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Effects of Alzheimer's and Parkinson's disease gene mutation on cell signaling

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Cover picture: Confocal image of a neuroblastoma cell stained with mitotracker orange (red), kinesin antibody (green) and DAPI (blue). Courtesy of Anna Sandebring.

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To Masoud

No man is an island

John Donne

ABSTRACT

Alzheimer's disease (AD) and Parkinson's disease (PD) are the major neurodegenerative disorders affecting the elderly. The discovery of genes causing familial forms of these disorders has contributed enormously towards our understanding of underlying pathogenic mechanisms. This thesis explores mechanisms by which Phospholipase C (PLC) mediated signaling is regulated by presenilin (PS) and parkin, these being genes in which mutations cause familial AD and PD respectively.

In **Paper I**, mouse embryonic fibroblasts (MEFs) lacking PS1, PS2 or both, were used to evaluate the dependence of PS on PLC and Protein Kinase C (PKC). Results revealed a dependence of both PS1 and PS2 on *m*-3M3FBS stimulated PLC activities. Also, PLC stimulated PKCα and PKCγ activities were dependent on the presence of both PSs. Protein levels of PKCα/γ were decreased in PS double knockout MEFs, while PKCδ levels were elevated in the same cells. These results were also verified by transfecting back PS and by using another cell line lacking PS. Furthermore PKCα levels were shown to be dependent on amyloid precursor protein (APP) and APP intracellular domain. It is concluded that PS modulate PLC and PKC activity and differently regulates PKC protein levels by both APP dependent and independent mechanisms.

Paper II explored the dependence of PS on Extracellular regulated kinase 1/2 (ERK1/2) activities. ERK1/2 has previously been implicated in the pathogenesis of AD by different mechanisms. Using MEF cells lacking PSs (same as in paper I) it was found that ERK1/2 activities were increased in PS1 or PS2 knockout MEFs. PKCα inhibition could reverse these elevated ERK1/2 activities. Results also revealed a lower PLC or PKC stimulation in PS double knockout cells. The total levels of ERK were only downregulated after phorbol-12,13-dibutyrate (PdBu) treatment in PS double knockout. These findings show that PS regulates ERK1/2 activity via a PKCα dependent manner. Lack of both PSs disrupts PLC/PKC signaling and this is also reflected in the lower downstream activation of ERK1/2.

In **Paper III,** PLCγ1 was found to interact with parkin, in cells transfected with parkin WT, mutants G328E and R42P. This interaction was also detected in cortical, striatal and nigral human brain homogenates. PLCγ1 protein levels were found to be higher in parkin knockout mice and lower in parkin WT cells. Also, parkin mutants cells showed a disrupted ubiquitination. In summary, PLCγ1 was identified as a novel substrate for parkin by using interaction and ubiquitination studies of PLCγ1 and parkin.

Paper IV describes functional studies that revealed an increased PLC phosphorylation and activity in parkin mutant cells. Also, intracellular calcium levels were elevated in these cells, which could be reversed by the PLC inhibitor Neomycin and ryanodine receptor inhibitor dantrolene, suggesting a deregulated PLC activity. Parkin WT was shown to be protective against 6-OH-dopamine toxicity. The toxicity seen in parkin mutants after 6-OH-dopamine, could be reversed by dantrolene treatment, suggesting that ryanodine receptor calcium deregulation contributes to toxicity.

In conclusion, these studies provide evidence that PS and parkin regulate PLC mediated signaling.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their roman numerals (I-IV).

I. **Nodi Dehvari**, Angel Cedazo-Mínguez, Ola Isacsson, Tatjana Nilsson, Bengt Winblad, Helena Karlström, Eirikur Benedikz, Richard F. Cowburn. *Presenilin dependence of phospholipase C and protein kinase C signaling*. Journal of Neurochemistry, 2007, 102, 848-57

II. **Nodi Dehvari**, Ola Isacsson, Bengt Winblad, Angel Cedazo-Mínguez, Richard F. Cowburn. *Presenilin regulates extracellular regulated kinase (Erk) activity by a protein kinase C alpha dependent mechanism*. Neuroscience Letters, 2008, 436, 77-80

III. **Nodi Dehvari** *, Anna Sandebring*, Amilcar Flores-Morales, Laura Mateos, Yin-Choy Chuan, Matthew S. Goldberg, Mark R. Cookson, Richard F. Cowburn, Angel Cedazo-Mínguez. *Parkin-mediated Ubiquitination Regulates Phospholipase C-gamma1*. Journal of Cellular and Molecular Medicine, 2008, in press

IV. Anna Sandebring*, **Nodi Dehvari** *, Monica Perez-Manso, Elena Karpilovski, Mark R. Cookson, Richard F. Cowburn, Angel Cedazo-Mínguez. *Parkin deficiency disrupts calcium homeostasis by modulating phospholipase C signaling*. Manuscript

* These authors contributed equally

CONTENTS

LIST OF ABBREVIATIONS

Aβ	Amyloid β-peptide
AD	Alzheimer's disease
AICD	APP intracellular domain
APP	Amyloid precursor protein
AR-JP	Autosomal recessive juvenile Parkinsonism
DAG	Diacylglycerol
ECL	Enhanced Chemiluminescence's
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERK1/2	Extracellular regulated kinase 1/2
FAD	Familial Alzheimer's disease
GPCR	G-protein coupled receptors
GSK-3β	Glycogen synthase kinase 3β
IBR	In-between-RING
IP_3	Inositol 1,4,5-triphosphate
IP_3 R	Inositol 1,4,5-trisphophate receptor
MEF	Mouse embryonic fibroblast
MPTP	*N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NFTs	neurofibrillary tangles
Pael-R	Parkin-associated endothelial-like receptor
PD	Parkinson's disease
PdBu	Phorbol-12,13-dibutyrate
PI	Phosphoinositide
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PM	Plasma membrane
PP	Phosphatase
PS	Presenilin
RING	Really Interesting New Gene
RTKs	Receptor Tyrosine Kinases
RyR	Ryanodine receptor
sAPP	secreted APP
SERCA	Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase
Snpc	Substantia nigra pars compacta
TBS-T	Tris-buffered solution containing 0.1% Tween
UBL	Ubiquitin-like sequence
WT	Wild-Type

INTRODUCTION

Life expectancy has risen and the elderly are an increasing segment of the population especially in the western industrialized nations. As such the health care needs of the elderly are continuously growing and are the subject of much research and debate. In normal aging there is a modest reduction in the mass and volume of the human brain, which may be due to die-back and death of neuronal cells. In the major neurodegenerative disorders of Alzheimer's and Parkinson's diseases, these changes are more profound in the brains of patients. The majority of Alzheimer's and Parkinson's disease cases are sporadic, although there are also rare familial variants where hundreds of different mutations in a small number of distinct genes have been found.

Alzheimer's disease

Alzheimer's disease (AD) is the most common cause of both neurodegeneration and dementia. Dementia affects 24 million individuals worldwide and AD is responsible for 50-70% of all dementia cases [1]. AD is primary characterized by progressive impairment of cognitive functions. Secondary symptoms include personality changes, decreased learning ability, shortened attention span, disorientation and eventual language loss [2].

The essential neuropathological features of AD are neuronal and synaptic loss, extracellular amyloid deposition in plaques and blood vessels, as well as intracellular neurofibrillary changes in the form of neurofibrillary tangles (NFTs), dystrophic neurites and neuropil threads composed of paired helical filaments containing hyperphoshorylated tau protein. Deposited amyloid is mainly composed of fibers of amyloid β-peptide (Aβ), which is formed by the proteolytic cleavage of the amyloid precursor protein (APP) [3].

AD is classified as either sporadic or familial. Three genes have been found in which mutations cause autosomal dominant familial AD (FAD), namely the

APP and presenilin 1 (PS1) and presenilin 2 (PS2) genes. Sporadic AD, which is the most common form, has also been associated with genetic predisposition, but appears to entail a number of gene polymorphisms that could increase the risk of developing disease [4]. The best established of these is the ε4 allele of the apoliporotein E gene. The disease phenotypes are similar for both sporadic and familial AD.

The exact causes and progression patterns of AD are still not fully understood. As discussed below the amyloid cascade is the dominant hypothesis upon which much recent research has been based.

Amyloid pathology

APP is a type 1 transmembrane protein ubiquitously expressed throughout the body. There are three different splice variants (APP-695, -751, -770) arising from a gene located on chromosome 21. APP can be processed by β-secretase followed by γ-secretase cleavage generating Aβ (Figure 1). This pathway is referred to as the amyloidogenic pathway while the non-amyloidogenic pathway starts by α-secretase cleavage of APP, followed by γ-secretase cleavage generating p3. Both these pathways also generate secreted APP (sAPP) forms and an APP intracellular domain (AICD) fragment [5]. sAPP has been associated with neuroprotection, learning and memory [6], while AICD translocates to the nucleus and couples to the nuclear adaptor protein Fe65 and regulates gene expression [7]. Also, the c-terminal part of APP has been shown to have neurotoxic properties [5].

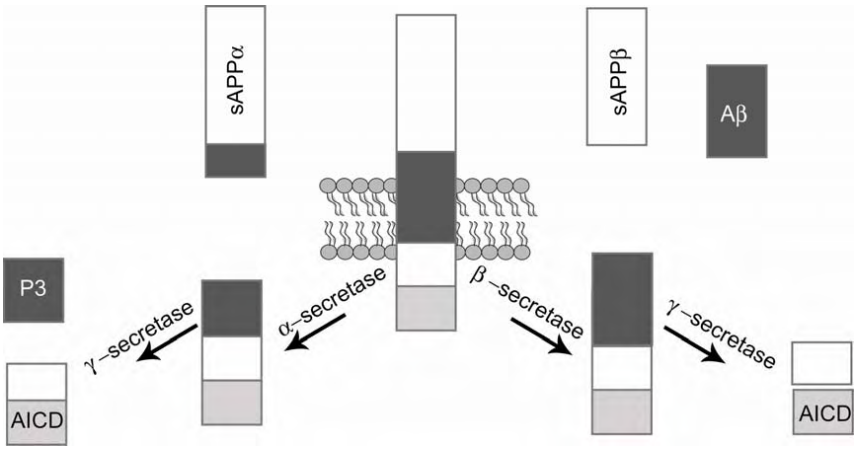

Figure 1. Proteolytic processing of APP. APP is pre-cleaved by α-secretase or β-secretase followed by γ-secretase cleavage, generating different products, as described in the text.

Aβ is the main component of amyloid plaques. The amyloid cascade hypothesis [8] states that an imbalance in production or clearance of Aβ plays an essential role in initiating AD. This theory is based on an initial increase of Aβ levels and the assembly of fibrils that are deposited in the plaques. Early studies demonstrated that the aggregation process of Aβ is essential for its toxicity, but later work has shown a correlation between the levels of soluble Aβ and the extent of synaptic loss and severity of cognitive impairment [9, 10]. Furthermore, Aβ-oligomers have been shown to inhibit hippocampal long-term potentiation [11, 12], to impair cognitive functions in rodents [13] and to result in synaptic loss in cultured hippocampal neurons [14, 15]. As such, much recent efforts have focused on Aβ oligomers, in some form, as being the neurotoxic species in AD. However, the relative importance of oligomers versus deposited Aβ for eventual disease symptoms remains an open question at present.

Tau pathology

Tau binds tubulin, thereby promoting microtubule assembly and stability. Hyperphosphorylated tau forms paired helical filaments (PHFs), the main

component of NFTs and other neurofibrillary changes. NFT accumulation is thought to start in the transentorhinal region of the brain and spreads to the hippocampus, amygdala and then the neocortex. This spread of pathology correlate well with the development of cognitive decline in AD [16]. Hyperphosphorylated tau is thought to lead to disrupted microtubules and resultant defective axonal transport, synapse loss and eventual cell death [17, 18].

Tau pathology in AD is believed to be caused by an imbalance of either protein kinase and/or phosphatase (PP) activity that are responsible for tau hyperphosphorylation. A number of protein kinases have been implicated in AD tau protein hyperphosphorylation, including Glycogen synthase kinase 3β (GSK-3β), Cyclin dependent kinase 5 (Cdk5), Extracellular regulated kinase 1/2 (ERK1/2), Calmodulin-dependent kinase II (CamKII), Protein kinase A (PKA) and Cell division cyclin 2 (Cdc2). PP-2A has been suggested to be the major phosphatase involved in dephosphorylation of tau in AD. The activity of PP2A has been shown to be reduced in brains of AD patients [19, 20].

Presenilin

Presenilins (PS) are 50 kDa proteins that contain nine transmembrane domains [21] and they have a wide tissue distribution [22]. Within cells, PSs are located mainly in the endoplasmic reticulum (ER) [23] and Golgi apparatus [24], but they have also been identified in the plasma membrane (PM) [25, 26], nuclear envelope [27] and mitochondria [28]. PS1 and PS2 were first identified because of their association to early onset, autosomal dominant FAD [29, 30]. The PS proteins display 67% amino acid sequence identity, and as such are highly homologous.

PS undergoes endoproteolytic processing to generate a 30 kDa N-terminal fragment (NTF) and a 20 kDa C-terminal fragment (CTF) [31]. PS CTF and NTF assemble to form the active γ-secretase complex of which Nicastrin, Aph-1 and Pen-2 proteins are also a part [31, 32]. PS provides the enzymatic activity of γ-secretase for processing of a number of type 1 transmembrane proteins including Notch and APP [33-35]. FAD PS1 and PS2 mutations are believed to

cause AD by altering APP cleavage resulting in increased production of longer more fibrillogenic forms of Aβ.

Besides providing γ-secretase enzyme activity, PS are involved in many cellular processes such as apoptosis, cell adhesion and calcium signaling. It has been shown that FAD PS mutations cause cell death or increase the sensitivity to cell death [36]. PS interacts with N-and E-cadherins at the cell surface stabilizing the cadherin-based adhesion complex [37]. PS also interacts with other proteins such as GSK3-β, β-catenin, tau and calcium-binding proteins such as calsenilin, calmyrin, syntaxin1A and sorcin [38-43]. PS also activates Phosphoinositide 3-kinase (PI3K)/AKT signaling pathway, thereby promoting cell survival. This has been shown to be suppressed by PS FAD mutations, leading to GSK-3 activation and subsequent tau phosphorylation [44]. A recent publication confirmed this in PS $^{-/-}$ neurons and also showed increased apoptotic caspase-3 activity by FAD PS mutations [45]. The modulation of calcium signaling is another important function for PS [46].

Presenilin and Calcium

Disruption of calcium homeostasis in AD was first suggested more than 20 years ago [47]. Since then it has been technically challenging to show direct calcium deregulation in brains of AD patients but an increase of calcium-dependent proteases has been reported [48]. Calcium deregulation has been postulated to be a causative factor in the disease. Calcium is involved in Aβ-induced synaptic dysfunction. Changes in calcium levels and dynamics have been reported by many different laboratories to alter metabolism and production of Aβ and it has been suggested that calcium dysfunction has a role in the initiation of sporadic AD [49]. Recently, a novel calcium-conducting channel was discovered in which polymorphisms were associated with increased risk for developing sporadic AD [50]. This novel channel, named Calcium Homeostasis Modulator 1 (CALHM1), was found to be expressed in all brain regions and localized both in the ER and PM.

Many FAD mutations in either the PSs or APP show perturbed calcium homeostasis. A range of cell model systems including skin fibroblasts from AD patients, xenopus oocytes and transgenic mice have been used to show that PS regulates calcium signaling. PS FAD mutations lead to enhanced release of calcium from ER by a mechanism suggested to be due to calcium overload. Likewise, knocking out PS also disrupts calcium signaling [51]. The ER contains two main types of calcium release channels, the inositol 1,4,5-triphosphate receptor (IP_3R) and ryanodine receptor (RyR). Calcium release by the IP_3R is stimulated by IP_3 that is formed after hydrolysis of plasma membrane phosphoinositide (PI) by the enzyme phospholipase C (PLC). RyR mediated calcium release is in itself stimulated by calcium ions, so called calcium-induced calcium release. IP_3 channel activity was shown to be increased in mutant PS1 and PS2 cells by using single-channel patch-clamp electrophysiology techniques [52]. Also, PS has been shown to interact with RyR via its N-terminus and the expression and activity of RYR was found to be upregulated by PS FAD mutations [53-57], with the outcome of enhanced cytosolic calcium. It has also been reported that PS may function as passive ER Ca^{2+} leak channels and that PS FAD mutations lose this function [58]. Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) has been shown to interact with PS and cells lacking PS have similar phenotypes to SERCA knockdowns [59], suggesting that PS can regulate calcium influx into the ER via SERCA.

Our group previously showed that FAD PS mutations in SH-SY5Y cells lead to enhanced calcium release from the ER and that this effect can be reversed by inhibiting PLC [60]. Basal and carbachol stimulated PLC activities were enhanced by the FAD PS1 ΔE9, L250S and M146V mutations. Pharmacological inhibition of γ-secretase and presenilin loss of function D257A and D385N mutations showed reduced PLC activity. Evidently, PS FAD mutations have a role in calcium regulation by multiple ways. All these findings suggest that endogenous PSs have an important impact on cellular calcium homeostasis.

Parkinson's disease

Parkinson's disease (PD) is the most common neurodegenerative movement disorder of the elderly. The number of PD patients over age 50 is between 4.1 and 4.6 million in western Europe's most and world's 10 most populous nations and will double by year 2030 [61]. The risk of developing PD between 55 and 85 years is 8.5 % and 7.7% for men and women, respectively [62]. PD is characterized by severe motor symptoms, including resting tremor, slowness of movement, muscular rigidity and bradykinesia [63].

In PD, there is a loss of dopaminergic neurons in the substantia nigra pars compacta (snpc), which is the main pathological hallmark and best linked with clinical motor symptoms. When the symptoms of PD first become evident more than 70% of these neurons have been lost. The surviving neurons have intracellular aggregates known as Lewy bodies [64]. Lewy bodies consist of lipids and proteins, of which the latter is predominantly α-synuclein [65]. Lewy bodies are also found in AD and other disorders such as diffuse Lewy body disease [63]. Other brain regions are also affected in PD, and in early stages it may affect the medulla oblongata, the lower region of the brain stem and gradually spreading upward through the basal ganglia into cortical areas [66].

PD has been considered for a long time to be a non-genetic disorder, but in recent years mutations in several genes have been linked to familial forms of PD. To date, 13 genetic loci, PARK1-13, have been found [67]. The first gene identified was that encoding for the α-synuclein protein [68]. Subsequently, the parkin, UCH-L1, PINK1, DJ-1, LRRK2, synphilin1 and NR4A2 genes have all been linked with familial forms of PD [69-76]. These genes have a mixed mode of inheritance both recessive and dominant and they could lead to disease by different suggested mechanisms, for example altered protein quality control (α-synuclein and parkin), mitochondrial dysfunction (PINK1 and DJ-1) and altered kinase activity (LRRK2) [77].

The involvement of calcium in the loss of dopaminergic neurons in Parkinson's disease

There are a number of theories as to why snpc dopaminergic neurons are preferentially lost in PD. One hypothesis declares that dopamine itself is the cause, which is based on the idea that oxidation of dopamine leads to production of free radicals that are damaging to cells. Other theories suggest that mitochondrial and proteasome dysfunctions are involved in PD. However, this does not rule out the selective vulnerability of snpc dopaminergic neurons. Nor do studies of the genes linked to familial forms of PD provide more clues as to why the snpc dopaminergic neurons are more susceptible to death in PD [78].

In contrast to other neurons, the snpc dopaminergic neurons are autonomously active. This pacemaking feature of the neurons could be a potential clue as to their vulnerability, leading to increased mitochondrial stress, accelerating cellular aging and death. Snpc dopaminergic neurons are physiologically different from other neurons because they generate action potentials autonomously without synaptic input. Instead of using Na^+ ions like most neurons, the snpc dopaminergic neurons rely on L-type Ca^{2+} channels [79, 80]. Snpc dopaminergic neurons have shown to have a lower level of the calcium-binding protein Calbindin that suggests a reduced calcium buffering capacity in these cells [81].

N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is an example of a non-genetic factor that can lead to PD. MPTP can evoke sustained elevations of intracellular calcium levels [82]. In this paradigm, it was reported that the calcium-activated protease calpain is activated and required for MPTP-induced death in mice. Calpains have also been shown to be elevated in brains of PD patients [83].

This higher metabolic rate that is dependent on high intracellular calcium levels increases the burden on the cell and makes it more vulnerable to environmental and genetic insults.

Parkin

Parkin is a ubiquitin-protein ligase (E3) [84] that is part of the ubiquitin/proteasome pathway responsible for protein degradation (Figure 2). Various deletion and point mutations of parkin have been found in autosomal recessive juvenile Parkinsonism (AR-JP) [69]. AR-JP linked to parkin is pathologically characterized by degeneration of dopamine neurons in the substantia nigra and locus coerulus and the absence of Lewy bodies, although there is one exception [85, 86].

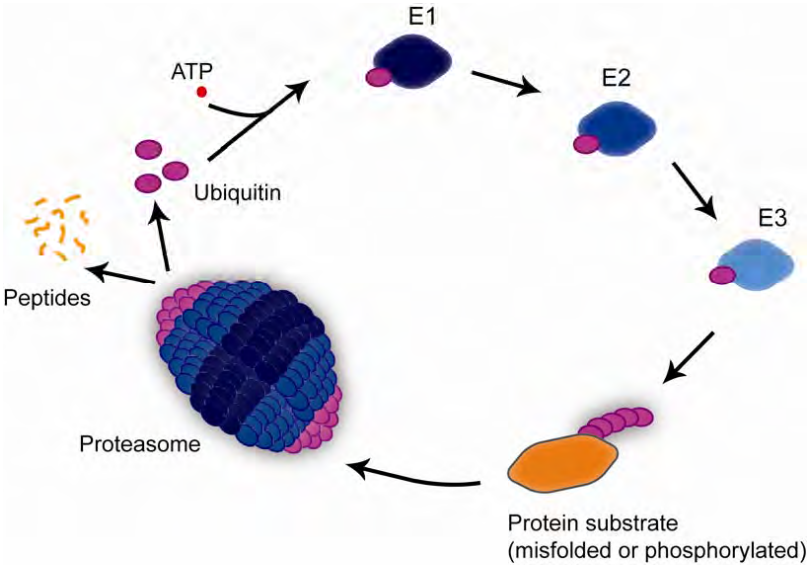

Figure 2. Schematic overview of the Ubiquitin/Proteasome pathway.
Polyubiquitination of a protein substrate leads to degradation of the target, while mono- or multi-ubiquitination leads to modulated functions of the protein target.

Parkin is a 52-kDa protein with a ubiquitin-like sequence (UBL) at the amino terminal region which interacts with Rpn10 in the proteasome cap [87]. This indicates that the UBL domain is responsible for bringing parkin close to the proteasome, directing the poly-ubiquitinated proteins for degradation. The AR-JP parkin R42P mutation disrupts this interaction because the mutation is

located in the UBL domain. Parkin has two RING (Really Interesting New Gene) finger motifs at the carboxy-terminal region separated by an in-between-RING (IBR) domain [88]. RING finger and IBR domains have been found in a number of E3 ligases [89, 90]. The RING-IBR-RING motifs are important for interaction with the substrates and the E2 conjugating enzymes UbcH7 and UbcH8 [91].

It has not been fully defined exactly how parkin ubiquitylates its targets. Other similar RING finger E3 ligases usually bring the ubiquitin bound to E2 to the substrate protein acting as scaffold. Parkin has several substrates, including the septins CDC-rel 1 and CDC-rel 2, cyclin E, p38 tRNA synthase, Pael-R, synaptotagmin XI and synphilin-1 [92-96]. Parkin has been found to localize to synaptic densities [97] and some of the substrate proteins are synaptic, implying that parkin could have a synaptic function.

It has been suggested that parkin loss of function (i.e. mutation in both alleles) leads to an accumulation of its substrates, which in turn result in cell death. It has been shown that overexpression of parkin substrate Pael-R lead to dopaminergic cell death which could be rescued by parkin but not by E3 inactive mutants [98]. To date, only one parkin substrate, aminoacyl-tRNA synthetase cofactor, p38/JTV-1, has been reported to accumulate in parkin knockout mice [99]. Parkin may also play a protective role in apoptotic cell death by delaying mitochondrial swelling and prevent cytochrome C release [100-102].

Phospholipase C mediated signaling

PLC mediated signal transduction processes lead to different effects in the cell, depending on different agents such as cell type, activation mode and the effects range from gene expression, differentiation, proliferation, motility, cell survival and death [103].

Phospholipase C

The minor phospholipid phosphatidylinositol 4,5-biphosphate (PIP_2) is hydrolyzed by PLC to generate diacylglycerol (DAG) and inositol 1,4,5-trisphophate (IP_3), which activates Protein kinase C (PKC) and induce the release of Ca^{2+} from internal stores respectively [104].

PLC has been the subject of extensive research and its role in signal transduction pathways is now well established. PLC activity was first described by Hokin and colleagues in 1953 [105] and 30 years later it was shown that the generation of IP_3 from hydrolysis of PIP_2, mobilized calcium from the intracellular stores [106]. Since then, 13 PLC isozymes have been identified in various tissues and organs. These different PLC isozymes are divided based on their structure and mode of activation into the subtypes PLC-β, -γ, -δ, -ε, -ζ and -η [103]. Most, PLC isozymes contain a pleckstrin homology (PH) domain, EF-hand motifs, catalytic X and Y domain, and C2 domain, with exceptions in some isozymes and splice versions. The highly conserved X and Y domains are composed of alternating α-helices and β-strands, located in between the EF-hand motifs and C2 domain as seen in Figure 3 [107].

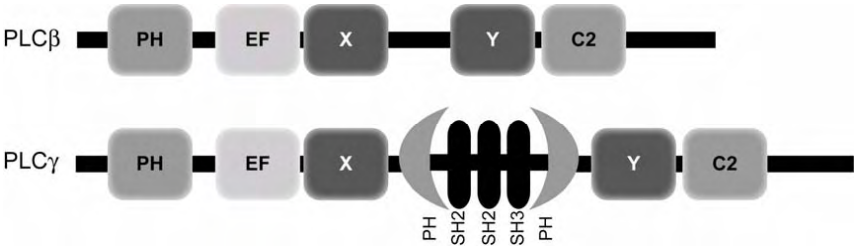

Figure 3. Domain structure of the different PLC isoforms, PLCβ and PLCγ, which have been studied in this thesis.

The studies described in this thesis have focused on PLC-β which is found mostly in cerebral cortex and hippocampus [108], and PLC-γ1 which is expressed in various tissues, including embryonic cortical structures, neurons, oligodendrocytes and astrocytes [109].

The activity of PLC isozymes is strictly controlled. In resting cells they are virtually inactive but after exposure to various extracellular stimuli they are immediately able to generate their messenger products [103].

PLC-β is activated by the G-protein coupled receptors (GPCR) [110]. The GPCRs act as detectors of a wide variety of stimuli such as hormones, neurotransmitters, light and odorants. These receptors span the plasma membrane and transduce their signals through G proteins, which are located at the cytoplasmic surface of membranes. G proteins consist of three subunits, an α-subunit which contains a site for guanine nucleotide binding and β- and γ-subunits that form a stable dimer. GDP is bound to inactive G proteins and when they are activated by the receptors GDP is exchanged for GTP. The G proteins hydrolyze slowly the bound GTP and thus catalyze their own inactivation. When the G proteins are active the β/γ-complex dissociates from the α-subunit and interacts with downstream effector molecules such as phospholipases, adenylyl cyclases and ion channels [111].

PLC-γ on the other hand is induced by Receptor Tyrosine Kinases (RTKs). The RTKs are transmembrane proteins with a protein tyrosine kinase domain at the cytoplasmic portion [112]. Growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factors (FGF) and nerve growth factor (NGF) bind to the extracellular domain of RTKs. Usually the growth factors that bind to the RTKs are dimeric molecules that bind two receptors simultaneously and promote the formation of stable receptor dimers. In this paradigm, receptor dimerisation leads to autophosphorylation of the intracellular tyrosine kinase domain. The phosphorylation of the tyrosine residues in turn precedes activation of kinase activity, autophosphorylation of other sites and phosphorylation of substrates. PLC-γ is among the substrates that bind to the phosphotyrosines with its SH2 domain, which facilitates its phosphorylation and subsequent activation. This association is also important for the membrane recruitment [113].

Protein kinase C

The serine/threonine PKC exists in multiple isoforms that are divided into three subclasses based on their second messenger mode of regulation namely conventional (α, βI, βII, γ), novel (δ, ε, η, θ) and atypical (λ, ζ) (Figure 4). The conventional PKCs are activated by the second messenger DAG and Ca^{2+}, while novel PKCs only respond to DAG and the atypical subclass respond to neither but sensitive to other phospholipids. All PKC isozymes consist of a C-terminal kinase core and an N-terminal regulatory portion [114].

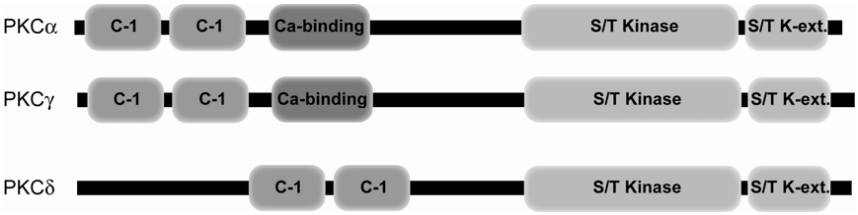

Figure 4. Multiples domains of the different PKC isoforms that were studied in this thesis, PKCα, PKCγ and PKCδ.

Before PKC can respond to lipid second messengers, the enzyme must be processed by phosphorylation. PKC is phosphorylated on the threonine residues at the kinase core by 3-phosphoinositide-dependent protein kinase-1 (PDK-1). This rate-limiting step of PDK-1 phosphorylation triggers the autophosphorylation on serine and/or threonine within the C-terminus making PKC catalytically competent and stable [115].

The regulatory moiety of PKC contains the pseudosubstrate sequence which is followed by cysteine-rich sequences occupying the phosphatidylserine and DAG docking sites [116]. The pseudosubstrate occupies the substrate-binding cavity of PKC and upon activation, the affinity of the pseudosubstrate for the catalytic site is reduced which allows PKC to bind and phosphorylate its substrates [117]. The activation mechanism is associated with translocation of PKC from one to another subcellular compartment. The second messengers DAG and Ca^{2+} initiate the membrane translocation and activation of PKC. The correct localization of PKC is mediated by the anchoring protein Receptor for

Activated C Kinase (RACK) which brings PKC close to the relevant substrates [118].

Extracellular regulated kinase

Both ERK 1 and 2 (44 and 42 kDa) isoforms are ubiquitously expressed and have the same substrate specificity. ERK1 knockout in mice are viable and fertile [119] while ERK2 knockout mice are lethal at embryonic stages [120], indicating that ERK2 can compensate for the most functions of ERK1 but not the other way around.

ERKs are serine/threonine kinases that are regulated by phosphorylation cascades organized in specific modules. A myriad of extracellular stimuli can induce the ERK cascade acting though cell surface receptors such as RTKs and GPCRs. These cell surface signals converge towards activation of small G-protein Ras that in turn recruits Raf family of ser/thr kinases (Raf-1, A-Raf, B-Raf). B-Raf is directly activated by Ras, while A-Raf and Raf-1 are first needed to be phosphorylated by membrane-bound tyrosine kinases, including c-Src. The signal is further amplified when MEK1 and 2 are activated by Raf phosphorylation at two serine residues [121]. MEK1/2 in turn, dually phosphorylate ERK1/2 on tyrosine and threonine residues of highly conserved motif Thr-Glu-Tyr (TEY) found within the activation loop.

Sequentially ERK1/2 phosphorylates numerous substrates including various membrane proteins (CD120a, Syk, and calnexin), nuclear substrates (SRC-1, Pax6, NF-AT, Elk-1, MEF2, c-Fos, c-Myc, and STAT3) and cytoskeletal proteins (neurofilaments and paxillin). Phosphorylation of ERK1/2 allows the ERK-MEK complexes dissociate and ERK1/2 to translocate into the nucleus, hence the nuclear substrates. ERK pathway is tightly regulated to ensure proper outcome of biological responses such as cell proliferation, differentiation and survival [122].

AIMS OF THE THESIS

PLC is an important upstream regulator of different cell fates such as survival and death and its involvement in the major neurodegenerative disorders AD and PD could be important to understand the underlying mechanism of pathogenesis. The overall aim with this thesis was to investigate the roles of presenilin and parkin on different aspects of PLC mediated signaling.

Paper I and II

To study the effect of presenilin on PLC, PKC and ERK1/2 activities.

Paper III and IV

To investigate whether PLCγ1 is a substrate for parkin and the effects of AR-JP parkin mutations on PLC and calcium signaling.

METHODOLOGY

Cell culture and transfections (Papers I-IV)

Fibroblasts generated from PS1, PS2 and PS1/PS2 knockout mice (provided by Bart De Strooper) were used in papers I and II. Blastocyst-derived embryonic stem cells lacking both PS1 and PS2 (BD8) or lacking PS2 (BD3) (obtained from Donoviel and St.George-Hyslop) and APP knockout fibroblasts and APP wild-type (WT) (provided by Ulrike Mueller) were also used in paper I. Dopaminergic human SH-SY5Y neuroblastoma cells were transfected with various expression vectors using Lipofectamin^{TM}2000 and siRNA knockdown by using DarmaFECT according to the manufacturer's instructions for the papers III and IV. All cells were cultured at 37°C, 5% CO_2, in Eagle's Minimal Essential Medium with Glutamax supplemented with 10% Fetal Bovine Serum. The cultures of stable neuroblastoma transfectants were supplemented with 200 μg/ml geneticin.

Brain tissue extraction and preparation (Paper III)

Whole brains from parkin knockout mice (gift from Jie Shen) [123] (*n*=4) were dounce homogenized on ice in 50 mM Tris-HCl (pH 7.4) plus 150 mM NaCl, 1% (v/v) Triton-X100 and complete protease inhibitors. Wild type littermates (n=4) were used as controls. Samples were centrifuged at 1000 x *g* and protein concentration of the supernatants containing soluble proteins were frozen until analysis by immunoblot.

Human brain (obtained from Huddinge brain bank) were homogenized by several strokes at 1500 rpm with a high torque motor-driven pestle on ice in 20 mM Tris-HCl, 137 mM NaCl, 2 mM EDTA, 2% Nonidet P-40, 2% Triton X-100 with protease inhibitor cocktail and the samples were centrifuged at 10000 x g for 10 min and the supernatant was used for co-immunoprecipitation.

Phosphoinositide hydrolysis assay (Papers I and IV)

Mouse embryonic cells (MEF) in paper I and Neuroblastoma cells in paper III were cultured in 35-mm dish and 150-mm dish respectively until 80%

confluence. One day prior to the experiment, medium was changed to serum free media containing 5μCi/ml myo-[2-^{3}H] inositol and incubated for 24 h. The MEF cells were treated with or without 50 μM *m*-3M3FBS for 20 min and the reactions stopped by adding 0.2M Perchloric acid followed by 1 h incubation on ice. The cells were harvested by scraping with a rubber policeman. Contents were centrifuged at 3000 x g for 15 min. Supernatants were transferred to glass centrifuge tubes and the pH adjusted by 1.5M KOH/75 mM HEPES to pH 7.2-7.6. Labeled inositol phosphates were separated from myo-[2-^{3}H] inositol by Dowex chromatography. In order to extract the lipid phases the pellets were mixed with HCl, H_2O and chloroform. The chloroform phases were removed, placed into scintillation vials and allowed to evaporate before determination of "lipid dpm" by scintillation spectroscopy. Results were expressed as dpm IPs/(dpm IPs + dpm lipid).

PI hydrolysis for the neuroblastoma cells was performed in a different manner. Krebs-Heinseleit bicarbonate buffer containing 10 mM LiCl (KHB/Li) was added to the cells after the 24 h incubation with myo-[2-^{3}H] inositol. The cells were harvested by scraping and the content was centrifuged at 15 000 rpm for 15 min. The pellet containing the cells was re-suspended in KHB/Li, gassed with 5% CO2, 95% O2 and added to glass centrifuge tubes. The tubes were incubated at 37°C for 30 min following addition of chloroform:methanol (1:2) and incubation on ice for 30 min. The soluble and lipid phase was separated by adding chloroform and water followed by vortexing and centrifugation. Dowex chromatography was used to separate the labeled inositol phosphates from myo-[2-^{3}H] inositol in the soluble phase and the lipid phase was directly added to scintillation vials.

Subcellular fractionation (Paper I)

In study I MEF cells were cultured until confluence in 15 cm^2 petri dishes and incubated with serum free media for 24 hours. Thereafter cells were treated with or without 1μM phorbol-12,13-dibutyrate (PdBu) or 50μM *m*-3M3FBS for 20 min. Cells were harvested by scraping in lysis buffer containing 20mM Tris-HCl pH 7.4, 0.32 M sucrose, 2mM EDTA, 50mM β-mecaptoethanol, protease

inhibitor cocktail, 50mM NaF and 0.2mM Na_3VO_4. Subsequently, cells were sonicated (12 s, 22 μ) and ultracentrifugation was performed at 100,000 x g for 30 minutes at 4°C as described previously [124]. Supernatants were designated as soluble fractions. Pellets were resuspended by sonicating 6 x 10 seconds in lysis buffer containing 0.5 % Trition X-100 and designated as particulate fractions.

Immunoblot analysis (Papers I-IV)

Protein concentrations were measured using BCA Protein Assay kit or Bradford for samples containing β-mercaptoethanol. 20 μg total protein amounts were resolved on 12 % acrylamide separation gels by SDS-PAGE. Proteins were transferred to ECL Nitrocellulose membranes by using trans-blot electrophoresis transfer. The membranes were blocked in 5% dried milk in Tris-buffered solution containing 0.1% Tween (TBS-T) for 1h at room temperature (RT) and probed with primary antibody (as seen in method section of each paper) at RT over night. Membranes were washed with TBS-T 10 min and 3 x 5 min. Secondary antibody (anti-rabbit or anti-mouse horseradish peroxidase-linked) was incubated at RT for 1 h. Membranes were washed again as above. Enhanced Chemiluminescence's (ECL) was used for protein detection.

Co-immunoprecipitation (Paper III)

The lysates containing 200 μg protein in 200 μl lysis buffer were pre-cleared first with Protein A/G-Sepharose beads. The lysates were immunoprecipitated overnight with 1 μg of primary antibody (as seen in method section for paper III). Thereafter, beads were added and lysates incubated for two hours at 4°C with rocking. The immune complexes were washed several times with lysis buffer and analyzed by immunoblot analysis.

Immunocytochemistry (Paper III)

Cells grown on glass coverslips were fixed in cold methanol for 10 min at ice and subsequently washed with PBS. Cells were permeabilized and unspecific

binding of antibodies were blocked by treating cells with buffer containing 5% BSA, 0.2% Triton X-100, 2% goat serum (in PBS) for 30 min. Primary antibodies PLCγ1 anti mouse IgG (1:100) and Parkin anti rabbit IgG (1:100) were incubated overnight at 4°C. Cells were washed several times with PBS prior to secondary Alexa Fluor 488 goat anti mouse IgG (1:1000) and Cy3 conjugated goat anti rabbit IgG (1:500) and DAPI (1:1000) and incubated for 1h at RT. Finally cells were washed several times with PBS and mounted with Fluorescent Mounting Medium on glass slides.

In vitro ubiquitin conjugation assay (Paper III)

PLC-γ1 and parkin immunoprecipitates were washed 3 times with washing buffer containing 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 25 μM N-Carbobenzoxy-Leu-Leu-Leucinal (MG132) , 10 nM dithiothreitol, 10 mM N-ethylmaleimide, protease inhibitor cocktail, 50 mM NaF, 1 mM Na_3VO_4. The immune complexes were analyzed by Immunoblot analysis and normalized to contain similar levels of PLC-γ1 and parkin. Thereafter, they were mixed and incubated together for 10 minutes at 30°C with agitation in a reaction buffer containing 10% (vol/vol) reticulocyte lysate, 1 μM dithiothreitol, 20 mM HEPES, 10 mM $MgCl_2$, 1 mM ATP, 1 mg/ml hexokinase, 20 mM deoxyglucose, 25 μM MG132 and His-tagged ubiquitin (5 μg/ml). The reaction was terminated by washing twice with washing buffer. The immune complexes were analyzed by Immunoblot analysis.

Intracellular Ca^{2+} measurements (Paper IV)

Intracellular Ca $^{2+}$ concentrations ($[Ca^{2+}]_i$) were essentially determined by using Fluo-3 as previously described [60, 125]. Basal $[Ca^{2+}]_i$ was measured in both Ca^{2+}-free PBS and phenol red-free MEM (containing 1 mM Ca^{2+}). For the experiments with neomycin or dantrolene, the agents were included in MEM without phenol red used during the 120 min of incubation period and also in the PBS for the 10 min incubation when basal $[Ca^{2+}]_i$ were measured.

MTT assay (Papers II and IV)

Cell viability was determined by the MTT assay. MTT powder was dissolved in MEM without phenol red at 0.3 mg/ml and then added to the cells. After 1 h at 37°C, the medium was removed and the formazan crystals were dissolved in isopropanol. Aliquots were moved to a 96-well plate and optical densities read at 540 nm in a Molecular Devices Spectra MAX 250 plate reader. For the experiments with dantrolene, cells were pretreated with 120 μM dantrolene for 30 min, followed by treatment with 120 μM 6OHDA for 6 h. Control cells received the equivalent amount of vehicle. Results were expressed as a percentage of values obtained for non-treated cells.

Statistical analyses (Papers I-IV)

The results were analyzed for statistical significance by ANOVA followed by Fisher's post-hoc test or student t-test. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Presenilin regulates phospholipase C, protein kinase C and extracellular regulated kinase signaling (Papers I and II)

PS mutations have been associated with calcium deregulation, which was also confirmed by our group showing cholinergic dependent calcium alterations in cells overexpressing different PS FAD mutations [126]. However, the exact mechanisms have not yet been elucidated. Cholinergic signaling has been shown to be impaired in AD [127]. PLCβ that is coupled to GPCR is essential for cholinergic signaling in hippocampal and cortical neurons [128].

The effect of PS on PLC-mediated signal transduction mechanisms was examined using mouse embryonic fibroblast (MEF) cells lacking PS1 or PS2 or both (papers I and II). The calcium alterations seen previously in PS FAD mutants were due to increased PLC activity [126]. Therefore the study was initiated by measuring PI hydrolysis (which reflects PLC activity) in the cells lacking PS, with and without the PLC activator *m*-3M3FBS. Results showed an increased PLC activity after stimulation with *m*-3M3FBS in PS wild-type (WT), PS1 and PS2 knockout MEFs but not in the PS double knockout MEFs. These findings demonstrated that both PSs are needed for full *m*-3M3FBS stimulated activity of PLC. By using the direct PLC activator *m*-3M3FBS, the data also revealed that PSs have an effect directly on PLC rather than on receptor coupling to the enzyme.

We continued studying the downstream effects of PLC by investigating PKC activity. PKC has been associated with AD, because of its positive effects on the non-amyloidgenic pathway and here we studied its role from another angle. We examined three PKC isozymes, specifically calcium-dependent PKCα and PKCγ and the calcium-independent PKCδ. Subcellular fractionation of MEF cell lysates into soluble and particulate fractions was used to determine the translocation of PKC following either *m*-3M3FBS activation of PLC or PdBu activation of PKC. PKCα was activated following *m*-3M3FBS in all cells but not

in PS double knockout MEFs verifying that PS is involved in the regulation of PLC activity. PKCγ was not activated following *m*-3M3FBS but the PS double knockout MEFs showed a lower basal and stimulated PKC activity. PdBu binds PKC at the same site as DAG and mimics its effect. PdBu treatment showed similar PKCα/γ activation in all cell types, which demonstrates that PKC activity per se, was not affected.

PKCδ activity could not be studied by using the subcellular fractionation approach because this isoform was predominantly located in the particulate fraction and therefore no changes could be detected after *m*-3M3FBS stimulation. By examining the different fractions separately PKCδ levels were shown to be higher in soluble fractions of PS1 knockout and PS double knockout MEFs as compared to the other cell types. PKCδ was downregulated by PdBu treatment in all cell types, which occurs after prolonged activation and the duration can differ for different isoforms, hence PKCα/γ were not affected at this time point. In order to elucidate if PS had any effects on PKC protein levels, total lysates were analyzed by immunoblotting. The results showed that both PKCα and PKCγ protein levels were lower in PS double knockout cells whereas PKCδ were higher in PS1 knockout and PS double knockout cells, suggesting that PS could modulate PKC expression. These findings also substantiate that different PKC isoforms have different roles in the cell. Reports have shown that PKCδ downregulation leads to tumor promotion and that upregulation induces apoptosis [129, 130].

Transfection of PS1 and PS2 to PS double knockouts and PS1 knockout MEFs gave a reversal effect on the PKC phenotype. Also, data generated in BD8 ($PS1^{-/-}PS2^{-/-}$) and BD3 ($PS1^{+/-}PS2^{-/-}$) cells confirmed these findings, suggesting that effects were not due to any cell line specific artifact.
It has been reported that AICD gene transcription can regulate ER calcium signaling [51]. Therefore, we also studied the effect of APP on PKC levels. APP knockout MEF cells had a lower level of PKCα in comparison to APP WT MEFs while the other PKC isoforms were not changed, suggesting that PKCα

is regulated in a different manner compared to the other isoforms. These findings also suggest an integrated signaling system, where AICD regulates the levels of PKC and in turn APP processing is regulated by PKC. PS also play an important role in this pathway by determining PKC levels.

Activation of PLC initiates a signal transduction cascade that also involves the downstream effectors ERK. This part of the signalling pathway was examined in paper II with a focus on the relevance of PS and PKCα. Data showed a higher basal ERK1/2 phosphorylation in PS1 and PS2 knockout MEFs, with no differences in ERK1/2 protein levels between the different cell lines. The enhanced ERK activity could be reversed by the specific PKCα inhibitor Gö6976. As seen in paper I there were no changes in PKCα levels or activity in PS1 and PS2 knockout cells suggesting the involvement of other signalling pathways that result in ERK becoming a better substrate for PKCα. We observed a lower activation of PLC (*m*-3M3FBS) and PKC (PdBu) stimulated ERK1/2 phosphorylation in PS double knockout MEFs, confirming the data obtained in paper I, that lack of both PSs leads to disrupted PLC/PKC signaling.

Parkin regulates Phospholipase C-gamma1 signaling and modulates calcium homeostasis (Papers III and IV)

The ubiquitin E3 ligase parkin is involved in regulation and degradation of different proteins and it is known that PLCγ1 is degraded in the proteasome after ubiquitination by another E3 ligase c-Cbl [131]. It has previously been reported that parkin modulates molecules in the same signaling pathway as PLC, including EGFR, PI3K and AKT by its substrate Eps15 [132]. Calcium deregulation has also been implicated in PD but this field has not been as much explored as in AD. So far, no reports have described the involvement of parkin and PLC mediated calcium deregulation. Therefore, we started by exploring whether PLC is a substrate for parkin.

Dopaminergic human neuroblastoma SH-SY5Y cells transfected with parkin WT, parkin G328E or parkin R42P were used for papers III and IV. Co-immunoprecipitation studies showed that parkin and PLCγ1 interact in these neuroblastoma cells as well as in human brain tissues derived from different regions. Immunoblot analysis of the brain homogenates from cortex, substantia nigra and striatum showed slightly reduced levels of PLCγ1. Parkin and PLCγ1 in neuroblastoma cells were also co-localized as shown using immunocytochemistry. A PM localization of both parkin and PLCγ1 was seen in parkin WT cells while the interaction in parkin G328E and R42P mutants appeared more scattered. Additional studies, such as subcellular fractionation need to be done for finding out whether or not mutations in parkin alter the localization of parkin and PLCγ1. Immunoblotting analysis revealed that PLCγ1 protein levels were lower in parkin WT cells, as compared to the other cell types. Also, PLCγ1 protein levels were higher in parkin-deficient as compared to WT mice. These findings, suggest that PLCγ1 protein levels are dependent on the parkin ubiquitin ligase activity.

This is the second potential parkin substrate that has been found to be accumulated in parkin deficient mice, signifying its importance. *In vitro* ubiquitination assays were then used to examine if parkin can ubiquitinate PLCγ1. Results showed less ubiquitinated PLCγ1 in the parkin G328E and R42P mutants as compared to in parkin WT cells, which imply a deficient catalytic activity of the mutants. Supplementary studies have to be done to distinguish the type of ubiquitination.

To further investigate the consequences of this, the same neuroblastoma cells were used to measure PLC activity by basal PI hydrolysis and by immunoblotting of pTyr^{783} in cells treated with EGF (paper IV). PLCγ1 is directly activated by EGF as described in the introduction. Parkin G328E and R42P mutant cells showed higher PLC activity as compared to NT and parkin WT cells. The downstream effect of PLC was investigated by measuring intracellular calcium levels. Basal calcium levels were higher in G328E and R42P parkin mutant cells in comparison to the other cells. Knockdown of

parkin by siRNA showed higher PLC activity and calcium levels, confirming that loss of functional parkin leads to perturbed calcium homeostasis. These findings are in line with the hypothesis that enhanced intracellular calcium levels could be the underlying cause of degeneration of snpc dopaminergic neurons in PD [133].

In order to clarify the mechanisms responsible for the enhanced intracellular calcium levels we explored the effects of direct and indirect calcium blockers. The PLC inhibitor neomycin and the RyR inhibitor dantrolene reversed the increased calcium levels, suggesting that differences were an outcome of altered PLC activity. Blocking plasma membrane L-and N-type Ca^{2+} channels with nimodipine and ω-conotoxin respectively gave on the other hand no significant effects. Treatment with thapsigargin (an inhibitor of the ER SERCA pump) also gave no changes in the different cell types.

Disruptive calcium homeostasis leads to activation of cascades that induce cell death. We therefore tested if the sensitization of parkin deficient cells with oxidized dopamine metabolite 6-OH-dopamine can be rescued by blocking calcium with the RYR antagonist dantrolene. Results were in agreement with previous data and showed that 6-OH-dopamine is less toxic in parkin WT cells. These toxicities in G328E and R42P parkin mutant cells could be reversed by dantrolene treatment. The exact mechanism by which parkin modulates the novel substrate PLCγ1 and calcium homeostasis is not yet fully established. PLCγ1 is probably together with other parkin substrates regulating intracellular calcium levels. The parkin substrate Pael-R which is in the same pathway as PLCγ1 [134] could be a potential candidate.

CONCLUSION

Papers I and II

We demonstrated that PLC and PKC activation are dependent on PS (Figure 5). Also, we found that PS1 can compensate for the loss of PS2 and vice versa. The lack of both PSs results in reduced PKCα and PKCγ activity and protein levels, and the regulation of PKCα was shown to be dependent on APP. In contrast, PKCδ protein levels were upregulated in absence of PS1, indicating different mechanisms of regulation of different PKC isoforms. The enhanced ERK1/2 activity seen in the absence of either PS1 or PS2 was dependent on PKCα. The lowered ERK1/2 activity in cells lacking both PSs is due to reduced PLC activity and lower levels and activity of PKCα.

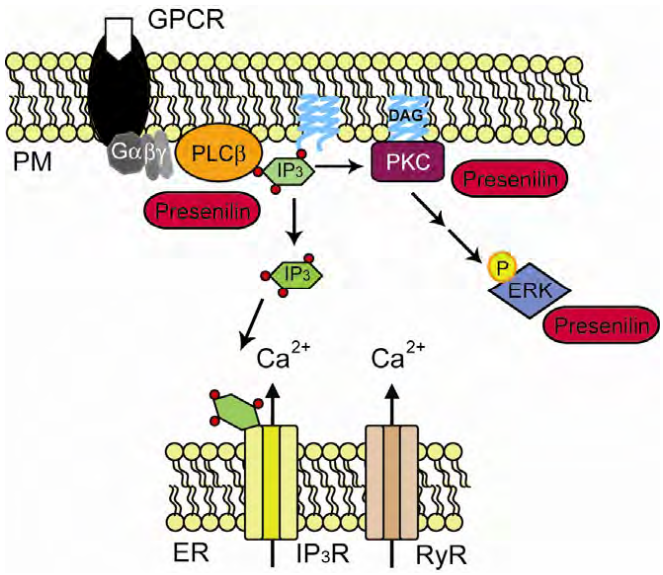

Figure 5. Schematic overview of the involvement of presenilin in the PLC mediated pathway

Papers III and IV

PLCγ1 is a substrate for parkin (Figure 6). PLCγ1 levels are dependent on parkin, as shown by overexpression and knockout models. Parkin G328E and R42P mutants show a defective ubiquitination of PLCγ1. PLC activity is also higher in parkin G328E and R42P mutants and parkin siRNA knockdown cells which was also reflected in the intracellular calcium levels. These enhanced calcium levels seen in the parkin mutants increased the sensitivity to the toxicity of 6-OH-dopamine, which was reversed by the blocking RyR. In conclusion, these data suggest that increased intracellular calcium levels due to altered PLC activity makes the cells more vulnerable to neurotoxins such as 6-OH-dopamine.

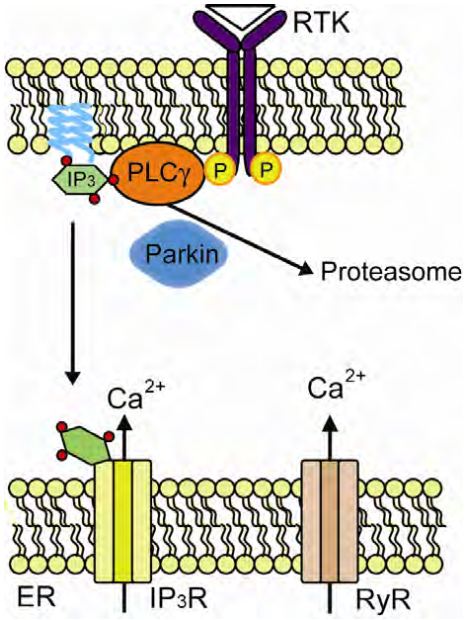

Figure 6. Schematic overview of the PLC mediated pathway and the contribution of parkin.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This thesis has focused on PS and parkin that are implicated in familial AD and PD respectively and their involvement in PLC mediated signaling. Regulation of signal transduction processes is fundamental for both survival and death in biological organisms. Deregulation of any element of the pathways can cause a sequence of events that could lead to cell death. Therefore, a detailed understanding of the AD and PD pathogenesis and underlying processes is important. Genetic findings have helped researchers to comprehend the pathogenesis of these neurodegenerative disorders and in support of this we have used them as tools for clarifying the signaling mechanisms that underlie the pathogenesis of these disorders.

An increasing body of evidence shows that PSs are important contributors to AD pathogenesis. Most PSs mutations lead to early-onset FAD. PS is the essential element of the γ-secretase complex, which activity leads to generation of Aβ. PSs are also crucial for various signal transduction pathways. In Papers I and II, we show that PSs regulate PLC activity and the downstream effectors PKC and ERK1/2. These pathways including GSK3 [135] have been shown to modulate Aβ generation and tau phosphorylation. PKC and ERK1/2 have both been previously associated with AD pathology. Tau candidate kinases (among them ERK1/2) have been shown to gain activity after calpain activation as a consequence of elevated intracellular calcium levels. Hence, PSs are likely involved in many crucial aspects of the pathogenesis of AD. It has also been suggested that PS1 contributes to NFT formation. Several PS1 mutations have been associated with fronto-temporal dementia (FTD) [136], which are characterized by the appearance of NFT and absence of Aβ deposition [137]. Furthermore, PS1 mutations have in some cases both been associated with NFT formation and Aβ deposition [138, 139].

Many different therapeutic strategies are under development to stop the progression of AD with an interest in γ-secretase modulators. It is important to develop inhibitors that will take into account all the different functions of PS and therefore it is also crucial to continue research and clarify the involvement of PS in other signaling pathways, other than just the cleavage of APP.

Parkin has been shown to be neuroprotective to toxic insults, especially in dopaminergic neurons. The accumulation of parkin substrates as a consequence of loss of parkin ubiquitin activity has been suggested to lead to cell death. Snpc dopaminergic neurons are specifically lost in brains of PD patients and it has been suggested that the high calcium burden could play a key role in the enhanced vulnerability of these cells. In papers III and IV we have identified PLCγ1 as a novel substrate for parkin, and disrupted calcium homeostasis in parkin deficient cells. It is known that deregulation of calcium homeostasis in neurons can over-activate proteases, induce mitochondrial dysfunction and provoke disruption of cytoskeleton.
Parkin may hold the key to establish new therapeutic interventions to modulate the enhanced vulnerability of snpc dopaminergic neurons to cell death. Researchers have suggested calcium channel blockers as a possible therapy for PD and continued research on finding the exact mechanisms for how parkin is involved in calcium homeostasis would be beneficial.

Generally, AD and PD are both affected by aggregating proteins with neuronal loss in specific regions of the brain. Moreover, they have both been associated with calcium deregulation and it has long been postulated to be an initiating cause for these neurodegenerative diseases. In conclusion, our studies suggest that PLC mediated signaling could be a common pathway affected in both disorders.

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