INVESTIGATING THE INTRACELLULAR MOLECULAR MECHANISMS OF PARKINSON’S DISEASE

By

PANAGIOTIS SOTIRIOU ATHANASOPOULOS

A thesis submitted for the fulfillment of the requirements for the Degree of Dr. rer. nat. (PhD)

From the Graduate School of Chemistry and Biochemistry (GSCB)

Ruhr University Bochum

March 2015

This research work was conducted at the Department of Molecular Neurobiochemistry, Faculty of Chemistry and Biochemistry, Ruhr University Bochum, under the supervision of Prof. Dr. Rolf Heumann and Prof. Dr. Stephan Hahn as a co-supervisor.
To my father
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>12</td>
</tr>
<tr>
<td>Part I</td>
<td></td>
</tr>
<tr>
<td>1. Introduction</td>
<td>13</td>
</tr>
<tr>
<td>1.1 PD in general</td>
<td>13</td>
</tr>
<tr>
<td>1.2 The LRRK2 protein; domains of LRRK2, founder mutations, epidemiology and possible mechanisms of LRRK2’s induced neurotoxicity in PD</td>
<td>14</td>
</tr>
<tr>
<td>1.3 Important phosphosites of the LRRK2 protein and their biological importance</td>
<td>17</td>
</tr>
<tr>
<td>1.4 Protein Phosphatase 2A in general</td>
<td>19</td>
</tr>
<tr>
<td>1.5 The role of PP2A in alpha-synuclein correlated PD</td>
<td>20</td>
</tr>
<tr>
<td>1.6 The role of PP2A regarding Tau phosphorylation levels and Alzheimer’s Disease</td>
<td>21</td>
</tr>
<tr>
<td>1.7 Phosphatases of LRRK2</td>
<td>22</td>
</tr>
<tr>
<td>1.8 Neuronal Ras activation is able to protect against chemically-induced parkinsonian mouse models</td>
<td>22</td>
</tr>
<tr>
<td>1.9 PP2A is able to activate the Ras pathway through dephosphorylation of Ksr and Raf</td>
<td>22</td>
</tr>
<tr>
<td>1.10 Aim of this study</td>
<td>24</td>
</tr>
<tr>
<td>2. Materials and Methods</td>
<td>26</td>
</tr>
<tr>
<td>2.1 Bacterial protein expression and GST pull down of the ROC protein</td>
<td>26</td>
</tr>
<tr>
<td>2.2 Staining of SDS gels by Coomassie Brilliant Blue R-250</td>
<td>27</td>
</tr>
</tbody>
</table>
2.3 GFP-TRAP for immunoprecipitation of GFP-fusion proteins
2.4 GST-RBD pull down
2.5 Immunoprecipitation of LRRK2 protein from SH-SY5Y and Hela cells
2.6 Confocal Microscopy and Proximity ligation assay for Hela cells
2.7 Immunohistochemistry
2.8 Cell culture transfection and cell lysis
2.9 Transfection reagents for secondary cell lines
2.9.1 GenJet for N2A cells
2.9.2 Xfect transfection reagent for Hela/HEK293T cells
2.9.3 Lipofectamine LTX for SH-SY5Y/HEK293T cells
2.10 Calcium phosphate transfection for cortical neurons
2.11 Protein determination by DCTM Protein Assay
2.12 SDS polyacrylamide gel electrophoresis
2.13 Protein transfer to nitrocellulose membrane
2.14 Western blotting
2.15 Primary antibodies
2.16 Given plasmids and shRNA plasmids targeting PP2Ac
2.17 Midi Prep plasmid purification
2.18 Agarose gel electrophoresis
2.19 DNA digestion by restriction enzymes
2.20 DNA ligation
2.21 Transgenic Mice and cortical neurons culture
2.22 Genotyping of BAC-LRRK2-G2019S mice
2.23 Insertion of WT-LRRK2 and R1441C-LRRK2 DNA sequence to pCDNA3.0 vector
2.24 PCR for GST-ROC-WT and GST-ROC-R1441C proteins
2.25 PCR for EGFP-ROC-WT and EGFP-ROC-R1441C proteins
2.26 Cell death and cell viability assay 42
2.27 Sodium selenate treatment for the Ras pathway activation 42
2.28 Mant GTPγS binding assay of the ROC proteins 43
2.29 Statistics 43

3. Results 44
3.1 The ROC domain of LRRK2 is sufficient for interaction with PP2A 44
3.2 LRRK2 and PP2Aa are interacting in the perinuclear region of Hela cells 48
3.3 Knockdown of the catalytic subunit of PP2A (PP2Ac, alpha and beta isoforms) results in aggravated cell death in transiently R1441C-LRRK2-transfected SH-SY5Y cells and G2019S-expressing cortical neurons 52
3.4 Activation of PP2A by sodium selenate partially rescues transiently R1441C-LRRK2-transfected SH-SY5Y cells, while there no statistically significant effect of sodium selenate in G2019S cortical neurons 54
3.5 Sodium selenate treatment is able to activate the Ras downstream pathway in SH-SY5Y cells and G2019S cortical neurons 58

4. Discussion 61
4.1 The ROC domain of LRRK2 is efficient to bind PP2A 61
4.2 The WT- and the R1441C-ROC of LRRK2 binds equally to PP2A, while LRRK2 interacts with PP2A in the perinuclear region of cells 61
4.3 Elimination of PP2Ac aggravates parkinsonian-cell death in neuronal models, while chemically-induced activation of PP2A protects from the toxic LRRK2 mutants 61
4.4 Correlation of shRNA-PP2Ac and sodium selenate systems with biological systems 62
4.5 Mechanisms of neuroprotection by sodium selenate in toxic LRRK2 parkinsonian models 62
4.6 Possible future experiments to be done 65
5. References

Part II

1. Introduction
   1.1 VDAC in general
   1.2 Role of the mitochondrial VDAC/VDAC-1 in the apoptosis pathway
   1.3 Plasma membrane VDAC-1 and its link to apoptosis
   1.4 Plasma membrane VDAC-1 and Ras neuroprotectivity
   1.5 Aim of the study

2. Materials and Methods
   2.1 Plasmids
   2.2 Knockdown of VDAC-1 -Western Blotting
   2.3 Ferri cyanide reductase assay
   2.4 Statistics

3. Results
   3.1 The ROC domain of LRRK2 is able to interact with VDAC/VDAC-1
   3.2 The plasma membrane VDAC-1 is the main enzyme responsible for the reductase activity of plasma membrane enzymes in HEK293T cells
   3.3 The ROC-WT protein is able to reduce the reductase activity of the plasma membrane enzymes of HEK293T cells, while the ROC-R1441C is also reducing the reductase activity levels of the plasma membrane VDAC-1 in a lesser extent
4. Discussion 85

4.1 Association of VDAC/VDAC-1 with the ROC domain of LRRK2 and reduction of plasma membrane VDAC-1 activity in the presence of WT ROC indicate a chaperone-like function of LRRK2 towards plasma membrane VDAC-1 85

4.2 Future experiments to be done 86

5. General hypothesis of the neuroprotective role of PP2A and pl-VDAC-1 in LRRK2-induced Parkinson’s Disease 89

6. References 91

7. Appendix 95

7.1 Curriculum Vitae 95

7.2 List of Publications 98

7.3 Invited Oral speaker in Conferences and Workshops 98

7.4 Poster speaker in Conferences and Workshops 98

7.5 Acknowledgments 98
## List of Figures

### Part I

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic representation of the domains of LRRK2</td>
<td>15</td>
</tr>
<tr>
<td>1.2</td>
<td>Schematic representation of multiple signaling pathways leading to PD</td>
<td>16</td>
</tr>
<tr>
<td>1.3</td>
<td>Phosphosites/phosphatases of the LRRK2 protein</td>
<td>18</td>
</tr>
<tr>
<td>1.4</td>
<td>Structure of the PP2A holoenzyme</td>
<td>19</td>
</tr>
<tr>
<td>1.5</td>
<td>Structure of alpha-synuclein, phosphorylation sites and role of the PP2A</td>
<td>20</td>
</tr>
<tr>
<td>1.6</td>
<td>Scheme of the Tau protein, its phosphosites and possible phosphatases that regulate its phosphorylation status</td>
<td>21</td>
</tr>
<tr>
<td>1.7</td>
<td>Dephosphorylation of Ksr in Ser392 and Raf in Ser259 by PP2A, can lead to MEK/Erk pathway activation</td>
<td>23</td>
</tr>
<tr>
<td>3.1</td>
<td>The ROC domain of LRRK2 and the full length LRRK2 associates with PP2A</td>
<td>45</td>
</tr>
<tr>
<td>3.2</td>
<td>Binding of the three PP2A subunits to ROC-WT versus ROC-R1441C and functionality of the ROC domains as GTPases</td>
<td>47</td>
</tr>
<tr>
<td>3.3</td>
<td>The LRRK2 antibody is specific for immunocytochemistry</td>
<td>49</td>
</tr>
<tr>
<td>3.4</td>
<td>The venus fused LRRK2 protein has the same localization with that of FLAG-WT LRRK2</td>
<td>50</td>
</tr>
<tr>
<td>3.5</td>
<td>The endogenous LRRK2 is associating with endogenous PP2Aa in Hela cells</td>
<td>51</td>
</tr>
<tr>
<td>3.6</td>
<td>The endogenous LRRK2 is associated with endogenous PP2Aa in the substantia nigra region of littermate mice</td>
<td>52</td>
</tr>
</tbody>
</table>
Figure 3.7  Transient knockdown of the PP2Ac subunit results in aggravation of cell death in transiently transfected with R1441C LRRK2- SH-SY5Y cells  

Figure 3.8  Sodium selenate has a neuroprotective effect in transiently transfected with R1441C LRRK2- SH-SY5Y cells  

Figure 3.9  Transient knockdown of the PP2Ac subunit results in aggravation of cell death in G2019S cortical neurons, while treatment with sodium selenate is not able to reduce apoptosis/cell death in a significant extent  

Figure 3.10 Sodium selenate is able to activate the Ras downstream pathway in SH-SY5Y cells and G2019S cortical neurons  

Figure 4.1 Schematic representation of the role of PP2A activation in LRRK2-induced cell death  

Part II

Figure 1.1  3-dimensional structure of VDAC-1  

Figure 3.1  The GST-ROC-R1441C binds less to VDAC compared to GST-ROC-WT  

Figure 3.2  The GST-ROC-R1441C binds less to VDAC-1 compared to GST-ROC-WT  

Figure 3.3  pl-VDAC-1 is the main responsible enzyme for the ferricyanide reductase activity of the HEK293T cells in their plasma membrane  

Figure 3.4  Expression of ROC-WT in HEK293T cells is able to reduce pl-VDAC-1’s reductase activity, while ROC-R1441C restores it partially
Figure 5.1 General scheme of PP2A and pl-VDAC-1 protecting from mutant LRRK2-induced neurotoxicity 90

List of Tables

Part I

Table 1.1 Autosomal dominant and recessive genes correlated with PD 14
Table 1.2 Statistics of familial PD or sporadic PD patients in different countries 15

List of Important Abbreviations

AD Alzheimer’s Disease
PD Parkinson’s Disease
l-Dopa levodopa
LRRK2 Leucine-rich repeat kinase 2
AD Autosomal dominant
AR Autosomal Recessive
ROC Ras of Complex
ARM Armadillo repeats region
ANK Ankyrin repeats region
N-terminal Amino terminal
C-terminal Carboxy terminal
CIP  alkaline calf intestinal phosphatase
LRR  Leucine-rich repeat
fPD  familial Parkinson’s Disease
sPD  sporadic Parkinson’s Disease
PINK-1  PTEN-induced putative kinase 1D
S1403  Serine 1403
T1503  Threonine 1503
WT  Wild type
PP1  Protein Phosphatase 1
PP2A  Protein Phosphatase 2A
PP2Aa  PR65 regulatory subunit of PP2A
PP2Ab  Regulatory subunit of PP2A
PP2Ac  Catalytic subunit of PP2A
OA  Okadaic Acid
shRNA  Short hairpin RNA
PLA  Proximity Ligation Assay
bp  Base pairs
BSA  bovine serum albumin
IPTG  Isopropyl β-D-1-thiogalactopyranoside
6-OHDA  6-hydroxydopamine
MPP+  1-methyl-4-phenyl pyridinium
GST  Glutathione-S- transferase
DNA  Deoxyribonucleic acid
RBD  Ras Binding complex of Raf
DMEM  Dubelcco’s Modified Eagles Medium
kDa  Kilodalton
hr  Hour
PAA  Polyacrylamide
PBS
phosphate buffered saline

PCR
Polymerase chain reaction

EGFP
Enhanced Green Fluorescent Protein

mRNA
messenger RNA

RNA
Ribonucleic acid

RT
Room Temperature

SDS
Sodium dodecyl sulfate

SDS PAGE
Polyacrylamide gel electrophoresis

TBS
Tris buffered saline

TBS-T
TBS with Tween 20

TEB
Tris/ EDTA/ Borate buffer

TEMED
N, N, N’, N’-Tetramethyl ethylenediamin

U
Units

UV
ultraviolet

E.Coli
Escherichia coli

EDTA
Ethylenediamintetraacetic acid

VDAC
Voltage Dependent Anion Channel

OMM
Outer mitochondrial membrane

PTP
Permeability transition pore

SynRas
synapsin-promoter driven Ras expression

mt-VDAC-1
Mitochondrial VDAC-1

pl-VDAC
Plasma membrane VDAC-1

Keywords
Parkinson’s Disease ● LRRK2 ● R1441C ● G2019S ● ROC ● PP2A ● Neurodegeneration ● Neuroprotection ● Sodium Selenate ● Ksr ● VDAC ● NADH reductase activity ● Apoptosis ● chaperone
Abstract

Mutations in the leucine-rich repeat kinase 2 (LRRK2) are the leading cause of genetically inherited Parkinson’s disease (PD). Two of the common found mutations namely R1441C and G2019S, mediate their toxic effects through their increased kinase activity. Furthermore, the kinase activity of LRRK2 is regulated through some essential serine and threonine autophosphorylation residues that are located in the LRR, ROC and kinase domains. In this study we identified Protein Phosphatase 2A (PP2A), as an interacting partner of LRRK2. Additionally we were able to demonstrate that only the ROC domain is needed to interact with the three subunits of PP2A in SH-SY5Y cells and in Hela cells. We have shown that the alpha subunit of PP2A (PP2Aa) is interacting with LRRK2 in the perinuclear region of Hela cells. We also investigated the physiological role of PP2A in SH-SY5Y cells transiently expressing R1441C-LRRK2, by silencing the catalytic subunit of PP2A (PP2Ac). As a result, cell death which was induced by R1441C-LRRK2 was significantly aggravated. Finally, pharmacological activation of PP2A by sodium selenate in SH-SY5Y cells transiently transfected with R1441C-LRRK2, showed a partial neuroprotection from the mutation. All these data suggest that PP2A is a new interacting partner of LRRK2 and reveal the importance of PP2A as a potential therapeutic target in PD.

The second part of my PhD Thesis focuses in VDAC-1, which is a protein also correlated with PD mitochondrial induced cell death. Initially we found that the ROC domain of LRRK2 is the domain, through which the interaction with LRRK2 takes place. When comparing the binding of the ROC-WT to the VDAC versus the ROC-R1441C, we noticed that the ROC-WT protein binds in a much bigger extent to the total VDAC levels, compared to the ROC-R1441C protein. Additionally, using reductase assays, plasma membrane VDAC-1 seems to be accountable for the majority of the reductase activity in HEK293T cells. After expressing ROC-WT or R1441C proteins in HEK293T cells, the total reductase activity levels in ROC-WT transfected HEK293T cells was reduced, compared to ROC-R1441C transfected cells. These data suggest that the ROC/LRRK2 protein could possibly bind the plasma membrane-VDAC-1 as a chaperone and reduce its reductase enzyme properties. On the other hand, in the presence of R1441C mutation, ROC-R1441C cannot bind plasma membrane VDAC-1 so efficiently and this leads to elevated reductase activity levels of plasma membrane VDAC-1.
Part I

1. Introduction

1.1 PD in general

Parkinson’s Disease (PD) is one of the most prevalent neurodegenerative diseases, second most common after Alzheimer’s Disease. PD patients account for 1-2% of individuals over 60 years of age. The motor syndrome of PD is characterized by akinesia, rigidity, rest tremor and postural disturbances, but it is also increasingly recognized that non-motor syndromes, including autonomic and cognitive disturbances, are also characteristic symptoms of the disease. A typical characteristic of the PD is the loss of dopaminergic neurons of the substantia nigra, resulting in a less production of the neurotransmitter dopamine in the striatal projection areas of these neurons. One of the most efficient clinical treatments for PD patients is currently, a dopamine replacement treatment using levodopa (L-Dopa), which ameliorates motor syndroms. Levodopa is the precursor to dopamine. However regarding non motor symptoms, they might be based to a different pathological basis, and therefore they are incompliant to dopaminergic drugs (Poewe, 2009). PD is characterized in a cell level, by intraneuronal inclusions of various shapes and subcellular accumulations of proteins, such as alpha synuclein and LRRK2 (Lewy bodies).

Depending on the family history of PD patients, there two basic groups of patients; the sporadic patients and the familial patients. For the familial patients, different genes have been found to be correlated with PD. Mutations in these genes are able to influence the disease risk (Gasser et al. 2009). As shown in Table 1.1, different genes could affect familial PD, being active as autosomal or recessive genes. The Leucine-rich-repeat kinase 2 (LRRK2) has been found to be one of these genes.
### Genetic loci and genes associated with monogenic forms of Parkinson’s Disease (PARK loci)

<table>
<thead>
<tr>
<th>Locus</th>
<th>Inheritance</th>
<th>Gene</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARK1</td>
<td>AD</td>
<td>SNCA</td>
<td>First PD gene identified; protein is major component of lewy bodies</td>
</tr>
<tr>
<td>PARK2</td>
<td>AR</td>
<td>PRKN</td>
<td>Mutated gene is most common cause of AR-JP</td>
</tr>
<tr>
<td>PARK3</td>
<td>AD</td>
<td>SPR?</td>
<td>Gene not known with certainty</td>
</tr>
<tr>
<td>PARK4</td>
<td>AD</td>
<td>SNCA</td>
<td>Duplications and triplications of the SNCA gene</td>
</tr>
<tr>
<td>PARK5</td>
<td>AD</td>
<td>UCHL1</td>
<td>Role uncertain</td>
</tr>
<tr>
<td>PARK6</td>
<td>AR</td>
<td>PINK1</td>
<td>Mutated gene is the second most common cause of AR-JP</td>
</tr>
<tr>
<td>PARK7</td>
<td>AR</td>
<td>DJ-1</td>
<td>Rare</td>
</tr>
<tr>
<td>PARK8</td>
<td>AD</td>
<td>LRRK2</td>
<td>Mutated gene is most common cause of dominant PD</td>
</tr>
<tr>
<td>PARK9</td>
<td>AR</td>
<td>ATP13A2</td>
<td>Mutations cause complex phenotype with parkinsonism, spasticity and dementia</td>
</tr>
<tr>
<td>PARK11</td>
<td>AD</td>
<td>GIGYF2?</td>
<td>Role of gene not yet certain</td>
</tr>
<tr>
<td>PARK13</td>
<td>AD?</td>
<td>OMI/HTRA2</td>
<td>Allelic to neuroaxonal dystrophy; two patients with mutant gene with adult-onset dystonia-parkinsonism described</td>
</tr>
<tr>
<td>PARK14</td>
<td>AR</td>
<td>PLA2G6</td>
<td>Mutated gene causes early-onset severe phenotype with spasticity and dementia</td>
</tr>
</tbody>
</table>

Table 1.1: Autosomal dominant and recessive genes correlated with PD (Table is from Gasser, 2009, modified- Copyright licence obtained from publishing group). AD: autosomal dominant, AR: autosomal recessive.

### 1.2 The LRRK2 protein; domains of LRRK2, founder mutations, epidemiology and possible mechanisms of LRRK2’s induced neurotoxicity in PD.

LRRK2 (also known as dardarin) is a complex protein of 2527 amino acids (around 286kDa) containing several functional domains (Fig. 1.1). The two most important domains are the Ras of complex domain (ROC), which is a GTPase binding domain, and the kinase domain, which is phosphorylating downstream targets of LRRK2 in S/T residues. The ARM domain at the N-terminal region of LRRK2 is an armadillo repeats region, and after that comes the ankyrin repeat region (ANK) and a leucine rich repeat domain (LRR). Then comes the GTPase ROC domain followed by its associated C-terminal of ROC (COR). Finally a WD40 repeat region is located at the C-terminus of the LRRK2 protein. The presence of all these interaction protein domains (ARM, ANK, LRR and WD40) triggers the hypothesis that LRRK2, except being a kinase or a GTPase protein could act as a scaffold protein for additional signaling pathways (Mata et al. 2006). However due to the fact that each of the interaction protein domains mentioned above (Andrade et al. 2001), bind to completely
different families of proteins, further research has to be made in order to see if and for which family of proteins, LRRK2 could act as a scaffold subunit. It has been shown that mutations in the LRRK2 gene cause autosomal dominant parkinsonism (Zimprich et al. 2004). More than 40 missense mutations have been reported that are located in the LRRK2 sequence. Three of the most common mutations identified in PD patients are the R1441C/G and the G2019S, found in the ROC and kinase domain of LRRK2, respectively. R1441C and G2019S induce neurotoxicity, due to the fact that the kinase activity of the mutant LRRK2 (G2019S or R1441C) is increased dramatically (Smith et al. 2006, West et al. 2005). Due to the neurotoxic effects of LRRK2 through its kinase activity, LRRK2 was suggested to be a therapeutic target for PD (Tan et al. 2011, Gillardon et al. 2013). Mutations in G2019S and R1441C/G account for a large number of PD patients sporadic or familial internationally (Table 1.2).

![Schematic representation of the domains of LRRK2](modified figure from Taymans and Baekelandt, 2014).

### Prevalence of specific LRRK2 mutations in different populations worldwide regarding Parkinson’s Disease

<table>
<thead>
<tr>
<th>Country</th>
<th>fPD Prevalence</th>
<th>sPD Prevalence</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germany</td>
<td>10%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>15%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UK</td>
<td>1,8%</td>
<td>G2019S</td>
<td></td>
</tr>
<tr>
<td>Spain/Portugal</td>
<td>5%</td>
<td>G2019S/R1441G</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>6%</td>
<td>G2019S</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>30%</td>
<td>13%</td>
<td>G2019S in Ashkenazim</td>
</tr>
<tr>
<td>North America</td>
<td>37%</td>
<td>41%</td>
<td>G2019S in Arabs</td>
</tr>
</tbody>
</table>

Table 1.2: Statistics of familial PD or sporadic PD patients in different countries (Table taken from Gasser, 2009 and modified- Copyright licence obtained from publishing group). LRRK2 G2019S and R1441G founder mutations, except contributing in familial PD, are also responsible for a significant number of sporadic PD cases around the world. fPD: familial PD, sPD: sporadic PD.

Mutant versions of LRRK2 have also been implicated to induce mitochondrial destabilization, resulting in mitochondrial induced apoptosis (Wang et al. 2012, Gasser 2009). Additionally it has been shown for R1441C/G, I2020T and Y1699C mutants, that they are accumulate within cytosolic pools resembling inclusion bodies, while in the case of wild type LRRK2 or other mutants no
accumulation in cytosolic pools is observed (Nichols et al. 2010). Finally mutant LRRK2 could induce destabilization of alpha synuclein and lead to alpha-synuclein Lewy bodies and mitochondrial destabilization (Fig. 1.2).

![Diagram of multiple signalling pathways leading to PD](image)

Fig. 1.2: Schematic representation of multiple signalling pathways leading to PD. Genes such as alpha synuclein, can lead to neuronal cell death. The mutations that are found in alpha synuclein could be point mutations, overexpression of the protein or RNA/protein mishandling. Neuronal death can be achieved through different cellular pathways such as the formation of Lewy bodies, or alpha synuclein oligomerisation, which subsequently could lead to mitochondrial damage, proteasomal and lysosomal dysfunction. Emphasis is also given to LRRK2. Mutant LRRK2 protein could also cause mishandling of a-synuclein, or alterate essential cellular functions, which could result in extensive accumulation of a-synuclein and mutant LRRK2 aggregates. Additionally there are been lately publications where they suggest that in the presence of mutant LRRK2, mitochondrial-induced cell death is observed (therefore the connection of LRRK2 and mitochondrial damage is not speculative/dashed line). Also the importance of parkin, PINK1 and GBA is shown and their connection to neuronal cell death. Solid lines indicate interactions supported by published literature, while dashed lines represent more speculative mechanisms (Figure taken from Gasser, 2009- Copyright licence obtained from publishing group).
1.3 Important phosphosites of the LRRK2 protein and their biological importance

Recent mass spectrometric data of LRRK2 protein revealed an important number of potential phosphorylation sites of LRRK2 (Fig. 1.3). These sites (such as S1403, T1404, T1503, T1967, T1969) are located in the ROC, COR or kinase domain of LRRK2 and could serve as indicators of LRRK2 kinase activity (Greggio et al. 2009, Kamikawaji et al. 2009, Pungaliya et al. 2010, Webber et al. 2011). In agreement with the fact that many phosphorylation sites of LRRK2 are in its ROC domain, another mass spectrometric study identified two major clusters of LRRK2 phosphorylation sites, being located in the N-terminus of LRRK2 protein and at the ROC domain of LRRK2 (Gloeckner et al. 2010). The review from Lobbestael et. al. 2012, gives a total overview of the phosphorylation and in vitro autophosphorylation sites of LRRK2. Focusing on LRRK2’s residue T1503, it has been demonstrated that it is an autophosphorylation site for LRRK2, and that removal of this residue by an alanine, resulted in a great decreased GTP binding and kinase activity, meaning that phosphorylation at this site is able to affect LRRK2’s kinase activity (Webber et al. 2011). The same case seems to be for T1967, an autophosphorylation site of LRRK2. When T1967 is substituted by an alanine, LRRK2’s activity is reduced (Kamikawaji et. al. 2009). Another important autophosphorylation site of LRRK2 is S1292 (Sheng et al. 2012). Sheng et al. demonstrated first by mass spectrometry and by an antibody against S1292 phosphorylation, that the phosphorylation of this residue is increased in the mutant G2019S-LRRK2 (which exhibits increased kinase activity) compared with the WT-LRRK2, while experiments in which the kinase dead D1994A-LRRK2 was used, showed no S1292 phosphorylation. This suggests that the autophosphorylation of LRRK2 in S1292 residue, is an important readout for LRRK2’s kinase activity. Additionally N1437H, R1441G and R1441C familial mutations of LRRK2 demonstrated higher S1292 phosphorylation levels, compared to the WT-LRRK2. Furthermore, when S1292 phosphorylation in G2019S primary embryonic hippocampal neurons was decreased due to LRRK2 kinase inhibitors, the neurite outgrowth defects of the mutant neurons were ameliorated, demonstrating that downregulation of LRRK2 activity results in neuroprotection (while LRRK2’s S1292 phospholevels are decreased in the presence of the inhibitor) .

Additionally constitutive phosphorylation sites, such as S910, Ser935, S955 and S973, were also found in the ANK-LRR interdomain region of LRRK2 (Gloeckner et al. 2010, Nichols et al. 2010, Lobbestael et al. 2012) and were shown to be mediated by the kinase activity of LRRK2 (Doggett et al. 2012). In contrast to the last hypothesis, Ito et al. 2014, showed by using a kinase inactive mutant form of LRRK2 (T2035), that the mutant was still undergoing basal phosphorylation in S910, S935, and S955, compared to the phosphorylation levels of S910, S935 and S955 of the WT-LRRK2. In agreement with the work of Ito et al., another study by Van Craenenbroeck et al. 2014, suggested that the phosphorylation of S935 is not exclusively due to LRRK2’s kinase activity, after testing LRRK2’s S935 levels by using different LRRK2 inhibitors. Therefore the phosphorylation status of S910, S935, S955, S973 does not seem to be regulated exclusively through LRRK2’s kinase activity. The S910 and
S935 phosphorylation status of LRRK2 has been described to be important for the interaction of LRRK2 with 14-3-3 proteins and subsequently LRRK2’s subcellular localization (Nichols et al. 2010).

All the above work suggest that the phosphorylation state of LRRK2 is probably regulated by a plethora of kinases but also phosphatases. Especially the regulation of serine or threonine residues such as T1503 or T1967, could act as an essential readout but also regulator of LRRK2’s kinase activity and subsequently its neurotoxic effect. Additionally as described above, reduction of other residues’ phosphorylation status such as S1292 in nervous systems, results in neuroprotection in terms of neurite outgrowth defects. Therefore the hypothesis emerges that there could be a phosphatase(s) that could mediate the phosphorylation state of LRRK2.

Fig. 1.3: Phosphosites/phosphatases of the LRRK2 protein: The different domains of the LRRK2 protein are shown with distinct colours. The mutations accounting for the majority of familial PD are shown with black letters. R1441C and G2019S mutations are located to the ROC and kinase domain respectively. Identified phosphosites by mass spectrometry sites are also indicated. The majority of the phosphosites are located at the ROC domain. S1292 phosphorylation is increased in the presence of okadaic acid (OA), a specific inhibitor of Protein Phosphatase 2A (PP2A), while Protein Phosphatase 1 (PP1) is dephosphorylating S910, S935, S955, and S973 (Figure taken from Taymans and Baekelandt, 2014).
1.4 Protein Phosphatase 2A in general

Protein Phosphatase 2A (PP2A) is a S/T phosphatase which is ubiquitously expressed in mammalian cells and it has shown to be very important for cell processes such as the cell cycle process. PP2A is a holoenzyme, consisting of the core enzyme, which is a 36 kDa catalytic subunit (or PP2Ac) and a regulatory subunit (PP2Aa or PR65) of molecular mass of 65 kDa. To this core enzyme a final third B regulatory subunit will bind. So far four different B subunits have been identified, the B, B’, B”, B’’’ families. A schematic description of the holoenzyme and the different subunits of PP2A are shown in Fig. 1.4. Regarding the PP2Aa and the PP2Ac, each subunit expresses two isoforms, the α and the β isoform. Every time the core enzyme is formed only one of the two isoforms can participate per core enzyme molecule. The α and the β isoform of PP2Ac share 97% identity in their primary sequence (Janssens and Goris, 2001). It also has been reported that, attempts to overexpress functional PP2Ac in mammalian cells with standard gene transfer techniques have long been unsuccessful (Green et al. 1987). However the activation of PP2A can be achieved with chemical reagents (will be extensively discussed in the following parts of the Thesis).

Fig. 1.4: Structure of the PP2A holoenzyme: The three different subunits of PP2A and its variations are shown in the upper schematic representation. PR65/A (or PP2Aa) is the scaffold subunit, PR55/B (or PP2Ab) is the regulatory subunit and the C (or PP2Ac) is the catalytic subunit of the phosphatase. In mammalian organisms, the A and the C are expressed by two genes (α and the β isoforms). Only one of the two isoforms participates each time per holoenzyme. ; the B/PR55 subunits are consisted by four genes; the B’/PR61 family are encoded by five genes (α, β, γ, δ, and ε); the B’’ family has three genes, namely PR48, PR59 and the splice variants PR72 and PR130; SG2NA and striatin belong to the B’’’ subunit family. Depending on which B subunit binds two the A and the C, the holoenzyme is localised to different compartment of the cell (photo modified from Janssens and Goris, 2001).
1.5 The role of PP2A in alpha-synuclein correlated PD

The phosphorylation status of alpha synuclein has shown to be important in PD cases. PD-aged brain studies have shown that the phosphorylation levels of alpha-synuclein in specific residues such as S87, S129 or/and at tyrosine residues such as Y125, Y133, Y136 (Fig. 1.5), are key sites for PD identification. The phosphorylation of the Y125 residue has shown to be inversely correlated with PD pathology. On the other hand, high phosphorylation levels of S129 are correlated with PD cases. Indeed it has been shown that alpha-synuclein which is found in Lewy bodies in postmortem brains of PD patients, is highly phosphorylated in S129, showing a connection between PD and S129 phosphorylation (Anderson et al. 2006, Fujiwara et al. 2002, Taymans and Baekelandt, 2014). Additionally, PP2A has been shown previously to dephosphorylate α-synuclein in vitro at S129 (Lee et. al 2011). Since pS129 could be important for the advancement of PD, and PP2A can dephosphorylate this residue, PP2A could be an attractive target of alpha synuclein-induced PD therapeutics.

Fig. 1.5: Structure of alpha-synuclein, phosphorylation sites and role of the PP2A. The different protein domains of the alpha-synuclein protein are shown with different colours. Different phosphorylation sites are shown. With red colour the S129 phosphosite is emphasized. S129 is shown to be upregulated in different PD cases. Protein phosphatase 2A is shown to dephosphorylate S129, and therefore being a possible drug target for alpha-synuclein PD cases. (Figure taken from Taymans and Baekelandt, 2014).
1.6 The role of PP2A regarding Tau phosphorylation levels and Alzheimer’s Disease

Tau has been a microtubule protein being most known for being associated with diseases such as Alzheimer’s Disease (AD), and other neurological diseases such as progressive supranuclear palsy, corticobasal degeneration, and some cases of frontotemporal lobar dementia. Although Tau is mostly associated to dementias, the tau gene has also been identified as a risk factor for PD via genome wide association studies (Sharma et al. 2012). Tau phosphorylation levels in specific Tau residues in AD and PD patients are higher compared to normal patients (Taymans and Baekelandt 2014). A possible explanation for this correlation would be that Tau has affinity for interaction with microtubules. Buee et al. 2000, have shown that an increased phosphorylation state of Tau can influence microtubules, cell viability but also synaptic functions. PP2A among other phosphatases has been found to dephosphorylate Tau in different phosphosites (Bennecib et al. 2000, Liu et al. 2005, Kuszczyk et al. 2009, Martin et al. 2009) (Fig. 1.6). This comes in agreement with the finding that the activity of PP2A is reduced in AD brains compared to healthy ones (Voronkov et al. 2011, Martin et al. 2013).

![Diagram of Tau phosphorylation](image)

Figure 1.6: Scheme of the Tau protein, its phosphosites and phosphatases that regulate its phosphorylation status. Different S/T phosphosites are shown within the structure of the Tau protein. Different phosphatases have shown to dephosphorylate Tau. Finally with red letters are the mutations correlated with frontotemporal dementia and Parkinson’s Disease (Photo taken from Taymans and Baekelandt, 2014).
1.7 Phosphatases of LRRK2

Protein phosphatase 1 (PP1) has already been shown to interact with and dephosphorylate LRRK2 in S910, S935, S955 and S973 (Lobbestael et al. 2013). Another attractive candidate phosphatase for dephosphorylating LRRK2 is PP2A. This hypothesis is strengthened by the work of Reynolds et al. 2014, where in the presence of calyculin A (a PP1 and PP2A inhibitor), but also with okadaic acid (selective PP2A inhibitor) the S1292 levels of the R1441G and G2019S mutant LRRK2 are increased to the same extent. This means that most probably PP2A is the phosphatase regulating dephosphorylation for S1292. On the other hand, calyculin A but not okadaic acid (OA) is able to inhibit dephosphorylation of S935 (Lobbestael et al. 2013), meaning that PP2A is not probably the phosphatase dephosphorylating the S935 (Fig. 1.3).

1.8 Neuronal Ras activation is able to protect against chemically-induced parkinsonian mouse models

Previously it has been extensively shown by other members of our lab (Heumann et al. 2000), that constitutive activation of Ras in transgenic mice could rescue dopaminergic substantia nigra neurons from neurotoxin-induced neurodegeneration (through 6-OHDA or MPP+ treatment), while additionally the cell soma of the brain was increased in a 14.5%, compared to the littermate mice which were used as controls. Additionally choline acetyltransferase and tyrosine hydroxylase activities were increased, as well as neuropeptide Y expression. Especially tyrosine hydroxylase is a key enzyme for the production of L-Dopa, a precursor of dopamine. Due to the fact that the activation of the Ras pathway in general results to proliferation, the transgenic Ras mouse was expressing Ras under a synapsin promoter, meaning that it should expressed only in neurons which do not divide. Therefore Ras activation in a mouse model seems to be neuroprotective against chemically-induced parkinsonian models, such as 6-OHDA.

1.9 PP2A is able to activate the Ras pathway through dephosphorylation of Ksr and Raf

Previous literature has also shown that PP2A could positively regulate Ras-mediated signaling in Caenorhabditis elegans experimental model (Sieburth et al. 1999). Activation of the Ras pathway has been extensively shown before to protect from chemically-induced parkisonian models (Heumann et al. 2000). A more detailed insight to the mechanism of how PP2A activates the Ras pathway is shown
in the review of Raabe and Rapp, 2003 (Fig. 1.7). It seems that dephosphorylation of Ksr at S392 and of Raf at S259 is essential for displacement of the proteins through 14-3-3 proteins to the plasma membrane, where the MEK/ERK pathway is active.

Fig. 1.7: Dephosphorylation of Ksr in S392 and Raf in S259 by PP2A, can lead to MEK/Erk pathway activation (A) In non-stimulated cells, 14-3-3 is bound to phosphorylated Raf (serines 259 and 621) and Ksr (serines 297 and 392). As a result both complexes remain in the cytoplasm, with Raf being inactive. The kinase which is phosphorylating Ksr at positions S297 and S392 is C-TAK1 (Muller et al. 2001). The A scaffold subunit and the catalytic C subunit of PP2A is in a complex with Raf and Ksr, while the regulatory subunit of PP2A (B) is not participating in the complex. At the same time, another complex is formed, comprised of Ksr and the inactive form of MEK. (B) Treatment with growth factors leads to activation of the PP2A holoenzyme. The B regulatory subunit of PP2A can bind the A and C subunits, and after this step the PP2A is able to dephosphorylate Raf at S259 and Ksr at S392. As a result of these dephosphorylations, 14-3-3 is dissociated from these sites, resulting in the recruitment of both proteins in the membrane. According to studies mentioned below, Raf can be active while
it is bound to the membrane, only when it will finally interact with phospholipids, bind to Ras-GTP, and finally re-associates with 14-3-3 (Dhillon et al. 2002, Hekman et al. 2002). Finally Raf, MEK and Erk will form an active complex at the membrane and the Erk pathway will be activated (Figure taken from Raabe and Rapp, 2003-Copyright licence obtained from publishing group).

Therefore since it has been shown earlier that the Ras pathway activation is neuroprotective in parkinsonian models, it would be reasonable to hypothesize that the activation of PP2A through the chemical substance sodium selenate, and subsequently the activation of the Ras/MEK/Erk pathway could be one of the mechanisms to reduce apoptosis in parkinsonian models.

1.10 Aim of this study

Only indirect experiments have been done (Reynolds et al. 2014), where correlation of PP2A with LRRK2’s phosphorylation levels is shown, through the addition of a chemical such as okadaic acid, which is inhibiting PP2A.

Our work is first focused to investigate if LRRK2 interacts with the PP2A holoenzyme using different cellular systems. We plan to do that using biochemical and fluorescent methods. If this would be the case, we would focus to see through which domain is the LRRK2 able to bind PP2A.

Afterwards using short hairpin RNA plasmids against the catalytic subunit of PP2A, we would inactivate the PP2A holoenzyme (since it would be catalytically active), and look if the silencing of PP2A aggravates or protects PD-induced cell death in neuronal cultures. We intend to use shRNAs against PP2Ac and not okadaic acid, due to the fact that the shRNAs are more specific than okadaic acid which could affect more signaling pathways as a chemical substance. On the other hand, although it has been shown from previous work that the PP2Ac overexpression has proven to be challenging in mammalian cells, we would like to overexpress PP2Ac if possible, or use chemical reagents which could activate PP2A enzymatically. Therefore we would crosscheck our shRNA data, using this time a PD system where PP2A is hyper activated. Finally if the elimination or the overexpression of PP2A could have any results in terms of neurodegeneration or neuroprotection, we would like to find the mechanism of how PP2A could affect the molecular mechanisms of PD. The mechanism of the neuroprotection of PP2A, could be done through modulation of LRRK2 or its downstream targets’ phosphorylation status or/and PP2A could activate other already known neuroprotective pathways, such as the activation of the Ras/MEK-Erk1/2 pathway.
To conclude, since none has so far shown a direct interaction between LRRK2 and PP2A, our current data focuses on the identification of PP2A as an interacting partner of LRRK2 and the impact of PP2A in R1441C/G2019S-induced parkinsonian models in terms of neurotoxicity and cell viability.
2. Materials and Methods

2.1 Bacterial protein expression and GST pull down of the ROC protein

GST empty (pGex-4T-1 plasmid), GST-ROC-WT and GST-ROC-R1441C plasmids were transformed in BL21 E. Coli bacteria. 1µg of plasmid was given to 50 µl bacteria for 30 minutes at 4°C. Afterwards, the bacteria were heat-shocked at 42°C for 45 seconds and then they were placed back to 4°C for 3 minutes. Afterwards, 600µl of SOC medium was given and the bacteria were incubated for 45 minutes at 37°C. Then bacteria were plated in agar plates containing ampicillin and chloramphenicol at 37°C for bacteria selection overnight. Next day, single bacteria colonies were picked and LB medium was given with ampicillin and chloramphenicol, in order to have a bacteria culture. At 0.6 OD, a final concentration of 0.5mM Isopropyl β-D-1-thiogalactopyranoside/IPTG (R0392, Thermoscientific, Germany) was given to the culture to induce protein expression. After 3-5 hrs of IPTG induction, bacteria were centrifuged for 10 minutes at 4000rpm at room temperature and the pellet was kept at -20°C overnight. Using the OD of the bacteria as a loading control, 1ml of culture was kept at 0 hr and 5 hrs after induction of IPTG, and a volume of water (according to the OD) was added together with 4x Laemmlli. Then the samples were subjected to an SDS gel followed by Coomassie staining to verify protein expression of the GST-ROC proteins.

Later on 20 ml of 1x PBS, containing 0.5% Triton-X100 and protease inhibitors cocktail (#p2714-1BTL, Sigma, Germany) was given to dissolve the pellet and lyse the cells. Using a microfluidizer, bacteria were lysed at 4°C (15-20x repeats of microfluidizer). Afterwards, the lysate was centrifuged at 8000 rpm, at 4°C for 30 minutes. The supernatant was taken (30µl of supernatant were kept to verify the protein expression). The supernatant then was incubated with glutathione beads (for 300ml of bacteria culture, briefly 700µl of beads were used) and the mixture was rotated for 4 hrs at 4°C, to enable the glutathione beads to bind the GST fused proteins. Later on, the supernatant was discarded (while a small portion of the supernatant was kept to check the quantity of the unbound GST proteins) after centrifugation at 3000 rpm for 2 minutes at 4°C. Using the OD of the bacteria as a loading control, 1 ml of culture was kept at 4°C, and the glutathione beads were washed 5 times with 1x PBS containing 0.5% Triton-X100 and protease inhibitors. At the end, beads were resuspended in 700µl 1x PBS containing 0.5% Triton-X100 and protease inhibitors. 20µl of the beads resuspension were used for verification of binding of the GST proteins to the beads through Coomassie staining. The stock protein beads binding the protein were kept at -20°C for a maximum period of 6 months. For the pull down of PP2A, 1 full T-75 flask of SH-SY5Y cells was used for 2 pull downs, while approximately 50 µl of glutathione beads were used to pull down PP2A from the cell lysate. SH-SY5Y cells were lysed with 1x PBS, containing 1% Triton-X100 and protease inhibitors and later on incubated (by continuous rotating) with the glutathione beads for 3,5 hrs at 4°C. After incubation the beads (bound to the GST proteins and the proteins being fished form the SH-SY5Y lysate) were washed 5 times with
0.5% Triton-X100 and protease inhibitors and the samples were subjected to an SDS gel electrophoresis and Western blotting.

**SOC-medium:**

- 2% (w/v) Bactotrypton
- 0.5% (w/v) Yeast extract
- 10 mM NaCl
- 2.5 mM KCl
- 10 mM MgCl2
- 20 mM Glucose

### 2.2 Staining of SDS gels by Coomassie Brilliant Blue R-250

To visualize the bacterially expressed proteins which were resolved by gel, the gel was placed in Coomassie Brilliant Blue R-250 staining solution and incubated for 1 hr to overnight at room temperature on a shaker. Afterwards, the gel was washed with a mixture of 10% acetic acid and 40% methanol in distilled water (Destain/washing solution). Rinse mixture was replaced several times while continuously shaking the gel, until proteins showed a deep blue color whereas otherwise leaving a clear gel. Gels have been either scanned or protein bands extracted for subsequent mass spectrometry analysis.

**Coomassie staining solution:**

- 0.25% (w/v) Coomassie Blue
- 50% (v/v) Methanol
- 10% (v/v) Acetic acid

**Destain/washing solution:**

- 10% (v/v) Acetic acid
- 40% (v/v) Methanol
2.3 GFP-TRAP for immunoprecipitation of GFP-fusion proteins

For the GFP-TRAP the following solutions were used:

**Lysis Buffer:**
- 10 mM Tris/Cl pH 7.5
- 150 mM NaCl
- 0.5 mM EDTA
- 0.5% Triton X-100

**Dilution Buffer/Wash Buffer:**
- 10 mM Tris/Cl pH 7.5
- 150 mM NaCl
- 0.5 mM EDTA

Procedure:

For the GFP-TRAP immunoprecipitation of PP2A using GFP-ROC-WT or GFP-ROC-R1441C, 2-3 wells of a 6-well plate of Hela cells were transfected with Lipofectamine LTX or Xfect (PT5003-2, Clontech, Germany), allowing 80-90% transfected cells. Afterwards the cells were lysed with 200 μl ice-cold lysis buffer and the sample was constantly resuspended by gentle pipetting. Afterwards the mixture was incubated/rotated on ice for 30 minutes with extensively pipetting every 10 minutes. Then cell lysate at 14000 g for 10 minutes at 4°C. The supernant containing the GFP-fused proteins was transferred to a precooled tube and 300 μl dilution buffer was added to the lysate. At the same time, 20-30μl of GFP beads were equilibrated, after being washed 3 times with 500 μl ice cold dilution buffer. To discard the dilution buffer, beads were centrifuged at 2700 g for 2 minutes at 4°C. Then the lysate of the cells was given to the beads and rotation of the mixture took place for 2 hrs at 4°C to enable efficient protein binding. Afterwards the mixture was centrifuged at 2700 g for 2 minutes at 4°C. Supernatant was removed and the beads were washed three times with 500μl ice cold wash buffer. Afterwards 80μl of 2x Laemmli buffer were used to elute bound proteins to the GFP beads by heating of the mixture at 95°C for 20 minutes. By centrifugation at 2700 g at RT for 2 minutes, the
eluted proteins in 2x Laemmlsi (without the beads) could be collected and subjected for SDS gel
electrophoresis and Western blotting.

2.4 GST-RBD pull down

Purified GST fused RBD (Ras binding domain of Raf) protein was designed/clone and produced by
Dr. Christoph Goemanns. The detailed experimental protocol is described by Leske et al. 2009 (PhD
Thesis). After incubation of 30 minutes at 4 °C with glutathione agarose beads (#745500.10,
Macherey-Nagel, Germany), the RBD protein-bound to the glutathiene beads solution was washed
with 3 times 0,5% Triton-PBS and incubated with cell lysate (SH-SY5Y). Later the samples were
loaded on a SDS gel and Western blotting followed to show the purification of the GST-ROC bound
proteins and their interacting proteins such as Ras.

2.5 Immunoprecipitation of LRRK2 protein from SH-SY5Y and Hela cells

For immunoprecipitation of LRRK2 we used the kit from Sigma Aldrich (IP50, Germany) and an anti-
mouse LRRK2 antibody (SIG-39840, Covance, Sigma). Initially a T-75 flask of 90% confluent SH-
SY5Y or Hela cells was used for 2 immunoprecipitation samples. The lysate buffer which was used is
included in the immunoprecipitation kit from Sigma (1x IP buffer). For one immunoprecipitation
sample the cells should be lysed in 600µl 1x IP buffer (10x protease inhibitors should be extra added).
Then 5µl of LRRK2 antibody were given and the mixture was incubated for 5 hrs rotating at 4°C. At
the same time 30µl Protein G agarose beads were used per sample and were washed three times with
1ml of cold 1x IP buffer (without protease inhibitors). After 5 hrs of lysate-antibody incubation, the
beads were also given to the lysate/antibody mixture and incubated for additional 4 hrs. Afterwards
the mixture was centrifuged at 12000 g for 2 minutes at 4°C and the supernatant was discarded. Then
the beads were washed twice with 1x IP buffer containing 0,1%SDS. Next they were washed 4 times
with 1x IP buffer. Finally the last wash was with 0,1x IP buffer. Afterwards 40µl of 1x Laemmlsi were
given to the beads and incubated for 20 minutes at 95°C. Finally after centrifugation the LRRK2
complex of proteins was in the 1x Laemmlsi without the beads. Then the solution was subjected for
SDS-PAGE gel and Western blotting.
2.6 Confocal Microscopy and Proximity ligation assay for Hela cells

Hela cells being 70% confluent and grown in glass slides, were washed twice with 0.2% BSA in 1x PBS, and fixed with 4% paraformaldehyde for 10 minutes. Afterwards they were washed twice with PBS and incubated with 50 mM NH₄Cl for 10 minutes at RT. After aspiration of NH₄Cl, cells were washed once with PBS and 0.2% Triton-X100 was given to the cells for permeabilisation for 5 minutes. Afterwards cells were blocked with 0.2% BSA in 1x PBS for 1hr. Then primary antibodies were diluted in 0.2% BSA in 1x PBS (for LRK2 1/200, for PP2Aa 1/50 working dilutions) for 1hr at RT. Then the antibody was washed 3 times with 0.2% BSA in 1x PBS. Later on secondary alexa antibodies were used (1/400 working dilutions) in 0.2% BSA in 1x PBS for 45 minutes (kept in dark). Alexa Fluor 555 donkey anti rabbit IgG (H+L) (#A31572, Invitrogen, Germany) and Alexa Fluor 488 goat anti mouse IgG (H+L) (#A11001, Invitrogen, Germany) were used as secondary antibodies. Immunocytochemistry samples were observed and photographed at 63x magnification under a confocal TCS SP2 Leica microscope (Wetzlar, Germany) using Leica confocal software. Hoechst 33342 (#14533, Sigma, Germany) was used to visualize the nucleus.

The proximity ligation assay was performed with Duo link system kit (Olink, Sigma, Germany). For the proximity ligation assay (PLA) the same steps apply with that of fluorescence microscopy, until the primary antibodies step. Then instead of normal secondary antibodies, two PLA probes were used (anti Rabbit Plus, Anti-Rabbit Minus, provided by the kit). The probes were diluted 1:5 in the same solution like the primary ones (0.2% BSA in 1x PBS or antibody diluent provided by the kit). Per each sample, 40µl of reaction was required. The mixture should be made 20 minutes before primary antibody removal and stay for these 20 minutes at RT. After the primary antibody was removed and washed thrice, PLA probes solution was given to the cells for 60 minutes at 37°C. Then samples were washed two times for 5 minutes with Buffer A (provided by the kit), while gently agitating. Then the ligation stock solution was diluted 1:5 in water and the ligase enzyme was also diluted 1:40 in the same solution, and the full solution was given to the slides-containing cells for 30 minutes at 37°C (40µl/slide, ligase stock and enzyme provided by the kit). After 30 minutes, slides were washed twice (2min/wash) with Buffer A. Then the amplification stock solution was diluted 1:5 in water, while also the polymerase enzyme was also diluted 1:80 in the same solution. Then the full solution was given to the slides for 100 minutes at 37°C (polymerase stock and enzyme provided by the kit). Then slides were washed twice (10 minutes/wash) with 1x wash buffer B and 1minute with 0.01x wash buffer B. Then the cells were rinced in water instantly and dried while at the end they were mounted with Hoechst 33342 solution, in order to visualize the nucleus (#14533, Sigma, Germany). For the
proximity ligation assay, the samples were observed at a 63x magnification under an X51 Olympus microscope (Hamburg, Germany) using cell sens standard software (Olympus, Hamburg, Germany). All the fluorescence photos were cut and merged using the software Image J and all the figures were further processed using CorelDRAW X5. Additionally, Image J was used to change the fluorescence signal in the PLA from red (Texas Red dye) to green for better visualization.

4% PFA:

4% (w/v) Paraformaldehyde

In 1x PBS

2.7 Immunohistochemistry

The immunohistochemistry experiments were conducted by Dr. Zoe Bichler’s lab, NNI, Singapore. Slides of the substantia nigra region of littermate mice were used as samples. The same antibodies used in immunocytochemistry were used in the same dilutions.

2.8 Cell culture transfection and cell lysis

Human neuroblastoma SH-SY5Y cells were cultured in DMEM high glucose (Sigma, D6546, Germany)/Ham’s F12 (#E15-817, GE Healthcare, Austria) 1:1 ratio, supplemented with 10% FCS, 2mM glutamine, 100 units penicillin and 100µg/ml streptomycin at 37 °C with 5% CO₂. N2A and HEK293T cells were cultured in DMEM high glucose supplemented with 10% FCS, 2mM glutamine; 100 units penicillin and 100µg/ml streptomycin at 37 °C with 5% CO₂. Hela cells were cultured with DMEM high glucose supplemented with 10% FCS, 2mM glutamine, 100 units penicillin and 100µg/ml streptomycin with 10% CO₂. HEK293T cells, SH-SY5Y and Hela cells were transfected with using various transfection protocols (see below). Protein expression was validated 48hrs after transfection with Western blotting or immunocytochemistry/fluorescence. Afterwards cells were washed three times with cold phosphate buffered saline (PBS) and incubated with 1% triton X-100 and protease inhibitor cocktail (#p2714-1BTL, Sigma, Germany) in 1x PBS for 10 minutes at 4°C while vortexing. After centrifugation at 14000 rpm, the supernatant was extracted, 4x Laemmli buffer was added, and the sample was boiled at 95°C for 5 minutes.
2.9 Transfection reagents for secondary cell lines

2.9.1 GenJet for N2A cells

Cells were plated 18 to 24 hrs prior to transfection so that the monolayer cell density reached to the optimal 70% confluency at the time of transfection. Complete culture medium with serum and antibiotics (1.2 ml) was freshly added to each well 60 minutes before transfection. For each well, add 0.5 ml of complete medium with serum and antibiotics freshly ~60 minutes before transfection. For each well of a 6-well plate, 2 μg of DNA were diluted into 100 μl of serum-free DMEM with high glucose. The mix was then vortexed. Additionally 3 μl of GenJet reagent (SL100489-N2A, SignaGen/Tebu-bio, Germany) were diluted into 1000 μl of serum-free DMEM with high glucose. This mix was also vortexed. Then the diluted GenJet reagent was added immediately to the diluted DNA solution all at once and vortexed directly afterwards. Then the final mix was incubated at RT for 15 minutes and finally added dropwise to the cells. 5hrs after addition of the transfection mix, the medium was exchanged with fresh. 24-48hrs after, cells were subjected for transfection efficiency.

2.9.2 Xfect transfection reagent for Hela/HEK293T cells

One day prior to the transfection, cells were plated in 1 ml of complete growth medium so that the cells would be 50–70% confluent at the time of transfection. Next day two mixtures were made. The first mixture contained 5 μg of DNA plasmid and a volume of Xfect reaction buffer (PT5003-2, Clontech, Germany) was used to reach 100μl. The second mixture contained 1.5 μl Xfect polymer and 98.5 μl Xfect reaction buffer. After vortexing both solutions, the polymer-containing solution was added to the DNA solution and the final mix was vortexed in medium speed for 10 seconds and then incubated for 10 minutes at RT. Finally the mixture was added to the cells. 4 hrs later the medium was replaced with fresh one. 24-48hrs after, the transfection efficiency was checked.

2.9.3 Lipofectamine LTX for SH-SY5Y/HEK293T cells

For the HEK293T cells, the same protocol suggested from the company (15338-100, Life Technologies, Germany) was followed. However the protocol for transfecting SH-SY5Y cells had to be optimized. Initially we were focused on transfecting a well of a 6-well plate, and then the quantities for the reagent were analogical to the surface of each well (e.g. if we need 6μl for a well of a 6-well plate with 5x more surface of a 24-well plate, then the lipofectamine that should be given to the 24-well plate should be 5x less or 1.2 μl). At the time of the transfection the SH-SY5Y cells should be
80-90% confluent. Then two solutions would follow. The first solution would contain 1,2 µg of plasmid and 1,2 µl of PLUS reagent and 120µl of OPTI-MEM reduced medium serum (31985-062, Life Technologies, Germany). Then this solution would be pipetted 3 times gently and incubate for 10 minutes at RT. Then a second solution containing 6µl lipofectamine and 114 µl OPTI-MEM reduced medium serum would be made, and afterwards pipetted 3 times. Afterwards the DNA solution should be added to the lipofectamine, pipetted up and down for 3 times, and this total mix should be incubated for 30 minutes at RT. Finally the total mix would be added to the cells. After 24-48 hrs the transfection efficiency of SH-SY5Y cells could be determined (25-30%).

2.10 Calcium phosphate transfection for cortical neurons

The transfection efficiency of cortical neurons with calcium phosphate is already explained in Dr. Sebastian Neumann’s PhD Thesis (Neumann et al. 2010). Briefly one day before transfection the medium was exchanged for cortical neurons. Next day 3µl plasmid (1 µg/µl) were given to 33,4 µl sterile water. Afterwards 3,6 µl CaCl2 (2,5mM) and 40 µl 2x BBS-buffer were given dropwise to the plasmid-water solution. After vortexing and 5 minutes incubation the mix would be given to the neurons after removing 750 µl medium from the total 1,5 ml medium. This 750 µl would be kept and given later back to the neurons. After 3 hrs incubation, the medium containing the transfection solution would be removed and then neurons would be washed three times. Then a fresh volume of 750 µl of NB/B27 medium together with the 750 µl which were kept earlier, would be given to the neurons. 24-48 hrs later the transfection efficiency could be determined (4-5%).

2.11 Protein determination by DCTM Protein Assay

Protein amounts of the lysed samples were quantified a Bio-Rad DCTM Protein Assay kit (#500-0111 Biorad, Germany). The Bio-Rad DCTM Protein Assay is a colorimetric assay to determine protein concentration following detergent lysis. Initially, standardized BSA (bovine serum albumin) solutions with 0.1 mg/ml, 0.2 mg/ml, 0.5 mg/ml, 1 mg/ml, 2 mg/ml, and 5 mg/ml have been prepared. 2 µl of sample or BSA standard were diluted with 20 µl solution A’ (consisting of 1 ml Solution A and 20 µl Solution S) and 200 µl Solution B in a 96-Well plate. The extinction at 620 nm was measured after 15-20 min incubation at room temperature in dark. Subsequently, protein concentration was determined on the basis of the BSA standard curve.
2.12 SDS polyacrylamide gel electrophoresis

Proteins were resolved according to their molecular weight by one-dimensional, discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE; Miniprotein; Bio-Rad). Therefore, the samples were diluted in 4x SDS buffer, heated to 95°C for 10 min, and loaded onto the tris-glycine gel comprised of the resolving gel (6-15%, Tris, pH 8.8) and a stacking gel (5%, Tris, pH 6.8). The proteins were separated by applying a constant electric field at 90 Volt being increased after 20 minutes to 150 volt.

<table>
<thead>
<tr>
<th>Stacking gel:</th>
<th>(5%) Resolving gel:</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4 ml H₂O</td>
<td>6.5 ml H₂O</td>
</tr>
<tr>
<td>1.5 ml Upper Tris, pH 6.8</td>
<td>3.75 ml Lower Tris, pH 8.8</td>
</tr>
<tr>
<td>1 ml 30% Polyacrylamide (PAA)</td>
<td>3 ml PAA</td>
</tr>
<tr>
<td>60 μl Ammonium persulfate (APS)</td>
<td>50 μl APS</td>
</tr>
<tr>
<td>6 μl TEMED</td>
<td>15 μl TEMED</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Upper Tris:</th>
<th>Lower Tris:</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M Tris/HCl, pH 6.8</td>
<td>0.5 M Tris/HCl, pH 8.8</td>
</tr>
<tr>
<td>0.4% (w/v) SDS</td>
<td>0.4% (w/v) SDS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SDS buffer:</th>
<th>SDS PAGE running buffer (1x):</th>
</tr>
</thead>
<tbody>
<tr>
<td>8% (w/v) SDS</td>
<td>1x TG</td>
</tr>
<tr>
<td>40% (w/v) Glycerin</td>
<td>1% (w/v) SDS</td>
</tr>
<tr>
<td>20% β-Mercaptoethanol</td>
<td>In H₂O</td>
</tr>
<tr>
<td>250 mM Tris/HCl, pH 6.8</td>
<td></td>
</tr>
</tbody>
</table>
30% Polyacrylamide:
30% Acrylamid
0.8% Bis-Acrylamid

2.13 Protein transfer to nitrocellulose membrane

Subsequently to SDS PAGE, gels were removed from the glass plates and equilibrated in transfer buffer to immediately proceed with protein transfer to nitrocellulose membrane (GE Healthcare, 10401396, Germany). Wet transfer was done by the help of the Mini Trans-Blot® Cell from Bio-Rad. For this purpose, gel and membrane were sandwiched between 2 sponges and 2 WhatmanTM paper (sponge/ paper/ gel/ membrane/ paper/ sponge) and are clamped tightly together, while being covered by transfer buffer. The sandwich was submerged in transfer buffer to which an electric field was applied, leading to migration of the negatively charged proteins towards the membrane, where they bound to. Transfer was done either for 2 hrs at 120 V under cooling by a cooling power pack, which was exchanged after 1 hr, or overnight at 25 V and room temperature. To check for successful transfer, membranes have been washed in Tris buffered saline-Tween (TBS-T see below) solution and incubated with Ponceau red solution on an agitator for 5 minutes. The membrane was washed extensively in water until the protein bands were well-defined. Repeated washing with TBS-Tween completely destained the membrane again.

**Transfer buffer:**
25 mM Tris/HCl, pH 8.6
192 mM Glycine
20% (v/v) Ethanol
0.04% (w/v) SDS

**Ponceau red solution:**
2% (w/v) Ponceau S red
30% (v/v) trichloroacetic acid

**TBS-T:**
50 mM Tris, pH 7.6
150 mM NaCl
0,05% (v/v) Tween-20

2.14 Western blotting

During primary but also secondary antibody incubation, membranes were washed with Tris buffered saline (TBS) – 0.1% Tween but also blocked with 5% non-fat milk, which was diluted in TBS- 0,1%T. Detection was achieved with the primary antibodies described above, and peroxidase conjugated anti-rabbit IgG (#A4416, Sigma, Germany) and anti-mouse IgG (#A1654, Sigma, Germany) secondary antibodies, followed by incubation with super signal west pico chemilunescent substrate (#34080, Thermoscientific, Germany) and supersignal west femto trial kit (#34094, Thermoscientific, Germany). The X-ray film (Hyperfilm ECL, GE Healthcare) was developed manually, exposing a film with suitable size between several seconds to minutes. Exposed films have been immersed first in developing solution and then in acetic acid, fixing solution and finally rinsed with distilled water.

Blocking solution:

5% (w/v) non-fat dried milk

in TBS-T

2.15 Primary antibodies

Mouse LRRK2 monoclonal antibody (#SIG-39840, Covance, Germany) and rabbit polyclonal PP2A A subunit (#2039, Cell Signaling Germany), rabbit polyclonal PP2A B (#4953, Cell Signaling, Germany) and rabbit polyclonal PP2A C (#2038, Cell Signaling, Germany) were used for immunocytochemistry, Western blotting and immunoprecipitation-co-immunoprecipitation. The PP2Aa antibody is able to detect the two isoforms of the scaffold subunit of PP2A (PPP2R1A and PPP2R1B genes), the PP2Ab antibody is detecting the α isoform of PR55 (PPP2R2A gene), while the PP2Ac antibody detects the both two isoforms of the catalytic subunit of PP2A (PPP2CA and PPP2CB genes). Normal mouse IgG (#12-371, Millipore, Germany) was used as a negative control for immunoprecipitation of endogenous LRRK2. Rabbit polyclonal GST (#AB3282, Millipore, Germany) was used to detect the GST-fused ROC domain of LRRK2 in the pull down assay, while rabbit
polyclonal GFP (#A11122, Invitrogen, Germany) was used to detect the GFP-tagged ROC domain of LRRK2. Mouse monoclonal FLAG antibody (#F1804, Sigma, Germany) was used to check the expression levels of FLAG tagged LRRK2. Polyclonal rabbit cleaved caspase-3 antibody (#9661, Cell Signalling, Germany) was used for Western blots of cortical neurons in order to verify neuroprotectivity after sodium selenate induction. Phospho-p44/p42 MAPK (Erk1/2) (T202/T204) antibody (#4370, Cell Signalling, Germany) and p44/42 MAPK (ERK1/2) antibody (#4695, Cell Signalling, Germany) were used in order to check the activation of the MAPK pathway. N-, K-, H-Ras antibody (#05-516, Millipore, Germany) was used for pulling down Ras with the GST-RBD protein. Phospho-Ksr-1 (Ser392) antibody (#4951, Cell Signalling, Germany) and Ksr-1 antibody (#4640, Cell Signalling, Germany) were used to investigate the effect of PP2A dephosphorylation on Ksr-1.

2.16 Given plasmids and shRNA plasmids targeting PP2Ac

The H2B-mRFP plasmid was kindly provided by Dr. Goemanns, (Molecular Neurobiochemistry, Bochum, Germany) (King et al. 2008). Prof. Finkbeiner (Gladstone Institutes, US), provided us with the pGW1-Venus and pGW1-Venus tagged WT LRRK2 plasmids. The shRNA plasmids against the two isoforms of the PP2Ac subunits were designed and manufactured by GeneCopoeia USA-Tebu-Bio France. The shRNA plasmid targeting the alpha isoform of PP2Ac (target sequence GGTAACCAAGCTGCAATCA for human DNA and GGAGCTGGTTATACCTTTG for mouse DNA sequence) was cloned in psi-mH1 plasmid under H1 promoter. The plasmid also contained mCherryFP as a reporter marker under CMV promoter. The shRNA plasmid targeting the beta isoform (target sequence CCACTTACAGCTTTAGTAG for human and mouse DNA sequence) was cloned to a psi-H1 plasmid, under H1 promoter. The plasmid also contained eGFP as a reporter marker under CMV promoter. After co-transfection of the two shRNAs in HEK293T cells and SH-SY5Y cells, puromycin was used as a selection marker.

2.17 Midi Prep plasmid purification

High yield, transfection-grade plasmid DNA, deriving from 100 ml bacteria liquid culture, was purified by using the NucleoBond® Xtra Midi kit (#740410.10), purchased from Macherey-Nagel. The protocol that was used was the same with the one suggested by the manufacturer. After isolation, DNA was dissolved in distilled water. The DNA concentration was determined by using a 260/280 nm photometer absorbance.
2.18 Agarose gel electrophoresis

DNA fragments were separated and visualized by using agarose gel electrophoresis. Various gels were made using 1%-2.5% agarose (w/v) according to varying DNA fragment sizes. The DNA samples were mixed with 5x DNA sample buffer and loaded wells in an agarose gel, sank in 1x TEB buffer solution. For the separation of different DNA fragment sizes, 80 mA current was applied to the gel/TEB and afterwards ethidium bromide solution was given, so that the DNA fragments could be visualized under UV light.

TEB buffer:
- 50 mM Tris/HCl pH 8.0,
- 50 mM Boric acid
- 1 mM EDTA

DNA sample buffer:
- 25% (w/v) Glycerin
- 0.25% (w/v) Bromophenol blue
- 74 mM EDTA

EtBr staining solution:
- 0.5-1 µg/ ml EtBr in TEB buffer

2.19 DNA digestion by restriction enzymes

After PCR and successful production of the PCR fragment, 1µg of the PCR product was digested2 units [U] of the corresponding enzyme following the protocol suggested by the manufacturer. PCR products have been separated by agarose gel electrophoresis and the DNA fragment having the requested size was purified by using a gel extraction kit (#K0691, Thermoscientific, Germany). The cloning vectors were treated with the same enzymes, separated by agarose gel electrophoresis and purified as mentioned above for the insert (PCR fragment). Finally, treatment with 1 Unit alkaline calf intestinal phosphatase (CIP) led to removal of 5’ phosphate groups of the linearized DNA and prevented re-ligation of empty vector-DNA.
2.20 DNA ligation

A mix of final volume of 15µl was made, using 1-2 Units of T4 DNA ligase, while the insert amount to the vector was given at a 3:1 ratio. This ratio is always dependent on the length of the insert and the vector but in general worked for the ROC ligation. The mix was incubated at RT overnight.

2.21 Transgenic Mice and cortical neurons culture

C57Bl6 mice overexpressing LRRK2-G2019S (#012467, Jackson Laboratory, USA) and WT-LRRK2 (#012466, Jackson Laboratory, USA) were characterized by Li et al. (2010). Cortical neurons cultures were prepared from the brains of the two strains. The procedure of cortical culture preparations and the transfection protocol with calcium phosphate was previously described by Karassek et al. 2010.

2.22 Genotyping of BAC-LRRK2-G2019S mice

BAC-LRRK2-G2019S mice express a mutant form of mouse leucine-rich repeat kinase 2 (LRRK2-G2019S) associated with autosomal dominant, late-onset Parkinson's disease directed by the endogenous Lrrk2 promoter/enhancer regions on the BAC transgene. Primers 5’-CAA ATG TTG CTT GTC TGG TG-3’ (Fwd control) and GTC AGT CGA GTG CAC AGT TT (Rev control) were used to PCR an irrelevant band of that of the –BAC-LRRK2 gene, as an internal positive control for the total PCR procedure (around 300 bp). Primers 5’-GAC TAC AAA GAC GAT GAC GAC AAG-3’ (Trans Fwd) and 5’- CTA CCA CCA CCC AGA TAA TGT C-3’ (Rev Trans) were used to amplify the BAC-LRRK2 gene if the mouse would be transgenic (expected band around 600bp). Then the following PCR took place:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume/sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>2.8µl</td>
</tr>
<tr>
<td>10x polymerase buffer</td>
<td>1.5µl</td>
</tr>
<tr>
<td>DNTPs (1.25mM stock.)</td>
<td>1.5µl</td>
</tr>
<tr>
<td>Trans Fwd Primer (100µM stock)</td>
<td>1.5µl</td>
</tr>
</tbody>
</table>
Trans Rev Primer (100µM stock) 1,5µl
Control Fwd Primer (100µM stock) 1,5µl
Control Rev Primer (100µM stock) 1,5µl
Taq polymerase (Genecraft/Biotherm GC002-0100) 0,2µl
DNA 3µl
Total 15µl

**PCR temperature steps:**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation (1)</td>
<td>10 minutes-95°C</td>
</tr>
<tr>
<td>Denaturation (2)</td>
<td>1 minute-95°C</td>
</tr>
<tr>
<td>Annealing (3)</td>
<td>1 minute-65°C</td>
</tr>
<tr>
<td>Elongation (4)</td>
<td>1 minute-72°C</td>
</tr>
<tr>
<td>Final extension step (5)</td>
<td>10 minutes-72°C</td>
</tr>
<tr>
<td>Cooling (6)</td>
<td>4°C</td>
</tr>
</tbody>
</table>

The cycle of step 2,3,4 was repeated 36 times in order to get the PCR products.

### 2.23 Insertion of WT-LRRK2 and R1441C-LRRK2 DNA sequence to pCDNA3.0 vector

WT and R1441C human DNA LRRK2 cDNA sequences were kindly obtained by Prof. Schulz (Neurological Clinic, Aachen, Germany). 3x FLAG tagged-WT-LRRK2 and -R1441C-LRRK2 DNA sequences were subcloned into the pcDNA3.0 vector, through restriction from Drosophila expression vectors (pPFW) with XhoI and KpnI restriction enzymes and they were kindly offered by Prof. Hovemann (Molecular Cell Biochemistry, Bochum, Germany, unpublished data).
2.24 PCR for GST-ROC-WT and GST-ROC-R1441C proteins

The ROC domain of LRRK2 was initially produced from PCR using FLAG-WT and R1441C-LRRK2 plasmids as template, then ligated to the pGEM-T easy vector (#A1360, Promega, Germany) according to the manufacturer’s suggested protocol. BamHI and XhoI restriction sites were designed during the PCR fragment, so that the ROC gene will be at the same reading frame with the plasmid in order to express a GST-fused protein. After midi prep of the pGEM-t-easy vector containing the ROC-WT and ROC-R1441C, it was restricted using BamHI and XhoI. Finally, the ROC DNA fragment was subcloned to a pGEX-4T-1 backbone plasmid, resulting in a GST-fused ROC-WT/R1441C protein. The 5’-GGGG GGATCCAAGGCTGTGCCTTATAACCGAATGAAAC and the 3’-AGTCCCTCGAGTTAGAATTAAGGCTCTCGTTTATGATGG primers were used. The phusion high fidelity polymerase (#M0530S, NEB, Germany) was used to amplify the ROC DNA sequence. Products were analysed by agarose gel electrophoresis. After subcloning the DNA sequence into pGEX-4T-1 plasmid, sequencing was used to verify the cloning. The fused GST tag was located at the amino terminal part of the ROC protein.

**PCR temperature steps for ROC:**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation (1)</td>
<td>10 minutes-95°C</td>
</tr>
<tr>
<td>Denaturation (2)</td>
<td>1 minute-95°C</td>
</tr>
<tr>
<td>Annealing (3)</td>
<td>1 minute-65°C</td>
</tr>
<tr>
<td>Elongation (4)</td>
<td>1 minute-72°C</td>
</tr>
<tr>
<td>Final extension step (5)</td>
<td>10 minutes-72°C</td>
</tr>
<tr>
<td>Cooling (6)</td>
<td>4°C</td>
</tr>
</tbody>
</table>

The cycle of step 2,3,4 was repeated 35 times in order to get the PCR products.

2.25 PCR for EGFP-ROC-WT and EGFP-ROC-R1441C proteins

The ROC domain of LRRK2 was initially produced from PCR using FLAG-WT and R1441C-LRRK2 plasmids as template, then ligated to the pGEM-T easy vector (#A1360, Promega, Germany) according to the manufacturer’s suggested protocol. BspEI and HindIII restriction sites were designed during the PCR fragment, so that the ROC gene will be at the same reading frame with the plasmid in order to express an EGFP-fused protein. After midi prep of the pGEM-t-easy vector containing the ROC-WT and ROC-R1441C genes, it was restricted using BspEI and HindIII restriction enzymes.
Finally the ROC DNA fragment was subcloned to an EGFP-C1 backbone plasmid, resulting in a GFP-fused ROC-WT/R1441C protein. The 5’- GGGG TCCGGA AAGGCTGTGCTTTAACCAGAATGAAAC and the 3’- CCCCAAGCTTTTAGAAATTAAGGCTCTCGTTTATGATGG primers were used. The Phusion high fidelity polymerase (NEB, M0530S, Germany) was used to amplify the ROC DNA sequence. Products were analysed by agarose gel electrophoresis. After subcloning the DNA sequence into the EGFP-C1 plasmid, sequencing was used to verify the cloning. The fused GFP-tag was located at the amino terminal part of the ROC protein. For the PCR the same steps temperature and times were used as in the GST-ROC PCR.

2.26 Cell death and cell viability assay

For the cell death assay, HEK293T cells were used to evaluate the silencing of PP2Ac (70-75% transfection efficiency). The cells that integrated the shRNAs for the two PP2Ac isoforms were visualized by fluorescence microscopy through mCherryFP and eGFP reporter genes. Afterwards, a 3 days selection with puromycin (4µg/µl) followed, where the majority of the non-transfected cells died (90-95% transfected cells remaining). Then cells were washed three times with PBS and lysed as described above. For the SH-SY5Y cells the same procedure took place with a lower puromycin concentration (2µg/µl), then were re-transfected with full length FLAG-WT or FLAG-R1441C LRRK2/H2B-mRFP in a ratio 10:3. Cell death was visualized by fragmentation of the nuclei (Hoechst 33342, Germany). Sodium selenate (#S8295, Sigma Aldrich, Germany) was diluted in water and added to SH-SY5Y cells 12 h after post-transfection with FLAG-WT or FLAG-R1441C LRRK2/H2B-mRFP in a ratio 10:3 in a concentration of 50µM. After 12 h treatment, sodium selenate was removed and cells were washed 3 times with PBS. After 48h transfection, cell death was analysed by counting fragmented nuclei (Hoechst 33342, Sigma, Germany). Regarding the sodium selenate treatment for cortical neurons, sodium selenate was given at a concentration of 35µM for 12 hrs. After treatment, neurons were washed 3 times with PBS and new medium was provided. 12hrs after PBS exchange, lysates were prepared for Western blot or neurons were fixed with 4% paraformaldehyde and treated with Hoechst 33342 to check nuclei fragmentation.

2.27 Sodium selenate treatment for the Ras pathway activation

SH-SY5Y cells and cortical neurons cultures were treated with 50µM and 35µM sodium selenate respectively for 1hr. Afterwards they were washed with 3 times PBS and lysed with PBS-1% Triton containing proteinase inhibitors (#p2714-1BTL, Sigma, Germany). Later the samples were loaded on a SDS gel and Western blotting followed to show the activation of the MAPK pathway through sodium selenate, or the binding of Ras activated protein to the GST-RBD of Raf.
2.28 Mant GTPγS binding assay of the ROC proteins

Fluorescence measurements were performed at 25°C with a Kontron SFM25 fluorospectrometer (Kontron, Zürich, Switzerland) and a non-hydrolysable Mant-GTPγS analogue (#NU-209, Jena Bioscience, Germany). The excitation and the emission wavelengths were 366 and 435 nm respectively. Mant-labelled nucleotide (1µM) was incubated with GST, GST-ROC-WT, and GST-ROC-R1441C proteins (2µM) -bound to glutathione beads for 5 minutes. The fluorescence of protein-free solution containing 1µM Mant GTPγS was used as a reference value. After incubation, the samples were centrifuged for 1 min. at 1000rpm, and the supernatant was used to measure the fluorescence. The fluorescence detected, responded to the fluorescence of Mant GTPγS which was not bound to the protein. Deduction of the supernatant value from the reference value, indicated the amount of the Mant GTPγS, which was bound to the protein.

2.29 Statistics

Comparison of the different treatments affecting the populations of cells (construct dependent) was done by One-way or Two-way ANOVA tests followed by Bonferroni post-hoc comparisons (*** p< .001, ** p< .01, * p< .05). Equality of variances has been verified with Levene test, and Welch T test or Dunnett Test for unequal variances have been considered if unequal variances were considered.
3. RESULTS

3.1 The ROC domain of LRRK2 is sufficient for interaction with PP2A

Initially previous lab members searched for new candidate interacting proteins of LRRK2 using LRRK2’s ROC domain. Therefore they used bacterially expressed GST fused ROC-WT protein, which was incubated with mouse brain lysate (3-months old C57Bl6 mouse). Afterwards the protein complex was further analyzed using LC-MS Multi-Dimensional Protein Identification Technology (MudPIT) and the alpha isoform of PP2Aa (PPP2R1A gene, accession code: Q76MZ3 for mouse) was identified (Jacob Wright et al. 2010). To verify this finding in a human-like cellular context we further used Western blotting. GST-fused ROC-WT and ROC-R1441C proteins were incubated with SH-SY5Y lysate. Both ROC-WT and ROC-R1441C proteins were able to associate with the three subunits of PP2A after GST pull down and Western blotting (Fig. 3.1a). Furthermore there seemed to be no difference in the binding activity of PP2A between ROC-WT and ROC- R1441C (Fig 3.2a, 3.2b). The functionality of the GST-ROC-WT and GST-ROC-R1441C proteins, in terms of GTP binding, was further validated with fluorescent mant GTPγS binding assay (Fig. 3.2c).

Next, we investigated whether this interaction was dependent of cellular context. Therefore the ROC proteins were reconstructed in GFP fused vectors, and Hela cells were used. Similarly, the ROC-WT and the ROC- R1441C proteins were able to interact with all the three PP2A subunits (Fig. 3.1b). In addition, we investigated whether PP2A subunits were also associating with the full length LRRK2. As shown in Fig. 3.1c, the endogenous full length LRRK2 was immunoprecipitated in SH-SY5Y cells, and we were able to co-immunoprecipitate the PP2Aa and PP2Ac subunits. We were also able to show that full length endogenous LRRK2 in Hela cells could be immunoprecipitated, and that PP2Ac could be co-immunoprecipitated (Fig. 3.1d). The specificity of the LRRK2 antibody for Western blotting was also investigated (Fig. 3.1e).
Figure a shows a gel with bands for Anti-PP2Aa, Anti-PP2Ab, Anti-PP2Ac, and Anti-GST. Ponceau staining is also present.

Figure b displays Western Blots for GFP-TRAP and Lysate with bands for Anti-PP2Aa, Anti-PP2Ab, Anti-PP2Ac, and Anti-EGFP.

Figure c illustrates Western Blots for IP-1G, IP-LRRK2, and SH-SY5Y with bands for Anti-LRRK2, Anti-PP2Ac, and IgG.

Figure d shows Western Blots for IP-1G, IP-LRRK2, and whole lysate with bands for Anti-LRRK2 and Anti-PP2Ac.

Figure e presents Western Blots for untransfected, PDHA1, WT-LRRK2, and R1441C with bands for Anti-LRRK2, Anti-FLAG, Anti-Tubulin, and Anti-EGFP.
Fig. 3.1: The ROC domain of LRRK2 and the full length LRRK2 associates with PP2A. (a) The GST fused ROC domain of LRRK2 (WT or R1441C) is sufficient to interact with the three subunits of PP2A in SH-SY5Y cells as shown after GST pull down and Western blotting. (b) Similarly when the ROC domain is GFP-tagged and transfected in Hela cells, the ROC domain interacts with the Hela PP2A subunits after GFP-TRAP purification and Western Blotting. (c) The endogenous full length LRRK2 is able to co-immunoprecipitate PP2Aa and PP2Ac subunits in SH-SY5Y cells after immunoprecipitation of LRRK2 and Western Blotting. PP2Ab could not be detected, because of the high background of the antibody’s heavy chain (PP2Ab has a molecular weight of 52 kDa while the heavy chain is at 50kDa) (d) The endogenous full length LRRK2 in Hela cells, when immunoprecipitated, is able to co-immunoprecipitate PP2Ac. (e) The LRRK2 antibody is specific for Western blotting. Endogenous LRRK2 is not detected in HEK293T cells with different LRRK2 antibodies due to very low mRNA LRRK2 levels as shown before (Biskup et al. 2007). Therefore the FLAG tagged WT and R1441C LRRK2 plasmids were expressed. While in the control cells there was no signal, we could detect a band at around 280 kDa for WT and R1441C LRRK2 either using the FLAG antibody, or the LRRK2 antibody. Additionally we could also detect a lower band at around 100 kDa which could be degraded LRRK2 protein.
Fig. 3.2: Binding of the three PP2A subunits to ROC-WT versus ROC-R1441C and functionality of the ROC domains as GTPases. Tina software was used to measure the band intensity of the three subunits of PP2A from Fig. 1a. (a) Means and SEM of the optical densities (raw data- densities of each PP2A subunit are divided by the density of their loading control), and (b) means and SEM of the normalized data of three independent pull down experiments for each PP2A subunits. Data were normalized to the respective “GST-Empty” control. ***p<.001 as compared to the respective GST-Empty with one-way ANOVA test (“domain” GST-Empty, GST-ROC-WT or GST-ROC-R1441C as in-between factor) for each of the PP2A subunits. “n.s.” stands for “non significant”. Two-ways ANOVA test with “subunit” (PP2Aa, b, or c), and “domain” (GST-Empty, GST-ROC-WT or GST-ROC-R1441C) as in-between factors showed an effect of both factors (“subunit” effect F(2, 27)=21.918, p<.001; “domain” effect F(2,27)=27.547, p<.001). Two-ways ANOVA followed by Bonferroni post-hoc multiple comparisons indicated that the control “GST-Empty” differed from the two other domains at p<.001, while GST-ROC-WT and GST-ROC-R1441C did not differ with statistical significance (p=1.000 or p=.617 for B. and C. respectively).
The GST-ROC-WT and the GST-ROC-R1441C proteins are functional as GTPases. Fluorescent Mant GTPγS was incubated with GST, GST-ROC-WT and GST-ROC-R1441C and then the fluorescence of the Mant GTPγS, which was bound to the proteins is shown on the graph. The fluorescence value of protein-free solution containing 1µM Mant GTPγS, was set at 100%. Data are means ± SEM. ***p<.001 with One-way ANOVA followed by Bonferroni post-hoc comparisons.

3.2 LRRK2 and PP2Aa are interacting in the perinuclear region of Hela cells

LRRK2 is mainly a cytoplasmic protein (West et al., 2005). Previous literature has shown that it also localizes in the nuclear envelope in neurons. (Alegre-Abarrategui et al., 2008). Initially we checked the specificity of our LRRK2 antibody (Fig. 3.3, Fig. 3.4). In agreement to the previous findings we observed the same pattern for endogenous LRRK2 expression in Hela, or for exogenous FLAG-WT-LRRK2/Venus-WT-LRRK2, transfected in HEK293T cells (Fig. 3.5a, Fig. 3.3, Fig. 3.4). On the other hand, PP2Aa and PP2Ac could be localized in the nucleus, but also in the cytoplasm, depending on the cell cycle stage and the PP2Ab subunit association (Turowski et al 1995, Janssens and Goris 2001). Therefore we investigated in which cellular region the two proteins interact in Hela cells. We found that the majority of PP2A was localized in the nucleus rather than in the cytoplasm (Fig. 3.5a). Despite the fact that LRRK2 was in close proximity around the nucleus with PP2Aa, no interaction was detected with confocal microscopy (Fig. 3.5a). However using the proximity ligation assay we were able to observe the interaction of endogenous LRRK2 with PP2Aa in the perinuclear region of Hela cells (Fig. 3.5e, 3.5f). By using the substantia nigra region from littermate WT mice, we were able to conduct immunohistochemistry and confocal microscopy using the LRRK2 and PP2Aa antibodies. We were able to observe a colocalisation of the two proteins without the help of PLA (Fig. 3.6).
Fig. 3.3: The LRRK2 antibody is specific for immunocytochemistry. HEK293T cells were used as a control cell line, since no endogenous LRRK2 protein can be detected in HEK293T cells. In (a), LRRK2 and Alexa 488 anti-mouse antibodies were used in untransfected HEK293T cells, in order to detect any LRRK2, which was not the case. Photos were taken at 63x magnification with a normal fluorescence microscope (b) The same experiment was conducted with that of (a), using a 63x magnification and a confocal microscope. (c) HEK293T cells were first transfected with the FLAG-WT-LRRK2 plasmid and 48hrs after they were subjected to immunohistochemistry with 63x magnification (normal fluorescence microscope). (d) The same experiment was conducted with that of (c), using a 63x magnification and a confocal microscope.
Fig. 3.4: The venus fused LRRK2 protein has the same localization with that of FLAG-WT LRRK2. HEK293T cells were transfected initially with pGW1-venus protein using a normal microscope at 63x magnification (a) or confocal at 63x magnification (b), or with pGW1-venus-WT LRRK2 with normal microscope at 63x (c), or confocal at 63x (d). The venus-WT LRRK2 had similar pattern with FLAG-tagged LRRK2 (Fig. 3.3c, 3.3d).
Fig. 3.5 The endogenous LRRK2 is associating with endogenous PP2Aa in Hela cells. (a) Representative confocal images of Hela cells stained with LRRK2 and PP2Aa antibodies. Both proteins are localized in close proximity in the perinuclear region. However no clear co-localisation could be visualized. Confocal microscopy is used with 63x magnification. (e) Proximity ligation assay demonstrated the association of endogenous LRRK2 with the PP2Aa subunit. As controls, in the second lane (a) both primary antibodies were omitted as a control. In (b) and (c), only PP2Aa and only LRRK2 primary
antibodies were omitted respectively. The nucleus is stained with Hoechst 33342 (blue colour), while the interaction is shown with green colour. 63x magnification was used with a normal fluorescence microscope. (f) Quantification of (b), (c), (d) and (e). Data are means ± SEM. ### p<.001 with One-way ANOVA followed by post-hoc comparisons. Bonferroni post-hoc comparisons (equality of variances verified) showed that the number of dots is significantly increased in PLA done with both antibodies as compared to each of the other groups (one of the LRRK2 or PP2A antibodies alone, or without any antibody).

Fig.3.6: The endogenous LRRK2 is associated with endogenous PP2Aa in the substantia nigra region of littermate mice. This photo is taken by our collaborators in Singapore, Dr. Zoe Bichler and Mrs Sally Dong Qianying. By using immunohistochemistry and confocal microscopy, they could verify our previous data (Fig. 3.5) in a more neuronal model. The two proteins seem to interact in the perinuclear region of the cells. The same antibodies were used with these of Fig. 3.5.

3.3 Knockdown of the catalytic subunit of PP2A (PP2Ac, alpha and beta isoforms) results in aggravated cell death in transiently R1441C-LRRK2-transfected SH-SY5Y cells and G2019S-expressing cortical neurons

Next, we investigated whether the silencing of the two isoforms of the catalytic subunit of PP2A would affect the survival of SH-SY5Y cells, which were transfected with the FLAG-R1441C-LRRK2 protein. While SH-SY5Y cells, transfected with WT-LRRK2, did not show any significant difference whether being treated with the mock or the shRNA-PP2Ac, there was an aggravated cell death in the R1441C transfected SH-SY5Y cells, when treated with the shRNA-PP2Ac (Fig. 3.7). Similar data were obtained, when knocking down PP2Ac in primary cortical neuron cultures. The cortical neurons were obtained from transgenic mice, overexpressing WT or G2019S-LRRK2 (Fig. 3.9a, 3.9b).
Fig. 3.7: Transient knockdown of the PP2Ac subunit results in aggravation of cell death in transiently transfected with R1441C LRRK2- SH-SY5Y cells. (a) Evaluation of shRNAs targeting the two isoforms of PP2Ac in HEK293T cells. Cells were treated with empty shRNA plasmid as a control. (b) SH-SY5Y cells were transfected with shRNA-PP2Ac or empty shRNA vector, selected for 3 days with puromycin, and re-transfected with WT or R1441C-LRRK2/H2B-mRFP (10:3 ratio). 48 h after transfection with LRRK2 plasmids, cell death was measured by observing fragmented nuclei, through Hoechst 33342 staining. Data are means ± SEM. *p<.05, **p<.01, ***p<.001 with One-way ANOVA followed by post-hoc comparisons-equality of variances verified. ### p<.001 as compared to each of the other constructs (pCDNA or WT-LRRK2), with two way ANOVA test with “construct” and “shRNA” as in between factors followed by Bonferroni post-hoc comparisons. (c) Fluorescent microscopic pictures of (b). Three SH-SY5Y cells are transfected with shRNA for the alpha (mCherryFP) and the beta (eGFP) isoform of PP2Ac. However only the two upper cells are also re-transfected with LRRK2/H2B-mRFP due to the enhanced red staining in the nucleus, coming from the H2B-mRFP. (d) Western blotting
showing the WT-LRRK2 and R1441C-LRRK2 protein expression levels in SH-SY5Y cells. R1441C-LRRK2 seems to be less expressed than WT-LRRK2 which could be explained by increased degradation of R1441C-LRRK2 compared to WT-LRRK2 (Greene et al. 2014). (e) WT-LRRK2 and H2B-mRFP are co-transfected in the same cells. HEK293T cells, not expressing endogenous LRRK2, were co-transfected with FLAG-WT-LRRK2 and H2B-mRFP in a 10:3 ratio. Afterwards immunocytochemistry followed with LRRK2 antibody, to detect the cells that were transfected with WT-LRRK2 (the FLAG antibody was not detecting LRRK2 protein properly in immunocytochemistry). 84.06% of HEK293T cells (SD 1.1) are co-transfected with WT-LRRK2 and H2B-mRFP.

3.4 Activation of PP2A by sodium selenate partially rescues transiently R1441C-LRRK2-transfected SH-SY5Y cells, while there no statistically significant effect of sodium selenate in G2019S cortical neurons

Next we investigated the possible effect of PP2A activation on neuroprotection in two neuronal model cell lines. Sodium selenate was previously shown to specifically activate PP2A (Corcoran et al. 2010). As shown in Fig. 3.8, after treatment of SH-SY5Y cells with sodium selenate for 12h, R1441C LRRK2-induced cell death was partially rescued. Similarly after preparing mice-derived G2019S cortical neurons, sodium selenate treatment showed a tendency of reduction of apoptosis (through cleaved-caspase-3, Fig. 3.9c, 3.9d), compared to the littermate mice (Control G2019S), however this reduction was not statistically significant (Fig. 3.9d). Similar results were obtained after using as an apoptosis readout fragmented nuclei (Fig. 3.9e).
Fig. 3.8: Sodium selenate has a neuroprotective effect in transiently transfected with R1441C-LRRK2- SH-SY5Y cells. (a) SH-SY5Y cells were transfected with WT or R1441C-LRRK2/H2B-mRFP (10:3 ratio). 12 h after transfection they were treated with 0µM, or 50µM sodium selenate. 48 h after transfection, cell death was measured by counting fragmented nuclei, through Hoechst 33342 staining. Data are means ± SEM. *p<.05, **p<.01, ***p<.001 with One-way ANOVA with “construct” as factor, followed by post-hoc comparisons and Student T Test within construct comparison (sodium selenate effect)-equality of variances verified. Two way ANOVA test with “construct” and “sodium selenate” as in between factors followed by Bonferroni post-hoc comparison showed a sodium selenate effect at p<.01 and a construct effect at p<.001. Bonferroni post-hoc comparisons (equality of variances verified) showed that the construct R1441C is different from the other two construct with p=.002 towards WT-LRRK2 and p<.001 towards the pCDNA empty vector. (b) Visualisation of (a). The first row shows SH-SY5Y cells, transfected with R1441C and H2B-mRFP and treated with 0µM sodium selenate. Dead SH-SY5Y cells are indicated with white arrows, and fragmented nuclei were visualized either by Hoechst 33342 or H2B-mRFP. In the second row, SH-SY5Y cells are also transfected with R1441C-LRRK2 and H2B-mRFP, but treated with 50 µM Sodium selenate. 20x magnification is used. (c) Enlargement of the parts of (b), indicated with white box.
Fig. 3.9: Transient knockdown of the PP2Ac subunit results in aggravation of cell death in G2019S cortical neurons, while treatment with sodium selenate is not able to reduce apoptosis/cell death in a significant extent. (a) Evaluation of shRNAs targeting the two isoforms of PP2Ac in mouse neuroblastoma N2A cells. Cells were treated with empty shRNA plasmid as a control. (b) Cortical neurons were derived from G2019S overexpressing transgenic mice, but also from WT overexpressing mice, including in both cases cortical neuron cultures from their littermates as a control. Afterwards cells were transiently transfected with the two shRNAs targeting the isoforms of the mouse PP2Ac (validated from figure 6A) or the empty shRNA vector. After 48 hrs post-transfection, cell death was measured after observing fragmented nuclei, through Hoechst 33342 staining. Stats: Data are means ± SEM. *p<.05, **p<.01, ***p<.001 with Student T Test (single comparison within groups) or One way ANOVA followed by post-hoc comparisons (with groups as in between factor). Equality of variances has been verified with Levene test. Two way ANOVA test with “Group” and “shRNA” as in between factors followed by Bonferroni post-hoc comparison showed a group and a shRNA treatment effects with the Group 2 (G2019S) being the only one differing from the three others with p=.001. (c) Western blots of G2019S cortical neuron samples or their littermates (Control G2019S) and statistical analysis of the band intensities of cleaved caspase 3/tubulin (d), demonstrates that after treatment with 35µM sodium selenate for 12 hrs, there is no significant statistical difference between treated or non-sodium selenate treated neurons (G2019S or Control G2019S). However, there seems to be a tendency for reduction of apoptosis, after treatment of G2019S-LRR2 neurons with sodium selenate (p=.244), compared to the littermate neurons, where the apoptosis rate is almost the same (p=1.000), with or without sodium selenate treatment. For (d), the average value of cleaved caspase 3 band intensity/tubulin intensity was set as 1-fold, and all the other values were normalized. (e) Similar results were obtained by counting fragmented nuclei in the presence or absence of sodium selenate in cortical neurons. Data are means ± SEM. No statistical differences were found.

In our effort to identify the mechanism of the neuroprotective effects of PP2A activation through sodium selenate in parkinsonian neuronal models such as cortical neurons and SH-SY5Y cells, we overexpressed WT and R1441C LRRK2 in HEK293T cells. The reason for that experiment was that since LRRK2 interacted with PP2A, it would be quite possible that PP2A could dephosphorylate LRRK2 in S/T residues. Afterwards we transfected the HEK293T cells with shRNAPP2Ac plasmids or their empty control plasmids and proceeded to immunoprecipitation of LRRK2. Purified LRRK2 was afterwards subjected to phosphoproteomics to trace differences in phosphorylation pattern between WT LRRK2 or R1441C LRRK2 and PP2Ac (shRNAPP2Ac) knockdown versus endogenous PP2A (shRNA empty plasmid). Unfortunately the phosphorylation mass spectrometry experiment failed to identify differences in the phosphorylation sites of LRRK2 in the presence or absence of PP2A catalytic subunit.

Afterwards we transfected WT versus R1441C LRRK2 in SH-SY5Y cells and then treated these cells with and without sodium selenate. Again immunoprecipitation of LRRK2 followed, and we still failed to detect through phosphoproteomics any phosphorylation residue of LRRK2.
3.5 Sodium selenate treatment is able to activate the Ras downstream pathway in SH-SY5Y cells and G2019S cortical neurons

Our next goal was to observe if PP2A activation (through sodium selenate treatment in an early time point such as 1hr) in SH-SY5Y cells or cortical neurons, could activate the Ras neuroprotection pathway as previously shown in literature. Initially we used the same concentration of sodium selenate, where we have seen the neuroprotection effects for SH-SY5Y cells (50 µM) and cortical neurons (35µM), to investigate whether this neuroprotection is partially explained by activation of the Ras pathway. For this experiment we checked the phosphorylation levels of Erk1/2, using as a control the basal protein levels of Erk1/2. Since the increase of the Erk1/2 phosphorylation levels is an early effect, we tested different time points/treatments of SH-SY5Y cells with sodium selenate. After treating the cells with 1hr sodium selenate, we were able to observe a difference in the phospho-levels of Erk1/2 (Fig 3.10a). Due to the fact that the loading controls from Fig. 3.10a were affected by the stripping process, we reproduced this result (Fig. 3.10b). Indeed after 1h treatment, there was a 2,75-fold increase (error bar value: ±0.49) of phospho-Erk1/2 levels treated with sodium selenate, compared with the non-treated sample. Another assay to verify the possibility that the Ras pathway is activated after sodium selenate treatment, is to check if the Ras binding domain (RBD) of Raf is able to bind more to Ras. The prerequisite for this assay is that activation of sodium selenate would be upstream of the Ras pathway, meaning that Ras should be already activated, and not acting lower than the Ras protein. Alternatively sodium selenate (or PP2A) could activate the Ras pathway downstream of Ras, but in case we could observe a Ras protein activation (through RBD binding), this could be explained by a positive feedback loop from a downstream protein to Ras protein. Indeed we used the GST fused RBD protein and incubated it with SH-SY5Y lysate. For the SH-SY5Y cell lysate there were two conditions; one set of cells has been previously treated with 50 µM of sodium selenate for 1h, while the other with water (diluent of sodium selenate) for 1h. Indeed there was a 1,65-fold increase (error bar value: ±0.06) of RBD-Raf to the Ras protein from the treated with sodium selenate SH-SY5Y cells, compared to the control sample (Fig. 3.10c). As explained extensively in introduction (Fig. 1.7), the activation of MEK-Erk pathway could be achieved through dephosphorylation of Ksr-1 at the critical residue S392 through PP2A. Since the Ras pathway seems to be activated in SH-SY5Y cells by sodium selenate, which is a PP2A chemical activator, we tested whether treatment of SH-SY5Y cells with sodium selenate would also dephosphorylate/decrease the S392 levels of Ksr-1. Indeed the S392 phospholevels of Ksr-1 were reduced 3-fold, compared to the untreated cells (Fig. 3.10d). Finally, since we also observed neuroprotection in the presence of sodium selenate in G2019S cortical neurons, we also investigated whether there was an activation of the Ras pathway through phospho-Erk1/2 levels. There seemed to be no remarkable difference between non-treated or treated with
sodium selenate littermate cortical neurons, while there was a 1.31-fold increase (error bar value: ±0.21) of the phosphor-Erk1/2 levels-treated with sodium selenate, compared to the G2019S non-treated neurons. (Fig. 10e). What however was interesting, was that non-treated with sodium selenate cortical neurons have a 2.43-fold (error bar value: ±1.03) increased phosphor-Erk1/2 levels, compared to non-treated with sodium selenate G2019S neurons (Fig. 10e). This indicates that it is possible that in parkinsonian models, such as overexpressing G2019S mice, the increase of cell death (due to the G2019S LRRK2 mutation) could be explained partially from the downregulation of the Ras neuroprotective pathway. At the same time we did not notice a decrease in the phosphorylation of Ksr-1 Ser392 in G2019S neurons after 1h sodium selenate treatment (Fig. 3.10f).

Fig 3.10: Sodium selenate is able to activate the Ras downstream pathway in SH-SY5Y cells and G2019S cortical neurons. (a) Different incubation times for sodium selenate (50 µM) have been tested in order to observe when/if there is a difference in phosphorylation levels of Erk1/2. After 1hr or 3hr treatment there seems to be an increase of the phospho-Erk1/2 levels (taking into account the basal pERK as a loading control). (b) SH-SY5Y cells, when treated with sodium selenate for 1hr, indeed showed increase of their phosphor-Erk1/2 levels. The intensities of the bands were measured using Image J, and the values from the phospho-Erk1/2 bands were divided to the corresponding band intensities of the basal Erk1/2 protein levels (Anti-pERK), which were used as a loading control. Later on, the phospho-Erk1/2 band intensity value/pERK1/2 band intensity value for the non-treated with sodium selenate samples was set as 1-fold. The sodium selenate treated phospho-Erk1/2 levels were then 2.75-fold higher (with an error bar value of ± 0.49) compared to the non treated samples. In (c), the RBD-domain of Raf is able to bind more efficiently Ras from SH-SY5Y lysate-previously treated with sodium selenate. Using as 1-fold the band intensity of the Ras protein bound to GST-RBD protein in the non-treated SH-SY5Y lysate, we
could observe that the Ras binding to the GST-RBD protein was 1.65-fold higher (with an error bar value of ±0.06), when Ras protein was coming from SH-SY5Y lysate-treated with sodium selenate. (d) Ksr-1 seems to be dephosphorylated by PP2A through sodium selenate in SH-SY5Y cells. After 1h sodium selenate treatment, Ksr-1 seems to be dephosphorylated in S392, while the non sodium selenate-treated cells have 3-fold higher S392 phospholevels (1 compared to 0.32 with an error value of ±0.045 - the basal Ksr-1 proteins levels were used as a loading control and the average of the pS392 band intensity/Ksr-1 of the untreated cells values was set to 1). (e) The Ras pathway is suppressed in G2019S cortical neurons. Littermate or G2019S neurons were treated with 35μM sodium selenate for 1h. While the is no remarkable difference between the Erk1/2 phosphorylation levels of littermates cultures treated with or without sodium selenate (non treated phosphoErk1/2/pErk1/2 band value was set as 1-fold, sodium selenate treated phosphoErk1/2/pErk1/2 value was slightly reduced at 0.95-fold with error band ± 0.15), there was a slight increase in the phosphorylation levels of sodium selenate treated G2019S cortical cultures versus the non-treated G2019S neurons (1.31-fold increase with an error bar of ± 0.21). Additionally we were be able to observe a difference between the Erk1/2 phosphorylation levels of non-sodium selenate treated littermate neurons and non-sodium selenate treated G2019S neurons (when the phosphoErk1/2/pErk1/2 band value of the non-sodium selenate treated G2019S cortical neurons was set as 1-fold, there was a 2.43-fold increase in the non-sodium selenate treated littermate neurons phosphoErk1/2/pErk1/2 levels with an error bar value of ±1.03. (f) After 1h sodium selenate treatment, there seems to be no dephosphorylation (due to PP2A activation) in littermate cortical neurons lysates. In contrast in the G2019S samples, not only seems to be a dephosphorylation, but a high increase in Ksr-1 S392 phosphorylation.
4. Discussion

4.1 The ROC domain of LRRK2 is efficient to bind PP2A

Previous studies have shown that proteins such as the death-associated protein kinase (DAPk) are interacting with the B’ regulatory subunit of PP2A, using their ROC domain as a main docking site (Carlessi et al. 2011). This demonstrates that the binding of LRRK2 to the PP2A seems to be more a ROC effect than a property of LRRK2 protein itself. Here we investigated whether LRRK2 also interacts with PP2A through its ROC domain. We show that the PP2A holoenzyme (subunits a,b,c) co-immunoprecipitates with LRRK2 through its ROC domain in SH-SY5Y and Hela cells. A number of LRRK2 interacting proteins have been described, including 14-3-3, ArfGAP1, Rac1 and Wnt signaling pathway protein disheveled (Chan et al. 2011, Muda et al. 2014, Sancho et al. 2009, Xiong et al. 2012). Our finding that the ROC domain of LRRK2 is sufficient for binding to PP2A is consistent with the previous observations showing that also other proteins specifically interact with this domain, i.e. death associated protein kinase (DAPk) and microtubules (Carlessi et al. 2011, Gandhi et al. 2008, Law et al. 2014).

4.2 The WT- and the R1441C-ROC domain of LRRK2 binds equally to PP2A, while LRRK2 interacts with PP2A in the perinuclear region of cells

We found that the mutation R1441C in LRRK2 did not affect the binding of PP2A to LRRK2-ROC. The R1441H/C/G mutation may trap ROC in a more persistently activated state which is compatible with the hypothesis of a constitutive PP2A binding to ROC independently of its activation state (Li et al. 2007, Liao et al. 2014,). Furthermore, the interaction of the two proteins takes place close to the nuclear envelope in Hela cells and cells of the substantia nigra from mice brains. This was expected since LRRK2 was localized in the cytoplasm and PP2A is localized to variable intracellular sites which depends on differentiation-induced phosphorylations of regulatory subunits B yet is mainly found in the nucleus, in this study (verified by confocal microscopy) (McCright et al. 1996).

4.3 Elimination of PP2Ac aggravates parkinsonian-cell death in neuronal models, while chemically-induced activation of PP2A protects from the toxic LRRK2 mutants

To investigate a possible relevance of PP2A for modulating the toxicity of R1441C-LRRK2 in SH-SY5Y cells, the two isoforms of the catalytic subunit of PP2A were silenced. Our data show that the knockdown of PP2Ac results in increased cell death of SH-SY5Y cells expressing R1441C-LRRK2 but not in WT-LRRK2 transfected SH-SY5Y cells. Additionally we observed the same aggravation of cell death in primary cortical neurons derived from mice overexpressing G2019S-LRRK2 (Li et al., 2010). Conversely, we used sodium selenate to see if there was a neuroprotection in SH-SY5Y cells and cortical neurons, as it has been described as an activator of PP2A, (Corcoran et al., 2010). Sodium
selenate was indeed able to partially rescue R1441C-LRRK2-induced cell death in SH-SY5Y cells, however in G2019S neurons we did not observe a dramatic effect in the presence of sodium selenate. Thus, our results show that a modulation of PP2A activity will selectively affect survival of cells expressing mutant LRRK2-R1441C but not the wild type LRRK2.

4.4 Correlation of shRNA-PP2Ac and sodium selenate systems with biological systems

In physiology, PP2A activity is not regulated by shRNA or selenate but by phosphorylation of the PP2A B subunits. These consist of four distinct families of regulatory subunits, B55 (B), B56 (B’), PR72 (B”’), and PR93 PR110 (B”’’) which are differentially expressed in many cell types (McCright et al. 1996). Inhibition of PP2A activity was shown to occur by protein kinase Ca (PKCa) – dependent phosphorylation of subunit B56α at Ser41 (Kirchhefer et al. 2014). Conversely, activation of PP2A has been described after B56 phosphorylation by protein kinase A in response to dopamine D1 receptors in striatal slices (Ahn et al. 2007). It remains to be investigated if regulation of PP2A activity by dopaminergic neurotransmission involving the activation of protein kinases may modulate the susceptibility to toxic stimuli evolving from R1441C-LRRK2 or G2019S-LRRK2 mutations. Interestingly, R1441C/G/H mutation of LRRK2 impair PKA phosphorylation of LRRK2 and disrupt its interaction with 14-3-3 resulting in an enhancement of toxicity (Muda et al. 2014).

4.5 Mechanisms of neuroprotection by sodium selenate in toxic LRRK2 parkinsonian models

Unfortunately, the mechanism of the selenate/PP2A-mediated rescue from R1441C – LRRK2 induced cell death remains unknown. PP2A could either directly dephosphorylate and thereby inactivate toxic LRRK2 kinase activity or may affect the LRRK2 downstream signaling such as toxic tau phosphorylation regulating microtubules dynamics (Kawakami et al. 2012). Furthermore, PP2A enhances the signaling of the small GTPase Ras by dephosphorylating the scaffolding complex Ksr-1 and contributes to cell survival and growth in Val12 transformed Cos cells via Rho guanine nucleotide exchange factor (Cullis et al. 2014, Ory et al. 2003,). It remains to be analysed if PP2A enhances Ras signaling activity which protects from lesion-induced degeneration in the brain (Heumann et al. 2000). However it is important to mention that others have shown contradictory data to that of Heumann et al. 2000, and show more specifically that LRRK2-G2019S expressing neurons result in an increase of phospho ERK levels, and that this increase accounts partially responsible the G2019S PD phenotype (Reinhardt et al. 2013). Initial experiments in our lab have shown that in SH-SY5Y cells, short-time treatment with sodium selenate, results in activation of the Erk1/2, which is a downstream target of Ras pathway. Additionally by pulldown, we could show that Ras itself is already activated in SH-SY5Y cells, upon sodium selenate treatment. Similar experiments in cortical neurons showed a similar tendency but not so significant such as for SH-SY5Y cells. For Ksr-1 phosphorylation, while we
observed a dramatic decrease of pS392-Ksr-1 in the presence of sodium selenate in SH-SY5Y cells, we did not observe any dramatic difference in mouse cortical neurons. Different sodium selenate time points and concentrations have to be tested (except 1hr and 35µM and 50µM sodium selenate). The final scheme demonstrating our data and the possible mechanism is shown below (Fig. 4.1). Taken together as of date, with the exception of this study, no others have demonstrated that PP2A is an interacting protein of LRRK2, and LRRK2 is establishing this interaction through its ROC domain. In conclusion, these data shed novel information on a potential regulator of LRRK2’s downstream signaling activity, and show PP2A as a potential therapeutic target of LRRK2 for PD.

i) No Ras/Erk1/2 activation through PP2A (no neuroprotection)

ii) LRRK2 kinase activity (increased cell death)

iii) no dephosphorylation of LRRK2’s downstream targets by PP2A (increased cell death rate)
b

Low Ras/Erk1/2 activation through PP2A

normal LRRK2 kinase activity (low cell death rate)

normal dephosphorylation of LRRK2’s downstream targets (low cell death rate)

i

ii

? (i)

? (ii)

? (iii)

?


c

↑↑ Ras/Erk1/2 activation through PP2A (neuroprotection)

↑↑ PP2A

↑↑ LRRK2 kinase activity (neuroprotection)

↑↑ dephosphorylation of LRRK2’s downstream targets (neuroprotection)

? (i)

? (ii)

? (iii)
Fig. 4.1: Schematic representation of the role of PP2A activation in LRRK2-induced cell death. (a) (i) When PP2A is inhibited, the phosphorylation of LRRK2’s important residues such as S1292 is increased (okadaic acid treatment-Reynolds et al 2014). Hyperphosphorylation of other critical residues such as T1503 could increase LRRK2’s kinase activity and explain the aggravated cell death levels that we observe in the presence of shRNA-PP2Ac. ii) Activation of the Ras pathway should not take place. iii) Downstream targets of LRRK2 could not be dephosphorylated by PP2A. (b) Under normal conditions PP2A could dephosphorylate LRRK2 in basal levels, and cell death levels would not be so dramatic (regarding WT LRRK2). ii) Ras pathway is slightly activated by PP2A while, iii) PP2A could be dephosphorylating LRRK2’s downstream targets in an extent. (c) When PP2A is activated (through sodium selenate for example), it could dephosphorylate LRRK2 much more than in (b) and that could result in decreased kinase activity of LRRK2 and subsequently explain the neuroprotection that we see in the presence of sodium selenate in LRRK2-induced parkinsonism. Another explanation for the neuroprotection is (ii) the activation of the Ras pathway and its downstream targets Erk1/2. Finally PP2A (iii) could dephosphorylate dramatically downstream targets of LRRK2, resulting in neuroprotection. The dashed lines represent speculated hypotheses. (The structure of LRRK2, shown in the figure, is obtained by Taymans and Baekelandt, 2014 and further modified).

4.6 Possible future experiments to be done

As extensively described above, the mechanism of how the presence and activation of PP2A (through sodium selenate treatment) in LRRK2-induced parkisonian cells, has neuroprotective effects, using different apoptosis assays, remains still unclear. Preliminary data (Fig. 3.10) show that PP2A could activate Ras and its downstream targets Erk1/2, which have been shown to potentially be neuroprotective in mice. Although the preliminary experiments in SH-SY5Y cells look very promising, we did not observe such a dramatic difference in cortical neurons derived from mice brains, which is a better neuronal model. Therefore different concentrations of sodium selenate should be used in the treatment of cortical neurons and additionally different treatment times, due to the fact that in cortical neurons the Ras activation through sodium selenate might be an earlier or a later effect compared with the same situation in SH-SY5Y cells. Also the Ksr-1 experiment (Fig. 3.10 e, 3.10 f) should be repeated in cortical neurons using different sodium selenate concentrations and treatment times. If the Ras pathway is activated through PP2A, it is quite possible that it could happen through Ksr-1 dephosphorylation (supported by Fig. 1.7-introduction, and our data in SH-SY5Y cells-Fig. 3.10 d-results). Finally what is very interesting to our findings and requires further investigation is the inactivation of Erk1/2 in G2019S mice compared to the littermate ones. If this finding could be reproduced with other experiments such as Ras activation (through RBD pull down), then there would be a direct connection of familial PD model (G2019S mouse) with suppression of the Ras pathway.
This finding would fit into our previous results where we have seen that Ras activation rescues chemically-induced PD mice.

Another explanation for PP2A neuroprotection would be the direct dephosphorylation of LRRK2 through PP2A on essential S or T residues. For this reason as also described above, we performed phosphoproteomic experiments in HEK293T and SH-SY5Y cells, treated with shRNA-PP2Ac or sodium selenate. Unfortunately we were not able to observe a difference in the phosphorylation status of LRRK2 (data not shown) and in some cases not even able to detect LRRK2 itself as a protein. Therefore the phosphoproteomic experiments should be repeated. Additionally there are commercial phospho-LRRK2 antibodies which could show if there is a difference in the phosphorylation of a specific LRRK2 residue. For example S1292 could be a good candidate to test, since it has been already shown to be sensitive to okadaic acid treatment (Reynolds et al. 2014). We have already tested phosphoantibodies of LRRK2 from different companies emphasizing on T1503 (at that time S1292 antibodies were not commercially available and pT1503 decrease results in reduction of LRRK2’s kinase activity), after immunoprecipitating exogenous WT or mutant LRRK2, but the experiments were not successful. This could be due to the difficulty to immunoprecipitate such a big protein as LRRK2 or because the antibody was not good. To conclude, mass spectrometric experiments together with phospho-LRRK2 antibodies experiments could be done to observe whether the the suppression and most importantly the activation of LRRK2 results in differences in the phosphorylation status of LRRK2.

Another hypothesis for the neuroprotection findings of LRRK2-induced parkinsonism through PP2A activation, would be that PP2A could not (only) dephosphorylate LRRK2 but its downstream targets, since LRRK2 has S/T kinase activity. For this experiment, immunoprecipitation experiments and mass spectrometric measurements should be done.

Other experiments with less priority would be to repeat the biochemical and fluorescent data in the mice models. For example the PLA technique could be tested in the substantia nigra sections to visualize the interaction of LRRK2 and PP2Aa. The substantia nigra section is selected because the dopaminergic neurons are situated in that region, however in order to prove directly that the colocalisation/Confocal- interaction-PLA of LRRK2 and PP2Aa is taking place in dopaminergic neurons, we would need to include a marker such as tyrosine hydroxylase. Therefore any new experiments in substantia nigra should also include tyrosine hydroxylase staining.
5. References


Part II

1. Introduction

1.1 VDAC in general

The Voltage dependent anion channel protein (VDAC) in early literature is described with the word porin (Benz et al. 1994). It has three isoforms namely VDAC-1, VDAC-2, VDAC-3. All of these three were initially found in the outer mitochondrial membrane (OMM). The three VDAC isoforms are sharing a 60-70% amino acid homology (Sampson et al. 1996, Sampson et al. 1997). However VDAC-1 is shown to some eukaryotic cells to be much more abundant compared to the other two isoforms (De Pinto et al. 2010). VDAC is shown to regulate the energy balance between the mitochondria and the remaining compartments of the cell, through transporting anions, cations but also ATP (Colombini et al. 1994, Benz et al. 1994, Gincel et al. 2001, Rostovtseva et al. 1997). The structure of VDAC-1 is also been identified and shown below (Fig. 1.1, Bayrhuber et al. 2008).

Fig. 1.1: 3-dimensional structure of VDAC-1. The presentation of the human VDAC-1 is shown and its scheme looks similar to a pore-like structure. The β-strands are marked with their respective numbers. (Figure taken from Bayrhuber et al. 2008).
1.2 Role of the mitochondrial VDAC/VDAC-1 in the apoptosis pathway

VDAC is considered to be a general pro-apoptotic protein marker. There are many different models, in which VDAC is participating to apoptosis. For example, it has been shown that VDAC is a component of the permeability transition pore (PTP), which is activated through apoptotic stimuli (Shoshan-Barmatz et al. 2006, Shoshan-Barmatz et al. 2003, Crompton et al. 1999, Bernandi et al. 1999, Lemasters et al. 2007, Tsujimoto et al. 2007). The PTP is found in the outer mitochondrial membrane and low molecular weight substances between the cytosol and the interior of the mitochondria are exchanged through it. Except VDAC, the adenine nucleotide translocator (ANT) and the matrix protein cyclophilin D (cypD) are also participating in the PTP formation (Reddy, 2013).

In another model, specifically VDAC-1 and the pro-apoptotic Bax protein could build a pore in the OMM, through which the cytoplasm c is released into the cytoplasm (Shimizu et al. 1999, Shimizu et al. 2000). Additionally, a third model suggests that if the VDAC-1 pore would remain closed, there would be no exchange of ATP and ADP between the cytoplasm and the mitochondria, resulting to the destabilization and collapsing of the mitochondrial membrane. This would finally result to the release of the cytochrome c from the mitochondria to the cytosol (Rostovtseva et al. 2005, Vander Heiden et al. 2000). Finally it would be possible that the OMM pores could be consisted of VDAC-1 oligomers, which could release further apoptotic proteins and thus resulting in a positive apoptotic feedback loop (Shoshan-Barmatz et al. 2006, Shi et al. 2003, Zalk et al. 2005).

VDAC-1, as mentioned above, is an important transporter of the OMM and a regulator of the apoptosis. Human VDAC-1 and its homologs in mouse and yeast have been identified as an essential apoptotic regulator. Experiments in different cell lines, independent of the origin species, showed that when VDAC-1 was overexpressed, apoptosis was induced. This demonstrates that the expression levels of VDAC-1 are an important regulator of the mitochondrial-induced apoptosis (Zaid et al. 2005, Godbole et al. 2003, Lu et al. 2007). On the other hand expression of a VDAC-1 N-terminus-deletion mutant in a VDAC stable knock down cell line, was able to inhibit release of cytochrome c and subsequently prevent apoptosis, induced by various stimuli. This shows that the N-terminus of VDAC-1 is essential for VDAC-1-induced apoptosis (Abu-Hamad et al. 2009).

1.3 Plasma membrane VDAC-1 and its link to apoptosis

The isolation and characterization of the plasma membrane VDAC-1 (pl-VDAC-1) took place in 1989 (Thinnes et al. 1989). Using human B-lymphocytes, they were able to isolate a 31-kDa protein from the plasma membrane fraction. This protein consisted of 282 amino acids and originally given the
name “porin 31HL” (Thinnes et al. 1989, Kayser et al. 1989). However, later on porin 31HL was shown to be identical with the human mitochondrial VDAC-1 (mt-VDAC-1). More specifically the amino acid sequence of the human porin 31HL was 100% identical to that of the human mt-VDAC-1 (Blachy-Dyson et al. 1993, Stadtmuller et al. 1999). Therefore porin 31HL was named plasma membrane VDAC-1 (pl-VDAC-1).

However in mice, there is a small difference in amino acid sequence between the mt- and the pl-VDAC-1 that could explain their different sublocalisation. Through alternative splicing, the pl-VDAC-1 contains one extra N-terminal hydrophobic 13-amino acid signal peptide which leads pl-VDAC-1 to the membrane (Buettner et al., 2000). After the pl-VDAC-1 reaches its destination, the signal peptide is cleaved (Buettner et al., 2000). Similarly to mt-VDAC, pl-VDAC-1 is also correlated with apoptosis. Pl-VDAC-1 is a NADH-ferricyanide reductase activity enzyme (Baker et al. 2004). When cells are ready to undergo staurosporin-induced apoptosis, the reductase activity of the pl-VDAC-1 is increased. Antibodies targeting the pl-VDAC-1 have shown to inhibit the reductase activity of the pl-VDAC-1 and subsequently the staurosporin-induced apoptosis (Akanda et al. 2008). The same conclusions were drawn from neural stem cells (Marin et al., 2007).

1.4 Plasma membrane VDAC-1 and Ras neuroprotectivity

The neuroprotective effects of synapsin promoter-driven Ras (synRas) transgenic mice have been extensively shown in the past literature by different working groups. Previous experiments demonstrated that mt-VDAC-1 seems to be a regulator of apoptosis. Additionally Neumann et al. 2010, have shown that neuroprotectivity in SynRas mice could be achieved through decrease in the expression levels of the pl-VDAC-1 and subsequently to its reductase enzymatic activity.

Initially endogenous VDAC-1 protein levels were shown by Neumann et al. 2010, to be decreased in SynRas mice-derived hippocampi neurons, compared to littermate-derived neurons. Additionally, when RT-PCR was performed, only the pl-VDAC-1 mRNA levels were decreased in synRas-derived cortices and hippocampi of adult mice, compared to the littermate-derived neurons, while the mt-VDAC-1 mRNA levels remained unaffected. Furthermore, a significant decreased NADH reductase activity was shown in primary cortical cultures of SynRas mice, compared to the littermate ones. In contrast, overexpression of VDAC-1 in PC12 cells induced cell death, while this cell death was prevented by co-expression of Val12-Ha-Ras. All the above data show that the pl-VDAC-1 has decreased protein levels and therefore decreased reductase activity in primary cortical neurons-derived from SynRas mice, resulting to neuroprotectivity, by inhibiting apoptosis through pl-VDAC-1.
Therefore the question which arises is whether pl-VDAC-1 is also regulating LRRK2-induced neurotoxicity, through its elevated pl-VDAC-1 reductase activity levels. That would show that pl-VDAC-1 is an important protein-switch, which either protects or leads to apoptosis in parkinsonian models through its reductase activity, depending on the activated upstream cascade signal.

1.5 Aim of the study

Initially we were interested to repeat the results of previous lab members, showing that the ROC domain of LRRK2 is able to interact with VDAC/VDAC-1 (through biochemical and mass spectrometric studies). Additionally we included the parkinsonian ROC-R1441C protein and investigated whether we could observe a difference in the binding properties of the ROC proteins towards total VDAC.

Additionally we would like to knockdown VDAC-1 by shRNA, and see the contribution of plasma membrane VDAC-1 regarding the cell’s reductase activity.

Finally probable differences in the binding affinity of the ROC-WT versus the ROC-R1441C towards VDAC/VDAC-1 and at the same time differences in the reductase activity levels of the plasma VDAC-1, could link directly LRRK2 mutations such as R1441C and apoptosis and shed light to LRRK2-induced cell death.
2. Materials and Methods

2.1 Plasmids

The cloning and the expression of GST-ROC-WT and GST-ROC-R1441C were previously described extensively. The plasmid pSuper Puro containing shRNA for human VDAC-1 (together with its control), was kindly offered by Dr. Sebastian Neumann. The targeting sequence for degrading the mRNA of VDAC-1 was 5’- AAAGTGACGGGCAGTCTGGAA – 3’ and was described extensively by Abu-Hamad et al. 2006). WT and R1441C human DNA LRRK2 cDNA sequences were kindly obtained by Prof. Schulz (Neurological Clinic, Aachen, Germany). 3x FLAG tagged-WT-LRRK2 and -R1441C-LRRK2 DNA sequences were subcloned into the pcDNA3.0 vector, through restriction from Drosophila expression vectors (pPFW) with XhoI and KpnI restriction enzymes and they were kindly offered by Prof. Hovemann (Molecular Cell Biochemistry, Bochum, Germany, unpublished data). The details for the construction of GST-ROC-WT and GST-ROC-R1441C plasmids are already described in section 2.24 of Part I.

For the construction of FLAG-ROC-WT and FLAG-ROC-R1441C plasmids, a PCR using 5’- AACCAACCGG TTA AAGGCTGTGCTTTATAACCAGATG-3’ forward and 5’- AGTCC CTGGAG TTA GAAATTAAGGCTCTCGTTTATGATGG-3’ reverse primer were used, in order to amplify the ROC domain of LRRK2. The same cycle steps for PCR were used with that of the PCR for the GST-ROC plasmid (Part I, section 2.24). The 3xFLAG was already inserted in pCDNA3.0 in the N-terminal part of the future inserted protein. After digestion of the PCR fragment with AgeI and XhoI and ligation to the 3x-FLAG-pCDNA3.0 plasmid (3xFLAG is in frame with the inserted ROC), we were able to get the FLAG-ROC-WT and FLAG-ROC-R1441C plasmids/proteins.

2.2 Knockdown of VDAC-1-Western Blotting

HEK293T cells were transfected with the pSuper Puro plasmid containing a targeting sequence for silencing VDAC-1 or the empty plasmid as a control. Lipofectamine LTX plus reagent was used for the transfection. Afterwards, transfected cells with the shRNA-VDAC-1 were selected with puromycin (2µg/µl) for 48hrs. Rabbit total VDAC antibody (#4866, cell signaling, Germany) was used to detect total protein levels of VDAC in Western Blotting.
2.3 Ferricyanide reductase assay

The ferricyanide reductase assay was previously extensively described by Akanda et al. 2008 regarding neuronal cells. Because of different cell populations, the conditions of the experiment were slightly changed. HEK293T cells were harvested and incubated in 1 ml buffer, containing 50 mM Tris-HCl, pH 8.0 and 500 μM β-NADH and 500 μM potassium ferricyanide, for 1 hr. Afterwards cells were spun down and the concentration of remaining ferricyanide was assessed, using a spectrophotometer, at 420 nm.

2.4 Statistics

Students T-test (two-tailed distribution/two sample equal variance) was used to calculate the significant difference between the measurements (*** p< .001, ** p< .01, * p< .05).
3. Results

3.1 The ROC domain of LRRK2 is able to interact with VDAC/VDAC-1

Initially we investigated whether VDAC is an interacting partner of LRRK2. Using a GST-ROC-WT and GST-ROC-R1441C proteins as a bait for a pull down, we were able to identify VDAC as an interacting partner of ROC-WT or R1441C. However VDAC seemed to interact much more with the ROC-WT than ROC-R1441C (Fig. 3.1). The upper data were confirmed by mass spectrometry (Fig. 3.2), whether a similar difference of binding between ROC-WT and ROC-R1441C towards VDAC-1 was noted.

Fig. 3.1: The GST-ROC-R1441C binds less to VDAC compared to GST-ROC-WT (as shown by GST pull down). The GST fused ROC domain of LRRK2 (WT or R1441C) is sufficient to interact with the VDAC protein, which is pulled down after using SH-SY5Y cell lysate. However ROC-R1441C binds less efficiently to VDAC than ROC-WT.
Fig. 3.2: The GST-ROC-R1441C binds less to VDAC-1 compared to GST-ROC-WT. Verification of the pull down experiment (Fig. 3.1) by mass spectrometry specifically for VDAC-1. VDAC-1 in the first spectrum seems to bind in the same complex with ROC-WT in a great extent, while ROC-R1441C is bound to much less VDAC-1 (second spectrum, peak with bold is representing VDAC-1). The sequence of an identified peptide for VDAC-1 is shown on top of the figure. The mass spectrometry measurement was conducted by our collaborator Mr. Shariful Islam and Dr. Marcus Kroeger.
3.2 The plasma membrane VDAC-1 is the main enzyme responsible for the reductase activity of plasma membrane enzymes in HEK293T cells

Since pl-VDAC-1 is known to be a NADH-ferricyanide reductase enzyme, which is involved in transmembrane redox regulation, different working groups (i.e. Akanda et al. 2008) have established reductase assays where they could measure the pl-VDAC-1 activity in neurons. Additionally pl-VDAC-1’s increased reductase activity is directly linked with apoptosis (Elinder et al. 2005). However a question which rises for this assay, would be that it is a general reductase assay not specifically measuring only VDAC-1’s reductase activity. More specifically, there is not only pl-VDAC-1 in the membrane of the cells which is a reductase enzyme. For this reason, HEK293T cells were transfected with shRNA-pl-VDAC-1, resulting in efficient silencing of VDAC-1 (Fig. 3.3a). Afterwards ferricyanide assay followed. As shown in the graph below (Fig. 3.3b), the total ferricyanide activity of HEK293T cells, is dramatically reduced to 5-fold (from 100% to 20%), in the absence of pl-VDAC-1. This verifies that the ferricyanide assay, is a proper assay for measuring the reductase activity of pl-VDAC-1, since pl-VDAC-1 is mainly responsible for the total reductase activity of the plasma membrane enzymes of HEK293T cells.

![Fig. 3.3: pl-VDAC-1 is the main responsible enzyme for the NADH-ferricyanide reductase activity of the HEK293T cells in their plasma membrane. (a) HEK293T cells were transfected with shRNA targeting the pl-or mt- VDAC-1 enzyme. As seen,](image-url)
compared to the control, VDAC-1 was efficiently silenced in the presence of the shRNA. VDAC-2 and VDAC-3 could not be detected, meaning that VDAC-1 is in extreme abundance in HEK293T cells. (b) Afterwards, the NADH-ferricyanide reductase assay for the plasma membrane enzymes of HEK293T cells was measured. The student’s T-test was used for calculating the significant difference.

3.3 The ROC-WT protein is able to reduce the reductase activity of the plasma membrane enzymes of HEK293T cells, while the ROC-R1441C is also reducing the reductase activity levels of the plasma membrane VDAC-1 in a lesser extent

Since we wanted to investigate the impact of the ROC-WT versus the ROC-R1441C in pl-VDAC-1’s activity, we transfected HEK293T cells with FLAG-ROC-WT and FLAG-ROC-R1441C, and used the full length LRRK2-WT and R1441C versions as positive controls. Interestingly, when the ROC-WT protein is expressed, the activity of pl-VDAC-1 is dramatically reduced. On the other hand, when ROC-R1441C is expressed, the activity of pl-VDAC-1 is increased compared with the ROC-WT, but still less compared to untransfected HEK293T cells (Fig. 3.4). An explanation for this observation is that ROC-WT and subsequently LRRK2-WT is binding to pl-VDAC-1 (Fig. 3.1 shows binding for the total VDAC levels, Fig. 3.2 shows reduced binding for VDAC-1) and acts as a chaperone, inhibiting pl-VDAC-1’s enzymatic activity and therefore being pro-apoptotic. On the other hand, when ROC-R1441C is present, it loses partially its ability to bind pl-VDAC-1. Therefore pl-VDAC-1 can act as a pro-apoptotic protein in a higher extent in the presence of the mutant R1441C. For the full versions of LRRK2-WT and R1441C, the similar pattern for pl-VDAC-1 activity was obtained but with not such a big difference between them. It is important to mention that for this experiment, HEK293T cells were chosen, because they don’t express endogenous LRRK2. In case we would use another more appropriate cell population (i.e. neuronal-like)-but expressing endogenous WT-LRRK2 we might had the risk that the LRRK2-access VDAC could bind only to the endogenous WT-LRRK2 and not to the ROC-R1441C or LRRK2-R1441C, due to their difference in their binding capacity to VDAC (Fig. 3.1, Fig. 3.2).
Fig. 3.4  Expression of ROC-WT in HEK293T cells is able to reduce pl-VDAC-1’s reductase activity, while ROC-R1441C restores it partially. FLAG-ROC-WT; FLAG-ROC-R1441C, full length FLAG-LRRK2-WT and full length FLAG-LRRK2-R1441C were transfected to HEK293T cells. Afterwards, all the above combinations were assayed to ferricyanide reductase activity. The student’s T-test was used in order to identify the significant difference between the statistical values.
4. Discussion

4.1 Association of VDAC/VDAC-1 with the ROC domain of LRRK2 and reduction of plasma membrane VDAC-1 activity in the presence of WT ROC indicate a chaperone-like function of LRRK2 towards plasma membrane VDAC-1

Mitochondrial or plasma membrane VDAC/VDAC-1 has been shown extensively by others to play an important role in the apoptosis mechanism of the cells. On the contrary, in neuroprotective models such as the synRas transgenic mouse, the expression of the plasma membrane VDAC-1 is shown to be downregulated (Neumann et al. 2010). Therefore it would be reasonable to hypothesize that especially the plasma membrane VDAC-1 is an important switch, deciding whether the cell will go to apoptosis or it will survive by increase or decrease of VDAC-1’s protein levels respectively.

Initially we tested whether VDAC/VDAC-1 associates with PD associated proteins such as WT-LRRK2 or R1441C-LRRK2, using the ROC domain as a possible interacting domain of LRRK2. Indeed different isoforms of VDAC (Fig. 3.1) and more specifically VDAC-1 (Fig. 3.2), mitochondrial or plasma membrane, seemed to interact with the ROC domain of LRRK2. However what was interesting was that the ROC-WT domain of LRRK2 seemed to interact in a bigger extent with total VDAC/VDAC-1 compared to the mutant ROC-R1441C domain of LRRK2. The interaction of LRRK2 with VDAC is already shown by Cui et al. 2011. However they claim that the interaction of LRRK2 with VDAC is through LRRK2’s ANL-LRR domain. We in contrast have shown through GST pull down assay and mass spectrometry that the ROC domain of LRRK2 is responsible for the interaction with VDAC/VDAC-1. Our last finding comes in agreement with experiments done by previous members of our lab (Jacob et al. 2010).

After showing that the total NADH-ferricyanide reductase activity of the plasma membrane enzymes in HEK293T cells is mainly due to the plasma membrane VDAC-1 (Fig. 3.3), we measured the reductase activity of HEK293T cells (we used HEK293T cells due to the fact that they express very low endogenous LRRK2) in the presence of FLAG-ROC-WT or FLAG-ROC-R1441C proteins. While the ROC-WT protein was able to significantly lower the basal reductase levels of HEK293T cells, ROC-R1441C managed to lower the reductase activity of the cells but in a lesser extent compared to the WT-ROC (Fig. 3.4). These data are supported by using as positive controls the full length-FLAG-WT-LRRK2 and the FLAG-LRRK2-R1441C, where not the same extreme effects are seen, but it seems to be the same tendency in terms of affecting VDAC-1’s reductase properties. These results together with the pull down results (Fig. 3.1, Fig.3.2) lead us to hypothesize that in the healthy situation, where WT-LRRK2 is present, it acts as a chaperone and is able to bind efficiently plasma
membrane VDAC-1, and inhibits it from acting as a reductase enzyme, and therefore inhibiting/reducing apoptosis. However in the presence of the mutant R1441C-LRRK2 and R1441C-LRRK2-induced PD, the mutant LRRK2 is not able to bind so efficiently the plasma membrane VDAC-1 and therefore VDAC-1 is able to act as a reductase and induce apoptosis.

4.2 Future experiments to be done

For the experiments where we showed that the WT-ROC is decreasing the reductase activity of the pl-VDAC-1, we used HEK293T cells. While HEK293T cells are not neuronal-like cells, they have the advantage that they have minimal levels of endogenous LRRK2. Basically they are considered to be as a LRRK2 knock-out cell line. In the situation of having R1441C-ROC protein expressed, and if R1441C-ROC would indeed bind pl-VDAC-1 less than the endogenous WT-LRRK2/WT-ROC of LRRK2, we would never manage to see an effect of the ROC-R1441C, because the endogenous LRRK2 would compete and finally bind to the accessible pl-VDAC-1 instead of ROC-R1441C. Therefore the HEK293T cells are a good model to test binding of LRRK2 towards pl-VDAC-1, but not good to conduct neuroprotection/apoptosis experiments in the presence of LRRK2/ROC-WT and LRRK2/ROC-R1441C and compare it with the decrease/increase of the reductase activity of pl-VDAC-1, due to the fact that they are not neuronal-like and sensitive to LRRK2-induced neurodegeneration. For the neuroprotection/neurotoxicity experiments, neuronal-like cells such as SH-SY5Y cells, stably knocking down their endogenous LRRK2 should be used. The shRNA sequence targeting LRRK2 should not be complementary to the sequence within the ROC domain of LRRK2. Afterwards the ROC-WT or the ROC-R1441C should be expressed and the reductase activity of the cells could be measured, together with viability/apoptosis assays, upon expression of the forementioned proteins. Alternatively, dopaminergic or cortical neurons, derived from a LRRK2 knock-out mouse could be used, in order to perform the same experiment.

Another experiment that could be done, would be to use other mutant versions of PD associated LRRK2 proteins. Since it seems that the ROC domain is responsible for the interaction, and that a mutant such as R1441C is capable of partially disrupting the interaction with pl-VDAC-1, the first candidate proteins would be PD-correlated mutations of LRRK2, located in the ROC domain. However, it would be also interesting to test other full length LRRK2 mutants, such as G2019S, since apoptosis in neuronal models in the presence of this mutation (and others) is increased compared to WT-LRRK2 and pl-VDAC-1 could also participate in this phenotype.

Furthermore another interesting point would be to reproduce Neumann’s et al. previous findings, meaning that in neuroprotective models such as this of the Ras mouse the protein levels of pl-VDAC-1 are getting decreased, leading to less apoptosis and explaining the neuroprotectivity which is observed.
These experiments could be done not only in a NMRI mouse background (data obtained from Neumann et al. 2010), but in different mice backgrounds such as C57Bl6, in order to strengthen our already existing findings. However we would like to investigate, whether the levels of pl-VDAC-1 are getting increased in PD models. Our last hypothesis is supported by already published work, which demonstrates that the total VDAC-1 protein levels have already been shown to be upregulated in brain tissue samples derived from Alzheimer’s Disease patients (Manczak and Reddy 2012, Reddy, 2013). Therefore it would be interesting to isolate and culture cortical neurons from G2019S-LRRK2 overexpressing mice and measure the levels of pl-VDAC-1 by RT-PCR, using as a positive control littermate mice or WT-LRRK2 overexpressing mice. If the levels of VDAC-1 would be increased compared to the control samples, that would indicate that the pl-VDAC-1 is a general switch in PD neuronal systems which decides the faith of the cell, meaning whether it will go to apoptosis or it will live. More interestingly, in case the pl-VDAC-1 levels would be increased in G2019S cortical neurons, and would be followed by apoptosis (Part I, Fig. 3.8c, d, e) compared to the controls, the next step would be to cross the G2019S mice with the synRas mice and see if the apoptosis levels would be decreased, compared to the G2019S mouse cortical neurons. If that would be the case, we would additionally test if the pl-VDAC-1 mRNA levels would be downregulated due to the neuronal Ras expression. If our hypothesis would be true we would be able to show that the Ras protects against LRRK2-induced parkisonian models (G2019S mouse) in terms of apoptosis, and that this protection can be explained due to downregulation of the protein levels of pl-VDAC-1, which under normal conditions leads the cells to apoptosis.

As mentioned, using the expression of the Ras pathway, could lead to the prevention of apoptosis in PD models, if the apoptosis is regulated through the pl-VDAC-1 protein levels. Another therapeutic strategy towards the decrease of the pl-VDAC-1 reductase activity and subsequently cell death, is already described by Akanda et al. 2008. Briefly, hippocampal neurons were incubated with antibodies against VDAC-1. More specifically they have added the antibodies in the medium solution of the cells, meaning that the antibodies would bind only to the pl-VDAC-1 and not to the mt-VDAC-1. Afterwards the neurons were treated with apoptotic agents, In the presence of the VDAC-1 antibodies, pl-VDAC reductase activity was blocked and apoptosis was reduced, when compared with untreated cells. Therefore the inhibition of pl-VDAC-1’s reductase activity by antibodies, is resulting in less apoptosis. Therefore we would like to test the same hypothesis in G2019S neurons, which have elevated apoptosis levels compared to their littermates, and test similar antibodies, compared to the antibodies used by Akanda et al. 2008, in order to block pl-VDAC-1. Alternatively we could design new antibodies targeting specifically the N-terminus of VDAC-1, which has been shown to mediate VDAC-1 induced apoptosis. If the apoptosis rate would be reduced in the presence of the VDAC-1/N-terminus VDAC-1 antibodies, this would prove that the apoptosis in a PD model is regulated through
the pl-VDAC-1 reductase activity, but would also be the start for a new therapeutic strategy towards LRRK2-induced parkinsonism.
5. General hypothesis of the neuroprotective role of PP2A and pl-VDAC-1 in LRRK2-induced Parkinson’s Disease

As extensively described in the first part of my thesis, mutations in LRRK2 gene result in familial Parkinson’s disease. Two of the most common LRRK2 mutations are R1441C and G2019S. We have shown, using different cell systems such as SH-SY5Y cells or cortical neurons, that the expression of these LRRK2 mutants leads to extensive cell death (Fig. 3.7a and 3.8a for SH-SY5Y cells/R1441C mutation, and 3.9b, 3.9c, 3.9d for cortical neurons/G2019S mutation), and therefore we were able to mimic the parkinsonic clinical phenotype in a cellular level. However we showed by using sodium selenate, a PP2A activator, that the LRRK2-induced cell death can be partially rescued (Fig. 3.8a and Fig. 3.9c, 3.9d, 3.9e). Subsequently, a possible mechanism through which PP2A is neuroprotective, is the activation of the Ras pathway by PP2A (Fig. 3.10).

At the second part of the thesis, VDAC-1 was found also as an interacting partner of LRRK2. As the pl-VDAC-1 was shown by previous collaborators (Neumann et al. 2010) to have decreased protein levels in a neuroprotective model such as the Ras mouse, we believe that the protein levels of pl-VDAC-1 could be an essential regulator of the LRRK2-induced apoptosis. It remains to be investigated if the pl-VDAC-1 protein levels are upregulated in Parkinson’s Disease models. In case our last hypothesis would be true, the downregulation of the pl-VDAC-1 protein levels in LRRK2-PD models could be a target for a therapeutic strategy against LRRK2 PD.

A total overview of PP2A and pl-VDAC-1 neuroprotective role in LRRK2-induced Parkinson’s Disease is shown below (Fig. 5.1).
Fig. 5.1: General scheme of PP2A and pl-VDAC-1 protecting from mutant LRRK2-induced neurotoxicity. i) When WT-LRRK2 is expressed the neuron is healthy. However, (ii) when there are LRRK2 mutations such as R1441C and G2019S, there is a significant cell death in the neuronal population leading to neurodegeneration. This neurodegeneration can be (iii) partially rescued, using sodium selenate, an activator of PP2A. PP2A in turn is activating the Ras pathway. Pl-VDAC-1 protein levels could be essential in LRRK2-induced cell death, since pl-VDAC-1 is a pro-apoptotic protein, and in neuroprotective models such as the Ras mouse, there is a decrease of the pl-VDAC-1 protein levels compared to the littermates (Neumann et al. 2010). The neuron figures were taken from http://stroke.nih.gov (know stroke campaign 26.02.2015). The LRRK2 figure is modified and taken from Taymans and Baekelandt, 2014.
6. References


7. Appendix

7.1 Curriculum Vitae

PERSONAL INFORMATION

First Name: Panagiotis
Last Name: Athanasopoulos
Born: July 6th, 1984 in Athens, Greece
Nationality: Greek
Phone Number: + (30)-6948327669, +(49)15784333922
E-mail: athanasopoulospanag@hotmail.com
Adress: Palaiopanagia Nafpaktou, 30300, Nafpaktos, Greece

EDUCATION

2011-todate: PhD student, Ruhr University of Bochum, Bochum, Germany

2007-2009: Master student, “Biomolecular Sciences” Prestige Master Programme, Graduate School of Life Sciences, University of Utrecht, The Netherlands

2002-2007: Bachelor student, Department of Biochemistry and Biotechnology at the University of Thessaly, Greece. Bachelor’s grade: 7.09/10.00

1999-2002: 1st High School of Nafpaktos, Nafpaktos, Greece. Grade: 17.7/20.0

SCHOLARSHIPS

2011-2014: ITN Marie Curie Fellowship, 7th Framework Programme, European Commission

2009-2010: B.S.R.C Alexander Fleming Postgraduate Scholarship
RESEARCH EXPERIENCE

October 2011-todate: PhD student, Molecular Neurobiochemistry Group, Faculty of Chemistry and Biochemistry, Ruhr University Bochum, Bochum, Germany. Title: Investigating the molecular role of LRRK2 protein in Parkinson’s disease models. Supervision by Prof. R. Heumann

January 2010-August 2010: Research Assistant, DNA Repair Group, Institute of Molecular Biology and Genetics, BSRC Fleming, Athens, Greece and Toxicogenetics Group 1, LUMC, Leiden University, Leiden, The Netherlands. Title: “Transcription-interference and associated DNA damage responses in mammalian cells.” Supervision by Dr. M. Fousteri

September 2009-December 2009: Research Assistant, Toxicogenetics Group 1, LUMC, Leiden University, The Netherlands. Title: “Role of 53BP1 in Transcription-Coupled Repair.” Supervision by Dr. M. Fousteri and Prof. L.H.F. Mullenders

January 2009-July 2009: Minor Master Thesis Research Project, Experimental Oncology Group, Utrecht University, The Netherlands. Title: “Regulation of Polo-like kinase and Aurora A in DNA damage”. Supervision by Prof. R.H. Medema. Grade: 8.0/10.0

February 2008-December 2008: Major Master Thesis Research Project, Membrane Enzymology research group, Utrecht University, The Netherlands. Title: “Role of CDC50 proteins in P4 ATPase-catalysed phospholipid transport”. Supervision by Dr. J.C.M. Holthuis. Grade: 7.0/10.0

Title: “Identification of the 5382insC mutation of BRCA1 gene in Greek sporadic breast/ovarian cancer patients using MS-PCR”.
Supervision by Dr. D. Yannoukakos. Grade: 10.0/10.0

VISITING SCIENTIST TRAINING STATIONS

March 2013: Visiting scientist in Behavioural Neuroscience Laboratory, National Neuroscience Institute, Singapore, Singapore. Supervisor: Dr. Zoe Bichler

June 2012: Visiting scientist in Cell Biology Department, Institut Curie, Paris, France.
Supervisor: Dr. Christophe Lamaze

June 2011: Visiting scientist in Biotechnological Company Silantes Gmbh, Munich, Germany. Supervisor: Dr. Hermann Heumann

MILITARY SERVICE

November 2010-August 2011: Hellenic Airforce.

WORKING EXPERIENCE

December 2010-August 2011: General Hospital of Hellenic Airforce, Athens, Greece, performing routine exams at the Immunology laboratory.

July-August 2004: Practical Internship, Biotech Company “AcronGenomics” – Eurogenet Labs, Athens, Greece. Title: “Studying the expression of hERR 1-4 and Ep-Cam genes in tumors”. Supervision by Dr. El. Georgakopoulos
7.2 List of Publications


7.3 Invited Oral speaker in Conferences and Workshops


7.4 Poster speaker in Conferences and Workshops (Selected poster presentations)


25-30 May 2013. ESF/EMBO symposium Molecular Perspectives On Protein-Protein Interactions, Pultusk, Poland. Title: “Identification of PP2A as an interacting partner of LRRK2”.

7.5 Acknowledgments

Initially I would like to thank Prof. Dr. Rolf Heumann, for accepting me into his lab and giving me the chance to do research in a very interesting project and conduct my PhD in Bochum. Additionally I would like to thank him for allowing me to do experiments that were coming to my mind, and by this way making me more critical and creative for my project. Additionally I am obliged to him for his support with financial matters such as chemical orders and attending conferences, since I felt that my research was not delayed through these matters. Finally I would like to thank him for encouraging me at some difficult times of my PhD and for his interest and help with my future career.

I would also like to thank Prof. Dr. Stephan Hahn for being my second supervisor and his support.
I would also like to thank Dr. Sebastian Neumann, not only for his support in the lab and his ideas for the project, but for also encouraging and advising me during my PhD time. Additionally I am grateful to Mrs Sabine Laerbusch for all her help during these 3 years.

I would like to thank present and previous members in the lab such as Daniela Damen, Koushik Chakrabarty, Christoph Goemanns, Nadine Ackermann, Anja Ehrkamp, Veena Nambiar Potheraveedu, Peter Wolff, Hendrik Schöneborn, Fabian Raudzus for their support in the lab, their interesting ideas and the great time that I had in Germany. Additionally I would like to thank Mrs Anja Koch, Mrs Jacinta Essling and Mrs Christiane Gelhaus for all the help with non-academic obligations, which helped me a lot during my time in Germany and made things much more easier for my daily life and I was concentrated to my research work.

I would like to thank Dr. Zoe Bichler for her help with the statistics and for hosting me in Singapore in order to work with the WT-LRRK2 overexpressed mouse, Mrs Sally Dong Qianying for doing the confocal experiment in the substantia nigra from the mice brains and Mrs Dini Nurul Binte Abdul Rahim for her support at the time of my research stay in Singapore.

I would like to thank Dr. Marcus Kroeger and Mr. Shariful Islam for the mass spectrometric work and Prof. Dr. Christian Herrmann, Mr. Sergii Shydlovskii and Mrs Semra Ince for their help with the GTP binding assay. I would also like to thank Prof. Katrin Marcus, Dr. Stefan Helling and Mrs. Jing Chen for the mass spectrometric experiments.

I am grateful also to the GSCB for all the support during my PhD and advices and TRANSPOL (MARIE CURIE ITN programme) for funding me.

I would like to thank my friends Yota, Dimitris, Christos, Giorgos, Tzihad and Sandra for their personal support during all these three years.

Finally I would like to thank my parents, Maria my Godparents and Konstantinos, Aggelos and Panagiotis for their support to me during my whole life.