THE PRECLINICAL STUDY OF THE EXPRESSION CHANGES OF PGC-1α AND SIRT GENES

SUMMARY OF Ph.D. THESIS

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I – INTRODUCTION

Chronic diseases are becoming increasingly serious health care problems in developed countries as the life expectancy increases. In addition to the most common chronic cardiovascular diseases and cancers, neurodegenerative diseases are also having a significant influence on the health financing system. To date, several common clinical and molecular similarities have been identified amongst these neurodegenerative conditions. However, it seems that mitochondria may be one of the most important common subcellular hotspots behind the different conditions. For this reason, significant research activity has been directed to the characterization of mitochondrial dysfunction and the identification of potential neuroprotective molecular targets. During the above-mentioned process, amongst others two interdependent metabolic master regulator families have been identified, namely the peroxisome proliferator-activated receptor-γ coactivator (PGC)- and the silent information regulator 2 homologues (Sirtuin)-family.

There are three members of the PGC-family: (1) peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), (2) peroxisome proliferator-activated receptor-γ coactivator 1β (PGC-1β) and (3) PGC-related coactivator (PRC). PGC-1α is the most widely tested member. PGC-1α was identified around 20 years ago as a cold-inducible coactivator of adaptive thermogenesis in brown adipose tissue (BAT). This master regulator participates in the gluconeogenesis of liver besides adaptive thermogenesis (BAT) and furthermore, in the fiber-type switching of skeletal muscle (type II to type I) as well and stimulates mitochondrial biogenesis and fatty acid β-oxidation. The complexity of the PGC-1α-system is enhanced by the fact that more than 10 isoforms are currently known, resulting from alternative splicing and promoter usage. These molecular processes are often coupled. The two main groups of the splicing variants are the full-length (FL-Pgc-1α(1/-a); 797 amino acid (AA)) and the N-terminal truncated (NT-Pgc-1α(-a); 270 AA) isoforms. The shorter, but also active NT-Pgc-1α isoform is resulted from the insertion of an in-frame stop codon (31 base pair (bp)) between exon 6 and 7. From the perspective of the promoters, in addition to the canonical proximal (or reference) (PP; REF-Pgc-1α) and alternative promoters (AP), tissue-specific promoters have also been described (brain-specific (BP; CNS-Pgc-1α) and liver-specific promoters (LP)). Currently there is only a limited amount of data about the isoform specific distribution of these in the brain. Pgc-1α was widely tested in the context of the neurodegenerative disorders. The models of *Pgc-*

1α deficiency resulted in the acceleration of the neurodegenerative mechanism, however, the stimulation of gene expression slowed it down.

Sirtuins are mainly NAD^+-dependent deacetylases, which react with the acetyllysine residues of various regulated proteins. Currently seven Sirtuin subtypes (Sirt1-7) have been identified. These subtypes show different subcellular localization. Like Pgc-1α, Sirtuins are responsible for regulating many proteins too. Sirtuins play a very important role in the regulation of genomic stability, tumor suppression and cellular energetic processes. Furthermore, the isoforms of Sirt1 and Sirt3 make the Sirtuin-system much more complex. These isoforms are originated from the alternative splicing and promoter usage similar to that of the PGC-system. Besides the full-length form of Sirt1 (Sirt1-Fl), two additional isoforms can be produced with specially designed PCR (polymerase chain reaction) primers: Sirt1-Δ8 (lack of exon 8) and Sirt-ΔE2 (lack of exon 2)). Sirt3 has also three transcript variants (Sirt3-M1 (AA (1-334)), -M2 (AA (15-334)) and -M3 (AA (88-334)), which produce three protein subtypes with various length of the N-terminal region. Experimental data showed that Sirt3-M1 and -M2 splice variants are mainly localized in the mitochondria, while -M3 in the nucleus. Several studies to date have raised the role of Sirtuins in neurodegeneration.

Cumulated data suggest that the above detailed two important neuroprotective systems are not independent. In the cytoplasm there is an interaction between PGC-1α and SIRT1. During this molecular linkage, SIRT1 deacetylates PGC-1α, which leads to the activation of the downstream targets and the promotion of mitochondrial biogenesis, oxidative phosphorylation and energy production. Furthermore, SIRT1 stimulates the promoter of *Pgc-1α* gene in special environmental context (fasting, calorie (dietary) restriction (CR (DR)).

The regulatory role of the PGC- and Sirtuin-systems in various metabolic pathways, as well as the potential neuroprotective effect led to several scientific efforts to modulate these molecules. These efforts can be divided into three main groups: (1) – environmental activation (cold exposure, training, CR (DR)), (2) – pharmacological activation (e.g. resveratrol) and (3) – genetic manipulation (e.g. overexpression, knockout (KO) animals). The effect of the environmental temperature on the PGC- and Sirtuin-systems was tested in the brown- and white adipose tissue (BAT, WAT), and in the skeletal muscle as well. However, there is only limited data available about the effect on the brain. Regarding the central nervous system (CNS), it is known that Trios *et al.* (2003) could not detect any Pgc-1α level alteration in the brain of adult (18–20-week-old) male C57Bl/6J mice after 4 hours (4°C) of cooling. The impact of exercise on PGC- and Sirtuin-systems was also widely tested, however, the diversity of the models and

the training strategies make the interpretation and comparison of these results complicated. It is proved that training elevates the level of PGC-1α and SIRT1 in the skeletal muscle. Steiner *et al.* (2011) found that after 8 weeks of treadmill training Pgc-1α and Sirt1 mRNA level elevations were detectable in different brain regions of mice. However, many contradictory data also available in the scientific literature.

Parkinson's disease (PD) is clinically characterized by bradykinesia, tremor and/or muscle rigidity. Behind this clinical constellation there is the cumulation of several complex pathological molecular processes which result in the loss of dopaminergic neurons in the substantia nigra (SN). At the time of diagnosis, around 30% of dopaminergic neurons have already died. One of the most widely known model of PD is the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxin (mitochondrial complex I inhibitor) treatment. Previously performed animal experiments hypothesized a potential neuroprotective effect of PGC-1α against MPTP-induced neuronal cell damage. In *Pgc-1α* deficient animals the MPTP-induced damage was more robust, and the oxidative damage was more pronounced compared to wild-type (wt) controls. However, the overexpression or the pharmacological activation (e.g. RESV) showed a potentially neuroprotective elevation after MPTP treatment. Two studies have tested the potential neuroprotective effect of *Sirt1* overexpression against MPTP. In conclusion, it seems that the *Sirt1* overexpression did not alleviate the toxic effect of MPTP.

Huntington's disease (HD) is an autosomal dominantly inherited neurodegenerative disease, which is caused by the expansion of CAG repeats in the IT15 gene encoding huntingtin protein (*Htt*). Previous works demonstrated that mutant huntingtin protein (*mHtt*) inhibits the function of PGC-1α via transcriptional dysregulation. In the brain, striatal degeneration was demonstrated in transgenic models of HD. Histological studies proved that the striatal medium spiny neurons are more vulnerable in HD, but the interneurons are relatively spared. Török *et al.* (2015) was the first, who estimated the brain region specific distribution of FL- and NT-Pgc-1α isoforms and tested the mRNA levels of REF- and CNS-Pgc-1α promoters in 8-, 12- and 16-week-old N171-82Q mice. They found that the FL-Pgc-1α mRNA level was significantly downregulated in the striatal and cortical samples of 8-week-old animals. The NT-Pgc-1α level was, however, upregulated in the striatal and cortical samples of 16-week-old animals. Török *et al.* (2015) also tested the cerebellum (Crb), where there was a strong significance of elevation regarding both isoforms. They hypothesized that it is a compensatory phenomenon. The association between SIRTs and HD was also widely studied. However, the published data about the Sirt1/SIRT1 mRNA and protein changes are somewhat inconsistent.

The data detailed above are seemingly incomplete. There is a lack of experimental data focusing on the role of the PGC- and Sirtuin-systems in different brain regions. Furthermore, increasing data suggest that isoforms resulting from alternative promoters and splicing usage may have an important biological role. We think that it is important to start the detailed characterization these systems in each neurodegenerative model, through which we will be able to identify novel therapeutic molecular targets.

II – AIMS

The aims of the work were:

(1) To determine the effect of cold exposure (4°C; 200 or 900 min) on the PGC- and Sirtuin-systems (FL-, NT-, CNS-, REF-Pgc-1α, Sirt1, Sirt3-M1/M2/M3) in different brain areas important in the regulation of motor functioning (striatum (Str), cortex (Ctx), cerebellum (Crb)) in C57Bl/6J mice.

(2) To determine the effect of exercise training (2x30 min/day; 5 or 12 days) on the PGC- and Sirtuin-systems (FL-, NT-, CNS-, REF-Pgc-1α, Sirt1, Sirt3-M1/M2/M3) in different brain areas important in the regulation of motor functioning (Str, Ctx, Crb) in C57Bl/6J mice.

(3) To determine the effect of MPTP (5x1 i.p. inj. (15 mg/kg) for 1 day; dissection 90 min or 1 week after the last injection) on the PGC- and Sirtuin-systems (FL-, NT-, CNS-, REF-Pgc-1α, Sirt1, Sirt3-M1/M2/M3) in different brain areas important for neurodegeneration (Str, Ctx, Crb) in C57Bl/6J mice.

(4) To assess the mRNA expression pattern of Sirt1 and three isoforms of Sirt3 in the Str, Ctx and Crb using the N171-82Q tg mouse model of HD. Furthermore, to assess the effect related to the presence of the transgene and the possible effect of aging and gender.

III - MATERIALS AND METHODS

ANIMALS - In the cold exposure and exercise training experiments 20-week-old female (C57Bl/6J) mice were involved. During the MPTP study 12-week-old male (C57Bl/6J) mice were treated. To test the effect of transgene on the PGC- and Sirtuin-systems, 8-, 12- and 16-week-old N171-82Q and their control (B6C3) wt mice with identical genetic background (female and male animals distributed equally) were used. All the tg animals were originally obtained from Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed in cages under standard conditions with 12-12 h light-dark cycle and free access to food and water. The experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC).

TREATMENT PROTOCOLS -

(1) *COLD EXPOSURE* - Animals were randomly divided into four groups (n = 7–8 in each group). The first group was kept at 4°C for 40 min/day, for 5 days (200 min), the second one was kept 4°C for 180 min/day for 5 days (900 min). After the cold exposure, mice were placed back under standard conditions (22–24°C). The third and fourth groups were control groups and were housed at 22–24°C in the same room.

(2) *EXERCISE TRAINING* was performed using a rotarod. The mice were randomly allocated into four groups (n = 5–8 in each group). The first and second groups were the training groups. The mice were placed on the rotarod for a 2-session period (9.00 a.m., 4.00 p.m.) for 5 days (first group) or 12 days (second group) after a short learning period. The standard speed was 5 RPM for 30 min. Prior to each training session, the mice were transported to the testing room for an acclimatization period of at least 30 min. The third and fourth groups were control groups.

(3) *MPTP TREATMENT* - MPTP was dissolved in phosphate-buffered saline (PBS; pH adjusted to 7.4) and was administered intraperitoneally (i.p.). Animals were randomly divided into four groups (n = 7–8 in each group). The first and second groups received i.p. injection of 15 mg/kg body weight MPTP 5 times at 2 h intervals. The third and fourth groups served as the respective control groups and were injected with 0.1 M PBS according to the above-detailed treatment regimen.

SAMPLE HANDLING - In the cold exposure and the exercise training experiments ninety minutes after the last session, the animals were deeply anesthetized with isoflurane (Forane; Abott Laboratories Hungary Ltd., Budapest, Hungary) and their brains were dissected immediately. In the MPTP experiments the animals in the first group were deeply anesthetized with isoflurane and the brains were dissected ninety minutes following the last MPTP injection (acute treatment – acute (day 1) assessment), while animals in the second group were deeply anesthetized and dissected only one week later (acute treatment – subacute (day 7) assessment). In the third and fourth (control) groups the dissection was made respective to the above-detailed acute and subacute groups. In the N171-82Q experiment, the animals were anesthetized and dissected when they reached the 8-, 12-, and 16 weeks of age (the control groups were followed this method as well). The method of dissection was the same for each experiment. During the process the brains were rapidly removed on ice and immediately halved at the midline and then both hemispheres were further cut to obtain the Str, Ctx and Crb. All the samples were stored at -80°C until the real-time polymerase chain reaction (RT-PCR).

RT-PCR ANALYSIS - Total RNA was isolated from Str, Ctx and Crb with Trizol reagent according to the manufacturer's protocol. The concentration of RNA was measured with a MaestroNano spectrophotometer, and the integrity of RNA was randomly tested by gel electrophoresis using 1% agarose gel. cDNA was generated from 1 μg of total RNA with random hexamer primers and reverse transcriptase according to the Revert Aid First Strand cDNA Synthesis Kit protocol. cDNA was kept at -20°C until further use. RT-PCR was performed with a CFX 96 Real-Time System (Bio-Rad, USA) to detect changes in mRNA expression, using various primer pairs at a final volume of 20 μl. The following Pgc-1α and Sirtuin primers were used: FL-Pgc-1α: forward - ex4/ex5 and reverse - ex6/ex7; NT-Pgc-1α: forward - ex4/ex5 and reverse - ex6/7a; CNS-Pgc-1α: forward - exB4 and reverse - ex3; Ref-Pgc-1α: forward - ex1a/ex2 and reverse - ex3; Sirt1: forward - ex7/ex8 and reverse - ex8; Sirt3-M1: forward - ex1b and reverse - ex3; Sirt3-M2: forward - ex1b and reverse - ex3; Sirt3-M3: forward - ex2 and reverse - ex3. Target gene expression was normalized to the endogenous control gene 18S rRNA (Applied Biosystems, USA). Relative expression was calculated by the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001).

STATISTICS - All statistical calculations were performed with the use of the freely available R software (R Development Core Team). The distribution of data populations was checked with the Shapiro–Wilk test, and Levene test was also performed for the analysis of the homogeneity of variances. To assess the differences between Pgc-1α- and Sirtuin gene expression levels in

all brain areas relative to their respective control groups, approximative (10,000 random permutation) two sample Fisher-Pitman permutation test was applied in case of cold exposure and exercise training experiments. In the MPTP experiments two-sample t-tests via Monte-Carlo permutation (with 10,000 random permutations) were performed. In the HD experiment in several cases the data were diverged from Gaussian distribution and the variances were not equal. For that reason we applied the Scheirer-Ray-Hare test to determine the differences between the investigated factors and their interaction as well. Afterwards, we carried out permutation t-tests as post hoc analysis for pairwise comparison and type I errors from multiple comparisons were controlled with false discovery rate. As some of the possible comparisons would not have yielded meaningful information regarding the *a priori* decided presumptions, a maximum of 9 pairwise comparisons were implemented in case of each subtype or isoform analyzed by each brain region. We calculated the gene expression level of Pgc-transcripts in all brain areas relative to FL-Pgc-1α and CNS-Pgc-1α control Str groups. For Sirtuins we compared Sirt1 expression levels to control striatal Sirt1-Fl, and all Sirt3 isoforms to control striatal Sirt3-M1 groups. The differences were considered significant when the p values were less than 0.05.

IV – RESULTS

COLD EXPOSURE - There were no detectable changes in the levels of Pgc-1α transcripts in the different brain areas after 200 min or 900 min cold (4°C) exposure. After 200 min of cold exposure there were also no detectable changes in the levels of Sirt1 and Sirt3-M2 transcripts in any brain regions, but Sirt3-M1 levels elevated in the Ctx (ctrl: 1.26 ± 0.49; EX: 1.97 ± 0.60; $p = 0.036$), whereas Sirt3-M3 levels decreased in the Crb (ctrl: 0.16 ± 0.05; EX: 0.10 ± 0.03; $p = 0.027$). 900 min of cooling resulted in the relative decrease of cortical Sirt1 (ctrl: 1.14 ± 0.31; EX: 0.66 ± 0.24; $p = 0.008$) and striatal Sirt3-M1 (ctrl: 1.04 ± 0.30; EX: 0.72 ± 0.21; $p = 0.029$) expression levels.

EXERCISE TRAINING - After 5-day-long rotarod training there was not present any change in the Pgc-1α isoforms in any of the investigated areas. However, the 12 day exercise training resulted in significant increases in all investigated isoforms (FL-Pgc-1α, NT-Pgc-1α, CNS-Pgc-1α and REF-Pgc-1α) mRNA expression in the Crb (FL-Pgc-1α: ctrl: 1.32 ± 0.20; EX: 1.59 ± 0.19; $p = 0.024$; NT-Pgc-1α: ctrl:0.29 ± 0.04; EX: 0.38 ± 0.04; $p = 0.0002$; CNS-Pgc-1α:

ctrl:1.35 ± 0.23; EX: 1.80 ± 0.32; p = 0.003, REF-PgcC-1α: ctrl: 0.21 ± 0.03; EX: 0.30 ± 0.02; p = 0.0003). With regards to the Str and the Ctx, no other significant differences were detected. From the perspective of Sirtuins, after 5 days of rotarod training, cortical Sirt1 levels were found to be elevated (ctrl: 0.78 ± 0.10; EX: 0.97 ± 0.16; p = 0.042), but the other isoforms stayed unchanged. However, 12 days of exercise training resulted in the increase of both Sirt3-M1 and -M2 mRNA expression in the Crb (Sirt3-M1: ctrl: 0.79 ± 0.18; EX: 1.28 ± 0.30; p = 0.002; SIRT3-M2: ctrl: 0.33 ± 0.09; EX: 0.50 ± 0.10; p = 0.007). We did not find differences in Sirt1 and Sirt3-M3 levels in any other brain areas.

MPTP TREATMENT - Ninety minutes following the last MPTP injection of the acute treatment of MPTP, the FL-Pgc-1α and NT-Pgc-1α expression significantly increased in the Str (FL-Pgc-1α: ctrl: 0.97 (0.92–1.04); EX: 1.47 (1.21–1.83); p = 0.0048; NT-Pgc-1α: ctrl: 0.44 (0.40–0.49); EX: 0.70 (0.56–0.78); p = 0.019), Ctx (FL-Pgc-1α: ctrl: 0.96 (0.91–1.06); EX: 1.23 (1.15–1.43), p = 0.009; NT-Pgc-1α: ctrl: 0.46 (0.43–0.48); EX: 0.69 (0.59–0.71); p = 0.0012) and Crb (FL-Pgc-1α: ctrl: 1.50 (1.27–1.90); EX: 2.40 (2.07–2.76); p = 0.013; NT-Pgc1α: ctrl: 0.67 (0.48–0.86); EX: 1.21 (1.14–1.44); p = 0.009). Furthermore, MPTP-induced increases in CNS-Pgc-1α expression were also significantly larger in all investigated brain regions compared to the controls (Str: ctrl: 1.03 (0.88–1.11); EX: 1.38 (1.34–1.78); p = 0.0069; Ctx: ctrl: 0.91 (0.80–0.98); EX: 1.41 (1.24–1.42); p = 0.0048; Crb: ctrl: 1.51 (1.20–1.98); EX: 2.77 (2.34–3.17); p = 0.019). However, there was not any difference between the REF-Pgc-1α levels in the Str (ctrl: 0.11 (0.10–0.12); EX: 0.11 (0.95–0.12)); Ctx (ctrl: 0.11 (0.11–0.12); EX: 0.09 (0.08–0.10)) and Crb (ctrl: 0.21 (0.20–0.29); EX: 0.28 (0.24–0.29)) of MPTP-treated and control mice. One week following the last injection in the acute treatment regimen, there was not any significant change either in the FL-, NT-, CNS- or in the REF-Pgc-1α levels between the control and the MPTP-treated animals in any brain area.

There were no detectable changes in the levels of Sirtuin transcripts in the different brain areas after ninety minutes following the last MPTP injection. However, 1 week following the last injection a slight significance was found in all of the Crb Sirt3 isoforms (Sirt3-M1: ctrl: 1.11 ± 0.29; EX: 1.44 ± 0.14; p = 0.015; Sirt3-M2: ctrl: 0.33 ± 0.08; EX: 0.44 ± 0.05; p = 0.021; Sirt3-M3: ctrl: 0.03 ± 0.01; EX: 0.044 ± 0.01; p = 0.018). However, these significances disappeared using the Bonferroni correction.

N171-82Q EXPERIMENT - We could not detect any significant difference between male and female mice regarding the expression any of the assessed Sirtuin isoforms either in the wt or in the tg groups, so they were pooled for further analyses. Furthermore, in respect of Sirt1 and

Sirt3 expression, no interaction was found between the presence of the transgene and aging. Focusing on their separate effects, there was a significant elevation of Sirt1 expression in all the cortical and cerebellar samples of 8-, 12- and 16-week-old tg animals compared to wt mice (Ctx (8-week-old: $p = 0.0029$; 12-week-old: $p = 0.0018$; 16-week-old: $p = 0.0029$); Crb (8-week-old: $p = 0.0052$; 12-week-old: $p = 0.0054$; 16-week-old: $p = 0.0065$), but not in the Str. Regarding the effect of aging on Sirt1 expression levels, we detected significant increase only in the Crb of tg group at 16 weeks of age (8- vs. 16-week-old: $p = 0.0245$; 12- vs. 16-week-old: $p = 0.0316$). There was no detectable change in Sirt3-M1 expression in the Str and Ctx of any age groups (8-, 12-, 16-week-old). In contrast, there was a clear elevation of Sirt3-M1 mRNA expression in cerebellar samples of all age groups of tg animals compared to wt mice (8-week-old: $p = 0.0024$; 12-week-old: $= 0.0024$; 16-week-old: $p = 0.0024$). We could not observe age-related effect in the Sirt3-M1 isoform in either group. Regarding Sirt3-M2 we could not detect any difference between wt and tg groups in the striatal and cortical samples, but we could identify a significant elevation in the Crb of tg animals in each age group compared to wt controls (8-week-old: $p = 0.0021$; 12-week-old: $p = 0.0012$; 16-week-old: $p = 0.0021$). When assessing the effect of aging on Sirt3-M2 expression levels, we detected significant decrease only in the Ctx of wt group at 16 weeks of age (8- vs. 16-week-old: $p = 0.038$; 12- vs. 16-week-old: $p = 0.038$). The expression of Sirt3-M3 elevated in each striatal and cerebellar tg group, but only in the 16-week-old group in the tg Ctx compared to wt mice (Str (8-week-old: $p = 0.0097$; 12-week-old: $p = 0.0054$; 16-week-old: $p = 0.0097$); Ctx (16-week-old: $p = 0.0032$); Crb (8-week-old: $p = 0.002$; 12-week-old: $p = 0.0006$; 16-week-old: $p = 0.0016$). In the striatal Sirt3-M3 samples there was detectable decrease of expression by 12 weeks of age in both wt and tg animals (8- vs. 12-week-old (wt-wt): $p = 0.0243$; 8- vs. 12-week-old (tg-tg): $p = 0.036$).

V – DISCUSSION

Mitochondrial dysfunction is one of the most relevant aspects of the pathomechanism of neurodegenerative disorders. Consequently, any attempt to maintain or restore the function of the mitochondrial system can be considered potentially neuroprotective. Many previously published scientific data suggest that two interdependent metabolic master regulator families, namely PGC- and Sirtuin-family could be relevant targets in this context. However, the detailed characterization of these systems has not yet been fully done. Therefore, the aim of the current researches was to investigate the function of these systems in detail (brain region and isoform-specific mRNA expression changes) in different environmental conditions (cold exposure, training exercise), and in toxin (MPTP) and transgenic models (N171-82Q) of PD and HD, respectively.

The effect of cooling on skeletal muscle and adipose tissue (especially on BAT) was extensively studied. Overall, the performed studies – although different experimental protocols (temperature, cooling period, different age and types of murine models) were used – suggest that important elements of the PGC- (e.g. FL-, NT-isoforms) and Sirtuin-systems (Sirt1, -2, -3, -6) are cold-sensitive. There is only limited available data related to the alterations of these molecules in the central nervous system. Tritos *et al.* (2003) could not demonstrate any Pgc-1α mRNA alteration in the adult (18-20-week-old, male) murine (C57BL/6J) brain after 4 h cold (4°C) exposure. According to the results of the previous experiment, we could not demonstrate any alteration in the level of Pgc-1α isoforms (FL-, NT-, CNS-, REF-Pgc-1α) in any brain area after cold exposure (either in 200 min or in 900 min protocols) as well. On the other hand, short (200 min) cold exposure elevated the level of cortical Sirt3-M1 mRNA level and decreased the cerebellar Sirt3-M3 level. Long exposure (900 min) resulted in a decline in cortical Sirt1, and striatal Sirt3-M1 levels. As an explanation, we presume that this cold-challenge regimen was not effective in decreasing the core body temperature sufficiently. We think that the early compensatory mechanisms in BAT and skeletal muscle may protect the brain against the effect of cold exposure. In conclusion, it seems that the Sirtuin-system may be more responsive to the cold stimulus than the PGC-system.

The impact of exercise on PGC- and Sirtuin-systems was widely tested, however, the diversity of the models and the training strategies makes the interpretation and comparison of these results complicated. In the skeletal muscle there is a clear tendency of elevation regarding

different Pgc-1α isoforms. The rate of increase depends on the applied training method and its intensity. It seems that during training with different intensities there is a complex program (including promoter) shifting in the gene expression regulation. Some members of the Sirtuin-family were also tested in order to find a correlation between training intensity, the age of animals and Sirtuin expression in the skeletal muscle. In summary, there is a detectable increase in the level of Sirt1 and Sirt6 after exercise. In contrast to the cold exposure, there are some, but mainly inconsistent data in the literature about the exercise-induced PGC- and Sirtuin alterations in the CNS. Steiner *et al.* (2011) tried to estimate the brain region-specific expression changes of these systems after 8 weeks of treadmill training (8-week-old ICR animals). There was an obvious elevation in both systems in many brain regions (e.g. Ctx, hippocampus, frontal lobe). However, they did not attempt to detect the isoforms. In contrast to Steiner's findings, Gusdon *et al.* (2017) found no alteration in the PGC-1α and SIRT3 protein levels after 17-day-long training either in young or old mice (examined brain regions: Ctx, Str). Many other experiments were made with different protocols, however, the results remained controversial. Thus, during our research we tried to resolve the inconsistencies between the previously published studies and investigated the PGC- and Sirtuin isoforms in two different training protocols. The 5-day-long training period (short-term) did not cause alterations in Pgc-1α transcripts in any brain region. Contrarily, the 12-day-long (long-term) training period induced changes in all isoforms of the PGC-system in the Crb which is consistent with the results of Steiner *et al.* (2011). In the Sirtuin-system, the 5-day-long training also did not cause mRNA level alterations, but the long-term exercise resulted in the cerebellar elevation of Sirt3-M1 and Sirt3-M2 mRNA levels. These results suggest that very short-term exercise is unable to induce the PGC- and Sirtuin-systems. Contrarily, the 12-day-long training period induced changes in the Crb. We assume that the prominent cerebellar Pgc- and Sirtuin activation is connected to the development of synaptic plasticity between Purkinje cells. It leads to better motor coordination and integration of movements. Lucas *et al.* (2015) found in their research that there is a decrease in cell number and firing rate between the Purkinje cells in *Pgc* knockout mice, which finding further strengthens our assumption.

One of the most widely accepted models of PD are the MPTP (mitochondrial complex I inhibitor) toxin experiment. From the perspective of PGC-system, a previously published article suggests that the overexpression and the pharmacological stimulation (RESV) of *Pgc-1α* expression could be protective against MPTP toxicity. Furthermore, in the *Pgc-1α* knockout mice, there is a pronounced susceptibility of dopaminergic neurons against this toxin. However,

it seems that the MPTP toxin induces only a short-term compensatory reaction in the PGC-system. In our experiments, we tested the brain region and isoform specific reaction of PGC-system to MPTP toxin injection. We found that in the acute regimen, all the tested isoforms were elevated in all brain regions compared to the control animals. However, we could not detect this upregulation effect after 7 days of the last injection. We presume that MPTP is not highly selective, which explains the cerebellar activation as well. The effect of MPTP on Sirtuin-system is much less characterized. Two studies tested the potential neuroprotective effect of *Sirt1* overexpression against MPTP neurotoxicity. The conclusion of these experiments was that *Sirt1* overexpression did not alleviate the toxic effect of MPTP. There is a lack of scientific data about the effect of MPTP on other members of the Sirtuin family. However, it seems that in *Sirt3* and *Sirt5* null mice the MPTP-related toxic effect is more pronounced. We found only a slight, but following normalization, not significant activation of cerebellar Sirt3 (mitochondrial) isoforms in the chronic experiment, which could be explained by the potential slower activation of the cerebellar Sirtuin-system elements.

Sirtuins are surely involved in the neurodegenerative process in HD, however, there are controversial results regarding their role. Tulino *et al.* found a significant decrease in striatal Sirt1 mRNA expression from 4 to 9 weeks in the wt group, whereas cerebellar Sirt1 mRNA expression increased significantly by 9 and 14 weeks of age in the same control group in experiments with R6/2 mouse model of HD (MRN: 204). The presence of the transgene seemingly did not affect Sirt1 mRNA expression. Another research group (Reynolds *et al.*) measured Sirt1 mRNA levels in the whole-brain samples of 5, 8, 11- (MRN: 144) and 8-, 12-week-old (MRN: 182) R6/2 mice. In the 5-, 8- and 11-week-old mice (MRN: 144) there was a significant increase in the mRNA levels of all tg groups. Aging did not affect the values and accordingly there was no significant interaction between age and the presence of the transgene. Regarding the only female cohort with 182 mean CAG repeat size, the significant increase could be observed only in the 8-week-old group. Due to the different CAG repeats, ages, brain regions and gender composition, the comparability of these results is limited. During the research, there was no significant differences between genders in respect of the above-mentioned aspects, and therefore, gender issues seemingly did not introduce bias into the studies of Tulino *et al.* and Reynolds *et al.* Similar to the study of Tulino *et al.*, there was no detectable effect of the transgene in the Str in any age groups, but a marked increase in Sirt1 expression was demonstrated in cortical and cerebellar samples of tg animals compared to wt controls in all age groups – similar to that was found by Reynolds *et al.* when applying whole

brain samples. The magnitude of difference increased only by 16 weeks of age, and again, similarly to the latter study, no significant interaction was found between aging and the presence of the transgene. Aging-related increase in Sirt1 mRNA expression either in striatal or in cerebellar samples of wt mice, as found by Tulino *et al.*, could not be confirmed by our study. Although data suggest that the induction of mitochondrially acting Sirt3 may be capable of exerting beneficial effects in a HD model, the expression pattern of Sirt3 mRNA isoforms has never been studied in any HD model. Similar to that was found in case of Sirt1-Fl, a remarkable increase of cerebellar expression of all Sirt3 isoforms could be observed in tg animals compared to wt controls in the current study. The striatal expression of Sirt-M3 in all age groups and the cortical expression of Sirt3-M3 by 16 weeks of age were found to be increased. However, the relative expression level of Sirt3-M3 mRNA is considerably lower compared to that of the other two isoforms. The expression level of cortical Sirt3-M2 in wt mice and striatal Sirt3-M3 in wt and tg mice decreased by 16 and 12 weeks of age, respectively. The pattern of expression changes in the Crb regarding any of the assessed SIRT subtypes and isoforms strongly resemble to the Pgc-1α expression changes (either of its full length or N-terminal fragment) as we have shown in a previous study using the same animal model of HD. The reason behind the same pattern may be that Sirtuins are upstream regulators of Pgc-1α expression. Although the Crb is known not to be the primarily affected structure in HD, there is an increasing evidence of its involvement in the pathomechanism of the disorder. A considerable loss of Purkinje cells was demonstrated in some HD patients with predominant motor symptoms, the extent of which may become more pronounced in patients with higher CAG repeat number. Furthermore, there is no clear relationship between the disease stage and the degree of Purkinje and granular cell loss, and the degree of cerebellar degeneration is quite variable. The exact background of this variability, involving the sparing of alterations even in some human cases, is not known and needs further elucidation. Nevertheless, some studies proved cerebellar hypermetabolism in HD with a presumed compensatory role for the dysfunction in the fronto-striato-thalamic motor circuit. The significant elevations in cerebellar Pgc-1α and Sirt mRNA expressions in the N171-82Q HD model may be considered as an important part of this compensatory cerebellar hypermetabolism. Furthermore, the increased Sirt3 mRNA expression indicates the involvement of mitochondrial activation as well. The beneficial role of the SIRT-PGC-1α axis in intact cerebellar functioning may be further supported by the finding that FL-Pgc-1α knockout mice demonstrated reactive astrogliosis in cerebellar nuclei, whereas the Str and Ctx were almost totally spared.

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(For references to the statements in the thesis booklet, see the dissertation.)

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