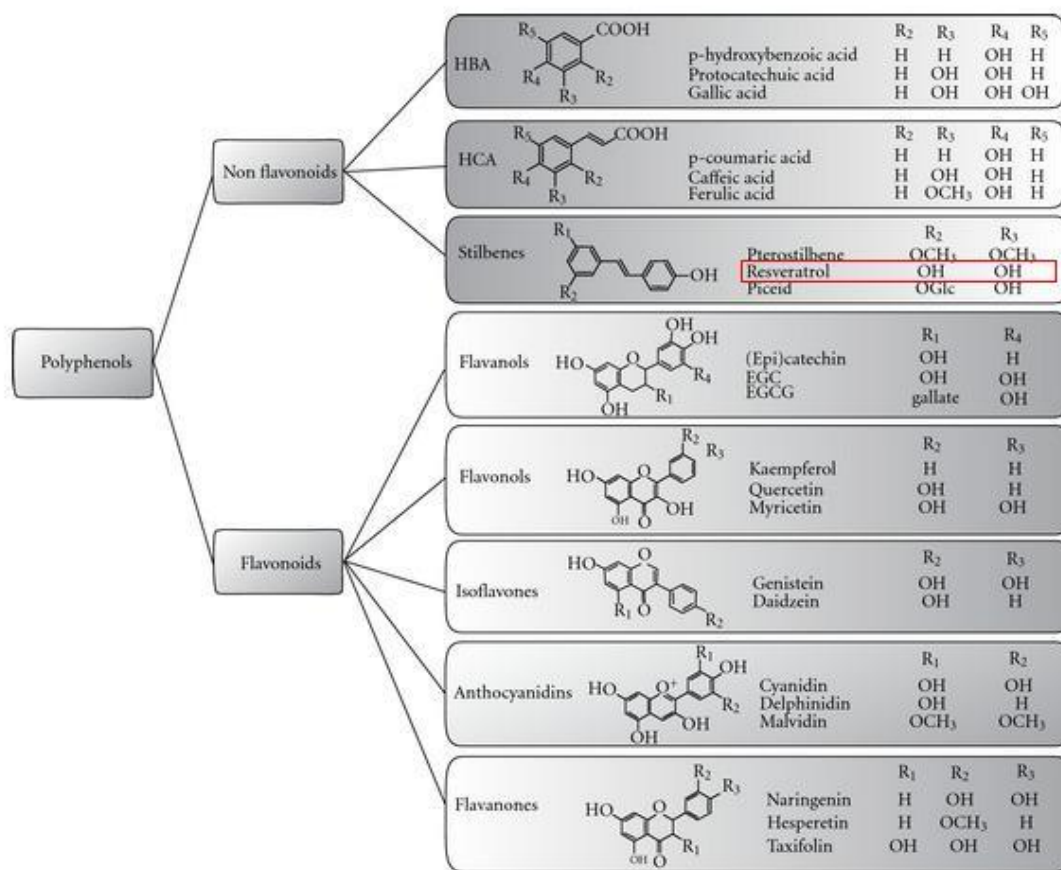


Figure 3: The two main groups of polyphenols

The two main groups of polyphenols are flavonoids and non flavonoids. Within the group of non flavonoids a huge category is stilbenes. In our study we used resveratrol, a stilbene with hydroxyl functional groups associated to the aromatic rings. (Vauzour, 2012)

Within the non flavonoids' group a huge category is stilbenes. Stilbenes possess a 1,2-diarylethenes structure based on the C₆-C₂-C₆ backbone and are usually synthesized in plants in response to infection or injury. Resveratrol (3,5,4'-trihydroxy-stilbene), the main stilbene, can be found in cis or trans configurations. Major dietary sources of resveratrol include grapes, wine and peanuts.

When polyphenols are applied either via intravenous or oral route, the biggest question is if they are capable to overpass the blood-brain barrier or not. Using *in vitro* models, initial studies have demonstrated that polyphenols permeation through the barrier is dependent on the degree of lipophilicity of each compound, so less polar polyphenols capable of greater brain uptake than the more polar ones. (Youdim, et al., 2003) Studies suggest that polyphenols usually localize in the brain at levels below 1 nmol/g tissue and usually accumulates in a nonregion-specific manner. ¹⁴C-labelled grape polyphenols did not show any regional differences in ¹⁴C accumulation from anterior to posterior slices of the brain. (Janle, et al., 2010) Although resveratrol accumulates in a low level in the brain there are already publications where its beneficial effects are discussed. Resveratrol has been reported to be effective in the experimental autoimmune encephalomyelitis with rises in IL-17/IL-10 ratio and with repressed macrophage IL-6 expression. (Imler & Petro, 2009) It was observed to protect PC12 cells against H₂O₂-mediated oxidative stress (Chen, Jang, Li, & Surh, 2005) and to attenuate cerebral ischemic injury in rat. (Ren, Fan, Chen, Huang, & Yang, 2011) It has been shown that SIRT1 is also activated by resveratrol resulting in cell survival, but it is still under investigation if this activation happens in a direct or an indirect way. Indirect way could happen through a signaling cascade involving cyclic adenosine monophosphate (cAMP), exchange proteins activated by cyclic AMP (Epac1) and 5' AMP-activated protein kinase (AMPK). (Park, et al., 2012)

To sum up the previous facts: sirtuins may have the potential to elongate life. To obtain this it seems to be useful to elevate the level of consumed polyphenols. Although eating antioxidants have several well-documented advantages, for a healthy body everyone should do one more thing: to have some regular exercise!

1.3 Exercise – The way to a long and healthy life

If we want to look for the origin of human body and human genetics we need to look further back a few thousand years. Originally we were built up to walk and run tens of miles a day seeking for fruits to collect or animals to hunt. During the past few thousand years life became much easier, or actually too easy. In developed countries people take up a huge protein and sugar surplus day by day. On the other hand we forgot about our in-built locomotion needs. Of course there are honorable exceptions for those who practice some kind of regular exercise. Statistically a cleft is about to open up between these two types of people, so in biology there was a need of a new model which can illustrate the aforementioned differences.

Lauren G. Koch and Steven L. Britton generated a rat model which is close enough to characterize the biological differences between the two extremities. They undertook a large-scale selective breeding program to develop rat lines that would diverge widely for intrinsic aerobic capacity. Six generations of selection produced lines that differed in running capacity by 171%, with most of the change occurring in the high capacity of running line. (Koch & Britton, 2001) Four years later Wisløff et al. characterized the 11th generation of the same model. By this time the low capacity runners (LCR) and high capacity runners (HCR) differed in running capacity by 347%. LCR animals on the other hand started to represent the average person with metabolic syndrome. They even showed all the risk factors linked to metabolic syndrome: weight gain, high blood pressure, reduced endothelial function, hyperinsulinaemia and increased triglyceride concentration in blood. (Wisløff, et al., 2005) In 2008 I had the chance to work with 24 low capacity of running (LCR) and 24 high capacity of running (HCR) male rats from the 22nd generation. Compare to previous works with this animal model I did not focus on cardiac nor skeletal muscle traits. I tried to map the differences between the central nervous system of these animals, especially the brain and hippocampus region.

Several studies reflect that there are huge benefits of regular exercise in the central nervous system. Falone et al. reports that exercise reversed the age-related decline in the level of SIRT1. (Falone, et al., 2012) In their experience hippocampus undergoes significant redox imbalance during the first period of the exercise program, but it seems that this imbalance might have an important role in preparing the cellular environment

for the subsequent beneficial modifications. Szabo refers that voluntary exercise may engage proteasome function to benefit the brain after trauma. (Szabo, Ying, Radak, & Gomez-Pinilla, 2010) Marosi experienced that long-term exercise treatment reduced oxidative stress in the hippocampus of aging rats. Exercise induced an up-regulation of SOD-1 and Glutathione peroxidase (GPx) enzymes, p-AMPK and PGC-1 α , that can be related to an improved redox balance in the hippocampus. These results suggest that long-term physical exercise can comprise antioxidant properties and by this way protect neurons against oxidative stress at the early stage of aging. (Marosi, et al., 2012) Radak et al. published that exercise can induce neurogenesis via neurotrophic factors, increase capillarization, decrease oxidative damage, and enhance repair of oxidative damage. Exercise is also effective in attenuating age-associated loss in brain function, which suggests that physical activity-related complex metabolic and redox changes are important for a healthy neural system. (Radak, et al., 2013) In 2005 Adlard proved that increased physical activity decreased the Amyloid beta (A β) protein levels in an Alzheimer disease mouse model. Already 1 month of exercise impacted learning and memory according to a Morris water maze task. (Adlard, Perreau, Pop, & Cotman, 2005) In 2010 Radak provided an overview of the positive impacts of exercise on Alzheimer's disease. According to the review regular physical activity increases the endurance of cells and tissues to oxidative stress, vascularization, energy metabolism, and neurotrophin synthesis, all important in neurogenesis, memory improvement, and brain plasticity. (Radak, et al., 2010)

It is well-proved that exercise has beneficial effects on the central nervous system. On the other hand it is also well-known that exercise generates a huge population of reactive oxygen species due to the increased oxygen consumption. It is still under investigation how training has such beneficial effects despite the ROS.

1.4 Reactive oxygen species (ROS) - Enemies within ourselves?

Reactive oxygen species is a general term for molecular oxygen-derived molecules that are reactive species or that are converted easily to reactive species. Many of them are free radicals. There are at least 4 primary sources of free radicals formed endogenously within living organisms:

- peroxisomal oxidation (Aliev, et al., 2008)
- respiratory generation of ATP using oxygen (Beckman & Ames, 1998)
- cytochrome P450 enzymes
- cells which use a mixture of oxidants to overcome an infection (Ames, Shigenaga, & Hagen, 1993)

Oxygen-derived free radicals are highly reactive chemical species involved in a variety of disorders. Superoxide anion (O_2^-), hydroxyl radical (OH^\cdot), and hydrogen peroxide (H_2O_2) are known as reactive oxygen species. As mentioned, they are mostly produced in the mitochondria during the reduction of molecular oxygen to water. Large amount of evidence has shown the important roles of ROS in cell proliferation, homeostasis, intracellular signaling, angiogenesis, and modifications of the extracellular matrix... etc. On the other hand ROS are described as harmful products and capable of DNA mutations, lipid peroxidation and protein oxidation. All these can lead to inflammation and cell death. (Zhu, Su, Wang, Smith, & Perry, 2007) Fortunately, there are several endogenous antioxidant defense mechanisms, like antioxidant enzymes (catalase, glutathione peroxidase or superoxide dismutase) and non-enzymatic antioxidants (vitamin E, ascorbic acid...). This is important because accumulation of misfolded proteins is a common feature in multiple human diseases, especially in the nervous system. Neurons are particularly sensitive to oxidative stress; therefore the brain is more vulnerable to reactive oxygen species-induced damage due to its high rate of oxygen consumption and high polyunsaturated lipid content. Prevention is very important because regular training increases endurance of cells to oxidative stress, vascularization, energy metabolism and neurotrophin synthesis which can be seen via improved memory and brain plasticity. In connection with Alzheimer disease Dumont and colleagues demonstrated that the overexpression of MnSOD reduced amyloid plaques, improved memory function and protected synapses. (Dumont, et al., 2009) Of course it would be

more convenient to look for a therapy which does not require genetic intervention. Drugs which have antioxidant property do and will have attention. Including but not limited to: statins, alkaloids, catapol and the big family of polyphenols. These molecules have the capacity to chelate metal ions and to directly quench free radical species. (Perron & Brumaghim, 2009)

Unfortunately antioxidants are not enough to avoid every danger which threatens our cells. Sometimes the amount of free radicals is too high to deal with and molecules of the cells' get damaged. There have to be mechanisms to repair these damages.

1.5 OGG1 – Repair mechanisms during life

DNA damage occurs in daily life and is aggravated following metabolic and oxidative stresses. Accordingly, DNA repair is essential to maintenance of genomic integrity and cellular viability. The severity of DNA damage varies from single base damage to double-stranded DNA breaks. The most common threat which can appear is oxidation. Reactive oxygen species can originate both from extracellular and intracellular sources. Among the four bases of DNA guanine has the lowest redox potential, thus it is prone to oxidation resulting 7,8-dihydro-8-oxoguanine (8-oxoG) formation. This lesion is particularly mutagenic because in addition to its ability to form a Watson-Crick pairing with cytosine, 8-oxoG has the ability to form a stable Hoogsteen pair with adenine. This can lead to G:C→T:A transversion after replication. (Kuchino, et al., 1987) Because of the high mutagenic potential during evolution arose a special enzyme to cut out 8-oxoG. It is 8-oxoguanine DNA glycosylase (OGG) which catalyses the first step of base excision repair in the case of an oxidated guanine.

OGG belongs to the helix-hairpin-helix superfamily of enzymes. OGGs can be divided into three subfamilies: OGG1, OGG2, AGOG. The majority of OGG1 enzymes are found in eukaryotes, OGG2 mostly appears in bacteria and archaea, while AGOG is exclusively found in archaeal organisms. (Robey-Bond, Barrantes-Reynolds, Bond, Wallace, & Bandaru, 2008) Human OGG1 exists in two different splice variants. While hOGG1 α can be found in the cytoplasm, nucleus and mitochondria, hOGG1 β is only expressed in mitochondria. (Nishioka, et al., 1999) OGG1's activity can be modified during post-translational changes: OGG1 is phosphorylated *in vitro* by CDK4 (Cyclin-dependent kinase 4), resulting in a 2.5-fold increase in the 8-oxoG/C incision activity of OGG1. C-Abl tyrosine phosphorylates OGG1 *in vitro*; however, this phosphorylation event does not affect OGG1 8-oxoG/C incision activity. (Hu, Imam, Hashiguchi, de Souza-Pinto, & Bohr, 2005) On the other hand OGG1 is acetylated on Lys338/Lys341 by p300 which also increased its activity. (Bhakat, Mokkaapati, Boldogh, Hazra, & Mitra, 2006)

The excision activity of OGG1 is quite important because accumulation of 8-oxoG in brain has been implicated in neurodegeneration (Lovell & Markesbery, 2007) (Wang, Markesbery, & Lovell, 2006) (Aguirre, Beal, Matson, & Bogdanov, 2005) It was

recently reported that aging results in increased levels of 8-oxoG in the hippocampus, which was associated with decreased level of acetylation of the most powerful repair enzyme of 8-oxoG, OGG1. (Radicella, Dherin, Desmaze, Fox, & Boiteux, 1997) Importance of OGG1's acetylation is underlined by data showing that exercise increases the acetylated OGG1 levels in muscle of young individuals. (Bori, et al., 2012) Efficient DNA repair has been shown to protect against neurodegeneration and thus amplifies the significance of DNA damage repair in the nervous system. (Liu, et al., 2011)

In contrast to these Stuart et al. proved that OGG1 null mice do not exhibit abnormal phenotype. (Stuart, Bourque, de Souza-Pinto, & Bohr, 2005) These animals accumulated 8-oxoG in mitochondrial DNA 9- to 20-fold higher than wild type, but it does not seem to raise disadvantages. (de Souza-Pinto, et al., 2001) It has even been found that OGG1-deficient mice are resistant to inflammation, implicating involvement of OGG1 in pro-inflammatory signaling. (Touati, et al., 2006) (Mabley, et al., 2005) In 2012 Boldogh et al. published for the first time several lines of evidence that OGG1 is able to bound free 8-oxoG, thus interacting with Ras family GTPases that initiates a signaling cascade. (Boldogh, et al., 2012)

In this context it might be possible that OGG1 is needed to be deactivated sometimes. To decrease its activity one option is deacetylation, so there might be a connection between OGG1 and SIRT1 deacetylase.

2. Objectives of the study

The aim of the study was to test how regular exercise can overcome the health risks which occur at metabolic syndrome. An animal model from the Michigan University was ideal for this purpose. As I mentioned in the “Introduction”, there is a model in which rat lines were developed that diverge widely for their intrinsic aerobic capacity. This is not the first time when artificial selection was used to investigate such question in exercise. (Swallow, Carter, & Garland, 1998) But as we know this is the first time when researchers selected animals for a very long time (more than ten years passed, which is quite long compare to the life-span of rats) for the final goal to determine the genetic components of aerobic capacity. Of course the 22nd generation, which I worked with, is not enough yet to reach that goal, but ideal to get conclusions about the extremities the two rat types typify. From this point of view observations on this model may reveal mechanisms, which can mean new information after all about us.

The laboratory, where most of the experiments were conducted, has a special interest in addition to sport sciences. Since their discovery, sirtuins have stood in a main focus in many of the investigations. No doubt that sirtuins are a very old and conservative protein family which on the other hand is barely known by modern biology. This experiment was the first in this laboratory when we attempted to get information about sirtuins not only in a descriptive way, but we tried to enlarge their effects by administering a well-known activator: resveratrol.

During my PhD years I had the opportunity to spend some time at the University of Texas. In those days I learnt how to work with cell cultures and I could test my hypothesis on cell culture. According to the observations on rat brain we presumed a connection between sirtuins and OGG1 repair enzyme, so my last hypothesis arose from this topic.

Hypotheses:

1. Regular physical activity and resveratrol treatment will enhance the cognitive function of both rat strains.
2. Our aim was to illustrate that the cognitive enhancement was caused via sirtuins and neurotrophic factors in the brain which overall can be seen in neurogenesis.
3. Training and/or resveratrol will compensate the differences which come from the genetic origin of the animals.
4. Sirtuins can deacetylate OGG1 protein and this might moderate its activity.

3. Materials and methods

3.1 Origin of the rats

Most of my results are based on the testing of a special type of rats. These Sprague-Dawley rats are artificially selected for intrinsic aerobic endurance running capacity by Lauren G. Koch and Steven L. Britton, who built up a new model to investigate the genetic factors of aerobic endurance. This model building is based on a large scale selective breeding program. Briefly they started the program from 96 male and 96 female rats. Each rat in the founder population was of different parentage. They each were provided food and water ad libitum and placed on a 12:12 hours light-dark cycle. The protocol for estimation aerobic running capacity required 2 weeks and was started when the rats were 10 weeks old. The first week consisted of introducing each rat to the treadmill (5 minutes, 10 m/min, 15° slope). During the second week, each rat was evaluated for maximal endurance running capacity on five consecutive days. The slope was constant 15°, and the starting speed was 10 m/min. Treadmill velocity was increased by 1 m/min every 2 minutes and each rat was run until exhausted. Using the criterion of single best day, the 13 lowest and 13 highest capacity rats of each sex were selected from the founder population and randomly paired for mating. At 10 weeks of age the offsprings were introduced to the treadmill and subsequently tested for running capacities as described above. The prearranged schedule of matings followed a simple sequence based on assigned family number (1 to 13). I.e.: Founder population - 1x1, 2x2, 3x3... 1st Generation - 1x2, 2x3, 3x4... 2nd Generation - 1x3, 2x4, 3x5... With the use of this technique they could decrease the rate of inbreeding and increased the overall response to selection. (Koch & Britton, 2001) I had the chance to work with 24 low capacity of running (LCR) and 24 high capacity of running (HCR) male rats from the 22nd generation.

3.2 Protocols in the animal house

The rats were arrived in September 2008 and were housed 2 per cage. The first week was taken up with adaptation. The animals were provided water and food ad libitum and we kept a 12:12 hours light dark cycle with the light cycle coinciding with daytime. They were randomly assigned to groups as follows: Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL), Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH). All the investigations took 15 weeks and were carried out according to the requirements of the Guiding Principles for Care and Use of Animals in the European Union, approved by the local ethics committee.

3.2.1 Maximal oxygen uptake measurement and training

The first two weeks consisted of teaching the rats how to run on the treadmill. The goal was to run for 10 minutes at a speed 10 m/min on a 5° slope. These days the animals usually slid off the back of the belt so they had to be picked up and moved forward. The failure to run caused the rats to fall onto a 10 x 10 cm electric shock grid that delivered 1.0 mA (3 Hz). Alternatively, at the end of the belt they could be shot with some air. Finally the rats learned to run on the treadmill. This amount of exposure to treadmill running is likely below that required to produce a significant change in their aerobic capacity.

After the learning period each animal's maximal oxygen uptake was measured with the use of a special rat ergospirometer system (Piston Medical Ltd. Hungary). Briefly the first step was to calibrate the machine and put the rat inside. At the first 10 minutes the machine measures the calm VO_2 value. Then we turned on the treadmill inside and started 5 minutes of warm up. From the 15th minute we increased the speed of the treadmill by 5m/min every 3rd minutes. This measurement was kept until: 1: the rat's VO_2 did not change when speed was increased, 2: the rat could not keep the position on the belt of the treadmill, 3: the respiratory quotient ($\text{RQ} = \text{VCO}_2/\text{VO}_2$) >1. The VO_2 measurement was repeated on every 2nd week and the training was set up according to the VO_2 values.

The initial parameters at the training were 10 m/min, 30 minutes, on a 5% slope. Then based on the level of VO₂ max, the speed corresponding to the 60% VO₂ max was determined and used for daily training for 1 hour five times a week.

3.2.2 Drug treatment and corresponding tests

During the 15 weeks of the procedure the animals treated with resveratrol got 100 mg/body mass kg resveratrol solution (made of sterile DW) per os on every 2nd day. The body weight of the animals was measured every week. The blood sugar of the animals was defined once in every month from a drop of blood which was collected from the tail vein. From this drop blood sugar was measured with a quick test.

3.2.3 Balance test

The balance and coordination of the rats was also determined using a rotarod test, in which the rodent is placed on a horizontally oriented, rotating cylinder (rod) suspended above a cage floor, which is low enough not to injure the animal, but high enough to induce avoidance of fall. Rodents naturally try to stay on the rotating cylinder, or rotarod, and avoid falling to the ground. The length of time that a given animal stays on this faster and faster rotating rod is a measure of their balance, coordination, physical condition, and motor-planning. The test measures the functions like balance and coordination of the subjects; especially in testing the effect of experimental drugs. (Jones & Roberts, 1968)

3.2.4 Behavioral tests

Behavioral tests are meant to measure cognitive ability of rodents. The Novel Object Recognition (NOR) task was used to evaluate cognition, particularly recognition memory, in rodent models. This test is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one. The choice to explore the novel object reflects the use of learning and recognition memory. The Novel Object Recognition task is conducted in an open field arena with two different kinds of objects. Both objects are generally consistent in height and volume, but are different in shape and appearance. During habituation, the animals are allowed to explore an empty arena.

(It is also called Open field test where the exploration rate can be expressed in numbers because the latency, the grooming, the line-crossing, etc. is counted and the time is measured in every action.) Twenty-four hours after habituation, the animals are exposed to the familiar arena with two identical objects placed at an equal distance. The next day, the rats are allowed to explore the open field in the presence of the familiar object and a novel object to test long-term recognition memory (as shown on Figure 4.). The time spent exploring each object as well as their discrimination index percentage is recorded. This test is useful for assessing impaired cognitive ability in transgenic strains of mice and evaluating novel chemical entities for their effect on cognition.



Figure 4: Novel object recognition test

Y Maze Spontaneous Alternation is a behavioral test to measure the willingness of rodents to explore new environments. Rodents typically prefer to investigate a new arm of the maze rather than return to one that was previously visited. Many parts of the brain, including the hippocampus, septum, basal forebrain, and prefrontal cortex, are involved in this task. Testing occurs in a Y-shaped maze with three opaque plastic arms at a 120° angle from each other (as shown on Figure 5. in our animal house.). After introduction to the center of the maze, the animal is allowed to freely explore the three arms. Over the course of multiple arm entries, the subject should show a tendency to enter a less recently visited arm. The number of arm entries and the number of triads are recorded in order to calculate the percentage of alternation. An entry occurs when all four limbs are within the arm. This test is used to quantify cognitive deficits in transgenic strains of rodents and evaluate novel chemical entities for their effects on cognition.



Figure 5: Y maze test

The Passive Avoidance task is a fear-aggravated test used to evaluate learning and memory. In this test, subjects learn to avoid an environment in which an aversive stimulus (such as a foot-shock) was previously delivered. The animals can freely explore the light and dark compartments of the chamber and a mild foot shock is delivered in one side of the compartment. (Figure 6 shows the free exploration before the foot shock in our animal house.) Animals eventually learn to associate certain properties of the chamber with the foot shock. The latency to pass the gate in order to avoid the stimulus is used as an indicator of learning and memory. The Passive Avoidance task is useful for evaluating the effect of novel chemical entities on learning and memory as well as studying the mechanisms involved in cognition. We measured short time (after 24 hours) and long time memory (after 10 days).

<http://sbfnl.stanford.edu/cs/bm/lm/>

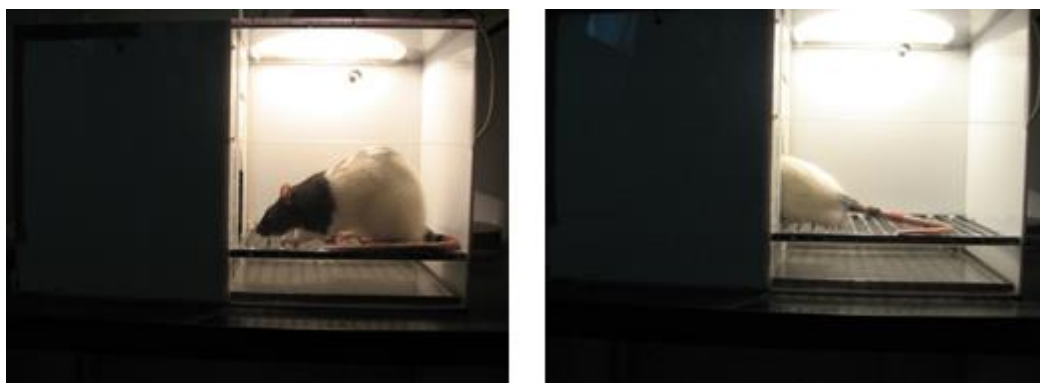


Figure 6: Passive avoidance test

In order to detect new cell formation, BrdU was injected into each animal for the last four weeks of the program.

At the end of the experiments the animals were sacrificed two days after the last exercise session to avoid the metabolic effects of the final run. Half of the brain was used for histochemistry. From the other half the hippocampus and the frontal lobe was excised and frozen in liquid nitrogen.

3.3 Protocols in the laboratory

3.3.1 Tissue separation

For protein analysis a piece of frontal lobe tissue was separated according to the followings:

The mass of every tissue piece before thawing was measured, and 1 ml of 4°C cold lysis buffer was added.

Lysis buffer contains: 137 mM NaCl (sodium-chloride Sigma-Aldrich #S3014), Tris-HCl pH: 8.0, (prepared and pH adjusted previously from Tris salt (Sigma-Aldrich #T1503), 1% NP40 (NonidetP-40 Fluka BioChemica #74385), 10% glycerol (Sigma-Aldrich #G5516), 1 mM PMSF (phenylmethylsulfonyl fluoride Sigma-Aldrich #78830), 10 µg/ml aprotinin (Sigma-Aldrich #A6279), 1 µg/ml leupeptin (Sigma-Aldrich #L8511), 0.5 mM sodium-vanadate (Sigma-Aldrich #590088).

The tissue was smashed in the lysis buffer on ice with a tissue homogenizer. Then the homogenate was shaken on ice for 30 minutes and centrifuged for 15 minutes, 4°C, 15300 RPM (Revolutions per minute, Sigma 2K15 centrifuge, Rotor#: 12148). Finally the supernatant was collected and the pellet was discarded.

The protein concentration of the samples was measured according to the Bradford method with a kit (Bio-Rad DC #500-0002). The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. (Bradford, 1976) The dye exists in three forms: cationic (red), neutral (green), and anionic (blue). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form ($A_{\max} = 470 \text{ nm}$). However, when the dye binds to protein, it is converted to a stable unprotonated blue form ($A_{\max} = 595 \text{ nm}$). It is this blue protein-dye form that is detected at 595 nm in the assay using microplate reader. For protein standard bovine serum albumin was used (blank, 1, 2, 4, 8, 15, 20, 25 µg/ml). The protein concentrations were measured in duplicates. Each standard and unknown sample solution was measured into microplate wells. 1x dye reagent was added to each well, mixed with the pipette and shook gently for 5 minutes. Then samples were

measured with the microplate reader (Thermo Scientific) and quantified with the help of Ascent Software. According to the results every sample was diluted to the same concentration.

3.3.2 Western blot

For western blot assays every sample was diluted 1:1 with Laemli buffer. The resolving gels were between 6-15% {10%: 40% distilled water = DW, 33% acrylamide/bis-acrylamide (Sigma-Aldrich #A6050), 25% Tris-HCl pH: 8.8, 10% SDS (sodium dodecyl sulfate Sigma-Aldrich #L3771), 0.1 g/ml ammonium persulfate (Sigma-Aldrich #A3678), 0.04% N,N,N',N'-Tetramethylethylenediamine = TEMED (Sigma-Aldrich #T9281)} and the stacking gel was 10% {70% DW, 16.5% acrylamide/bis-acrylamide, 12.5% Tris-HCl pH: 6.8, 10% SDS, 0.1 g/ml ammonium persulfate, 0.1% TEMED}. Usually 20-50µg protein/well was loaded plus the protein bench mark (Life Technologies # 10748-010). For the electrophoresis a Bio-Rad electrophoresis system was used. The electrophoresis was ready after approx. 1.5 hours, and it needed 1x running buffer {10x Running buffer: 30.3 g Tris powder, 144 g glycine (Sigma-Aldrich #G8898), 10% SDS + DW fill to 1000ml}. The blotting to the membrane (Millipore, Immobilon PVDF) was made by Bio-Rad Mini blotting system and took 50 minutes with transfer buffer {3.03 g Tris powder, 14.4 g glycine, 100 ml methanol (Molar Chemicals Ltd. #05730) + DW fill to 1000 ml}. After blotting the membranes were blocked between 1-12 hours with 5% nonfat dry milk in 1x TBST {1% 2 M Tris-HCl pH: 7.4, 8.8 g NaCl (Sigma-Aldrich #S7653), 0.1% Tween20 + DW fill to 1000 ml}. Then the primary antibody was dissolved according to the manufacturers' protocol into 5% milky TBST or 1% bovine serum albumin (BSA) TBST. The membranes were soaked in the primary antibody solutions between 1-12 hours (See list of the primary antibodies in Table 2.). After the incubation with the primary antibody the membranes were washed in TBST 3 times and soaked into the secondary antibody solution {according to the manufacturers' protocol the secondary antibody was also solved into 5% milky TBST or 1% BSA TBST}. After incubation with the secondary antibody the membranes were washed in TBST at least 3 times. Labelled protein bands were revealed with the use of Pierce ECL Western Blotting Substrate (Thermo Scientific). For detection, membranes were exposed to x-ray films. Finally the x-ray films were

scanned and the protein densities were quantified using ImageJ (National Institute of Health, USA). On every membrane β -actin was used as internal control.

Table 2: Antibodies in the western blots

Antibody	Producer & Catalogue number	Concentration
SIRT1	Abcam, ab53517	1:500
Acetylated Lysine	Cell Signaling, #9441	1:1000
Carbonylated proteins (dinitrophenylhydrazine)	Oxyblot Kit, Millipore #S7150	1xDNPH (according to the kit's manual)
NAMPT	Abcam, ab37299	1:500
BDNF	Santa Cruz, sc-546	1:1000
PAR	Calbiochem, #AM80	1:500
OGG1	Abcam, ab204	1:500
Acetylated OGG1	Abcam, ab93670	1:1000
β -Actin	Santa Cruz, sc-81178	1:2000

3.3.3 SIRT1 activity assay

Nowadays several SIRT1 activity assay kits are available on the market. During the laboratory measurements there was only the CycLex kit (#CY-1151) which we could use. All the recipes can be found in the kit's manual. For this measurement a piece of the frontal lobe was homogenized in Lysis buffer, vortexed and kept on ice for 15 minutes. The samples were spun through a sucrose cushion at 1 300 g for 10 minutes at 4°C. The nuclei pellet was washed in 10 mM Tris HCl pH: 7.5, 10 mM NaCl. Then the pellet was suspended in 100 μ l extraction buffer and sonicated for 30 s. After 30 minutes of incubation on ice the samples were centrifuged for 10 minutes at 15 000 RPM. The supernatant's protein concentration was assessed by Bradford method as described above.

CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit measures the activity of SIRT1 by the basic principle of changing a SIRT1 reaction into the activity of the protease. In order to measure the enzyme activity of SIRT1, which is the NAD^+

dependent histone deacetylase, this kit is designed so that the activity of NAD⁺ dependent histone deacetylase can be measured under existence of Trichostatin A, which is the powerful inhibitor of histone deacetylase other than SIRT1. In this kit, fluorophore and quencher are coupled to amino terminal and carboxyl terminal of substrate peptide, respectively, and before reaction of deacetylase, the fluorescence can not be emitted. However, if SIRT1 performs deacetylation, substrate peptide will become cut by the action of protease added simultaneously, quencher will separate from fluorophore, and fluorescence will be emitted. Deacetylase enzyme activity is measured by measuring this fluorescence intensity by a fluorometer (Fluoroskan Ascent Microplate Fluorometer #5210480)

For the assay solutions were mixed into the microplate wells: Assay buffer, Fluoro-Deacetylated Peptide, NAD, TSA, my enzyme sample or recombinant SIRT1 and finally LEP which initiates the reaction. “No enzyme control”, “no NAD control” and positive control (recombinant protein) was used as well. The machine measured the excitation at 340 nm and emission at 440 nm in every 5th minute for 3 hours.

3.3.4 PCR

For the PCR measurements half of the hippocampus was used.

RNA separation was made with RNA NucleoSpin kit (Macherey-Nagel #740955.50) according to the kit's manual. Shortly the tissue was homogenized in Buffer RA1 with a Teflon homogenizer. Then β -mercaptoethanol was added and vortexed vigorously. The lysate was cleared by filtration through the violet filter via centrifuging it at 11 000 g for 1 minute. Then 70% ethanol was added, mixed well and filtered through the blue filter via centrifugation at 11 000 g for 30 seconds. Membrane Desalting Buffer was added and centrifuged again for 1 minute at 11 000 g. DNase reaction mixture was pipetted onto the filter membrane and incubated for 15 minutes at room temperature. Then the filter membrane was washed once with Buffer RAW2 and twice with Buffer RA3. Finally the RNA was collected into RNase-free water via centrifugation at 11 000 g for 1 minute.

The cDNA synthesis was made with cDNA Synthesis kit (BIOLINE #BIO-65026) according to the kit's manual. Briefly 1 µg RNA was mixed with 1 µl Oligo (dT)₁₈ and Random Hexamer, 1 µl 10 mM dNTP and was filled up to 10 µl with DEPC-treated water. The samples were incubated at 65°C for 10 minutes and placed on ice for 2 minutes. 10 µl reaction mix was added {4 µl 5x RT Buffer, 1 µl RNase Inhibitor, 0.25 µl Reverse Transcriptase and 4.75µl DEPC-treated water} to the primed RNA and samples were held at 42°C for 30 minutes. Finally the reaction was terminated by incubating the samples at 70°C for 15 minutes.

The geNorm Housekeeping Gene Selection kit (Primerdesign #ge-SY-6 rat) was used to determine the appropriate housekeeping gene. According to the guideline reactions were elicited with βAKT, YWHAZ, UBC, ATP5B, CYC1, GAPDH primers. Finally the geNorm analysis was shown that βAKT is a suitable housekeeping gene.

Afterwards cDNAs were diluted {20 µl cDNA + 180 µl DEPC-water} and RT-PCR was made with βAKT primer. The cDNAs were diluted to the same concentration, and this was checked via agarose gel electrophoresis. For this and every following RT-PCR reactions 5 µl cDNA, 10 µl ImmoMix (BIOLINE #BIO-25020), 1 µl of the reverse-forward primer mix (See list of the primers in Table 3.), 1 µl SYBR green (QIAGEN) and 3 µl DEPC-treated water was used. RT-PCR measurement was performed on Rotor-Gene 6000 real-time system (Corbett Life Sciences) and gene expression levels were determined by delta CT method with the help of the Rotor-Gene software. The thermocycling profile conditions used were: 95 °C for 10 minutes, 95 °C for 10 seconds, 60 °C for 15 seconds, 72 °C for 20 seconds. 35 cycles were used in case of each primer. Each run was finished with a melt phase (50-95 °C).

Table 3: Genes and primers in PCR

Genes	Primers
Sirt1	f 5' TCGTGGAGACATTTTAAATCAGG 3' r 5' GCTTCATGATGGCAAGTGGG 3'
Sirt3	f 5' GTCGGGCATCCCTGCCTCAAAGC 3' r 5' GGAACCCTGTCTGCCATCACGTCAG 3'

Sirt4	f 5' ACCCTGAGAAGGTCAAAGAGTTAC 3' r 5' TTCCCCACAATCCAAGCAC 3'
Sirt6	f 5' GAGGAGCTGGAGCGGAAGGTGTG 3' r 5' GGCCAGACCTCGCTCCTCCATGG 3'
Creb	f 5' GCCTCTGGTGATGTACAAACATACC 3' r 5' GGGAGGACGCCATAACAACACTC 3'
β -Actin	f 5' GCTCGTCGTCGACAACGGCTC 3' r 5' CAAACATGATCTGGGTCATCTTCT 3'

3.3.5 Immunofluorescence and immunohistochemistry

For histochemistry measurements half of the brain was fixed with paraformaldehyde (Sigma-Aldrich #47608) embedded into paraffin and cut with microtome into 5 μ m slides.

For the detection of neurogenesis the sections were de-paraffinated with xylol, rehydrated with ethanol solutions and washed 3 times with PBS. The sections were needed to be digested in DNase I (Sigma-Aldrich #DN25) for 10 minutes and for antigen retrieval citrate buffer pH: 6.0 (Sigma-Aldrich #W302600) was used. The slides were blocked in normal goat serum (Sigma-Aldrich #G9023) Triton X-PBS for 60 minutes and washed 3 times in PBS. Then BrdU primary antibody 1:200 (BD Pharmingen #555627) was added after solved in the blocking solution and incubated overnight at 4°C. Next morning the slides were washed 3 times in Triton X-PBS and the secondary Alexa Fluor 546 antibody 1:200 (Life Technologies) was applied after solving it in the blocking solution and incubated the slides at room temperature for 30 minutes. After the washing steps I added the anti-Neuronal Nuclei (NeuN) Alexa 488 conjugated antibody 1:100 (Millipore #MAB377X) and incubated the slides overnight again. Next morning after the washing steps Hoechst 33342 stain was used in 1:1000 (Molecular Probes #H3570) for 10 minutes. Slides were washed twice in DW and mounted with Gel Mount (Sigma-Aldrich #G0918). Microscopy was performed on Zeiss ELYRA Superresolution Microscopy. Colocalization was visualized by superimposition of green, red and blue images using Zeiss LSM Image Browser Version 4.2.0.121 All measurements were done on coded slides, so during the evaluation I was

blind to the animal groups. The level of neurogenesis was quantified as reported earlier in a publication of our laboratory. (Koltai, et al., 2011)

For the acetylated OGG1 detection Mouse specific HRP/DAB detection kit (Abcam #ab64264) was used. So according to the Abcam protocol the sections were deparaffinated with xylol, rehydrated with ethanol solutions and washed 3 times with PBS. After the hydrogen peroxide block for antigen retrieval citrate buffer (pH: 6.0) treatment was used then slides were washed 3 times in PBS. Protein block was applied and incubated for 10 minutes at room temperature. Samples were washed 4 times and the acetylated OGG1 antibody (Abcam #ab93670) or the OGG1 antibody (affinity purified mouse anti-OGG1 antibody generated against a synthetic peptide, C-DLRQSRHAQEPPAK, representing the C-terminus of OGG1, acquired from Antibodies-Online GmbH) was added and incubated overnight. Next morning after the required washing steps the biotinylated goat anti-mouse IgG was applied and incubated for 10 minutes at room temperature (RT). In the next phase the Streptavidin Peroxidase was also added for 10 minutes and the sections were rinsed in PBS 4 times. Finally the DAB chromogen and the substrate were added for 10 minutes and the results were improved with a 1 minute Hematoxylin staining. After the tap water washing the slides were mounted with 1:1 glycerol: DW. The density of the AcOGG1 was determined with ImageJ software.

3.4 Protocols of the cell culture

For the cell culture experiments I chose an easily manageable cell type from the ATCC collection: HCT116. This is a human colorectal carcinoma cell line, which feeds on McCoy's 5a Modified Medium + 10% fetal bovine serum + 1% penicillin-streptomycin. (All the solutions are available sterile at Life Technologies, Gibco) Cultures were incubated at 37°C, with 5% CO₂. The medium had to be changed in every 2 to 3 days. The cells were kept on Corning plastic surface (75 cm² flasks, 100 mm ø petri dish or 60 mm ø petri dish) from where they can be easily detached with 0.25% Trypsin-EDTA solution.

The cells were lysed for western blot using 1x RIPA buffer + 1% protein inhibitor cocktail + 2% NaF solution + 10% SDS. (Ingredients are available at Sigma-Aldrich). The western blot was made as mentioned above; except the protein ladder which was produced by Fermentas (#SM 01811). The following primary antibodies were used: AcOGG1 (1:500 Abcam #ab93670), SIRT1 (1:500 Abcam # ab53517), OGG1 (affinity purified mouse anti-OGG1 as mentioned above).

The cells were lysed for PCR studies using RLT buffer (Qiagen RNeasy kit #74104) and the RNA was separated according to the kit's handbook. The cDNA synthesis was similar to the BIOLINE kit, but Invitrogen's SuperScript III (#18080-300) was used this time. The housekeeping gene was GAPDH (Sigma-Aldrich).

To monitor the SIRT1's deacetylation activity on OGG1 SIRT1 was silenced via siRNA with the help of siSMART pool (Dharmacon #M-094699-01-0005). The cells were transfected using INTERFERin system (Polyplus transfection #409-10), reverse transfection, and 4 groups were made (Without siRNA, Control siRNA, siSIRT1, siSIRT3). Briefly, siRNA was diluted and incubated with the Interferin reagent for 15 minutes. Then the siRNA-interferin solution was diluted with McCoy's serum-free medium and applied onto the petri dish. Then the detached cells' solution was added, swirled and incubated for 5 hours at 37°C. After this 5 hours some full medium (McCoy's with serum) was added, also next day the medium was changed. The plates were harvested after 48 hours or after 72 hours.

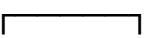
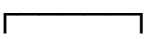
I also wanted to check whether the acetylation status of OGG1 changes after treatment of the cells with known SIRT1 activator/inhibitor. So resveratrol (100 μ M), nicotinamide (10 mM) and Trichostatin A (100 nM, as HDACI-II inhibitor) was applied on the cells. After washing the confluent HCT116 petri dishes with DPBS (+Ca, +Mg) they were covered with the reagents which were prepared in McCoy's medium (without serum) for 6 hours. As a control a petri dish was covered with simple McCoy's medium (without serum). Then the plates were harvested for western blot analysis.

3.5 Statistics

At the beginning the rats were randomly assigned to groups as follows: Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL), Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH).

In results: At those data which fit a Gaussian curve (according to a Shapiro Wilk's W-test) the statistical significance was assessed by ANOVA, followed by Tukey's posthoc test. The significance level was set at $p < 0.05$.

If the values don't fit the "bell curve" shape the statistical significance was assessed by Mann-Whitney U test. The significance level was also set at $p < 0.05$ everywhere.

For statistics Statistica 9.1 software was used. The differences between groups were shown with the help of "  " sign. The statistically significant differences were marked with the help of "  " plus a "*" sign.

4. Results

4.1 Results from the animal experiments

At the beginning a main goal was to measure the maximal oxygen uptake capacity ($VO_2\text{max}$) of the rats. These data were used to follow the progression of exercise and of course to adjust the running speed. If anyone compares the results of the first and the last measurements it is easy to recognize that every group which was trained shows development when compared to the untrained ones. As an example TrL reached ~40% better result than CL by the last week. (Details can be read in Nikolett Hart's paper, where the results are explained considering the molecular mechanisms of the muscle tissue. (Hart, et al., 2013)) There is no statistically significant difference between the groups except for, of course, the genetic feature that determines the low capacity of running (LCR) and the high capacity of running (HCR) groups.

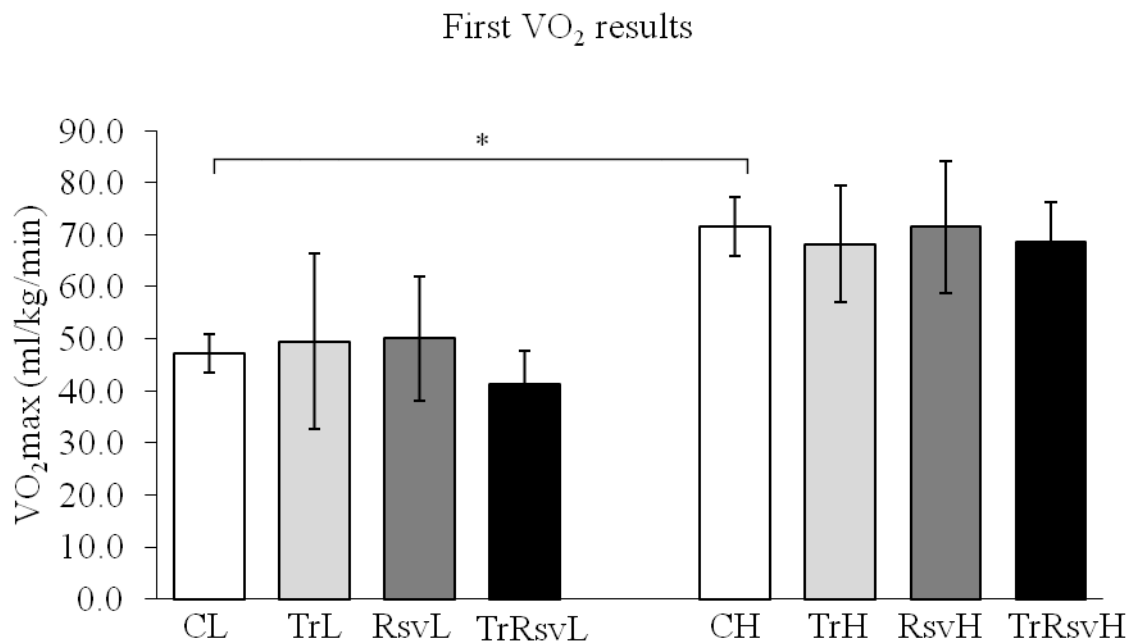

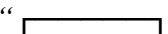


Figure 7: Maximal oxygen uptake results at the beginning of the study

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

The effect of the regular training is spectacular, because every trained group reached greater VO₂max results than the similar but untrained ones. These differences are significant in LCR groups, but only tendencies in HCR groups. It is normal to see the bigger differences in LCR animals, because they had worse fitness, than the HCR ones so they could show greater progression as a result of training.

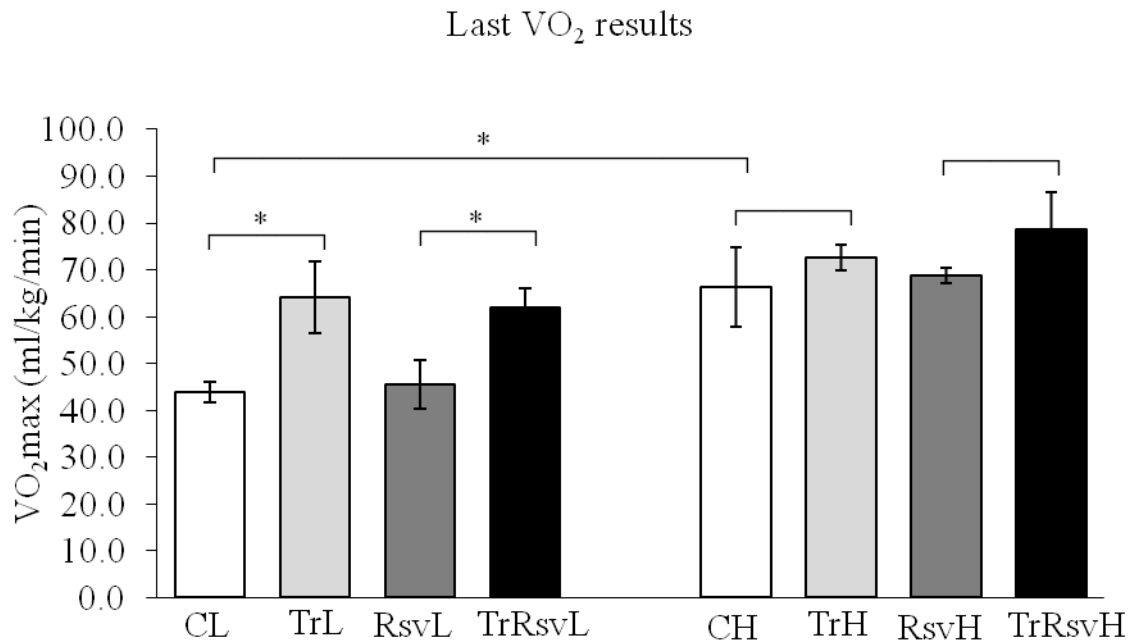
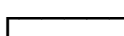
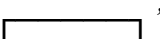


Figure 8: Maximal oxygen uptake results at the end of the study

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with:

“” plus a “” sign.*

We checked the body mass of the animals weekly, to track the changes which can come up because of the exercise or the resveratrol treatment. Finally the body mass of TrL group was significantly lower (470 ± 47 g) compared to CL (595 ± 38 g), while the difference was much smaller between CH and TrH (433 ± 21 g vs 403 ± 39 g). LCR groups had significantly higher body mass than corresponding HCR groups. The resveratrol treatment did not change significantly the body weight during the experimental period (results not shown).

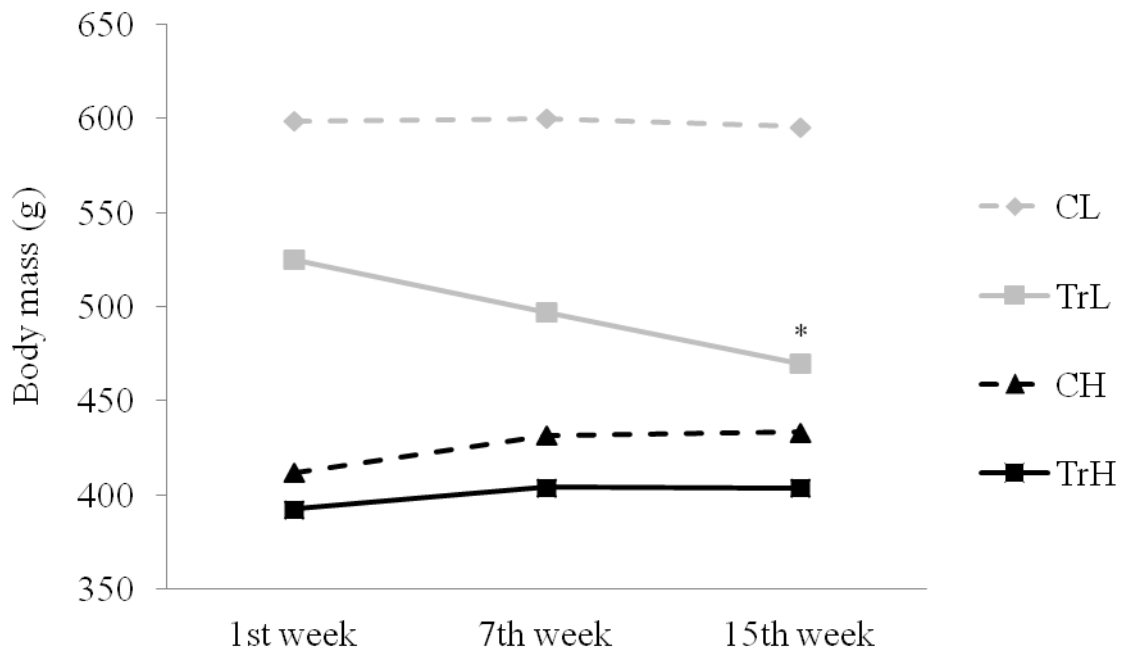


Figure 9: Changing of the body mass at control and trained groups

Control LCR (CL), Trained LCR (TrL)

Control HCR (CH), Trained HCR (TrH)

Statistically significant difference between CL and TrL was shown with: “” sign.*

We also measured the blood sugar levels of the animals. Previous studies referred to the fact that low aerobic running capacity is associated with high blood glucose values and insulin resistance among other signs of metabolic syndrome. Animals with low running capacity had higher blood glucose levels than animals with high running capacity. It is also proven that exercise had a meliorating effect on blood sugar especially at the LCR groups. On Figure 10. the trained low capacity of running groups had almost as low blood glucose levels as the high capacity of running animals.

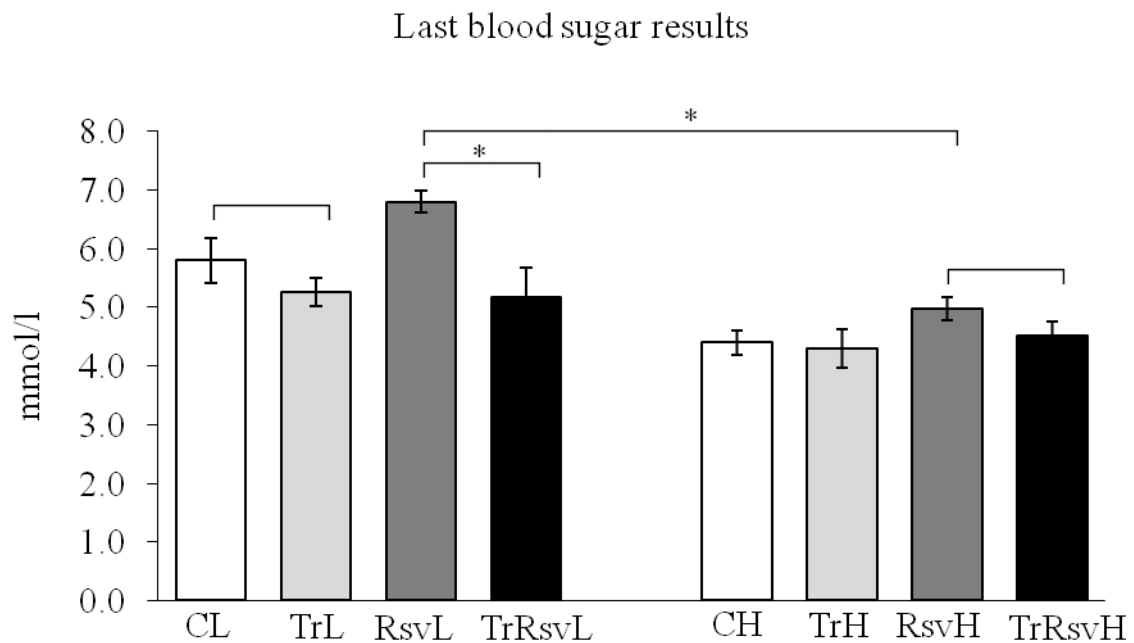
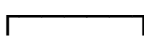
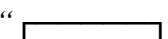


Figure 10: Blood sugar results at the end of the training period

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

The rotarod performance test is based on a rotating rod with forced motor activity being applied. The test measures parameters such as riding time (seconds) or endurance. Some of the functions of the test include evaluating the balance and the coordination of the subjects. Therefore the rotarod test was used to map the balance of the animals and check if training or resveratrol has a result on it or not. According to the results resveratrol raised the time the animals could spend on the barrel of the machine. Exercise had the same result but it was not statistically significant. The highest values could be observed at groups which had both of the treatments.

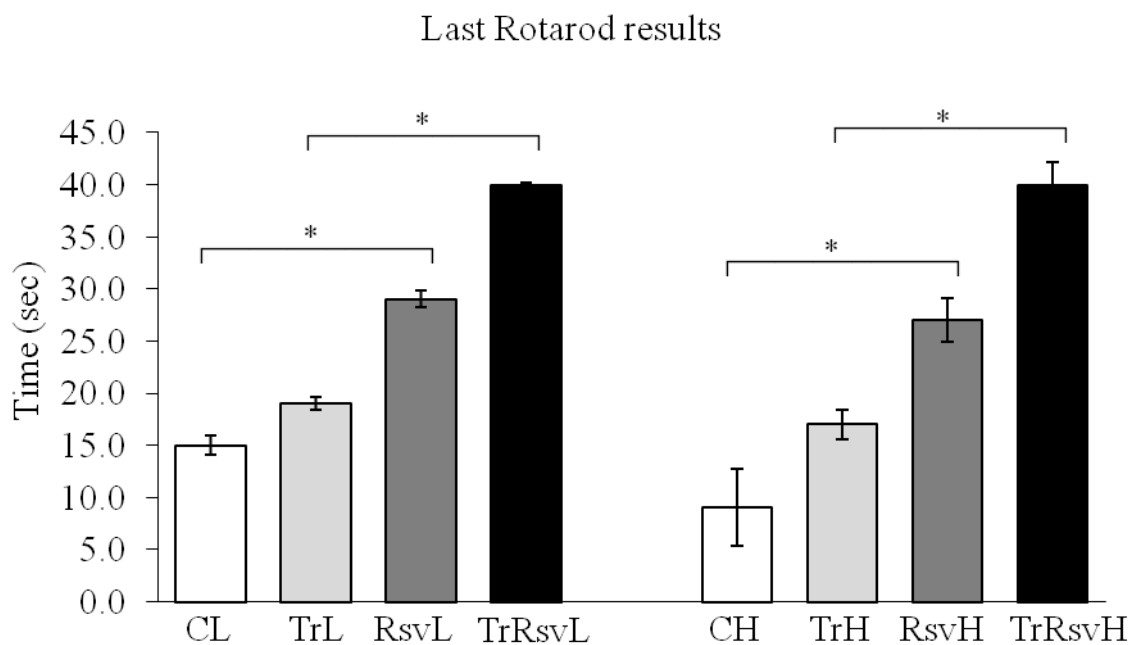


Figure 11: Results of the rotarod test by the end of the 15 weeks

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

The nervous system is responsible for behavior, and so behavioral analysis is the ultimate assay of neural function. Sensory tests may also be performed on an animal that is removed from its home cage to a new environment, for example an open field cage. Normally an animal in a novel environment ignores food in favor of making exploratory movements. These movements are quantifiable by counting latency time, line crossing, rearings...etc. Our data is shown in Figure 12-13.

The most remarkable difference in latency time was that LCR animals spent more time still when placed into the unknown cage. HCR animals started the exploration of the new environment faster.

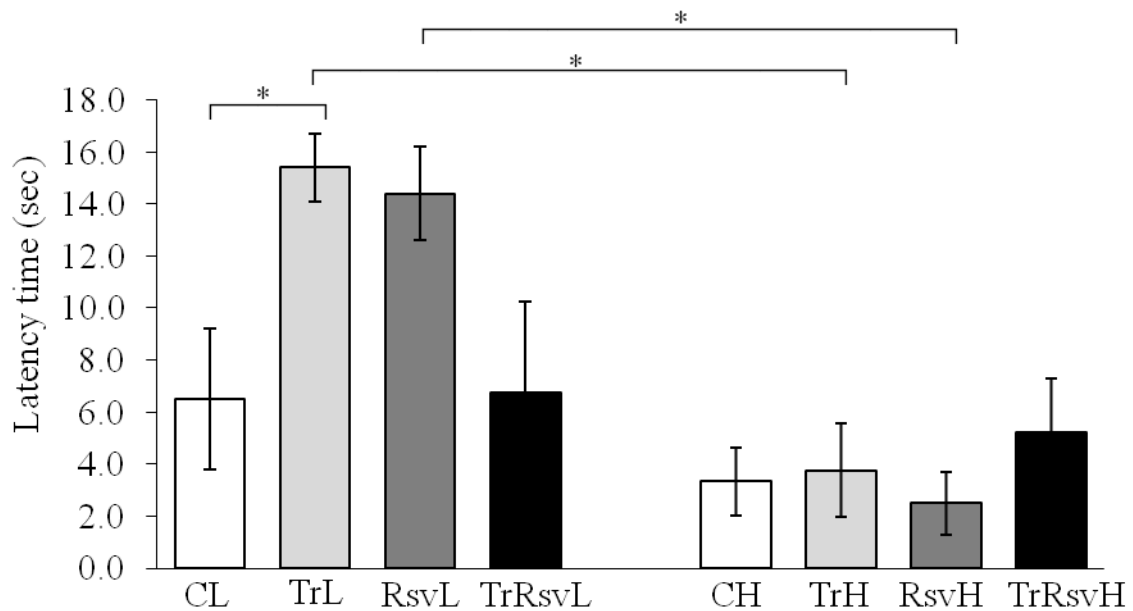
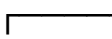
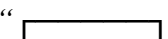


Figure 12: Latency times of the animal groups during an open field test

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

The exploration rate in the open field test can be calculated from line crossings and rearings which were made by the animals in the test field. HCR groups also reached greater values in exploration of the new cage. This difference is significant in the groups which were treated with resveratrol.

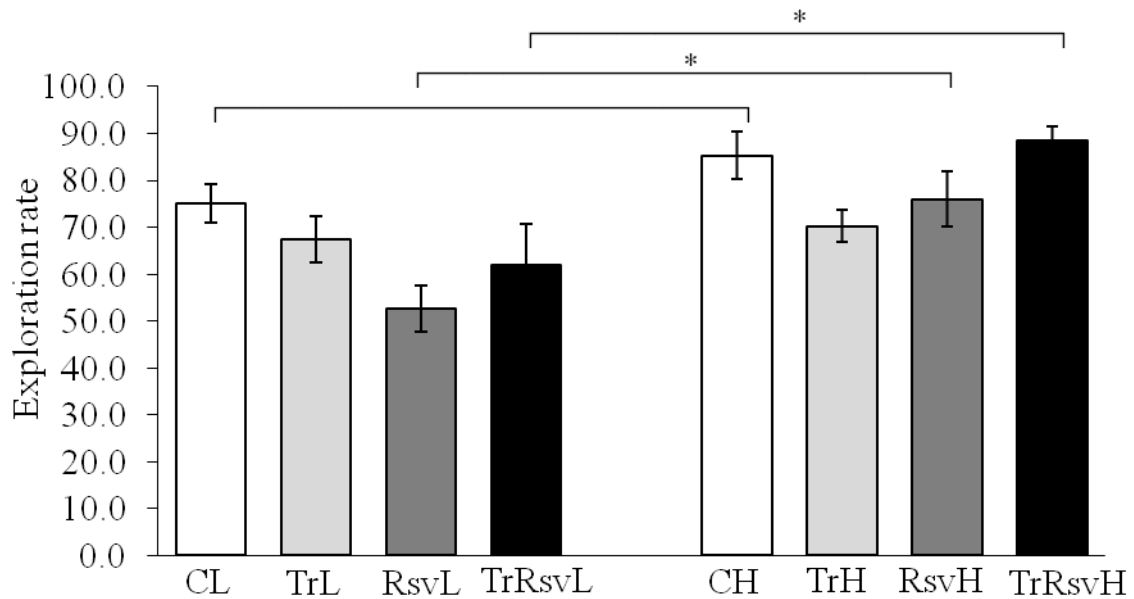
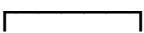
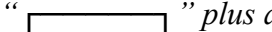


Figure 13: Exploration rate of the animal groups during an open field test

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

Anyway the open field test is necessary before the novel object recognition test. The animal should investigate the objects not the environment at this point to have an objective result.

During object recognition in a natural environment the time spent sniffing and examining objects placed in the animal's home or familiar environment is used as the measure of recognition. Rats recognize the old object as familiar so they spend more time discovering the new one. These results were shown with the help of percentages. (% = Time spent with the new object / all the time spent with both objects * 100) Exercised HCR groups spent the greater time periods at the new object compare to the LCR groups.

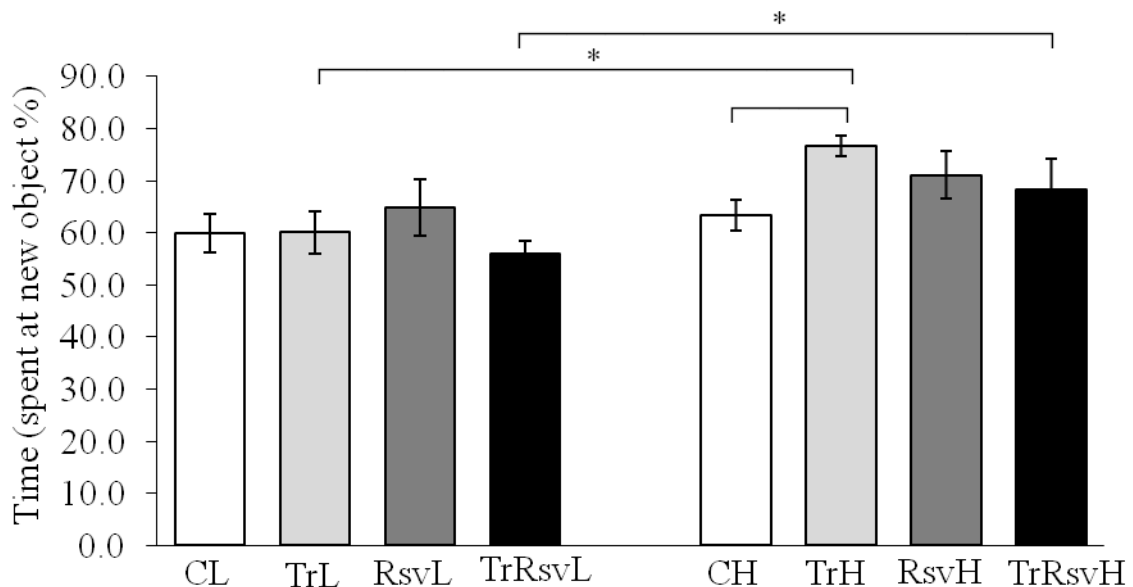

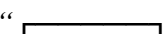


Figure 14: Results of the new object recognition test

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

Y maze test is used to quantify cognitive deficits in transgenic strains of rodents and evaluate novel chemical entities for their effects on cognition.

Unfortunately it was not useful with the rats. These animals were found to be too clever to check the arms of the Y shaped box again and again. When they were placed to the centre they only explored an arm maximum once. After this quick check (when they figured out there's nothing to worry about or to be excited for) they stayed in one arm or at the centre and spent the time with rearing and grooming.

So we could not collect useful data with this method, that's why no data is shown at this point. Later I found in literature that Y maze is rather used on mice or gerbils or maximum juvenescent rats.

Memory is described as being either short-term, to be used only for the moment, or long-term, to be used for long durations. Passive avoidance has been found to be a very sensitive measure of both types of memory (Figure 15-16.).

After the learning period (when they had the mild foot shock in the dark chamber), each group spent longer time at the light chamber before entering the dark one. The diagram (Figure 15.) shows that on the 1st day they were able to remember the bad experiences. Interestingly control HCR group had slightly better results than control LCR. It is also remarkable that only resveratrol treated groups reached the highest latency values.

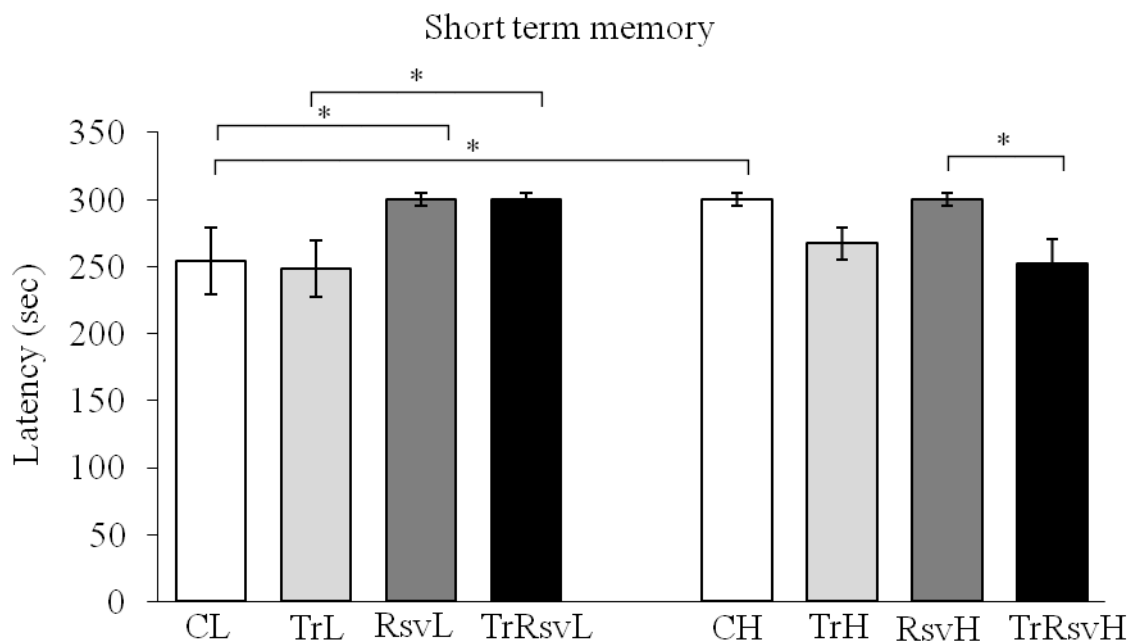

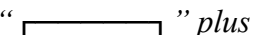


Figure 15: Short term memory was measured 24 hours after the learning period

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

At passive avoidance test by the 10th day of the test the differences became greater between the LCR and HCR animals. In control, exercised and resveratrol treated groups genetic differences seem to stand out (as shown on Figure 16.). LCR animals had worse long term memory than HCR groups. Only TrRsv gangs had the similar results.

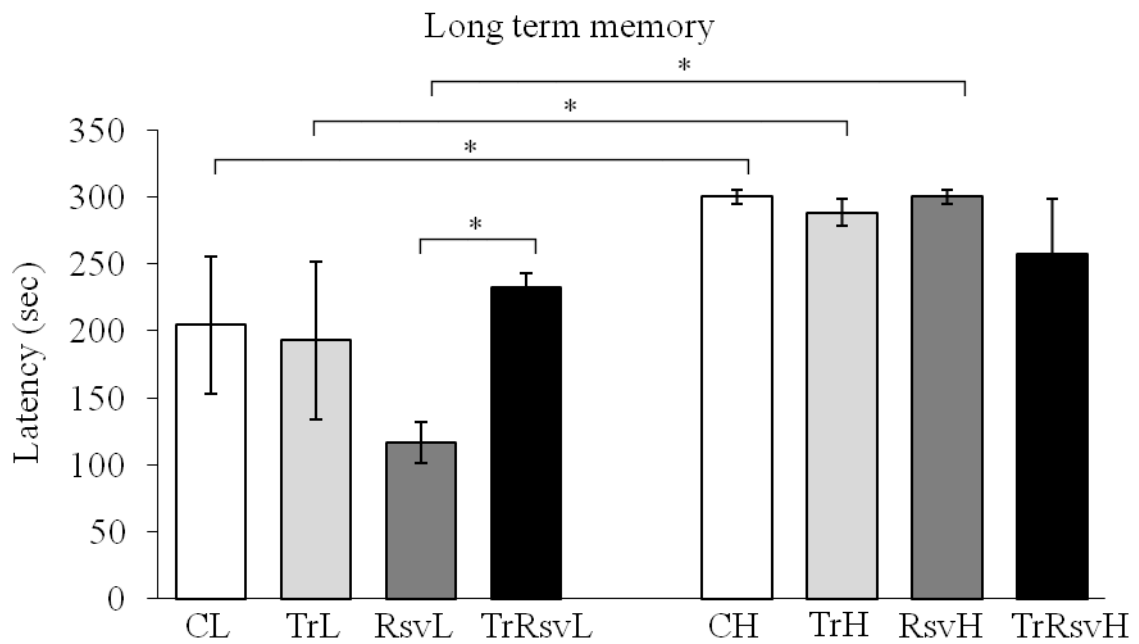

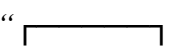


Figure 16: Long term memory was measured 10 days after the learning period
Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and
resveratrol treated LCR (TrRsvL)
Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and
resveratrol treated HCR (TrRsvH)
Differences were shown with: “” Statistically significant differences with:
“” plus a “” sign.*

4.2 Results from investigation of brain tissue

Animal behavior tests, especially the long term memory results suggested that we should look for cellular and molecular differences between the groups of LCR and HCR animals.

For a start we used immunofluorescence to test the number of the newly produced neurons in the hippocampus' gyrus dentatus region. The new cells were labeled with BrdU staining and NeuN that staining helped to count the neurons among the new cells. Resveratrol enhanced the new cell production in the hippocampal region. Training had the same effect but only at the HCR animals.

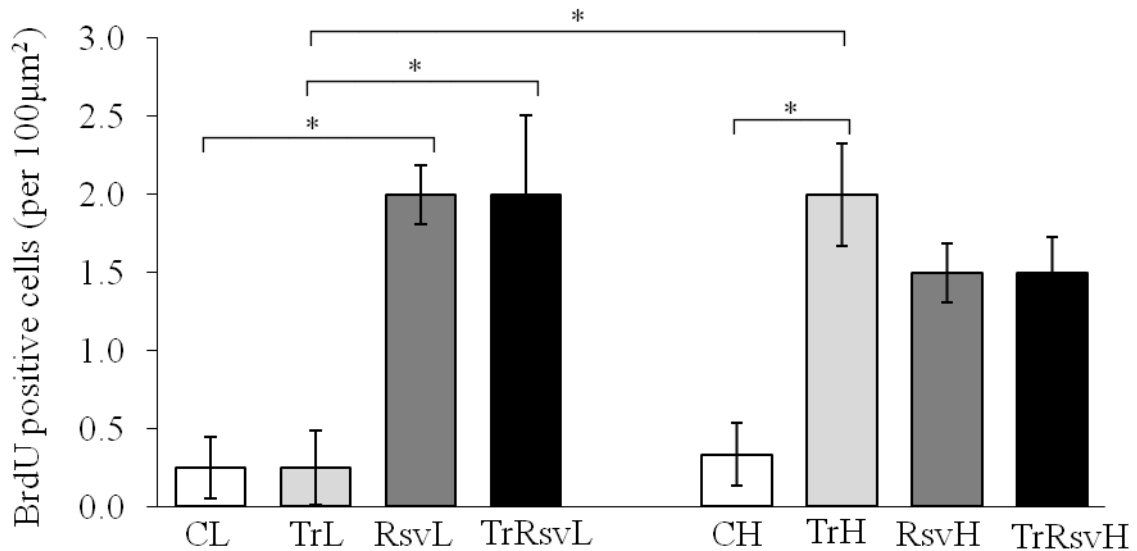

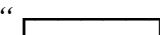


Figure 17: BrdU shows the new cells in the hippocampus

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

The results show new neurons in the hippocampus, especially after the resveratrol administration. Exercise only caused increasing at HCR rats.

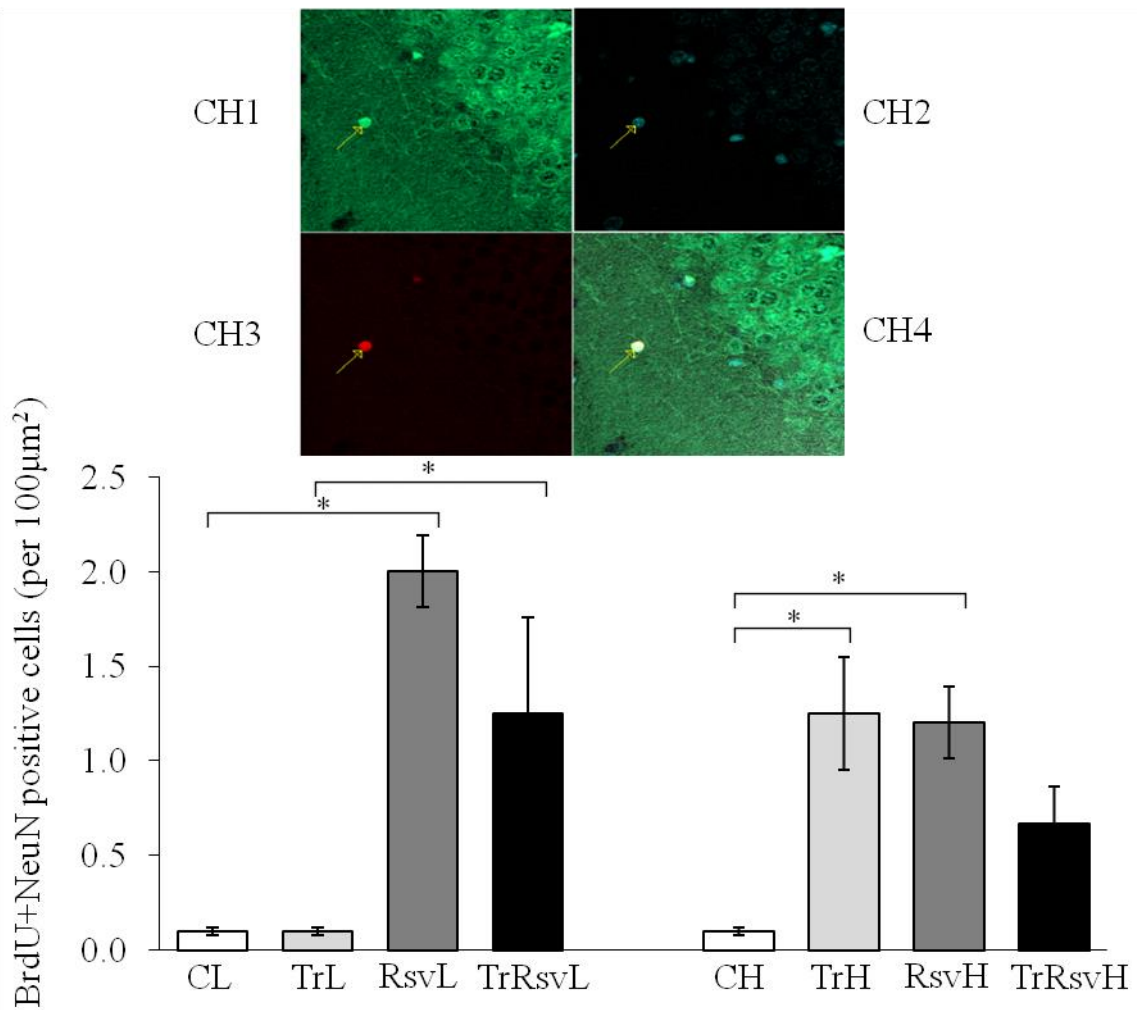
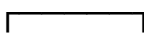



Figure 18: *BrdU+NeuN co-localization in the gyrus dentatus*

CH1: NeuN staining, CH2: nucleus staining, CH3: BrdU staining, CH4: co-localization
63x magnification

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

Since the immunofluorescent results showed a difference between the groups we checked the mRNA levels of most common sirtuin molecules.

SIRT1 is a nucleus located protein with deacetylase activity. Resveratrol is a well-known activator of sirtuins. Resveratrol made no difference compared to the control groups in the level of Sirt1 mRNA. But there were significant differences between RsvL-RsvH.

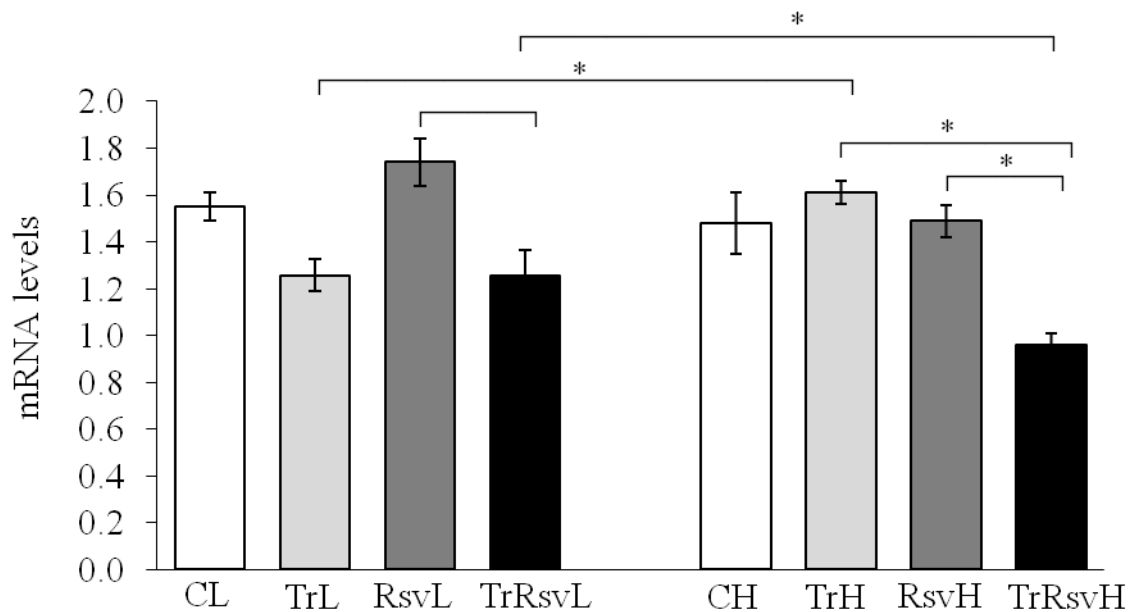
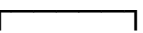
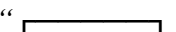


Figure 19: Sirt1 mRNA levels

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

SIRT3 is a mitochondria located protein with proven deacetylase activity. As far as we know from literature, resveratrol is not a direct activator of SIRT3 enzyme. But we wanted to check this statement. We found no differences between the groups at the Sirt3 mRNA levels.

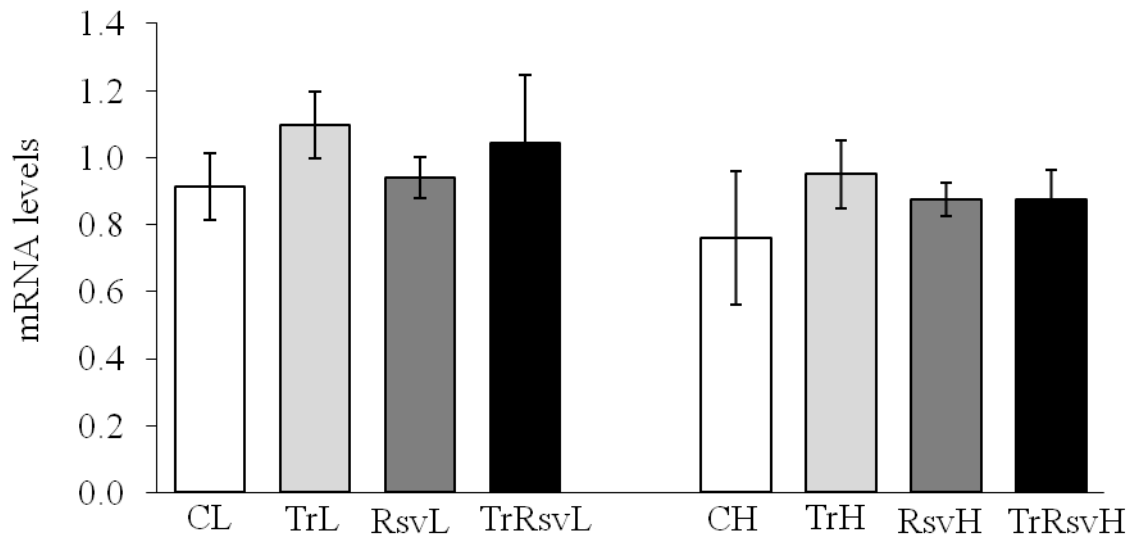

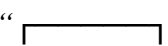


Figure 20: Sirt3 mRNA levels

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

SIRT4 protein is also located in the mitochondria. Compared to the previous sirtuins it has mainly just ADP-ribosyltransferase activity. We saw no difference in the Sirt4 mRNA levels among the groups. Neither training nor resveratrol changed the Sirt4 mRNA amounts.

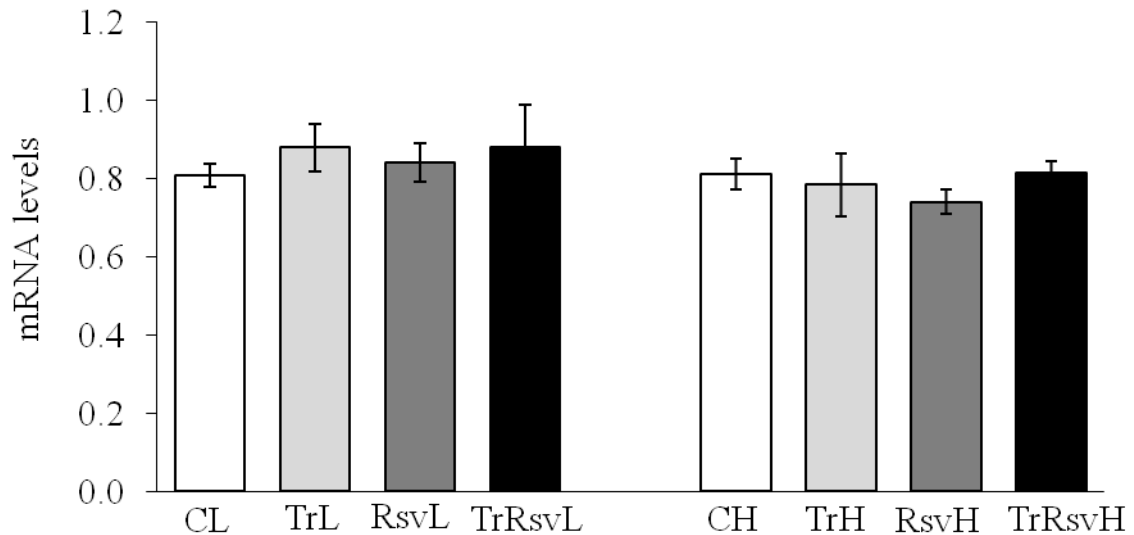
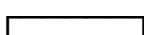



Figure 21: Sirt4 mRNA levels

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

SIRT6 is nucleus located and it has mainly just ADP-ribosyltransferase activity as the SIRT4 in the mitochondria. Because SIRT6 is a nuclear protein like SIRT1, it seemed reasonable to check if resveratrol has any affect on the amount of SIRT6 or not. Sirt6 mRNA level showed decrease at the exercised animals compared to the controls. There was also difference among the control groups which could be caused by the different genetic background. Resveratrol did not seem to modify the mRNA levels of Sirt6 in the treated groups.

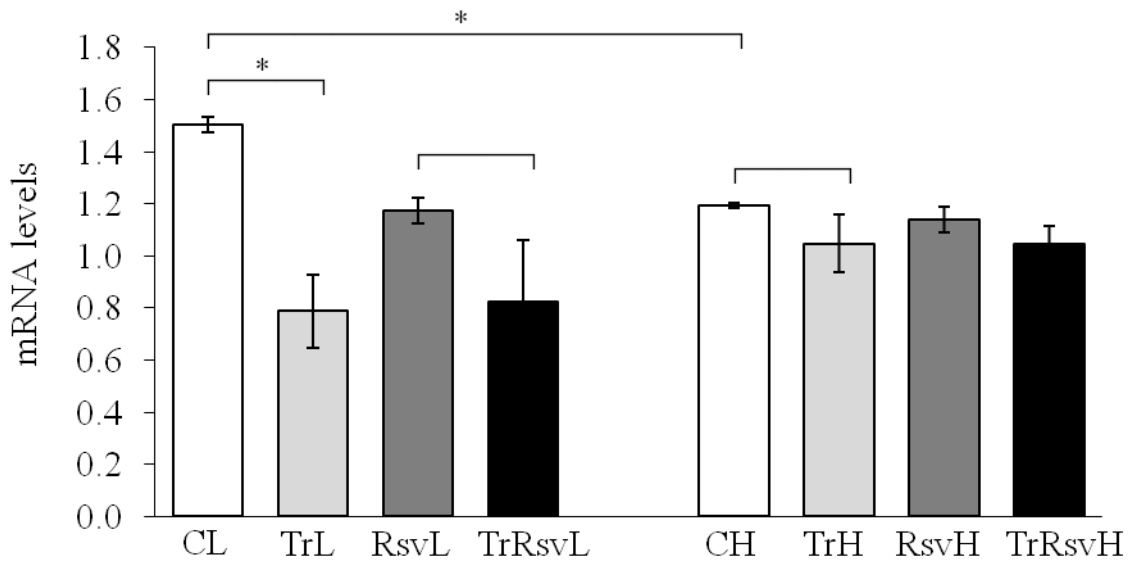




Figure 22: Sirt6 mRNA levels

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “*” sign.

Of course mRNA results do not show exactly the amount of active proteins in the tissues, so I measured the SIRT1 protein level via western blot. The most remarkable difference was between TrL-TrH. On the other hand resveratrol seemed to elevate slightly the SIRT1 amount in RsvL group but did not show the same elevation in RsvH animals.

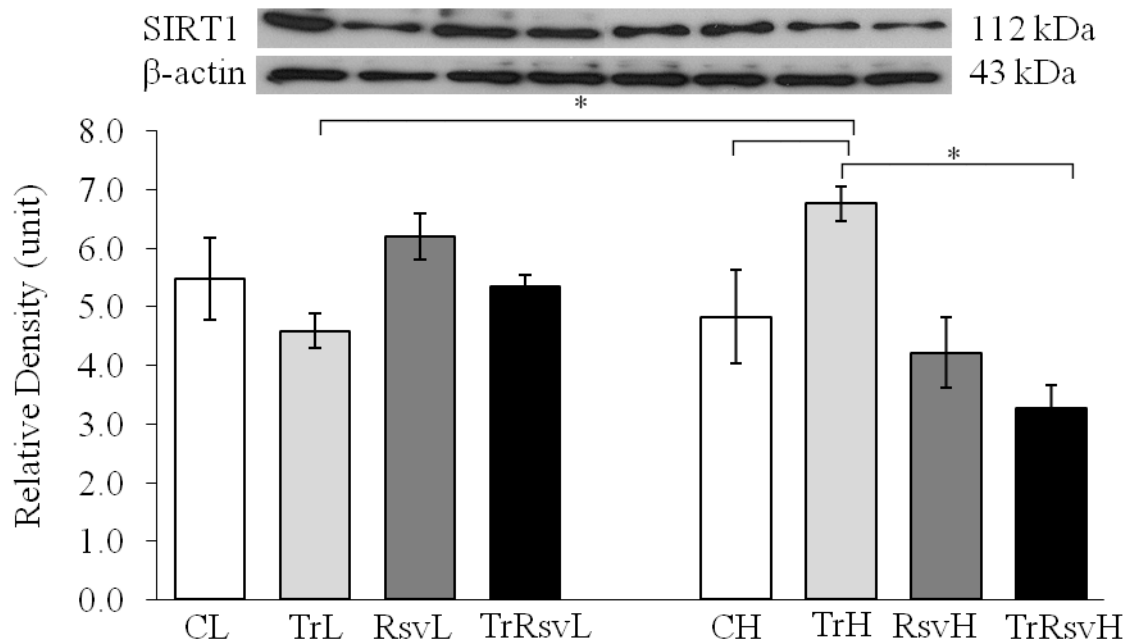
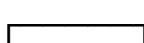
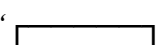


Figure 23: SIRT1 relative density in the brain tissue

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with:

“” plus a “” sign.*

There can be a big difference between a protein's level and activity, so I measured its activity in an artificial deacetylation reaction with a kit. Results are shown on Figure 24. The activity showed the same result as the western blot, so the TrH is significantly higher than TrL. Resveratrol also did not seem to change the activity of SIRT1 (neither in low capacity of running nor in high capacity of running animals) according to this measurement.

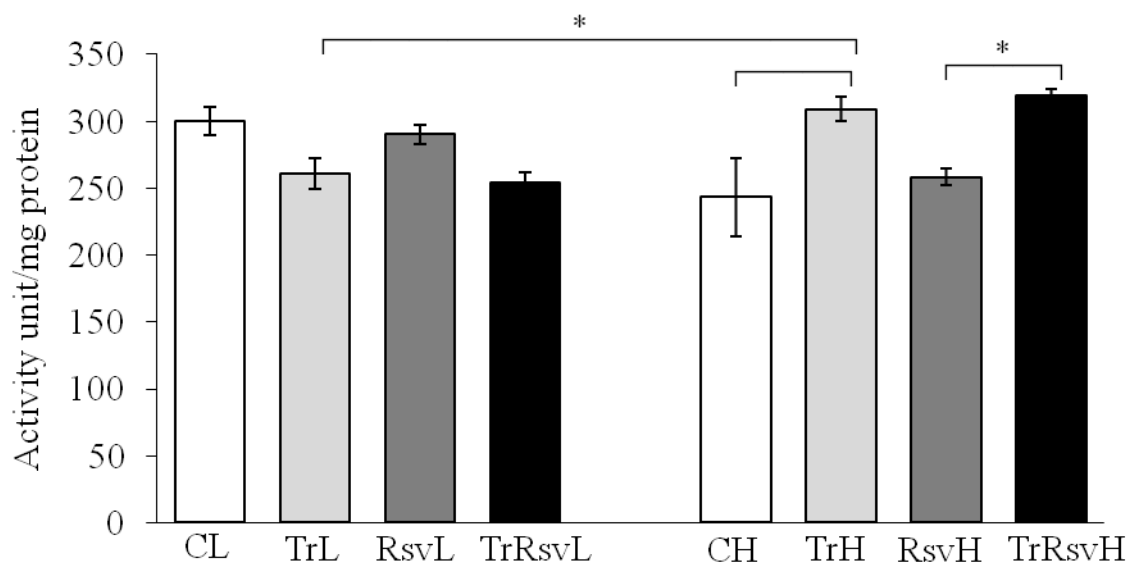
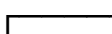
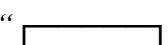


Figure 24: SIRT1 activity (in activity units where the results were divided with the protein amounts)

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

Sirtuins are NAD^+ dependent deacetylases. Inside the cell there are several targets which can be deacetylated. For a start I measured an overall acetylation with the help of the acetylated lysine western blot as it is shown on Figure 25. Both treatments significantly decreased the lysine molecules' acetylation levels. It shows that exercise and resveratrol had the same effect in deacetylation.

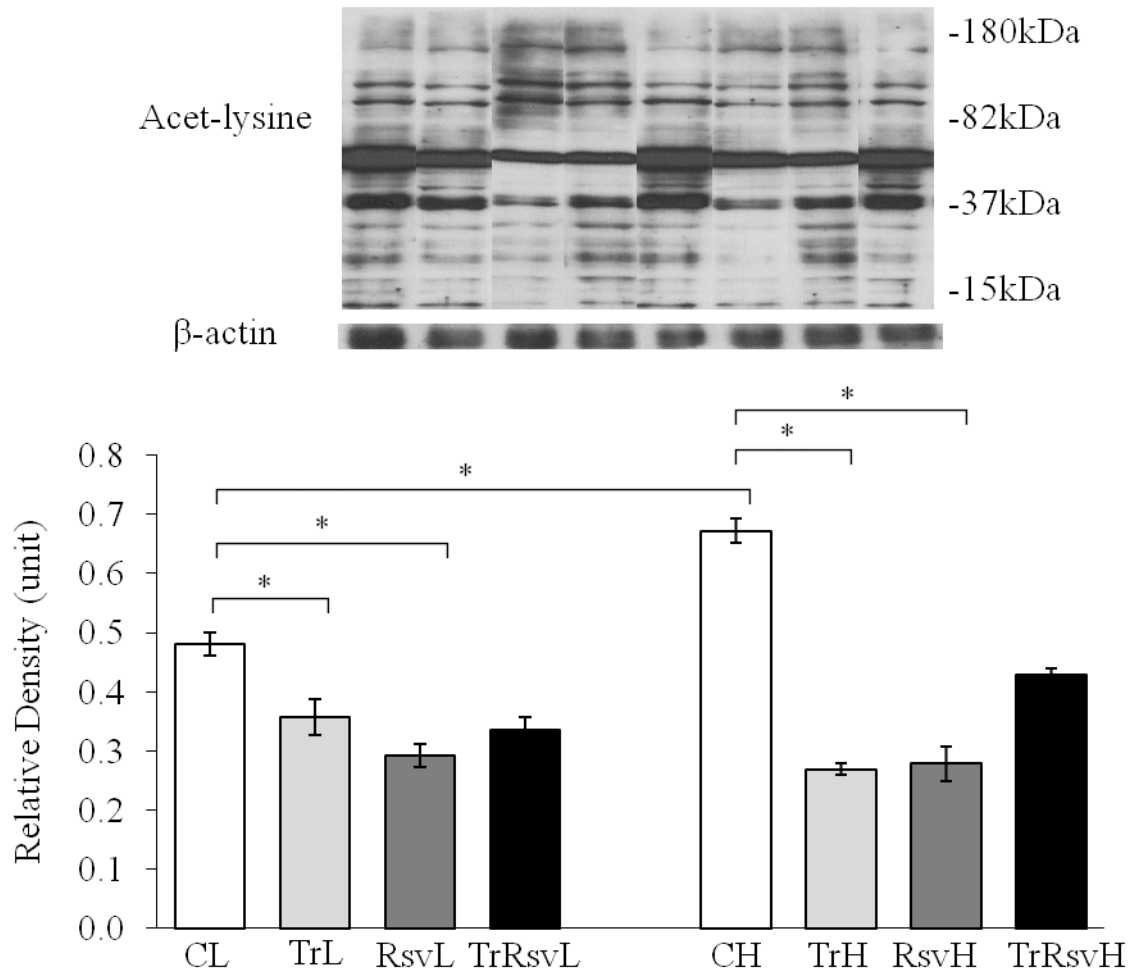
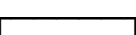
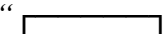


Figure 25: Acetylated lysine relative density

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “*” sign.

Deacetylation can change a protein's activity through changing its conformation. But carbonylation can change a protein's turn-over. The carbonyl level of proteins is used as a marker for oxidative stress. It has been shown that exercise elevates the level of carbonylated proteins because of the elevated ROS levels in the cells. This effect is clearly seen at HCR animals. However, resveratrol seems to ameliorate this disadvantage both in LCR and HCR animals.

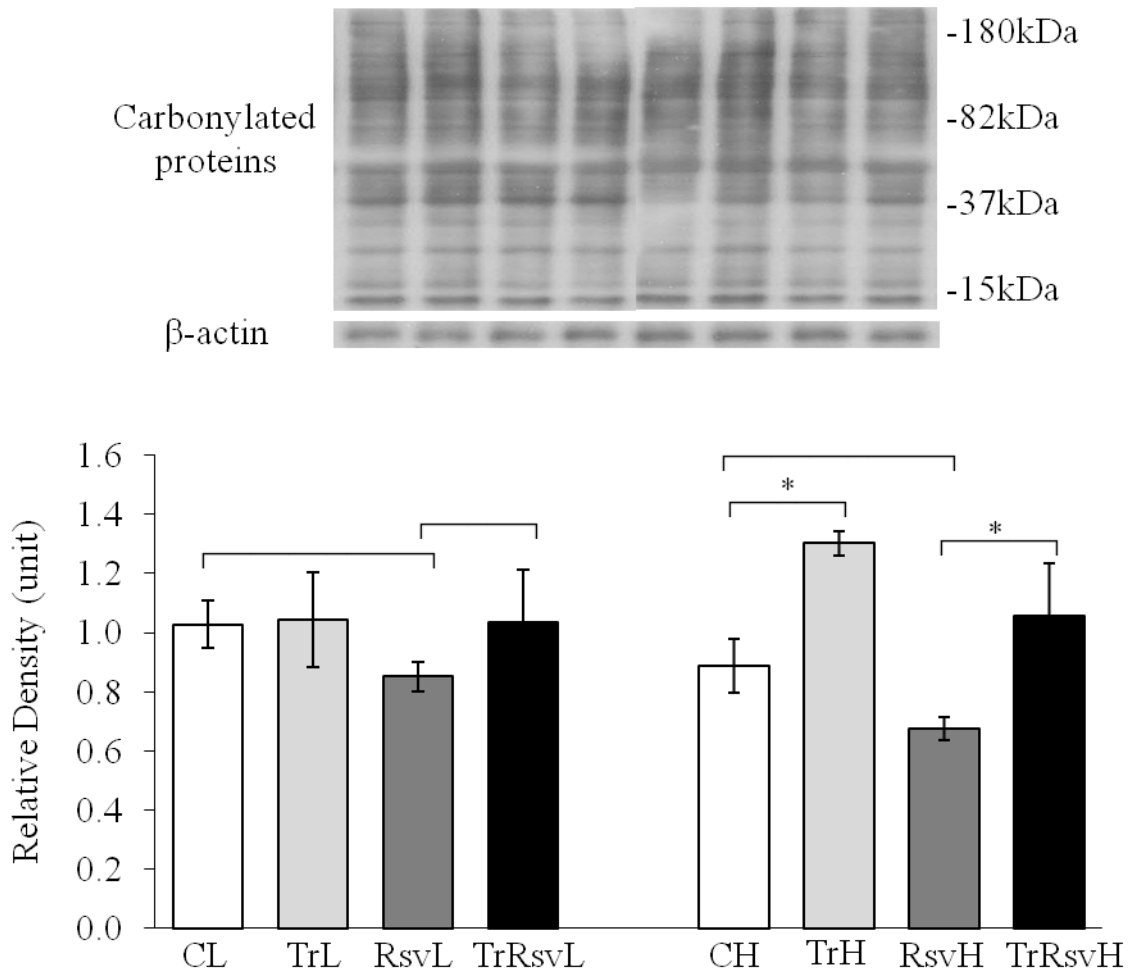
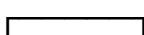
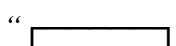


Figure 26: Carbonylated proteins relative density

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

While sirtuins deacetylate other molecules they use a NAD^+ which will hydrolyze and transfer the acetyl-group. Finally nicotinamide will be produced until it will be converted back into NAD^+ . For this conversion NAMPT (also known as PBEF) is required. According to the western blot results shown on Figure 27, the level of NAMPT protein is significantly higher in the resveratrol-treated groups. It suggests that resveratrol might elevate the NAMPT level through SIRT1. Training did not inflict the same results.

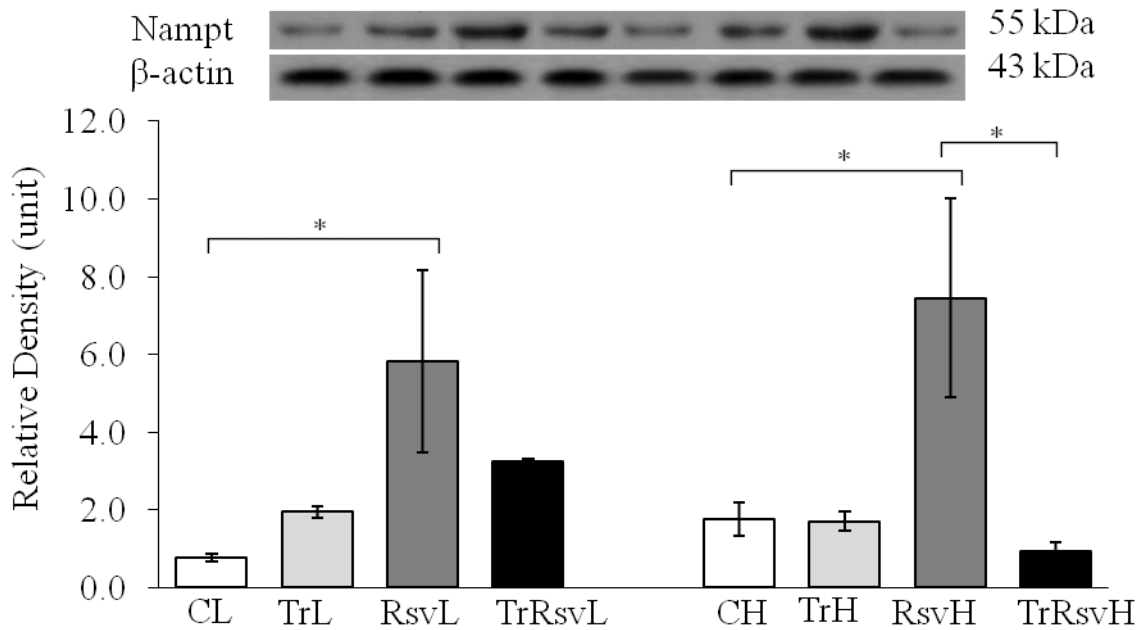
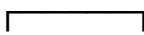
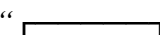


Figure 27: NAMPT relative density

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

CREB (cAMP response element-binding protein) is a cellular transcription factor. It binds to certain DNA sequences called cAMP response elements (CRE), thereby increasing or decreasing the transcription of the downstream genes (e.g.: BDNF). CREB has been shown to be integral in the formation of spatial memory, in neuronal plasticity and the formation of long-term memory in the brain. We measured the Creb mRNA levels as shown in Figure 28. It turned out that exercise increased the mRNA level of Creb spectacularly.

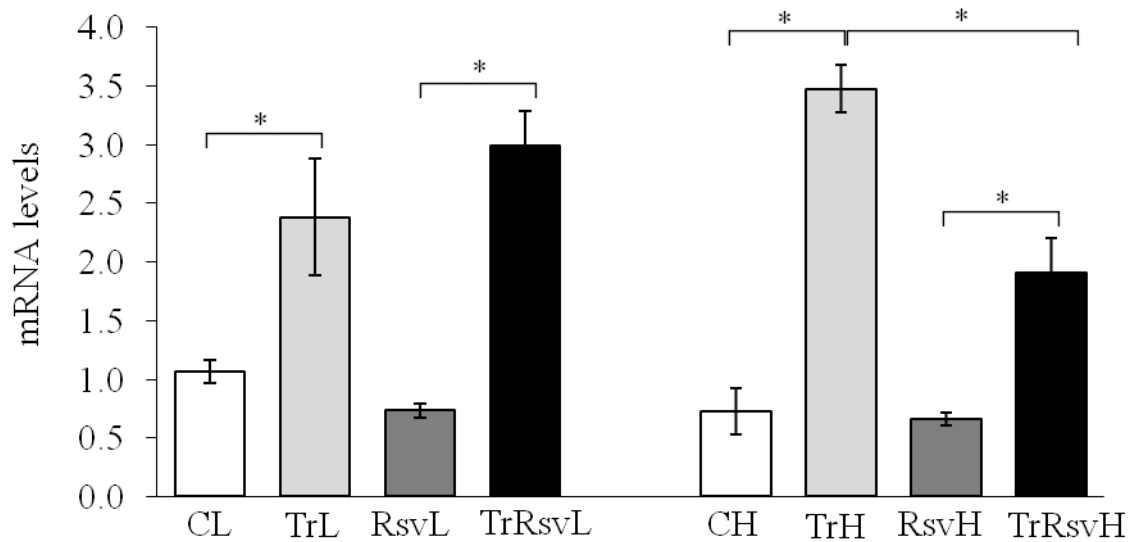


Figure 28: Creb mRNA levels

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “ [] ” Statistically significant differences with: “ [] ” plus a “” sign.*

Brain-derived neurotrophic factor, also known as BDNF acts on certain neurons of the central nervous system and the peripheral nervous system, helping to support the survival of existing neurons, and encouraging the growth and differentiation of new neurons and synapses. It is active in the hippocampus, the cortex, and the basal forebrain areas vital to learning, memory, and higher thinking. At low capacity of running type rats both resveratrol and training enhanced the BDNF protein's level. These effects were also detectable at high capacity of running type rats, but only significant at the TrH group.

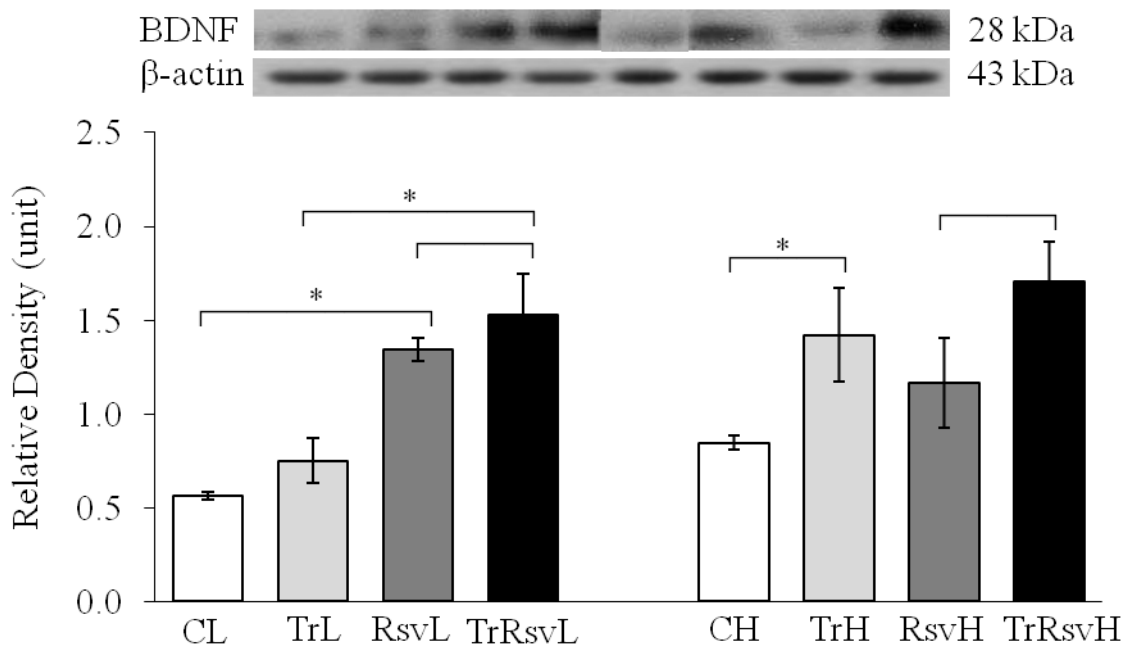

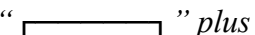


Figure 29: BDNF relative density

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

Poly(ADP)-ribose (PAR) is synthesized by the nuclear DNA repair enzyme poly(ADP)-ribose polymerase (PARP). PARP is selectively activated when DNA strands break and catalyze the addition of long branched chains of PAR to a variety of nuclear proteins. The amount of PAR formed in living cells with DNA damage is directly related to the extent of the damage (e.g. at oxidative stress). Interestingly, both in low and high running capacity animals, resveratrol decreased the level of PAR. On the other hand training with resveratrol supplementation enhanced the PAR level to the control animal's value.

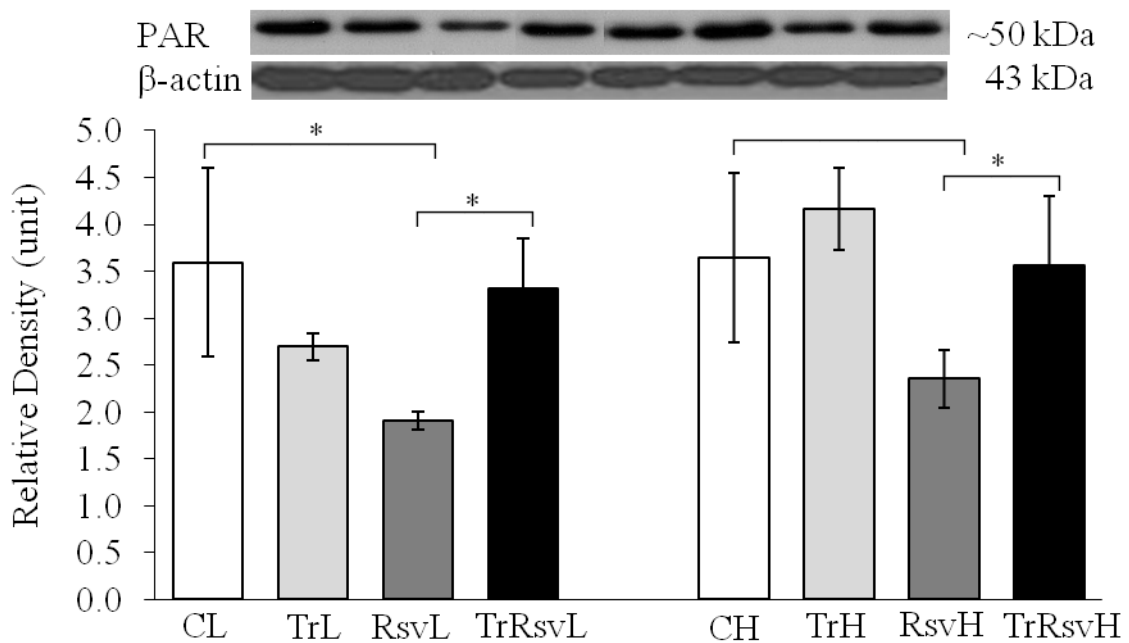

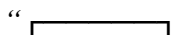


Figure 30: Poly (ADP)-ribose relative density

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “*” sign.

Decreased PAR levels at resveratrol treatment suggest repair mechanisms at the background. As written earlier, OGG1 is a base excision repair enzyme, which is responsible for the excision of 7,8-dihydro-8-oxoguanine (8-oxoG), a mutagenic base byproduct that occurs as a result of exposure to reactive oxygen species (ROS). OGG1 is a bifunctional glycosylase, as it is able to both cleave the glycosidic bond of the mutagenic lesion and cause a strand break in the DNA backbone.

Like PAR, OGG1's density is decreased at resveratrol treatment. Training had different results: at low running capacity animals training made no significant change compare to the controls, but there was huge difference between the LCR and HCR control groups. At high running capacity animals training increased the OGG1 level.

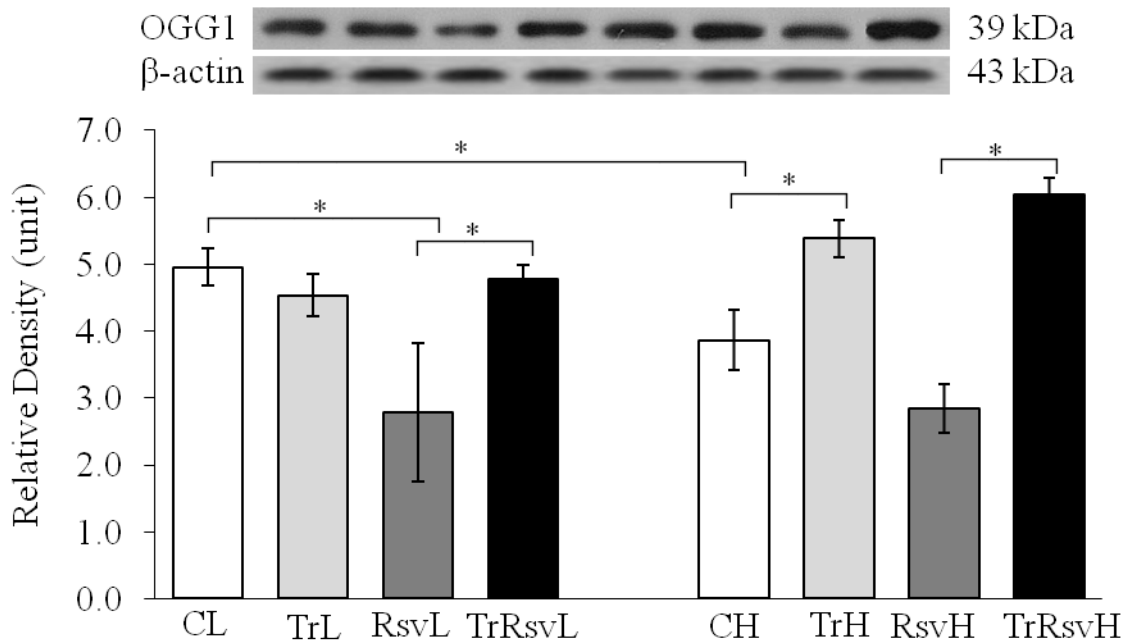
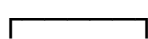
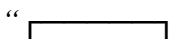


Figure 31: OGG1 relative density

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

OGG1's activity can be fine-tuned through posttranslational modification. One type of these potential modifications is acetylation-deacetylation. It seemed interesting to test if sirtuins are potent deacetylators of OGG1. Therefore I applied histochemistry staining on the half-brain slides. Acetylated OGG1 had higher concentration in almost every LCR groups than the parallel HCR ones. It correlated inversely with the passive avoidance's long term memory data.

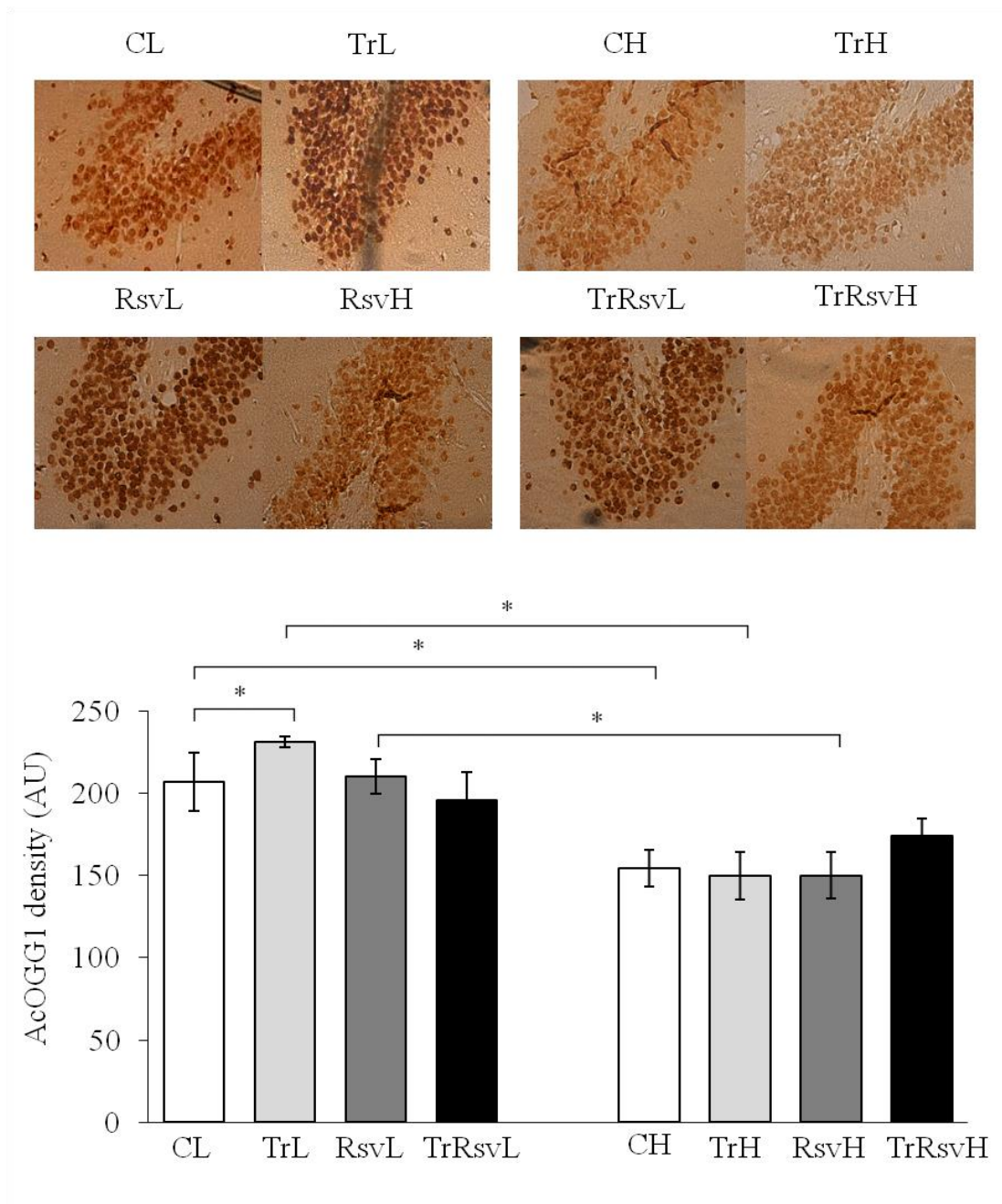
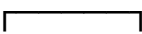



Figure 32: Acetylated OGG1 levels from histochemistry densities

Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL)

Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH)

Differences were shown with: “” Statistically significant differences with: “” plus a “” sign.*

4.3 Results from experiments on cell cultures

Data from the animal experiments foreshadowed some kind of connection between the memory results, resveratrol and the DNA repair via OGG1. To evaluate if there is a molecular link behind these findings I made tests on a simpler model system, the HCT116 cell culture.

I checked whether the acetylation status of OGG1 changes after treatment of known SIRT1 activator/inhibitor. So I applied resveratrol, nicotinamide and Trichostatin A as HDACI-II inhibitor. Resveratrol treatment decreased the amount of AcOGG1, but nicotinamide increased it. If the deacetylator is SIRT1 it makes sense that a SIRT1 activator (resveratrol) will decrease the acetylation state and a known inhibitor (NAM) will increase it. TSA does not have any effect because it only blocks HDAC I-II proteins.

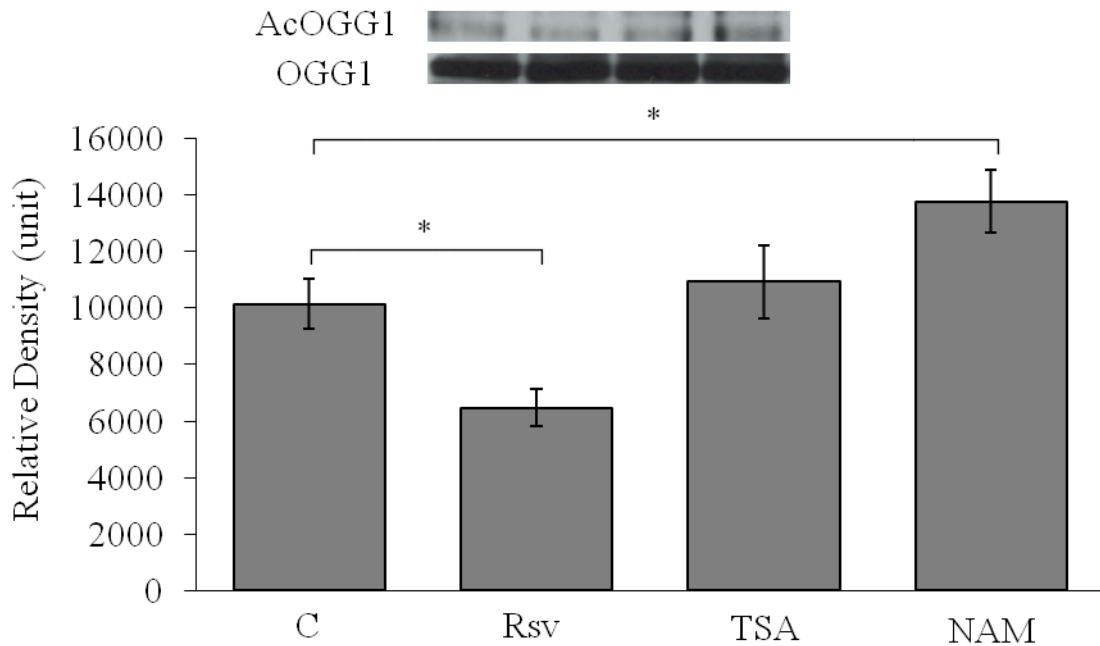
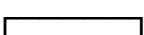


Figure 33: Acetylated OGG1 relative density

Control (C), Resveratrol treated (Rsv), Trichostatin A treated (TSA), Nicotinamide treated (NAM)

Statistically significant differences were shown with: “” plus a “” sign*

To prove the connection between the SIRT1 and OGG1 molecules I chose to silence the SIRT1 protein with siRNA technology. After the silencing I measured the relative density of acetylated OGG1. It was significantly higher in the group where SIRT1 was silenced with siRNA. siSIRT3 which was used as a “deacetylator control” had no effect on AcOGG1.

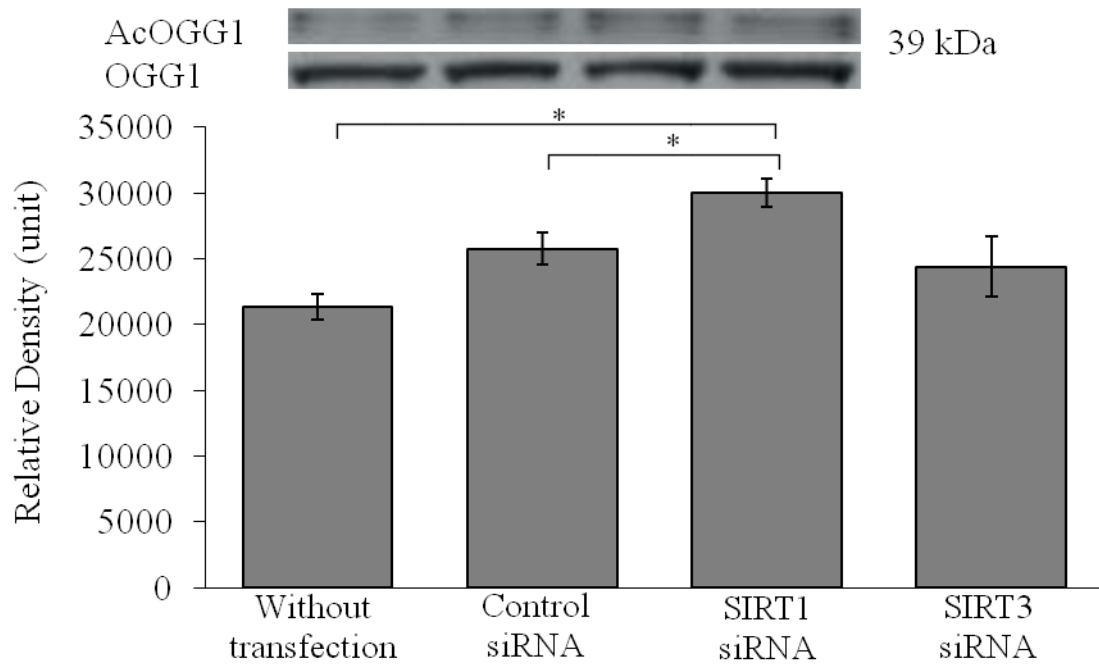
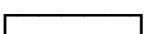


Figure 34: Acetylated OGG1 relative density after siRNA treatment

Statistically significant differences were shown with: “” plus a “” sign*

To verify these results I made PCR reactions, which are supposed to show the Sirt1 mRNA levels in the cells. Due to silencing the Sirt1 mRNA level was significantly decreased compared to the control level.

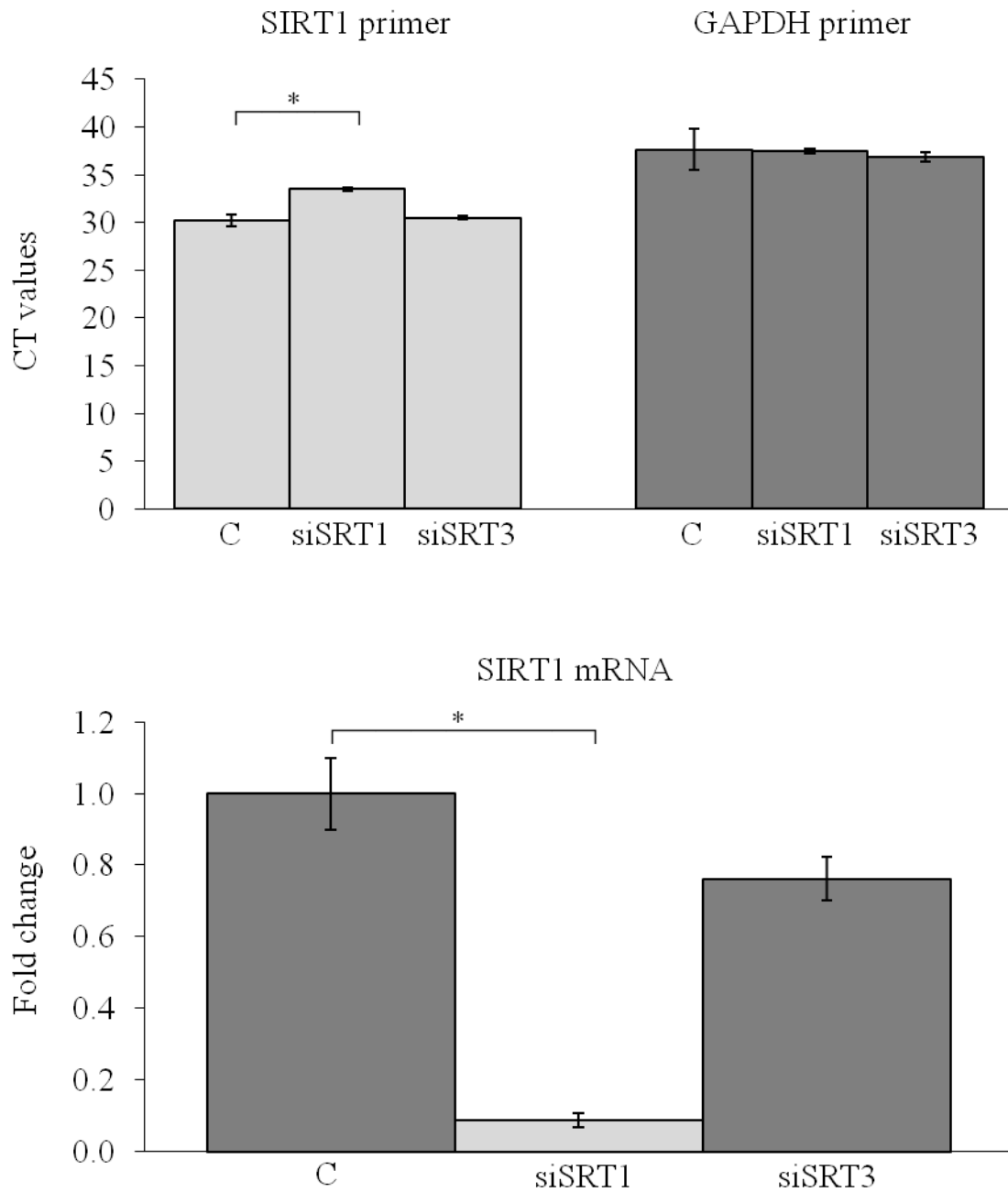
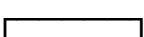


Figure 35: Sirt1 mRNA levels after silencing in the cells

Statistically significant differences were shown with: “” plus a “*” sign

5. Discussion

Although we measured VO₂max only for practical reasons (to adjust the training speed regularly), the results are quite interesting. We perceived 152% difference between the control groups (as seen on Figure 7.). This result is in accordance with Koch and Britton's results, because high running capacity animals have better capabilities in genetic compare to low running capacity animals. (Henderson, et al., 2002) The effectiveness of the training is also clearly visible at the end of the exercise program. Each exercised group produced higher VO₂max than the corresponding control (as seen on Figure 8.). These differences came up significant only at LCR animals, at HCR these are mainly tendencies. It is plausible that training's effectiveness is higher in LCR animals, because HCR animals were already at a higher level of fitness.

We likely can use the same idea at explanation of the body weight and blood sugar data. Koch, Britton and Wisløff presented that the signs of metabolic syndrome is evincible on LCR animals. (Koch, Britton, & Wisløff, 2012) Low running capacity animals had a greater body weight, and their body composition was also far from ideal. We did not measure any fat : muscle ratio, but there was a pronounced difference between low and high running capacity animals in the amount of the abdominal fat (just observation during the autopsy). It was also remarkable that TrL and TrRsvL groups had significantly less abdominal fat compare to CL. So, regular exercise already resulted in a leaner body shape after these few months (Figure 9.). In metabolic syndrome the blood glucose level is higher due to imbalanced glucose homeostasis. The glucose depots do not function well at skeletal muscles, which is in accordance with insulin resistance. Exercise could meliorate the blood glucose levels too (Figure 10.). We could not detect the same effect at resveratrol supplementation.

We certainly could see the effects of resveratrol in rotarod performance test. Rotarod test measures the balance and coordination of rodents. (Jones & Roberts, 1968) Both resveratrol and exercise enhanced the balance of the animals and the enhancement was cumulative (Figure 11.). In balance and coordination the main characters are the vestibular nuclei and cerebellum of course. In this study there was no space to go after this, but in 2011. Steiner and colleagues have previously demonstrated that exercise training increases brain mitochondrial biogenesis (via SIRT1 and PGC-1 α) in various

regions (i.e. cerebellum) and it results increased neuronal functions. (Steiner, Murphy, McClellan, Carmichael, & Davis, 2011)

It is also interesting to watch the results of cognitive tests all together. (Excluding Y maze test, because on our adult rats it did not give valid results. There was not enough alteration to measure an error : alteration ratio, so data is not shown.) HCR animals performed better in every task. They had the shorter latency and higher exploration in open field, they spent more time investigating a new object and they could remember the learnt things for longer time. Unfortunately in most cases neither resveratrol nor training could meliorate the disadvantages of low animals in these tasks. According to the literature exercise should increase cognitive function (Radak, et al., 2001), but in this case improved function is seems to be genetically inherited, not acquired with this few month of training.

The better cognitive function might be a result of some kind of cellular alteration. In most studies this alteration is neurogenesis. In our results high animal groups reacted to each type of treatment with neurogenesis, in contrast at low animals only resveratrol affected the show up of new neurons (Figure 18.). The neuronal changes do not follow correctly the cognitive data. NeuN staining can label the majority of neurons, but maybe the neurons which we detected were immature and could not affect cognitive function yet. (Patten, et al., 2013) As the literature reflects, BDNF is a sensitive marker, widely used to measure exercise's effect on cognitive function, especially in barely-invasive human studies. (Lee, et al., 2013) So we measured the amount of BDNF as well. It turned out that training elevated the BDNF levels in both animal types and there was a beneficial tendency at resveratrol too (Figure 29.). It is still under investigation, how SIRT1 is capable to change the levels of BDNF. In 2011 Jeong et al. provided a possible interaction. (Jeong, et al., 2011) They proved that SIRT1 can deacetylate and also activate TORC-1 which will increase BDNF expression through CREB. We measured the expression levels of Creb (Figure 28.) and data was in accordance with the BDNF levels. CREB also can verify exercise's beneficial effects through BDNF.

We checked if SIRT1 will also underpin these assumptions. Two signs were referring to this. The acetylated lysine levels were decreased in both high and low running capacity animals to every treatment, particularly to resveratrol treatment (Figure 25.).

Resveratrol is a well-known activator of SIRT1, so we hoped this polyphenol enlarged the deacetylase activity of SIRT1. On the other hand the level of PBEF (or NAMPT or visfatin) was markedly higher in resveratrol treated groups (Figure 27.). NAMPT is the key enzyme of NAD⁺ production from nicotinamide. As we thought an increased NAD⁺ production is the sign and protector of the increased deacetylase activity. Controversially the results did not prove this theory fully. After measuring Sirt1 mRNA, protein level and relative activity (Figure 19, 23, 24.) it is sure that SIRT1 was more active at exercised groups but only at high capacity of running type animals. (Other sirtuins did not become more active as well. Sirt3 and Sirt4 did not have elevated their mRNA expressions during the treatments (Figure 20-22.). And Sirt6's mRNA levels are happened to decrease to exercise which means that SIRT6 can not be the main deacetylator what we are looking for.) Resveratrol also did not raise the activity of SIRT1 spectacularly. Some publications say that resveratrol is not only a SIRT1 activator (or not a SIRT1 activator at all) but it has its own function probably as an antioxidant. (Pacholec, et al., 2010) The answer for this question is not in focus at this study, but we think that resveratrol both can increase SIRT1 activity and work as an antioxidant what we can see at results in the level of carbonylated proteins (Figure 26.). (Chung, et al., 2010)

The carbonyl level of proteins is used as a marker for oxidative stress. According to our data at resveratrol supplemented groups we measured lower carbonyl levels. It's a well-known fact that exercise increases protein's carbonyl amount through the enhanced level of ROS. This found to be true, especially at HCR animals. It might seem to be confusing because how can animals do better cognitive performance with higher level of damaged proteins? Radak et al. published an explanation in 2011, where they suggested that certain types of carbonyl groups could be important to stimulate protein turnover. (Radak , Zhao, Goto, & Koltai , 2011)

Reactive species also produce multiple oxidative DNA damage such as oxidized DNA bases, oxidized sugar fragments, abasic (AP) sites, and single-strand breaks (ssbs). Training increased BrdU incorporation into hippocampal cells in high performing animals (Figure 17.). On the other hand, we did not observe any indication for the S-phase and, thus, we considered that BrdU incorporation may represent DNA synthesis

due to repair processes of the oxidative base and strand lesions. (Elevated PAR results might mean the same on Figure 30, particularly because the PAR results show the same pattern as OGG1.) 8-oxoG is repaired via the base excision repair pathway that is initiated by the OGG1 (Hollenbach, Dhénaut, Eckert, Radicella, & Epe, 1999). Unexpectedly, in HCR rats there was a significantly lower Ogg1 expression in the hippocampus compared to LCR at both protein and RNA levels (Figure 31.). Intriguingly the activity-related post-translational modification of OGG1 (acetylation), was lower in high performing rats, when compared to LCR rats. These results appear to contradict previously published observations showing the imperative role of DNA damage repair in the hippocampal cells. (Jarrett, Liang, Hellier, Staley, & Patel, 2008) (Gredilla, Garm, Holm, Bohr, & Stevnsner, 2010)

We used a cell culture model to test if SIRT1 is the deacetylase of OGG1 or not. Nicotinamide, a SIRT1-specific inhibitor, caused the greatest increase in the acetylation of OGG1 (Figure 33.). Resveratrol an activator of SIRT1 decreased AcOGG1 levels and TSA (histone deacetylase inhibitor) had no significant effect on AcOGG1. Also silencing SIRT1 via siRNA increased the level of AcOGG1 (Figure 34.). Exercise in high running capacity groups increases the activity of SIRT1, leading to a decreased acetylation of OGG1, which implies a decreased enzymatic OGG1 activity and lower efficiency of 8-oxoG repair in the brain. It also has been reported earlier that, exercise increases DNA repair activity of OGG1 in human skeletal muscle from young individuals (Radak, et al., 2002) (Radak, et al., 2003) (Radak, Kumagai, Nakamoto, & Goto, 2007). It seems possible that OGG1's activity is differentially regulated in response to exercise, and that specifically its activity is transiently down-regulated in the brain, while upregulated in muscle. These observations raise the possibility that a delay in the repair of 8-oxoG lesions could be beneficial for brain function. As summarized before despite a genomic accumulation of 8-oxoG, Ogg1^{-/-} mice appeared to have a normal phenotype and showed an increased resistance to inflammation. Moreover, no organ defects were observed, and these Ogg1^{-/-} mice showed an increased tolerance to chronic oxidative stress (Arai, Kelly, Minowa, Noda, & Nishimura, 2006). These observations imply that the 8-oxoG base released from the genome of the brain cells (and not the transient 8-oxoG accumulation in DNA) could have a higher physiological/patho-physiological relevance compared to skeletal muscle.

Indeed, in 2012, Boldogh et al. shown that OGG1 binds its excision product, the 8-oxoG base. In complex with the 8-oxoG base, OGG1 interacts with the canonical Ras family members and induces guanine nucleotide exchange. Activated Ras then initiates signal transduction via Raf1-MEK1,2/ERK1,2, leading to the transcriptional activation of genes (Boldogh, et al., 2012). Activation of Ras and the MAPK pathway has been shown to cause apoptosis in neurons (Yang, et al., 2012). Therefore deactivation of OGG1 by SIRT1-mediated deacetylation could favor its control of the OGG1-initiated repair of DNA, but also imply an anti-apoptotic role of SIRT1 (as drawn on Figure 36.).

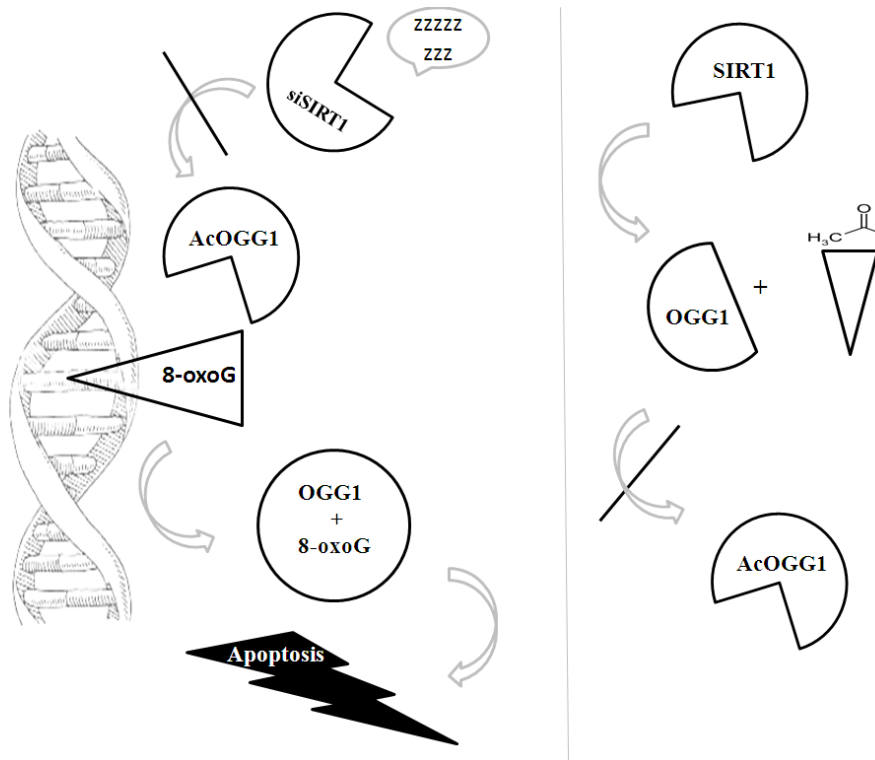


Figure 36: SIRT1 can deacetylate OGG1; it attenuates the repair, so apoptosis is avoidable

6. Conclusions

At the Objectives chapter I made up the aims of the study. Now it is time to review the four hypotheses I set up:

1. Regular physical activity and resveratrol treatment will enhance the cognitive function of both rat strains. **FALSE** Exercise had positive effects on the animals, but it was only seen in cognitive function at HCR animals. Resveratrol did not elevate the performance in cognitive tests.
2. Our aim was to illustrate that the cognitive enhancement was caused via sirtuins and neurotrophic factors in the brain which overall can be seen in neurogenesis. **PARTLY TRUE** At high running capacity animals the activity of SIRT1 was elevated, and we also could detect the elevated BDNF levels which enhanced the expression of Creb.
3. Training and/or resveratrol will compensate the differences which come from the genetic origin of the animals. **PARTLY TRUE** Training could overcome the signs of metabolic syndrome at LCR animals but it did not enhance the cognitive function compare to HCR animals. The meliorating results of resveratrol could not seen uniformly too.
4. Sirtuins can deacetylate OGG1 protein and this might moderate its activity. **TRUE** SIRT1 certainly can deacetylate OGG1, because at SIRT1-silenced cell cultures the levels of AcOGG1 was elevated. On the other hand AcOGG1 was also increased when nicotinamide was applied as a SIRT1 inhibitor, and AcOGG1 was decreased when SIRT1 activator (resveratrol) was applied.

According to the results it would be useful to do further investigations on the connection between SIRT1 and OGG1 protein through special circumstances. (For example, performing experiments on cell cultures from SIRT1 knock out mice.)

7. Summary

7.1 Summary in English

Koch and Britton carried out a long-term cross-breeding program with rats where the mating pairs were selected according to their running capacity. In each generation animals with the worst performance were chosen for inbreeding, and the same happened to animals with the best performance. Due to this procedure fundamental differences arose between the two types already at early generations. For example it was demonstrated that the animals from the “inactive” population showed the signs of metabolic syndrome (obesity, hypertension, high blood glucose levels, abnormal blood fat composition...etc.). I had the chance to study the effects of regular exercise and resveratrol on a group of animals which are from the 22nd generation of this model.

Exercise was successful because it elevated the animals’ maximal aerobic capacity, decreased the amount of abdominal fat and blood glucose levels. But we can’t detect the same with resveratrol alone. Surprisingly the “active” animals had better performance at cognitive tests.

We hypothesized neurogenesis and increased SIRT1 activity at the background of this result. The levels of acetylated proteins and NAMPT are also confirming the same. SIRT1 in “active” animals elevates the level of BDNF which will enhance the expression of Creb.

On the other hand we noticed alterations in the level of OGG1 repair enzyme. Our investigations proved that acetylated OGG1 protein has a lower concentration in the “active” population. We also could confirm on cell culture that SIRT1 protein is able to deacetylate OGG1, which causes decreased activity. For the first sight it can be surprising to mention the lower repair capacity as an advantage, but literature data was found that OGG1 can bind to the excised 8-oxoG, and together these two can initiate a signaling cascade which points to apoptosis.

In summary: we could prove the advantages of exercise again, unfortunately the results of resveratrol are could not be seen uniformly. At the “active” population we could detect molecular changes according to the literature and verify the connection between the SIRT1 and OGG1 repair enzyme.

7.2 Summary in Hungarian – Összefoglalás

Koch és Britton több éven át tartó keresztezési programjában patkányokat szelektált futási kapacitásuk alapján. Több generáción át egymás között keresztezték a legrosszabb teljesítményt nyújtó állatokat, ill. a legjobban futó egyedeket. Ennek következtében az állatok között alapvető különbségek keletkeztek. Az „inaktív” állatokról már a korai generációkban is leírták, hogy a rosszabb futási képesség mellett megfigyelhetőek rajtuk a metabolikus szindróma jelei (elhízás, magas vérnyomás, magas vércukorszint, kóros vérzsír-összetétel... stb.) Ezen két állatcsoport 22. generációján tanulmányoztuk a rendszeres testedzés és a szájon át adagolt rezveratrol hatásait.

Futási képesség tekintetében az edzés eredményes volt, hiszen javult az állatok maximális aerob kapacitása, csökkent a megfigyelhető testzsír mennyisége és a vércukor szintje. Ezzel szemben a rezveratrol önmagában nem volt elég hatásos a teljesítmény növelésére. A kognitív tesztek során az „aktív” genetikával bíró állatok szinte mindenben felülmúlták az „inaktív” példányokat.

Ennek háttérében az „aktív” állatoknál neurogenezist és a SIRT1 fehérje megnövekedett aktivitását sejtettük. Az acetilált fehérjék és a NAMPT fehérje mennyisége szintén alátámasztja ezt a feltevést. A SIRT1 az „aktív” állatokban emeli a BDNF mennyiségét, ezzel is fokozva a Creb expresszióját.

Mindemellett felfigyeltünk az OGG1 repair enzim megváltozott mennyiségére is. Vizsgálataink kimutatták, hogy az acetilált OGG1 fehérje alacsonyabb szinten van jelen az „aktív” állatok mintáiban. Ezt követően sejt kultúráján sikerült igazolni, hogy a SIRT1 fehérje az OGG1 potenciális deacetilálója, így csökkentve annak aktivitását. Bár első hallásra megdöbbentő lehet a repair mechanizmusok csökkenését előnyként felhozni, az OGG1 egyes források szerint a kivágott 8-oxoG molekulával komplexet képez, amely a kiindulási lépése a továbbiakban egy, az apoptózis irányába mutató jelsorozatnak.

Összességében elmondható: az edzés minden esetben eredményez jótékony változásokat, a rezveratrol hatásai azonban nem figyelhetők meg egységesen minden csoportban. Leginkább az „aktív” genetikájú állatoknál sikerült az irodalmi adatokkal is összecsengő molekuláris változásokat tetten érni és megfigyelni a SIRT1 hatását a repair folyamat egy enzimére. Ezt követően sejt kultúráján sikeresen igazolódtott az OGG1 és a SIRT1 fehérje kapcsolata.

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9. Bibliography of own publications

In connection with the thesis:

- **Sárga L**, Hart N, Koch LG, Britton SL, Hajas G, Boldogh I, Ba X, Radák Z (2013) Aerobic endurance capacity affects spatial memory and SIRT1 is a potent modulator of 8-oxoguanine repair. *Neuroscience* 252:326-36.

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- Radák Z, Hart N, **Sárga L**, Koltai E, Atalay M, Ohno H, Boldogh I (2010) Exercise plays a preventive role against Alzheimer's disease. *J Alzheimers Dis.* 20(3):777-83.

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Not in connection with the thesis:

- Hart N, **Sárga L**, Csende Z, Koltai E, Koch LG, Britton SL, Davies KJ, Kouretas D, Wessner B, Radák Z (2013) Resveratrol enhances exercise training responses in rats selectively bred for high running performance. *Food Chem Toxicol.* 61:53-9.

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- Marosi K, Bori Z, Hart N, **Sárga L**, Koltai E, Radák Z, Nyakas C Long-term exercise treatment reduces oxidative stress in the hippocampus of aging rats. *Neuroscience.* 226:21-8.

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