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

Ph.D. THESIS

ANDRÁS SALAMON, M.D.

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PUBLICATIONS DIRECTLY RELATED TO THE THESIS

I. Salamon A, Maszlag-Török R, Veres G, Boros FA, Vágvölgyi-Sümegi E, Somogyi A, Vécsei L, Klivényi P, Zádori D (2020) Cerebellar predominant increase in mRNA expression levels of Sirt1 and Sirt3 isoforms in a transgenic mouse model of Huntington's disease. Neurochem Res (accepted publication) (original paper; **IF** (2018): **2,782**)

II. Salamon A, Török R, Sümegi E, Boros F, Pesei ZG, Fort Molnár M, Veres G, Zádori D, Vécsei L, Klivényi P (2019) The effect of physical stimuli on the expression level of key elements in mitochondrial biogenesis. Neurosci Lett 698: 13-8 (original paper; **IF** (2018): **2,173**)

III. Török R, **Salamon A**, Sümegi E, Zádori D, Veres G, Fort Molnár M, Vécsei L, Klivényi P (2017) Effect of MPTP on mRNA expression of PGC-1α in mouse brain. Brain Res 1660: 20-6 (original paper; **IF**: **3,125**)

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I. Salamon A, Dézsi L, Radics B, Varga ET, Hortobágyi T, Tömösvári A, Vécsei L, Klivényi P, Rajda C (2020) CANOMAD syndrome with respiratory failure. Ideggyogy Sz 73: 141–4 (case report; **IF** (2018): **0,113**)

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III. Salamon A, Zádori D, Szpisjak L, Klivényi P, Vécsei L (2019) Neuroprotection in Parkinson's disease: facts and hopes. J Neural Transm (Vienna) 127: 821-9 (review article; **IF** (2018): **2,903**)

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V. Salamon A, Zádori D, Horváth E, Vécsei L, Klivényi P (2019) Zonisamide treatment in myoclonus-dystonia. Orv Hetil 160: 1353-7 (case report; **IF** (2018): **0,564**)

VI. Salamon A, Faragó P, Németh VL, Szépfalusi N, Horváth E, Vass A, Bereczky Z, Tajti J, Vécsei L, Klivényi P, Zádori D (2019) Multiple ischemic stroke in Osler-Rendu-Weber disease. Ideggyogy Sz 72: 65-70 (case report; **IF** (2018): **0,113**)

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LIST OF ABBREVIATIONS

AAV	adeno-associated virus
AceCS2	acetyl-CoA synthetase 2
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
AP	alternative promoter
APC	adenomatous polyposis coli protein
Aβ	amyloid beta protein
BAT	brown adipose tissue
BP	brain (CNS)-specific promoter
BSKO	brain-specific *Sirt1* knockout mice
CNS-Pgc-1α	central nervous system (brain)-specific promoter (mRNA)
CNS-PGC-1α	central nervous system (brain)-specific promoter (protein)
CPS1	carbamoyl phosphate synthetase 1
CR	calorie restriction
CREB	cAMP response element-binding protein (CREB)
ctrl	control
DR	dietary restriction
ERR	estrogen-related receptor
ex	exon
EX	exposed
FL-Pgc-1α	full-length PGC-1α (mRNA)
FL-PGC-1α	full-length PGC-1α (protein)
FOXO	forkhead box O
FTD	frontotemporal dementia
G6PD	glucose-6-phosphate dehydrogenase
GDH	glutamate dehydrogenase
GPx	glutathione peroxidase
H	histone
HD	Huntington's disease
HIF	hypoxia-inducible factor

HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2
HNF4α	hepatocyte nuclear factor
Hsp70	heat shock protein 70
Htt	huntingtin protein
i.p.	intraperitoneally
IDH	isocitrate dehydrogenase
IT15	interesting transcript 15
KO	knockout
K-RAS	Kirsten rat sarcoma 2 viral oncogene homolog protein
LCAD	long chain acyl CoA dehydrogenase
LP	liver-specific promoter
MAO-B	monoamine oxidase B
MEF2C	myocyte-specific enhancer factor 2C
mHtt	mutant huntingtin protein
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NF-κB	nuclear factor κB
NRF	nuclear respiratory factor
NT-Pgc-1α	N-truncated PGC-1α isoform (mRNA)
NT-PGC-1α	N-truncated PGC-1α isoform (protein)
PBS	phosphate-buffered saline
PD	Parkinson's disease
Pgc-1α	*Pgc-1α* gene
Pgc-1α	peroxisome proliferator-activated receptor-γ coactivator 1α (mRNA)
PGC-1α	peroxisome proliferator-activated receptor-γ coactivator 1α (protein)
Pgc-1β	peroxisome proliferator-activated receptor-γ coactivator 1β (mRNA)
PGC-1β	peroxisome proliferator-activated receptor-γ coactivator 1β (protein)
Pitx3	pituitary homeobox 3 protein
PP	proximal promoter

PPAR	peroxisome proliferator-activated receptor
PRC	PGC-related coactivator
RAR	retinoic acid receptor
REF-Pgc-1α	reference PGC-1α promoter (mRNA)
REF-PGC-1α	reference PGC-1α promoter (protein)
RESV	resveratrol
RNA Pol I	RNA polymerase I
ROI	reactive oxygen intermediate
RPM	revolutions per minute
RT-PCR	real-time polymerase chain reaction
SDH	succinate dehydrogenase
Sir2	silent information regulator 2
SIRT	homolog of the silent information regulator 2
Sirt1-FL(/Sirt1-Fl)	Full-length Sirt1 isoforms (mRNA)
SIRT1-FL (/SIRT1-Fl)	Full-length Sirt1 isoform (protein)
Sirt1-Δ8	SIRT1 isoform (lack of exon 8) (mRNA)
SIRT1-Δ8	SIRT1 isoform (lack of exon 8) (protein)
Sirt1-ΔE2	SIRT1 isoform (lack of exon 2) (mRNA)
SIRT1-ΔE2	SIRT1 isoform (lack of exon 2) (protein)
Sirt3-M1, -2, -3	SIRT3 transcript isoforms (mRNA)
SIRT3-M1, -2, -3	SIRT3 transcript isoforms (protein)
SN	substantia nigra
SOD	superoxide dismutase
TAF4	transcription initiation factor TFIID subunit 4
tg	transgene
TH	tyrosine hydroxylase
TNFα	tumor necrosis factor-alpha
TR	thyroid hormone receptor
UCP	uncoupling protein
VLCAD	very long chain acyl CoA dehydrogenase
WAT	white adipose tissue
wt	wild-type

SUMMARY

Neurodegenerative diseases are becoming an increasingly serious health care problem in developed countries as the life expectancy increases, while the pathomechanism of the neurodegeneration is not fully understood yet. It seems that mitochondrial dysfunction might play a crucial role in the development of this process. The PGC- and the Sirtuin molecular families can induce the mitochondrial biogenesis and to interact with the neurodegenerative mechanism. Currently there is only a limited amount of data focusing on the brain region specific alteration of the isoforms of these systems. Accordingly, the aim of these experiments was to determine the mRNA level of some important elements of the PGC- and Sirtuin-systems (FL-, NT-, CNS-, REF-Pgc-1α, Sirt1-Fl, Sirt3-M1/M2/M3) in three brain regions involved in the regulation of motor functions (striatum, cortex, cerebellum) after different stimuli. Following two cold exposure (4°C) protocols (200 and 900 min) we found no detectable changes of Pgc-1α transcripts in different brain areas. In contrast, after short cold exposure the cortical Sirt-M1 increased and the cerebellar Sirt3-M3 decreased. Longer cold exposure resulted in a relative decrease in the cortical Sirt1-Fl and striatal Sirt3-M1 levels. The effect of training was also tested in two different protocols. After 5 days training (short-term) no detectable change was identified in the PGC-system. From the perspective of Sirtuins, we found a cortical Sirt1-Fl mRNA elevation, but the other isoforms remained stable. Longer training period (12 days) resulted in a significant cerebellar activation in both molecular families (FL-, NT-, CNS-, REF-Pgc-1α, Sirt3-M1/M2). To test further the systems' behavior, we used the widely accepted MPTP toxin model of Parkinson's disease. The animals were treated with intraperitoneally administered MPTP (5x1 inj./day) and were dissected 90 min or 1 week after the last injection. In the PGC-system we identified a transient elevation in all tested brain regions. On the other hand, the effect of MPTP to the tested elements of Sirtuin-system was negligible. A transgenic animal model of Huntington's disease (N171-82Q) was also applied in the set of experiments. We found that in case of the striatum and cortex, the presence of the transgene resulted in a significant increase in Sirt3-M3 and Sirt1-Fl mRNA levels, respectively, whereas in case of the cerebellum the transgene resulted in increased expression of all the assessed subtypes and isoforms. In conclusion, we presume that our cooling protocol was ineffective to reduce the core temperature appropriately. In contrast, long-term training protocol

was able to induce prominently the cerebellar PGC- and Sirtuin-systems, which emphasize the importance of cerebellum from the view of neuroprotection. Furthermore, we conclude that MPTP induces the PGC-system not only in the striatum and cortex, but also in the cerebellum. The effect of MPTP on Sirtuin-systems seems to be slight. In the N171-82Q transgenic HD model the unequivocal cerebellar Sirtuin activation with presumed compensatory role suggests that the cerebellum might be another key player in HD in addition to the most severely affected striatum.

I - INTRODUCTION

Chronic diseases are becoming increasingly serious health care problems in developed countries as the life expectancy increases (Hajat and Stein, 2018). In addition to the most common chronic cardiovascular diseases and cancers, neurodegenerative diseases are also having a significant influence on the health financing system (Sambamoorthi *et al.*, 2015). Alzheimer's disease (AD) (prevalence (USA): 1200 per 100,000), Parkinson's disease (PD) (prevalence (USA): 300 per 100,000), frontotemporal dementia (FTD) (prevalence (USA): 14 per 100,000), amyotrophic lateral sclerosis (ALS) (prevalence (USA): 7 per 100,000) and Huntington's disease (HD) (prevalence (Caucasian population): ~ 5 per 100,000) are the relevant elements of the heterogeneous group of neurodegenerative disorders (Rawlins *et al.*, 2016; Relja, 2004). To date, several common clinical (e.g. the presence of Parkinsonian symptoms) and molecular similarities (e.g. accumulations of special type of proteins; selective neuronal damage) have been identified amongst these neurological conditions (Ahmad *et al.*, 2017; Snowden *et al.*, 1995). However, it seems that mitochondria may be one of the most important common subcellular hotspots behind the different conditions (Lezi and Swerdlow, 2012). For this reason, significant research activity has been directed to the characterization of mitochondrial dysfunction and the identification of potential neuroprotective molecular targets (Arun *et al.*, 2016; Bose and Beal, 2016; Cabezas-Opazo *et al.*, 2015; Costa and Scorrano, 2012; Shi *et al.*, 2010). 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 (Jęśko *et al.*, 2017; Johri *et al.*, 2013).

I/1 – THE PGC-SYSTEM

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) (Austin and St-Pierre, 2012). PGC-1α is the most widely tested from the perspective of neurodegeneration. PGC-1α was identified around 20 years ago as a cold-inducible coactivator of adaptive thermogenesis in brown adipose tissue (BAT) (Puigserver *et al.*, 1998). It is coded by *PPARGC1* gene (*Pgc-1α*) (13 exons; located on the 5th chromosome (mice)) (Esterbauer *et al.*, 1999; Liang and Ward, 2006). 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 (Puigserver et Spiegelman, 2003). The most important molecular targets are inter alia the followings: uncoupling proteins (UCPs), peroxisome proliferator-activated receptors (PPARs), reactive oxygen intermediate (ROI), defense enzymes (superoxide dismutases (SODs) and glutathione peroxidase (GPx)), thyroid hormone receptors (TRs), retinoic acid receptor (RAR), nuclear respiratory factors (NRFs), estrogen-related receptors (ERRs), myocyte-specific enhancer factor 2C (MEF2C), hepatocyte nuclear factor 4α (HNF4α) and forkhead box Os (FOXOs) (Lin *et al.*, 2002, 2005; McGill and Beal, 2006; Puigserver *et al.*, 1998; Puigserver and Spiegelman, 2003; St-Pierre *et al.*, 2006; Zhang *et al.*, 2009). The complexity of the PGC-1α-system is further enhanced by the fact that more than 10 isoforms are currently known, resulting from alternative splicing and promoter usage (Martínez-Redondo *et al.*, 2015; Johri *et al.*, 2011). 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 (Zhang *et al.*, 2009). 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 (Zhang *et al.*, 2009). 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)) (Martínez-Redondo *et al.*, 2015; Soyal *et al.*, 2012). Some research data suggest that different environmental or pharmacological stimuli result in a tissue-specific

expression pattern of the above-mentioned isoforms (Lochmann *et al.*, 2015; Wen *et al.*, 2014). Currently there is only a limited amount of data about the isoform specific distribution of these in the brain. In the murine brain total Pgc-1α was detected in the cortex, striatum, globus pallidus, substantia nigra and hippocampus (Tritos *et al.*, 2003). Interestingly in the mouse brain there is a relative dominance of NT-Pgc-1α isoform (Zhang *et al.*, 2009). Additionally, mitochondrial density is increased in cortical, midbrain and cerebellar Pgc-1α overexpressing neuronal cells (Wareski *et al.*, 2009). Pgc-1α was widely tested in the context of the neurodegenerative disorders. In the models of *Pgc-1α* deficiency resulted in the acceleration of the neurodegenerative mechanism, however, the stimulation of gene expression slowed it down (Róna-Vörös and Weydt, 2010). The *Pgc-1α* deficient mice developed HD-like phenomenon (hyperactivity, limb clasping and impaired thermoregulation), and furthermore, a spongiform striatal degeneration was observed in the brain of these animals (Lin *et al.*, 2004; Leone *et al.*, 2005). It is hypothesized that the mutant huntingtin (*mHtt*) interferes with the expression of *Pgc-1α* via binding to the promoter with cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) / transcription initiation factor TFIID subunit 4 (TAF4) complex (transcriptional repression) (Cui *et al.*, 2006). In the MPTP model of Parkinson's disease, the *Pgc-1α* deficient mice are more vulnerable to the toxin (St-Pierre *et al.*, 2006). On the other hand, the pharmacological (resveratrol (RESV)) or genetical overexpression of *Pgc-1α* protects the dopaminergic neurons against toxin-induced neurodegeneration (Mudò *et al.*, 2012).

I/2 – THE SIRTUIN-SYSTEM

Sirtuins are mainly NAD^+-dependent deacetylases, which react with the acetyllysine residues of various regulated proteins (Cen *et al.*, 2011). The molecularly highly conserved Sirtuins were first identified in *Saccharomyces cerevisiae* (Silent information regulator 2 (Sir2)) (Paraíso *et al.*, 2013; Zakhary *et al.*, 2010). One of the most studied properties of Sirtuins are their lifelong increasing characteristic, however, the results in different species are controversial (Blander and Guarente, 2004; Cen *et al.*, 2011). The effect of calorie restriction (CR) on Sirtuin-system is widely studied. It was showed that CR lead (through the modification of NAD^+/NADH ratio) to the activation of some Sirtuin subtypes (Qui *et al.*, 2010). Currently

seven Sirtuin subtypes (Sirt1-7) have been identified (Anekonda and Reddy, 2006; Cen *et al.*, 2011; Donmez, 2012; Kelly, 2010a, b; Nunomura *et al.*, 2007). These subtypes show different subcellular localization (Cen *et al.*, 2011). Sirt1, -6 and -7 are mostly localized in the nucleus, Sirt3, -4, -5 in the mitochondria, while Sirt2 in the cytosol (Cen *et al.*, 2011; Michishita *et al.*, 2005). Certain Sirtuins have additional enzymatic properties besides deacetylase function: SIRT2 – demyristoylation, SIRT4 and -6 – ADP-ribosylation, SIRT5 – demalonylation, desuccinylation (Paraíso *et al.*, 2013; Brenmoehl and Hoeflich, 2013). Like Pgc-1α, Sirtuins are responsible for regulating many proteins too. The main targets of the different Sirtuin subtypes are the followings: SIRT1 – histones (H1, -3, -4), tumor protein p53 (p53), nuclear factor-κB (NF-κB), FOXOs, hypoxia-inducible factors (HIFs); SIRT2 – H3, -4, α-tubulin, FOXOs, p53, NF-κB, HIFs, β-secretase, adenomatous polyposis coli protein (APC), Kirsten rat sarcoma 2 viral oncogene homolog protein (K-RAS); SIRT3 – H4, SODs, acetyl-CoA synthetase 2 (AceCS2), succinate dehydrogenase (SDH), 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), (very) long chain acyl CoA dehydrogenase ((V)LCAD); SIRT4 – glutamate dehydrogenase (GDH), heat shock protein 70 (Hsp70); SIRT5 – carbamoyl phosphate synthetase 1 (CPS1), HMGCS2, SOD1, VLCAD, cytochrome c, isocitrate dehydrogenase (IDH), glucose-6-phosphate dehydrogenase (G6PD); SIRT6 – H3, tumor necrosis factor-alpha (TNFα); SIRT7 – H3, RNA polymerase I (RNA Pol I), p53 (Hallows *et al.*, 2006; Schiedel *et al.*, 2018). From the molecular targets detailed above, it becomes evident that 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 primers: Sirt1-Δ8 (lack of exon 8) and Sirt-ΔE2 (lack of exon 2)). However, the biological role of these isoforms is questionable despite the dominant expression in the brain (Deota *et al.*, 2017; Lynch *et al.*, 2010). 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 (Bao *et al.*, 2010; Cooper *et al.*, 2009; Jin *et al.*, 2009; Yang *et al.*, 2010). Experimental data showed that Sirt3-M1 and -M2 splice variants are mainly localized in the mitochondria, while -M3 in the nucleus (Nogueiras *et al.*, 2012). Limited data (mostly murine models) are available for the brain distribution of each subtypes. SIRT1 is normally localized in the neuronal and glial cells of cerebellum, cortex, hippocampus and hypothalamus (Hadem *et al.*, 2019; Kelly, 2010b). SIRT2 has a prominent expression in the neurons and glias (especially in the

oligodendroglias and astrocytes) (cerebellum, spinal cord, striatum, hippocampus) (Gomes *et al.*, 2015; Kelly, 2010b; Maxwell *et al.*, 2011; She *et al.*, 2017). SIRT3 shows a pronounced species dependence, however, it seems to be mostly localized in the cortical neurons (Jęśko *et al.*, 2017; She *et al.*, 2017). SIRT4 is localized also in the cortex and in the hippocampus (She *et al.*, 2017). SIRT5 and -7 can be found in the frontal lobe neurons (She *et al.*, 2017). The effect of SIRT1 on neurodegeneration has been extensively tested. In AD SIRT1 reduces the production of Aβ peptide under oxidative stress in mouse hippocampus, and furthermore, it stimulates the autophagy to help removing the pathological proteins (Sun *et al.*, 2010; Zhang *et al.*, 2011). It seems that the total SIRT1 and -3 protein levels correlate with the disease stage of AD (Jęśko *et al.*, 2017). In a similar way, in PD, SIRT1 reduces the formation of α-synuclein aggregates as well as promotes the autophagy which lead to the removal of the pathological proteins (Donmez *et al.*, 2012; Lee *et al.*, 2008; Zhang *et al.*, 2011, 2012). In HD the pharmacological overexpression (RESV) of Sirtuins resulted in the improvement of the neuronal dysfunction in nematodes and mammalian neurons as well. In postmortem striatal and cortical brain samples of HD patients Sirt1 mRNA level was reduced (Baldo *et al.*, 2019; Parker *et al.*, 2005).

I/3 - INTERACTION BETWEEN THE PGC- AND SIRTUIN-SYSTEMS

Cumulated data suggest that the above detailed two important neuroprotective systems are not independent (Amat *et al.*, 2009; Nemoto *et al.*, 2005; Rodgers *et al.*, 2005). In the cytoplasm there is an interaction between PGC-1α and SIRT1 (SIRT1 connects to the central, regulatory region of PGC-1α) (Lin *et al.*, 2005; Zhong and Mostoslavsky, 2011). During this molecular linkage, SIRT1 (some data suggest that SIRT3 as well) deacetylates PGC-1α, which leads to the activation of the downstream targets and the promotion of mitochondrial biogenesis, oxidative phosphorylation and energy production (Brenmoehl and Hoeflich, 2013; Kong *et al.*, 2010; Nemoto *et al.*, 2005; Rodgers *et al.*, 2005). Furthermore, SIRT1 stimulates the promoter of *Pgc-1α* gene in special environmental context (fasting, calorie (dietary) restriction (CR (DR)) (Amat *et al.*, 2009; Dominy *et al.*, 2009; Kelly, 2010a; Nemoto *et al.*, 2005; Rasouri *et al.*, 2007).

I/4 – MODULATION OPPORTUNITIES

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. RESV) and (3) – genetic manipulation (e.g. overexpression, knockout (KO) animals). In the following paragraphs, only modulation options relevant to the current researches are detailed.

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. In the BAT, cold exposure (4°C for 5 h) led to a significant increase in both FL- and NT-Pgc-1α isoforms (6-week-old C57BL/6J male mice) (Zhang *et al.*, 2009). Sirt1 and -3 mRNA levels in BAT also showed continuous significant elevations to cold exposure (5°C for 0-12 h) and decreases if the room temperature was elevated (27.5°C for 16 h) (8-week-old C57BL/6 male mice) (Jokinen *et al.*, 2017; Shi *et al.*, 2005). A longer cooling period (1-3 days, 4°C) was able to induce (the peak value was on day 1) the Pgc-1α expression in retroperitoneal WAT of 3-month-old male rats as well (Jankovic *et al.*, 2015). On 5°C, Sirt2/SIRT2 (mRNA and protein) were also inducible in BAT after 6 hours of cooling (4-week-old C57BL/6 male mice) (Wang and Tong, 2009). 3 hours after the exercise (30 min), in the skeletal muscle of 9 physically active men (mean age: 25.8 years) the cooling of the vastus medialis (10°C) resulted in the elevation of the Pgc-1α level (Ihsan *et al.*, 2014). Consistent with these data, in the skeletal muscle of 4-month-old male rats a significant elevation of the PGC-1α protein level (from day 3 to day 45) was detectable as well (Stancic *et al.*, 2013). There was a significant increase after 6 hours of cooling on 4°C in the relative mRNA level of Pgc-1α in the skeletal muscle of *Sirt1* overexpressing transgenic (tg) mice (Gerhart-Hines *et al.*, 2011). Regarding the central nervous system (CNS), it is known that HD tg animal models (R6/2 and N171-82Q) are susceptible to hypothermia (Weydt *et al.*, 2006). 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 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 (interestingly the intensity of the training influences the promoter usage in the muscle) (Costa *et al.*, 2010; Huang *et al.*, 2015; Lochmann *et al.*, 2015; Wen *et al.*, 2014). 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 ICR (Institute of Cancer Research) mice (8-week-old) (Pgc-1α – musculus soleus, brainstem, cortex, frontal lobe, hippocampus, hypothalamus, midbrain; Sirt1 – cortex, frontal lobe, hippocampus, hypothalamus, midbrain). On the other hand, Lezi *et al.* (2013) found no alteration of Pgc-1α and Sirt1 mRNA levels in the whole brain samples of C57BL/6 mice (4-month-old) after 6 weeks of moderate intensity treadmill training. Lezi *et al.* (2014) in their next study applied a longer training period (8 weeks), but there was no detectable Pgc-1α mRNA alteration in the whole brain samples of the mice (C57BL/6; 18-month-old), whereas the nuclear PGC-α protein level significantly increased. The potential influencing role of the age of animals has arisen behind the incongruent data, and therefore, Gusdon *et al.* (2017) tried to clarify the question with a 17-day-long treadmill (with increasing intensity) protocol, where 2 representative age groups of C57BL/6 mice (only the striatum and cortex was processed) were used (4-week-old and 24-month-old). They found no alteration in the protein level either in the PGC-1α, or in the SIRT3 group. In young (5-week-old) rats (Sprague-Dawley) Bayod *et al.* (2011) found a cortical PGC-1α and SIRT1 and a hippocampal SIRT1 protein elevation after 36 weeks of training. Belvirani and Okudan (2018) found that in young (3-month-old) Wistar rats the Pgc-1α mRNA level was elevated in the hippocampus (90 days, free wheel running exercise). Nevertheless, this effect was no more detectable in older animals (20-month-old). In contrast, after 2 weeks of moderate intensity treadmill training there was a detectable elevation of PGC-1α protein level in the hippocampus of 12-month-old Wistar rats (Marosi *et al.*, 2012).

I/5 – ROLE OF PGC- AND SIRTUIN-SYSTEMS IN SOME MODELS OF NEURODEGENERATION

Parkinson's disease (PD) is clinically characterized by bradykinesia, tremor and/or muscle rigidity (Postuma *et al.*, 2015). 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) (Allain *et al.*, 2008; Mandel *et al.*, 2003). At the time of

diagnosis, around 30% of dopaminergic neurons have already died (Cheng *et al.*, 2010). The most important contributors of neurodegeneration are the followings: (1) MAO-B activity, (2) oxidative stress and reduced endogenous antioxidant capacity, (3) elevated iron level, (4) glutamatergic excitotoxicity, (5) abnormal protein aggregation, misfolding, (6) reduced level of trophic factors, (7) altered ion homeostasis and (8) neuroinflammation (Hirsch *et al.*, 2013; Koutsilieri and Riederer 2007; Majláth *et al.*, 2016; Mandel *et al.*, 2003; Salamon *et al.*, 2019; Zádori *et al.*, 2011, 2012, 2013). 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 (Langston, 2017). Previously performed animal experiments hypothesized a potential neuroprotective effect of PGC-1α against MPTP-induced neuronal cell damage (Mudò *et al.*, 2012). 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 (St-Pierre *et al.*, 2006). However, the overexpression or the pharmacological activation (e.g. RESV) showed a potentially neuroprotective elevation after MPTP treatment (Breidert *et al.*, 2002; Dehmer *et al.*, 2004; Mudò *et al.*, 2012). In contrast**,** Clark *et al.* (2012) found that the number of the tyrosine hydroxylase (TH) positive cells was unexpectedly reduced after 5 days of the last MPTP injection when unilateral SN *Pgc-1α* induction (adeno-associated virus (AAV), C57BL/6CR mice) was applied. This effect was associated with the loss of a transcription factor, namely pituitary homeobox 3 protein (Pitx3). Wang *et al.* (2019) also tested the effect of stereotactic injection of lentivirus (*Pgc-1α* overexpression or silencing) in C57BL/6 mice. After the injection of lentivirus the mice were treated for 2 weeks with MPTP. They found an increase in the number of the TH-expressing cells and an elevated mitochondrial density in SN of the *Pgc-1α* overexpressing group. The optimal timing of the dissection after the last MPTP injection is critical to be able to compare the experimental data. If the dissection was performed after 24 h of the last MPTP injection, the level of PGC-1α was elevated and after 72 h it was normalized (short-term compensatory reaction) (Swanson *et al.*, 2013). Two studies have tested the potential neuroprotective effect of *Sirt1* overexpression against MPTP toxicity (Kakefuda *et al.*, 2009; Kitao *et al.*, 2005). Kakefuda used neuron-specific enolase-driven *Sirt1* tg mice. In the tg mice there was no detectable neuroprotective effect against the toxin compared to the wt mice. Interestingly the tg animals exhibited a memory deficit (Kakefuda *et al.*, 2009). In line with these results Kitao *et al.* (2005) found that in the SN of *Sirt1*-transgenic (prion promoter-driven) mice the number of TH-positive neurons was comparable with that of wt littermates. In conclusion, it seems that the *Sirt1* overexpression did not alleviate the toxic effect of MPTP. Other members of the Sirtuin-system were also tested by Liu *et al.* (2015a, b). They have proved

that in *Sirt3* and *Sirt5* null mice the MPTP-induced nigrostriatal damage becomes dramatically pronounced.

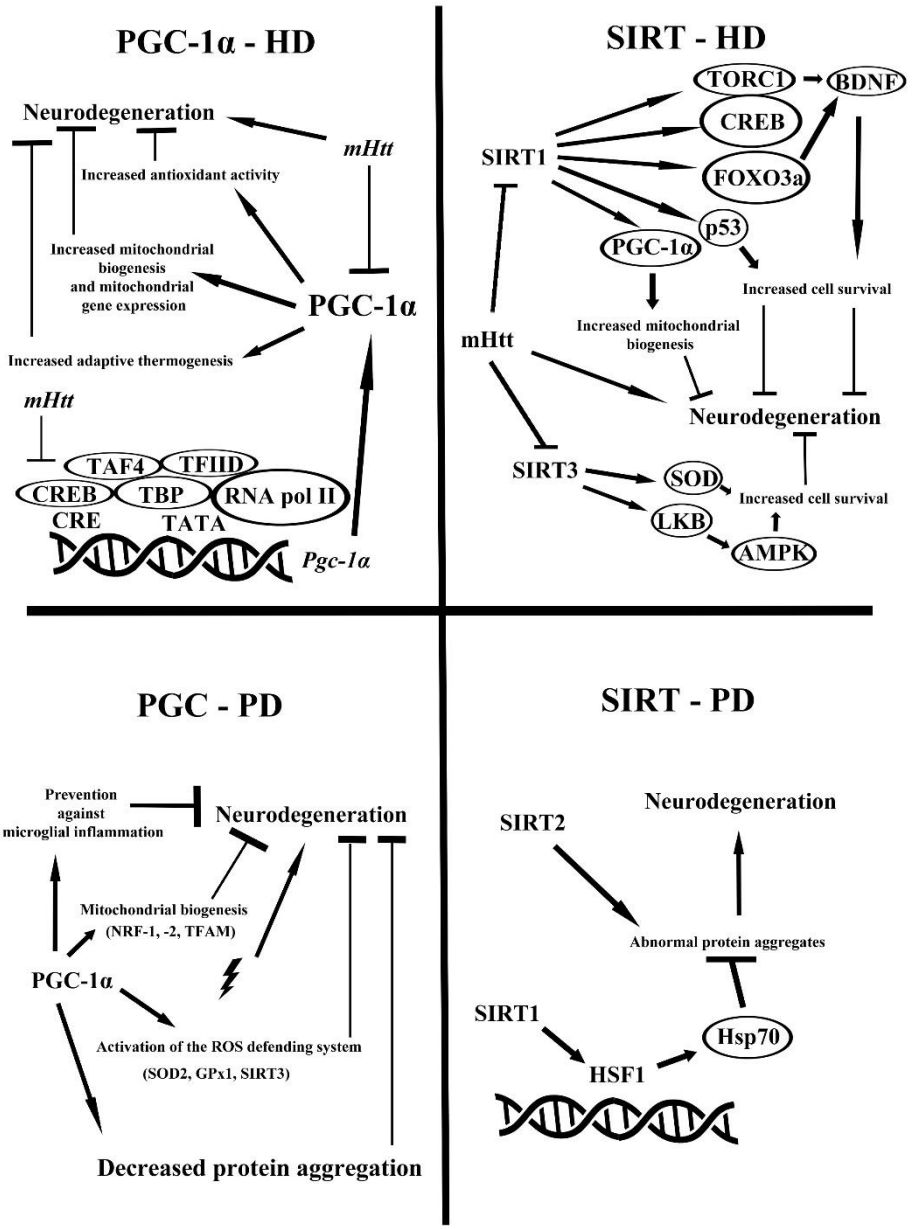

Figure 1 – Role of PGC- and Sirtuin-systems in Huntington's and Parkinson's diseases (Abbreviations: AMPK – 5' AMP-activated protein kinase; BDNF – Brain-derived neurotrophic factor; CRE – cAMP response element; CREB – cAMP response element-binding protein; FOXO3a – Forkhead box O3; GPx1 – Glutathione peroxidase 1; HD – Huntington's disease; HSF1 – Heat shock factor 1; Hsp70 – 70 kDa heat shock protein; LKB – Tumor suppressor serine/threonine-protein kinase; *mHtt* – mutant Huntingtin protein; NRF – Nuclear factor erythroid 2-related factor; p53 – TP53; PD – Parkinson's disease; RNA pol II – RNA polymerase type II; SOD – Superoxide dismutase; TAF4 – transcription initiation factor TFIID subunit 4; TATA – TATA box; TBP – TATA-box binding protein; TFAM – Transcription factor A; TFIID – Transcription factor II D; TORC1 – CREB-regulated transcription coactivator 1 (CRTC1)) (The following articles served as the basis for the figure: Herskovits and Guarente, 2013, 2014; Róna-Vörös and Weydt, 2010; McGill and Beal, 2006)

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*) (Schulte and Littleton, 2011; Vonsattel *et al.*, 1985). Previous works demonstrated that mutant huntingtin protein (*mHtt*) inhibits the function of PGC-1α via transcriptional dysregulation (Cui *et al.*, 2006; Jodeiri Farshbaf and Ghaedi, 2017). The Pgc-1α level was downregulated in the skeletal muscle samples of a transgenic HD mouse model and in human muscle biopsies as well, which refers to the potential role of the PGC-system in the muscle dysfunction in HD (Chaturvedi *et al.*, 2009). From the perspective of the brain, striatal degeneration was demonstrated in transgenic models of HD (Weydt *et al.*, 2006; Zádori *et al.*, 2011). Furthermore, in the nucleus caudatus of premanifest HD patients the mRNA level of Pgc-1α was significantly reduced (Cui *et al.*, 2006). Kim *et al.* (2010) confirmed these results and found a relation between the stage of HD and the level of PGC-1α protein in the nucleus caudatus (the immunoreactivity of PGC-1α was gradually decreased (by 70% in Grade 4)). Interestingly, the lentiviral mediated overexpression of *Pgc-1α* in the striatum of R6/2 mice resulted in the improvement of the phenotype (Cui *et al.*, 2006). Histological studies proved that the striatal medium spiny neurons are more vulnerable in HD, but the interneurons are relatively spared (Cui *et al.*, 2006). Johri *et al.* (2011) were the first, who tested the potential influence of NT-Pgc-1α in the pathogenesis of HD in transgenic murine models (R6/2, N171-82Q, mouse Q111 striatal cells). They found a depletion of the N-truncated isoform in the Q111 striatal cells. On the other hand, the NT-Pgc-1α level was upregulated in the striatal samples of older R6/2 and N171-82Q animals compared to the younger animals, which correlated with the human results. 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 a previously neglected (except the work of Steiner *et al.* (2011)) brain region, namely the cerebellum, where there was a strong significance of elevation regarding both isoforms. FL-Pgc-1α mRNA level was increased in the brains of 12- and 16-week-old animals, and furthermore, the NT-Pgc-1α level was elevated in all three age groups. They hypothesized that it is a compensatory phenomenon. The association between SIRTs and HD was also widely studied (Ajami *et al.*, 2017; Jeong *et al.*, 2011; Jiang *et al.*, 2011; La Spada, 2012; Naia and Rego, 2015; Tabrizi *et al.*, 2012). The published data about the Sirt1/SIRT1 mRNA and protein changes are somewhat inconsistent. In human brain

tissue and in R6/1 transgenic model the SIRT1 protein level was reduced (Hathorn *et al.*, 2011; Pallàs *et al.*, 2008). In R6/2 (mean CAG repeats number (MRN): 204) and HdhQ150 (MRN: 165) the SIRT1 activity was reduced in the striatum and cerebellum (Tulino *et al.*, 2016). The level of Sirt1/SIRT1 mRNA and protein were not affected by the presence of the transgene in the striatal samples of R6/2 mice. However, there was a decrease in the wt mice from 4 to 9 weeks (Tulino *et al.*, 2016). The changes of the protein and mRNA levels of Sirt1/SIRT1 was opposite in the cerebellum of R6/2 mice (the Sirt1 mRNA expression increased significantly by 9 and 14 weeks; the SIRT1 protein levels significantly decreased predominantly in wt mice by 14 weeks). In another model (HdhQ150) the SIRT1 protein level did not change (Tulino *et al.*, 2016). Reynolds *et al.* (2018) reported later a whole-brain study (R6/2 mice (MRN: 144)), where the Sirt1 mRNA level elevated in the 5-, 8- and 11-week-old tg animals. In contrast to these findings, in the brain of 12-week-old animals (MRN: 182) the Sirt1 mRNA elevation was no more detectable. The protein level of SIRT1 was also consistently elevated in the brain of both 8- and 12-week-old animals (Reynolds *et al.*, 2018). Jeong *et al.* (2011) crossed the brain-specific *Sirt1* knockout mice (BSKO; genotype: *Sirt1*flox/flox) with the R6/2 HD model mice to test the potential neuroprotective effect of SIRT1 in HD. It resulted in the augmentation of the neuronal cell damage in the striatum (Jeong *et al.*, 2011). In contrast, the overexpression of *Sirt1* (Sirt1-KI-R6/2) led to a longer survival time and less dominant neuropathological alterations (Jeong *et al.*, 2011). Jiang used other *Sirt1*-crossed models (N171-82Q and BAC HD), also demonstrating the deceleration of disease process (Jiang *et al.*, 2011). The available data are limited regarding the role of other Sirtuin isoforms (SIRT2-7). However, it seems that SIRT2 might worsen the disease process in HD (Chopra *et al.*, 2012; Maxwell *et al.*, 2011). Some authors suggest that SIRT3 has an opposite effect (Ahn *et al.*, 2008; Ajami *et al.*, 2017; Fu *et al.*, 2012; Neo and Tang, 2017). Fu *et al.* (2012) found that the striatal administration of a RESV dimer (ε-viniferin) was able to reduce the ROS level through SIRT3-mediated superoxide dismutase 2 (SOD2) induction in striatal progenitor cells (Hdh(Q111)).

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

III/1 – 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) and were approved by the local animal care committee (XI./846/2019, XXIV./352/2012).

III/2 – TREATMENT PROTOCOLS

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.

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.

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.

III/3 – 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 striatum, cortex and cerebellum. All the samples were stored at -80°C until the real-time polymerase chain reaction (RT-PCR).

III/4 – RT-PCR ANALYSIS

Total RNA was isolated from striatum, cortex and cerebellum with Trizol reagent according to the manufacturer’s protocol (Molecular Research Center, USA). 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 (Thermo Scientific, USA). 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 (Chang *et al.*, 2012; Lynch *et al.*, 2010; Yang *et al.*, 2010): FL-Pgc-1α (Pgc-1α-a/b/c, L-Pgc-1α): 5'-TGCCATTGTTAAGACCGAG-3' (forward; ex4/ex5) and 5'-TTGGGGTCATTTGGTGAC-3' (reverse; ex6/ex7); NT-Pgc-1α (NT-Pgc-1α-a/b/c): 5'-TGCCATTGTTAAGACCGAG-3' (forward; ex4/ex5) and 5'-GGTCACTGGAAGATATGGC-3' (reverse; ex6/7a (in-frame stop codon)); CNS-Pgc-1α: 5'-AATTGGAGCCCCATGGATGAAGG-3' (forward; exB4) and 5'-TCAAATGAGGGCAATCCGTC-3' (reverse; ex3); Ref-Pgc-1α (Pgc-1α-a and NT-Pgc-1α-a): 5'-TGAGTCTGTATGGAGTGACATCGAGTG-3' (forward; ex1a/ex2) and 5'-TCAAATGAGGGCAATCCGTC-3' (reverse; ex3); Sirt1: 5'-GCACTAATTCCAAGTTCTATACCC-3' (forward; ex7/ex8) and 5'-GTGGTACAGTTCTTTCAGGTG-3' (reverse; ex8); Sirt3-M1: 5'-TCAGACTGTGGGGTCCGGGAGTGTTA-3' (forward; ex1b) and 5'-CAACATGAAAAAGGGC-3' (reverse; ex3); Sirt3-M2: 5'-GACTGTGGGGTCCGGGAGGTGG-3' (forward; ex1b) and 5'-CAACATGAAAAAGGGC-3' (reverse; ex3); Sirt3-M3: 5'-GGCGTTTGGCGAGGACTA-3' (forward; ex2) and 5'-CAACATGAAAAAGGGC-3' (reverse; ex3). The thermal cyclic conditions were: Pgc-1α primers: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, and 60°C for 30 s. Sirt1: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, and 62°C for 30 s; Sirt3-M1 and Sirt3-M2: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, and 62.4°C for 30 s; Sirt3-M3: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, and 56.6°C for 30 s. 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).

III/5 – 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 striatum 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

IV/1 – 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 (Figure 2 and 3). 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 (Figure 4 A, B, C), but Sirt3-M1 levels elevated in the cortex (ctrl: 1.26 ± 0.49; EX: 1.97 ± 0.60; p = 0.036; Figure 4 B), whereas Sirt3-M3 levels decreased in the cerebellum (ctrl: 0.16 ± 0.05; EX: 0.10 ± 0.03; p = 0.027; Figure 4 C). 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; Figure 4 E) and striatal Sirt3-M1 (ctrl: 1.04 ± 0.30; EX: 0.72 ± 0.21; p = 0.029; Figure 4 D) expression levels.

IV/2 – 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 (Figure 5). 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 cerebellum (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; Figure 7 C). With regards to the striatum and the cortex, no other significant differences were detected (Figure 7 A, B). (The CNS-specific promoter was hardly detectable in the quadriceps muscle. However, the expression level of FL-Pgc-1α (ctrl: 1.01 ± 0.19; EX: 3.19 ± 1.25; p = 0.003), NT-Pgc-1α (ctrl: 0.10 ± 0.02; EX: 0.50 ± 0.19; p = 0.001) and REF-Pgc-1α (ctrl: 1.00 ± 0.11; EX: 1.69 ± 0.52; p = 0.016) mRNA was significantly elevated in the quadriceps muscle after 5 days of training.) 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 (Figure 6). However, 12 days of exercise training resulted in the increase of both Sirt3-M1 and -M2 mRNA expression in the cerebellum (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; Figure 7 F). We did not find differences in Sirt1 and Sirt3-M3 levels in any other brain areas (Figure 7 D, E).

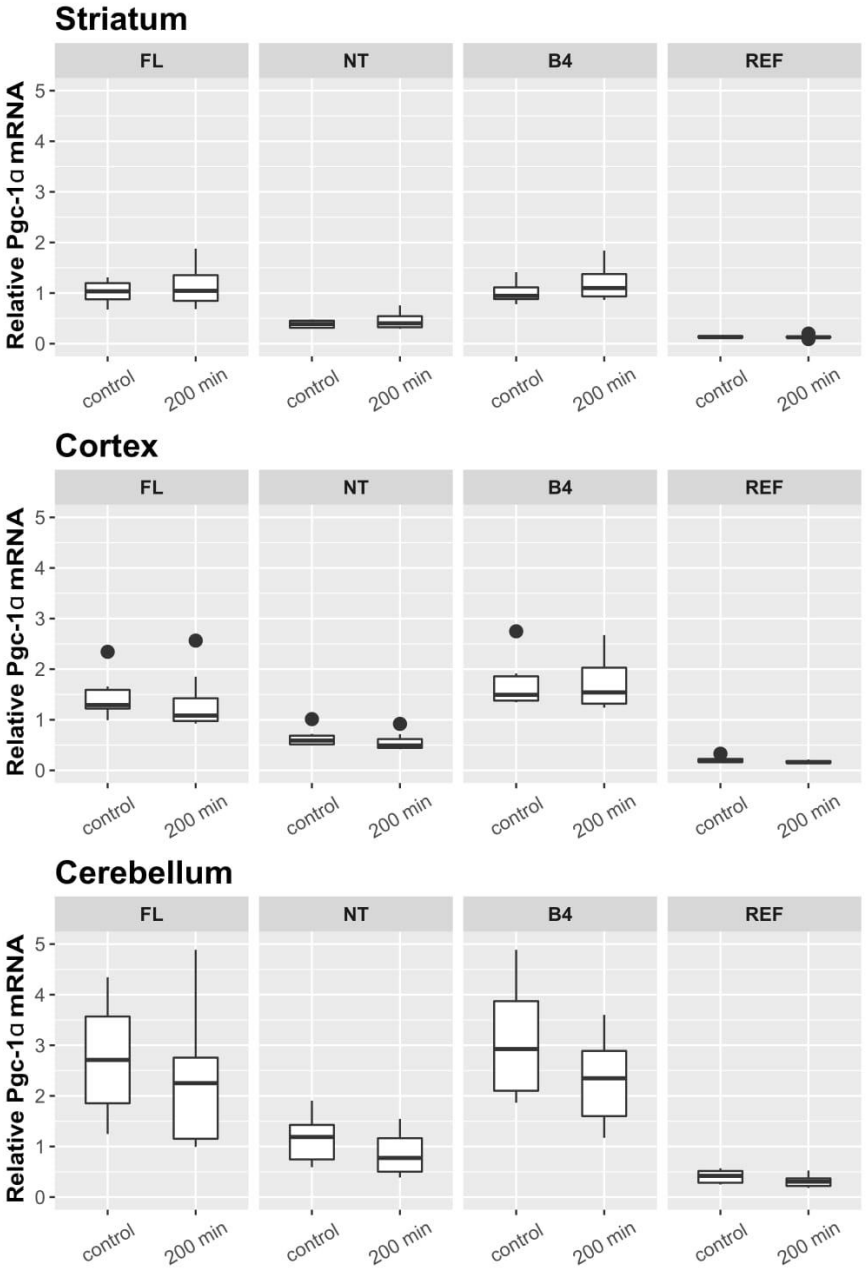

Figure 2. – Striatal, cortical and cerebellar relative mRNA expression levels of Pgc1-1α isoforms in mice after 200 minutes of cold exposure (4°C). Expression levels of the examined isoforms did not change. Values are plotted as medians and interquartile range; *200 min* – 200 minutes cold exposure; *FL* – FL-Pgc-1α; *NT* – NT-Pgc-1α; *B4* – CNS-Pgc-1α; REF – REF-Pgc-1α (Salamon et al., 2019)

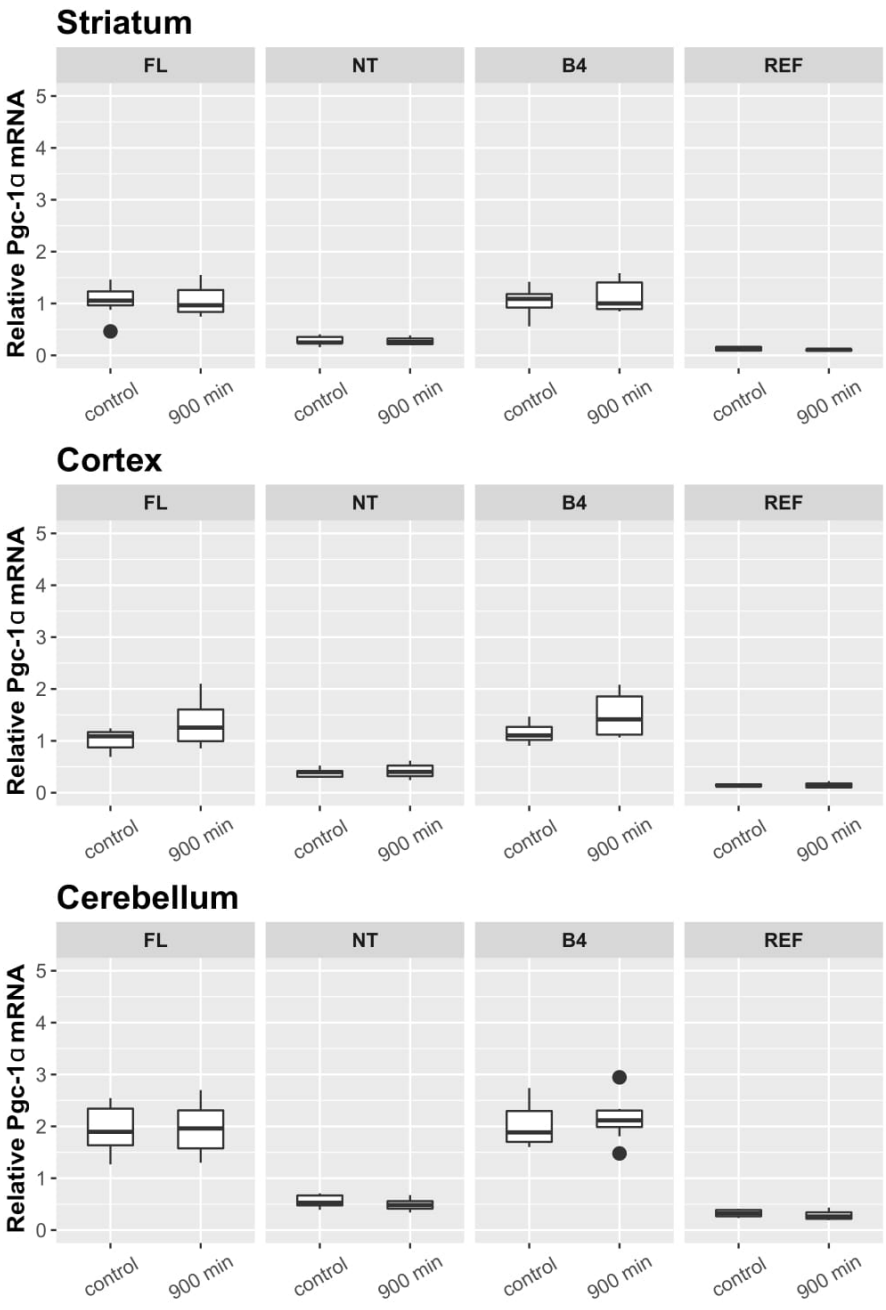

Figure 3. – Striatal, cortical and cerebellar relative mRNA expression levels of Pgc1-1α isoforms in mice after 900 minutes of cold exposure (4°C). Expression levels of the examined isoforms did not change. Values are plotted as medians and interquartile range; *900 min* – 900 minutes cold exposure; *FL* – FL-Pgc-1α; *NT* – NT-Pgc-1α; *B4* – CNS-Pgc-1α; REF – REF-Pgc-1α (Salamon et al., 2019)

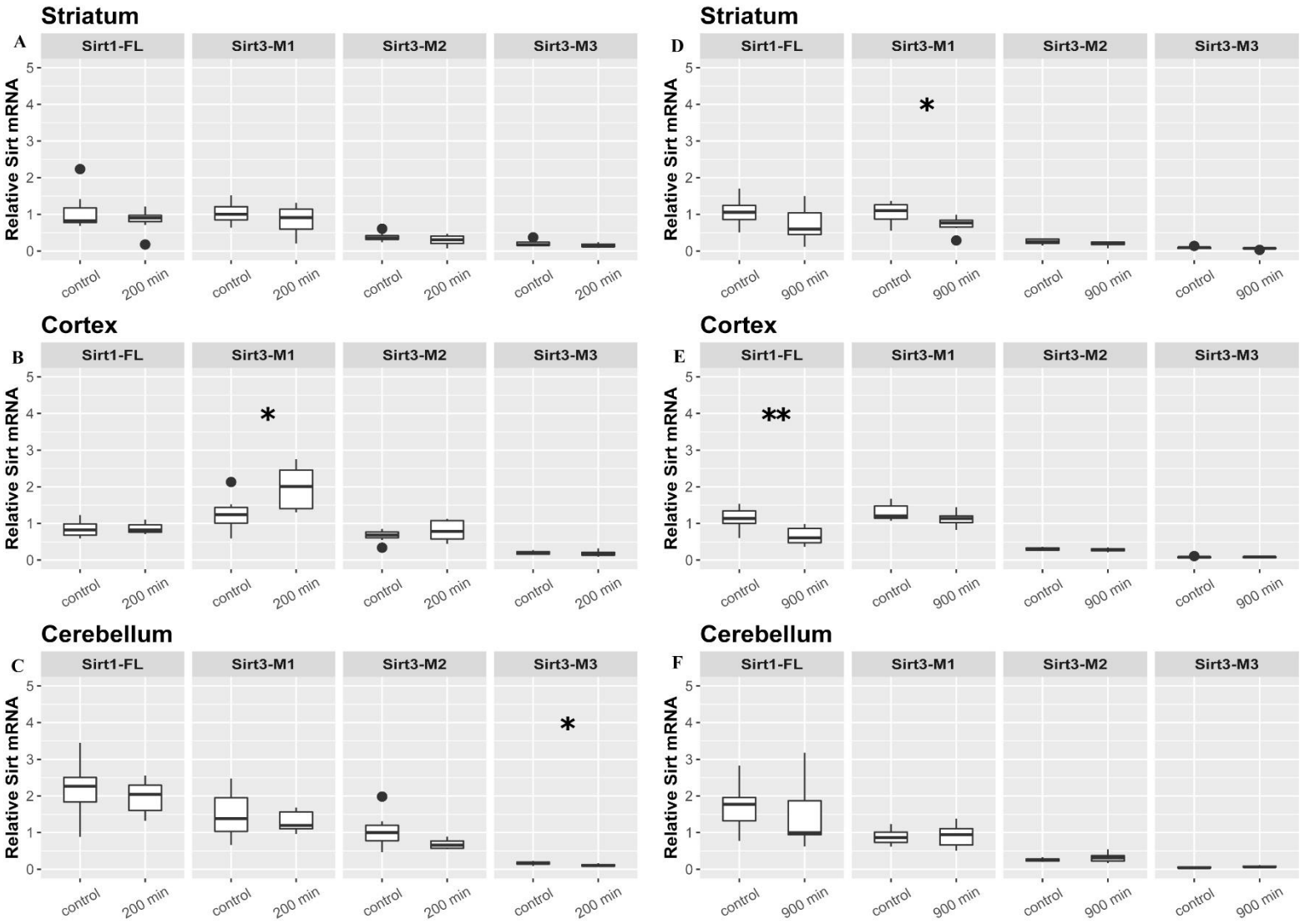

Figure 4. – Striatal, (A, D), cortical (B, E) and cerebellar (C, F) relative mRNA expression levels of Sirtuin isoforms in mice after 200 (A–C) and 900 (D–F) minutes of cold exposure (4 °C). After 200 min the Sirt3-M1 isoform was significantly upregulated in mouse cortex and cerebellar Sirt3-M3 was also significantly decreased. After 900 min the Sirt1-Fl isoform was significantly downregulated in mouse cortex and striatal Sirt3-M1 was also significantly decreased. Values are plotted as medians and interquartile range; *p < 0.05, **p < 0.01, ***p < 0.005; 200 – 200 min cold exposure (Salamon et al., 2019)

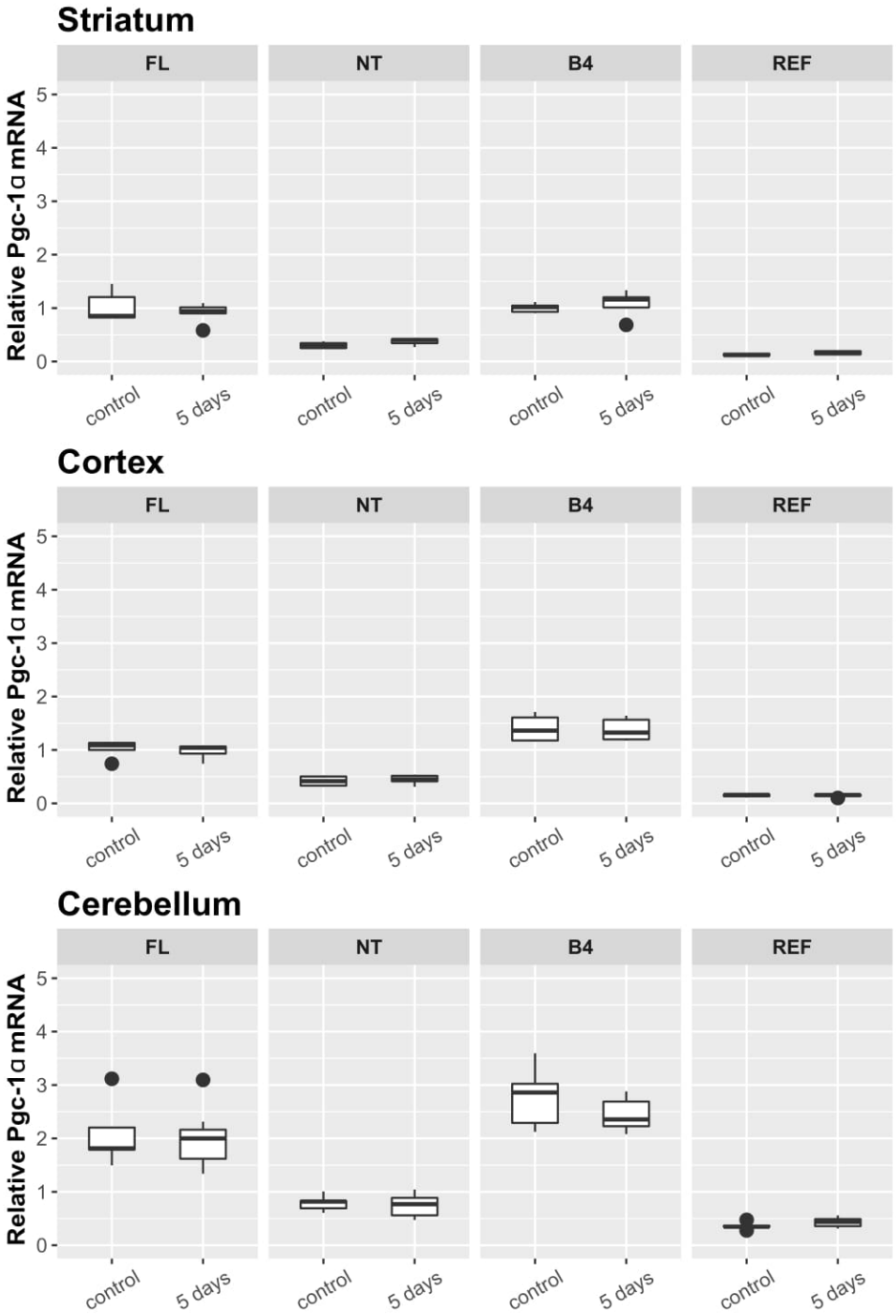

Figure 5. – Striatal, cortical and cerebellar relative mRNA expression levels of Pgc1-1α isoforms in mice after 5 days of rotarod training (5 RPM). Expression levels of the examined isoforms did not change. Values are plotted as medians and interquartile range; *5 days* – 5 days rotarod training; *FL* – FL-Pgc-1α; *NT* – NT-Pgc-1α; *B4* – CNS-Pgc-1α; REF – REF-Pgc-1α (Salamon et al., 2019)

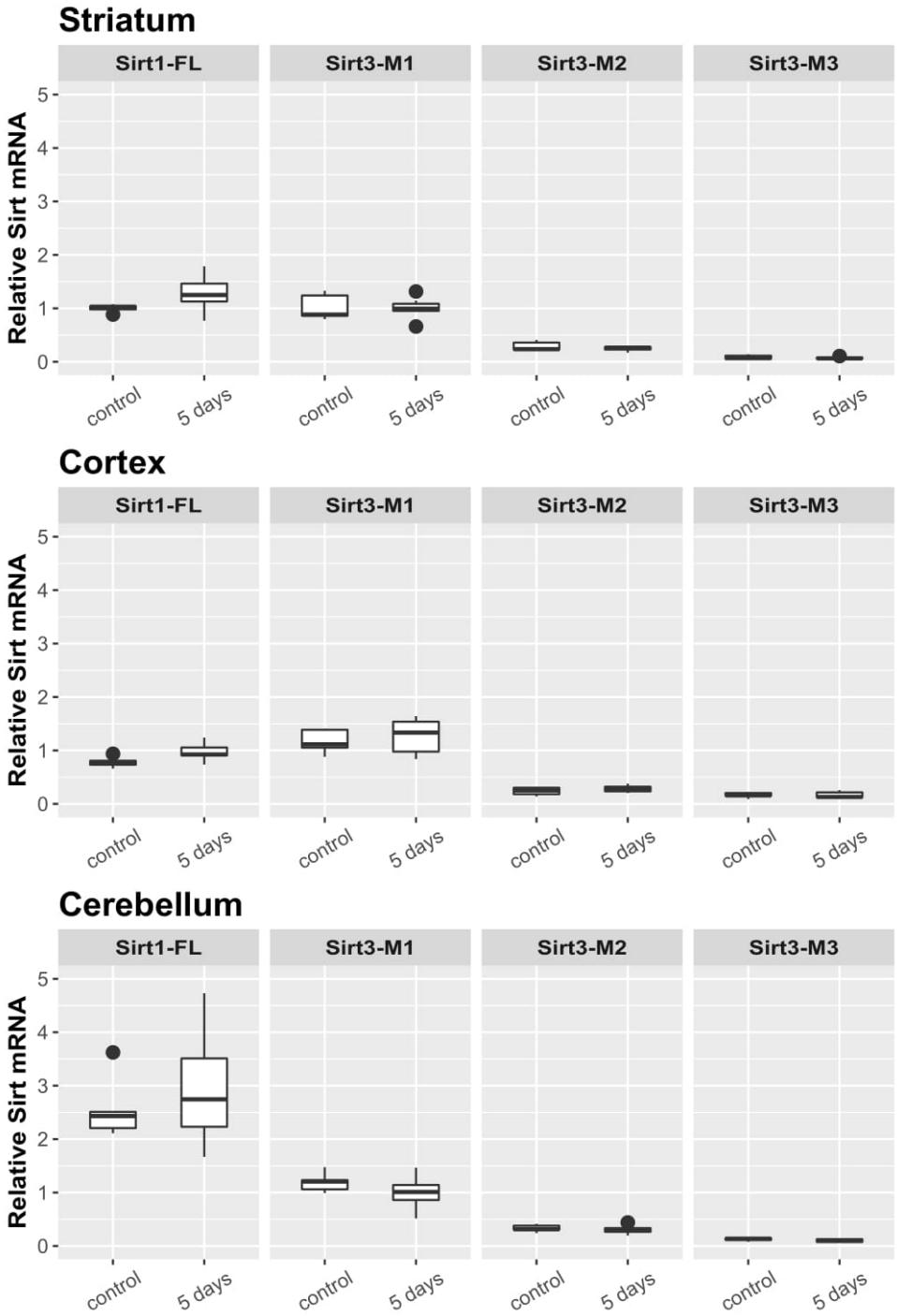

Figure 6. – Striatal, cortical and cerebellar relative mRNA expression levels of Sirtuin isoforms in mice after 5 days of rotarod training (5 RPM). Expression levels of the examined isoforms did not change. Values are plotted as medians and interquartile range; *5 days* – 5 days rotarod training; *FL* – FL-Pgc-1α; *NT* – NT-Pgc-1α; *B4* – CNS-Pgc-1α; REF – REF-Pgc-1α (Salamon et al., 2019)

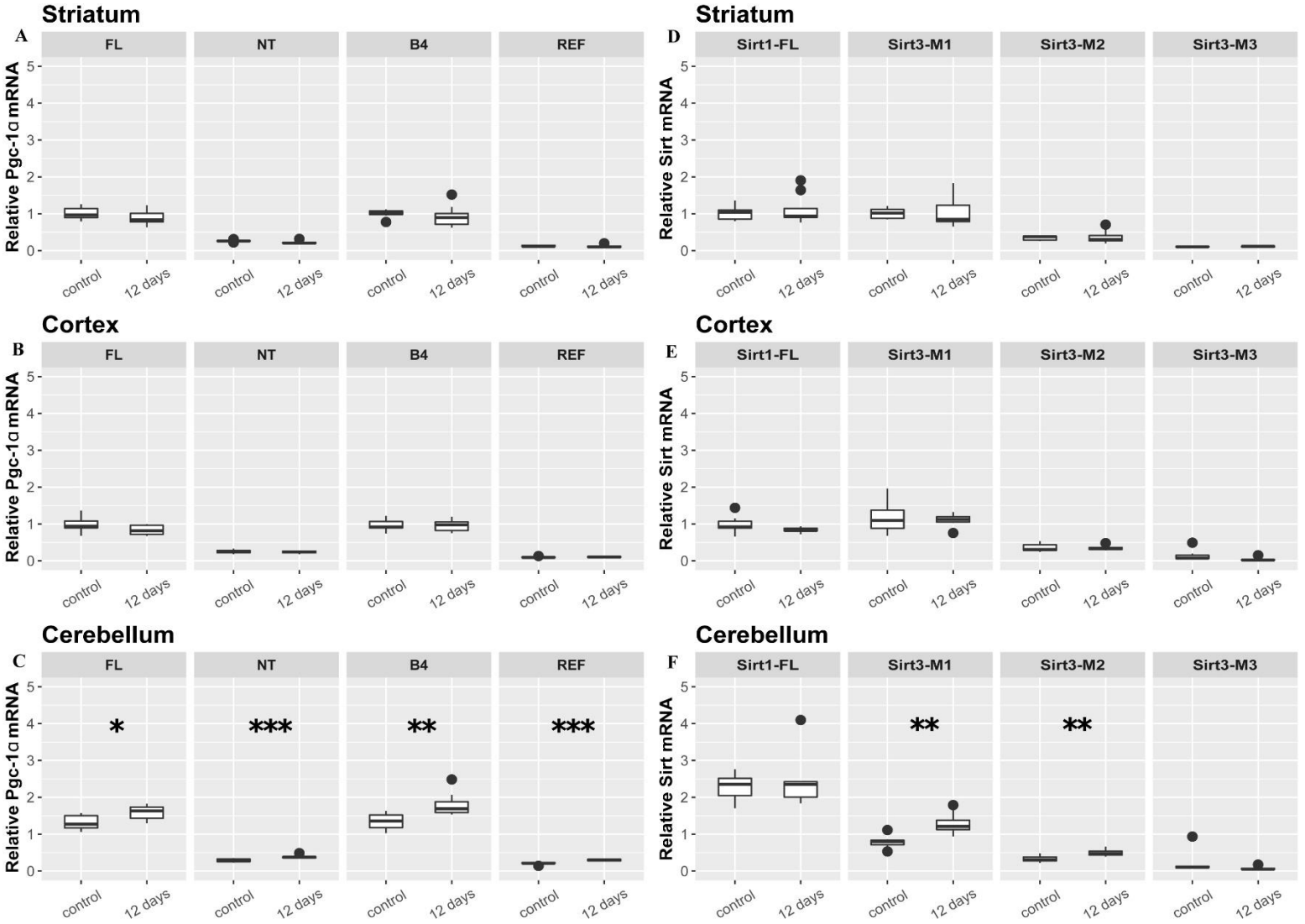

Figure 7. – Striatal (A, D), cortical (B, E) and cerebellar (C, F) relative mRNA expression levels of Pgc1-1α (A, B, C) and Sirtuin (D, E, F) isoforms in mice after 12 days of rotarod training (5 RPM). The FL-Pgc-1α, NT-Pgc-1α, CNS-Pgc-1α (B4), Reference promoter (REF), Sirt3-M1 and –M2 levels significantly increased in the cerebellum of exercised mice. Values are plotted as medians and interquartile range; *p < 0.05, **p < 0.01, ***p < 0.005; 12 days – 12 days rotarod training (Salamon et al., 2019)

IV/3 – 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 striatum (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), cortex (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 cerebellum (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) (Figure 8 A, B). Furthermore, MPTP-induced increases in CNS-Pgc-1α expression were also significantly larger in all investigated brain regions compared to the controls (striatum: ctrl: 1.03 (0.88–1.11); EX: 1.38 (1.34–1.78); p = 0.0069; cortex: ctrl: 0.91 (0.80–0.98); EX: 1.41 (1.24–1.42); p = 0.0048; cerebellum: ctrl: 1.51 (1.20–1.98); EX: 2.77 (2.34–3.17); p = 0.019) (Figure 8 C). However, there was not any difference between the REF-Pgc-1α levels in the striatum (ctrl: 0.11 (0.10–0.12); EX: 0.11 (0.95–0.12)); cortex (ctrl: 0.11 (0.11–0.12); EX: 0.09 (0.08–0.10)) and cerebellum (ctrl: 0.21 (0.20–0.29); EX: 0.28 (0.24–0.29)) of MPTP-treated and control mice (Figure 8 D). 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 (Figure 9).

There were no detectable changes in the levels of Sirtuin transcripts in the different brain areas after ninety minutes following the last MPTP injection (Figure 10). However, 1 week following the last injection a slight significance was found in all of the cerebellar 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; Figure 11). However, these significances disappeared using the Bonferroni correction (therefore, this is not indicated in the figure).

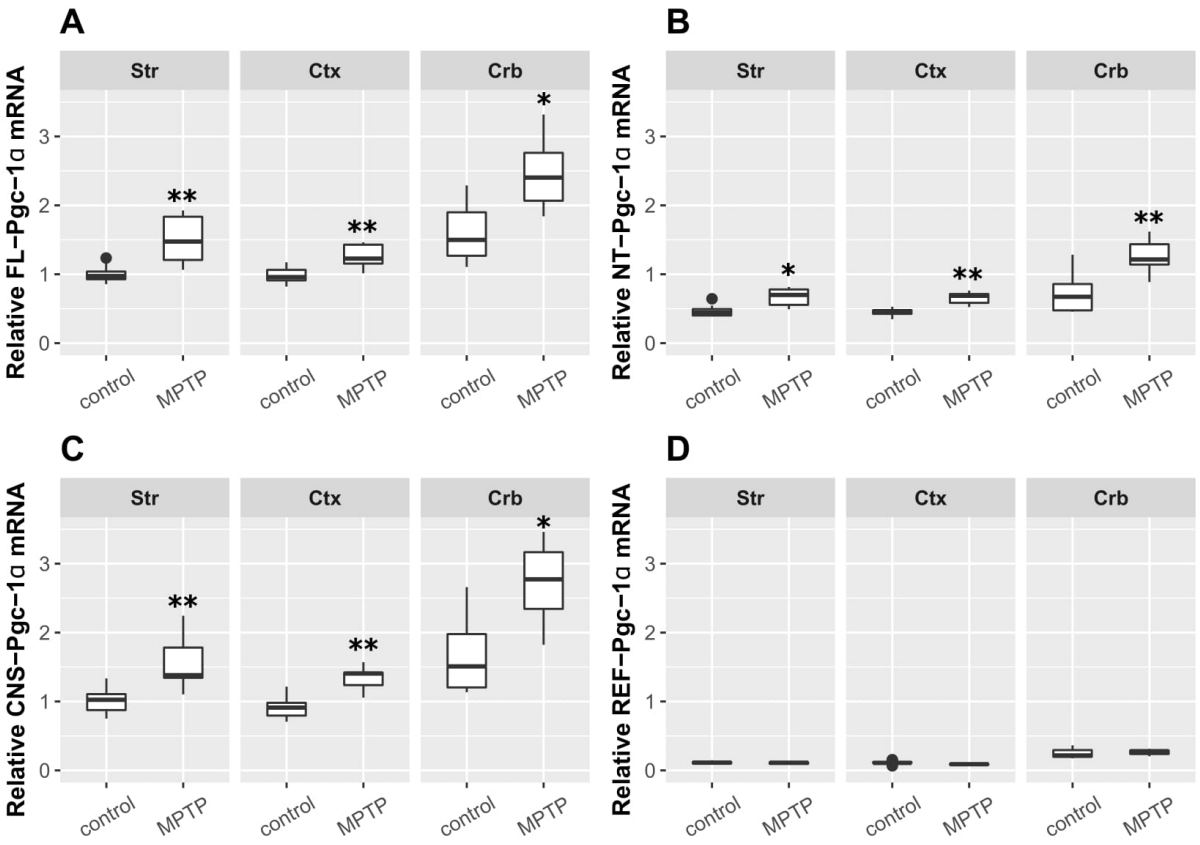

Figure 8. – The relative mRNA expression of Pgc-1α isoforms in the striatum, cortex and the cerebellum of mice 90 min after acute MPTP intoxication. The FL-Pgc-1 α, NT-Pgc-1α and CNS-Pgc-1α levels were significantly increased in the striatum, cortex and the cerebellum of MPTP-treated mice. (A, B, C respectively). The Ref-Pgc-1α expression did not change in any brain areas of MPTP-treated mice compared to the controls (D). Values are plotted as medians and interquartile range; *$p < 0.05$, **$p < 0.01$; *MPTP* MPTP-treated; *Str* striatum, *Ctx* cortex, *Crb* cerebellum. (Török et al., 2017)

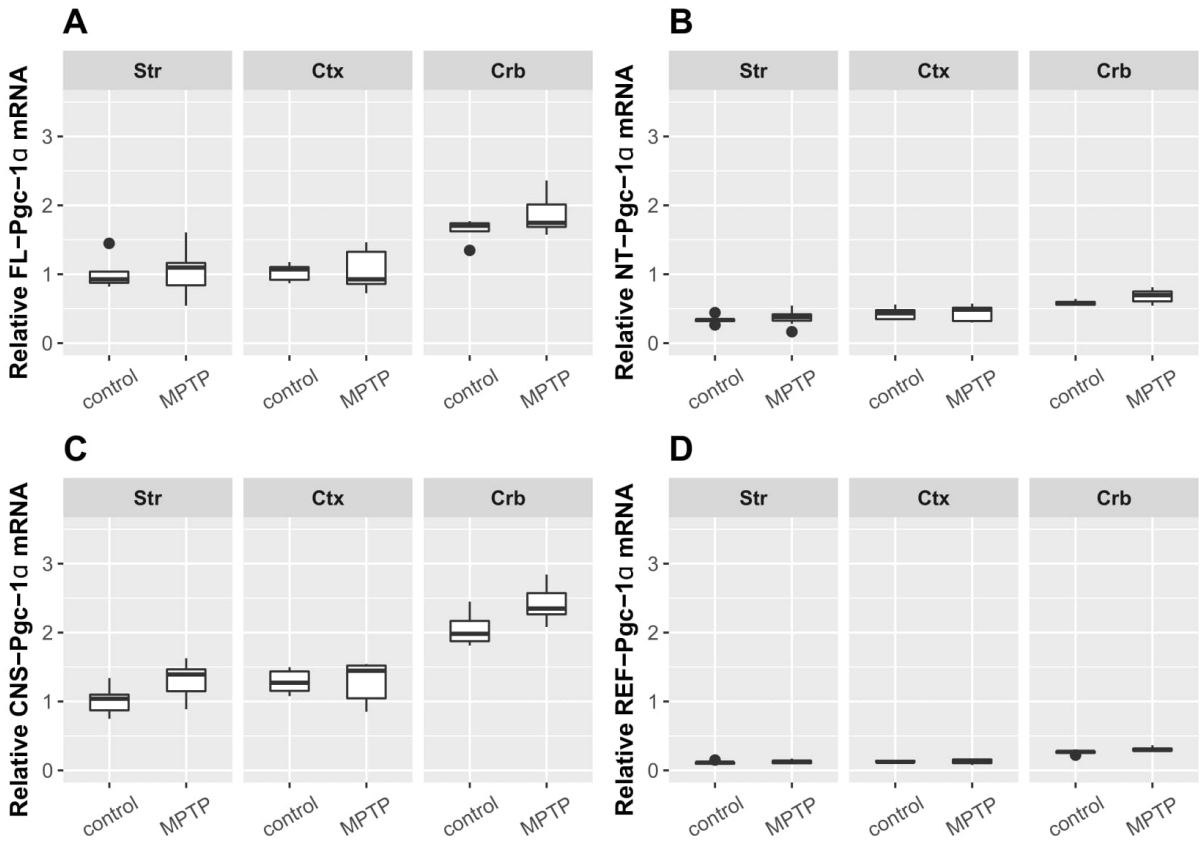

Figure 9. – The relative mRNA expression of Pgc-1α isoforms in the striatum, cortex and the cerebellum of mice 7 days after acute MPTP intoxication. The expression levels of the Pgc-1α isoforms did not change in any brain areas of MPTP-treated mice (A-D). Values are plotted as medians and interquartile range; *MPTP* MPTP-treated; *Str* striatum, *Ctx* cortex, *Crb* cerebellum. (Török et al., 2017)

IV/4 – 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 (cortex (8-week-old: $p = 0.0029$; 12-week-old: $p = 0.0018$; 16-week-old: $p = 0.0029$); cerebellum (8-week-old:

p = 0.0052; 12-week-old: p = 0.0054; 16-week-old: p = 0.0065), but not in the striatum (Figure 12). Regarding the effect of aging on Sirt1 expression levels, we detected significant increase only in the cerebellum 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 striatum and cortex 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; Figure 13). 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 cerebellum 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; Figure 14). When assessing the effect of aging on Sirt3-M2 expression levels, we detected significant decrease only in the cortex 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 cortex compared to wt mice (striatum (8-week-old: p = 0.0097; 12-week-old: p = 0.0054; 16-week-old: p = 0.0097); cortex (16-week-old: p = 0.0032); cerebellum (8-week-old: p = 0.002; 12-week-old: p = 0.0006; 16-week-old: p = 0.0016) (Figure 15). 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).

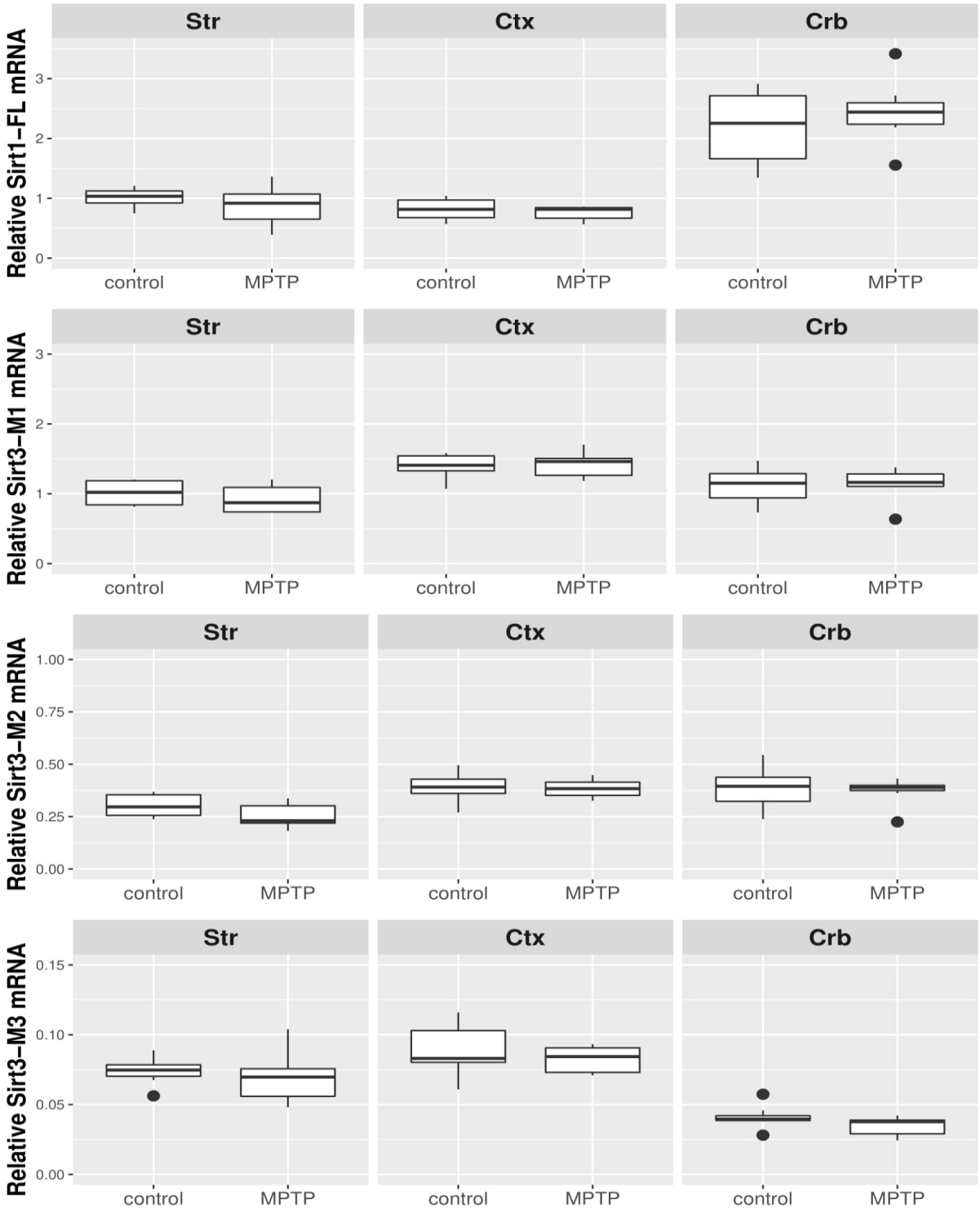

Figure 10. – The relative mRNA expression of Sirtuin isoforms in the striatum, cortex and the cerebellum of mice 90 min after acute MPTP intoxication. The expression levels of the Sirtuin isoforms did not change in any brain areas of MPTP-treated mice.Values are plotted as medians and interquartile range; *MPTP* MPTP-treated; *Str* striatum, *Ctx* cortex, *Crb* cerebellum. (unpublished results)

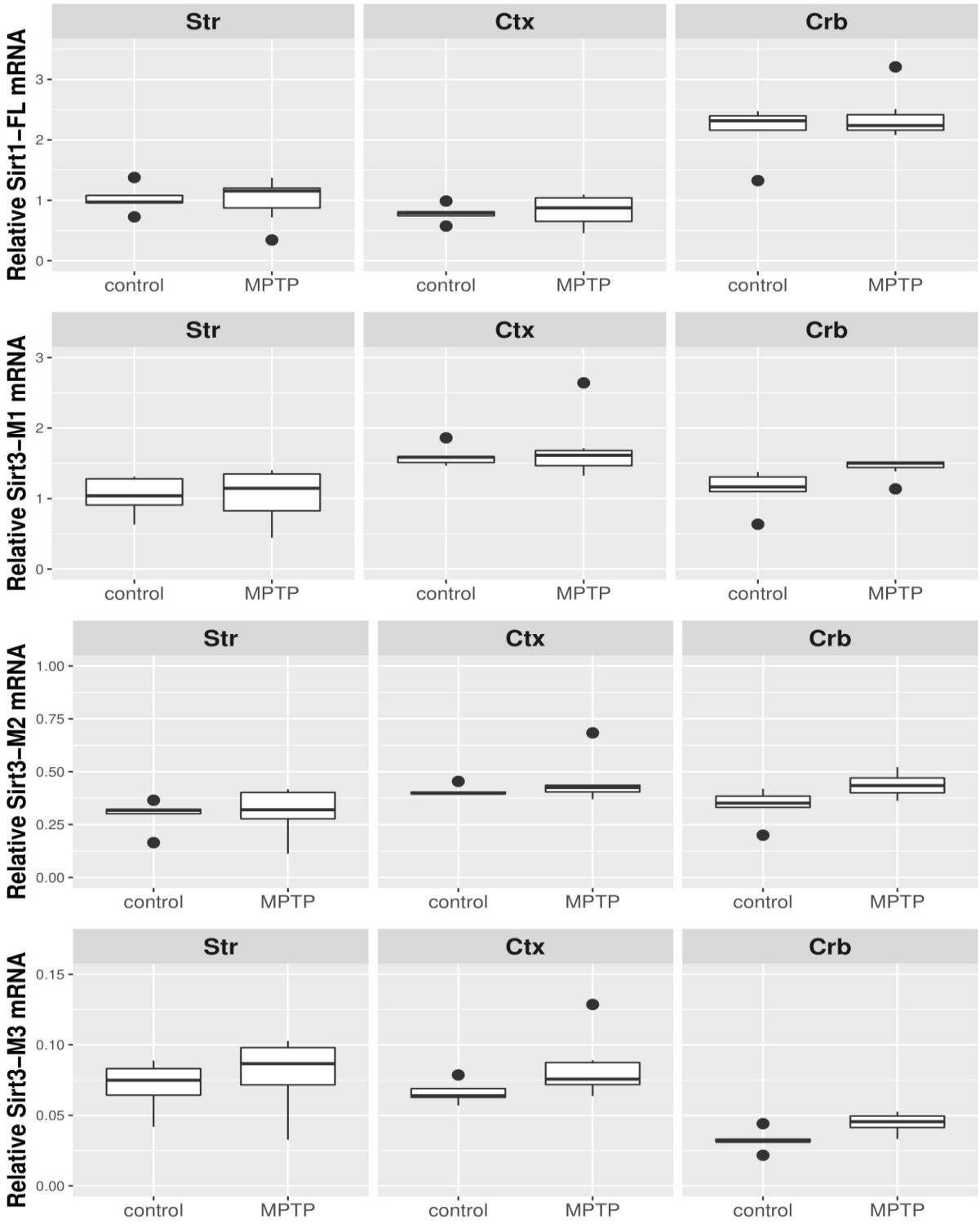

Figure 11. – The relative mRNA expression of Sirtuin isoforms in the striatum, cortex and the cerebellum of mice 7 days after acute MPTP intoxication. The expression levels of the Sirtuin isoforms did not change in any brain areas of MPTP-treated mice.Values are plotted as medians and interquartile range; *MPTP* MPTP-treated; *Str* striatum, *Ctx* cortex, *Crb* cerebellum. (unpublished results)

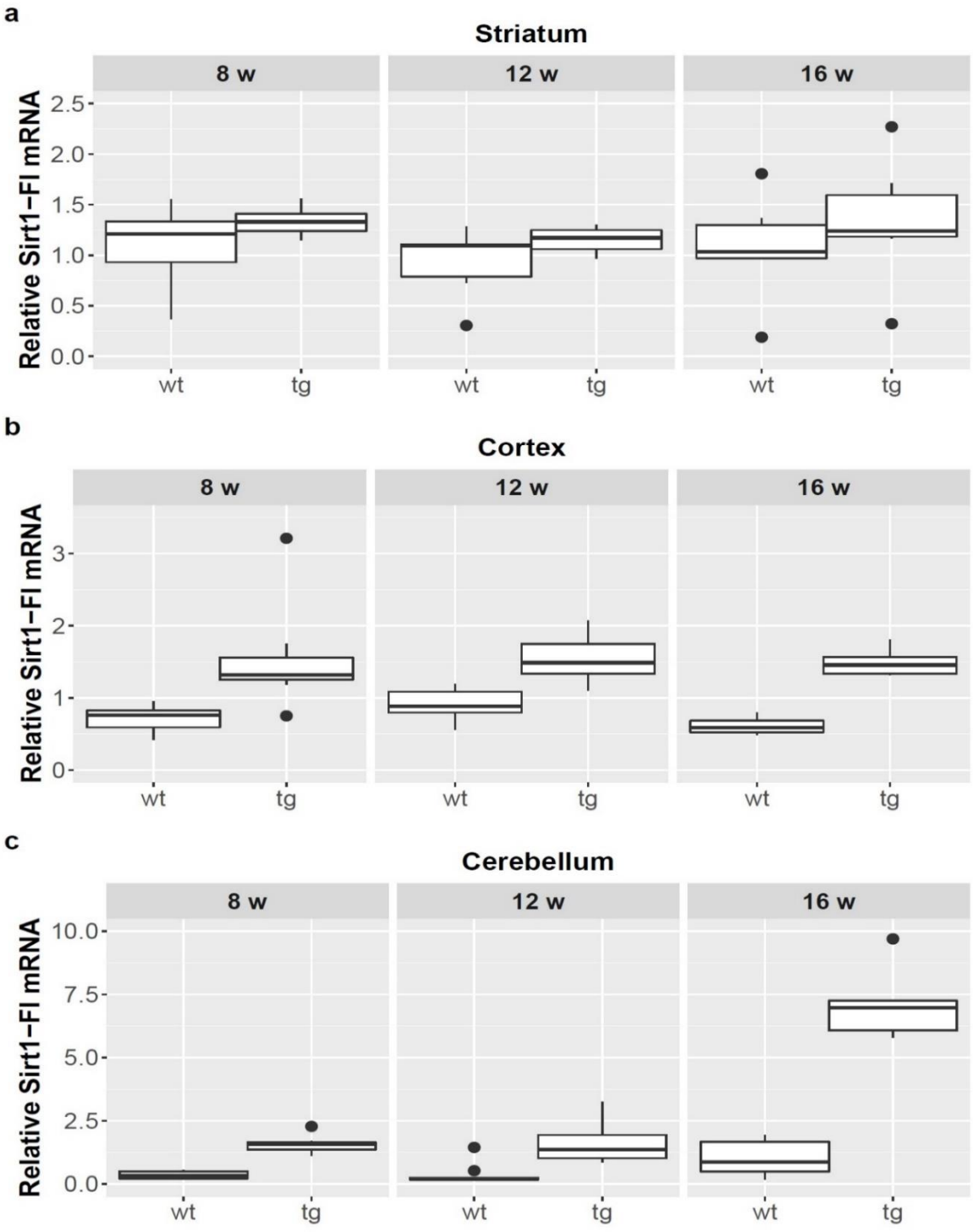

Figure 12. – Relative mRNA expression level of Sirt1-Fl in the striatum (a), cortex (b) and cerebellum (c) of N171-82Q transgenic and B6C3 wild-type mice of three age groups. The Sirt1-Fl level significantly elevated in all cortical and cerebellar samples in each age group of tg animals compared to wt mice. Aging caused a significant increase only in the cerebellum of tg group by 16 weeks of age. For clarity, the levels of significance were indicated separately in Figure 16. in a special table format. Values are plotted as medians and interquartile ranges; tg = transgenic, wt = wild-type, w = weeks

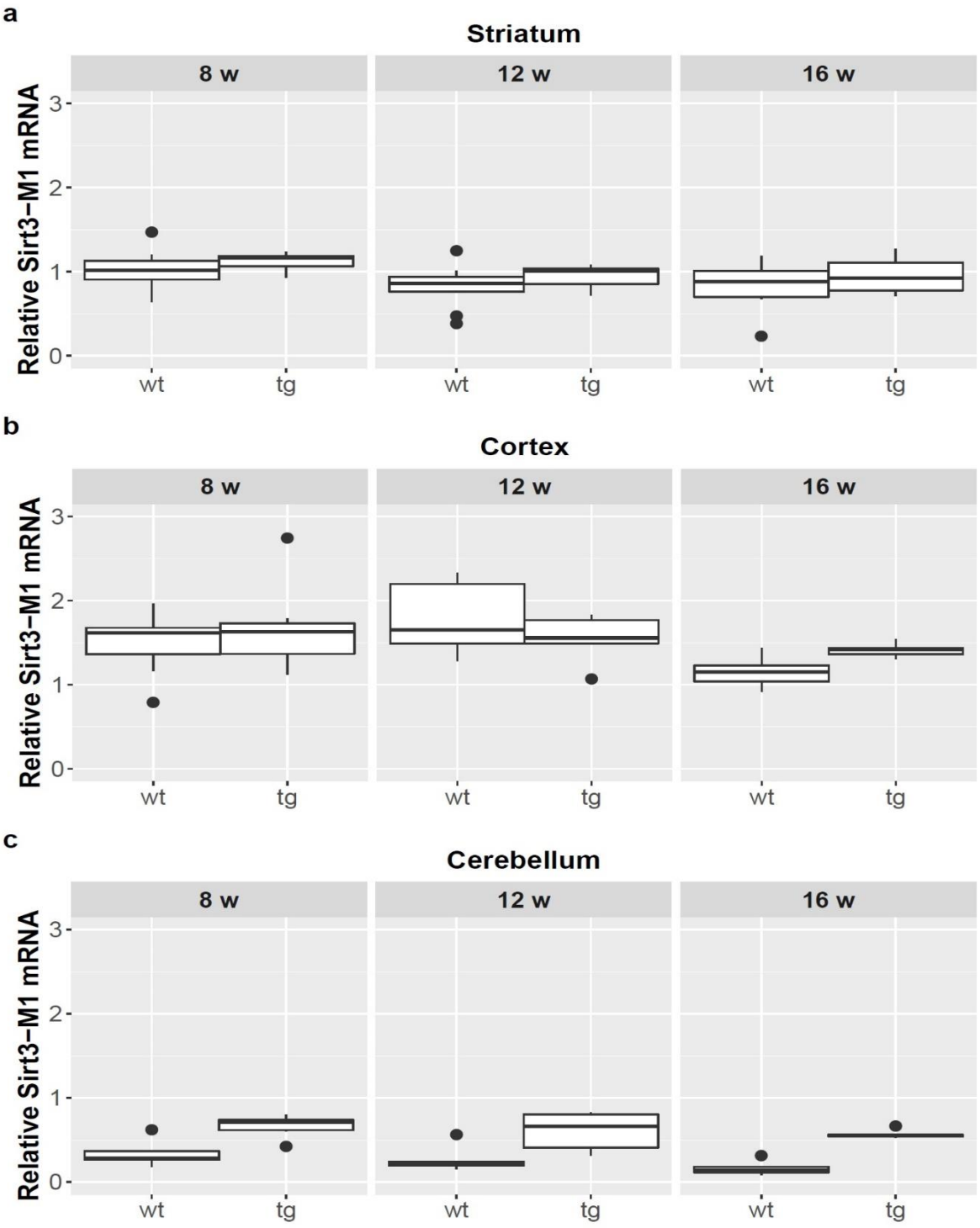

Figure 13. – Relative mRNA expression level of Sirt3-M1 in the striatum (a), cortex (b) and cerebellum (c) of N171-82Q transgenic and B6C3 wild-type mice of three age groups. The Sirt3-M1 level was significantly elevated in all cerebellar samples in each age group of tg animals compared to wt mice. For clarity, the levels of significance were indicated separately in Figure 16. in a special table format. Values are plotted as medians and interquartile ranges; tg = transgenic, wt = wild-type, w = weeks

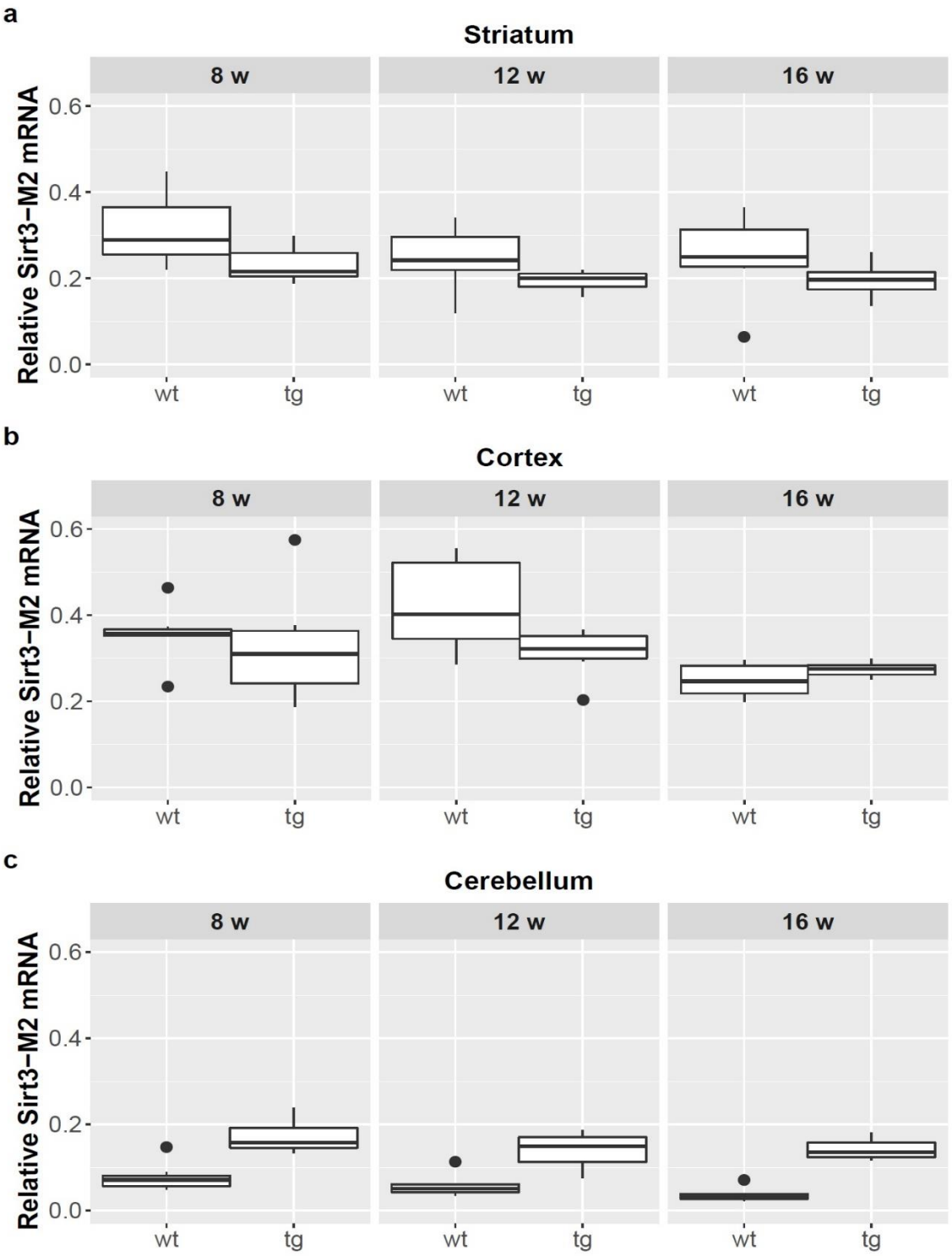

Figure 14. – Relative mRNA expression level of Sirt3-M2 in the striatum (a), cortex (b) and cerebellum (c) of N171-82Q transgenic and B6C3 wild-type mice of three age groups. The Sirt3-M2 level was significantly elevated in all cerebellar samples in each age group of tg animals compared to wt mice. Aging caused a significant decrease only in the cortex of wt group by 16 weeks of age. For clarity, the levels of significance were indicated separately in Figure 16. in a special table format. Values are plotted as medians and interquartile ranges; tg = transgenic, wt = wild-type, w = weeks

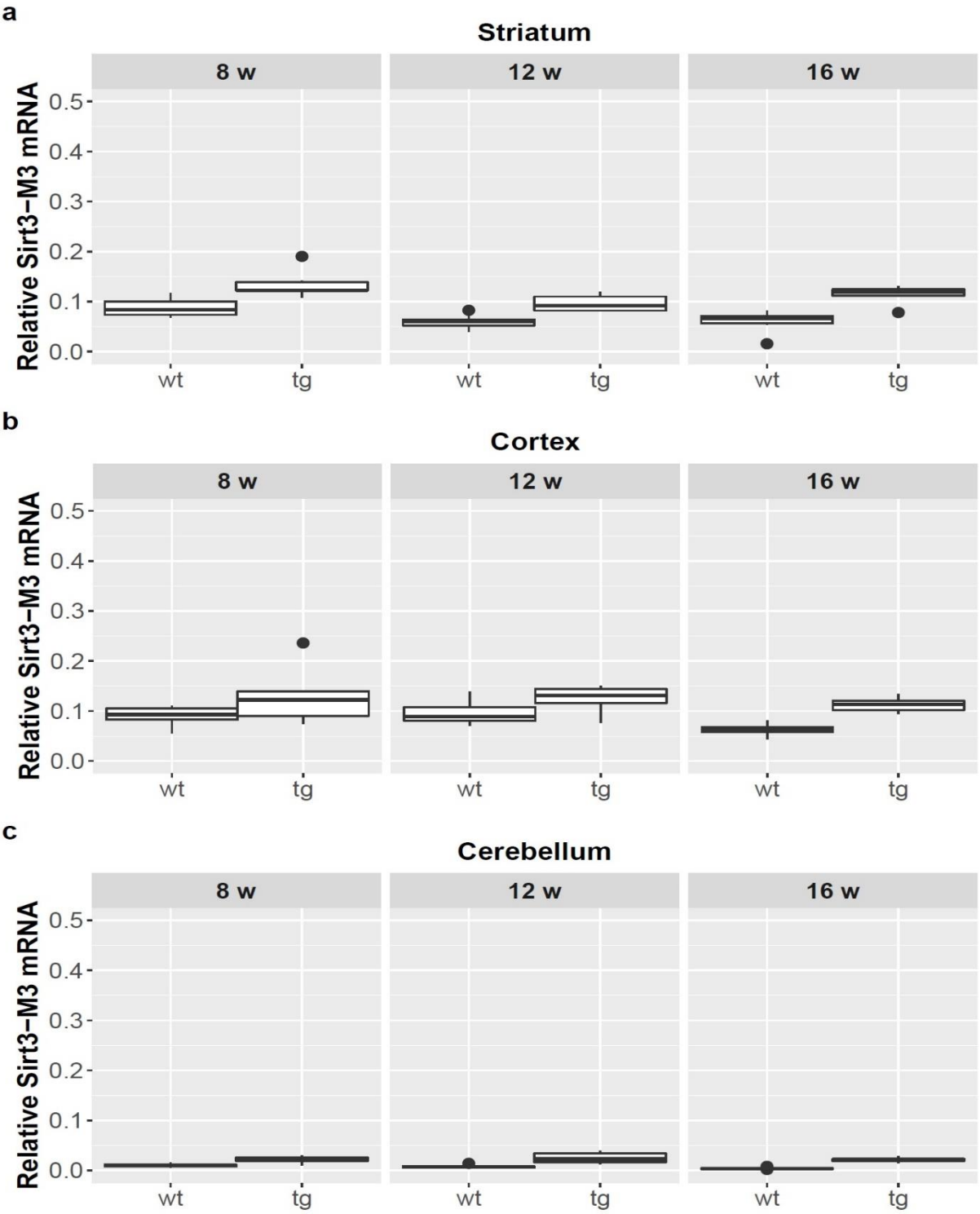

Figure 15. – Relative mRNA expression level of Sirt3-M3 in the striatum (a), cortex (b) and cerebellum (c) of N171-82Q transgenic and B6C3 wild-type mice of three age groups. The Sirt3-M3 level was significantly elevated in all striatal and cerebellar samples in each age group of tg animals compared to wt mice. The striatal expression decreased significantly by 12 weeks of age in both wt and tg animals. For clarity, the levels of significance were indicated separately in Figure 16. in a special table format. Values are plotted as medians and interquartile ranges; tg = transgenic, wt = wild-type, w = weeks

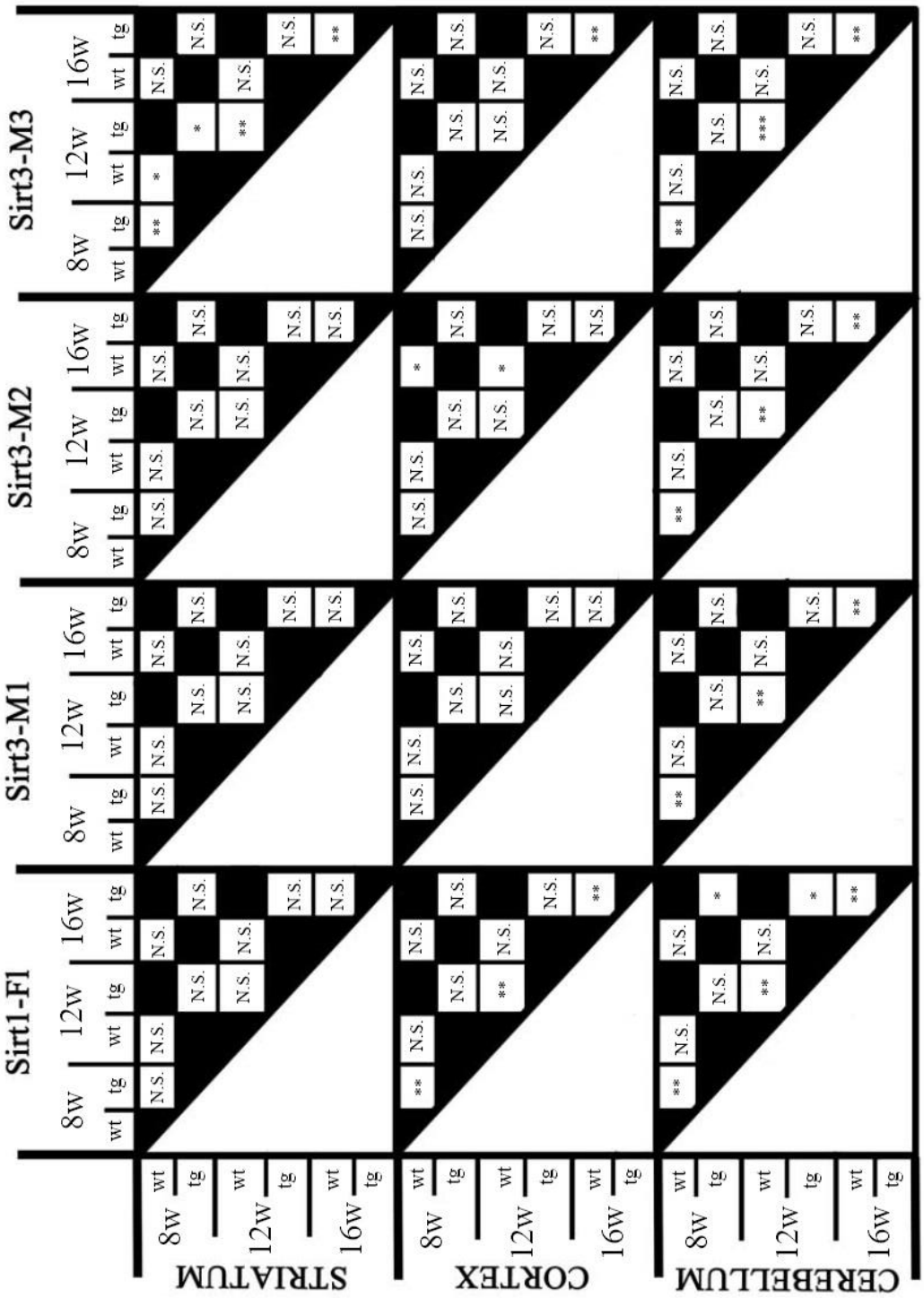

Figure 16. – This figure represents the effect of presence of transgene on the expression patterns (transgenic-wild-type comparisons) and on the time course (age-related alterations) and interaction as well. Regarding Sirt1 and Sirt3 expression, there were no significant interactions between the presence of the transgene and age. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; N.S. = not significant; tg = transgenic, wt = wild-type, w = weeks

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 (Chang *et al.*, 2012; Jankovic *et al.*, 2015; Jokinen *et al.*, 2017; Puigserver *et al.*, 1998; Shi *et al.*, 2005; Stancic *et al.*, 2013; Wang and Tong, 2009; Yao *et al.*, 2017; Zhang *et al.*, 2009). 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 (Costa *et al.*, 2010; Huang *et al.*, 2015; Lochmann *et al.*, 2015; Shi *et al.*, 2005; Tadaishi *et al.*, 2011; Wen *et al.*, 2014). 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 (Bayod *et al.*, 2012; Cheng *et al.*, 2015; Garcia-Valles *et al.*, 2013; Gusdon *et al.*, 2017; Holloszy, 1997; Huang *et al.*, 2015; Huang *et al.*, 2016; Koltai *et al.*, 2010; Shi *et al.*, 2005; Steiner *et al.*, 2011). 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. cortex, 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: cortex, striatum). Many other experiments were made with different protocols, however, the results remained controversial (Bayod *et al.*, 2011; Cheng *et al.*, 2015; Lezi *et al.*, 2013, 2014; Marosi *et al.*, 2012). 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 regions. Contrarily, the 12-day-long (long-term) training period induced changes in all isoforms of the PGC-system in the cerebellum 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 cerebellum. 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 (Breidert *et al.*, 2002; Dehmer *et al.*, 2004; Mudò *et al.*, 2012). Furthermore, in the *Pgc-1α* knockout mice, there is a pronounced susceptibility of dopaminergic neurons against this toxin (St-Pierre *et al.*, 2006). However, it seems that the MPTP toxin induces only a short-term compensatory reaction in the PGC-system (Swanson *et al.*, 2013). 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 (Kakefuda *et al.*, 2009; Kitao *et al.*, 2005). 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 (Liu *et al.*, 2015a, b). 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 (Naia and Rego, 2015; Neo and Tang, 2017; Reynolds *et al.*, 2018; Tulino *et al.*, 2016). 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) (Tulino *et al.*, 2016). 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 (Reynolds *et al.*, 2018). 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 (Reynolds *et al.*, 2018). 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.* (Reynolds *et al.*, 2018; Tulino *et al.*, 2016). Similar to the study of Tulino *et al.*, there was no detectable effect of the transgene in the striatum 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 (Fu *et al.*, 2012), 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 cerebellum 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 (Török *et al.*, 2015). The reason behind the same pattern may be that Sirtuins are upstream regulators of Pgc-1α expression (Cui *et al.*, 2006; Jodeiri Farshbaf and Ghaedi, 2017). Although the cerebellum 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 (Samson and Claassen, 2017). A considerable loss of Purkinje cells was demonstrated in some HD patients with predominant motor symptoms (Singh-Bains *et al.*, 2019), the extent of which may become more pronounced in patients with higher CAG repeat number (Hedjoudje *et al.*, 2018).

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 (Gutekunst *et al.*, 2002; Jeste *et al.*, 1984; Rodda, 1985). 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 (Rees *et al.*, 2014) with a presumed compensatory role for the dysfunction in the fronto-striato-thalamic motor circuit (Deckel, 1995; Squitieri *et al.*, 2003). The significant elevations in cerebellar Pgc-1α (Török *et al.*, 2015) 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 striatum and cortex were almost totally spared (Szalárdy *et al.*, 2013).

VI – CONCLUSION

Neurodegenerative diseases yield a growing health care problem, therefore, the identification of the potential novel aspects of the neurodegenerative mechanism could be a future target of drug design. From that reason we focused on the deep characterization of the PGC- and Sirtuin-system in different experimental protocols. Although the results of cold exposure were incongruent, but exercise training unequivocally could be an important potential activator of the cerebellar neuroprotective system including the PGC-Sirtuin axis. From the toxin experiment we concluded that the PGC-system is more sensitive to MPTP than the Sirtuins, however, not all isoforms were tested. In the N171-82Q model, in addition to the previously demonstrated activation of the cerebellar PGC-system, there was an obvious activation of cerebellar Sirtuin-system as well, which may emphasize the involvement of cerebellum in HD. However, in addition to the results detailed above, further studies are required to exactly clarify the role of the PGC- and Sirtuin-systems in the development of neurodegenerative processes.

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ORIGINAL PAPER

Cerebellar Predominant Increase in mRNA Expression Levels of Sirt1 and Sirt3 Isoforms in a Transgenic Mouse Model of Huntington's Disease

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Abstract

The potential role of Sirt1 and Sirt2 subtypes of Sirtuins (class III NAD^+-dependent deacetylases) in the pathogenesis of Huntington's disease (HD) has been extensively studied yielding some controversial results. However, data regarding the involvement of Sirt3 and their variants in HD are considerably limited. The aim of this study was to assess the expression pattern of Sirt1 and three Sirt3 mRNA isoforms (Sirt3-M1/2/3) in the striatum, cortex and cerebellum in respect of the effect of gender, age and the presence of the transgene using the N171-82Q transgenic mouse model of HD. Striatal, cortical and cerebellar Sirt1-Fl and Sirt3-M1/2/3 mRNA levels were measured in 8, 12 and 16 weeks old N171-82Q transgenic mice and in their wild-type littermates. Regarding the striatum and cortex, the presence of the transgene resulted in a significant increase in Sirt3-M3 and Sirt1 mRNA levels, respectively, whereas in case of the cerebellum the transgene resulted in increased expression of all the assessed subtypes and isoforms. Aging exerted minor influence on Sirt mRNA expression levels, both in transgene carriers and in their wild-type littermates, and there was no interaction between the presence of the transgene and aging. Furthermore, there was no difference between genders. The unequivocal cerebellar Sirtuin activation with presumed compensatory role suggests that the cerebellum might be another key player in HD in addition to the most severely affected striatum. The mitochondrially acting Sirt3 may serve as an interesting novel therapeutic target in this deleterious condition.

Keywords Huntington's disease · Transgenic · Sirt1 · Sirt3 · Sirtuin · Brain

Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease [66]. HD is caused by expansion of CAG repeats in the *IT15* gene encoding Huntington protein (Htt) which has an important role in the maintenance of cellular energy metabolism and mitochondrial function [50]. Previous works demonstrated that mutant Huntington protein (mHtt) inhibits the function of a key metabolic master regulator, namely peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α), which, amongst others, has an essential role in mitochondrial biogenesis [8, 25].

Sirtuins are class III NAD^+-dependent deacetylases [38]. Currently there are seven identified mammalian Sirtuin subtypes (SIRT1-7), which are localized in different cellular compartments (nuclear: SIRT1 (the mammalian orthologue of the yeast Silent information regulator 2 protein (Sir2)), -6, -7; mitochondrial: SIRT3, -4, -5; cytoplasmatic: SIRT2) [36]. In addition to the above-detailed subtypes, alternative splicing results in further isoforms of Sirtuins [31, 67]. Several molecular targets of Sirtuins, including the above-mentioned PGC-1α, were identified as participants of the regulation of energy metabolism, circadian rhythm, stress response, apoptosis and aging [38]. The association between SIRTs and neurodegenerative disorders, including HD, has been widely studied using these models [2, 22, 24, 27, 36, 56]. Calorie restriction is capable of increasing SIRT1 protein level in the brain, liver, hearth and white adipose tissue

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of mice [39], and also increases the lifespan in the N171-82Q transgenic (tg) mouse model of HD [11]. In contrast to these findings, exercise, which induces the expression of Sirt3-M1 and -M2 isoforms [47], did not elongate the lifespan in the same mouse model of HD [43]. Regarding Sirt1 mRNA and SIRT1 protein expression changes in HD the results are somewhat inconsistent: SIRT1 protein levels were found to be reduced in human brain tissue and in the R6/1 transgenic mouse model of HD as well [18, 41]. Tulino et al. found that SIRT1 activity becomes reduced in R6/2 (with a mean CAG repeat number (MRN) of 204) and *Hdh*Q150 (MRN: 165) mice, in the background of which they hypothesized altered phosphorylation status of SIRT1 via an 5′ AMP-activated protein kinase α1-related mechanism in the striatum and cerebellum [61]. Although the presence of the transgene did not affect either Sirt1 mRNA, or SIRT1 protein expression in the striatum of R6/2 mice, there was a significant decrease in mRNA expression from 4 to 9 weeks in wild-type (wt) animals [61]. In contrast to these findings, cerebellar Sirt1 mRNA expression increased significantly by 9 and 14 weeks of age in R6/2 mice, whereas SIRT1 protein levels significantly decreased predominantly in wt mice by 14 weeks of age. In *Hdh*Q150 and wt mice the SIRT1 protein level did not show any alteration either in the 2 or in the 22 months old animals [61]. A later whole-brain study reported the elevation of Sirt1 mRNA level in 5, 8 and 11 weeks old R6/2 mice (MRN: 144). However, up-regulation of Sirt1 was only observed in their 8, but not in 12 weeks old counterparts possessing a mean CAG repeat size of 182 [45]. Regarding SIRT1 protein expression, there was a consistent elevation in tg mice with 182 mean CAG repeats in both ages, and when examined at 12 weeks of age, without gender differences [45]. Besides the somewhat inconsistent findings obtained from the above-mentioned studies, the experiments influencing the expression of SIRT1 or of its orthologues from a therapeutic point of view yielded more controversial results. Parker et al. were the first who demonstrated that *Sir2* overexpression and resveratrol (RESV) treatment (one of the most important non-selective Sirtuin inducer) could delay the development of neuronal dysfunction in a *Caenorhabditis elegans* model of HD (Htt N-terminal fragment, 128Q) in vivo [42]. They also reported that RESV prevented the striatal neuronal cell death in *Hdh*Q111 knock-in mice [42]. To test the potential neuroprotective effect of SIRT1, Jeong et al. crossed a brain-specific *Sirt1* knockout mice (BSKO; genotype: $Sirt1^{flox/flox}$) with the R6/2 HD model mice [22]. They detected the exacerbation of the neuropathological aspects of HD indicated by lower striatal (neuronal) volume [22], whereas they found the opposite in SIRT1 overexpressing knock-in mice (Sirt1-KI-R6/2). These animals showed longer survival time (30% extension) and less prominent neuropathological alterations [22]. They proposed that the neuroprotective effect of SIRT1 is exerted through the activation of the cyclic AMP response element binding transcription factor-regulated transcription coactivator 1 factor, which leads to the enhancement of the brain-derived neurotropic factor-mediated neuroprotection [22]. Jiang et al. crossed *Sirt1* and N171-82Q or BAC HD transgenic mice which resulted in offsprings with decelerated disease progression and reduction of brain atrophy probably via the overexpression of *Sirt1* [24]. In contrast to these findings, the pharmacological inhibition of SIRT1 by selisistat exerted beneficial effects in both Drosophila and mouse models of HD and was found to be safe in human studies as well [52, 54].

SIRT2, another member of the Sirtuin family, is suspected to enhance the disease process in HD. Chopra et al. reported a beneficial effect of SIRT2 inhibition in R6/2 HD mice [7]. Previously published articles demonstrated that there is an age-dependent SIRT2 accumulation which results in microtubule deacetylation in mouse brain and spinal cord [33]. These alterations lead to the disruption of microtubule-associated cellular transport which is an important component of the pathogenesis of HD [10, 16]. However, it seems that the ablation of SIRT2 did not prevent the development of HD-related pathological mechanisms in R6/2 mice [5].

Similar to SIRT1, for which most of the results support a protective role in HD, SIRT3 is also proposed to have a beneficial effect regarding the pathogenesis of the disease [38], though the available data are limited. SIRT3 is involved in the regulation of fatty acid oxidation, urea- and amino acid pathways [2]. Striatal administration of a RESV dimer (ε-viniferin treatment) reduced ROS level through SIRT3-mediated superoxide dismutase 2 (SOD2) induction in striatal progenitor cells (*Hdh*(Q111)) [14]. Some authors suggested that SIRT3 could alleviate the pathological process in HD through the deacetylation of the mitochondrial complexes (I, II, V) as well [1, 2]. Furthermore, SIRT3 is implicated in autophagy regulation via its effect on chaperones [28].

Regarding the role of another Sirtuin isoforms (SIRT4-7) in the pathogenesis of HD, experimental data are lacking.

The aim of the current study was to further elucidate the role Sirt1 and Sirt3 isoforms with presumed beneficial effects in HD, because the available data are somewhat controversial regarding Sirt1, and no deep assessment was done regarding the M1, M2 and M3 isoforms of Sirt3. For that purpose the authors applied a multi-dimensional approach to simultaneously assess the effect of the transgene, the time course of the disease and their interaction as well in addition to the screening of regional and possible gender-related differences in the N171-82Q tg mouse model of HD.

Materials and Methods

Animals

8, 12 and 16 weeks old N171-82Q and as their control, B6C3 wt mice with identical genetic background (female and male animals distributed equally) were involved in this study (n = 6–7 in each group). The HD model mice originally came from Jackson Laboratories (USA). They were housed in cages under standard conditions with 12–12 h light-dark cycle. The food and water were freely available. The experiments were carried out in accordance with European Communities Council Directive (86/609/EEC) and were approved by the local animal care committee. All animals were euthanized via isoflurane overdose (Forane; Abott Laboratories Hungary Ltd., Budapest, Hungary).

Sample Handling

The brains were rapidly removed from the skull and both hemispheres were dissected into the following brain areas: striatum, cortex and cerebellum. The tissue samples were stored at − 80 °C until the RT-PCR analysis.

RT-PCR Analysis

To perform the RT-PCR analysis, total RNA was isolated from the striatal, cortical and cerebellar samples with Trizol reagent according to manufacturer's protocol (Molecular Research Center, USA). The RNA concentration was determined with MaestroNano spectrophotometer. The RNA integrity was certified by 1% gel electrophoresis. For cDNA synthesis 1 µg of total RNA, random hexamer primers and reverse transcriptase were used (Revert Aid First Strand cDNA Synthesis Kit; Thermo Scientific, USA). The synthesized cDNA was stored at − 20 °C. Real-time PCR analysis (CFX 96 Real Time System; Bio-Rad, USA) with various Sirtuin primers was performed in 20 µl final volume using syber green label (PCR Biosystems, USA) [47]. Primer sequences and the exact thermal cycling conditions are described in our previous work [47]. We used the 18S rRNA as endogenous control (Applied Biosystems, USA). To calculate the relative mRNA expression levels, we used the $2^{-\Delta\Delta Ct}$ method [30].

Statistics

All statistical calculations were performed with the use of the freely available R software (R Development Core Team). First, the relative mRNA expression levels were calculated separately regarding each gene (i.e., Sirt1 or Sirt3), but they were normalized to the striatal level of the main subtype or isoform (i.e., Sirt1-Fl and Sirt3-M1) of 8 weeks old wt mice in each case to allow a time course analysis of the changes of expression patterns. Then we checked the distribution of data populations with the Shapiro–Wilk test and the homogeneity of variances with the Levene's test. In several cases the data diverged from Gaussian distribution and the variances were not equal. For that reason, we applied the Sheirer-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. The results were considered significant when the corrected p values were greater than 0.05. The data were presented as median and 1st and 3rd quartiles (Figs. 1, 2, 3, 4, 5).

Results

We could not detect any significant difference between male and female mice regarding the expression of 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-Fl (full length) expression in all the cortical and cerebellar samples of 8, 12 and 16 weeks old tg animals compared to wt mice (cortex (8 weeks: $p = 0.0029$; 12 weeks: $p = 0.0018$; 16 weeks: $p = 0.0029$); cerebellum (8 weeks: $p = 0.0052$; 12 weeks: $p = 0.0054$; 16 weeks: $p = 0.0065$), but not in the striatum (Figs. 1 and 5). Regarding the effect of aging on Sirt1-Fl expression levels, we detected significant increase only in the cerebellum of tg group at 16 weeks of age (8 vs. 16 weeks: $p = 0.0245$; 12 vs. 16 weeks: $p = 0.0316$). There was no detectable change in Sirt3-M1 expression in the striatum and cortex of any age groups (8, 12, 16 weeks). 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 weeks: $p = 0.0024$; 12 weeks: $= 0.0024$; 16 weeks: $p = 0.0024$; Figs. 2 and 5). We could not observe any 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 cerebellum of tg animals in each age

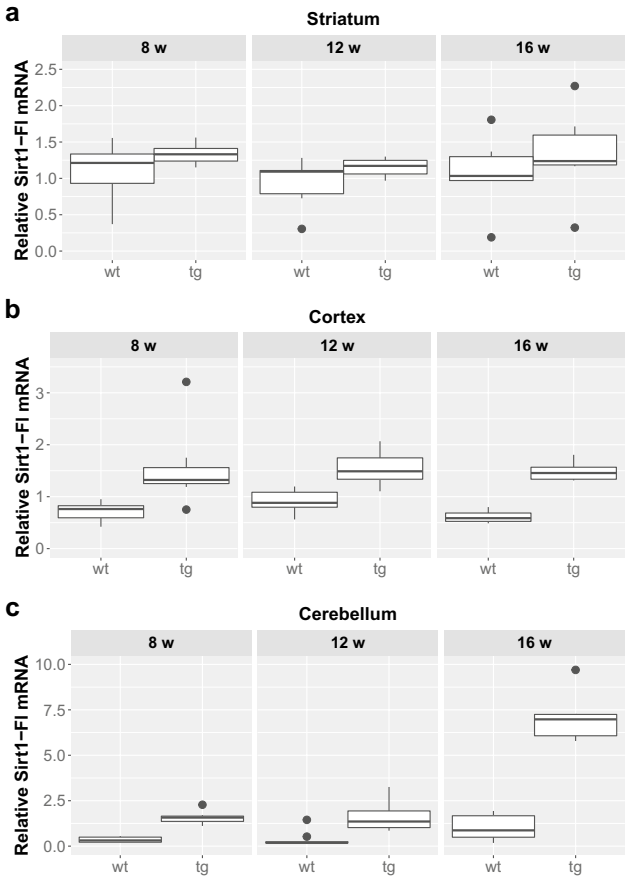

Fig. 1 Relative mRNA expression level of Sirt1-Fl in the striatum (**a**), cortex (**b**) and cerebellum (**c**) of N171-82Q transgenic and B6C3 wild-type mice of three age groups. The Sirt1-Fl level significantly elevated in all cortical and cerebellar samples in each age group of tg animals compared to wt mice. Aging caused a significant increase only in the cerebellum of tg group by 16 weeks of age. For clarity, the levels of significance were indicated separately in Fig. 5 in a special table format. Values are plotted as medians and interquartile ranges; *tg* = transgenic, *wt* = wild-type, *w* = weeks

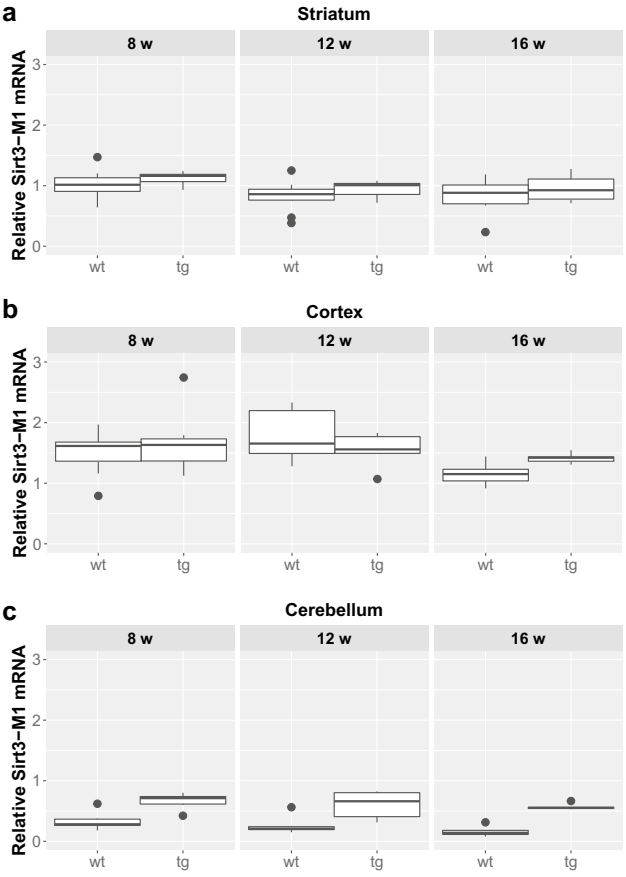

Fig. 2 Relative mRNA expression level of Sirt3-M1 in the striatum (**a**), cortex (**b**) and cerebellum (**c**) of N171-82Q transgenic and B6C3 wild-type mice of three age groups. The Sirt3-M1 level was significantly elevated in all cerebellar samples in each age group of tg animals compared to wt mice. For clarity, the levels of significance were indicated separately in Fig. 5 in a special table format. Values are plotted as medians and interquartile ranges; *tg* = transgenic, *wt* = wild-type, *w* = weeks

group compared to wt controls (8 weeks: p = 0.0021; 12 weeks: p = 0.0012; 16 weeks: p = 0.0021; Figs. 3 and 5). When assessing the effect of aging on Sirt3-M2 expression levels, we detected significant decrease only in the cortex of wt group at 16 weeks of age (8 vs. 16 weeks: p = 0.038; 12 vs. 16 weeks: p = 0.038). The expression of Sirt3-M3 elevated in each striatal and cerebellar tg groups, but only in the 16 weeks old group in the tg cortex compared to wt mice (striatum (8 weeks: p = 0.0097; 12 weeks: p = 0.0054; 16 weeks: p = 0.0097); cortex (16 weeks: p = 0.0032); cerebellum (8 weeks: p = 0.002; 12 weeks: p = 0.0006; 16 weeks: p = 0.0016) (Figs. 4 and 5). 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 weeks (wt-wt): p = 0.0243; 8 vs. 12 weeks (tg–tg): p = 0.036).

Discussion

HD is a neurodegenerative disorder, which has currently no curative treatment, but encouraging clinical trials are ongoing (e.g. antisense oligonucleotide treatment) [13, 56]. These therapeutic approaches target the pathological trinucleotide repeat expansion in the mutant hunting in mRNA, the extent of which has been demonstrated to have the strongest association with the age of disease onset and severity [12, 26, 37]. However, other important influencing factors of the disease have already been identified as well, which may serve as good candidates for the augmentation of beneficial effects obtained by the currently available therapies [32, 64].

Sirtuins are surely involved in the neurodegenerative process in HD, however, there are controversial results regarding their role [36, 38, 45, 61]. Further studies are needed to clarify these controversies and to better explore the role of

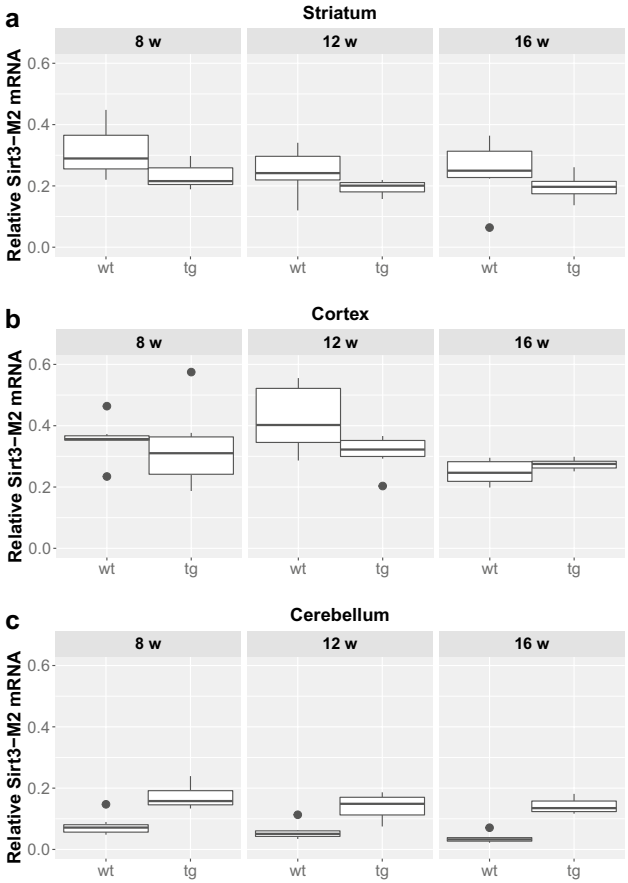

Fig. 3 Relative mRNA expression level of Sirt3-M2 in the striatum (**a**), cortex (**b**) and cerebellum (**c**) of N171-82Q transgenic and B6C3 wild-type mice of three age groups. The Sirt3-M2 level was significantly elevated in all cerebellar samples in each age group of tg animals compared to wt mice. Aging caused a significant decrease only in the cortex of wt group by 16 weeks of age. For clarity, the levels of significance were indicated separately in Fig. 5 in a special table format. Values are plotted as medians and interquartile ranges; *tg* = transgenic, *wt* = wild-type, *w* = weeks

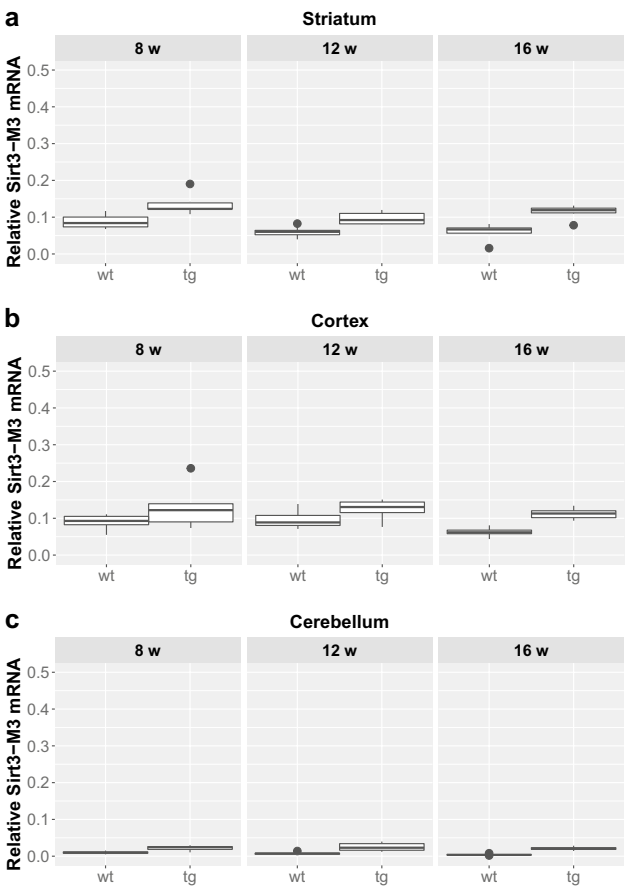

Fig. 4 Relative mRNA expression level of Sirt3-M3 in the striatum (**a**), cortex (**b**) and cerebellum (**c**) of N171-82Q transgenic and B6C3 wild-type mice of three age groups. The Sirt3-M3 level was significantly elevated in all striatal and cerebellar samples in each age group of tg animals compared to wt mice. The striatal expression decreased significantly by 12 weeks of age in both wt and tg animals. For clarity, the levels of significance were indicated separately in Fig. 5 in a special table format. Values are plotted as medians and interquartile ranges; *tg* = transgenic, *wt* = wild-type, *w* = weeks

different Sirtuin subtypes and isoforms obtained by alternative splicing [2, 22, 24, 27, 36, 56]. Accordingly, we aimed at contributing to the clarification of the controversies regarding Sirt1 and Sirt3 mRNA expression patterns by a better characterization of region- and aging-specific changes in the mRNA expression levels of Sirt1 and three Sirt3 isoforms using the N171-82Q tg mouse model of HD.

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) [61]. 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 weeks old (MRN: 182) R6/2 mice [45]. In the 5, 8 and 11 weeks old mice (MRN: 144) there was a significant increase in 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 weeks old group [45]. Due to the different CAG repeats, ages, brain regions and gender composition the comparability of these results is limited. Therefore, we find it important to get closer to the unbiased preclinical modelling of changes in Sirt1 mRNA expression in HD. Thus, we paid a special attention to study design regarding the following points: we used the N171-82Q tg model of HD, because the phenotype of these mice mimics better the majority of human cases with considerable similarities in pathological alterations as

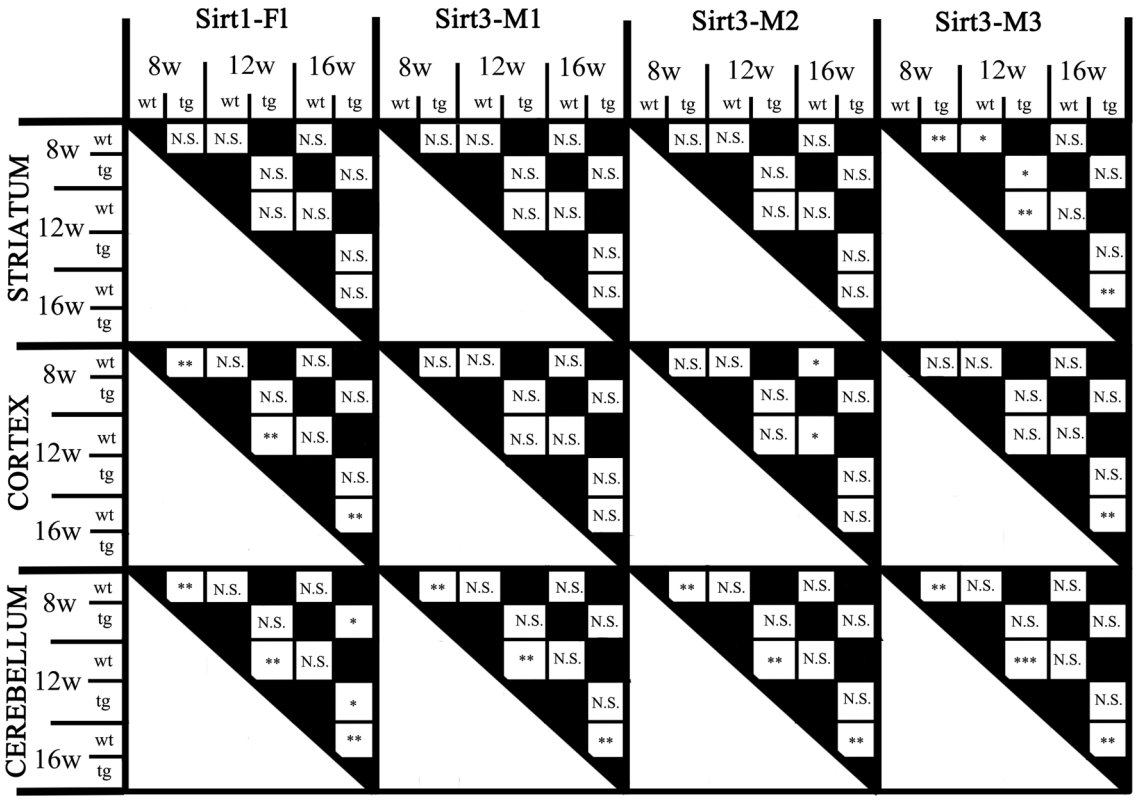

Fig. 5 The statistical delineation of the effect of the transgene and the time course of the neurodegenerative process on the expression pattern of Sirt1 and Sirt3 isoforms. In addition to the presented statistical differences, there were no significant interactions between the presence of the transgene and age. $*p<0.05$, $**p<0.01$, $***<0.001$; *N.S.* = not significant; *tg* = transgenic, *wt* = wild-type, *w* = weeks

well than the R6/2 tg mice, the symptoms of which mainly resemble that of juvenile HD cases. Furthermore, the authors have extensive previous experiences with this model [15, 35, 40, 59, 62, 63, 68, 69], which enables the drawing of indirect correlations between the characteristic features of disease progression with the time course of the changes in mRNA expression patterns. Characteristically, in N171-82Q mice the symptoms begin to develop at approximately 2 months of age and disease progression results in a mean survival time of 110–130 days [68, 69]. Accordingly, the study of mice at the age of 8, 12 and 16 weeks may represent early, moderate and advanced stages of the modelled disease. In addition to the assessment of the presence of transgene on Sirt mRNA expression changes in key structures (striatum, overlying cortex, cerebellum) of the regulation of motor functions, our study design involved the determination of the effect of aging and its interaction with the presence of the transgene with a view on gender-related effects as well. First of all, we found no significant differences between genders regarding either 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. [45, 61]. Similarly to the study of Tulino et al. [61], we found no effect of the transgene in the striatum regarding either 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 found by Reynolds et al. [45] 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 the striatal or cerebellar samples of wt mice, found by Tulino et al. [61], could not be confirmed by the current study.

Although data suggest that the induction of mitochondrially acting SIRT3 may be capable of exerting beneficial effects in a HD model [14], the expression pattern of Sirt3 mRNA isoforms has never been studied in any HD model. Similar to that 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, we have to note here that 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 cerebellum regarding any of the assessed Sirt subtypes and isoforms strongly resembles to that of 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 [58]. The reason behind the same pattern may be that Sirtuins are upstream regulators of PGC-1α expression [8, 25]. Similar cerebellar predominant PGC-1α activation was induced by long-term physical exercise [47] and by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment in its acute phase [60], but not by cooling, by short-term exercise [47], or by 3-nitropropionic acid (3-NP), the toxin used for modeling HD-associated striatal damage [58, 65].

Although the cerebellum is known not to be the primarily affected structure in HD, there is increasing evidence of its involvement in the pathomechanism of the disorder [48]. A considerable loss of Purkinje cells was demonstrated in some HD patients with predominant motor symptoms [51], the extent of which may become more pronounced in patients with higher CAG repeat number [19]. However, 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 [17, 23, 46]. The exact background of this variability, involving the sparing of alterations as well in some human cases, is not known and needs further elucidation. Nevertheless, some studies proved cerebellar hypermetabolism in HD [44] with a presumed compensatory role for dysfunction in the fronto-striato-thalamic motor circuit [9, 53]. The significant elevations in cerebellar PGC-1α [58] 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 striatum and cortex were almost totally spared [55]. Although data obtained from the above-mentioned toxin studies are somewhat controversial, this may be resolved by considering that in case of 3-NP the most vulnerable structures are the striatum and the hippocampus, and not the cerebellum [34], whereas MPTP is capable of inducing cerebellar degeneration as well in addition to its well-known deleterious effects on the nigrostriatal system [57]. However, the mRNA expression changes in Sirt1 and Sirt3 levels following MPTP intoxication were not significant either in its acute or chronic phase (unpublished data).

Regarding genotype-phenotype correlations, the first description of the applied N171-82Q transgenic mouse model of HD has already demonstrated that in addition to cortical, hippocampal, amygdalar and striatal involvement, an expressed deposition of intranuclear inclusions were present in cerebellar granule cells as well [49]. It was also verified by the authors, but no remarkable neuronal loss or reactive astrogliosis was found [40]. This may be explained by that the presence of abundant intranuclear inclusions does not compulsorily equals to severe neuronal damage, indeed, it was presumed to exert protective effects [3]. This may further support our hypothesis that coping mechanisms in the cerebellum, including inclusion body formation and enhanced expression pattern of the Sirt – PGC-1α axis, demonstrated by the current and previous studies [59] of the authors, may be capable of exerting rather neuroprotective than damaging effects. Accordingly, the progression of the decrease of coordination, which was described as one of the key features of this model and probably attributed to cerebellar dysfunction, could be observed from 3 months of age on [20]. However, the hypokinetic feature of N171-82Q transgenic mice, rather explained by the dysfunction of the basal ganglia, involving the striatum, was still present at 8 weeks of age [69]. The evidence from unbiased anatomical work-ups of the authors also favors the above-detailed hypothesis, i.e., more than 20% of striatal neurons contained intranuclear inclusions at 16 weeks of age, whereas no remarkable neuronal loss, but prominent neuronal atrophy could be detected at this age, responsible for the decrease of striatal volume and brain weight [69]. However, besides the well demonstrated atrophy of striatum, cortex, hippocampus, hypothalamus, thalamus and amygdala, the volume of the cerebellum was still preserved even at 18 weeks of age [6].

The characteristic pattern observed in preclinical findings could not be confirmed on human samples, where Sirt1, -2, and -3 mRNA levels were assessed, demonstrating elevated cortical and striatal Sirt1 and striatal Sirt2 expression in HD patients without any change regarding Sirt3 or the cerebellum [4]. However, this gene expression study was carried out on post-mortem samples obtained from multiple centers, and the postmortem delay of sample handling and other characteristics of the specimens were not given, so their effect on individual differences in mRNA decay cannot be ruled out introducing a bias into the results. Nevertheless, further human studies are needed to be able to better determine the human relevance of the current preclinical findings.

The major limitation of the current study is the lack of experiments on protein expression changes of the corresponding Sirtuins. However, the widely applied antibody-based methods in this topic (e.g. Western blotting) are semi-quantitative at best, mostly lacking appropriate sensitivity and specificity [29]. Although the application of deep proteomic analysis by mass spectrometry is appropriate, but

quite challenging [21], especially in case of brain samples with considerably high lipid content. Accordingly, this was out of the scope of the current study. Nevertheless, future experiments are needed to confirm the results of the present work at proteome level.

In conclusion, the current study demonstrates an unequivocal elevation of Sirt1 mRNA expression level in the cortical and cerebellar samples of HD transgenic animals compared to their age-matched wild-type littermates. An increase of these differences with aging could be observed only in the cerebellum of transgenic animals, presumably related to disease progression. Regarding the expression pattern of the mitochondrially acting Sirt3 isoforms (M1, M2, M3), which have not been assessed before in HD, a similar transgene-specific increase of their cerebellar level was observed, and only the striatum showed a likewise elevation of exclusively the M3 isoform regarding the other 2 assessed brain regions. Accordingly, the observed pattern of changes with a presumed compensatory role may draw attention to that the involvement of cerebellum in HD may be more pronounced than previously thought, yielding a novel target for therapeutic approaches aiming at symptom relief in that deleterious condition.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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Research article

The effect of physical stimuli on the expression level of key elements in mitochondrial biogenesis

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ABSTRACT

Proper mitochondrial function is crucial for intact cellular homeostasis. Mitochondrial dysfunction is clearly involved in the pathogenesis of most neurodegenerative- and age-related chronic disorders. The aim of this study is to stimulate cellular production of important compounds of mitochondrial biogenesis, namely in the peroxisome proliferator-activated receptor-gamma coactivator (PGC)- and Sirtuin (SIRT)-systems.

We studied the effect of cold challenge and training on the mRNA expression levels of some compounds of these systems in different brain areas of mice. With regard to the PGC-system, the mRNA levels of the full- and N-truncated isoforms, and those of the two promoters (brain-specific, reference) were measured. In case of Sirtuins, the mRNA levels of SIRT1 and SIRT3-M1/M2/M3 were assessed.

We found the following expression level alterations: cooling resulted in the elevation of cortical SIRT3-M1 levels and the decrease of cerebellar SIRT3-M3 levels after 200 min. 900 min of cold exposure resulted in the reduction of cortical SIRT1 and striatal SIRT3-M1 levels. A prominent elevation could be observed in the levels of all PGC-1α isoforms in the cerebellum after 12 days of training. The 12 days of exercise resulted in increased cerebellar SIRT3-M1 and SIRT3-M2 mRNA levels as well.

Although the efficacy of cooling core body and brain temperature is questionable, we found that training exerted a clear effect. The cause of the prominent cerebellar elevation of PGC-, and Sirtuin isoforms could be an increase in synaptic plasticity between Purkinje cells, which facilitates better motor coordination and more precise movement integration. We propose that these systems may serve as promising targets for future therapeutic studies in neurodegenerative diseases.

1. Introduction

A constant energy supply is crucial for proper tissue function. Mitochondria synthesize adenosine triphosphate (ATP), and play a role in adaptive thermogenesis, intracellular Ca^{2+} homeostasis, aging and cell death. Mitochondrial dysfunctions are implicated in the pathogeneses of neurodegenerative diseases.

Peroxisome proliferator-activated receptor-gamma (PPARγ) coactivator-1 alpha (PGC-1α) is a transcriptional coactivator that regulates mitochondrial biogenesis, energy homeostasis and adaptive thermogenesis [1,2]. Besides the full-length protein (FL-PGC-1α), multiple PGC-1α isoforms have been identified [3,4]. Alternative splicing between exons 6 and 7 of the *Pgc-1α* gene produces the N-truncated, shorter PGC-1α (NT-PGC-1α) isoform [5]. Besides the classical proximal promoter, novel tissue-specific PGC-1α isoforms have recently been described, including muscle-, liver- and central nervous system-specific (CNS-PGC-1α) isoforms [3,4,6,7].

Sir2-like proteins (Sirtuins) are mainly NAD^+-dependent deacetylases which play a prominent role in mitochondrial biogenesis [8,9]. Seven mammalian Sirtuin subtypes (SIRT1-7) that are present in different subcellular locations were identified [10]. Alternative promoter usage and splicing variability results in a wide range of Sirtuin isoforms. From the perspective of our research, the four most important subtypes are SIRT1 and SIRT3-M1, -M2 and -M3 [11–14].

It is well known that tissue-specific PGC-1α and Sirtuin alterations develop by challenging the energy homeostasis, e.g., with cold exposure and physical exercise [1,6,15–27]. Previous studies described that full length PGC-1α mRNA expression was elevated in mouse brown adipose tissue (BAT) and skeletal muscle following cold exposure [1]. It is also known that cold exposure shifts the transcription from PP to an

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alternative promoter in BAT [6]. Recent studies demonstrated that the mRNA levels of SIRT2 and SIRT3 in mice BAT were increased by cold exposure (3, 6, 12 h periods at 5 °C) and decreased if the environmental temperature was higher than thermoneutral (16 h period at 27.5 °C) [22]. SIRT6 mRNA and protein levels were also elevated in the brown and inguinal white adipose tissue of 8-week-old mice following 4 °C overnight cold exposure [25]. While there is increasing data about changes in these 2 systems in BAT following cooling, there is little data related to alterations in the brain. A study demonstrated that the brain mRNA level of PGC-1α did not change after 3 h or 12 h at 4 °C [26].

Increased PGC-1α expression was observed in skeletal muscles after physical exercise [24,27]. An isoform-specific expression pattern during exercise of different intensity was demonstrated, which is caused by a promoter shift [22,24]. It has been proven that PGC-1 mRNA levels increased in rats already on the first day of a 1-week training program where the rats trained on alternate days [20]. However, following a similar increase on the first day, an opposite, decreasing trend was observed with 4 days of consecutive training [20]. Studies reported that long-term intensive exercise training induced FL-PGC-1α expression and mitochondrial biogenesis in the whole brain, particularly in some brain areas of mice [23]. The 17-day-long training did not change PGC-1α protein levels in the examined brain regions (cortex, striatum), either in young or old mice [18].

Although exercise failed to extend life span in animal experiments, it seemingly had an influence in the Sirtuin-system [15–23]. Several studies have been carried out to investigate the correlation between training intensity, the age of animals and Sirtuin expression in the skeletal muscle, liver and heart [15,21]. The results of these studies can be summarized as an increase in SIRT1 and SIRT6 levels after exercise with different protocols [39]. Steiner and Bayod found an elevation of SIRT1 protein levels in specific brain regions of mice after exercise [15,23], whereas Lezi et al. found no difference in the expression level of SIRT1 in brains of mice following exercise [40]. With regards to another subtype of the Sirtuin-system, SIRT3, its level was found to be elevated in the brain of exercised mice [16].

It has already been demonstrated that there is a direct relationship between the Sirtuin- and PGC-systems [28,29]. These systems are implicated in neurodegenerative diseases, such as ALS, Huntington's, Parkinson's and Alzheimer's disease [30].

Although the expression of PGC-1α and Sirtuins have been already investigated in rodent brain with the above-mentioned environmental stimuli, the results are controversial [1,6,15–27]. From the perspective of the PGC-system, only the FL-PGC-1α and NT-PGC-1α were previously examined, whereas it is well established that in the brain, short-term cold exposure did not alter the level of the examined isoforms. Training elevated the levels of PGC-1α isoforms in intensity-, age- and duration-specific manners. However, there is no available data about the recently identified novel isoforms of PGC- and Sirtuin-systems in different brain regions. Accordingly, the aim of the current study was to assess the effect of cold challenge and training on the expression levels of FL-PGC-1α, NT-PGC-1α, CNS-PGC-1α, PP-PGC-1α, SIRT1, SIRT3-M1, -M2 and -M3 in the striatum, cortex and cerebellum of wild-type C57Bl/6 J mice.

2. Experimental procedure

2.1. Animals

20-week-old female C57Bl/6 J mice were involved in this study. The rationale for the use of female mice was that the PGC-system has a gender-specific expression pattern [31,32]. Weydt et al. detected an SNP (rs3736265, *PPARGC1A*) in patients with Huntington's disease which caused earlier disease onset only in men [32]. They also showed that there is an earlier disease onset and age of death in SOD1 transgenic ALS model of FL-PGC-1α deficient male mice [31]. Accordingly, these data indicate that the PGC-system mediated protective effects may be more active in females. With regards to the Sirtuin-system, it can be said that the overexpression of SIRT6 increases lifespan in transgenic male mice, but not in females. As a conclusion, the Sirtuin-system seems to be more active in female mice as well [33]. The 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) and were approved by the local animal care committee. All animals were euthanized via isoflurane overdose (Forane; Abott Laboratories Hungary Ltd., Budapest, Hungary).

2.2. Treatments and sample handling

2.2.1. 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. Ninety minutes after the last cold exposure the animals were deeply anesthetized with isoflurane and their brains were dissected immediately.

2.2.2. Exercise training

Exercise training was examined 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). The speed profile was standard 5 RPM for 30 min. Prior to training, 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. Ninety minutes after the last measurement, the animals were anesthetized, and the brains were dissected immediately.

2.3. RT-PCR analysis

Total RNA was isolated from striatum, cortex and cerebellum with Trizol according to the manufacturer's protocol. RNA concentrations were measured with a MaestroNano spectrophotometer, and the integrity of RNA was confirmed 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 (Thermo Scientific, USA). cDNA was kept at −20 °C until further use. Real-time PCR was performed with a CFX 96 Real TimeSystem (Bio-Rad, USA) to detect changes in mRNA expression, using various primer pairs at a final volume of 20 μl. We used previously described PGC-1α and Sirtuin primers [6,12,14]. (See the exact thermal cycling conditions in Supplementary File 1.). 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 [34].

2.4. Statistics

All statistical analyses were performed with the use of the R software (R Development Core Team). The Levene test was performed for the analysis of 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. We calculated the gene expression level of PGC-transcripts in all brain areas relative to FL-PGC-1α and CNS-PGC-1α control striatum groups. For Sirtuins we compared SIRT1 expression levels to SIRT1-FL,

and all SIRT3 isoforms to SIRT3-M1 control striatum groups. The differences were considered significant when the p values were less than 0.05.

3. Results

3.1. PGC-1α transcript levels

3.1.1. Cold exposure

There were no detectable changes in the levels of PGC-1α transcripts in the different brain areas after the total 200 min or 900 min cold exposure. The expression level of all the investigated transcripts was detected at room temperature and this expression was not altered by cold exposure (Supplementary Figs. 1 and 2).

3.1.2. Exercise training

The levels of PGC-1α transcripts did not show any change in the investigated brain areas after the 5-day-long rotarod training (Supplementary Fig. 3). However, the 12 day exercise training resulted in significant increases in FL-PGC-1α, NT-PGC-1α, CNS-PGC-1α and Ref-PGC-1α mRNA expression in the cerebellum (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-PGC-1α: ctrl: 0.21 ± 0.03; EX: 0.30 ± 0.02; p = 0.0003; Fig. 1 C). With regards to the striatum and the cortex, no other significant differences were detected (Fig. 1 A, B). To verify that the CNS specific promoter is only poorly expressed in peripheral tissues, the quadriceps muscle was used, demonstrating hardly detectable expression levels. However, the expression level of FL-PGC-1α (ctrl: 1.01 ± 0.19; EX: 3.19 ± 1.25; p = 0.003), NT-PGC-1α (ctrl: 0.10 ± 0.02; EX: 0.50 ± 0.19; p = 0.001) and Ref-PGC-1α (ctrl: 1.00 ± 0.11; EX: 1.69 ± 0.52; p = 0.016) mRNA was significantly elevated in the quadriceps muscle after 5 days of training.

3.2. Sirtuin transcript levels

3.2.1. Cold exposure

After 200 min of cold exposure there were no detectable changes in the levels of SIRT1 and SIRT3-M2 transcripts in any brain regions (Fig. 2), but SIRT3-M1 levels elevated in the cortex (ctrl: 1.26 ± 0.49; EX: 1.97 ± 0.60; p = 0.036; Fig. 2 B), whereas SIRT3-M3 levels decreased in the cerebellum (ctrl: 0.16 ± 0.05; EX: 0.10 ± 0.03; p = 0.027; Fig. 2 C). 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; Fig. 2 E) and striatal SIRT3-M1 (ctrl: 1.04 ± 0.30; EX: 0.72 ± 0.21; p = 0.029; Fig. 2 D) expression levels.

3.2.2. Exercise training

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 did not change (Supplementary Fig. 4).

However, 12 days of exercise training resulted in the increase of both SIRT3-M1 and SIRT3-M2 mRNA expression in the cerebellum (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; Fig. 1 F). We did not

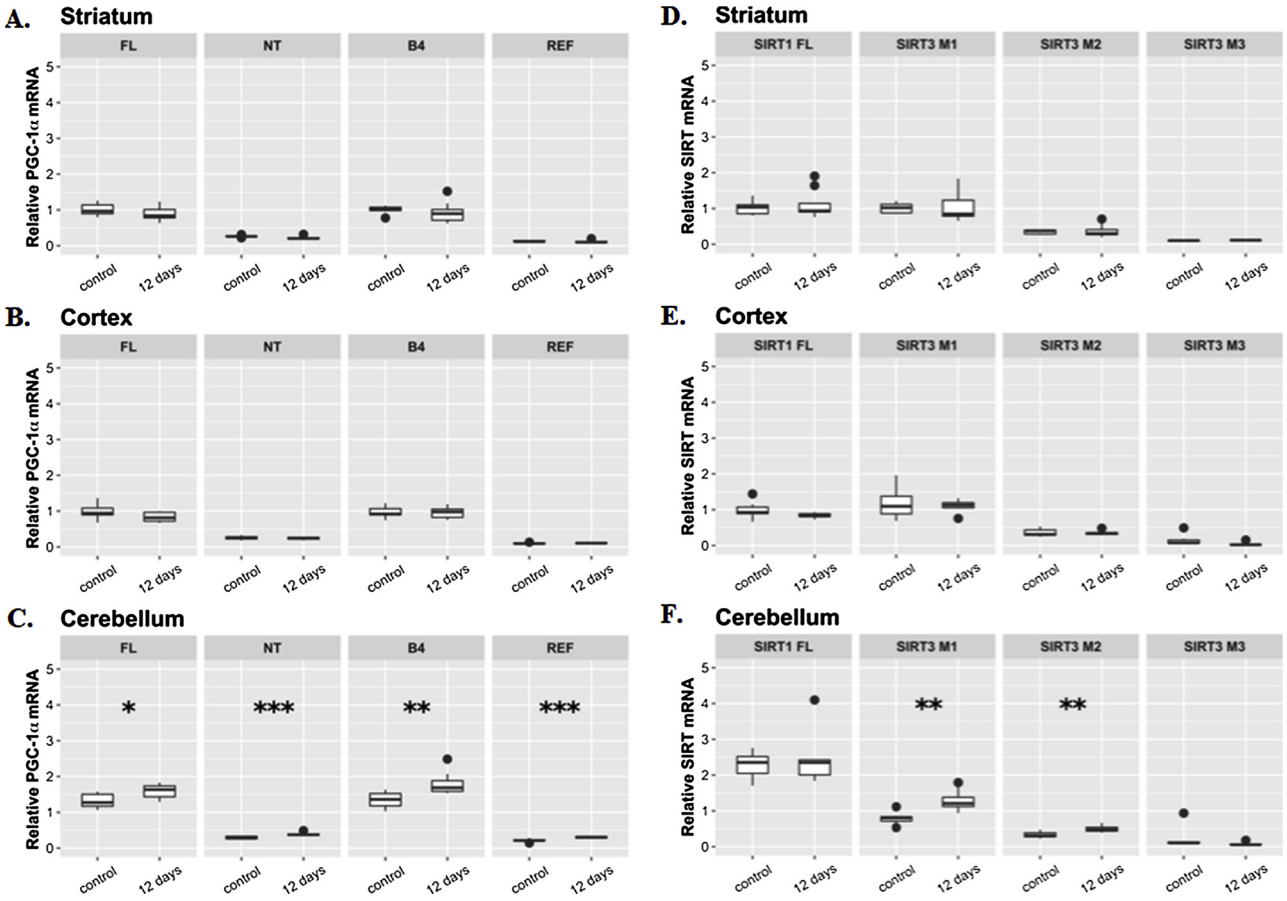

Fig. 1. Striatal (A, D), cortical (B, E) and cerebellar (C, F) relative mRNA expression levels of PGC1-1α (A, B, C) and Sirtuin (D, E, F) isoforms in mice after 12 days of rotarod training (5 RPM). The FL-PGC-1α, NT-PGC-1α, CNS-PGC-1a (B4), Reference promoter (REF), SIRT3-M1 and –M2 levels significantly increased in the cerebellum of exercised mice. Values are plotted as medians and interquartile range; *p < 0.05, **p < 0.01, ***p < 0.005; *12D* – 12 days rotarod training; *str* – striatum; *ctx* – cortex; *crb* - cerebellum.

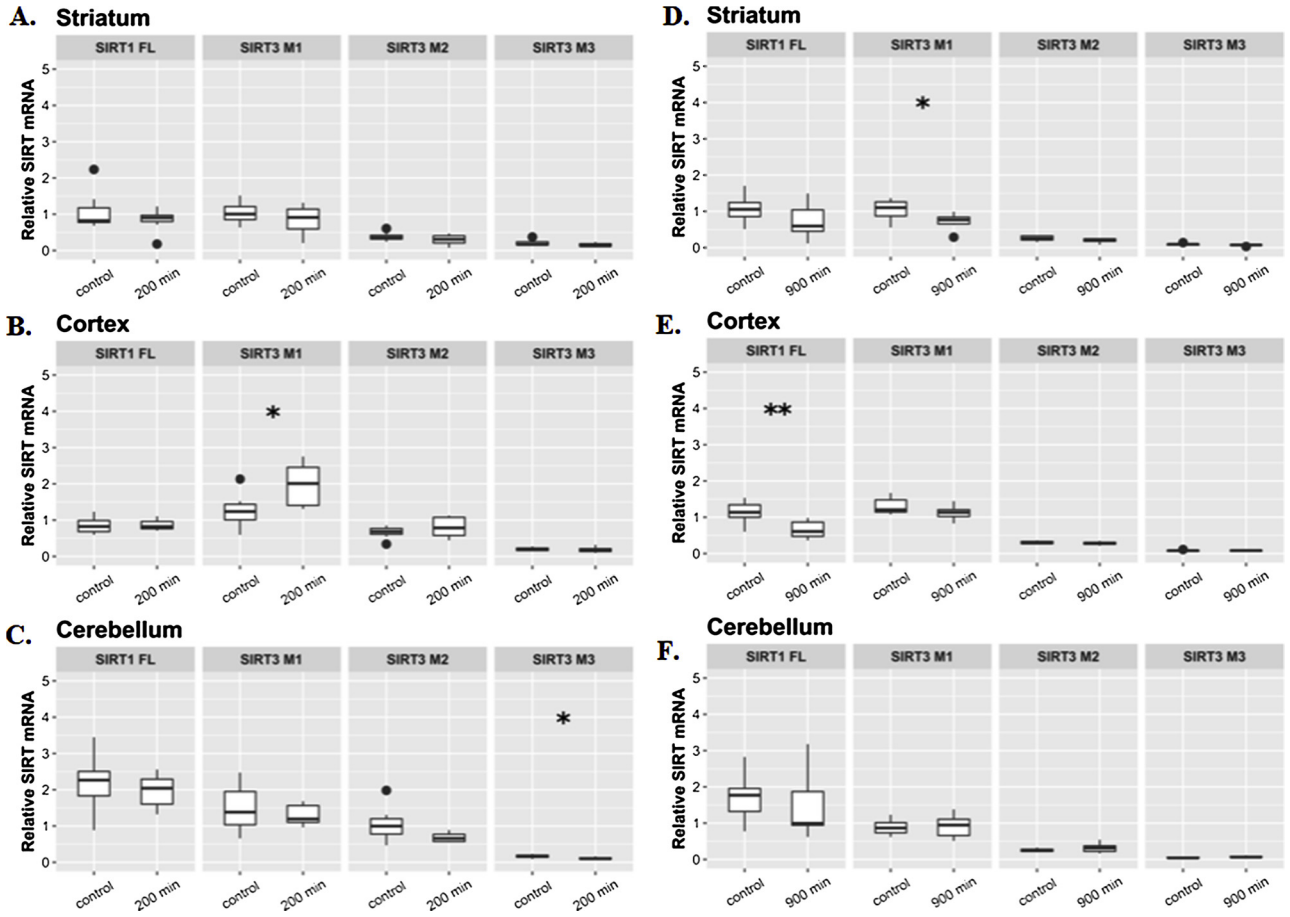

Fig. 2. Striatal, (A, D), cortical (B, E) and cerebellar (C, F) relative mRNA expression levels of Sirtuin isoforms in mice after 200 (A–C) and 900 (D–F) minutes of cold exposure (4 °C). After 200 min the SIRT3-M1 isoform was significantly upregulated in mice cortex and cerebellar SIRT3-M3 was also significantly decreased. After 900 min the SIRT1-FL isoform was significantly downregulated in mice cortex and striatal SIRT3-M1 was also significantly decreased. Values are plotted as medians and interquartile range; *p < 0.05, **p < 0.01, ***p < 0.005; *200* – 200 min cold exposure; *str* – striatum; *ctx* – cortex; *crb* - cerebellum.

find differences in SIRT1 and SIRT3-M3 levels in any other brain areas (Fig. 1 D, E).

4. Discussion

Maintenance of energy homeostasis is crucial for survival. PGC-1α and Sirtuins modulate mitochondrial biogenesis and other cellular mechanisms in the brain in response to physical exercise and cold exposure. In this study we investigated the isoform- and brain area-specific expression pattern of PGC-1α and Sirtuin following environmental stimuli.

The alteration of PGC-1α has already been examined in the brain, and previous findings suggested that very short-term cold exposure did not influence PGC-1α expression in the brain [26]. Accordingly, we also could not demonstrate any changes in the FL-PGC-1α, NT-PGC-1α, CNS-PGC-1α or Ref-PGC-1α levels in any brain area after cold exposure between the control and short- or long-duration cold-exposed animals.

However, there is currently no data about the effect of cooling on the levels of Sirtuin isoforms in the brain. We found that short exposure (200 min) elevated the level of the SIRT3-M1 isoform in the cortex, and decreased the SIRT3-M3 level in the cerebellum. The long exposure (900 min) revealed a decline in cortical SIRT1, and striatal SIRT3-M1 levels. As an explanation, we suppose that this cold-challenge regime is not effective in decreasing the core body temperature sufficiently and the early compensatory mechanisms in BAT and skeletal muscle protect the brain against cold exposure.

Previous studies reported that physical activity reduces the risk of dementia and Alzheimer's disease [35]. The possible effects of inactivity are impaired learning and memory functions, dementia and neurodegeneration [36]. It is well-known that exercise increases mitochondrial biogenesis via the up-regulation of PGC-1α and Sirtuin pathways in various tissues.

Previous studies demonstrated that metabolic stress occurring in the brain during exercise is similar to that known to stimulate mitochondrial biogenesis in muscle. Therefore, the effects of exercise training on PGC-1α have been examined in the brain as well, but the results are inconsistent. Lezi et al. could not detect any alteration of PGC-1α mRNA levels in young or old mice following exercise training. Contrarily, another group reported a considerable elevation of PGC-1α mRNA in different brain areas, but the training protocols were different [23].

From the perspective of Sirtuins it seems that in the brain there could be an elevation in the expression levels of SIRT1 and SIRT6, but the available data are controversial [15,16,23,40]. In this study, we investigated FL-PGC-1α, NT-PGC-1α, CNS-PGC-1α, and Ref-PGC-1α mRNA levels in two different training protocols. The 5-day-long training period did not cause alterations in PGC-1α transcripts in any brain regions. Contrarily, the 12-day-long training period induced changes in all isoforms of the PGC-system in the cerebellum. In the Sirtuin-system, the 5-day-long training also did not cause mRNA level alterations, but the long-term exercise resulted cerebellar elevation of SIRT3-M1 and SIRT3-M2 mRNA levels. These results suggest that very short-term exercise was unable to induce the PGC-1α and SIRT systems. Contrarily, the 12-day-long training period induced changes in the cerebellum, which seems to be consistent with our previously findings [37]. We hypothesize that the reason behind the prominent cerebellar

elevation of PGC-, and Sirtuin isoforms could be the increase in synaptic plasticity between Purkinje cells, which facilitates better motor coordination and more precise movement integration. Previous studies demonstrated that in the cerebellum of *Pgc* knockout mice there is a decrease in cell number and firing rate between Purkinje cells [18,38].

In conclusion we suggest that all PGC isoforms and SIRT3-M1,-M2 (i.e., the mitochondrial Sirtuins, except SIRT3-M3, which seems does not play important role in the cerebellum) take part in mitochondrial energy production, enhancing synaptic functioning. However, additional studies are needed to better understand the interaction between mitochondria and each PGC- and Sirtuin isoform in the cerebellum.

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Conflict of interest

The authors declare that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neulet.2019.01.003.

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Research report

Effect of MPTP on mRNA expression of PGC-1α in mouse brain

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ABSTRACT

The peroxisome proliferator-activated receptor-γ (PPARγ) coactivator 1α (PGC-1α) is a key regulator of mitochondrial biogenesis, respiration and adaptive thermogenesis. Besides the full-length protein (FL-PGC-1α), several other functionally active PGC-1α isoforms were identified as a result of alternative splicing (e.g., N-truncated PGC-1α; NT-PGC-1α) or alternative promoter usage (e.g., central nervous system-specific PGC-1α isoforms; CNS-PGC-1α). Achieving neuroprotection via CNS-targeted pharmacological stimulation is limited due to poor penetration of the blood brain barrier (BBB) by the proposed pharmaceutical agents, so preconditioning emerged as another option. The current study aimed to examine of how the expression levels of FL-, NT-, CNS- and reference PGC-1α isoforms change in different brain regions following various 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment regimens, including chronic low-dose treatment for preconditioning. Ninety minutes following the acute treatment regimen, the expression levels of FL-, NT- and CNS-PGC-1α isoforms increased significantly in the striatum, cortex and cerebellum. However, this elevation diminished 7 days following the last MPTP injection in the acute treatment regimen. The chronic low-dose administration of MPTP, which did not cause significant toxic effects in light of the relatively unaltered dopamine levels, did not result in any significant change of PGC-1α expression. The elevation of PGC-1α levels following acute treatment may demonstrate a short-term compensatory mechanism against mitochondrial damage induced by the complex I inhibitor MPTP. However, drug-induced preconditioning by chronic low-dose MPTP seems not to induce protective responses via the PGC-1α system.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons, and the presence of Lewy bodies in the substantia nigra (SN) pars compacta (Forno, 1996). Although the precise pathomechanism of PD is not fully understood, several molecular mechanisms of neuronal death were described in the pathogenesis, including mitochondrial dysfunction, energy deficit and oxidative stress (Bose and Beal, 2016). It is postulated that life-long cumulative low-dose exposure to mitochondrial toxins may contribute to the pathogenesis of certain neurodegenerative disorders (Harris and Blain, 2004). The delineation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced Parkinsonian symptoms yielded one of the first pieces of evidence that mitochondrial dysfunction is involved in PD pathogenesis (Forno et al., 1993). Accordingly, systemic MPTP administration has been widely used to study disease mechanisms in various *in vivo* animal studies (Javitch et al., 1985).

Besides environmental factors, several causative or susceptibility genes have been identified in PD, many of them having direct implications in mitochondrial dysfunction (Kalinderi et al., 2016). Peroxisome proliferator-activated receptor-gamma (PPARγ) coactivator-1 alpha (PGC-1α) is one of them, which may play a role in PD pathogenesis. PGC-1α is a multifunctional transcriptional coactivator of nuclear respiratory factors 1 and 2 (NRF-1, -2), estrogen-related receptors (ERRs) and PPARs amongst others, and hereby regulates mitochondrial function and biogenesis (Knutti and Kralli, 2001).

Analysis of human brain samples indicated that PD is associated with the increased methylation of PGC-1α promoter and the reduced expression of PGC-1α itself (Su et al., 2015) and its downstream-regulated genes in the SN of PD patients (Zheng et al., 2010). Furthermore, possible associations of PGC-1α poly-

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morphisms with PD risk, age of onset and longevity were described as well (Clark et al., 2011). Reduced expression of PGC-1α leads to enhanced α-synuclein oligomerization, too (Ebrahim et al., 2010), and accordingly, overexpression of PGC-1α produced neuroprotection against α-synuclein- and rotenone-induced neurotoxicity (Zheng et al., 2010).

Several PGC-1α isoforms were identified as a result of alternative splicing and alternative promoter usage (Martinez-Redondo et al., 2015). The proximal promoter of PGC-1α has been reported as an important key regulator in several neurodegenerative diseases, including PD (Su et al., 2015). With regard to alternative splicing, besides the full-length protein (FL-PGC-1α), the N-truncated PGC-1α (NT-PGC-1α) isoform was discovered, which is a shorter, but active isoform of PGC-1α (Zhang et al., 2009). Recent studies identified further different tissue-specific isoforms of PGC-1α, including central nervous system-specific isoforms (CNS-PGC-1α (Ruas et al., 2012; Soyal et al., 2012)). The novel CNS-specific isoforms originated from a new promoter located 587 kb upstream of exon 2 (Choi et al., 2013; Soyal et al., 2012). A recent study demonstrated that both PGC-1α reference gene and CNS-PGC-1α are downregulated in human PD brain and in experimental models with α-synuclein oligomerization, and that the pharmacological activation or genetic overexpression of PGC-1α reference gene reduced α-synuclein oligomerization and toxicity (Eschbach et al., 2015). In contrast, the loss of PGC-1α enhances the vulnerability of SN pars compacta dopaminergic neurons to α-synuclein toxicity (Ciron et al., 2015). These data suggest that PGC-1α downregulation and α-synuclein oligomerization form a vicious circle (Eschbach et al., 2015). Similarly to PD, certain mutations in amyotrophic lateral sclerosis inhibit the expression of CNS-specific isoforms, indicating this as a common finding in neurodegeneration (Bayer et al., 2017).

St-Pierre et al. described that PGC-1α-deficient mice are more sensitive to MPTP toxicity compared to the controls (St-Pierre et al., 2006). Interestingly, the sub-chronic administration of MPTP to mice resulted in the significant elevation of PGC-1α expression in the striatum after 24 h that was normalized following 72 h (Swanson et al., 2013). This may represent an adaptive mechanism to neurotoxicity. Accordingly, the protective effect of PGC-1α was demonstrated previously as well; pioglitazone- and resveratrol-induced activation of PGC-1α was protective against MPTP toxicity (Breidert et al., 2002; Dehmer et al., 2004). However, there is a seeming controversy with regard to the effect of genetically-induced overexpression of PGC-1α on MPTP neurotoxicity. On the one hand, the transgenic overexpression of PGC-1α was proven to be protective against MPTP (Mudo et al., 2012), on the other hand, the adenovirus vector-mediated overexpression of PGC-1α resulted in dopamine depletion in the SN (Ciron et al., 2012) and consequently enhanced susceptibility to MPTP (Clark et al., 2012). Clarification of this issue needs further studies.

Evidence suggests a beneficial role of PGC-1α stimulation in neurodegenerative disorders. However, CNS-targeted pharmacological stimulation is limited due to the poor penetration of the blood brain barrier (BBB) by the above-mentioned compounds, so preconditioning emerged as another option to achieve neuroprotection. It was previously demonstrated that the acute administration of the selective complex II inhibitor 3-nitropropionic acid (3-NP) increased the expression of both FL- and NT-PGC-1α isoforms in the striatum of C57Bl/6 mice (Torok et al., 2015). As the available data are limited with regard to the alteration of tissue-specific PGC-1α expression in the brain following MPTP administration, this study aimed to examine the expression levels of several PGC-1α isoforms in different brain regions following various MPTP treatment regimens. The hypothesis that low doses of MPTP may produce compensatory, protective alterations in the PGC-1α system was tested as well.

2. Results

2.1. Gene expression analysis

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 striatum (FL-PGC-1α: ctrl: 0.97 (0.92–1.04), MPTP: 1.47 (1.21–1.83), $p = 0.0048$; NT-PGC-1α: ctrl: 0.44 (0.40–0.49), MPTP: 0.70 (0.56–0.78), $p = 0.019$), cortex (FL-PGC-1α: ctrl: 0.96 (0.91–1.06), MPTP: 1.23 (1.15–1.43), $p = 0.009$; NT-PGC-1α: ctrl: 0.46 (0.43–0.48), MPTP: 0.69 (0.59–0.71), $p = 0.0012$) and cerebellum (FL-PGC-1α: ctrl: 1.50 (1.27–1.90), MPTP: 2.40 (2.07–2.76), $p = 0.013$; NT-PGC-1α: ctrl: 0.67 (0.48–0.86), MPTP: 1.21 (1.14–1.44), $p = 0.009$) (Fig. 1A, B). Furthermore, MPTP-induced increases in CNS-PGC-1α expression were also significantly larger in all investigated brain regions compared to the controls (striatum: ctrl: 1.03 (0.88–1.11), MPTP: 1.38 (1.34–1.78), $p = 0.0069$; cortex: ctrl: 0.91 (0.80–0.98), MPTP: 1.41 (1.24–1.42), $p = 0.0048$; cerebellum: ctrl: 1.51 (1.20–1.98), MPTP: 2.77 (2.34–3.17), $p = 0.019$) (Fig. 1C). However, there was not any difference between the Ref-PGC-1α levels in the striatum (ctrl: 0.11 (0.10–0.12), MPTP: 0.11 (0.95–0.12)), cortex (ctrl: 0.11 (0.11–0.12), MPTP: 0.09 (0.08–0.10)) and cerebellum (ctrl: 0.21 (0.20–0.29), MPTP: 0.28 (0.24–0.29)) of MPTP-treated and control mice (Fig. 1D).

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 (Fig. 2A–D).

Furthermore, the low-dose 12-day MPTP-treatment did not influence the expression levels of FL-PGC-1α, NT-PGC-1α, CNS-PGC-1α and Ref-PGC-1α in any brain region (Fig. 3A–D).

2.2. HPLC measurement

Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) values in the respective control groups of the 3 treatment regimens were compared to each other, and there were no significant differences. Therefore the values in these control groups were pooled for further comparisons with the MPTP-treated groups. MPTP administration caused significant reductions in striatal DA (ctrl: 8.08 ± 0.50, MPTP: 4.36 ± 0.92, $p = 0.0005$), DOPAC (ctrl: 2.57 ± 0.21, MPTP: 0.44 ± 0.08, $p = 3.78 * 10^{-8}$) and HVA (ctrl: 2.18 ± 0.12, MPTP 0.67 ± 0.11, $p = 5.12 * 10^{-10}$) levels compared to control values 90 min following its last administration in the acute treatment regimen (acute-1 day; Fig. 4). Moreover, a significant reduction in metabolite levels was also observed one week after the last injection in the acute treatment regimen (acute-7 days; Fig. 4) in the DA (ctrl: 8.08 ± 0.50, MPTP: 1.34 ± 0.43, $p = 4.86 * 10^{-8}$), DOPAC (ctrl: 2.57 ± 0.21, MPTP: 0.76 ± 0.15, $p = 7 * 10^{-6}$) and HVA (ctrl: 2.18 ± 0.12, MPTP: 0.81 ± 0.13, $p = 5.08 * 10^{-8}$) values in the striatum of the MPTP-treated mice compared to the control animals. However, chronic MPTP treatment resulted in significant reductions of only striatal HVA (ctrl: 2.18 ± 0.12, MPTP 1.40 ± 0.08, $p = 0.0005$) levels, striatal DA (ctrl: 8.08 ± 0.50, MPTP: 6.83 ± 0.48) and DOPAC (ctrl: 2.57 ± 0.21; MPTP 1.99 ± 0.23) levels were not decreased significantly (Fig. 4). Seven days following the acute treatment regimen DA levels significantly decreased compared to those data from samples obtained 90 min following the last MPTP injection in the acute treatment regimen ($p = 0.039$).

3. Discussion

PGC-1α is essential in normal mitochondrial function and its deficiency may contribute to neurodegeneration, while its stimula-

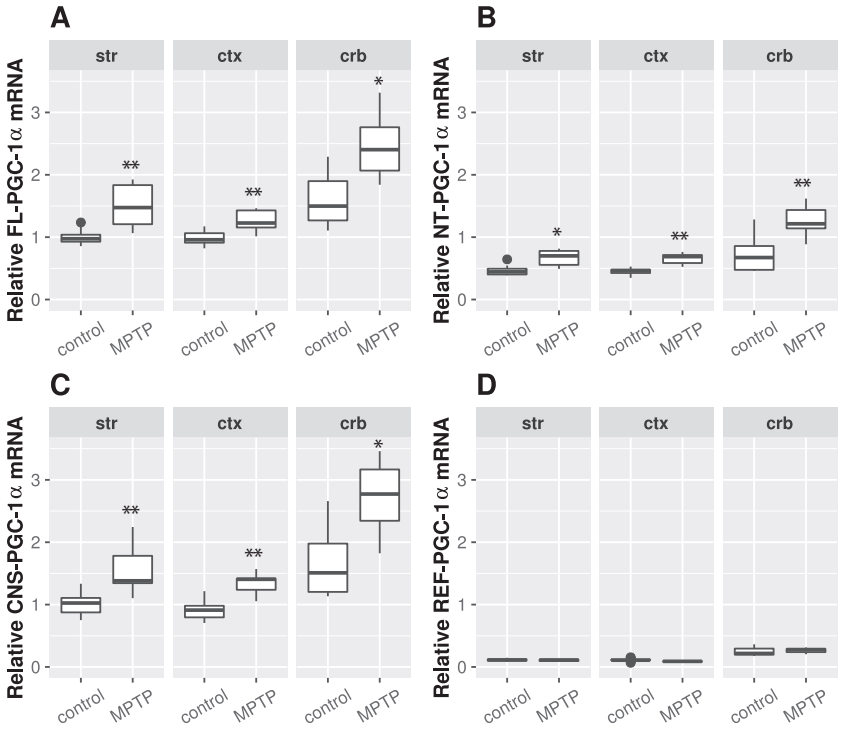

Fig. 1. The relative mRNA expression of PGC-1α isoforms in the striatum, cortex and the cerebellum of mice 90 min after acute MPTP intoxication. The FL-PGC-1α, NT-PGC-1α and CNS-PGC-1α levels were significantly increased in the striatum, cortex and the cerebellum of MPTP-treated mice. (A, B, C respectively). The Ref-PGC-1α expression did not change in any brain areas of MPTP-treated mice compared to the controls (D). Values are plotted as medians and interquartile range; $^{*}p < 0.05$, $^{**}p < 0.01$; *MPTP* MPTP-treated; *str* striatum, *ctx* cortex, *crb* cerebellum.

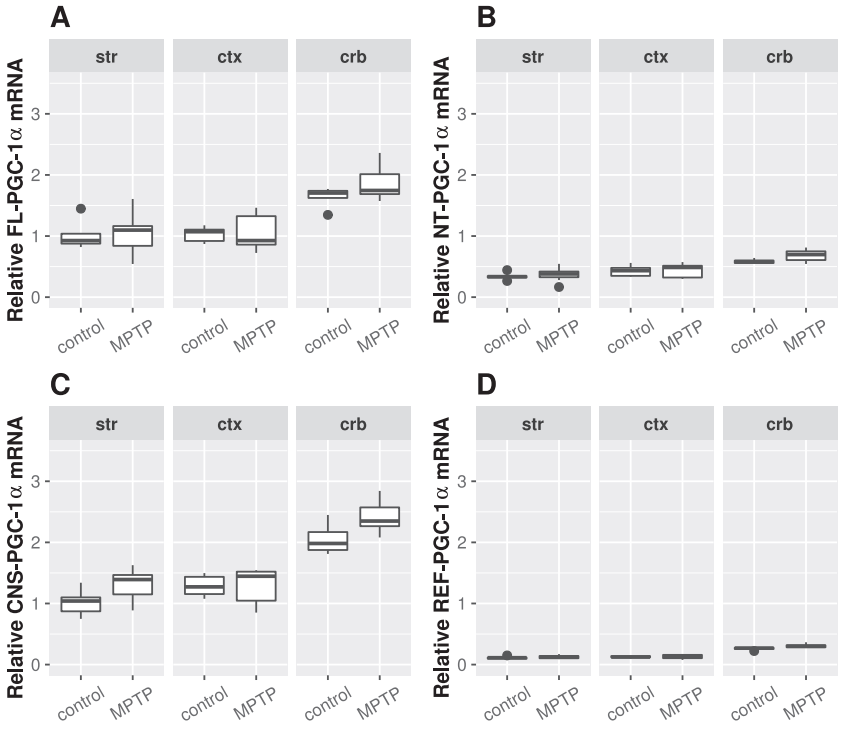

Fig. 2. The relative mRNA expression of PGC-1α isoforms in the striatum, cortex and the cerebellum of mice 7 days after acute MPTP intoxication. The expression levels of the PGC-1α isoforms did not change in any brain areas of MPTP-treated mice (A–D). Values are plotted as medians and interquartile range; *MPTP* MPTP-treated; *str* striatum, *ctx* cortex, *crb* cerebellum.

tion was demonstrated to be neuroprotective in certain models (Breidert et al., 2002; Dehmer et al., 2004; Eschbach et al., 2015; Mudo et al., 2012). Accordingly, the pharmacological induction of PGC-1α expression may be considered as a neuroprotective approach, but currently this possibility seems to be limited in light of the reduced BBB penetration of the potential pharmaceutical agents.

The aim of the current study was a thorough assessment of the expression of PGC-1α isoforms in various brain regions following different MPTP administration regimens, including a

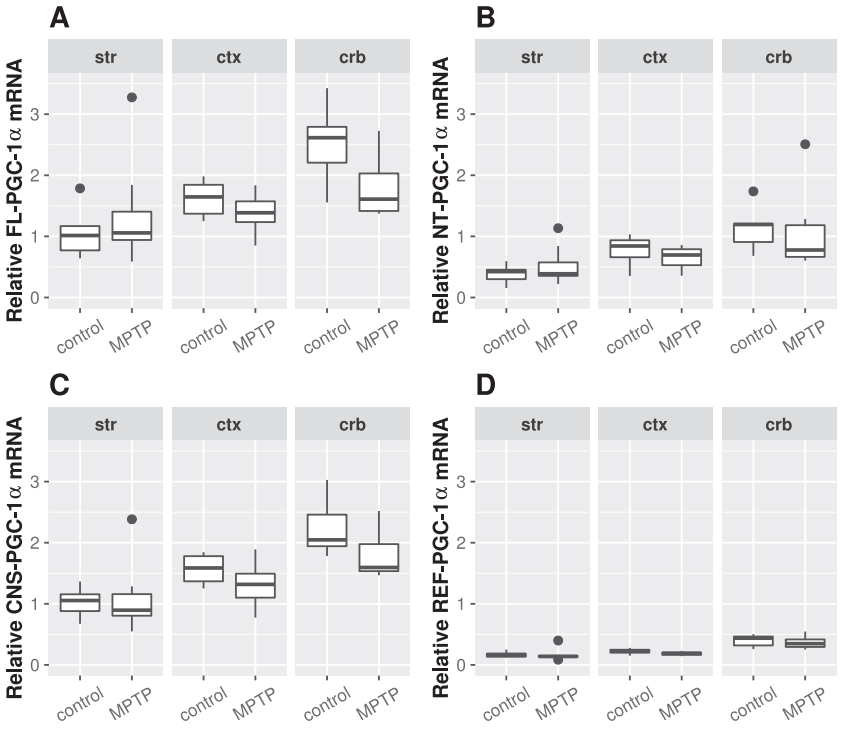

Fig. 3. The relative mRNA expression of PGC-1α isoforms in the striatum, cortex and the cerebellum of mice following a 12-day treatment with low-dose MPTP. The expression levels of the PGC-1α isoforms did not change in any brain areas of MPTP-treated mice (A–D). Values are plotted as medians and interquartile range; *MPTP* MPTP-treated; *str* striatum, *ctx* cortex, *crb* cerebellum.

low-dose chronic one, possibly mimicking drug-induced preconditioning.

Ninety minutes following the last MPTP injection of the high-dose acute treatment regimen of MPTP (75 mg/kg/day total dose) the expression level of FL-, NT- and CNS- PGC-1α isoforms increased significantly in the striatum, cortex and cerebellum. However, this elevation was diminished 7 days following the last MPTP injection in the acute treatment regimen. Torok et al. (Torok et al., 2015) demonstrated that the acute (90 min following a single dose injection of 100 mg/kg dose), but not the subacute (50 mg/kg twice daily for 5 days) 3-NP treatment regimen induced the overexpression of FL- and NT- PGC-1α isoforms mainly in the striatum (3-NP is a rather selective striatal neurotoxin (Brouillet et al., 2005)) similar to the results of the current study. Those findings were explained by a proposed reduced neuronal adaptive capability of the striatum following the neurotoxic insult. The above-mentioned results of the current study may also be explained by the propagation of the neurotoxic process following 7 days of the acute treatment regimen given the extent of decrease in striatal DA levels. The elevation of PGC-1α expression, especially that of the CNS-specific isoform, may indicate a short-term compensatory protective mechanism against mitochondrial dysfunction induced by the complex I inhibitor MPTP. It is hard to interpret the increased expression of PGC-1α in the cerebellum, which is not primarily affected in MPTP toxicity. However, several lines of evidence indicate that MPTP neurotoxicity is not highly selective to dopaminergic neurons; in specific circumstances systemic MPTP administration resulted in Purkinje cell loss (Takada et al., 1993). The involvement of the cerebellum in disease mechanisms of different neurodegenerative disorders such as amyotrophic lateral sclerosis, Huntington's disease (HD) and PD is frequently seen (Rees et al., 2014; Tan et al., 2016; Wu and Hallett, 2013). Furthermore, increasing evidence suggest that PGC-1α expression is associated with degenerative changes in the CNS, including cerebellum (Torok et al., 2015). It was hypothesized that the elevation of PGC-1α in the cerebellum is a compensatory mechanism against the energy deficit which may be an important factor underlying the relative resistance of cerebellar neurons against neurodegenerative processes in HD and in PD.

The drug-induced preconditioning by applying low-dose neurotoxic agents may stimulate neuroprotective mechanisms, resulting in the amelioration of neurodegenerative process. This approach has already been demonstrated to be beneficial in case of 3-NP: the low-dose of toxin treatment increased tolerance to ischemia and hypoxia in rats and gerbils (Horiguchi et al., 2003; Riepe et al., 1997; Wiegand et al., 1999). Although the exact mechanism is not fully understood, the overexpression of free radical scavenging enzymes may be involved: acute 3-NP treatment activated superoxide dismutase (SOD) and catalase (CAT) in several brain areas (Binienda et al., 1998). Similarly, an increase in SOD activity in the glial cells of the striatum and SN was observed following MPTP treatment (Kurosaki et al., 2004). The preconditioning by MPTP is not intended to suggest a future direct therapeutic approach, but rather aimed at finding key players which may help to alleviate the pathological alterations. The situation may be similar to ischemic preconditioning where the outcome in myocardial infarction may depend substantially on which medications were applied with an influence on preconditioning (Tomai et al., 1999). This may be especially important in light of the fact that environmental toxins could play a role in the pathogenies of idiopathic PD. The chronic low-dose administration of MPTP in the current study neither resulted in significant DA depletion (i.e. neurotoxic effect at biochemical level), nor in any significant change in PGC-1α expression. These data suggest that drug-induced preconditioning by MPTP may not evoke apparent responses in the PGC-1α system.

In conclusion the current study demonstrated that acute severe mitochondrial dysfunction initiated protection via elevating the expression of brain specific isoforms of the mitochondrial master regulator PGC-1α. However, low-dose chronic administration of

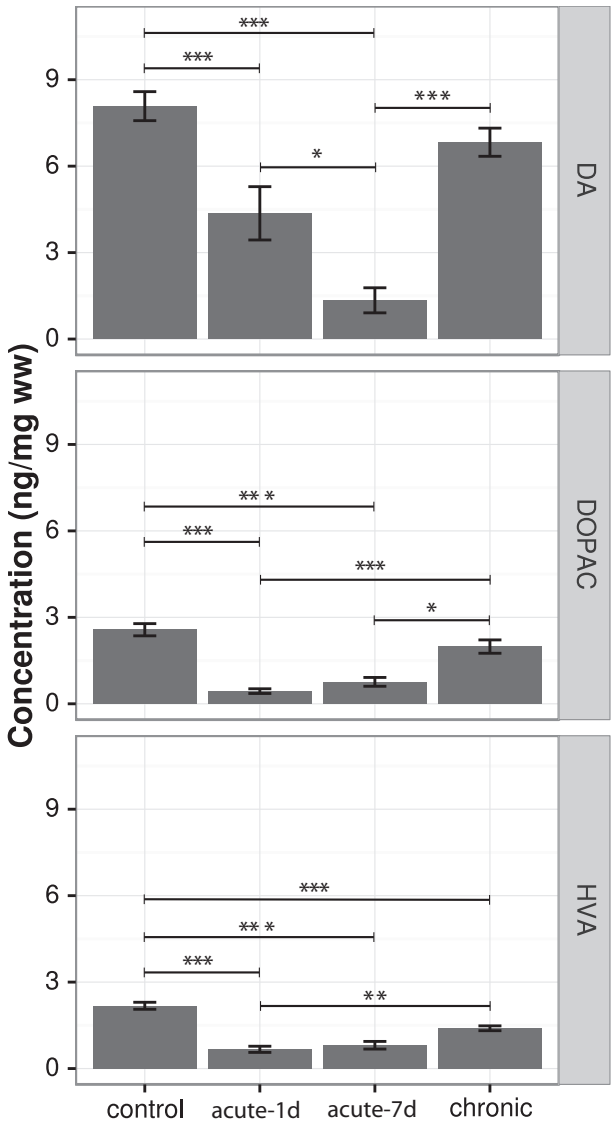

Fig. 4. Striatal dopamine, DOPAC and HVA concentrations of MPTP-treated mice in 3 different treatment regimens. Ninety minutes (acute-1d) and 7 days (acute-7d) after acute MPTP intoxication, DA, DOPAC and HVA levels significantly decreased in the striatum compared to the controls. The chronic (12 day) low-dose MPTP treatment did not influence the striatal level of DA and DOPAC, only HVA levels were significantly decreased. Values are plotted as means ± S.E.M; $^{***}p < 0.001$; *DA* dopamine, *DOPAC* 3,4-dihydroxyphenylacetic acid, *HVA* homovanillic acid.

mitochondrial toxin MPTP did not induce those protective mechanisms with the involvement of PGC-1α.

4. Experimental procedures

4.1. Animals

12-Week-old C57Bl/6J male mice were used in this study. The animal strain was originally obtained from Jackson Labs (Jackson Laboratories, Bar Harbor, ME, USA).

The animals were housed in cages and maintained under standard laboratory 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) and were approved by the local animal care committee.

4.2. Treatment and sample handling

MPTP was dissolved in phosphate-buffered saline (PBS; pH adjusted to 7.4) and was administered intraperitoneally (i.p.). Animals were randomly divided into six groups (n = 7–8 in each group). The first and second group received i.p. injection of 15 mg/kg body weight MPTP 5 times at 2 h intervals. The animals in the first group were deeply anesthetized with isoflurane (Forane; Abott Laboratories Hungary Ltd., Budapest, Hungary) 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 with isoflurane and the brains were dissected one week later (acute treatment – subacute (day 7) assessment). The mice in the third group were injected i.p. with 15 mg/kg body weight MPTP once a day for 12 days (low-dose chronic treatment). Ninety minutes following the last injection the animals were euthanized via isoflurane overdose as well. The fourth, fifth and sixth groups served as the respective control groups, and were injected with 0.1 M PBS according to the above-detailed treatment regimen. During the dissection process the brains were rapidly removed on ice and immediately halved at the midline. Following that, both hemispheres were further cut to obtain the striatum, cortex and cerebellum. Thereafter, these samples were stored at −80°C until the RT-PCR and HPLC analysis.

4.3. RT-PCR analysis

The left striatum, cortex and cerebellum were homogenized and Trizol reagent was used to extract RNA according to the manufacturer's protocol. The RNA was quantified spectrophotometrically, and the integrity of RNA was confirmed by gel electrophoresis using 1% agarose gel. 1 μg of total RNA was reverse-transcribed applying random hexamer primers and reverse transcriptase according to the RevertAid First Strand cDNA Synthesis Kit protocol (Thermo Fisher Scientific Inc., Marietta, OH, USA). cDNAs were kept at −20°C until further use.

Real-time PCR reactions were carried out in a 20 μl final volume.

The following, previously published primers were used: for FL-PGC-1α, 5′-TGCCATTGTTAAGACCGAG-3′ (forward) and 5′-TTGGG GTCATTTGGTGAC-3′ (reverse); for NT-PGC-1α, 5′-GGTCACTGGAA GATATGGC-3′ (reverse); for CNS-PGC-1α and Ref-PGC-1α, 5′-AAT TGGAGCCCCATGGATGAAGG-3′ and 5′-TGAGTCTGTATGGAGTGA CATCGAGTG-3′ (both forward), and 5′-TCAAATGAGGG CAATCCGTC-3′ (reverse), respectively (Chang et al., 2012; Soyal et al., 2012). qRT-PCR reaction conditions were 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, and 60°C for 30 s. Target gene expression was normalized to the endogenous control gene 18S rRNA (Applied Biosystems, Carlsbad, CA, USA). The relative expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

4.4. Dopamine measurement

DA and its metabolites, DOPAC and HVA were measured by reversed-phase chromatography from the right striatum of the MPTP-treated and the control animals, using an Agilent 1100 high-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, USA) combined with a Model 105 electrochemical detector (Precision Instruments, Marseille, France) under isocratic conditions. The striata were weighed and then homogenized in an ice-cold solution (750 μl) containing perchloric acid (70% wt/wt), sodium metabisulfite (0.1 M), disodium ethylenediaminetetraacetate (0.1 M), distilled water and 0.25 mM isoproterenol for 30 s. The homogenate was centrifuged at

12,000*g* for 10 min at 4°C. The supernatant was stored at −20°C until the analysis. The supernatants were measured with an Agilent 1100 high-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, USA) combined with a Model 105 electrochemical detector (Precision Instruments, Marseille, France) under isocratic conditions. In brief, the working potential of the detector was set at +750 mV, using a glassy carbon electrode and a Ag/AgCl reference electrode. The mobile phase containing sodium dihydrogenphosphate (75 mM), sodium octylsulfate (2.8 mM) and disodium ethylenediaminetetraacetate (50 μM) was supplemented with acetonitrile (10% v/v) and the pH was adjusted to 3 with phosphoric acid (85% w/w). The mobile phase was delivered at a rate of 1 ml/min at 40°C onto the reversed-phase column (HR-80 C18, 80 × 4.6 mm, 3 μm particle size; ESA Biosciences, Chelmsford, MA, USA) after passage through a pre-column (SecurityGuard, 4 × 3.0 mm I.D., 5 μm particle size, Phenomenex Inc., Torrance, CA, USA)). 10 μl aliquots were injected by the autosampler with the cooling module set at 4°C. With regard to method validation, the following parameters are reported briefly. The LOD and LLOQ for the investigated compounds in the brain samples were 2 ng/ml and 10 ng/ml, respectively. With regard to precision, the relative standard deviation was ⩽ 3.25% for the peak area responses and ⩽ 0.05% for the retention times. The recoveries ranged from 109 to 110%, 108 to 109% and 99 to 102% for DA, DOPAC and HVA, respectively.

4.5. Statistics

All statistical analyses were performed with the use of the 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. In case of gene expression analysis, due to the necessity of a large number of comparisons of data, two-sample *t*-tests *via* Monte-Carlo permutation (with 10,000 random permutations) were applied for RT-PCR results. In case of HPLC analysis, all the data exhibited normal distribution and equal variances were assumed, and therefore ANOVA was used with Bonferroni post hoc comparison. The null hypothesis was rejected when the corrected p values were < 0.05, and in such cases the differences were considered significant. FL- and NT-PGC-1α levels of gene expression of all brain areas were calculated relative to the levels of FL-PGC-1α gene expression in the striatum, whereas the CNS- and Ref-PGC-1α expression levels of all brain areas were calculated relative to the level of CNS-PGC-1α expression in the striatum. Data with Gaussian or non-Gaussian distributions were plotted as means (± S.E.M.) or medians (and interquartile range), respectively.

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Conflict of interest

The authors declare there is no conflict of interest.

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