DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

27/2016

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Neuroprotective Effects of PGC-1α Activators in Dopaminergic Neurons

MINERVA FOUNDATION INSTITUTE FOR MEDICAL RESEARCH AND MEDICUM
DEPARTMENT OF BIOCHEMISTRY AND DEVELOPMENTAL BIOLOGY
FACULTY OF MEDICINE
DOCTORAL PROGRAMME IN BRAIN & MIND
UNIVERSITY OF HELSINKI

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University of Helsinki

&

Doctoral Programme in Brain & Mind

Neuroprotective effects of PGC-1α activators in dopaminergic neurons

Johanna Mäkelä

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Medicine, University of Helsinki, in Lecture Hall 3, Biomedicum Helsinki, Haartmanikatu 8, Helsinki, on May 20th, 2016 at 12 noon

Helsinki 2016

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ISBN: 978-951-51-2083-0 (paperback)
ISBN: 978-951-51-2084-7 (PDF, http://ethesis.helsinki.fi)
ISSN: 2342-3161 (paperback)
ISSN: 2342-317X (PDF)
Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam Universitatis Helsinkiensis
Hansaprint
Helsinki 2016

"Begin to be now what you will be hereafter"

-William James

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV):

I. Transgenic expression and activation of PGC-1α protect dopaminergic neurons in the MPTP mouse model of Parkinson's disease
Mudò G, **Mäkelä J***, Di Liberto V*, Tselykh TV, Olivieri M, Piepponen P, Eriksson O, Mälkiä A, Bonomo A, Kairisalo M, Aguirre JA, Korhonen L, Belluardo N, Lindholm D.
Cell Mol Life Sci. 2012 Apr;69(7):1153-65.

II. Involvement of estrogen receptors in the resveratrol-mediated increase in dopamine transporter in human dopaminergic neurons and in striatum of female mice
Di Liberto V, **Mäkelä J**, Korhonen L, Olivieri M, Tselykh T, Mälkiä A, Do Thi H, Belluardo N, Lindholm D, Mudò G.
Neuropharmacology. 2012 Feb;62(2):1011-8.

III. Fibroblast Growth Factor-21 enhances mitochondrial functions and increases the activity of PGC-1α in human dopaminergic neurons via Sirtuin 1
Mäkelä J, Tselykh TV, Maiorana F, Eriksson O, Do HT, Mudò G, Korhonen LT, Belluardo N, Lindholm D
SpringerPlus 2014 3:2.

IV. Peroxisome proliferator-activated receptor-γ (PPARγ) agonist GW1929 is neuroprotective and stimulates PGC-1α expression and phosphorylation of cAMP responsive element (CREB) in human dopaminergic neurons by activating PKA/CREB
Mäkelä J, Tselykh TV, Kukkonen JP, Eriksson O, Korhonen LT, Lindholm D
Neuropharmacology, 2016 Mar;102:266-75

*Authors contributed equally

Author's contribution to the publications:

I. Contributed to the design of the experiments, performed cell culture maintenance and genotyping of mice, performed immunoblotting and immunohistochemistry experiments and participated in analyzing data and writing the manuscript.

II. Contributed to the design of the experiments, performed the experimental work with cell cultures, and participated in analyzing the data and writing the manuscript.

III. Contributed to the design of the experiments, performed most of the experimental work, analyzed the data and participated in writing the manuscript

V. Contributed to the design of the experiments, performed most of the experimental work, analyzed the data and participated in writing the manuscript

ABBREVIATIONS

6-OHDA	6-hydroxydopamine
Acetyl-CoA	Acetyl-coenzyme A
AD	Alzheimer's disease
Akt	Protein Kinase B/Akt
ALP	Autophagy-lysosome pathway
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine monophosphate
AMPK	AMP activated protein kinase
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
cAMP	Cyclic AMP
CBP/p300	CREB binding protein
CRE	cAMP response element
CREB	cAMP response element-binding protein
DA	Dopamine
DHE	Dihydroethidium
DAT	Dopamine transporter
DOPAC	3,4-dihydroxyphenylacetic acid
ER	Estrogen receptor
E2	17β-estradiol
FAD	Flavin adenine dinucleotide, oxidized form
$FADH_2$	Flavin adenine dinucleotide, reduced form
FGF	Fibroblast growth factor
FGF21	Fibroblast growth factor 21
GPx	Glutathion peroxidase
GSH	Glutathione, reduced
GSSH	Glutathione, oxidized
GW1929	N-(2-Benzoylphenyl)-O-[2-(methyl-2-pyridinylamino)ethyl]-L-tyrosine hydrate
HD	Huntington's disease
HGB	Human globulin
IMM	Inner mitochondrial membrane
IMS	Mitochondrial intermembrane space
L-DOPA	levodopa, l-dihydroxyphenylalanine
MAO B	Monoamine oxidase B
MAPK	mitogen activated protein kinase
MEF2	Myocyte enhancer factor 2
MPP^+	1-methyl-4-phenyl pyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA	Mitochondrial DNA
NA	Nicotinic acid
NAD^+	Nicotinamine dinucleotide, oxidized form
NADH	Nicotinamine dinucleotide, reduced form
NAM	Nicotinamide
Nampt	Nicotinamide phosphoribosyl transferase
ND1	NADH dehydrogenase subunit 1
NR	Nicotinamide riboside

NRF1	Nuclear respiratory factor 1
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
PD	Parkinson´s disease
PGC-1α	Peroxisome proliferator activated receptor gamma co-activator 1 alpha
PINK 1	Phosphate and tensin homolog (PTEN)-induced putative kinase-1
PPARγ	Peroxisome proliferator-activated receptor gamma
Prx	Peroxiredoxin
ROS	reactive oxygen species
RSV	Resveratrol, 3,4',5-trihydroxystilbene
SIRT1	Sirtuin 1
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SOD	Superoxid dismutase
SRC-1	Steroid receptor coactivator-1
TCA	Tricarboxylic acid cycle
TFAM	Mitochondrial transcription factor A
TG	transgenic
Trx	Thioredoxin
TrxR	Thioredoxin reductase
TZD	Thiazolidinedione
UPS	Ubiquitin-proteasome system
VMAT-2	vesicular monaomine transporter-2
WT	Wild type
WAT	White adipose tissue

ABSTRACT

Neurodegenerative diseases are characterized by the progressive loss of structure and function of neurons, but the underlying mechanisms for this are largely unknown. Disturbed cell signaling and protein metabolism as well as mitochondrial dysfunctions are thought to be involved in several neurodegenerative diseases. Mitochondria are the major source of energy in the cell, and they also regulate cell death. In brain, neurons are highly dependent on oxidative energy metabolism. Mitochondrial dysfunctions cause oxidative stress with an excess production of reactive oxygen species (ROS). In neurodegenerative diseases such as Parkinson's disease (PD), ROS are thought to contribute to the loss of dopaminergic neurons in substantia nigra pars compacta (SNpc), which leads to dopamine depletion in striatum. Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is a transcriptional co-activator that regulates mitochondrial biogenesis, ROS defense and respiration. The lack of PGC-1α has been shown to increase the sensitivity of neurons to oxidative stress and brain injuries.

In this study we show that increasing the expression of PGC-1α protects against toxin-induced oxidative stress in dopaminergic neurons. We show that PGC-1α expression in dopaminergic neurons can be modulated by resveratrol (RSV), fibroblast growth factor 21 (FGF21) and peroxisome proliferator-activated receptor γ (PPARγ) that are known to regulate metabolism in other tissues. The increase in PGC-1α expression and activation was linked to metabolic changes mimicing low energy levels in the cell, and an increase in SIRT1, a metabolic regulator sensing changes in energy levels. PGC-1α activation was further associated with an increase in mitochondrial respiration and antioxidant levels suggesting that the neuroprotective effect of PGC-1α was due to an improved capacity to combat oxidative stress. These results show that regulation of metabolism by PGC-1α activators could be a useful tool to prevent neurodegeneration in PD.

In addition to modulating PGC-1α, RSV was also found to increase the expression of dopamine transporter (DAT) in dopaminergic neurons of female mice. The increase in the level of DAT increases the uptake of dopamine, further indicating that RSV has beneficial effects in dopaminergic neurons. By affecting DAT, RSV also contributes to maintaining functional neurons, as a decline in DAT has been associated with degeneration of dopaminergic neurons. This effect on DAT expression was mediated by estrogen receptors, indicating that the effect of RSV differs between genders that should be considered if RSV is used as therapy for patients with PD.

1. INTRODUCTION

Mitochondria are considered to be the powerhouse of cells by providing most of the energy required for cellular functions. A common feature in ageing and age-related diseases such as neurodegenerative diseases is the failure of maintaining bioenergetics homeostasis, and mitochondrial dysfunction is strongly linked to the pathogenesis of neurodegenerative diseases. Under normal conditions mitochondria produce reactive oxygen species (ROS) as a product of the respiratory chain. ROS function as signaling molecules in the cell, and the cell has antioxidant mechanisms to prevent an excess production of ROS. Mitochondrial dysfunction leads to an excess production of ROS giving rise to oxidative stress, a situation where the normal antioxidant defense system can not combat the excess ROS produced in the cell. This leads to a situation where ROS can react with cellular macromolecules such as DNA, lipids and proteins, and cause damage to these molecules.

Parkinson's disease (PD) is an adult-onset neurodegenerative disease with the main age of onset being 55 years. The disease is characterized by progressive loss of dopaminergic neurons from substantia nigra pars compacta (SNpc) and the projections in striatum. The onset of the disease is gradual and motor symptoms are developed when 50-60% of the dopaminergic neurons are degenerated.

In PD, oxidative stress is thought to contribute to the pathogenesis of the disease. Oxidized macromolecules have been found in brain in post-mortem studies of patients with PD, and as well as accumulation of metals in substantia nigra, contributing to the production of ROS. The neurotransmitter dopamine can also contribute to the production of ROS in neurons when it is not stored in vesicles highlighting the importance of proper uptake and storage of dopamine in the cells.

Mitochondrial dysfunction has been implicated to have an important role in the pathogenesis of PD. Several genes that are linked to PD are involved in regulating mitochondrial function and quality control, and animal models with toxins blocking complex I in the respiratory chain mimics the symptoms of PD. Therefore, it would be of importance to be able to regulate mitochondrial quality control and function in the attempt to prevent dopaminergic neuron degeneration.

Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is a major regulator of mitochondrial biogenesis, respration and antioxidant defense. The expression of PGC-1α has been shown to be down-regulated in patients with PD and animal studies have shown that PGC-1α knock out increases neuron sensitivity to oxidative stress, revealing PGC-1α as a potential therapeutic target for PD. It would be of importance to be able to regulate the expression of PGC-1α in the brain, since a slight increase in PGC-1α expression has been shown to be beneficial for neuronal survival, whereas a high overexpression has turned out to be harmful for neurons as shown in animals. Metabolic regulators known to affect PGC-1α in other tissues such as liver, adipose tissue and muscle might also have similar effects in the brain.

In this work, I have used compounds known to regulate metabolism in other tissues to study the possible neuroprotective effect in dopaminergic neurons. By treating cells with resveratrol (RSV), fibroblast growth factor 21 (FGF21) or the peroxisome proliferator-activated receptor γ (PPARγ) agonist GW1929 the expression and activation of PGC-1α was increased and the mitochondrial function was improved in dopaminergic neurons. RSV also increased the expression of dopamine transporter (DAT) which could improve the dopamine uptake, and thereby improve the function of dopaminergic neurons. These results reveal potential compounds that could improve the survival of dopaminergic neurons by helping to combat oxidative stress and maintaining the dopamine uptake in dopaminergic neurons.

2. REVIEW OF LITERATURE

2.1. Mitochondrial structure and biogenesis

2.1.1. Evolution of mitochondria

Mitochondria are membrane bound organelles that are found in all eukaryotic cells and they are essential for life by producing the energy required for maintaining cellular processes (Duchen 2004). According to the endosymbiont hypothesis that was described in 1970 by Lynn Margulis, mitochondria evolved from a bacterial progenitor via symbiosis with an eukaryotic host cell (Margulis 1970). To support this theory, mitochondria contain its own DNA (mtDNA) which is maternally inherited (Duchen 2004). Mitochondrial DNA content has been reduced throughout evolution by gene transfer to the nucleus that encodes the majority of the mitochondrial proteins (Nunnari & Suomalainen 2012). By analyzing mitochondrial gene sequences the origin of mitochondria has been suggested to be related to the α-division of the Proteobacteria (Yang et al. 1985).

The endosymbiotic model has two different theories for the origin of the mitochondria, the archezoan scenario where the host was a hypothetical amitochondrial eukaryote, an archezoan, and the symbiogenesis scenario where the uptake of an α-Proteobacterium by an archaeal cell led to the formation of the mitochondria. The question that has been raised is whether mitochondria originated after the eukaryotic cell arose as assumed in the archezoan scenario or if the mitochondria had its origin at the same time as the formation of the eukaryotic cell as assumed in the symbiogenesis theory (Gray 2012).

2.1.2. Mitochondrial structure and dynamics

Mitochondria are double membrane organelles where the outer mitochondrial membrane (OMM) surrounds the inner mitochondrial membrane (IMM). The space between the two membranes is referred to as the intermembrane space (IMS) and the space surrounded by the IMM is called the mitochondrial matrix. The mitochondrial structure is shown in figure 1. The IMM is folded into cristae where a variety of mitochondrial membrane-bound enzyme systems such as the respiratory chain can be found (Duchen 2004, McBride et al. 2006).

Mitochondria are comprised of over 1000 proteins and the composition varies between tissue-specific needs (Friedman & Nunnari 2014). In humans, mtDNA is a circle of 16.6 kb double stranded DNA encoding for 13 proteins that all are components of the respiratory chain. In addition, mtDNA encodes for 24 other genes, two rRNAs and 22 tRNAs, that are needed for the synthesis of the 13 proteins in the respiratory chain. This is only a fraction of the proteins needed for mitochondrial function, and the majority of proteins required to build the mitochondrion are encoded by the nucleus (Gray et al. 1999, Duchen 2004, Olsen et al. 2015). Most of the nuclear encoded proteins are synthesized at ribosomes and targeted to mitochondria (Endo & Yamano 2010). In the OMM, translocases of the outer membrane (TOM) are responsible for the import of proteins to the mitochondria. Only unfolded proteins can be imported, and TOM acts together with cytosolic chaperones to allow transport of proteins to the IMS (Hood et al. 2003, Endo & Yamano 2010). At the IMM, translocases of the inner membrane (TIM) facilitates the movement of proteins to the matrix. The translocation of proteins across IMM is also dependent on a source of energy and a membrane potential to function properly (Hood et al. 2003).

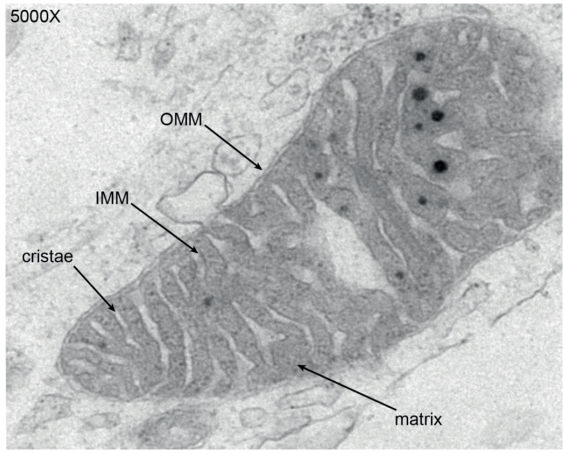

Figure 1. Mitochondrial structure. Electron microscope (EM) capture of mitochondria in dopaminergic neuron shows the different structures in mitochondria. Magnification 5000X. OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; cristae, folding of IMM; matrix, space surrounded by IMM.

Mitochondria are dynamic organelles that form networks that constantly undergo fission and fusion that affects the organelle size, number and shape (Itoh et al. 2013). The morphology of mitochondria varies between different cell types (Scorrano 2013). The changes in mitochondrial shape affect cellular functions such as Ca^{2+} signaling, generation of reactive oxygen species (ROS), neuronal plasticity and life span (Campello & Scorrano 2010, Scorrano 2013). Under normal conditions, mitochondrial fusion and fission are in balance. Fusion is needed for the distribution of different metabolites, proteins and mtDNA and the maintenance of electrical and biochemical connectivity. Fission is needed for cell division and mitochondrial quality control by elimination of damaged mitochondria (Kluge et al. 2013).

An increase in fusion and /or decrease in fission help to overcome low levels of stress such as starvation where fission is inhibited to protect the cell from mitophagy, whereas a decrease in fusion and/or increase in fission is observed during high levels of stress (van der Bliek et al. 2013). Loss of fusion can give rise to mitochondrial dysfunction, which might be caused by an impaired exchange of matricial material between mitochondria (Scorrano 2013). The fusion/fission machinery also affects mitochondrial shape which determines cellular fate during autophagy. Elongated mitochondria are protected from autophagic degradation, have more cristae and are capable of maintaining adenosine triphosphate (ATP) production and cell viability (Gomes et al. 2011). The fusion of the OMM is regulated by the two transmembrane GTPases mitofusin-1 (Mfn1) and mitofusin-2 (Mfn2) and the fusion of the IMM is regulated by optic atrophy protein 1 (Opa1). Mitochondrial fission on the other hand is mediated by dynamin-related protein 1 (Drp1) and fission 1 (Fis1) (Kluge et al. 2013).

Mitochondria are targeted for degradation by the recruitment of Parkin by phosphate and tensin homolog (PTEN) -induced putative kinase-1 (PINK1) to the mitochondria with low membrane potential to initiate the mitophagy of damaged mitochondria (Matsuda et al. 2010, Vives-Bauza & Przedborski 2011). PINK1 is rapidly degraded under steady-state conditions in a mitochondrial membrane potential-dependent manner, where loss of membrane potential stabilizes PINK1 mitochondrial accumulation (Matsuda et al. 2010). Mutations in PINK1 affect the translocation of Parkin to the mitochondria, leading to accumulation of damaged mitochondria in cells which may contribute to disease pathogenesis in mitochondria related diseases (Geisler et al. 2010).

PINK1 has also been shown to regulate mitochondrial dynamics, although the results are controversial. In rat dopaminergic neuronal cells, the overall effect of PINK1 overexpression promotes mitochondrial

fusion by reducing the levels of the fission proteins Drp1 and Fis1 and increasing the levels of the fusion protein Mfn2, whereas mutant PINK1 increases mitochondrial fission (Cui et al. 2010). Contrary to these findings, in rat hippocampal neurons the overexpression of PINK1 and Parkin results in increased mitochondria number and smaller mitochondrial size suggesting an increase in fission, whereas inactivation of PINK1 results in elongated mitochondria (Yu et al. 2011). This indicates that PINK1 and Parkin have a role in mitochondrial dynamics, but the effect varies between cell types.

2.1.3. Mitochondrial biogenesis

Mitochondrial biogenesis is a complex process that requires a coordinated transcription of both nuclear and mitochondrial genes (Hock & Kralli 2009). The mitochondrial content in a cell depends on the balance between mitochondrial biogenesis and mitophagy (Kluge et al. 2013). The biogenesis requires both replication and transcription of mtDNA as well as import of proteins and lipids to the existing mitochondrial reticulum (Puigserver & Spiegelman 2003, Hock & Kralli 2009, Kluge et al. 2013). The mitochondrial mass and function is regulated by nuclear-encoded factors in response to energy and growth demands (Scarpulla 2002). Some of the mitochondrial proteins are expressed in a tissue-specific manner, suggesting that some of the mitochondrial proteome is dedicated to specialized functions (Mootha et al. 2003).

The transcription of mitochondrial genes is under the regulation of a network of nuclear DNA-binding transcription factors that can be activated in response to physiological changes (Hock & Kralli 2009). Two transcription factors, nuclear respiratory factor 1 (NRF1) and nuclear respiratory factor 2 (NRF2) also called GA binding protein (GAPB) can bind to promoter regions of a wide variety of mitochondrial genes encoded by the nucleus, e.g. β-ATP synthase, cytochrome c, cytochrome c oxidase subunit IV, and mitochondrial transcription factor A (TFAM) among others (Evans & Scarpulla 1990, Virbasius et al. 1993). NRF1 activates the expression of components from the respiratory chain, mitochondrial ribosomal proteins, mitochondrial transporters and expression of mtDNA replication and expression (Scarpulla 2008). NRF2 also affects the expression of mitochondrial respiratory chain components and mtDNA replication (Scarpulla 2008).

TFAM is a nucleus-encoded transcription factor that translocates to the mitochondria and binds to the promoter region of mitochondrial genes (Scarpulla 2008). TFAM together with the mitochondrial transcription factor B (mtTFB) isoforms TFB1M and TFB2M regulate the transcription and replication of mtDNA thereby regulating the transcription of essential subunits of the respiratory chain (Gleyzer et al. 2005, Scarpulla 2008).

TFAM and NRF1 are known to be regulated by peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) (Wu et al. 1999). PGC-1α not only influences the transcription of NRF1, but it also coactivates its transcriptional activity (Wu et al. 1999, Puigserver & Spiegelman 2003). Activation of PGC-1α in response to changes in cellular energy levels and environmental changes increases the transcription of NRF1 and TFAM leading to increased mitochondrial biogenesis. This makes PGC-1α an important link between environmental or metabolic stimuli and mitochondrial biogenesis and respiration (Puigserver & Spiegelman 2003, Fernandez-Marcos & Auwerx 2011, Kluge et al. 2013).

Other nuclear transcription factors regulating mitochondrial protein expression are peroxisome proliferator activated receptors (PPAR) and estrogen related receptors (ERR). The PPARs affects mitochondrial uncoupling protein expression and genes involved in fatty acid oxidation, whereas the ERRs affects the transcription of genes that are involved in fatty acid oxidation, components of the respiratory chain and tricarboxylic acid cycle (TCA) cycle, mitochondrial dynamics and oxidative stress defense (Scarpulla 2002, Scarpulla 2008, Hock & Kralli 2009). Estrogen related receptor α (ERRα) has

also been suggested to be involved in the regulation of genes involved in the respiratory chain (Scarpulla 2011). Genes that are involved in the regulation of mitochondrial biogenesis are shown in figure 2.

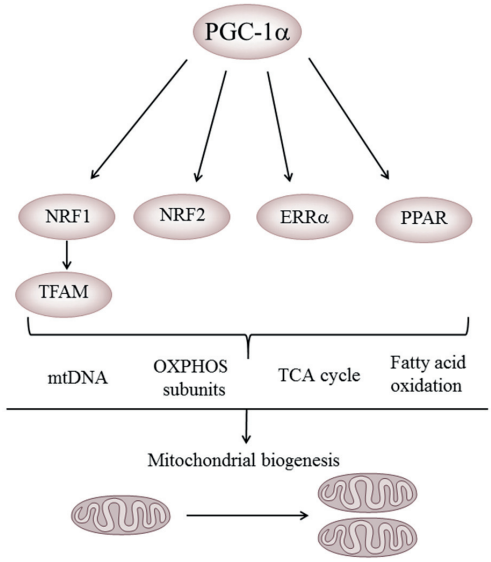

Figure 2. Genes involved in mitochondrial biogenesis. The figure is modified from (Komen & Thorburn 2014). PGC-1α, Peroxisome proliferator-activated receptor γ coactivator-1α; NRF, Nuclear respiratory factor; ERRα, Estrogen related receptor α; PPAR, Peroxisome proliferator-activated receptor; TFAM, Mitochondrial transcription factor A; mtDNA, mitochondrial DNA; TCAcycle, tricarboxylic acid cycle.

2.1.4. Mitochondrial function

Mitochondria are essential for living organisms by providing the energy required for cellular processes by conversion of nutrients to adenosine triphosphate (ATP) (Duchen 2004). Mitochondria are also involved in other cellular functions such as apoptosis, regulation of calcium and iron homeostasis, synthesis of metabolites, β-oxidation of fatty acids and in heme and phospholipid synthesis (Westermann 2010, Osellame et al. 2012, Scorrano 2013).

In apoptosis, cytochrome c is released from the mitochondrial IMS and it binds to apoptotic protease activating factor 1 (Apaf-1) and activates procaspase 9. This in turn activates caspase 3 and initiates the apoptotic pathway in the cell (Duchen 2004). Mitochondria are involved in iron homeostasis by synthesizing heme and hosting the synthesis of iron-sulphur (Fe-S) clusters which are components of hemoglobin and also found in the respiratory chain (Stehling & Lill 2013). Mitochondria regulate calcium homeostasis by Ca^{2+} uptake from the cytoplasm. Mitochondrial Ca^{2+} can stimulate ATP production, trigger the opening of the mitochondrial permeability transition pore and activate the cell death cascade. In neurons, mitochondrial Ca^{2+} uptake is involved in the control of neurotransmitter release (Abeti & Abramov 2015). Mitochondria generate reactive oxygen species (ROS) as a byproduct from the respiratory chain. The production of ROS is essential for cell signaling, but an excess production is harmful for the cell by causing damage to cellular componens such as proteins and DNA (Duchen 2004).

Mitochondrial dysfunction is associated with a large amount of inherited disorders and it is also implicated to be involved in several common pathologies from neurodegenerative diseases to cancer, cardiomyopathies, metabolic syndrome and obesity (Ylikallio & Suomalainen 2012, Nunnari &

Suomalainen 2012, Scorrano 2013). The increase in ROS production by mitochondria during ageing has been suggested to be the driving force behind ageing (Lane et al. 2015).

2.2. Mitochondrial energy production

2.2.1. Mitochondria as energy sensors

Energy homeostasis requires a controlled regulation of energy intake, storage and expenditure (Canto & Auwerx 2009). Mitochondria act as key regulators of energy homeostasis in the cell, where an increase in the need for energy is met by an increase in mitochondrial mass and oxidative phosphorylation to generate ATP (Duchen 2004). The NAD^+/NADH ratio, AMP/ATP ratio and acetyl-coenzyme A (acetyl-CoA) levels signal mitochondrial activity in the cell, which is recognized by various transcription factors, hormones, nuclear receptors and kinases (Lettieri Barbato et al. 2012).

Key sensors of cellular energy status are AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1). AMPK is activated in response to an increase in the AMP/ATP ratio and acts by phosphorylating a variety of target genes and upregulating catabolic pathways such as gluconeogenesis, autophagy and oxidative phosphorylation and inhibiting anabolic pathways such as cell growth and differentiation (Nunnari & Suomalainen 2012). SIRT1 senses the NAD^+/NADH ratio, and in response to elevated NAD^+ levels regulates the mitochondrial mass and oxidative phosphorylation via PGC-1α (Ng et al. 2015). AMPK indirectly modulates the activity of SIRT1 by regulating the NAD^+/NADH ratio (Canto & Auwerx 2009, Canto et al. 2009). During high nutritional load, high levels of ATP and NADH switch the metabolic balance towards lipid and glycogen storage, downregulation of mitochondrial biogenesis and an increase in the glycolytic ATP production (Nunnari & Suomalainen 2012). The mitochondrial energy production is summarized in figure 3.

2.2.1. Tricarboxylic acid cycle and respiratory chain

Mitochondria produce energy by generating ATP in an oxygen-dependent manner. The mitochondria can use sugars, fat and proteins to produce ATP by degrading the products to acetyl-CoA via different enzymatic systems (Ryan & Hoogenraad 2007). Sugars undergo glycolysis in the cytosol generating pyruvate that can enter the mitochondria and be converted to acetyl-CoA, fats are converted to acetyl-CoA by β-oxidation in the mitochondria and proteins can be converted by various systems to pyruvate, acetyl-CoA or directly to citric acid cycle intermediates (Osellame et al. 2012).

In the citric acid cycle, also called tricarboxylic acid (TCA) cycle or Krebs cycle, acetyl-CoA is transferred to oxaloacetate forming a six-carbon molecule of citrate. In a series of enzymatic steps, citrate is oxidized back to oxaloacetate, generating two molecules of CO_2 and passing electrons to the cofactors nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). The oxaloacetate can be used for another round of the TCA cycle, and the generated electrons in the form of NADH and $FADH_2$ are transferred to the respiratory chain (Osellame et al. 2012). Intermediates from the TCA cycle can be converted to amino acids and the neurotransmitters glutamate and γ-aminobutyric acid (GABA) (Nunnari & Suomalainen 2012).

The respiratory chain, also called the electron transport chain, consists of 4 multisubunit protein complexes (complex I-IV) located in the IMM, and the two diffusible factors cytochrome c and coenzyme Q10 that function as electron shuttles within the IMS. Although the mtDNA encodes 13 subunits of the respiratory chain which are core constituents of the respiratory chain complexes I-IV,

the majority of the subunits of the respiratory chain are encoded by the nucleus (Osellame et al. 2012, Galluzzi et al. 2012, Friedman & Nunnari 2014, Komen & Thorburn 2014).

The respiratory chain pumps protons from the matrix to the IMS, creating an electrochemical gradient that is used to drive the ATP-synthase /complex V that drives the phosphorylation of adenosine diphosphate (ADP) to generate ATP (Galluzzi et al. 2012). NADH from the TCA cycle donates two electrons to complex I of the respiratory chain, also called NADH: ubiquinone oxidoreductase or NADH dehydrogenase, and NADH is oxidized to NAD^+. Complex I is a L-shaped protein consisting of 45 subunits with a hydrophobic domain embedded into the IMM and a hydrophilic arm towards the matrix which functions as the binding site for NADH (Efremov et al. 2010). The electrons are passed to coenzyme Q via a series of Fe-S clusters, and linked to the electron transfer from NADH, four protons are passed from the matrix to the IMS. Complex II, also called succinate: ubiquinone oxidoreductase or succinate dehydrogenase, is the enzyme that reduces FAD to $FADH_2$ in the TCA cycle and is also a part of the respiratory chain. Complex II is located in the IMM and contains FAD as the prosthetic group. The complex transfers electrons to coenzyme Q, but no protons are pumped in this complex to the IMS (Lancaster & Kroger 2000).

Coenzyme Q can freely diffuse in the IMM and electrons from coenzyme Q are transferred to cytochrome c reductase or to complex III that passes the electrons to cytochrome c. Two protons that are obtained from the oxidation of coenzyme Q are transferred to IMS and an additional two protons are translocated from the matrix to the IMS (Crofts 2004). At complex IV or cytochrome c oxidase, four electrons passed through the chain are donated by four molecules of cytochrome c to the enzyme's iron/copper containing active site and two molecules of H_2O are generated from one O_2 molecule. At this complex an additional four protons are pumped from the matrix to the IMS (Belevich et al. 2006, Vilhjalmsdottir et al. 2015).

The electrochemical gradient generated by proton pumping from the matrix to the IMS is used to drive complex V or ATP-synthase, which drives the phosphorylation of ADP to ATP. The enzyme consists of two domains, the F_0 domain that spans the IMM and the F_1 domain facing the matrix. Complex V acts as a rotary molecular motor, where protons flow down the electrochemical gradient through the F_0 domain causing the rotation of the F_1 domain where ADP and phosphate binds, inducing the formation of ATP and thereby completing the energy production by oxidative phosphorylation. Each turn of the rotor produces 3 molecules of ATP and it is estimated that 3 to 5 protons are required for each molecule of ATP. In addition to generating ATP, the electrochemical potential generated by the respiratory chain is used for other mitochondrial functions, such as buffering Ca^{2+} through uptake by a uniporter in the IMM and mitochondrial protein import (Osellame et al. 2012, Friedman & Nunnari 2014).

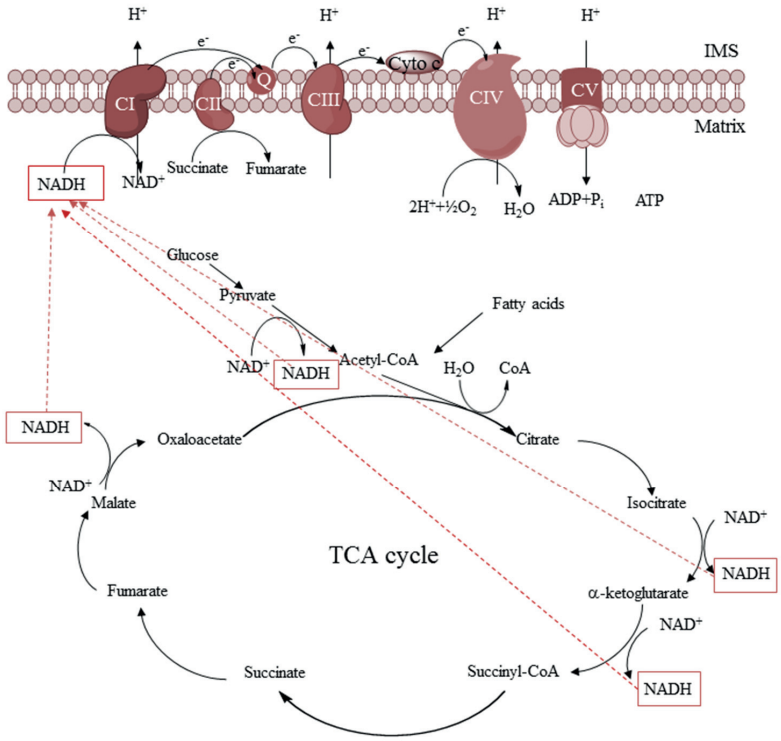

Figure 3. Mitochondrial energy production. The TCA cycle uses Acetyl-CoA from glucose and fatty acids to generate NADH by electron transfer to NAD^+ via a series of different enzymatic steps. NADH is converted to NAD^+ by complex I in the respiratory chain by electron transfer. In the respiratory chain, H^+ is pumped to the IMS creating a proton gradient that is used to drive Complex V and generate ATP from ADP and P_i. Figure modified from (Osellame et al. 2012). NADH Nicotinamine dinucleotide; Q, Coenxyme Q; Cyto c, cytochrome c;IMS, intermembrane space.

2.3. Free radical generation

Free radicals are defined as molecules containing one or more unpaired electrons in atomic or molecular orbitals, which usually causes the free radical to be highly reactive. Free radicals derived from oxygen are the most important class of radicals in living organisms (Valko et al. 2007). Molecular oxygen (O_2) itself is not a highly reactive molecule (Bartosz 2009), but it can form the highly reactive superoxide anion radical ($O_2^{\bullet-}$) by the addition of one electron. Free radicals containing oxygen, also called ROS, are products formed in normal cell metabolism. Low levels of ROS have beneficial effects in the cell and they are involved in cellular signaling pathways such as adaption to hypoxia, regulation of autophagy, immunity and ageing (Sena & Chandel 2012). An overproduction of ROS leads to oxidative stress that causes damage to cellular components such as DNA, proteins and lipids (Valko et al. 2007). The level of ROS is thought to increase during ageing (Payne & Chinnery 2015), and ROS has been suggested to contribute to diseases that are linked to mitochondrial dysfunction, among them neurodegenerative diseases such as Parkinson's disease (PD) (Nunnari & Suomalainen 2012).

Mitochondria are the main source of ROS production in the cell (Bartosz 2009). Seven sites in mammalian mitochondria are known to produce $O_2^{\bullet-}$ (Brand 2010), but the main sites where mitochondria generates $O_2^{\bullet-}$ are complex I and complex III in the respiratory chain, although there is an increasing amount of evidence that the main source of $O_2^{\bullet-}$ production is complex I, where the oxidation

of NADH provides electrons for the reduction of O_2 (McLennan & Degli Esposti 2000, Kussmaul & Hirst 2006, Hirst et al. 2008). The rate of $O_2^{\bullet-}$ generation is dependent on the concentration of O_2 and potential electron donors such as NADH (Kussmaul & Hirst 2006, Murphy 2009).

$O_2^{\bullet-}$ can be transformed into other free radicals via enzymatic reactions that convert $O_2^{\bullet-}$ into H_2O_2, by reacting with nitric oxide ($^{\bullet}NO$) to form peroxynitrite ($^{\bullet}ONOO^-$) or hydroxyl radicals ($^{\bullet}OH$) by the Fenton reaction in the presence of iron:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \qquad \text{(Fenton reaction)}$$

(Walling et al. 1975, Lipinski 2011, Kotiadis et al. 2014). Oxidative stress occurs at conditions where ROS are generated at a higher level than the defense system can neutralize ROS. These conditions occur when there is a disturbance in the equilibrium of prooxidant/antioxidant reactions, leading to damage on other cellular components that react with the excess ROS that is produced (Valko et al. 2007, Kotiadis et al. 2014). When mitochondria are respiring normally, the amount of $O_2^{\bullet-}$ formed from complex I is low, but inhibition of complex I by cellular damage, mutations, environmental toxins or by buildup of NADH levels by low ATP demand increases the production of $O_2^{\bullet-}$ (Lambert & Brand 2004, Murphy 2009).

2.4. Antioxidants

ROS production is a normal event in the cell, and cells have a mechanism for handling ROS produced during normal respiration. Antioxidants are molecules that prevent ROS from reacting with other cellular components by transferring electrons from ROS (Oyewole & Birch-Machin 2015). Antioxidants can be grouped into 2 major categories, endogenous and exogenous antioxidants, both being widely distributed in the body (Oyewole & Birch-Machin 2015). The endogenous antioxidants are synthesized in the body and consist of enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), peroxiredoxins (Prx) and catalase as well as nonenzymatic molecules such as α-tocopherol (vitamin E) glutathione and bilirubin (Dringen 2000, Engin 2009, Jansen & Daiber 2012), whereas exogenous antioxidants are obtained from the diet and include polyphenols, carotenoids, flavonoids and vitamins such as ascorbic acid (vitamin C) and retinoids (vitamin A) (Peng et al. 2014, Amara et al. 2015). The exogenous compounds are naturally occurring molecules that are capable of redox cycling (Amara et al. 2015). Polyphenols are the most abundant naturally occurring antioxidants in the diet, with the main sources being fruits, tea, coffee and red wine (Scalbert et al. 2005).

Antioxidant enzymes can eliminate ROS by converting ROS to less reactive molecules (Sena & Chandel 2012). In the cell, SOD converts $O_2^{\bullet-}$ to H_2O_2 (Kotiadis et al. 2014). There are three types of SOD in the cell; SOD1 (CuZnSOD) is located in the cytoplasm (Crapo et al. 1992), SOD2 (MnSOD) is in the mitochondrial matrix (Karnati et al. 2013) and SOD3 (ECSOD) is found in the extracellular matrix (Fattman et al. 2003). In mammalian cells, SOD2 is the essential $O_2^{\bullet-}$ detoxifying enzyme found in mitochondria (Candas & Li 2014), even though cytochrome c has also been shown to be able to detoxify $O_2^{\bullet-}$ (Korshunov et al. 1999, Andreyev et al. 2005). PGC-1α has been shown to have an important role in regulating the transcription of SOD2 (Valle et al. 2005, Lu et al. 2010).

The H_2O_2 that is generated by SOD can be further converted to H_2O by enzymes such as Prx, GPx and catalase (Andreyev et al. 2015). Catalase is one of the most efficient enzymes converting H_2O_2 to H_2O and O_2 and it is mainly found in the peroxisome (Kirkman & Gaetani 2007). The expression of catalase is low in the brain, and it may not be of importance in ROS defence, but in the liver catalase has an important role in ROS defense (Salvi et al. 2007).

Another mechanism to convert H_2O_2 to H_2O is by GPx (Sena & Chandel 2012). GPx converts H_2O_2 to H_2O by redox cycling where the reduced form accepts an electron from H_2O_2. The oxidized form of GPx then transfers an electron to reduced glutathione (GSH) to form the oxidized glutathione (GSSH) that can be converted back to its reduced form GSH by glutathione reductase (GR). GSH is an important antioxidant in the brain (Dringen 2000), and overexpression of GPx has been shown to protect neurons against oxidative stress (Dringen 2000, Wang et al. 2003, Smeyne & Smeyne 2013).

Prx are a family of thiol peroxidases that scavenge peroxides in the cell by converting H_2O_2 to H_2O by redox cycling (Cox et al. 2009). In mammals, Prx1, 2 and 6 are found in the cytoplasm, Prx4 in the endoplasmic reticulum, Prx5 in several different parts of the cell including peroxisomes and mitochondria, and Prx3 is located in the mitochondria (Cox et al. 2009). Prx3 has been suggested to be the major mitochondrial H_2O_2 scavenging enzyme (Cox et al. 2009) and it is estimated to be the target for up to 90% of the H_2O_2 produced in the mitochondrial matrix, whereas GPx1 contributes to the removal of approximately 9% of the H_2O_2 and other enzymes for 1% of the H_2O_2 produced (Andreyev et al. 2005, Cox et al. 2009).

The mitochondrial Prx activity is dependent on redox cycling of thioredoxin 2 (Trx2), where the oxidized form of Prx is reduced by Trx2 and the oxidized form of Trx2 is reduced by thioredoxin reductase 2 (TrxR2) in a NADPH- dependent manner (Arner & Holmgren 2000, Arner 2009). Different Trx isoforms are found in most organisms, and mitochondria have its own Trx system that works independently of the cytosolic system (Arner & Holmgren 2000). Trx2 and TrxR2 are found in the mitochondrial matrix in nearly all mammalian cells with a higher expression in cells with higher metabolic activity such as skeletal muscle, heart and brain (Spyrou et al. 1997, Rybnikova et al. 2000, Rohrbach et al. 2006). Trx2 has been shown to be under the transcriptional regulation of PGC-1α (Lu et al. 2010). Figure 4 summarizes the production of ROS and the reactions of the major antioxidant enzymes that occur in the cell.

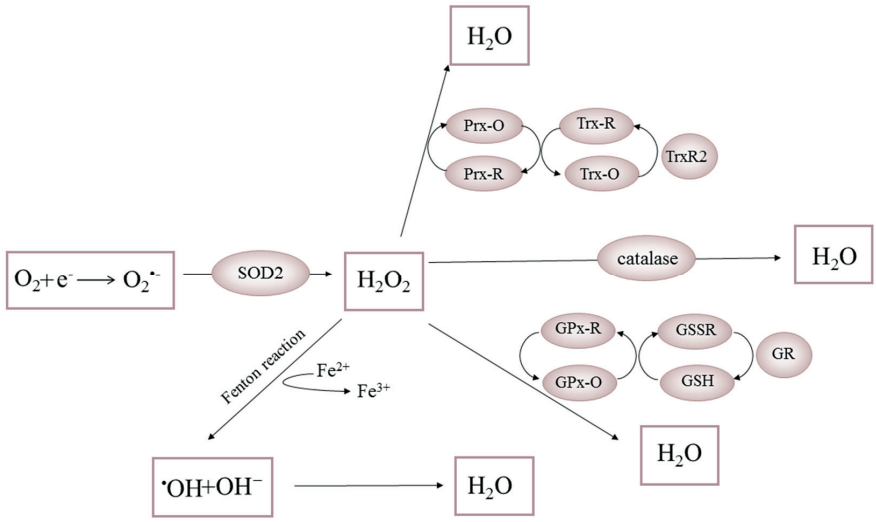

Figure 4. ROS formation and antioxidant enzymes. ROS can be produced when O_2 reacts with a free e^- to form $O_2^{\bullet-}$. SOD2 can convert $O_2^{\bullet-}$ to H_2O_2 that is further processed by redox reactions to generate H_2O. Figure modified from (Kotiadis et al. 2014). SOD2, superoxide dismutase2; Prx-O, Peroxiredoxin oxidized; Prx-R, Peroxiredoxin reduced; Trx-O, Thioredoxin oxidixed; Trx-R, thioredoxin reduced; TrxR2, thioredoxin reductase 2; GPx-O, glutathione peroxidase oxidized, GPx-R glutathione peroxidase reduced; GSSH glutathione oxidized; GSH, glutathione reduced; GR, glutathione reductase.

2.5. Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) expression and function

Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) is a key player in the regulation of energy metabolism in the cell, and it was first cloned from brown adipose tissue (BAT) as a cold-inducible coactivator of the nuclear receptors peroxisome proliferator-activated receptor-γ (PPARγ) and thyroid hormone receptor (Puigserver et al. 1998). PGC-1α is a member of the PGC-1 family together with Peroxisome proliferator-activated receptor-γ coactivator 1β (PGC-1β) and PGC-1-related coactivator (PRC). All of these family members share a high sequence homology in the N- and C-terminus with a transcriptional activation domain containing the hormone receptor interacting motif (LXXLL) in the N-terminus and a RNA-binding motif and serine-arginine rich domain in the C-terminus (Puigserver & Spiegelman 2003). The PGC-1α gene PPARGC1A is localized on chromosome 4p15.1-2 (Esterbauer et al. 1999).

The PGC-1α gene contains 13 exons and this gene encodes a protein of 798 amino acids with a molecular mass of 91 kDa (Esterbauer et al. 1999, Popov et al. 2015). The promoter of this canonical isoform of PGC-1α contains two transcription initiation sites 90 and 119 bp upstream of the transcription initiation codon ATG (Esterbauer et al. 1999). Alternative tissue-specific isoforms of PGC-1α have been shown to exist in muscle, liver and brain (Miura et al. 2008, Yoshioka et al. 2009, Felder et al. 2011, Soyal et al. 2012). In addition, a truncated form of PGC-1α (NT-PGC-1α) with a molecular weight of 35-38 kDa has been shown to exist in muscle, mouse brain and human heart (Zhang et al. 2009, Popov et al. 2015).

In addition to being a coactivator of PPARs and thyroid hormone receptors, PGC-1α has been found to directly co-activate other transcription factors, including glucocorticoid receptors, estrogen receptors (ER) and ERR and nonnuclear receptor transcription factors such as myocyte enhancer factor-2 (MEF2) and the family of forkhead O-box (FOXO) transcription factors (Canto & Auwerx 2009, Fernandez-Marcos & Auwerx 2011). A coactivator enhances the rate of transcription by interacting with transcription factors, but does not itself bind to DNA sequences. Coactivators usually exist as multiprotein complexes that contain proteins that mediate docking of transcription factors and others that mediate functions necessary for transcription (Puigserver & Spiegelman 2003). PGC-1α forms a complex with proteins that contain histone acetyl transferase activity, such as cyclic AMP (cAMP) response element (CRE) binding protein (CREB)-binding protein (CBP/p300) and steroid receptor coactivator-1 (SRC-1), as well as RNA binding polymerase II (Puigserver et al. 1999). This binding of CBP/p300 or SRC-1 to PGC-1α depends on the docking of transcription factors such as PPAR or NRF-1 to PGC-1α (Puigserver et al. 1999), suggesting that PGC-1α is activated when a transcription factor binds and induces a conformational change that can recruit SRC-1 and CBP/p300 to the complex (Puigserver & Spiegelman 2003).

PGC-1α has been found to be highly expressed in tissues with high energy requirements, such as BAT, heart, skeletal muscle, kidney, and brain, and is induced in conditions that require energy, such as cold, fasting and exercise (Puigserver et al. 1998, Esterbauer et al. 1999, Handschin & Spiegelman 2006, Canto & Auwerx 2009). Many genes that are linked to lipid oxidation and mitochondrial metabolism are under the control of PGC-1α, and PGC-1α increases mitochondrial biogenesis and respiration rates as well as the uptake and utilization of substrates for energy production (Canto & Auwerx 2009).

In adipose tissue, PGC-1α is involved both in the regulation of adaptive thermogenesis in BAT by inducing the expression of uncoupling protein-1 (UCP1) and in the adipocyte cell fate decision (Puigserver et al. 1998, Puigserver & Spiegelman 2003). In addition to BAT, skeletal muscle can also participate in cold-induced adaptive thermogenesis by mitochondrial uncoupling where the uncoupling is mediated by uncoupling protein-2 (UCP2) and uncoupling protein-3 (UCP3) (Wu et al. 1999, Wijers

et al. 2008, van den Berg et al. 2011). Figure 5 shows mitochondrial functions that are regulated by PGC-1α.

Enhanced mitochondrial biogenesis is essential for adaptive thermogenesis, and the core function of PGC-1α has been suggested to be regulation of mitochondrial biogenesis (Puigserver & Spiegelman 2003, Handschin & Spiegelman 2006). PGC-1α has been shown to strongly induce the expression of genes involved in mitochondrial biogenesis, such as NRF-1, NRF-2 and TFAM (Wu et al. 1999, Puigserver & Spiegelman 2003). In addition to mitochondrial biogenesis PGC-1α also regulates mitochondrial function by regulating oxidative phosphorylation by increasing ATP synthetase and the cytochrome c oxidase subunits COX II and COXIV (Puigserver et al. 1998, Wu et al. 1999). PGC-1α is also involved in the antioxidant defense by regulating the expression of antioxidant enzymes such as SOD2 and Trx2 (Valle et al. 2005, St-Pierre et al. 2006, Lu et al. 2010, Austin & St-Pierre 2012).

In the liver, PGC-1α is activated in response to glucagon during fasting (Fernandez-Marcos & Auwerx 2011) and it regulates hepatic gluconeogenesis by increasing the expression of enzymes involved in gluconeogenesis (Yoon et al. 2001). PGC-1α deficiency has been shown to cause defects in hepatic glucose production, although no alteration in PGC-1α-regulated genes involved in gluconeogenesis were observed (Burgess et al. 2006). PGC-1α is strongly linked to obesity and type 2 diabetes due to its ability to regulate cellular metabolism, and it has been shown to improve insulin sensitivity by increasing the expression of the insulin sensitive glucose transporter glucose transporter 4 (GLUT4) (Michael et al. 2001, Soyal et al. 2006).

In the brain, PGC-1α is expressed in several regions, including the olfactory bulb, cerebral cortex, hippocampus, striatum and substantia nigra (SN) (Tritos et al. 2003). PGC-1α has a role in neuronal development and function by affecting synaptic formation and maintenance (Cheng et al. 2012) as well as in neuroprotection by upregulating the expression of antioxidant enzymes (St-Pierre et al. 2006) and reducing inflammation (Nijland et al. 2014). PGC-1α has been implicated to have an important role in neurodegenerative diseases such as PD, Huntington's disease (HD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and in ischemic or excitotoxic insults (Cui et al. 2006, Qin et al. 2009, Zheng et al. 2010, Clark et al. 2011, Zhao et al. 2011, Puddifoot et al. 2012). In patients with PD, PGC-1α has been shown to be downregulated (Zheng et al. 2010, Eschbach et al. 2015) and a downregulation of PGC-1α has been shown to correlate with an increase in α-synuclein aggregation and a higher vulnerability of dopaminergic neurons to α-synuclein oligomerization in mice (Eschbach et al. 2015, Ciron et al. 2015). Also, brain specific isoforms of PGC-1α has been found in human brain, suggesting an important role of PGC-1α in neuronal function and neuroprotection (Soyal et al. 2012).

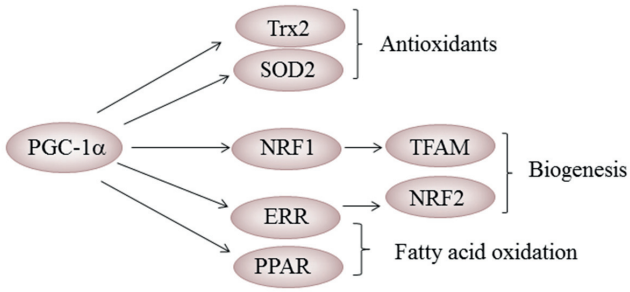

Figure 5. Mitochondrial functions regulated by PGC-1α. Figure modified form (Scarpulla 2011). PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; Trx2, thioredoxin 2; SOD2, superoxid dismutase 2; NRF, nuclear respiratory factor; ERR, estrogen related receptor; PPAR, peroxisome proliferator-activated receptor; TFAM, mitochondrial transcription factor A.

2.5.1. Regulation of PGC-1α gene expression

The transcription of PGC-1α can be regulated by a variety of mechanisms that vary between different tissues. The level of PGC-1α is tightly regulated and influenced by both environmental signals such as cold, fasting and physical activity, and cell-specific signals such as growth factors, hormonal signaling and cell energy levels (Houten & Auwerx 2004, Handschin & Spiegelman 2006, Fernandez-Marcos & Auwerx 2011, Lindholm et al. 2012). Figure 6 shows the regulation of PGC-1α gene expression.

These signals can activate different transcription factors that regulate the expression level of PGC-1α in the cell. At the PGC-1α promoter, the transcription factors CREB and activating transcription factor 2 (ATF2) binds to the CRE site, MEF2 binds to the MEF2 site and FOXO1 binds to the insulin response sequence (IRS), and all of these transcription factors enhance the transcription of PGC-1α (Puigserver & Spiegelman 2003, Fernandez-Marcos & Auwerx 2011).

In the muscle, PGC-1α expression is regulated by Ca^{2+}/calmodulin dependent protein kinase IV (CaMKIV) that activates CREB which binds to CRE, and p38 mitogen-activated protein kinase (p38 MAPK) that can activate MEF2 or ATF2. MEF2 can also be activated by calcineurin A (CnA) in muscle (Handschin et al. 2003, Fernandez-Marcos & Auwerx 2011). AMPK is also thought to increase the expression of PGC-1α in muscle, although the mechanisms are unclear. In addition, insulin signaling seems to be able to regulate PGC-1α expression in muscle (Fernandez-Marcos & Auwerx 2011). In BAT, an increase in cAMP by the sympathetic nervous system via β-adrenergic receptors activates protein kinase A (PKA) that in turn can activate CREB (Fernandez-Marcos & Auwerx 2011). In the liver, CREB is activated in response to glucagon that signals via cAMP (Herzig et al. 2001, Fernandez-Marcos & Auwerx 2011). In white adipose tissue (WAT), PGC-1α can also regulate its own transcription by coactivating the binding of PPARγ to its promotor region in PGC-1α (Hondares et al. 2006), and PGC-1α can bind to MEF2 to regulate the expression in an auto-regulatory loop (Handschin et al. 2003).

In the brain, the transcription of PGC-1α can be repressed by Parkin interacting substrate (PARIS), a substrate for the E3 ubiquitin ligase Parkin (Shin et al. 2011). Loss of Parkin function increases the accumulation of PARIS, leading to a downregulation of PGC-1α expression (Shin et al. 2011, Castillo-Quan 2011). Overexpression of PARIS leads to a decrease in mitochondrial biogenesis and a decline in the mitochondrial pool in the cell (Stevens et al. 2015).

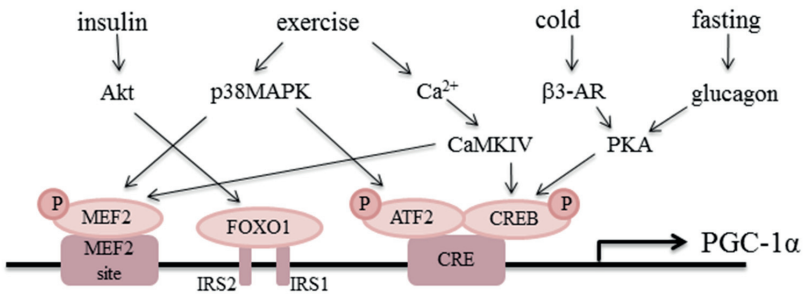

Figure 6. Regulation of PGC-1α transcription. Figure modified from (Fernandez-Marcos & Auwerx 2011). Akt, Protein Kinase B/Akt; p38MAPK, p38 mitogen-activated protein kinase; β3-AR, β-adrenergic receptor; CaMKIV, Ca^{2+}/calmodulin dependent protein kinase IV; PKA, protein kinase A; MEF2, myocyte enhancer factor 2; FOXO1, forkhead O-box; IRS, insulin response sequence ; ATF2, activating transcription factor 2; CREB, cyclic AMP response element binding protein; CRE, cyclic AMP response element; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α.

2.5.2. Regulation of Cyclic AMP response element-binding protein (CREB)

Cyclic AMP response element (CRE)-binding protein (CREB) is a transcription factor regulating the transcription of a wide variety of genes. It is one of the most widely expressed transcription factors and the first transcription factor that was shown to be regulated by phosphorylation (Mayr & Montminy 2001, Lonze & Ginty 2002). In brain, CREB is involved in the regulation of learning and memory, anxiety, neuronal plasticity, growth and survival, development of the nervous system and in neurodegeneration by regulating specific gene expression in response to external stimuli (Johannessen et al. 2004). CREB mediates the response to caloric restriction in neurons (Fusco et al. 2012) and PGC-1α expression has been shown to be regulated by CREB in patients with AD (Sheng et al. 2012). CREB has also been shown to be involved in the neuroprotection in the 6-hydroxydopamine model of PD (Chalovich et al. 2006, Ahmed et al. 2013).

CREB belongs to the CREB family transcription factors together with cAMP response element modulator (CREM) and activating transcription factor 1 (ATF1). There is a high degree of similarity between these transcription factors and they can all bind to the same cis-regulatory element CRE. In the brain, there are a wide variety of genes that contains CRE sequences, including growth factors and genes involved in neurotransmission, gene transcription and cellular metabolism (Mayr & Montminy 2001, Lonze & Ginty 2002). Recruitment of the co-activators CREB-binding protein (CBP) and p300 after phosphorylation of CREB at serine 133 strongly enhances CREB-dependent transcription (Johannessen et al. 2004). CREB can also be phosphorylated at other serine residues, and it has been shown to be phosphorylated at Ser98, Ser108, Ser111, Ser114, Ser117, Ser121, Ser129, Ser142, Ser143 and Ser156 having various effects on CREB activated transcription (Johannessen et al. 2004).

The activation of CREB in response to external stimuli can be mediated by the second messengers cAMP or Ca^{2+} (Lonze & Ginty 2002, Paramanik & Thakur 2013). CREB can also be activated by other pathways in response to growth factor signaling via receptor tyrosine kinase signaling or in response to stress, such as the p38MAPK pathway, the ERK pathway and PI3-kinase/Akt signaling (Lonze & Ginty 2002). An increase in intracellular Ca^{2+} causes Ca^{2+} to bind to calmodulin (CaM) that can further activate CaMKI, CaMKII and CaMKIV that all can phosphorylate CREB, although CaMKIV is the most important Ca^{2+}-activated CREB kinase (Lonze & Ginty 2002).

Several protein phosphatases regulate phosphorylation of CREB either by directly dephosphorylating CREB or by controlling the enzymatic activity of CREB kinases (Johannessen et al. 2004). During fasting, an increase in glucagon leads to an increase in cAMP and activation of CREB with an induction of the gluconeogenetic program via PGC-1α (Herzig et al. 2001).

The phosphorylation of CREB at Ser133 was originally shown to be mediated by activated PKA in response to increased cAMP levels in the cell (Gonzalez & Montminy 1989). cAMP levels rise in cells in response to adenylyl cyclase (AC) activity which is regulated by G protein coupled receptors (GPCR). A rise in cAMP levels causes a conformational change of PKA and activates PKA (Lonze & Ginty 2002, Kamenetsky et al. 2006, Steegborn 2014). AC is the enzyme that synthesizes cAMP from ATP in the cell (Kamenetsky et al. 2006). There are nine transmembrane isoforms of AC that are directly activated by G proteins to generate cAMP. In addition, cAMP can also be generated by the soluble AC that is regulated by bicarbonate and Ca^{2+} (Hanoune & Defer 2001, Kamenetsky et al. 2006). The expression of different isoforms of AC varies depending on the tissue, with all nine transmembrane isoforms found in the brain (Simonds 1999).

2.5.3. Regulation of PGC-1α activity

The level of PGC-1α is tightly regulated in the cells in response to changes in energy levels (Handschin & Spiegelman 2006). An increase in the expression of PGC-1α is associated with improvement in metabolic function and has been shown to have beneficial effects in tissues with high metabolic rate such as liver, muscle and brain (Handschin & Spiegelman 2006). Overexpression of PGC-1α in high quantities has been reported to have unfavorable effects in brain by decreasing cell viability in dopaminergic neurons (Ciron et al. 2012) and muscle by decreasing insulin sensitivity (Choi et al. 2008). This highlights the importance to tightly regulate the expression of PGC-1α to maintain its beneficial effects on metabolism (Lindholm et al. 2012).

The regulation of PGC-1α activity is mediated by its expression level but also by posttranslational modifications such as phosphorylation, acetylation, ubiquitination, methylation and GlcNAcylation. The posttranslational modifications affect the activity and stability of the protein or the interaction of PGC-1α with other proteins such as the repressor p160MBP (Canto & Auwerx 2009, Fernandez-Marcos & Auwerx 2011).

PGC-1α can be phosphorylated by AMPK, p38 MAPK and Akt at different sites (Fernandez-Marcos & Auwerx 2011). In addition to regulating the phosphorylation of PGC-1α, AMPK can also induce PGC-1α transcription (Jager et al. 2007). The phosphorylation by AMPK increases the activity of PGC-1α (Jager et al. 2007), whereas phosphorylation by Akt inhibits PGC-1α activity in response to insulin (Li et al. 2007). The phosphorylation by p38 MAPK increases PGC-1α activity by increasing protein stability and disrupting the interaction between PGC-1α and its repressor p160MBP in muscle (Puigserver et al. 2003). The activation of p38 MAPK has also been shown to increase the transcription of PGC-1α (Knutti et al. 2001).

Acetylation of PGC-1α also regulates the activity of the protein. The acyltransferase GCN5 can acetylate PGC-1α and inhibit its activity (Lerin et al. 2006), whereas the histone deacetylase SIRT1 deacetylates PGC-1α and increases its activity (Rodgers et al. 2005, Rodgers et al. 2008). Both GCN5 and SIRT1 act as energy sensors in the cell, and therefore the energy demands of the cell affect the activity of PGC-1α (Fernandez-Marcos & Auwerx 2011). GCN5 is induced by caloric excess (Coste et al. 2008) whereas SIRT1 is activated at low energy levels (Houtkooper et al. 2010). The regulation of PGC-1α acetylation is shown in figure 7.

Ubiquitination of PGC-1α by the E3 ubiquitin ligase Skp1/Cullin/F-box-cell division control 4 (SCF^{Cdc4}) leads to proteasomal degradation, thereby reducing the expression of the protein (Olson et al. 2008). The methylation of PGC-1α by the protein arginine methyltransferase 1 (PRMT1) on the other hand enhances PGC-1α transcription (Teyssier et al. 2005). PGC-1α has also been shown to be GlcNAcetylated by O-linked N-acetylglucosamine transferase (OGT), which leads to the stabilization of PGC-1α (Housley et al. 2009, Ruan et al. 2012).

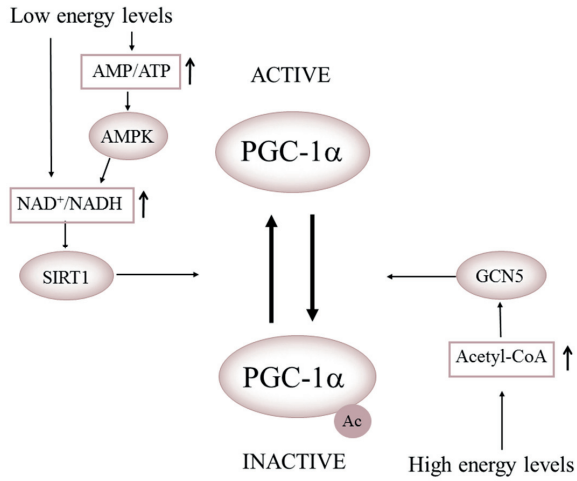

Figure 7. PGC-1α regulation by high and low energy levels. Figure modified from (Fernandez-Marcos & Auwerx 2011). AMPK, AMP activated protein kinase; SIRT1, Sirtuin1; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α.

2.5.4. Sirtuins

Mammalian Sirtuin family members are proteins with key roles in whole-body metabolic homeostasis and longevity (Canto & Auwerx 2012, Guarente 2013). The Sirtuin family consists of seven paralogs, SIRT1-SIRT7, that differ in their subcellular localizations as well as in their enzymatic activities (Canto & Auwerx 2012). The localization of the different SIRT paralogs differs in the cell. SIRT1 is usually nuclear, although it can be cytoplasmic in some cell types, SIRT2 is localized in the cytoplasm, SIRT3, SIRT4 and SIRT5 are mitochondrial proteins and SIRT6 and SIRT7 are nuclear (Alcain & Villalba 2009, Canto & Auwerx 2012). The function also differs between the different paralogs, SIRT1 and SIRT5 are deacetylases, SIRT4 is a mono-ADP-ribosyl transferase, SIRT2, SIRT3 and SIRT6 show both activities and SIRT7 is thought to act as a deacetylase (Canto & Auwerx 2012).

2.5.4.1. SIRT1

SIRT1 activity changes in response to changes in energy requirements and is increased in situations of energy or nutrient stress (Canto & Auwerx 2012). SIRT1 is the mammalian ortholog of the yeast silent information regulator 2 (Sir2) that was first discovered as a regulator of lifespan (Canto & Auwerx 2012), and it is a NAD^+ dependent class III histone deacetylase (Imai et al. 2000, Blander & Guarente 2004). SIRT1 affects mitochondrial biogenesis and function by regulating the activity of PGC-1α (Rodgers et al. 2005, Gerhart-Hines et al. 2007). SIRT1 has been implicated as a potential therapeutic target in neurodegenerative diseases because of its ability to sense metabolic changes in the cell (Outeiro et al. 2008, Ng et al. 2015).

SIRT1 is expressed in response to nutrient availability (Rodgers et al. 2005). The transcription of SIRT1 can be regulated by CREB (Noriega et al. 2011, Fusco et al. 2012) and p53 depending on nutrient availability (Kanfi et al. 2008). SIRT1 expression is induced by caloric restriction in different tissues including brain, but the changes in SIRT1 expression during caloric restriction are tissue specific (Cohen et al. 2004, Chen et al. 2008). SIRT1 can for example in response to caloric restriction activate fat mobilization by repressing genes controlled by PPARγ that mediate fat storage in the cell (Picard et al. 2004).

During caloric restriction, NADH levels are decreased yielding an increase in the NAD^+/NADH ratio, and changes in NAD^+ levels act as a metabolic sensor leading to an increase in SIRT1 activation (Lin et al. 2004, Houtkooper et al. 2010, Canto & Auwerx 2012). AMPK can also increase NAD^+ levels by enhancing fatty acid oxidation and thereby increase SIRT1 activity (Canto et al. 2009).

NAD^+ biosynthesis starts from the essential amino acid L-tryptophan but NAD^+ can also be synthesized from other precursors that are taken up from the diet, such as nicotinic acid (NA), nicotinamide (NAM) and nicotinamide ribosine (NR) (Houtkooper et al. 2010). The rate-limiting enzyme in the biosynthesis of NAD^+ is NAM phosphoribosyltransferase enzyme (Nampt) that catalyzes the conversion of NAM to NAM mononucleotide (NMN) (Revollo et al. 2004). The expression of Nampt is regulated by nutrient restriction and cell stress, and Nampt levels as well as NAD^+ levels are increased during fasting (Yang et al. 2007), providing NAD^+ for SIRT1 activity. At states of excess energy, NAM inhibits both Sir2 and SIRT1 *in vitro* (Bitterman et al. 2002).

SIRT1 can also be pharmacologically activated. RSV is the most potent naturally occurring SIRT1 activator but also other plant polyphenols that share structural similarity with RSV can activate SIRT1 (Alcain & Villalba 2009). Other small molecular compounds structurally unrelated to RSV has also been shown to activate SIRT1 with similar effects on mitochondrial function (Villalba & Alcain 2012).

2.6. Resveratrol

Resveratrol (3,4',5-trihydroxystilbene, RSV) is a naturally occurring compound with antioxidant and anti-inflammatory properties that can be found in the skin of grapes and in red wine (Baur & Sinclair 2006, Sun et al. 2010). RSV is thought to be able to prevent or slow down the progression of several different diseases, such as cancer (Jang et al. 1997), cardiovascular disease (Meng et al. 2014) and metabolic syndrome (Mendez-del Villar et al. 2014).

RSV has been shown to be able to cross the blood-brain barrier (Wang et al. 2002) and in brain RSV is thought to have a protective effect in several different neurodegenerative disorders such as ischemia, PD, AD and ALS (Sun et al. 2010). In the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) animal models of PD, RSV has been shown to have a protective effect against striatal dopaminergic neuron degeneration which might be mediated by the antioxidant capacity of RSV (Blanchet et al. 2008, Khan et al. 2010). RSV has also been reported to have neuroprotective effects in excitotoxicity, after brain injury and in ischemia (Baur & Sinclair 2006, Ates et al. 2007, Della-Morte et al. 2009).

The antioxidant effect of RSV is thought to be due to its properties as a scavenger of ROS (Leonard et al. 2003). RSV has also been shown to increase the expression of mitochondrial antioxidants and thereby reducing oxidative stress in cells (Fukui et al. 2010, Kairisalo et al. 2011).

The mechanism of action for RSV is still unclear, but RSV mimics caloric restriction in obese humans (Timmers et al. 2011). In line with this, RSV has been shown to activate SIRT1 (Howitz et al. 2003, Lagouge et al. 2006) and increase the activity of PGC-1α and its downstream genes such as NRF-1, TFAM, UCP3 and UCP1, thus increasing mitochondrial function (Lagouge et al. 2006).

The structure of RSV is related to the synthetic estrogen diethylstilbestrol, and RSV has been suggested to be an ER agonist (Gehm et al. 1997, de la Lastra & Villegas 2007) that can bind to both ERα and ERβ (Bowers et al. 2000). The neuroprotective effect of RSV in ischemia could be mediated by the activation of ER (Saleh et al. 2013).

2.7. Fibroblast growth factors

Fibroblast growth factors (FGF) are a family of growth factors that are essential for embryonic development and metabolic regulation. Human FGF family consisits of 22 members having intracrine, paracrine or endocrine functions depending on the FGF (Itoh & Ornitz 2011). Of the FGFs, FGF20 has been implicated to have a neuroprotective role in PD (Lindholm et al. 2015).

2.7.1. Fibroblast growth factor 21

Fibroblast growth factor 21 (FGF21) is a multifunctional metabolic regulator that belongs to the fibroblast growth factor 19 subfamily together with FGF19 and FGF23. It was first found to be expressed in the liver (Nishimura et al. 2000) and later reported to be expressed also in WAT (Muise et al. 2008), BAT (Hondares et al. 2011), muscle (Izumiya et al. 2008) and pancreas (Johnson et al. 2009). Lacking the heparin-binding domain that binds the protein to the extracellular matrix, FGF21 can function as an endocrine hormone entering the circulation (Goetz et al. 2007). In the liver, FGF21 expression is induced by PPARα during prolonged fasting (Galman et al. 2008) whereas in adipose tissue, FGF21 expression is induced by PPARγ (Muise et al. 2008). In the heart, FGF21 expression was recently shown to be under the influence of the SIRT1 pathway (Planavila et al. 2015).

FGFs signal through the FGF receptor tyrosine kinase family that has four members, FGFR1-FGFR4. In addition, there are splice variants for FGFR1, FGFR2 and FGFR3 encoded b and c, depending on the isoform (Kuro-o 2008). FGF21 signals through the FGFR1c, 2c or 3c but fails to directly act with the FGF receptors and requires the single transmembrane protein βKlotho as a cofactor for signaling (Kharitonenkov et al. 2005, Ogawa et al. 2007).

FGF21 has been shown to be involved in the regulation of blood glucose levels, uptake of glucose in adipocytes and regulation of body weight in rodents (Kharitonenkov et al. 2005, Badman et al. 2009, Chau et al. 2010). Overexpression of FGF21 has been shown to extend lifespan in mice (Zhang et al. 2012). In addition, FGF21 has been linked to increased thermogenic activity (Hondares et al. 2010) and it has been shown to increase the expression of mitochondrial genes and enhance mitochondrial function in adipocytes (Chau et al. 2010).

The increase in mitochondrial function in adipocytes is mediated by activation of the AMPK-SIRT1-PGC-1α pathway by increasing the concentration of NAD^+ (Chau et al. 2010). In the liver, FGF21 also require PGC-1α for lipid metabolism (Potthoff et al. 2009). FGF21 can phosphorylate CREB in adipose tissue, which contributes to an increase in PGC-1α expression (Wu et al. 2011), although there are also studies indicating that FGF21 does not increase the expression of PGC-1α but affects it via posttranslational modifications (Fisher et al. 2012). Elevated levels of FGF21 in serum have been suggested to be a biomarker of mitochondrial disease in humans (Suomalainen et al. 2011, Crooks et al. 2014)

FGF21 has been implicated to be a starvation hormone due to its effects on glucose and lipid metabolism (Canto & Auwerx 2012, Woo et al. 2013) but elevated levels of FGF21 are found in humans with metabolic syndrome (Zhang et al. 2008, Cuevas-Ramos et al. 2010), indicating that there is a possible FGF21 resistance (Fisher et al. 2010).

FGF21 has not been studied extensively in the brain. It is known to pass the blood-brain barrier by simple diffusion in physiologically relevant concentrations (Hsuchou et al. 2007, Liang et al. 2014), and FGF21 has a neuroprotective effect against excitotoxicity injury in primary neurons (Leng et al. 2015). It has also recently been shown to have a neuroprotective effect in hippocampal neurons in ageing mice by increasing the activity of antioxidant enzymes and thereby reducing ROS (Yu et al. 2015).

2.8. Peroxisome proliferator activated receptors (PPAR)

Peroxisome proliferator activated receptors (PPAR) are involved in the regulation of genes that regulate adipogenesis, lipid metabolism, maintenance of metabolism and inflammation. They belong to the nuclear receptor superfamily of ligand inducible transcription factors, and three PPARs are found in mammals, PPARα, PPARβ/δ and PPARγ. PPARs regulate transcription by binding to PPAR-responsive regulatory elements as heterodimers together with retinoid X receptors (RXR) (Ahmadian et al. 2013).

After ligand binding, the PPARs undergo conformational changes to allow the recruitment of co-activator proteins (Yki-Jarvinen 2004). PGC-1α is a co-activator that binds upon activation to PPAR to promote gene transcription (Puigserver et al. 1998). PPARs can regulate gene transcription either by transactivation that is DNA-dependent and involves binding to PPAR response elements of target genes or by transrepression that interferes with other transcription factor pathways in a DNA-independent manner (Yki-Jarvinen 2004).

The function and tissue distribution of the different PPARs differs *in vivo*. PPARα is expressed in the liver, heart and BAT where it functions as an activator of fatty acid oxidation pathways, whereas PPARβ/δ is expressed in many tissues and has a crucial role in key metabolic tissues such as the liver, skeletal muscle and heart where it affects the fatty acid oxidation, and PPARγ is highly expressed in adipose tissue where it regulates adipogenesis and functions as a modulator of whole-body lipid metabolism and insulin sensitivity (Abbott 2009, Ahmadian et al. 2013, Corona & Duchen 2015). Studies in knock out animals revealed PPARγ to be important for the formation of adipose tissue (Barak et al. 1999). In addition to adipose tissue, PPARγ is also expressed in the gastrointestinal tract, kidney, heart placenta, pancreas and brain (Dubois et al. 2000, Abbott 2009).

2.8.1. Peroxisome proliferator activated receptor γ (PPARγ)

Two different isoforms of PPARγ has been found, PPARγ1 and PPARγ2, which differ in size by 30 amino acids in the N-terminus (Tontonoz & Spiegelman 2008). PPARγ1 is expressed in a variety of tissues, but the expression of PPARγ2 is restricted to adipose tissue, although the expression of PPARγ2 can be induced in other tissue by a high fat diet (Vidal-Puig et al. 1996, Ahmadian et al. 2013).

Natural ligands for PPARγ are unsaturated fatty acids, eicosanoids, oxidized phospholipids and nitroalkenes. The prostaglandin 15-deoxy-delta-12,14-prostaglandin J_2 (15d-PGJ_2) is the most potent and the most commonly used naturally occurring ligand for PPARγ. Upon ligand binding, the conformational change allows co-activators such as PGC-1α to bind and induce the transcription of genes (Heneka & Landreth 2007, Chen et al. 2012, Corona & Duchen 2015).

The activity of PPARγ can be regulated by posttranslational modifications such as phosphorylation, SUMOylation, ubiquitination, GlcNAcylation or acetylation (Choi et al. 2014). Phosphorylation of PPARγ can either increase or decrease the activity depending on which site is phosphorylated and by which signaling mechanism is involved (Choi et al. 2014). SUMOylation decreases the activity of PPARγ and interestingly, FGF21 can decrease the SUMOylation of PPARγ and thereby increase the transcriptional activity of PPARγ (Dutchak et al. 2012). Acetylation decreases the activity of PPARγ and deacetylation of PPARγ by SIRT1 can promote browning of WAT (Qiang et al. 2012). GlcNAcylation is thought to decrease the transcription activity of PPARγ (Ji et al. 2012), and ubiqitination induces degradation of PPARγ (Hauser et al. 2000).

In brain, PPARγ has been found to be expressed in several different cell types such as neurons, astrocytes, microglia and oligodendrocytes (Bernardo et al. 2000, Moreno et al. 2004, Bernardo et al. 2009). By activating PPARγ with different ligands, some beneficial effects preventing neurodegeneration has been

observed. This has been thought to be due to the ability to reduce neuroinflammation (Schintu et al. 2009, Swanson et al. 2011) and some agonists has been shown to influence the expression of anti- and proinflammatory cytokines in microglia (Pisanu et al. 2014). To further support the neuroprotective effect of PPARγ in brain, treatment with PPARγ antagonists has been shown to cause neuronal loss, indicating the importance of PPARγ in neuronal survival (Martin et al. 2012)

In addition to the ability to reduce neuroinflammation, PPARγ agonists have also been shown to be protective in the MPTP mouse model of PD by increasing the levels of antioxidant enzymes and decreasing the amount of ROS (Martin et al. 2012) and by decreasing neuroinflammation (Carta et al. 2011). Other studies have also shown that by activating PPARγ, the mitochondrial function has improved in neuroblastoma cells (Corona et al. 2014, Chiang et al. 2014).

Thiazolidinediones (TZD) are PPARγ agonists that increase insulin sensitivity and reduce free fatty acids and triglycerides in the blood (Yki-Jarvinen 2004). TZD have been used in type 2 diabetes therapy because of their beneficial effects on blood glucose and lipid content (Yki-Jarvinen 2004), and TZD can also act by directly affecting mitochondria (Feinstein et al. 2005). Of the TZD, Troglitazone was the first to be developed, but it has been withdrawn from the market because of hepatotoxicity (Yki-Jarvinen 2004). Rosiglitazone and Pioglitazone were also developed for use as type 2 diabetes drugs, but Rosiglitazone has been withdrawn form market because of its adverse side effects and possible involvement in heart failure, leaving Pioglitazone as the only available drug, although it does also have potentially dangerous side effects (Consoli & Formoso 2013).

Of the TZDs, Pioglitazone has been shown to cross the blood-brain barrier (Chang et al. 2015), but there is a debate whether Rosiglitazone does cross the blood-brain barrier or not (Landreth et al. 2008). Both Rosiglitazone and Pioglitazone have been suggested to have a role in preventing neurodegeneration in AD, but the results for Rosiglitazone are controversial. In animal models of PD, treatment with TZD has been shown to have beneficial effects on neuroinflammation (Patrone et al. 2014).

In the attempt to reduce the negative effects of TZD, other PPARγ agonists have been developed, such as N-(2-Benzoylphenyl)-L-tyrosine linked PPARγ agonists, F12016, N-acetylfarnesylcysteine, T2384, LT175 and the antibiotic ionomycin, and they have been found to have similar effects on adipogenesis, insulin sensitivity and blood glucose level (Henke et al. 1998, Li et al. 2008, Bhalla et al. 2011, Zheng et al. 2013, Gilardi et al. 2014, Liu et al. 2015). The PPARγ agonist MDG548 has also been shown to be neuroprotective by reducing neuroinflammation and increasing ROS defense in the MPTP model (Lecca et al. 2015).

N-(2-Benzoylphenyl)-L-tyrosine linked PPARγ agonists were synthesized and tested for their ability to bind PPARγ in *in vitro* assays, and some structurally novel PPARγ agonists were identified. Of these compounds, N-(2-Benzoylphenyl)-O-[2-(methyl-2-pyridinylamino)ethyl]-L-tyrosine hydrate (GW1929) was found to be a potent selective PPARγ agonist (Henke et al. 1998).

GW1929 was shown to have the same effect on blood glucose levels, free fatty acid and triglyceride levels and glycosylated hemoglobin levels as well as the whole body insulin sensitivity than Troglitazone in Zucker diabetic fatty rats (Brown et al. 1999). GW1929 was found to have a higher potency and higher selectivity to bind to PPARγ when compared to the TZD Troglitazone, and when comparing the serum concentration of the drugs, GW1929 was more potent to reduce blood glucose levels than Troglitazone (Brown et al. 1999). Although L-tyrosine-based PPARγ ligands have a higher potency to bind and activate PPARγ, this does not translate into an improved antidiabetic efficacy compared with the TZD (Picard & Auwerx 2002).

In brain, GW1929 has been shown to have a protective effect in ischemia-reperfusion. This protective effect is thought to be because of its ability to reduce inflammation in the brain (Kaundal & Sharma

2011, Kaundal & Sharma 2011). GW1929 has also been shown to protect against apoptosis in primary neocortical cells (Wojtowicz et al. 2014).

2.9. Estrogen receptor

The steroid hormone estrogen mediates its function via the estrogen receptor α (ERα) and estrogen receptor β (ERβ) that are nuclear receptors involved in the regulation of many physiological processes in humans, such as cell growth, reproduction and development (Tcherepanova et al. 2000, Jia et al. 2015). 17β-estradiol (E2) is the most potent ER ligand produced in the body, but the E2 metabolites estrone and estriol have also been found to be weak agonists for ER (Heldring et al. 2007). In addition to being a ligand for ERα and ERβ, estrogen can also signal via the G-protein coupled estrogen receptor (GPER/GPR30) by non-genomic mechanisms (Kim et al. 2015). Abnormal function of the ERs can lead to the development of a variety of different diseases, including cancer, metabolic and cardiovascular diseses, neurodegeneration and inflammation (Jia et al. 2015).

ERα is mainly expressed in the uterus, ovary, breast, kidney, bone, WAT and liver whereas ERβ is found in the ovary, central nervous system, cardiovascular system, lung, male reproductive organs, prostate, colon, kidney and the immune system (Jia et al. 2015). In brain, both ERα and ERβ are expressed in adult rat with ERβ -containing cells being more widely spread throughout the brain than those expressing ERα, but the expression pattern varies between gender and species (Kalita et al. 2005, Heldring et al. 2007).

E2 is the main endogenous ligand for ER, but in addition to estrogens, environmental contaminants such as pesticides, xenoestrogens, polycyclic aromatic hydrocarbons and phthalates show affinity for the ER (Bolger et al. 1998). Also phytoestrogens such as RSV (Gehm et al. 1997, de la Lastra & Villegas 2007) that are found in plants have biologically relevant estrogenic action in humans (Heldring et al. 2007). ER signaling can be blocked with either pure antagonists or by blocking estrogen synthesis. Tamoxifen can act either as an ER agonist or antagonist depending on tissue (Krishnan et al. 2000, Jordan 2003), whereas ICI 182 780 (fulvestrant) is a pure ER antagonist that binds and inhibits ER and also promotes the degradation of the receptor (Van Den Bemd et al. 1999, Howell et al. 2000, Marsaud et al. 2003).

E2 has been shown to have beneficial effects in metabolic dysfunction and oxidative stress (Ahmed & Hassanein 2012, Mauvais-Jarvis et al. 2013, Paterni et al. 2014). E2 has also been implicated to affect mitochondrial biogenesis and decrease oxidative stress via GPR30 in cardiac muscle (Sbert-Roig et al. 2016). PGC-1α has been shown to be a coregulator of ER, further indicating the involvement of ER in metabolic regulation (Tcherepanova et al. 2000).

ER has been implicated to have a role in neurodegeneration. ER has been shown to be protective by mediating anti-inflammatory, antioxidant and neurotrophic effects (Lee et al. 2014). In AD, women have a higher incidence than men, and the decline in estrogen levels are thought to have a role in the disease, as the activation of ER is thought to protect agains amyloid β accumulation (Lee et al. 2014, Paterni et al. 2014). PD is more common in men than women, implicating a neuroprotective role of estrogen in the disease (Al Sweidi et al. 2012), and neuroprotective effects of E2 has been observed in the MPTP and 6-hydroxydopamine models of PD (Callier et al. 2002, Ramirez et al. 2003, Murray et al. 2003, Liu & Dluzen 2007). E2 has also been shown to enhance dopamine (DA) synthesis (Sarvari et al. 2014) and to modulate both DA receptors and dopamine transporter (DAT) (Morissette & Di Paolo 1993, Bosse et al. 1997, Landry et al. 2002). The changes in DAT and DA receptor density did not change the mRNA levels, suggesting a non-genomic activity of E2 (Le Saux et al. 2006, Al Sweidi et al. 2012). In line with

this, recent studies implicate that the neuroprotective effect of E2 could be mediated by GPR30 (Bessa et al. 2015).

2.10. Mitochondria in neurodegeneration

Neurons have a high energy requirement in order to function correctly (Lin & Beal 2006). Even though the brain in an adult human represents about 2% of the total body weight, it consumes about 20% of the oxygen and calories that are consumed by the body (Raichle & Gusnard 2002). Energy consumption of the brain depends on glucose and its complete oxidation in mitochondria via TCA cycle and electron transport chain (Albarracin et al. 2012). In neurons, mitochondria produce not only ATP to meet the energy requirements, but also TCA intermediates that serve as building blocks in the synthesis of the neurotransmitters glutamate and GABA (Lettieri Barbato et al. 2012). Mitochondria can also control neurotransmitter release by modulating the flux of Ca^{2+} (Nunnari & Suomalainen 2012, Abeti & Abramov 2015). The regulation of mitochondrial dynamics, biogenesis and mitophagy is needed to maintain mitochondrial function in the cell and are crucial for functional recovery of neurons after injury (Hagberg et al. 2014).

Mitochondrial dysfunction is implicated to have a role in the pathogenesis of several neurodegenerative diseases, but it is debated whether mitochondrial dysfunction and oxidative stress are involved in the onset of neurodegenerative diseases or if they are consequences of neurodegeneration (Mancuso et al. 2006). Mitochondrial dysfunction with failure to maintain energy levels and oxidative stress has been implicated to have a role in PD, AD, HD and multiple sclerosis (MS) as well as in ALS (McCoy & Cookson 2012, Guedes-Dias et al. 2015, Grimm et al. 2015, Haider 2015, Palomo & Manfredi 2015).

Mitochondrial dysfunction is strongly linked to PD where mutations in genes involved in mitochondrial function are linked to the pathogenesis of the disease. Patients have also been reported to have a decrease in complex I function in the respiratory chain and an increase in ROS production as well as an imbalance in Ca^{2+} homeostasis, an increase in mtDNA damage and impaired mitochondrial quality control (McCoy & Cookson 2012, Ryan et al. 2015).

In AD, oxidative stress is observed already at early stages of the disease. The production of Amyloid β plaques has been suggested to be enhanced by mitochondrial ROS production, and Amyloid β can induce oxidative stress, neuroinflammation and disturbed Ca^{2+} homeostasis which contributes to neuronal death. Amyloid β has also been suggested to disturb mitochondrial dynamics resulting in an increase in fragmented mitochondria (Grimm et al. 2015).

In HD, mutant Huntingtin (mHtt) causes excitotoxicity, synaptic dysfunction, defects in intracellular transport, autophagy and mitochondrial dysfunction. mHtt is suggested to have direct interactions with mitochondria giving rise to impaired Ca^{2+} homeostasis, disrupted trafficking and fragmented mitochondria and impaired mitophagy. mHtt is also thought to repress the transcription of PGC-1α, further linking mitochondrial dysfunction to the pathogenesis of the disease (Guedes-Dias et al. 2015).

In ALS, mutations in SOD1 are found in 20% of the familial cases, strongly linking changes in the antioxidant defence and ROS to the progression of the disease. Accumulation of abnormal mitochondria has been observed in patients with ALS, and mitochondrial quality control is thought to be involved in the pathogenesis of the disease. In ALS, the mitochondrial dysfunction contributes to defective bioenergetics as well as changes in Ca^{2+} homeostasis and altered mitochondrial morphology and impaired trafficking (Palomo & Manfredi 2015).

In patients with MS, oxidized DNA, lipid and protein molecules are found in the active MS lesions, and mitochondrial dysfunction is thought to contribute to the loss of myelin sheets by increasing ROS

production. Other sources of ROS are inflammation-induced production of ROS and free iron from myelin sheets. Also, a high ratio of mtDNA mutations are observed in patients with MS, further contributing to the loss of energy production and an increase in ROS in these patients (Haider 2015).

2.11. Parkinson's disease

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder after AD with a prevalence of about 1% in people over 60 years of age (de Lau & Breteler 2006). The disease was first described by James Parkinson in 1817 in *An Essay on the Shaking Palsy* where he described it as a movement disorder (Parkinson 1817). The disease got its name "Parkinson's disease" later by Jean-Martin Charcot (Goetz 2011).

PD is an adult-onset neurodegenerative disease with the mean age of onset being 55 years and the incidence increases with age from being 20/100 000 to 120/100 000 at age 70 (Dauer & Przedborski 2003). 95% of the cases are sporadic with a non-genetic cause and 5% are considered being familial or genetically inherited (Fitzgerald & Plun-Favreau 2008). The incidence is about 1.5 times higher in men than women (Twelves et al. 2003, Wooten et al. 2004).

The main neuropathological finding is the loss of dopaminergic neurons in substantia nigra pars compacta (SNpc) and the projections to striatum, together with the presence of insoluble protein inclusions called Lewy bodies (Dauer & Przedborski 2003, Wirdefeldt et al. 2011). The Lewy bodies are proposed to first affect the lower brain stem or olfactory nucleus and from there spread throughout the brain, which allows the pathology of the brain to be classified into different stages called Braak stages (Braak et al. 2003). The onset of the disease is gradual and the earliest symptoms might be unnoticed or misinterpreted for a long time (Lees et al. 2009). Clinical symptoms are resting tremors, rigidity, bradykinesia and postural problems, although the symptoms vary between patients (Kansara et al. 2013, Thenganatt & Jankovic 2014). The diagnosis of PD is usually clinical, and an autopsy is needed for confirmation of the disease, since no validated biomarkers are currently available (Miller & O'Callaghan 2015). At the time that symptoms develop approximately 50-60% of the dopaminergic neurons in SN are lost and about 80-85% of the dopamine content in striatum is depleted (Wirdefeldt et al. 2011). Non-motor symptoms that are suggested to be linked to PD include sleep disturbances, olfactory dysfunction, neurobehavioral disturbances, constipation and other autonomic dysfunction (Wu et al. 2011). Different subtypes of PD have been identified, including young-onset PD, late-onset PD, postural instability and gait difficulty PD and tremor-dominant PD (Thenganatt & Jankovic 2014).

The etiology of PD is complex, and it is thought to involve both genetic and environmental factors (Wirdefeldt et al. 2011). Ageing is a major risk factor for developing neurodegenerative diseases (Rodriguez et al. 2015), but other risk factors such as head trauma (Harris et al. 2013), toxicant exposure (de Lau & Breteler 2006), mitochondrial dysfunction (Schapira 2007, Schapira 2008), oxidative stress (Segura-Aguilar et al. 2014, Blesa et al. 2015), type 2 diabetes, (Hu et al. 2007, Cereda et al. 2011, Cereda et al. 2013), obesity (Hu et al. 2006) and accumulation of transition metals such as copper and iron in SN that cause oxidative damage (Di Monte 2003) are linked to PD. Neuroinflammation is also a possible cause of PD, with activated microglia contributing to the pathogenesis (Pisanu et al. 2014), and α-synuclein aggregation and dysfunction of protein degradation are other possible causes of PD (Spillantini et al. 1997, Malkus et al. 2009). Recent studies also suggest the involvement of the gut in PD development (Derkinderen et al. 2014, Holmqvist et al. 2014, Scheperjans et al. 2015). Neuroprotective factors that are linked to PD is lifelong high estrogen exposure (Lees et al. 2009, Gatto et al. 2014), cigarette smoking, caffeine (Di Monte 2003, Lees et al. 2009) and the use of nonsteroidal anti-inflammatory drugs (NSAID) (Chen et al. 2003). Several different genetic mutations have been

linked to PD, among them mutations in α-synuclein, PINK1, Parkin, Leucine-rich repeat kinase 2 (LRRK2), protein deglycase DJ1 (DJ1) and ATPase type 13A2 (ATP13A2) (Fitzgerald & Plun-Favreau 2008). Figure 8 shows a summary of potential causes of PD.

2.11.1. Parkinson's disease therapies

The currently available medical treatment targets motor symptoms. Standard therapies include the administration of the dopamine precursor levodopa (L-DOPA) together with a dopadecarboxylase inhibitor and a catechol-O-methyltransferase inhibitor to prevent degradation of DA (Obeso et al. 2010). L-DOPA is effective in reducing motor symptoms (Fahn & Parkinson Study Group 2005) but dyskinesia has been reported to be a side effect of the treatment, affecting the quality of life (Chapuis et al. 2005, Cenci 2014). Other therapies include administration of DA agonists, monoamine oxidase B (MAO B) inhibitors, anticholinergics and antiglutamatergics or deep brain stimulation (Obeso et al. 2010). The medical treatment of patients with PD usually starts when motor symptoms occur, but at this stage a significant amount of neurons has already been lost. Therefore it would be of importance to find biomarkers to be able to detect the disease and start the treatment earlier (Kansara et al. 2013). Clinical trials have shown that the quality of life can be improved with early diagnosis combined with exercise and appropriate therapy to treat motor symptoms as well as non-motor symptoms of PD (Jankovic & Poewe 2012).

Several different approaches has been used to try to develop new drugs for treatment of PD targeting mitochondrial dysfunction, endoplasmatic reticulum stress, protein aggregation and neuroinflammation as well as the use of neurotrophic factors and neuropeptides (Lindholm et al. 2015). Antidiabetic drugs have been shown to affect metabolism in the brain, as well as neuroinflammation and neuron regeneration (Patrone et al. 2014). Naturally occurring polyphenols such as RSV has neuroprotective effects by reducing oxidative stress (Sun et al. 2010), and neuropeptides such as pituarity adenylate cyclase-activating peptide (PACAP) has been shown to have neurotrophic effects and protect against neuroinflammation (Takei et al. 1998, Delgado et al. 2003, Makela et al. 2010).

Growth factors including FGF, paletelet-derived growth factor (PDGF) and vascular endothelial growth factors (VEGF) have also been implicated to have a protective role in dopaminergic neuron degeneration by promoting cell survival and neurogenesis as shown in animal models. These growth factors could potentially have a role in the treatment of PD because of their ability to influence cell survival in PD (Lindholm et al. 2015).

Neurotrophic factors have been studied as potential candidates for treatment of patients with PD because of their ability to slow down the progress of symptoms of PD in *in vivo* models and they also show both neuroprotective and neurorestorative properties (Voutilainen et al. 2015, Domanskyi et al. 2015). Of the neurotrophic factors, the glial cell-line derived neurotrophic factor (GDNF) and neurturin (NRTN) are the most studies ones and phase II clinical studies have been done, but GDNF did not show any clinical benefit and NRTN showed only modest benefit which may be due to the very limited diffusion of the proteins in the brain (Voutilainen et al. 2015). Cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) diffuse better in brain tissue than GDNF (Domanskyi et al. 2015) and they have been shown to be neuroprotective by regulating endoplasmic reticulum stress and the unfolded protein response inside the cell, and promote dopaminergic neuron survival by acting on plasma membrane receptors. They have also been shown to have neuroprotective effects in toxin induced animal models of PD (Lindholm & Saarma 2010).

2.11.2. Molecular mechanisms in Parkinson's disease pathology

A number of different intracellular pathways have been implicated to contribute to the pathogenesis of PD, but no single pathway has been able to explain the range of pathologies found in the brain of PD patients (Winslow & Rubinsztein 2011). Molecular mechanisms underlying the cause of PD include mitochondrial dysfunction, oxidative stress, Lewy body formation, endoplasmic reticulum stress, impaired protein degradation and neuroinflammation (Malkus et al. 2009, Lindholm et al. 2015). Many correlations have been established that oxidative stress is an underlying cause of PD, although conclusive proof for this theory is lacking (Malkus et al. 2009). This theory is however supported by the finding of an increased amount of oxidized proteins, lipids and DNA in post-mortem brain of PD patients compared to age-matched disease-free subjects (Jenner & Olanow 1996).

Oxidative reactions derived from the production of ROS can induce both the formation of protein inclusions and neurodegeneration, both being hallmarks of PD pathology (Malkus et al. 2009). An increase in the rate of ROS production together with a decline in the efficiency of antioxidants to remove ROS contribute to oxidation of cellular biomolecules such as proteins, lipids and DNA (Malkus et al. 2009). Protein degradation, lipid turnover and DNA repair serve as protective mechanisms to sustain cellular homeostasis by repairing or removing oxidized biomolecules (Malkus et al. 2009).

Protein degradation serves as a defense mechanism against accumulation of toxic proteins which can interfere with normal cellular function and viability (Betarbet et al. 2005). The ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway (ALP) are the two major pathways responsible for protein degradation in the cell (Rubinsztein 2006). Blocking of UPS and ALP pathways shows an increase in the accumulation of α-synuclein aggregates in cells, indicating that protein degradation may have an important role in PD pathology (Wang et al. 2015).

The UPS is the main pathway for short-lived protein degradation in the cytosol and nucleus and for misfolded proteins in the endoplasmic rerticulum that accumulate during endoplasmic reticulum stress (Ross & Pickart 2004, Rubinsztein 2006), and it is a pathway for detoxification of damaged proteins by targeting them for degradation in the proteasome (Betarbet et al. 2005). Autophagy is induced in the cell during conditions of stress, such as starvation (Rubinsztein 2006). Autophagy has an impact on several pathways that have been implicated to be involved in neurodegeneration, as it is a pathway of protein degradation as well as a way for disposal of dysfunctional mitochondria (Winslow et al. 2010). Dysfunction of the UPS contributes to protein aggregation and increased levels of ROS in the cell (Winslow & Rubinsztein 2011).

Neuroinflammation has been linked to the pathogenesis of PD with activated microglia in the SN contributing to the loss of dopaminergic neurons. Activated microglia contribute to neuroinflammation by secreting pro-inflammatory cytokines such as interleukin-1β, interferon-γ and tumor necrosis factor α (TNFα) (Ouchi et al. 2009). It has been suggested that aggregated α-synuclein contributes to the activation of microglia in dopaminergic neurons, further implicating the role of neuroinflammation in PD (Zhang et al. 2005). Microglia might therefore be a potential therapeutic target in PD which could be modulated by neuropeptides such as PACAP or neurotrophic factors (Lindholm et al. 2015).

2.11.3. Genes involved in Parkinson's disease

The finding of the first mutations responsible for PD showed that PD can be inherited (Polymeropoulos et al. 1996), and further studies showed that mutations in other genes also were linked to the disease, showing that PD is a genetically heterogeneous disease (Klein & Westenberger 2012). In addition to causing the inherited form of PD, genetic mutations also contribute to 3-5% of the sporadic cases of the disease (Klein & Westenberger 2012). The well validated genes linked to PD are listed in table 1.

Of the well validated genes linked to PD, mutations in Parkin, PINK1 and DJ-1 are responsible of the majority of autosomal recessive early-onset cases of PD (van der Merwe et al. 2015). Mutations in α-synuclein and LRRK2 are found in the autosomal dominantly inherited form of PD (Sundal et al. 2012).

Locus	Gene	Function	Disorder	Inheritance
PARK1, PARK4	SNCA	Involved in synaptic vesicle formation	Early onset PD	Autosomal dominant; sporadic
PARK2	parkin	E3ligase	Juvenile and early onset PD	Autosomal recessive; sporadic
PARK6	PINK1	Mitochondrial kinase	Early onset PD	Autosomal recessive
PARK7	DJ-1	Involved in oxidative stress response	Early onset PD	Autosomal recessive
PARK8	LRRK1	Protein kinase	Late onset PD	Autosomal dominant; sporadic
PARK9	ATP13A2	P-type transport ATPase	Kufor-Rakeb syndrome	Autosomal recessive

Table 1. Well validated genes involved in PD. Modified from (Lesage & Brice 2009, Schulte & Gasser 2011, Klein & Westenberger 2012, Lesage & Brice 2012). SNCA, α-synuclein; PINK1, PTEN induced putative kinase 1; DJ-1,Protein deglycase DJ-1; LRRK2, Leucine rich repeat kinase 2; ATP13A2, ATPase Type 13A2.

Mutations in Parkin are responsible of the majority of the juvenile cases of PD (Lesage & Brice 2012). Parkin is an E3 ubiquitin ligase and the identification of loss of function of this gene strongly implicates the involvement of the UPS in the pathogenesis of PD (Rubinsztein 2006, Malkus et al. 2009). Parkin also affects mitochondrial biogenesis by enhancing TFAM mediated transcription (Kuroda et al. 2006) as well as cellular energy metabolism (Periquet et al. 2005), and in Parkin-mutant cells the mitochondrial complex I activity has been shown to be reduced (Mortiboys et al. 2008).

Mutations in PINK1 are the second most common genetic cause of PD (Lesage & Brice 2012). PINK1 is a mitochondrial-associated protein that phosphorylates mitochondrial proteins in response to cellular stress and thereby protects against mitochondrial dysfunction and apoptosis (Valente et al. 2004). PINK1 also has a role in autophagy by enhancing the basal and starvation-induced autophagy (Michiorri et al. 2010). Interestingly, PINK1 and Parkin function in a common pathway where PINK1 phosphorylates Parkin that is recruited to mitochondria for mitophagy (Deas et al. 2011). This suggests that mitochondrial quality control is of importance in the pathology of PD (Vives-Bauza & Przedborski 2011). Also, DJ-1 affects the mitochondrial quality control by interacting with the PINK1/Parkin pathway in response to ROS (Joselin et al. 2012), and DJ-1 deficiency shows mitochondrial defects contributing to oxidative stress-induced cellular death (Irrcher et al. 2010).

Mutations in α-synuclein have been implicated as a cause of PD (Polymeropoulos et al. 1997) and α-synuclein has been shown to be the main component in Lewy bodies, the pathological hallmark for PD (Spillantini et al. 1997, Mezey et al. 1998). Mutations and multiplications of α-synuclein contribute to a dominantly inherited PD (Polymeropoulos et al. 1997, Singleton et al. 2003). α-synuclein can form oligomers, fibrils and large aggregates in response to overexpression, exposure to changes in pH, oxidative stress and by interacting with DA (Gallegos et al. 2015). Mitochondrial function has been shown to be disrupted by the interaction of α-synuclein with the mitochondria, giving rise to cytochrome c release, alterations in cellular Ca^{2+} levels, an increase in ROS and a decrease in mitochondrial membrane potential (Parihar et al. 2008, Parihar et al. 2009). α-synuclein has also been suggested to be a transcriptional modulator of PGC-1α, thereby affecting mitochondrial function (Siddiqui et al. 2012). The potential use of α-synuclein as a biomarker for PD has been implicated, and an increase in phosphorylated α-synuclein/ α-synuclein ratio in cerebrospinal fluid has been found to correlate with PD (Stewart et al. 2015).

Mutations in LRRK2 are the most common genetic cause of late-onset PD (Zimprich et al. 2004, Paisan-Ruiz et al. 2013). LRRK2 is a multifunction protein with kinase activity (Ryan et al. 2015), and

mutations in LRRK2 have been shown to affect immune system cells, autophagy, trafficking of vesicles, intracellular Ca^{2+} levels, as well as α-synuclein phosphorylation (Cherra et al. 2013, Orenstein et al. 2013, Esteves et al. 2014). LRRK2 also affects mitochondrial function by regulating fusion/fission and mitophagy (Ramonet et al. 2011, Cherra et al. 2013, Ryan et al. 2015).

Mutations in ATP13A2 have been suggested to be the cause of Kufor-Rakeb syndrome, a form of autosomal recessive parkinsonism (Ramirez et al. 2006). ATP13A2 is expressed mainly in the brain (Ramirez et al. 2006, Yang & Xu 2014) where it regulates lysosomal function (van Veen et al. 2014). Loss of function mutations have been shown to result in accumulation of α-synuclein in neurons (Usenovic et al. 2012), but also affecting the mitochondrial quality control, resulting in increased oxidative stress (Gusdon et al. 2012, Grunewald et al. 2012).

In addition to the well-verified genes linked to PD, there are also other putative genes that have been linked to PD (Lesage & Brice 2009, Klein & Westenberger 2012). Using genome wide association studies, additional genes have been found to be associated with PD (Lesage & Brice 2012). The putative genes linked to PD are listed in table 2.

Locus	Gene	Function	Disorder	Inheritance
PARK 3	Unknown	Unknown	Late onset PD	Autosomal dominant
PARK5	UCHL1	Ubiquitin-protein hydrolase	Late onset PD	Autosomal dominant
PARK10	Unknown	Unknown	Late onset PD	Unclear
PARK11	GIGYF2	Involved in cellular insulin and insulin-like growth factor response	Late onset PD	Autosomal dominant
PARK12	Unknown	Unknown	Late onset PD	Risk factor
PARK13	Omi/HTRA2	Mitochondrial-targeted serine protease	Late onset PD	Autosomal dominant or risk factor
PARK14	PLA2G6	A2 Phospholipase	Early onset dystonia-parkinsonism	Autosomal recessive
PARK15	FBXO7	Component of the E3 ubiquitin protein ligase complex	Early onset parkinsonian-pyramidal syndrome	Autosomal recessive
PARK16	Unknown	Unknown	Late onset PD	Risk factor
PARK17	VPS35	Component of the retrograde cargo-selective complex	Late onset PD	Autosomal dominant
PARK18	EIF4G1	Component of eIF4F complex involved in the recruitment of mRNA to the ribosome	Late onset PD	Autosomal dominant
	GBA	Beta-glucocerebrosidase	Early onset PD	Risk factor

Table 2. Putative genes linked to PD. Modified from (Lesage & Brice 2009, Schulte & Gasser 2011, Klein & Westenberger 2012, Lesage & Brice 2012). UCHL1, ubiquitin carboxyl-terminal esterase L1; GIGYF2, GBR10 interacting GYF protein 2; Omi/HTRA2, serine protease HTRA2, mitochondrial; PLA2G6, phospholipase A2, group VI; FBXO7, F–Box protein 7; VPS35, Vacuolar protein sorting-associated protein 35; EIF4G1, Eukaryotic translation initiation factor 4 gamma1; GBA, beta-glucocerebrosidase.

2.11.4. Role of mitochondria in Parkinson's disease

Mitochondria have a central role in age-related neurodegenerative diseases, and mitochondrial dysfunction is a common theme in these diseases. Many of the genes associated to the pathogenesis of PD are also implicated to have a role in the mitochondrial function (Lin & Beal 2006). An impaired energy metabolism and redox homeostasis are considered hallmarks of brain ageing, and therefore the mitochondrial energy-transducing capacity is important to maintain neuronal function (Yin et al. 2014). It is possible that the DA neurons that are lost in PD have specific energy requirements, which supports the involvement of mitochondria in the pathogenesis of PD (McCoy & Cookson 2012, Pissadaki & Bolam 2013). Midbrain neurons have also been shown to produce more H_2O_2 in response to complex I inhibition by rotenone, suggesting a higher vulnerability of these neurons to complex I dysfunction

(Sanders et al. 2014). The mitochondrial mass differs between different neuronal types, and it has been shown to be low in DA neurons in SN, which might contribute to the increased vulnerability of these neurons (Liang et al. 2007). The genetic causes of PD are also strongly related to mitochondrial dysfunction, with all well validated genes influencing the mitochondrial function in DA neurons (Ryan et al. 2015). A meta-analysis done with patients with PD shows a decrease in the expression of PGC-1α and mitochondrial genes. This data further supports the involvement of mitochondria in the pathogenesis of PD (Zheng et al. 2010). Studies in mice shows that knocking out TFAM can give rise to PD-like symptoms, further supporting the importance of mitochondria in PD (Ekstrand et al. 2007).

Oxidative stress due to an increase in the generation of ROS by mitochondrial dysfunction, DA metabolism and neuroinflammation is closely related to the pathogenesis of PD (Hwang 2013). Studies of post-mortem brain samples from patients with sporadic PD indicate that ROS are important in the development of the disease (Jenner & Olanow 1996). The mitochondrial electron transport chain generates most of the ROS (95-98%) produced in the cell during aerobic metabolism (Albarracin et al. 2012). The ROS production has been shown to have a correlation with the risk of developing neurodegenerative diseases. Studies of certain continent-specific clusters of polymorphism termed mtDNA haplogroups have revealed that partial uncoupling of mitochondria is linked to an increased longevity and a decreased risk of neurodegeneration, partially by reduced ROS production (Tanaka 2002). Mitochondrial dysfunction is also linked to sporadic PD, the inhibition of complex I in the respiratory chain is a central cause of sporadic PD by increasing ROS production, causing α-synuclein aggregation, inhibition of the proteasome and causing neurons to be vulnerable to glutamate excitotoxicity (Dawson & Dawson 2003, Hashimoto et al. 2003).

2.11.5. Generation of reactive oxygen species in dopaminergic neurons

ROS are generated as a byproduct in complex I of the respiratory chain, and under normal conditions the cell's defense mechanisms can take care of the excess ROS produced in the cell (Murphy 2009). ROS are also needed for cellular mechanisms such as metabolic adaption, immune cell activation and autophagy (Sena & Chandel 2012). ROS can also be generated by other mechanisms, such as DA degradation (Guillot & Miller 2009, Dias et al. 2013) and by the Fenton reaction, where iron (Fe) and H_2O_2 generates the highly reactive $^{\bullet}OH$ (Walling et al. 1975, Lipinski 2011, Kotiadis et al. 2014).

Neurons are dependent on iron as a cofactor for some enzymes involved in regulation of metabolism, the electron transport chain and in the synthesis of neurotransmitters (Moos & Morgan 2004). An excess of iron in neurons might contribute to the production of ROS, and in patients with PD the concentration of iron in SN has been found to be higher than in controls, which could lead to neurodegeneration in PD (Oakley et al. 2007, Dashtipour et al. 2015). In a Fenton reaction, iron and H_2O_2 have also been found to stimulate the aggregation of α-synuclein, further contributing to the pathogenesis of PD (Hashimoto et al. 1999).

Environmental factors contribute to PD (de Lau & Breteler 2006) and exposure to heavy metals such as iron, manganese, copper, lead or zink can lead to a generation of ROS in the brain (Lai et al. 2002). The drug MPTP and the herbicide paraquat as well as the pesticide rotenone are mitochondrial complex I inhibitors, contributing to the production of ROS in dopaminergic neurons that is associated with PD (Betarbet et al. 2000).

2.11.6. Dopamine synthesis and degradation

Dopamine (DA) is a neurotransmitter that controls many functions, such as movement, cognition, mood and reward (Vaughan & Foster 2013). DA is synthesized in the cytosol from the amino acid tyrosine in two steps; first tyrosine is hydroxylated to L-DOPA by tyrosine hydroxylase (TH) in a reaction that requires oxygen, and in the second step, L-DOPA is decarboxylated to form DA by aromatic amino acid decarboxylase (AADC) (Segura-Aguilar et al. 2014).

TH is the rate limiting enzyme in the synthesis of the catecholamines DA, epinephrine and norepinephrine, catalyzing the transformation of tyrosine to L-DOPA (Daubner et al. 2011). TH is activated by phosphorylation at four different Serine residues by a variety of protein kinases (Dunkley et al. 2004) and TH catalyses the hydroxylation of tyrosine to L-DOPA together with tetrahydrobiopterin (BH4), O_2 and Fe^{2+} (Dunkley et al. 2004). DA, epinephrine and norepinephrine are all able to inhibit TH activity (Daubner et al. 2011) and overexpression of soluble α-synuclein has been shown to reduce the activity of TH *in vitro*, suggesting that the loss of soluble α-synuclein could contribute to increased synthesis of DA and its metabolites (Perez et al. 2002).

The synthesized DA is immediately taken up into monoaminergic synaptic vesicles by vesicular monaomine transporter 2 (VMAT2) that is localized in the membranes of the vesicles (Segura-Aguilar et al. 2014). The uptake of DA into vesicles prevents the accumulation of DA in the cytosol as well as the oxidation of DA into dopamine *o*-quinone in a reaction that generates superoxide radicals (Guillot & Miller 2009, Dias et al. 2013). The *o*-quinone formed can be further oxidized and the products formed can affect cellular processes that are linked to PD. These processes include mitochondrial dysfunction by interfering with complex I and III in respiratory chain and the oxidative phosphorylation in complex V, stabilizing protofibril formation of α-synuclein, preventing the function of DJ-1, affecting protein degradation by interfering with the proteasome system and by inducing oxidative stress (Segura-Aguilar et al. 2014). DA oxidation also leads to the formation of neuromelanin, a dark pigment that is composed of a melanic structure bound to peptides and lipids (Segura-Aguilar et al. 2014), and high levels of neuromelanin in SN is linked to an excess of DA in the cytosol (Zucca et al. 2014).

Upon stimulation, DA is released into the intersynaptic space to interact with postsynaptic DA receptors. DA clearance from the intersynaptic cleft is mediated by DAT that is localized in the membrane of dopaminergic neurons (Segura-Aguilar et al. 2014). The reuptake of DA is another source of cytosolic DA that is also stored in vesicles by VMAT2 (Segura-Aguilar et al. 2014).

In the cytosol, DA can be degraded by deamination by the enzyme monoamine oxidase (MAO) to form 3,4-dihydroxyphenylacetaldehyde, NH_3 and H_2O_2. In a second step, aldehyde dehydrogenase forms 3,4-dihydroxyphenylacetic acid (DOPAC) in a reaction that requires NAD^+ (Segura-Aguilar et al. 2014). Two different isoforms of MAO are found in brain, MAO A is mainly expressed in catecholaminergic neurons (Thorpe et al. 1987) and MAO B is found in serotonergic and histaminergic neurons, astrocytes and radial glia (Kumar & Andersen 2004). MAO are found at the outer membranes of mitochondria in neurons and glial cells and they are the primary enzymes involved in the degradation of biogenic amines such as catecholamines in the brain, thereby influencing the concentration of neurotransmitter amines (Kumar & Andersen 2004). Inhibition of MAO B is used as a therapeutic approach to restore DA in neurons in patients with PD, both as monotherapy and in combination with L-DOPA (Robakis & Fahn 2015). The catalysis of substrate by MAO B gives rise to H_2O_2 contributing to oxidative stress in the cells (Kumar & Andersen 2004). MAO B also catalyzes the formation of 1-methyl-4-phenylpyridinium (MPP^+) from MPTP, and therefore it has an important role in the MPTP-induced degeneration of dopaminergic neurons (Heikkila et al. 1984).

DA can also be degraded by catechol-*ortho*-methyltransferase (COMT) that methylates DA to yield 3-methoxytyramine (3-MT). The same enzymes that degrade DA then further processes 3-MT by

oxidative deamination by MAO to yield 3-methoxy-4-hydroxyphenylacetaldehyde that is further oxidized by aldehyde dehydrogenase to yield homovanillic acid (HVA). COMT can also catalyze the formation of HVA from DOPAC (Segura-Aguilar et al. 2014).

Neurotoxins have been implicated to affect DA flux in the cell. Rotenone seems to affect the DA compartmentalization whereas paraquat is associated with DA breakdown into metabolites (Qi et al. 2014). MPP^+ in turn seems to affect the MAO activity and increasing the level of DA in the cytosol (Choi et al. 2015).

2.11.6.1. Dopamine transporter

Dopamine transporter (DAT) is a plasma membrane glycoprotein that translocates released DA from the extracellular space into the presynaptic neuron (Vaughan & Foster 2013) and thereby terminates the action of DA on the DA receptors (Miller et al. 1999). Once inside the cell, DA is immediately packed into vesicles by VMAT2 (Vaughan & Foster 2013). The overall transport capacity of DAT depends on its surface density which is regulated by posttranslational modifications (Vaughan & Foster 2013). The expression levels decrease with age (Salvatore et al. 2003, Cruz-Muros et al. 2009), and in patients with PD, the expression of both DAT and VMAT2 has been found to be reduced (Harrington et al. 1996).

The transcription of DAT has been suggested to be regulated by the nuclear receptors Nurr1 and ERRγ (Sacchetti et al. 2001, Lim et al. 2015). The expression level of the protein has been shown not to correlate with mRNA levels, indicating that there is a translational regulation of protein expression (Gonzalez-Hernandez et al. 2004). Posttranslational modifications are also important for protein function, and DAT activity has been shown to be dependent on its glycosylation status with the glycosylated form being more efficient in DA transport (Li et al. 2004). The expression pattern of glycosylated DAT in the human brain shows a correlation with neuron degeneration (Afonso-Oramas et al. 2009). DAT activity can also be modulated by other posttranslational modifications, binding partner interactions and by cholesterol and membrane raft association to allow neurons to modulate the uptake by DAT (Vaughan & Foster 2013). The posttranslational modifications include phosphorylation, palmityolation and ubiquitination (Vaughan & Foster 2013). Interestingly, α-synuclein can interact with DAT, reducing dopamine uptake (Wersinger & Sidhu 2003).

Many toxic substances are substrates for DAT because of the resemblance with DA, and therefore toxic substances can accumulate in dopaminergic neurons (Miller et al. 1999). The neurotoxin MPTP that has been converted to MPP^+ by MAO B in glial cells can be transported into dopaminergic neurons by DAT (Bove & Perier 2012). DAT is required for MPTP toxicity, as mice lacking DAT do not show any alteration in dopaminergic neuron viability in MPTP treated or untreated mice (Gainetdinov et al. 1997), while mice over-expression DAT are highly sensitive to MPTP treatment (Masoud et al. 2015). There is also a correlation with DAT expression and the cellular damage caused by MPTP (Sanghera et al. 1997), although the uptake of MPTP into vesicles by VMAT2 is also of importance in combating MPTP-induced neurotoxicity (Gainetdinov et al. 1998).

Since DAT is exclusively found in dopaminergic neurons, it serves a good marker for dopaminergic neurons (Miller et al. 1999) and *in vivo*, DAT imaging is a potentially useful marker for nigrostriatal dopaminergic neuron degeneration (Kraemmer et al. 2014). It seems that DAT is of importance in dopaminergic cell survival, although an increase in DA uptake by DAT can lead to oxidative stress and neuronal loss in neurons that routinely handle DA (Masoud et al. 2015).

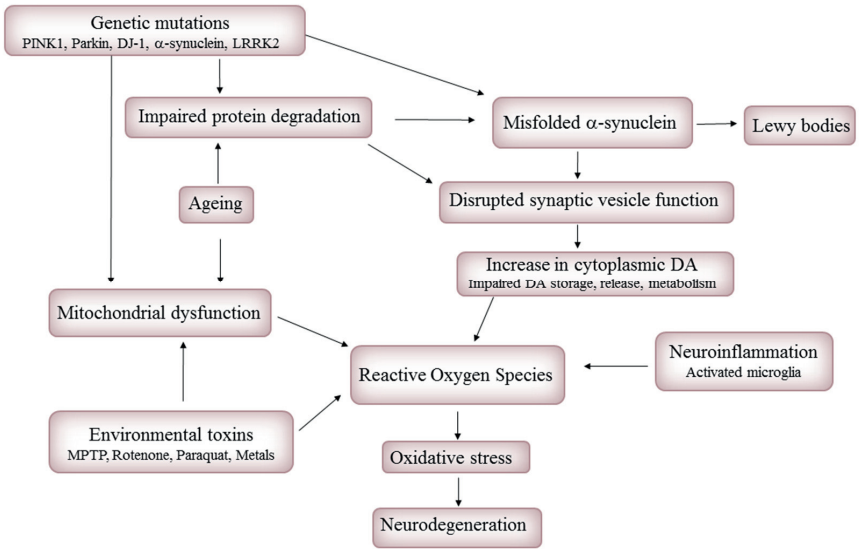

Figure 8. Summary of causes of Parkinson's disease. Modified from (Lotharius & Brundin 2002, Malkus et al. 2009, Blesa et al. 2015).

2.11.7. Animal models of Parkinson's disease

Animal models are used as tools to study the pathogenesis of PD in order to increase the knowledge of molecular mechanisms leading to the degeneration of dopaminergic neurons (Blesa & Przedborski 2014). Animal models have been able to replicate almost all the hallmarks of PD including oxidative stress and dopaminergic neuron degeneration, and have been useful for testing new strategies for neuroprotection or neuroregeneration (Blesa et al. 2012).

The currently existing animal models can be divided into two main groups, toxic and genetic models (Blesa et al. 2012, Blesa & Przedborski 2014). The neurotoxins MPTP, 6-OHDA, rotenone and paraquat cause irreversible symptoms of PD and share the common feature of causing dopaminergic neuron degeneration and oxidative stress. Of these, MPTP and 6-OHDA are the most commonly used (Blesa et al. 2012, Blesa & Przedborski 2014). Inhibitors of the ubiquitin-proteasome system and autophagy such as Lactacystin and MG-132 also mimic symptoms of PD in rodents (Xie et al. 2010).

The genetic models include mutations in genes linked to PD such as α-synuclein, LRRK2, PINK1, Parkin, DJ-1 and ATP13A2. The genetic models may simulate the mechanisms underlying the familial form of PD better, but there are often phenotypes that are quite different than the human conditions (Blesa & Przedborski 2014). The MitoPark mouse model with TFAM knocked out in DAT expressing neurons has also been shown to be a useful tool in PD showing symptoms that develop slowly with age (Ekstrand et al. 2007, Galter et al. 2010).

2.11.7.1. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model is a widely used model to study PD (Meredith & Rademacher 2011, Blesa & Przedborski 2014). MPTP was discovered in 1983 as a by-product in the attempt to synthesize the synthetic heroin analog 1-methyl-4-phenyl-4-propionpiperidine (MPPP). MPTP is a lipophilic molecule that can cross the blood-brain barrier, producing irreversible

symptoms of PD in humans including all the characteristic symptoms such as tremor and rigidity (Ballard et al. 1985), although MPTP treatment of monkeys did give rise to increased α-synulcein levels but not Lewy body formation (Halliday et al. 2009). MPTP has been used to model PD in several different species, and the most common are mouse and monkey, although the choice of mouse strain should be done carefully as different mouse strains react differently to the MPTP treatment (Bove & Perier 2012). Interestingly, rats have been shown to be resistant to MPTP treatment (Chiueh et al. 1984).

MPTP is metabolized to the actual toxin MPP^+ in a two-step reaction in glial cells containing MAO B that converts MPTP to 1-methyl-4-phenyl-2,3,dihydropyridinium (MPDP). MPDP is further oxidized to MPP^+ and released to the extracellular space from where it enters the dopaminergic neurons via DAT, making the dopaminergic neurons vulnerable to MPP^+ (Smeyne & Jackson-Lewis 2005, Meredith & Rademacher 2011, Bove & Perier 2012). Once inside the dopaminergic neuron, MPP^+ can accumulate in synaptic vesicles via the vesicular monoamine VMAT2 (Speciale et al. 1998) or in the mitochondrial matrix by diffusion through the IMM where it acts by inhibiting complex I in the respiratory chain (Smeyne & Jackson-Lewis 2005) as shown in figure 9.

The loss of dopaminergic neurons after exposure to MPP^+ is thought to be due to an increase in ROS production (Cleeter et al. 1992, Lotharius & O'Malley 2000, Obata et al. 2001). Both the inhibition of complex I by MPP^+ and the accumulation of MPP^+ into vesicles contribute to the increase in ROS production. Complex I inhibition reduces the energy production in dopaminergic neurons and generates an excess production of ROS (Cleeter et al. 1992, Wu et al. 2003), whereas MPP^+ accumulation in vesicles causes displacement of DA from the vesicles to the cytoplasm or the extracellular space where it can undergo oxidation and produce ROS (Lotharius & O'Malley 2000, Obata et al. 2001).

2.11.7.2. The rotenone model

Rotenone has been used as a model for PD in rat (Betarbet et al. 2000) and in mice (Pan-Montojo et al. 2010). Rotenone is commonly used as a pesticide and because it is a hydrophobic compound it can pass cell membranes easily (Talpade et al. 2000, Betarbet et al. 2000). Rotenone treatment induces symptoms of PD such as loss of motor function and degeneration of dopaminergic neurons in SN (Blesa et al. 2012), and it can give rise to α-synuclein accumulation and Lewy body formation in long-term treatment with a low dose in rat and mice (Betarbet et al. 2000, Pan-Montojo et al. 2010).

Rotenone causes dopaminergic neuron degradation by blocking complex I in the mitochondrial respiratory chain (Betarbet et al. 2000) and increases the production of ROS (Radad et al. 2006) (figure 9). Although rotenone is not specific for dopaminergic neurons, these neurons are highly sensitive to rotenone toxicity (Ahmadi et al. 2003). The oxidative damage caused by complex I inhibition is thought to contribute to cell death in rotenone-treated dopaminergic neurons (Testa et al. 2005) rather than ATP depletion (Betarbet et al. 2000, Sherer et al. 2003). Also, DA could contribute to cell degeneration in rotenone treated dopaminergic neurons by increasing the production of ROS in the cell (Sakka et al. 2003, Sai et al. 2008). The antioxidant α-tocopherol has been shown to protect dopaminergic neurons against rotenone-induced cell degeneration, further supporting the theory that rotenone-induced cell degeneration is due to an increase in ROS (Sherer et al. 2003).

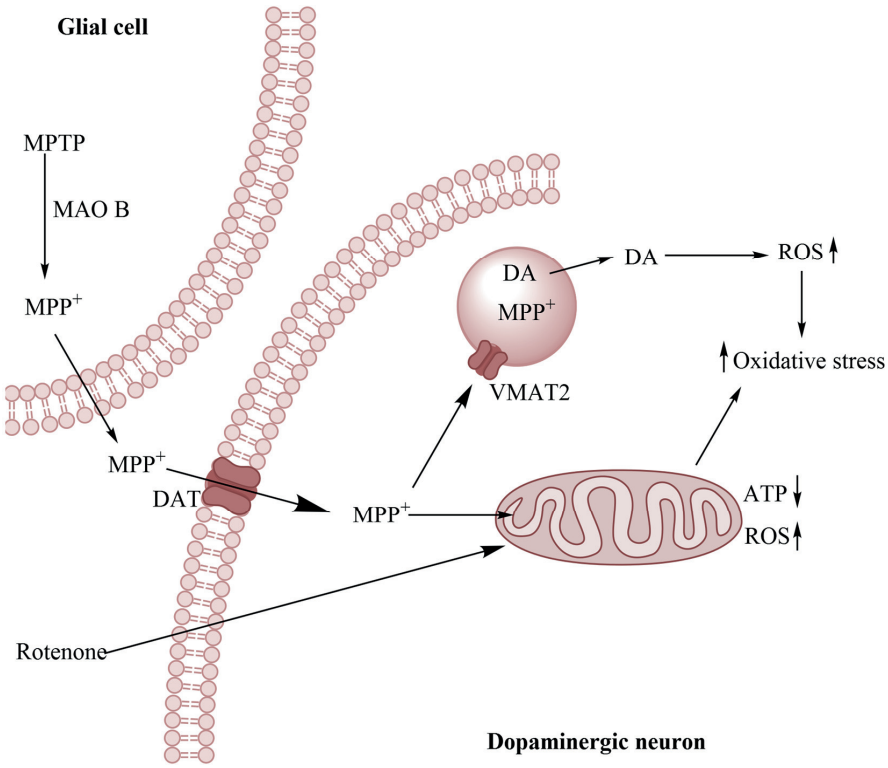

Figure 9. Mechanism of action of the neurotoxins MPTP and rotenone in PD. Modified from (Bove & Perier 2012). MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+, 1-methyl-4-phenylpyridinium; MAO B, monoamine oxidase B; DAT,dopamine transporter; VMAT2, vesicular monoamine transporter 2; DA, dopamine; ROS, reactive oxygen species.

3. AIMS OF THE STUDY

The overall aim of the study was to investigate the neuroprotective effects of PGC-1α during oxidative stress in dopaminergic neurons. The aim was also to search for compounds that could affect the expression of PGC-1α and be beneficial for dopaminergic neuron function and survival by modulating mitochondrial function. Compounds that are known to regulate metabolism in other tissues were used as potential candidates in the search for regulators of PGC-1α expression and activation in dopaminergic neurons.

The specific aims were:

I) To clarify the neuroprotective effect of overexpressing PGC-1α in mouse nigrostriatal dopaminergic neurons against MPTP induced cell death.

II) To clarify the neuroprotective effect of resveratrol in dopaminergic neurons *in vivo* and *in vitro*.

III) To clarify the effect of FGF21 on PGC-1α expression and mitochondrial function in human dopaminergic neurons.

IV) To study the mechanism of the regulation of PGC-1α expression by the PPARγ agonist GW1929 and the effect on mitochondrial function in human dopaminergic neurons.

4. MATERIALS AND METHODS

4.1. Animal experiments (I, II)

All animal experiments were approved by the local ethical committee and accomplished in accordance with the European Communities Council Directive (86/609/EEC). Adult male and female C57BL/6 mice were obtained from local stocks. Female mice were used independently of estrous cycle (II).

Generation of PGC-1α transgenic mice:
To generate PGC-1α transgenic (TG) mice (I) the PGC-1α cDNA with a Flag-tag was cloned into the *Thy*1.2 expression cassette with the Thy1.2 promoter driving the PGC-1α transgene expression in brain neurons. The transgenic mice were generated at the Uppsala Transgenic Facilities at Uppsala University, Sweden using standard techniques. The genetic background of the mice was CBA x C57BL/6 and they were backcrossed to C57BL/6 for several generations to produce stable PGC-1α transgenic mouse lines. Controls were obtained from the same breeding.

Drug treatments:
MPTP treatment: The MPTP treatment was done by injecting three times 14 mg/kg MPTP intraperitoneally (i.p.) at the time points 0 h, 1.5 h and 3 h. A fourth injection using 7 mg/kg MPTP was given at the time point 4.5 h (I).

RSV treatment: 20 mg/kg RSV was first dissolved in 50% DMSO/50% ethanol, and then diluted in saline to a final volume of 0.2 ml and injected i.p. Mice receiving cotreatment with RSV and MPTP were pretreated with RSV 30 min before first MPTP injection (I). Additional treatments with RSV were done 12 h, 24 h, 48 h and 72 h after the first MPTP treatment, and mice were sacrificed 7 days after the first MPTP injection. Mice receiving RSV treatment for the analysis of DAT expression were injected daily with 20 mg/kg RSV i.p. for four days. Brains were collected eight days after the first injection (II).

Tamoxifen treatment: 1 mg/kg Tamoxifen dissolved in ethanol and diluted in sesame oil to the volume 50 µl was injected subcutaneously (s.c.). When mice were cotreated with Tamoxifen and RSV, they were pretreated with Tamoxifen for 1.5h followed by the RSV treatment. The treatment was done for four following days, and the mice were sacrificed 8 days after the first treatment (II).

ICI 182,780 treatment: 2 mg/kg, ICI 182, 780 was injected s.c. Mice receiving co-treatment with RSV were pretreated with ICI 182,780 followed by RSV treatment after 1.5 h. The treatment was repeated for four following days, and the brains were collected 8 days after the first treatment (II).

4.2. Cell cultures (I-IV)

Cell types used in this work are listed in table 3:

Cell type	Origin	Publication
MESC2.10	human	II, III, IV
SN4741	mouse	I, II
PC6.3	rat	I
Primary glial cells	rat	III
Huh7	human	III

Table 3. List of cell types used in experiments.

Human MESC2.10 cells

Human mesencephalon neuronal precursor cells (MESC2.10) were cultured in poly-lysine (Sigma) coated flasks in proliferation medium containing Dulbecco's Modified Eagle Medium (DMEM)/F12 medium (Gibco) supplemented with B27 (Gibco), penicillin-streptomycin and human basic FGF (Peprotech). For differentiation, cells were plated on poly-lysine and laminin (Sigma)-coated plates in proliferation medium with a change to differentiation medium containing DMEM/F12, B27, penicillin-streptomycin and 1 µg/ml tetracyclin (Sigma) the following day. Cells were differentiated 5 days before treatment and fresh medium was added every second day.

Mouse SN4741 cells

Mouse SN4741 dopaminergic cells were cultured in DMEM containing phenol red in the presence of 10% fetal calf serum and penicillin-streptomycin. One day before experiments, the medium was changed to DMEM supplemented with B27 for experiments with estrogen receptor. Cells were treated with 10 µM RSV and 1 µM 17β-estradiol alone or in combination with 2 µM ICI 182,780 to inhibit ER.

Rat primary glial cells

Rat primary glial cells were prepared from postnatal day 1-2 brain cortex by dissociating and washing the tissue by centrifugation. Cells were resuspended in DMEM/F12 containing 10% fetal calf serum and penicillin-streptomycin and cultured for up to three weeks with an addition of medium two times a week. Cells were lysed for Western blot analysis.

Human Huh7 cells

Human Huh7 hepatic cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum and penicillin-streptomycin. Cells were lysed for Western blot analysis.

Rat PC6.3 cells

Rat PC6.3 cells were cultured in RPMI-1600 medium supplemented with 5% fetal calf serum and 10% horse serum, 100 mM Na-glutamine and penicillin-streptomycin.

4.3. Western blotting (I-IV)

MESC2.10 cells or tissue from mouse brain was washed with ice-cold phosphate buffered saline (PBS) and lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA and 50 mM Tris-HCl pH=7.4) with an addition of 1% sodium dodecyl sulfate (SDS) and protease inhibitor cocktail (Roche). 30 µg of protein was run on SDS-PAGE, transferred to nitrocellulose membrane and blocked in 5% nonfat milk- TBS- 0.1% Tween 20 (TBS-T) for 1 h in room temperature (RT). The primary antibody was diluted in 5% nonfat milk- TBS-T and added to the membrane overnight in +4˚C. The following day the membrane was washed three times with TBS-T and the appropriate HRP conjugated secondary antibody was added for 1 h at RT. Primary antibodies are listed in table 4. Densitometric analysis was done using ImageJ software.

4.4. Immunoprecipitation (I, III, IV)

Immunoprecipitation was done using Protein G agarose for MESC2.10 cells and Sepharose A for SN4741 cells. Cells were lysed in RIPA buffer supplemented with protease inhibitor cocktail. The lysates were prepurified with Protein G agarose (Roche) or Sepharose A and incubated with 2 µg of PGC-1α antibody over night. 50 µl of Sepahrose A was added for 2 h or 50 µl of Protein G agarose was added for 6 h to bind immune complexes. Beads were washed three times with RIPA buffer. The beads were boiled and proteins subjected to immunoblotting using anti-acetylated lysine or anti-PGC-1α antibodies.

4.5. Immunocytochemistry (IV)

Differentiated cells were fixed with 4% paraformaldehyde (PFA), 20 min RT and blocked for 1 h using 5% BSA in PBS/0.1% Triton-X100 (PBS-Tx). Anti-DAT (1:200, Novus Biologicals) was added and the cells were incubated o/n at +4˚C. The following day, cells were washed with PBS-T and secondary Alexa 594 fluorescent antibody (1:500, Invitrogen) was added for 1h at RT. The cells were washed and mounted with Mowiol mounting media (Sigma). Images were captured using fluorescent microscope Leica DM4500B.

Target protein	Method	Publication nr	Supplier	Dilution	Species
Acetylated lysine	WB	III, IV	Cell Signaling Technology	1:1000	Mouse
Catalase	WB	II	Abcam	1:500	Rabbit
COX IV	WB	II, III	Abcam	1:2000	Mouse
DAT	WB, IHC	I, II	Santa Cruz Biotechnology	1:100-1:200 IHC, 1:800 WB	Rat
DAT	WB, ICC	I, IV	Novus Biologicals	1:500 WB 1:200 ICC	Rabbit
DYKDDDDK Tag	WB, IHC	II	Cell Signaling Technology	1:1000 WB	Rabbit
FGF21	WB	III	Novus biologicals	1:2000	Rabbit
HO-1	WB	II	Stressgen	1:500	
Nampt/PBEF	WB	III	Abcam	1:1000	Rabbit
NeuN	IHC	II	Chemicon	1:300	Mouse
NRF1	WB	II	Molecular probes	1:1000	Rabbit
NRF1	WB	IV	Abcam	1:1000	Rabbit
PGC-1α	WB, IP	III, IV	Calbiochem	1:1000-1:2000	Mouse
PGC-1α	WB	II	Cell Signaling Technology	1:1000	Rabbit
SIRT1	WB	III, IV	Cell Signaling Technology	1:1000	Mouse
SIRT1	WB	II	Abcam	1:2000	Goat
SOD1	WB	II	Santa Cruz Biotechnology	1:5000	Rabbit
SOD2	WB	II, III, IV	AbFrontier	1:5000-1:30000	Mouse
TFAM	WB	III, IV	Abcam	1:1000	Rabbit
TH	WB, IHC	I, II	Chemicon	1:1000 IHC, 1:2000 WB	
Trx2	WB	II, III	AbFrontier	1:1000	Rabbit
XIAP	WB	II	BD Bioscience	1:5000	Mouse
β-Actin	WB	II, III, IV	Sigma	1:2000-1:5000	Rabbit
β-Actin	WB	I	Santa Cruz Biotechnology	1:6000	Rabbit
TH	IHC	II	Covance		Mouse
TH	WB,IHC, ICC	II, III, IV	Cell Signaling Technology	1:1000 WB 1: 500 IHC 1:500 ICC	Rabbit
p-CREB (Ser133)	WB	IV	Cell Signaling Technology	1:1000	Rabbit
CREB	WB	IV	Cell Signaling Technology	1:1000	Rabbit

Table 4. Antibodies with dilutions used in experiments.

4.6. Immunohistochemistry (I, II)

Frozen sections:

Brains were fixed in 4% PFA in PBS for three days and immersed in 10% sucrose solution for one day and 20% sucrose for an additional day. Brains were then frozen in cooled isopentane. 20 µm thick sections were washed in PBS followed by treatment with 0.4% hydrogen peroxidase in PBS for 20 min. The sections were washed in PBS and blocked in 5% BSA-PBS-Tx for 10 min. Primary antibody was added and incubated over night at +4˚C. The following day the sections were washed and incubated

with biotinylated secondary antibody for 1 h, RT followed by 1 h incubation with a streptavidin horseradish peroxidase-complex. After washing in PBS and 0.1 M Tris-HCl buffer pH=7.4 for 10 min, the reaction was developed using 2.5% 3,3-diaminobenzidine and 0.04% hydrogen peroxide. The reaction was stopped with 0.1 M Tris-HCl, dehydrated and mounted. The quantification of fiber densities was done using ImageJ software.

Paraffin embedded sections:
Paraffin embedded sections were dewaxed using xylene and rehydrated in decreasing ethanol concentrations and water. Sections were boiled in 10 mM citrate, pH=6, for antigen retrieval and blocked in 5% BSA- PBS-Tx 1 h, RT and incubated with primary antibody over night at +4˚C. The sections were washed and the appropriate secondary fluorescent antibody Alexa 488 or Alexa 594 (Invitrogen) was added for 1 h, RT followed by counterstaining with Hoechst 33342 and mounting. The images were captured using fluorescent microscope Leica DM4500B. Primary antibodies used are listed in Table 4.

4.7. Cell viability (II, IV)

Mouse SN4741 cells were incubated in 96-well plates and treated with 400 µM or 800 µM of MPP^+ in the absence or presence of 5 or 10 µM RSV for 48 h (I) or differentiated MESC2.10 cells were pretreaterd with 1 µM GW1929 for 24 h followed by 100 µM H_2O_2 treatment for an additional 24 h (IV). Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay by adding MTT solution to the cells for 2 h at +37˚C. The medium was removed and the dye absorbed by the cells was dissolved in isopropanol containing 40 mM HCl. The absorbance was measured at 560 nm using Labsystems Multiscan MS Version 3.0 spectrophotometer.

4.8. Luciferase assay (II-IV)

Cells were transfected with 0.5µg of PGC-1α promoter constructs linked to luciferase reporter. Three different constructs were used, the intact PGC-1α promoter (PGC) or constructs with mutations in the CRE site (PGCΔCRE) or the MEF site (PGCΔMEF) of the promoter. The pGL3 basic promoter was used as control. Cells were transfected using Transfectin reagent (Biorad) for SN4741 cells and PC6.3 cells (I) and Fugene HD Transfection Reagent (Promega) for MESC2.10 cells (III, IV). 0.02 µg of Renilla luciferase pRL-TK was co-transfected to control transfection efficiency. 24 h after transfection cells were stimulated with RSV for an additional 24 h (I) or cells were differentiated for 2 days followed by 24 h treatment with 50 ng/ml FGF21 (III) or 1µM GW1929 (IV). Cells were lysed in Passive lysis buffer and luciferase activity was measures using Dual-Luciferase reporter Assay (Promega) according to manufacturer's protocol using GLOMAX 20/20 luminometer (Promega). The values for firefly luciferase were normalized to Renilla.

4.9. Quantitative PCR (I-IV)

Total RNA was extracted using Lipid tissue RNeasy kit for brain tissue or RNeasy kit for cells (QIAGEN). 0.5µg RNA was used for cDNA synthesis using SuperScript VILO cDNA synthesis kit (Invitrogen) and the product was diluted 1:5. 2 µl of cDNA was used for the analysis together with 200 nM of the forward and reverse primers. Quantitative -PCR amplification was performed using Sybrgreen (Applied Biosystems) and the AbiPrism 700 Sequence detector (I, II) or SYBR Green Master Mix (Roche) and the Light Cycler 480 II instrument (Roche) (III, IV). The cycling conditions were denaturation at 95 ˚C for 10 min followed by 40 cycles of denaturation at 95 ˚C for 10 s, annealing at 60 ˚C for 30 s and extension at 72 ˚C for 45 s. All samples were run in triplicates. Melting curve analysis

was done to ensure single product amplification. Treshold cycle (Ct) values were calculated using the default second derivate maximum method which is built in the Light Cycler 480 II instrument. The gene expression ratio was calculated using the $2^{-\Delta\Delta Ct}$ method. Primers used are listed in Table 5. β-Actin was used as an internal control gene.

4.10. Mitochondrial DNA copy number (III, IV)

DNA was isolated from differentiated MESC2.10 cells using QIAamp DNA Mini Kit (QIAGEN) according to manufacturer´s protocol. Quantitative PCR was done using 1X SYBR Green Mastermix (Roche) with an addition of 5ng DNA and 200 nmol/l of forward and reverse primers for NADH dehydrogenase subunit 1(ND1) gene for mtDNA and human globulin (HGB) gene for nuclear DNA content. Quantitative PCR amplification was performed using Light Cycler 480 II instrument (Roche) with the thermal cycling conditions were 95 ˚C for 10 min followed by 40 cycles at 95 ˚C for 15 s and 60 ˚C for 1 min. Each sample was run in triplicates and water was used as negative control. Primers used are listed in Table 5.

Gene	Forward primer	Reverse primer	Publication nr
DAT	5´-CCAGCTACAACAAGTTCACCAA-3´	5´-AGAAGCTCGTCAGGGAGTTG-3´	II
SOD2	5´-GACCCATTGCAAGGAACAA-3´	5´-GTAGTAAGCGTGCTCCCACAC-3´	I
ND-1	5´-CCTAAAACCCGCCACATCT-3´	5´-GAGCGATGGTGAGAGCTA AGGT-3´	III
HGB	5´-GTGCACCTGACTCCTGAGAGA-3´	5´-CCTTGATACCAACCTGCCCAG-3´	III
β-Actin	5'-TCC TTC CTG GGC ATG GAG-3'	5'-GAT GTC CAC GTC ACA CTT CA-3'	IV
SIRT1	5'-TAA TTC CAA GTT CCA TAC C-3'	5'-ATT CAC ACA CTA ACC TAT-3'	IV
PGC-1α	5'-CAA ACC AAC AAC TTT ATC TCT TCC-3'	5'-ACA CTT AAG GTG CGT TCA ATA GTC-3'	IV
ADCY6	5'-CCT CCA TTG CCA ACT TCT CT-3'	5'- CGC TCC TCG CTG ATA ATC TC-3'	IV

Table 5. Primer sequences.

4.11. Isolation of mitochondria and measurement of respiratory control (I)

Brain tissue from wild-type C57Bl/6J and PGC-1α TG mice were minced into pieces in isolation medium (10mM Hepes-K pH=7.4, 1 mM EGTA and sufficient sucrose to obtain an osmolarity of 300 mOsm). The samples were homogenized and centrifuged at 800 g for 8 min. To obtain a mitochondrial pellet, the supernatant was centrifugated 2 times at 10,000 g for 10 min and the pellet containing mitochondria was suspended in 300 µl of isolation medium. Mitochondria were used for measurements within 4 h from isolation.

To measure the mitochondrial respiration mitochondria were suspended in a medium containing 125 mM KCl, 10 mM Hepes-K pH=7.4, 2 mM MgCl, 1 mM Pi and 100 µM EGTA. To start the respiration 10 mM malate and 10 mM glutamate or 10 mM succinate were added. The respiratory control ratio was determined by addition of ADP. Mitochondrial respiration was measured using a Clark-type electrode (Yellow Springs Instruments, USA).

The mitochondrial membrane potential was measured by adding 0.5 µM of the fluorescent dye tetramethylrhodamine (TMRM) to the measurement medium and using a Cary Eclipse Fluorescence Spectrophotometer (Varian, USA). The excitation was set at 550 nm and emission at 575 nm.

4.12. Relative oxygen consumption analysis (III, IV)

MESC2.10 cells were plated on Seahorse XFe 96-well plates and differentiated for 5 days before stimulation with FGF21 50 ng/ml (III) or 1 μM GW1929 (IV) for 24 h. The medium was changed to non-buffered DMEM pH=7.4 for 1 h prior to measurement and cells were incubated in a non CO_2 supplemented incubator.

XF cell mito stress kit (Seahorse Bioscience) was used for analysis of oxygen consumption with a Seahorse XFe96 analyzer (Seahorse Bioscience) with an addition of 1 μM Oligomycin, 0.8-1 μM Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) and 1 μM Rotenone and Antimycin A. 3x3 min cycles were run for every measurement before adding the following compound. Values were normalized to the protein content of the wells.

4.13. NAD^+/NADH assay (III)

Differentiated MESC2.10 cells were stimulated with FGF21 50 ng/ml for 24 h prior to measurement of NAD^+/NADH levels using NAD^+/NADH assay (Abcam) according to manufacturer's protocol. The absorbance was measured using Multiscan MS Version 3.0 spectrophotometer at 450 nm.

4.14. ROS measurements (I)

SN4741 cells were treated with 100 μM MPP^+ or 5-10 μM RSV for 24 h followed by a loading of the cells with 10 μM dihydroethidium (DHE) for 15 min. Cells were centrifugated at 1500 rpm for 3 min and suspended in PBS. The levels of ROS were measured immediately using fluorescence-activated cell sorter Aria (FACS; BD Biosciences). The number of DHE-positive cells was measured with excitation at 488 nm and emission at 595 nm.

4.15. Electron microscopy and mitochondrial density analysis (III)

Differentiated MESC 2.10 cells were stimulated for 24 h and fixed with 2.5% glutaraldehyde in PBS for 1 h RT and washed two times 1 h with H_2O. Postfixation was done using 1% osmiumtetroxide. Thin sections were stained with lead citrate and uranyl acetate and the sections were viewed using Jeol JEM-1400 transmission electron microscope (Jeol Ltd., Tokyo, Japan) equipped with Gatan Orius SC 1000B bottom mounted CCD-camera (Gatan Inc., USA). Mitochondria were manually marked and the relative surface area was calculated using ImageJ software.

4.16. High-pressure liquid chromatograpy (HPLC) (I)

DA and DOPAC concentrations were determined in mouse striatal tissue. Mouse striatal tissue was dissected and immediately frozen. The samples were homogenized in 0.5 ml homogenization solution containing 0.2 M $HClO_4$ supplemented with an antioxidant solution containing oxalic acid, acetic acid and L-cysteine in ratio 6:1. Samples were centrifuged at 20 800g for 35min at 4˚C, the supernatant was transferred to 0.5 ml Vivaspin® filter concentrators (Vivascience AG, Hannover, Germany) and centrifuged at 8600 g for 35 min at +4˚C. The filtrate was measured by HPLC with electrochemical detection.

A Spherisorb1 ODS23 lm, 4.6 3 100 mm^2 column (Waters, Milford, MA) was used and kept at 50˚C with a column heater (Croco-Cil, Bordeaux, France). The mobile phase consisted of 0.1 M NaH_2PO_4,

350 mg/l octane sulfonic acid, 3.5–5% methanol and 450 mg/l EDTA. pH was set to 2.7 using H_3PO_4. A pump (ESA Model 582 Solvent Delivery Module; ESA, Chelmsford, MA) equipped with a pulse damper (SSI LP-21, Scientific Systems, State College, PA) provided a flow rate of 1 ml/min. The filtrate (60 µl) was injected into chromatographic system with a CMA/200 autoinjector (CMA, Stockholm, Sweden). The detection of dopamine and DOPAC was done using ESA® CoulArray Electrode Array Detector and the concentrations were calculated using CoulArray® for windows software® (ESA, Chelmsford, MA). The concentrations were calculated as pg/g of tissue.

4.17. cAMP measurements (IV)

MESC 2.10 cells were plated on 24-well plates and differentiated for 2 days before treatment with GW1929 for 3 h or Forskolin for 30 min prior to the measurement of cAMP levels. cAMP measurements were done using cAMP GLo assay (Promega) according to manufacturer's protocol, but without phosphodiesterase inhibitors in the induction media. The luminescence was measured using Glomax 96 microplate luminometer (Promega).

4.18. Statistical analysis (I-IV)

All experiments were performed at least three times. Statistical analysis was done using Student's t-test when comparing two groups and one-way ANOVA followed by Bonferroni's post hoc test when comparing three or more groups. A p value $p<0,05$ was considered statistically significant.

5. RESULTS AND DISCUSSION

5.1. Involvement of PGC-1α in neuroprotection in the MPTP mouse model of Parkinson's disease

5.1.2. Characterization of PGC-1α transgenic mice

Mitochondrial dysfunction and oxidative stress have been linked to neurodegenerative diseases including PD (Lin & Beal 2006). PGC-1α is considered a master regulator of mitochondrial function (Puigserver & Spiegelman 2003, Canto & Auwerx 2009) and PGC-1α knock out mice have been shown to be vulnerable to oxidative stress induced by MPTP treatment as well as excitotoxicity (St-Pierre et al. 2006). Exercise has been shown to have a neuroprotective effect in the MPTP model (Zigmond & Smeyne 2014), and PGC-1α is known to be activated during exercise (Canto & Auwerx 2009), suggesting that PGC-1α might be involved in the neuroprotection against MPTP induced cell death. We wanted to study the possible neuroprotective effect of PGC-1α overexpression in dopaminergic neurons. A TG mouse was generated overexpressing Flag tagged PGC-1α under the control of the Thy-1 promoter to drive expression in neurons (Caroni 1997) to study the possible neuroprotective effect of PGC-1α *in vivo*.

Phenotypic analysis of the mice showed that PGC-1α expression was increased in the nigrostriatal system in TG mice when compared to wildtype (WT) mice (I/Fig.1B) and immunostaining using anti-Flag antibody showed that the TH-positive dopaminergic neurons in SN expressed Flag-PGC-1α in TG mice but was not present in WT mice (I/Fig 1C), further confirming that the generation of the TG mouse strain was successful. As PGC-1α has been shown to influence the expression of mitochondrial antioxidants to combat oxidative stress (St-Pierre et al. 2006), the levels of mitochondrial proteins were analyzed. Immunoblotting of SN revealed an increase in the antioxidant enzymes SOD2 and Trx2 in TG mice compared to WT and an elevated level of COX IV was also observed in TG mice compared to WT, indicating an increase in mitochondrial function in TG mice (I/Fig 1E). The increase in protein levels was due to an increase in gene transcription, as shown for SOD2 and COX IV using quantitative PCR (I/Fig 1D). Our data shows that an overexpression of PGC-1α leads to changes in gene expression for mitochondrial genes in SN.

5.1.3. PGC-1α transgenic mice are protected against MPTP-induced dopaminergic neuron degeneration

To study the possible neuroprotective effect of PGC-1α overexpression we treated mice with the neurotoxin MPTP. Treatment of WT mice with MPTP is known to reduce the number of TH-positive neurons in the SN (Thomas & Beal 2007) and this was also the case in our study. In PGC-1α TG mice however, treatment with MPTP did not significantly reduce the amount of TH-positive cells, indicating a neuroprotective effect of PGC-1α overexpression (I/Fig 2A,B). The number of NeuN positive cells did not alter in TG mice after MPTP treatment (I/Fig 2C), while MPTP treatment did decrease the number of NeuN positive cells in WT (I/Fig 5D), further supporting neuroprotective effect of PGC-1α in MPTP-treated mice.

TH levels in striatum were analyzed in two separate TG mouse lines with similar expression of PGC-1α. Immunoblots showed a decrease of TH in WT but not in the TG mouse lines after MPTP treatment, and similar results were obtained with both TG mouse lines. Measuring the levels of DA and the metabolite DOPAC in WT and TG mice after MPTP treatment showed that MPTP treatment decreased the striatal concentration of DA and DOPAC in WT mice as expected (Heikkila et al. 1984). The reduction in DA and DOPAC levels by MPTP treatment were smaller in TG mice, indicating that TG

mice were significantly more resistant to MPTP treatment (I/Fig 3A,B). These results show that overexpression of PGC-1α prevents changes in DA and DOPAC in striatum showing a beneficial effect on the functional state of the nigrostriatal system.

Our results show that the TG expression of PGC-1α protects dopaminergic neurons against MPTP-induced cell degeneration (figure 10). This data is further supported by studies showing the beneficial effect of PGC-1α in cell stress (St-Pierre et al. 2006, Lu et al. 2010, Makela et al. 2016). A meta-analysis of PD patients showed decreased levels of PGC-1α and its downstream genes, indicating that PGC-1α is involved in the pathogenesis of the disease and might serve as a possible target for early intervention in PD (Zheng et al. 2010).

The currently available toxin-induced animal models of PD cause an acute degeneration of dopaminergic neurons. This does not mimic the development of the disease in patients where a slow degeneration of the dopaminergic neurons is observed. The use of the MPTP model of PD does however mimic the symptoms of PD (Ballard et al. 1985), but of the currently available models the MPTP model is useful to study potential neuroprotective mechanisms.

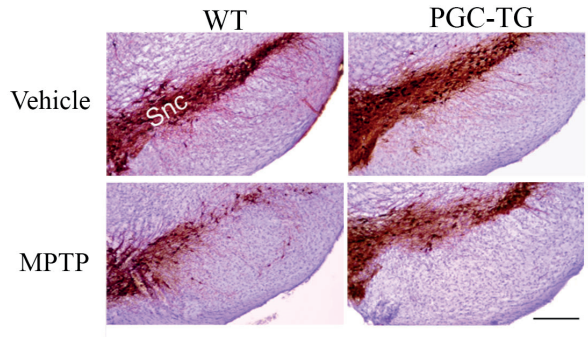

Figure 10. Immunostaining of TH-positive cells in SN of TG and WT mice. MPTP treatment showed a smaller decrease in TH-positive cells in TG mice compared to control, indicating that PGC-1α has a neuroprotective effect in these cells. Snc, substantia nigra; WT, wildtype; PGC-TG, PGC-1α transgenic; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

5.1.4. Mitochondrial respiration is increased in isolated brain mitochondria from PGC-1α transgenic mice

PGC-1α regulates the expression of mitochondrial genes, including proteins of the respiratory chain (Puigserver et al. 1998, Wu et al. 1999). It has previously been shown that overexpression of PGC-1α in cultured neurons increased the mitochondrial capacity and protected cells against mitochondrial loss (Wareski et al. 2009). We isolated mitochondria from the brains of TG and WT mice. Measurements of mitochondrial oxygen consumption showed an increase in the respiratory control rate in mitochondria from TG mice compared to mitochondria from WT mice, indicating that mitochondria from TG mice have a higher ATP production capacity than mitochondria from WT mice. The relatively small difference in respiratory control rate might be due to the fact that mitochondria were isolated from whole brain, whereas the TG expression of PGC-1α is driven by the neuron-specific Thy1 promoter (Caroni 1997), leading to a relatively small level of overexpression of PGC-1α. Therefore it would be of interest to isolate neurons from these mice and analyze the respiratory capacity of neuronal mitochondria *in vitro*.

PGC-1α has previously been shown to mainly localize in GABAergic neurons with only weak expression in midbrain (Cowell et al. 2007). Immunostaining with Flag-antibody did show a co-

localization of PGC-1α and TH in SN (I/Fig 1C) in TG mice revealing an overexpression in dopaminergic neurons. It is possible that endogenous PGC-1α levels in dopaminergic neurons are too low to be neuroprotective but higher levels of PGC-1α can have neuroprotective effects, although an extensive overexpression of PGC-1α in rat brain has been shown to be harmful for neuronal survival (Ciron et al. 2012). Since PGC-1α increases the mitochondrial respiration, it may be possible that an extensive overexpression of PGC-1α gives rise to an increase in ROS generated by the respiratory chain (Lindholm et al. 2012).

5.2. Resveratrol has neuroprotective effects in dopaminergic neurons *in vivo*

5.2.1. Resveratrol protects against MPTP-induced cell death in dopaminergic neurons

PGC-1α can be regulated by posttranslational modifications such as deacetylation by SIRT1 (Rodgers et al. 2005, Rodgers et al. 2008). RSV is a naturally occurring polyphenol (Baur & Sinclair 2006, Sun et al. 2010) that can activate SIRT1 and PGC-1α (Lagouge et al. 2006). RSV has previously been shown to have neuroprotective effects in dopaminergic neurons in different systems (Okawara et al. 2007, Chao et al. 2008, Blanchet et al. 2008) as well as in glutamate excitotoxicity, after brain trauma and ischemia (Baur & Sinclair 2006, Ates et al. 2007, Della-Morte et al. 2009). We were interested in the neuroprotective effects of RSV and analyzed the effect of RSV in mice treated with MPTP as described in the methods section. The treatment scheme is shown in figure 11.

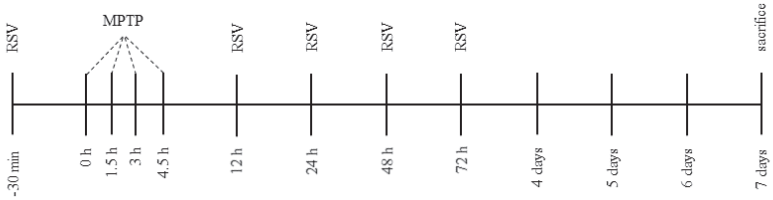

Figure 11. Treatment scheme of MPTP and RSV injections to C57BL6 mice.

In WT mice, treatment with MPTP showed a decrease in the number of TH-positive cells in SN compared to vehicle treated mice (I/Fig 5A). This reduction of TH-positive cells was partly counteracted when mice were co-treated with RSV (20 mg/kg) (I/Fig 5B,C). The number of NeuN positive cells in SN corresponded to the number of TH-positive cells, showing that RSV was largely neuroprotective after MPTP treatment (I/Fig 5D). Similar results were obtained in striatum when TH and DAT levels were analyzed, although the protective effect of RSV was not as high in striatum as observed in SN (I/Fig 5E-H). These results shows that RSV has a neuroprotective effect in DA neurons in SN and it also partially protects against MPTP-induced neurodegeneration in striatum of mice, which is in line with previous studies (Okawara et al. 2007, Chao et al. 2008, Blanchet et al. 2008). The mechanism of action for RSV in the brain is not known, and the difference in the protective effect between SN and striatum might be because RSV affects cell bodies and nerve terminals differently, although this needs to be studied.

In line with the data obtained from TG mice, we also observed an increase in SOD2 and Trx2 levels in RSV-treated mice (I/Fig 5I,J), indicating that RSV may reduce oxidative stress by increasing antioxidants. RSV has been shown to increase the expression of mitochondrial antioxidants *in vitro* (Kairisalo et al. 2011), and RSV is also known to affect PGC-1α (Lagouge et al. 2006). Since PGC-1α regulates the expression of mitochondrial antioxidants (Austin & St-Pierre 2012), the neuroprotective effect of RSV could be mediated by affecting PGC-1α expression.

5.2.2. Mechanisms of resveratrol-mediated neuroprotective effects

To study the mechanism of RSV action, we used cultured dopaminergic SN4714 cells. In line with the data obtained from mice, treatment of these cells with 400 μM and 800 μM MPP^+ showed a decrease in cell viability which was counteracted by co-treatment with 5 μM or 10 μM of RSV (I/Fig 6A). MPP^+ is known to produce ROS by inhibiting complex I in the respiratory chain (Smeyne & Jackson-Lewis 2005). In line with this, we observed an increase in ROS production when cells were treated with 100μM MPP^+. Treatment with 5 μM or 10 μM RSV was able to decrease the production of ROS in these cells (I/Fig 6B).

As the decrease in oxidative stress in RSV-treated cells may be due to an increase in antioxidant enzymes, we measured the protein levels of SOD2 and Trx2. In line with the data obtained from RSV-treated mice, RSV-treated cells showed an elevated protein level of both SOD2 and Trx2 compared to untreated cells (I/Fig 6C,D). Both SOD2 and Trx2 have been shown to be regulated by NFκB signaling that can be induced by X-chromosome linked inhibitory apoptosis protein (XIAP) (Kairisalo et al. 2011). We did not observe any changes in XIAP in RSV-treated cells (I/Fig 6F) or in SOD1, catalase or heme oxygenase (HO-1) which are reported to combat oxidative stress and located in the cytoplasm, peroxisome and endoplasmatic reticulum (Crapo et al. 1992, Stocker & Perrella 2006, Kirkman & Gaetani 2007). This suggests that the effect of RSV is on mitochondrial function that could be mediated by PGC-1α as both SOD2 and Trx2 are target genes for PGC-1α.

An increase in SIRT1 was also observed in RSV-treated cells (I/Fig 6C,D) and RSV was able to reduce the degree of acetylation of PGC-1α, showing an activation of PGC-1α by RSV treatment (I/Fig 6E). SIRT1 responds to metabolic changes in the cell (Canto & Auwerx 2012). SIRT1 activation has been shown to be neuroprotective in AD and ALS models (Qin et al. 2006, Kim et al. 2007), and an increase in SIRT1 expression could also be neuroprotective in PD. SIRT1 is a NAD^+-dependent deacetylase (Imai et al. 2000), and caloric restriction increases SIRT1 activity by increasing NAD^+ levels (Cohen et al. 2004, Lin et al. 2004, Houtkooper et al. 2010). RSV has been shown to affect AMPK levels in neurons (Dasgupta & Milbrandt 2007), and AMPK can increase NAD^+ levels by fatty acid oxidation (Canto et al. 2009). RSV could activate SIRT1 via AMPK and NAD^+, but this remains to be studied.

The finding that RSV increases PGC-1α levels (I/Fig 6C,D) raised the question if there is also an effect on PGC-1α gene transcription. We used PGC-1α promoter constructs linked to a luciferase reporter gene and data showed that the gene activity was enhanced by 80% in SN4741 cells treated with 10μM RSV (I/Fig 7A). Treating neuronal PC6.3 cells with different concentrations of RSV also showed that the gene activity was enhanced in a dose dependent manner (I/Fig 7B). When using PGC-1α promoter constructs with mutations in the MEF site or in the CRE site, the increase in gene activity by RSV treatment was abolished (I/Fig 7A). This shows that the PGC-1α gene activity in neuronal cells is enhanced by RSV and involves the binding of various transcription factors to the promoter.

Taken together, our data show that RSV could be a potential candidate to target the SIRT1/PGC-1α system with a neuroprotective effect in patients with PD. Our results show that RSV can increase both the level and activation of PGC-1α in dopaminergic cells, leading to an increase in antioxidant enzymes and the capacity to combat oxidative stress. The results are summarized in figure 12. The cellular mechanisms how RSV affects PGC-1α transcription remains to be clarified, as the transcription can be affected by multiple pathways (Handschin et al. 2003, Fernandez-Marcos & Auwerx 2011). RSV has been reported to affect CREB and ATF2 (Thiel & Rossler 2016) as well as MEF2 pathways (Gracia-Sancho et al. 2010), all of which may be involved in the transcriptional activation of PGC-1α. The use of RSV as a therapeutic agent would also require careful analysis of its kinetics and biological actions in humans.

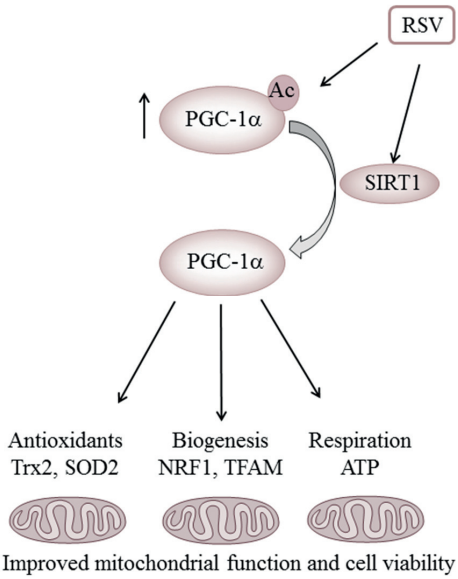

Figure 12. Summary figure. RSV increases PGC-1α transcription and activation, leading to an improved mitochondrial function and cell viability. RSV, resveratrol; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; SIRT1, Sirtuin1; Trx2, thioredoxin2; SOD2, superoxide dismutase 2; NRF1, nuclear respiratory factor1; TFAM, mitochondrial transcription factor A.

5.2.3. Resveratrol treatment enhances DAT protein levels in striatum of female mice

While studying the neuroprotective effect of RSV on MPTP-treated mice (I), we observed that DAT levels were increased in striatum after RSV treatment in female but not male mice. Mice were treated with RSV (20mg/kg i.p.) for four days and tissue was analyzed 8 days after the first RSV injection. Data shows that RSV significantly increased DAT levels in striatum of female mice compared to vehicle treated female mice, as shown by immunoblotting of tissue samples (II/Fig 1A). Multiple bands of DAT appeared on the blot which might represent glycosylated forms of DAT (Li et al. 2004), although this was not further analyzed. Quantification of immunohistochemical stainings showed a significant increase in DAT levels in female mice after RSV treatment compared to vehicle treated female mice (II/Fig 2A,B), further verifying the observed increase in DAT levels in female mice. When analyzing tissue samples from male mice, we found that RSV did not increase DAT levels is striatum of male mice as compared to male controls (II/Fig 1B). The expression levels of DAT in untreated female and male mice did not differ (II/Fig 1C), showing that the basal level was the same in both genders.

ER have been shown to be present in rat brain (Shughrue et al. 1997, Laflamme et al. 1998, Zhang et al. 2002, Creutz & Kritzer 2002). The striking structural similarity of RSV and the synthetic estrogen diethylstilbestrol (Gehm et al. 1997), and studies showing that RSV could act as an ER agonist binding to both ERα and ERβ (Gehm et al. 1997, Bowers et al. 2000, Mueller et al. 2004, Wu et al. 2008, Robb & Stuart 2011) suggest that RSV might mediate its effect via ER.

DAT levels were not significantly increased when measured immediately after the last RSV treatment in female mice (II/Fig 1D). This suggests that RSV could mediate the effect by activating non-genomic ER pathways such as mitogen activated protein kinase (MAPK) signaling (Klinge et al. 2005) and phosphatidylinositol-3-kinase (PI3K) signaling (Pozo-Guisado et al. 2004). TH levels did not change in

striatum after RSV treatment when analyzed by immunoblotting (II/Fig 1E), indicating that the effect was on DAT levels and not a change in DA neuron content.

5.2.4. The effect of resveratrol on DAT expression is mediated by estrogen receptors

To confirm that RSV mediated the effect on DAT via ER, we used ICI 182, 780, a selective antagonist for ERα and ERβ (Van Den Bemd et al. 1999, Howell et al. 2000). Female mice were pretreated with ICI 182, 780 (2 mg/kg s.c.) followed by RSV treatment (20 mg/kg i.p.) 1.5h later for four days, and mice were sacrificed 8 days after the first treatment. Pretreatment with ICI 182, 780 blocked the effect of RSV on DAT levels in striatum. ICI 182, 780 alone did not affect DAT levels in striatum compared to control (II/Fig 3A). This shows that ER are involved in the up-regulation of DAT after treatment with RSV (figure 13), but the data did not reveal whether RSV acts via ERα or ERβ.

The finding that RSV increases the level of DAT in female mice is in line with previous studies showing that estrogen can increase the expression of DAT in mice (Jourdain et al. 2005) and that ovariectomy modulated the level of DAT in rat brain (Bosse et al. 1997). Estrogen has been shown to affect DAT activity *in vitro* by binding to ERα (Watson et al. 2006), and in postmenopausal women estrogen replacement therapy increased DAT levels (Gardiner et al. 2004). A decline in DAT levels is related to PD (Harrington et al. 1996), and a recent study demonstrated that among PD patients, women had a higher DAT activity in striatum than men (Lee et al. 2015), indicating a possible role of ER in the regulation of DAT levels. Also, the lower incidence of PD in women (Twelves et al. 2003, Wooten et al. 2004) indicates that ER could have a role in preventing neurodegeneration by maintaining DA neuron function. The lack of ER increases the vulnerability to MPTP treatment in mice (Al-Sweidi et al. 2011) and RSV has been shown to protect against MPTP-induced cell death via both ERα and ERβ (Saleh et al. 2013), indicating an important role for RSV in PD, possibly by restoring the uptake capacity of DA to maintain neuronal function and prevent ROS generation.

Tamoxifen can function as an ER antagonist or agonist, depending on the tissue that is targeted (Krishnan et al. 2000, Jordan 2003). Tamoxifen has been shown to be able to mimic the effect of estradiol by increasing DAT levels in striatum of ovarectomized rat (Le Saux & Di Paolo 2006). Because of its possible effects on ER, we used tamoxifen to further study the involvement of ER in the regulation of DAT in mice. Female mice were pretreated with tamoxifen (1 mg/kg s.c.) followed by RSV treatment (20 mg/kg i.p.) 1.5h later for four days. Mice were sacrificed 8 days after the first treatment. One group received tamoxifen only, one group received RSV only and one were injected with vehicle. Surprisingly, tamoxifen did not change the levels of DAT in striatum compared to vehicle as shown by WB, suggesting no agonist effect of tamoxifen (II/fig 3B). Tamoxifen treatment did not counteract the increase of DAT in RSV-treated mice either, suggesting no antagonist effect of tamoxifen (II/Fig 3B). Opposite to our expectations, our results showed that tamoxifen alone or in combination with RSV did not have any effect on DAT levels in striatum of female mice. This may be due to different methods for analyzing DAT expression or that tamoxifen has different effects in different species.

Figure 13. Resveratrol (RSV) increases Dopamine transporter (DAT) expression by action on Estrogen receptor α/β (ER α/β).

5.2.5. Resveratrol increases DAT expression in cell cultures

RSV was also able to increase the level of DAT *in vitro*. We used both mouse SN4741 dopaminergic cells and human MESC2.10 cells from embryonic ventral midbrain that were differentiated to dopaminergic cells. DAT levels were increased in mouse SN4741 cells after 10 μM RSV treatment. Treatment with 1 μM E2 also showed an increase in DAT in these cells, although the effect was smaller, while pretreatment with ICI 182, 780 blocked the effect of RSV on DAT shown by WB (II/Fig 4A). Similar results were obtained with MESC2.10 cells (II/Fig 4B). We also observed that the mRNA levels were increased in differentiated MESC 2.10 cells after a 16 h treatment with 10 μM RSV, indicating that RSV increases DAT gene expression (II/Fig 4C). Taken together, these results are in line with the data obtained from our in vivo results and further indicate involvement of ER in the regulation of DAT levels after RSV treatment.

The mechanism how RSV mediates the effect on DAT remains to be studied. RSV may activate non-genomic pathways as has been previously reported for estrogen and other phytoestrogens (Pedram et al. 2002, Beyer et al. 2002, Jeng et al. 2009), or it may be a direct interaction with the ERs ERα, ERβ or with the membrane receptor GPR30 (Maggiolini & Picard 2010). Here we did not address the issue of DAT activation by an increase in protein glycosylation (Li et al. 2004). This would also be of interest to study to clarify whether RSV acts by increasing the expression levels but also by affecting the glycosylation and thereby the activation of DAT.

Our data shows that RSV mediates functions in DA neurons to combat oxidative stress which could be beneficial for the treatment of PD, not only by affecting the expression of PGC-1α. By regulating the expression of DAT, RSV can affect DA levels in the synaptic cleft as DAT is the key mediator of DA uptake (Gainetdinov & Caron 2003). DA uptake and storage in vesicles is of importance since DA can contribute to the production of ROS in the cell (Guillot & Miller 2009, Dias et al. 2013). Oxidative stress can affect DA uptake (Berman et al. 1996) and as RSV can combat ROS production as a scavenger (Leonard et al. 2003) and by increasing PGC-1α-mediated antioxidant levels (I) it is possible that RSV not only affect the expression of DAT but also the activity.

5.3. FGF21 induces the expression of PGC-1α and enhances mitochondrial function in human dopaminergic neurons

5.3.1. FGF21 increases the expression and activity of PGC-1α

FGF21 is a growth factor that functions as a metabolic regulator by affecting glucose and lipid metabolism, and it has been shown to increase the expression of mitochondrial genes and enhance mitochondrial function in adipocytes (Kharitonenkov et al. 2005, Badman et al. 2009, Chau et al. 2010). PGC-1α is a master regulator of mitochondrial function by regulating the gene expression of mitochondrial genes (Houten & Auwerx 2004, Lin et al. 2005), and FGF21 has been shown to increase the expression of PGC-1α in adipose tissue (Wu et al. 2011). Other studies did not show any change in PGC-1α expression levels in adipocytes after FGF21 treatment, but an increase was observed in the expression of genes regulated by PGC-1α, suggesting that FGF21 has an impact on posttranslational modifications of PGC-1α (Fisher et al. 2012). The effect of FGF21 in brain has not been extensively studied, but there are recent studies showing that FGF21 has a neuroprotective effect against excitotoxic injury in primary neurons and in ageing (Leng et al. 2015, Yu et al. 2015). Since mitochondrial dysfunction has been strongly linked to neurodegenerative diseases (Mancuso et al. 2006) we wanted to study the possible effect of FGF21 on PGC-1α and mitochondrial function in dopaminergic neurons.

In this study, we used human MESC2.10 cells from embryonal midbrain. These cells were differentiated to dopaminergic neurons in culture as described in (Lotharius et al. 2002). WB analysis of cells

differentiated for six days showed expression of both TH and DAT which are considered to be markers of dopaminergic neurons (III/Fig 1A). The expression levels of TH and DAT did not differ between untreated cells and cells treated with FGF21, and no morphological changes were observed either between treated and untreated cells.

The expression level of PGC-1α was analyzed by immunoblotting after 24 h treatment of differentiated cells with 50 ng/ml FGF21. Data shows that FGF21 significantly increases the expression level of PGC-1α (III/Fig 1B). These results are in line with findings in adipose tissue (Wu et al. 2011). We were interested to see if the increase in PGC-1α protein level was due to an increase in gene expression. To study this, we used a PGC-1α promoter linked to a luciferase reporter gene. Treatment with FGF21 50 ng/ml for 24 h increased the activity of the PGC promoter but not the pGL3-basic promoter that was used as control (III/Fig 1C). Quantitative PCR also confirmed an increase in mRNA levels of PGC-1α after treatment with FGF21 for 24 h (data not shown). FGF21 has been shown to increase the transcription of PGC-1α by phosphorylating CREB (Wu et al. 2011). Our results show that the transcription of PGC-1α was increased in cells treated with FGF21, although we did not further study the mechanism behind this. It is possible that CREB mediates the elevated levels of PGC-1α gene transcription, but this remains to be studied.

As FGF21 has been implicated to have a role in posttranslational modification of PGC-1α (Fisher et al. 2012), we wanted to see if treatment of differentiated cells with FGF21 also had an impact on the activity of PGC-1α. To analyze whether there was an increase in the activity of PGC-1α the acetylation of the protein was analyzed by immunoprecipitation. Our results show that the degree of acetylation was reduced in cells treated with FGF21 for 24 h compared to untreated cells indicating a higher activity of the protein (III/Fig 1D). Our data indicatse that FGF21 has a similar effect in adipocytes and in dopaminergic neurons in culture where treatment with FGF21 for 24 h increases both the expression and activity of PGC-1α.

5.3.2. FGF21 increases NAD^+ levels and SIRT1 expression

Posttranslational modifications of PGC-1α are known to modulate the activity of the protein (Houten & Auwerx 2004). Deacetylation by SIRT1 is a known posttranslational modification affecting PGC-1α activity (Rodgers et al. 2005, Rodgers et al. 2008, Fernandez-Marcos & Auwerx 2011) and FGF21 has been shown to affect the AMPK-SIRT1-PGC-1α pathway in adipocytes (Chau et al. 2010). As our results show that PGC-1α is deacetylated when treated with FGF21, it was of interest to see if the expression of SIRT1 was affected after treatment with FGF21. Data show that protein levels of SIRT1 was elevated in FGF21-treated dopaminergic neurons (III/Fig2A).

SIRT1 is a NAD^+ dependent deacetylase (Imai et al. 2000), and therefore we were interested to see if there are changes in NAD^+ levels in FGF21-treated cells. NAD^+ levels are crucial in the control of metabolic reactions (Yang et al. 2007), and SIRT1 functions as an energy sensor of the cell mediating cellular responses to changes in energy requirements (Houtkooper et al. 2010, Canto & Auwerx 2012). Nampt is the rate limiting enzyme in NAD^+synthesis, and it has been previously shown to be regulated by nutrient deprivation (Revollo et al. 2004, Yang et al. 2006, Yang et al. 2007). In liver, FGF21 is induced by prolonged fasting (Galman et al. 2008) and overexpression of FGF21 has been shown to increase longevity (Zhang et al. 2012). This suggests that FGF21 might have a role in regulating NAD^+ levels that influence the activity of SIRT1.

We observed that there was an increase in Nampt in FGF21-treated cells after 24 h of stimulation compared to untreated cells (III/Fig 2B). Analysis of the NAD^+/NADH ratio also showed that NAD^+ levels were elevated after FGF21 treatment (III/Fig 2C). These data suggests that FGF21 can modulate

the activity of SIRT1 by affecting Nampt and NAD^+ levels. By pretreating cells with the SIRT1 inhibitor NAM (20 μM) the effect of FGF21 on the acetylation of PGC-1α was counteracted, and NAM alone did increase the degree of acetylation of PGC-1α as expected (III/Fig 2D). Taken together, these data suggests that FGF21 influences the Nampt/SIRT1 pathway which leads to an activation of PGC-1α in these cells (figure 14).

5.3.3. FGF21 increases the levels of mitochondrial antioxidants

Activation of PGC-1α affects the expression of a variety of different genes, among these mitochondrial antioxidants (St-Pierre et al. 2006). We showed in the TG mice overexpressing PGC-1α that the expression of mitochondrial antioxidants were increased in SN of these animals (I). Therefore we wanted to see if treatment with FGF21 was able to increase the expression of antioxidants in these cells. In line with recent reports from heart muscle (Planavila et al. 2015) our results showed that both SOD2 and Trx2 were significantly upregulated after 24 h FGF21 treatment, suggesting that FGF21 via PGC-1α could increase antioxidants in these cells (III/Fig 3A-C). This indicates that FGF21 could have a neuroprotective effect against oxidative stress in dopaminergic neurons by increasing the expression of antioxidants.

5.3.4. FGF21 stimulates mitochondrial respiratory capacity but not copy number

To see if FGF21 had an effect on mitochondrial function in addition to the increase in antioxidant defense, we wanted to analyze the effect of FGF21 on mitochondrial respiratory capacity. The respiratory capacity was analyzed using Seahorse XFe^{96} and the obtained data showed an increase in the basal respiration as well as a higher maximal respiratory capacity compared to untreated cells (III/Fig 4A,B). These data indicates that FGF21 not only increases the antioxidant defense, but also the mitochondrial respiratory capacity. The improved respiratory capacity was most likely due to the activation of the SIRT1/PGC-1α pathway in a similar manner that has been observed in muscle cells (Gerhart-Hines et al. 2007).

PGC-1α is known to regulate mitochondrial biogenesis (Wu et al. 1999, Puigserver & Spiegelman 2003), and we wanted to see if there was an increase in the amount of mitochondria in the cells. TFAM and COX IV were used as a mitochondrial markers. Immunoblotting did not show any changes in the levels of these proteins after treatment with FGF21 compared to control (III/Fig 4C) suggesting that there were no changes in mitochondrial copy number. To further confirm that there were no change in copy number, we analyzed the ratio between mtDNA and nuclear DNA by quantitative PCR as well as the morphology of mitochondria using electron microscopy and measured the relative area of mitochondria. Data obtained showed no change in the ratio of mtDNA to nuclear DNA between treated and untreated cells (III/Fig 4D), indicating an equal number of organelles in FGF21-treated and untreated cells. The relative mitochondrial areas showed no difference either (III/Fig 4 E,F). These data indicate that the increase in mitochondrial efficacy is not related to an increase in the number of mitochondria in the cells. It is possible that changes in mitochondrial quality control and mitophagy might contribute to maintaining a stable level of functional mitochondria, but this remains to be studied.

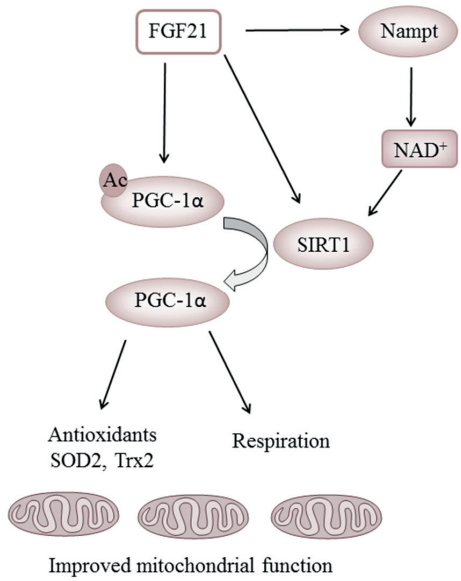

Figure 14. Summary figure. FGF21 increases PGC-1α expression and activation which improves mitochondrial function in dopaminergic neurons. FGF21, fibroblast growth factor 21; Nampt, Nicotinamide phosphoribosyl transferase NAD^+, Nicotinamine dinucleotide, oxidized form; SIRT1, sirtuin1; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; SOD2, superoxide dismutase 2; Trx2, thioredoxin2.

5.3.5. FGF21 is expressed in brain

The expression of FGF21 in the brain has not been extensively studied. It is known that members of the FGF family are expressed in the central nervous system, and FGFs are thought to have important functions during development but also in the mature brain (Mudo et al. 2009, Itoh & Ornitz 2011). To study whether FGF21 is present in the brain, we performed immunoblotting from tissue samples from different regions of mouse brain. Our results showed that FGF21 was detected in different brain regions, including SN and striatum. Also, *in vitro* analysis of primary glial cultures from neonatal rat brain did show an expression of FGF21 in these cells. Human hepatocyte Huh7 cell line was used as a positive control for FGF21 expression as FGF21 is known to be expressed in hepatocytes (Nishimura et al. 2000). This data suggests that glial cells can produce FGF21 *in vitro*, but it requires further studies to determine which cells express FGF21 in the brain.

FGF21 has been shown to pass the blood brain barrier (Hsuchou et al. 2007), and it is possible that FGF21 could be synthesized in the liver and exert its effect on neurons by entering the brain by crossing the blood brain barrier. It is of great interest to see whether FGF21 could also be expressed in the brain, and there contribute to sensing the energy requirements of the cells and regulate metabolism. It would also be of importance to study the localization of the receptors that mediate FGF21 signaling in the brain. β-Klotho is known to be required as a cofactor for FGF21 signaling (Suzuki et al. 2008, Kharitonenkov et al. 2008) and analysis of the localization of FGF receptors and βKlotho by in situ hybridization and immunohistochemistry might reveal the target cells in FGF21 signaling in the brain.

The ability of FGF21 to enhance the mitochondrial respiratory capacity and altering gene expression pathways regulated by PGC-1α in dopaminergic neurons suggests that FGF21 may be of value in neuroprotection in PD. It would be of interest to determine which cells express FGF21 in the brain and also study the potential benefits of FGF21 in different neurological diseases.

5.4. The PPARγ agonist GW1929 affects PGC-1α expression via cAMP-PKA-CREB pathway and improves mitochondrial function in human dopaminergic neurons

5.4.1. PPARγ agonist GW1929 increases the protein level and activity of PGC-1α

PPARγ is a transcription factor that regulates genes involved in glucose and lipid metabolism (Chen et al. 2012, Ahmadian et al. 2013). PPARγ agonists are used in treatment of type 2 diabetes due to their ability to increase insulin sensitivity and regulate cellular metabolism (Yki-Jarvinen 2004). PPARγ agonists have also been shown to be neuroprotective in both rotenone-induced and MPTP-induced mouse models of PD by increasing antioxidants and reducing ROS in the brain (Martin et al. 2012, Aleshin & Reiser 2013, Corona et al. 2014, Mounsey et al. 2015). PGC-1α was first described as a coactivator of PPARγ in adipose tissue (Puigserver et al. 1998), and later the PGC-1α gene has been shown to be a direct target of TZD in BAT and WAT (Hondares et al. 2006).

In the search for compounds that could act as PGC-1α activators in neurons PPARγ agonists might be of importance. As TZD has been reported to have severe side effects (Consoli & Formoso 2013), we used a non-TZD compound GW1929 as PPARγ agonist to study the effect on PGC-1α and the underlying mechanism for PGC-1α expression. In this study, we used MESC2.10 cells that were differentiated to dopaminergic neurons. Treatment with 1 μM GW1929 did not alter the expression of DAT or TH in cells that were differentiated for 6 days (IV/Fig 1A,B).

Treatment with GW1929 for 24 h showed an increase in PGC-1α levels in a concentration dependent manner (IV/Fig 1B,C). GW1929 was also able to activate PGC-1α as shown by the reduction in acetylation (IV/Fig 1D,E). This shows that GW1929 is able to increase the expression and activation of PGC-1α in these cells. The results are in line with previous studies showing that the TZD Rosiglitazone can increase the expression of PGC-1α (Corona et al. 2014). SIRT1 levels were also increased in GW1929 treated cells (IV/Fig 1F,G), indicating that SIRT1 activates PGC-1α.

5.4.2. GW1929 increases the transcription of PGC-1α via CRE

The effect of GW1929 on PGC-1α transcription was studied using quantitative PCR and the results showed an increase in PGC-1α levels in cells treated with 1 μM GW1929 for 24h after 6 days of differentiation (IV/Fig 2A,B). It is possible that GW1929 acts directly on PGC-1α transcription by binding to the PPAR site in the promoter (Hondares et al. 2006), but our results suggest that GW1929 increases the transcription of PGC-1α by activating CREB. PGC-1α promoter activity measurements with cells differentiated for 2 days prior to GW1929 treatment showed that the activity was increased in GW1929-treated cells when employing the wildtype PGC-1α promoter linked to luciferase reporter gene, but not with the PGC-1α promoter with a mutation in the CRE site (IV/Fig 2C). This suggests that GW1929 stimulates the expression of PGC-1α via the CRE sequence in the promoter. CREB has been shown to increase the expression of SIRT1 (Noriega et al. 2011, Fusco et al. 2012), and we also observed an increase in SIRT1 mRNA levels in GW1929-treated cells (IV/Fig 2A,B). This further indicates a possible CREB activation in GW1929 treated cells.

5.4.3. GW1929 activates the cAMP-PKA-CREB pathway

The strong indication of the involvement of CREB in the regulation of PGC-1α expression gave rise to the question if GW1929 could affect the phosphorylation of CREB. Data showed that addition of GW1929 increased the phosphorylation of CREB after 0.5 h reaching a peak at 3 h (IV/Fig 3A,B). Co-treatment with the PKA inhibitor H89 blocked the phosphorylation of CREB (IV/Fig 3C,D) as well as the level of PGC-1α (IV/Fig 3E,F), suggesting involvement of the CREB pathway in the regulation of

PGC-1α expression that is in line with previous studies (Handschin et al. 2003, Fusco et al. 2012). Measurement of cAMP levels revealed a small but significant increase in GW1929-treated cells compared to control (IV/Fig 3G) suggesting that the cAMP-PKA pathway is involved in the phosphorylation of CREB. The increase in cAMP level after GW1929 treatment showed a delay compared to forskolin treatment, suggesting that the effect could be mediated by changes in protein levels in the cell. The cAMP level is known to be increased by the activity of ACs (Lonze & Ginty 2002, Kamenetsky et al. 2006, Steegborn 2014) and co-treatment with the AC inhibitor SQ22536 reduced the level of CREB phosphorylation in cells treated with GW1929 (IV/Fig I,J), suggesting that AC is increasing the level of cAMP that acts as a CREB activator via PKA.

The AC6 isoform was recently shown to be induced by the PPARγ-activator Rosiglitazone (Desch et al. 2010). Treating the cells with GW1929 did increase mRNA levels of AC6 (IV/Fig 3H). These results suggest that AC6 expression may contribute to the effect of GW1929 on cAMP levels, but further studies are required to confirm this. It is also possible that other AC subunits have PPARγ binding sites, but this is not known.

The finding that GW1929 can increase cAMP levels and induce the transcription of PGC-1α is of importance for neuroprotection in dopaminergic neurons. The increase in cAMP levels has been suggested to mimic caloric restriction by direct binding of cAMP to SIRT1, which increases the activity of SIRT1(Wang et al. 2015), further suggesting an important role of cAMP in neuroprotection by contributing to the activation of PGC-1α and increased mitochondrial function.

5.4.4. GW1929 increases mitochondrial biogenesis and respiration

In line with data obtained from our TG mice (I), we observed an increase in mitochondrial biogenesis in GW1929-treated cells. The levels of NRF1 (IV/Fig 4A,B) and TFAM (IV/Fig 4C,D), two major transcription factors regulating biogenesis (Wu et al. 1999), were increased in cells treated with GW1929 compared to untreated cells. GW1929 also increased the mtDNA copy number (IV/Fig 4E), further showing an effect on mitochondrial biogenesis. These results are in line with results showing that PPARγ agonists does promote mitochondrial biogenesis in non-neuronal cells (Bogacka et al. 2005, Rong et al. 2007, Rong et al. 2011). The increase in mitochondrial biogenesis is most likely due to an increase in PGC-1α expression and activation, but further studies with inhibitors or silencing of PGC-1α would be needed to confirm this.

To study whether the increase in mitochondrial copy number improved the mitochondrial efficacy mitochondrial respiration in differentiated cells was analyzed. Treatment with GW1929 for 24 h did show an increase in the basal respiration compared to untreated cells (IV/Fig 4F,G), but the change in maximal respiratory capacity and the spare respiratory capacity was not statistically significant. The increase in basal respiration indicates that there is an increase in the bioenergetic capacity of these cells. The increase could be due to the increase in mitochondrial copy number, or PGC-1α might increase the expression of some specific proteins in the respiratory chain and thereby increase the efficiency of the respiratory chain. This issue was not studied here, but PGC-1α is known to increase the expression of components of the respiratory chain (Wu et al. 1999).

5.4.5. GW1929 increases mitochondrial antioxidants and protects against oxidative stress

We have previously shown that PGC-1α increases the expression of the mitochondrial antioxidants SOD2 and Trx2 (I, III). PPARγ activation has also been shown to be neuroprotective in the MPTP model of PD by increasing the level of antioxidants (Martin et al. 2012). In line with this, treatment of

differentiated cells with GW1929 for 24 h did increase the expression of both SOD2 and Trx2 (IV/Fig A-D). To further study possible effects to combat oxidative stress in dopaminergic neurons, we treated cells with H_2O_2 or the complex I inhibitor rotenone that induces the production of ROS (Radad et al. 2006). GW1929-treated cells were more resistant to both H_2O_2 and rotenone-induced cell death compared to controls (IV/Fig 5E,F), suggesting that GW1929 can be neuroprotective by upregulating the expression of antioxidant enzymes to combat oxidative stress.

PGC-1α-deleted mice have been shown to be vulnerable to oxidative stress (St-Pierre et al. 2006), and treatment with PPARγ agonists could have a protective role in neurodegenerative diseases by affecting the expression and activation of PGC-1α and enhancing mitochondrial function and antioxidant defense in dopaminergic neurons. A summary is shown in figure 15. These data are supported by findings showing that compounds used for treatment of type 2 diabetes, such as TZD, have neuroprotective potential in neurodegenerative diseases (Patrone et al. 2014), and the PPARγ agonists have been shown to have beneficial effects in treatment of PD (Aviles-Olmos et al. 2013, Carta & Simuni 2015).

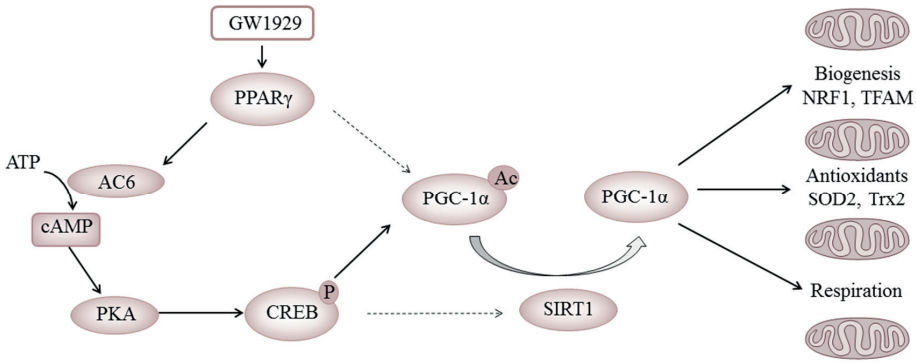

Figure 15. Summary figure. PPARγ increases cAMP levels and affects PGC-1α expression via CREB to enhance mitochondrial function and protect against oxidative stress. cAMP, cyclic AMP; AC6, adenylyl cyclase 6; PKA, protein kinase A; CREB, cyclic AMP response element binding protein; PPARγ, peroxisome proliferator-activated recepror γ; PGC-1α, peroxisome proliferator-activated recepror γ coactivator-1α; SIRT1, sirtuin1; NRF1, nuclear respiratory factor1; TFAM, mitochondrial transcription factor A, SOD2, suoeroxide dismutase2, Trx2, thioredoxin 2.

6. CONCLUSIONS AND FUTURE PROSPECTS

The aim of this work was to clarify the possible beneficial effects of PGC-1α in dopaminergic neurons and to search for compounds that could act as PGC-1α activators in these cells. In this work, we show that transgenic expression of PGC-1α has a neuroprotective effect on MPTP-induced dopaminergic neuron death in mice. This may be due to the increase in mitochondrial respiratory capacity and antioxidant enzymes that was observed in PGC-1α TG mice. Regulating the expression and activation of PGC-1α could serve as a useful tool in preventing degeneration of dopaminergic neurons in PD. However, the increase in PGC-1α needs to be carefully controlled, since a high overexpression has been shown to contribute to cell death.

Compounds that are able enhance the expression of PGC-1α in dopaminergic neurons would be of interest in the development of therapies for patients with PD. We showed that RSV was able to mimic the effect of PGC-1α overexpression in mice treated with MPTP. The beneficial effect of RSV on neuronal survival was thought to be mediated by PGC-1α, as treatment of neuronal cells with RSV was able to increase the expression and activation of PGC-1α as well as increase the expression of proteins that are known to be regulated by PGC-1α.

RSV was also capable of increasing the expression of DAT in female but not male mice, suggesting that the effect could be mediated by ER. The same effect on DAT expression was observed in cells treated with estrogen, indicating that ER might serve as mediators of DAT expression. By increasing the expression of DAT the reuptake of DA in cells is more efficient and this could prevent DA to be involved in the production of ROS, but also help maintain the cognitive functions of the patient. The decline in DAT levels is related to PD, and by increasing the expression of DAT RSV might not only protect against oxidative stress but also improve dopaminergic neuron viability by maintaining the function of the neurons. If using RSV as a therapeutic compound in PD, it should be taken into account that the neuroprotective effect of RSV might differ depending on the gender of the patient as we here show that DAT expression is mediated by ER.

We also observed that FGF21 is able to regulate the expression of PGC-1α, as well as the activation of PGC-1α, by affecting SIRT1 expression and the NAD^+/NADH ratio in dopaminergic neurons, and thereby improve mitochondrial function and antioxidant defense in these cells. This implies that therapies affecting the level of FGF21 might serve as a treatment for patients with PD and other neurodegenerative diseases. FGF21 did not affect the mitochondrial biogenesis, but the protective effect could be due to the increase in antioxidant defense. The lack of increase in mitochondrial biogenesis might also be due to an increase in mitochondrial quality control, but this remains to be studied.

Mitochondrial function and PGC-1α expression was also improved by activating PPARγ with the non-thiazolidinedione ligand GW1929. GW1929 was able to increase the level of cAMP and activate the PKA-CREB pathway, leading to an increased PGC-1α expression. GW1929 was also able to increase the expression of SIRT1. This might be due to the activation of CREB, although it remains to be clarified. The increase in PGC-1α expression and activation in DA neurons treated with GW1929 showed an increase in the expression of antioxidant enzymes, and ROS-induced cell degeneration was also reduced in GW1929-treated cells compared to control.

PPARγ and FGF21 have been shown to be regulating each other in adipose tissue. It could be of interest to study if there would be an additive effect by treating dopaminergic neurons with both compounds simultaneously. The positive effect of these metabolic regulators on dopaminergic neuron survival shows that drugs used in metabolic diseases, such as type 2 diabetes drugs, might also have a role in preventing the progression of PD and other neurodegenerative diseases.

Taken together, the results show that PGC-1α has neuroprotective effects in toxin-induced models of PD and the PGC-1α activators have beneficial effects in dopaminergic cell survival. It is, however, of importance to find biomarkers for early intervention in the progress of the disease. At the state when motor symptoms are visible the majority of dopaminergic neurons are already degenerated. If the diagnosis could be done at an earlier stage, it could be possible that treatments targeting mitochondrial dysfunction and oxidative stress would be beneficial for the patient. Different ways of fine-tuning the antioxidant defense and preventing the formation of ROS is of importance in preventing the pathology of PD to proceed. Compounds that are able to regulate the expression of PGC-1α in dopaminergic neurons might have an important role in the development of therapies to combat the loss of dopaminergic neurons in patients with PD.

7. ACKNOWLEDGEMENTS

This thesis was carried out in Minerva Foundation Institute for Medical Research and Department of Biochemistry and Developmental Biology, Faculty of Medicine, University of Helsinki. The work was financially supported by the the Minerva Foundation, Svenska Kulturfonden, Finnish Parkinson Foundation, Magnus Ehrnrooth Foundation and Oskar Öflund Foundation and by the general grants to the Lindholm group from Sigrid Juselius Foundation and Academy of Finland.

I would like to thank my supervisor Professor Dan Lindholm for giving me the opportunity to do my doctoral thesis in his research group. I am grateful to Dan for the guidance and support I have received on becoming a scientist. I would also like to thank Professor Poul Henning Jensen for being my opponent and my thesis reviewers and Professor Kid Törnqvist and Docent Mikko Airavaara for giving me helpful comments on my thesis. I also wish to thank Professor Tomi Taira and Professor Kid Törnqvist for making it possible for me to finalize my thesis.

I want to acknowledge the co-authors for their contribution: Dr. Timofey Tselykh, Dr. Ove Eriksson, Dr. Jyri Kukkonen, Dr. Minna Kairisalo, Dr. Annika Mälkiä, M.Sc. Hai Thi Do, Dr. Petri Piepponen and from Italy Dr. Guiseppa Mudò, Dr. Natale Belluardo, Dr. Alessandra Bonomo, Dr. Valentina Di Liberto, Dr. Florinda Maiorana, Dr. Melania Olivieri and Dr. Jose A Aguirre.

I wish to thank all the past and present members of the Lindholm lab and all the people at Minerva, it has been a pleasure to work with all of you. I wish to thank Laura for helping with many practical things in the lab, especially when I first started to work at Minerva. I would also like to thank Vesa for always being supportive and encouraging and Carita and Cia for all the help with all the practical issues during the years. I want to thank Kristiina for all the technical assistance and Eeva for all the help I have received in the lab and for being the one who knows how to solve almost any problem. I wish to thank Noora for the great company both in the lab and during different kinds of sports activities and Raili and Alise for the nice company at conferences abroad. I want to thank Celine and Tima for all the help I have received during the years we have worked together and Annukka for the help with my thesis. I also wish to thank Kata and Riikka for all the coffee break discussions during my years at Minerva.

I wish to thank my friends, Nina, Anna, Titti, Linn and Sussu, for all the fun moments during all these years, and for reminding me that there is a life outside science. A special thank you to Nina for all the discussions, support and help you have given me, I will always be thankful for that.

A special thank you to my family, mum and dad for your love and support and for always believing in me, and to Seba, Minna, Max, Axel and Robin for bringing so much joy to my life.

Finally, I would like to express my deepest love and gratitude to Jari. Thank you for your endless love and support and for always being there for me, even at my weakest moments. You have showed me that I can do so much more than I ever thought was possible. The sky is the limit when I am with you.

Helsinki, May 2016

Johanna

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Helsinki 2016 ISSN 2342-3161 ISBN 978-951-51-2083-0