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TRANSLATIONAL STUDIES OF MOLECULAR INTERACTIONS IN PARKINSON'S DISEASE

Ellen Hertz

Karolinska Institutet

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Translational Studies of Molecular Interactions in Parkinson's Disease
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Ellen Hertz

Principal Supervisor:
Prof. Per Svenningsson
Karolinska Institutet
Department of Clinical Neuroscience

Co-supervisor(s):
Prof. Ellen Sidransky
National Institute of Health
Department of National Human Genome Research Institute

Assoc. Prof. Vladana Vukojević
Karolinska Institutet
Department of Clinical Neuroscience

Prof. Thomas Perlmann
Karolinska Institutet
Department of Cell and Molecular Biology

Prof. Maciej Machaczka
Karolinska Institutet/ University of Rzeszów
Department of Clinical Science and Education/ Faculty of Medicine

Opponent:
Dr. Aylin Hanyaloglu
Imperial College London
Department of Metabolism, Digestion and Reproduction

Examination Board:
Prof. Jerker Widengren
Royal Institute of Technology
Department of Applied Physics

Prof. Robert Fredriksson
Uppsala University
Department of Pharmaceutical Biosciences

Prof. Lou Brundin
Karolinska Institutet
Department of Clinical Neuroscience

To my family with love

ABSTRACT

Interactions between molecules are the basis of cellular function. In disease, these tightly regulated systems are often disturbed. Parkinson's disease (PD) is recognized by protein aggregates in the brain of erroneously interacting molecules, directly linking the clinical picture to the molecular mechanisms. The currently incomplete understanding of the mechanisms behind PD pathogenesis impedes the development of disease-modifying treatment and new targets are needed since today's dopaminergic treatment does not fully alleviate the symptoms. G protein-coupled receptors (GPCRs) are a wide group of transmembrane receptors usually targeted by drugs due to their ability to convey extracellular information into definite cellular responses. This Thesis focuses on protein interactions, from cellular models of a Parkinson-linked GPCR, GPR37, to detection of nanoaggregates in serum from PD patients as a sign of disease.

The main findings relate to the interactome of GPR37 as a factor regulating cell survival. GPR37 has been suggested to accumulate and cause dopaminergic cell death in PD when improperly folded but is able to elicit cytoprotective function when correctly matured and trafficked to the cell surface. We report that GPR37 interaction with ganglioside GM1-enriched lipid rafts and a proposed ligand prosaposin affects trafficking of GPR37 to the plasma membrane. Since the role of lipids in PD pathogenesis is increasingly acknowledged and GM1 has been suggested to slow down PD progression in clinical studies, we further studied the mechanism of the GPR37-GM1 interaction. We propose that exogenous GM1 treatment increase cellular resistance to a neurotoxin partly through a GPR37-dependent mechanism. This suggests yet another molecular mechanism of GM1 cytoprotection. Moreover, GPR37 has been suggested to be a modulator of dopaminergic transmission why we investigated the proposed interaction with dopamine D2 receptor (D2R) and GPR37 in live cells. The levels of heterodimerization where generally low in our cellular system. However, it could be augmented both by chaperone treatment, inducing trafficking of GPR37, and by clinically used dopamine agonist treatment. Shifting the heterodimerization level of GPCRs is known to alter molecular response. Therefore, the physiological outcome of this interaction needs to deciphered to understand effects and side effects of dopamine agonist treatment.

We also investigate improper protein interactions as a potential biomarker in serum from PD patients by detection of β-sheet enriched nanoaggregates. We report a higher detected frequency of nanoamyloids in patients compared to healthy controls and a bimodal distribution of amyloid size in serum. However, the frequency of nanoamyloids did not correlate robustly with neither disease progression or disease symptoms. Potentially this is due to heterogeneity, both clinical and molecular, in the disease. This emphasizes the need of understanding the molecular interactions in a clinical context.

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I. Gregorsson Lundius E, Vukojević V, **Hertz E**, Stroth N, Cederlund A, Hiraiwa M, Terenius L, Svenningsson P. GPR37 Protein Trafficking to the Plasma Membrane Regulated by Prosaposin and GM1 Gangliosides Promotes Cell Viability.
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II. **Hertz E**, Terenius L, Vukojević V, Svenningsson P. GPR37 and GPR37L1 Differently Interact With Dopamine 2 Receptors in Live Cells. *Neuropharmacology* (2019) 152, 51-57

III. **Hertz E.**, Saarinen M., Svenningsson P. GM1 is Cytoprotective in GPR37-Expressing Cells and Down Regulates Signaling.
Manuscript

IV. **Hertz E.***, Tiiman A*, Terenius L, Vukojević V, Svenningsson P. Increased Amyloid Nanoplaques in Serum from Patients with Parkinson's Disease.
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ADDITIONAL PUBLICATIONS BY THE AUTHOR

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CONTENTS

LIST OF ABBREVIATIONS

α-syn	α-synuclein
$A_{2A}R$	adenosine receptor 2A
AC	adenylyl cyclase
AD	Alzheimer's disease
ALP	autophagy-lysosome pathway
ATP	adenosine 5' triphosphate
BB	bombesin
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
COMT	catechol-O-methyltranseferase
CSF	cerebrospinal fluid
CtxB	cholera toxin β-subunit
D2R	dopamine receptor 2
D2R	dopamine receptor 2-long
D2RS	dopamine receptor 2-short
DAT	dopamine transporter
eGFP	enhanced green fluorescent protein
ER	endoplamatic reticulum
ET	endothelin
FCS	fluorescence correlation spectroscopy
FCCS	fluorescence cross-correlation spectroscopy
FDA	Food and Drug Administration
FRET	Förster/fluorescence resonance energy transfer
f_{SEO}	frequency of single event occurance
GPCR	G protein-coupled receptor
GCase	glucocerebrosidase
G protein	guanine nucleotide-binding protein
GRK	G protein-coupled receptor kinase
H&Y	Hoehn and Yahr
HA	head activator

HTRF	homogeneous time-resolved fluorescence
iPSC	induced pluripotent stem cells
IP_3	inositol triphosphate
ICD	impulse control disorder
iPSC	induced pluripotent stem cells
KO	knock out
LB	Lewy bodies
MAO-B	monoamine oxidase B
MDS	Movement Disorder Society
MEM	maximum entropy method
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NfL	neurofilament light
NMS	non-motor symptoms
NPD1	neuroprotection D1
OVE	observation volume element
PD	Parkinson’s disease
PKA	protein kinase A
PLC	phospholipase C
PSAP	prosaposin
RCCA	relative cross-correlation amplitude
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SBD	sfingolipid binding domain
SN	substantia nigra
SNpc	substantia nigra pars compacta
ThT	thioflavin T
UPDRS	unified Parkinson’s disease rating scale
UPR	unfolded protein response
UPS	ubiquitin-proteosome system
VTA	ventral tegmental area

1 INTRODUCTION

Proteins, the main effectors of the cell, act in complex molecular networks that are interdependent of each other. After sequencing the human genome and mapping all proteins to specific tissues, dynamic interactions between molecules add a new layer of complexity. To map the protein interactome and comprehend the outcome of interactions is a major scope of today's biological research[1]. Dysregulation in the spatial and temporal control of a protein's interactome may lead to, among other problems, protein aggregation and disturbed signaling cascades. This Thesis focuses on proteins linked to cell survival in Parkinson's disease (PD), their interactions and relevance, from mechanism to clinical implications.

PD is a common neurodegenerative disorder marked by a progressive degeneration of dopaminergic neurons in the substantia nigra (SN) and accumulation of protein aggregates in the remaining neurons. The accompanying reduction of dopamine level in the striatum is associated with the motor deficits characteristic of PD. Present dopamine-replacing therapies bring symptomatic relief of motor symptoms. However, treatment of non-motor symptoms (NMS) is unsatisfactory, side-effects are disabling, and, most importantly, the treatment has no slowing effect on the underlying cell death. To date the events leading to dopaminergic degeneration are only partly elucidated. To understand the dysfunctional cellular and molecular mechanisms is key to develop future disease modifying treatments.

1.1 G PROTEIN-COUPLED RECEPTORS (GPCRS)

G protein-coupled receptors (GPCRs) are the largest group of cellular surface receptors in the human genome including at least 800 transmembrane receptors[2]. Based on sequence homology, members of this super-family are allotted into 5 classes; rhodopsin-like (class A), secretin-like (class B1), adhesion receptors (class B2), metabotropic glutamate-like (class C) and frizzled/taste2 receptors (class F)[2,3,]. Embedded in cellular membranes, GPCRs share a 7-α-helical transmembrane domain structure with an extracellular amino-terminal domain and an intracellular carboxyl-terminal tail (Fig. 1A). They convey a myriad of extracellular information, transducing signals from photons to neurotransmitters, into elicit versatile cellular responses. They are often pictured as ideal targets for drugs due to their ability to specifically bind ligands and allosteric modulators and they are involved in almost all physiological processes. To date, about 1/3 of drugs approved by Food and Drug Administration (FDA) target a GPCR, underscoring the enormous role that GPCRs have on human health and disease[4]. However, approximately 140 GPCRs, including GPR37 which is central in this Thesis, have still not been linked to an endogenous ligand and are therefore known as "orphan receptors"[5]. Little is known of the physiological effects of these receptors and they are therefore an unexploited source for future treatments.

1.1.1 GPCR-mediated signaling cascades

Key in flexibility of GPCR-mediated signaling is the wide span of intracellular interaction partners including guanine nucleotide-binding proteins (G proteins) and G protein independent scaffold proteins[3]. In the canonical pathway, signal transduction depends on receptor-mediated activation of heterotrimeric G proteins, composed of three subunits Gα, Gβ and Gγ[6]. The classification of G proteins depends on the Gα subunit which are divided into four classes; $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$. Ligand-induced activation induces conformational changes of the receptor's intracellular domains[3]. The coupling to G proteins trigger exchange of Gα-associated GDP to GTP which allows Gα to dissociate from Gβγ. Gα regulates key effectors which in turn generate second messengers capable of inducing distinct signaling cascades to propagate and amplify the signal. $G\alpha_s$ and $G\alpha_{i/o}$ regulates adenylyl cyclase (AC) activity which catalyzes adenosine triphosphate (ATP) to the second messenger cyclic adenosine monophosphate (cAMP). $G\alpha_{q/11}$ modulate phospholipase C (PLC) to change inositol triphosphate (IP_3) levels (Fig. 1B). IP_3 in turn binds calcium pumps in the endoplasmic reticulum (ER) and thereby regulates intracellular Ca^{2+} levels. The G protein elicited signal is terminated through the intrinsic GTPase activity of Gα which hydrolyzes GTP to GDP allowing Gα to re-associate with Gβγ. Upon GPCR activation, the receptor's cytoplasmic loops and C-terminal tail are phosphorylated by G protein coupled receptor kinases (GRK) and β-arrestin is recruited. This both blocks the binding heterotrimeric G proteins and initiate receptor internalization[3]. Thereby, the signaling pathway is terminated. In short, this is the traditional view of a GPCR-mediated signaling event.

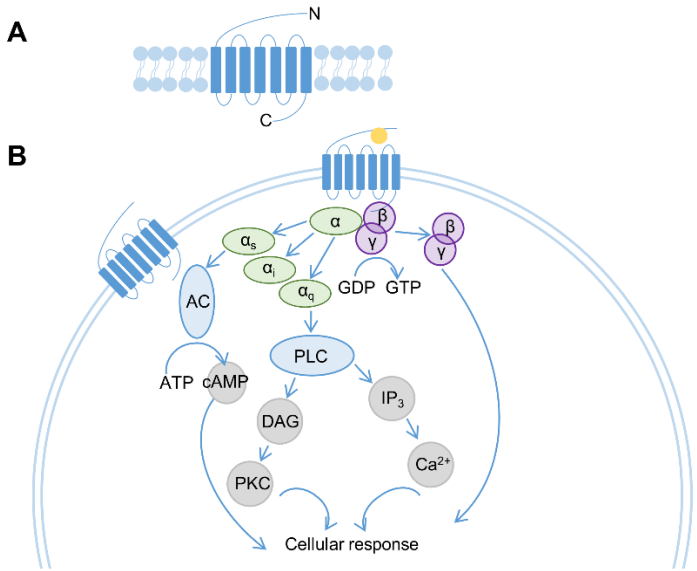

Figure 1. Schematic drawing of GPCR structure and function A) Structure of a GPCR with the extracellular N-terminal domain, 7 trans-membrane helixes and an intracellular C-terminal tail.
B) The initial steps in the canonical GPCR mediated signaling pathway where the different Gα subunits regulates key effectors, e.g., adenylyl cyclase (AC) and phospholipase C (PLC) which in turn generate second messengers.

Today's understanding of GPCR activation and signaling is more detailed and complex. One GPCR can couple to different Gα-subunits depending on cellular situation, eliciting various downstream effects[3]. In addition, Gβγ subunit can induce separate signaling cascades. Furthermore, there are a number of G protein independent pathways where the β-arrestins are central. Historically, the β-arrestins are mainly studied as negative regulators of GPCR signaling. However, scaffolding β-arrestin can initiate a number separate signaling cascades, possibly due to the protein's variability of active conformations[6]. These pathways are initially

separate from G protein dependent but include similar signaling cascades at a different time points[7]. This paradigm shift of GPCR-mediated signaling needs further studies to determine if the arrestin-dependents pathways are truly G protein-independent or requires initial G protein activation[8]. Furthermore, some GPCRs can simultaneously bind β-arrestin and G proteins[9]. Today it is evident that there is an intricate intertwining between the G protein dependent and G protein independent signaling pathways.

This plethora of different signaling pathways has evolved into the concept of biased agonism. A GPCR should not be regarded as an on-off switch. Instead, it has multiple distinct active confirmations[3]. An agonist can preferentially stabilize specific confirmations and thereby trigger a subset of the existing intracellular cascades, or the full range. The biased receptor response can be induced by a ligand, splice form alterations in the receptor or by relative expression levels of the transducer proteins in different cell types[7]. Understanding how biased signaling is regulated could make it possible to tailor drugs that induce only the desirable pathway in the targeted cell type[3].

In addition to biased activation, GPCR's signaling intensity are also regulated by expression levels, cellular localization and hence, cellular trafficking. When activated, the receptor is, in general, internalized as explained above. This process is followed either by recycling the receptor to the plasma membrane or degradation in the endosomal-lysosomal system. However, GPCRs are subjected to different fates after internalization, depending on arrestin recruitment and receptor phosphorylation. In addition, there is emerging evidence of intracellular GPCR signaling. GPCRs have been found on vesicles, mitochondria and the nuclear membrane[10]. Furthermore, endosomal internalization can lead to endosomal signaling, both G protein dependent and β-arrestin dependent. This further increases the spatio-temporal regulation of GPCR signaling[3, 11].

1.1.2 GPCR interactions

GPCRs can form protein complexes with other proteins. This adds an additional layer of complexity to signaling pathways since the interactions can allosterically modulate the signaling outcome[12]. Studies of this molecular integration developed in the 80s when the first reports of receptor-receptor interactions were published[13]. Dimerization was first recognized for GPCRs in the metabotropic-glutamate family and in 1998, heterodimerization was shown to be required for a functional GABAB receptor[14]. Today there is strong evidence also for dimerization in the rhodopsin-like and secretin-like receptor families[12, 15]. The interactions are well documented in heterologous systems but there are still difficulties to develop proper methods to study naïve systems. Homo- and heterodimers can modulate the pharmacological response and induce G protein coupling change from $G\alpha_s$, to $G\alpha_{i/o}$ or towards β–arrestin signaling[12,16]. Since the dimers have different properties than the monomer, the number of possible ligand-receptor responses are increasing. The balance between monomers, dimers and larger oligomers determine the final cellular response which can be fine-tuned by shifting the equilibrium[3]. Levels of dimerization between GPCRs induced by pharmacological tools are studied in this Thesis.

GPCRs are embedded in a lipid membrane. The environment affects both lateral organization and allows for protein-lipid interactions[17]. The membrane's properties including fluidity, composition, and thickness affects the arrangement of GPCRs. Assembly is, e.g, promoted if the hydrophobic portion of the GPCR is thicker than the plasma membrane since dimerization of two GPCRs then reduces the exposed hydrophobic area[18]. On a larger scale, the plasma membrane is compartmentalized into dynamic highly-ordered domains called lipid rafts, characterized by enrichment of cholesterol and sphingolipids including the ganglioside GM1. These hubs constrain essential signaling molecules, including GPCRs, into close proximity and thereby organize the bioactivity[17]. Apart from influencing GPCRs' micro-environment, both cholesterol and sphingolipids can directly interact with GPCRs via typical binding motifs in transmembrane domains and extracellular loops[19, 20]. Membrane cholesterol can modulate GPCRs, e.g. adenosine receptor 2A ($A_{2A}R$), by binding into the orthosteric binding pocket and hence act as a conventional ligand[21, 22]. In addition, just like protein-protein interactions, protein-lipid interactions can change preferred G protein-coupling[23]. Recently published simulation data of 28 GPCRs reveals specific lipid interactions for all GPCRs examined, although with a unique profile for each receptor[24]. This enforces the general feature that lipids intimately regulate GPCRs, either via direct interactions or lipid bilayer properties or possibly by a combination of both mechanisms[21].

In summary, GPCRs is a large and diverse group of transmembrane proteins which by remarkable specificity can orchestrate cellular response. The signaling cascades are functionally pleiotropic intricately regulated by trafficking, biased signaling and permanent or transient molecular interactions. GPCR regulation is hence not an on/off phenomenon but rather an integrated, cell-specific outcome. To understand and utilize this characteristic is desirable when designing drugs since the tissue and local environment specificity possibly can reduce side effects[7]. The following sections present the GPCRs relevant to PD that are investigated in this Thesis.

1.1.3 Dopamine receptors

The neurotransmitter dopamine controls a variety of physiological functions by acting on five GPCRs. In addition, dopamine receptors are well-established targets in clinical pharmacology treating PD as well as neuropsychiatric diseases. Due to their central role in human physiology the receptors are well characterized thus far.

1.1.3.1 Structure and expression of dopamine receptors

There are five closely related GPCRs exerting the physiological effects of dopamine, D1-D5, each one encoded by a separate gene. Depending on structural, pharmacological and biochemical properties, they are divided into D1-class receptors (D1 and D5) and D2-class receptors (D2, D3 and D4). D1-class receptors are expressed exclusively postsynaptically, couple to $G\alpha_s$ and their respective genes do not have introns. Class D2, on the opposite, couple to $G\alpha_{i/o}$, D2 and D3 are localized both pre- and postsynaptically and the genes include introns[25]. Due to the presence of introns, alternative splicing is feasible and have been described for all

D2-class receptors. The most studied example is an 87-base-pair exon in D2 which is alternatively spliced forming D2-short (D2RS) and D2-long (D2RL). D2RS is mainly expressed presynaptically and hence act as an autoreceptor while D2RL is mostly postsynaptic[26]. These variants are studied in this Thesis.

Dopamine receptors are expressed broadly both in the central nervous system (CNS) as well as in the periphery. In the brain, D1 and D2 are vastly expressed in the nigrostriatal and mesolimbic areas including the striatum, the substantia nigra (SN), and ventral tegmental area (VTA), as well as in cortex, hippocampus and the amygdala. Expression of D3 is more restricted, with the highest levels in limbic areas and lower expression in striatum, SN and VTA. D4 and D5 are expressed in low levels in, e.g., frontal cortex, SN and thalamus for D4 while D5 is expressed in the prefrontal cortex, premotor cortex as well as in SN and hippocampus[25].

1.1.3.2 Signaling pathways of dopamine receptors

As stated above, principally D1-class receptors couple to $G\alpha_s$ whereas D2-class receptors mainly couple to $G\alpha_i$ in their canonical action on cAMP-mediated signaling. cAMP levels regulate protein kinase A (PKA) activity which in turn phosphorylates DARPP-32. DARPP-32 is an integrator of neuronal transmission and has been extensively studied due to its pivotal role in dopamine signaling[27]. However, dopamine receptors are now known to fine-tune their response by activating a variety of signaling pathways[28]. These involve both alternative G protein coupling and non-G protein mediated mechanisms. Examples include; D1-class receptors regulating Ca^{2+} levels via $G\alpha_q$ coupling[28], β-arrestin-2 dependent late phase D2R signaling as well as transactivation of RTKs[25]. This more complex understanding of dopamine receptor transduction enables future progress in drug development aiming at post-receptor signaling targets.

1.1.3.3 Molecular interactions of dopamine receptors

Oligomerization of GPCRs is now widely established and dopamine receptors can form a wide range of homo- and heteroreceptors[28, 29]. These receptor interactions are suggested to modulate the response of dopamine activation which is important to disentangle therapeutic effects and side effects of dopaminergic treatments. In addition, due to heterodimerization non-dopaminergic treatment strategies can potentially improve dopaminergic effects. One of the mostly studied interactions is between $A_{2A}R$ and D2R in the striatum. On the molecular level, the interaction shifts D2R signaling from $G\alpha_i$ towards β-arrestin signaling[30]. In animal models of PD, $A_{2A}R$ antagonists decrease dyskinesia, a well-known side effect of PD treatment[31]. Hence, modulation of $A_{2A}R$ can possibly fine-tune dopaminergic signaling and reduce disabling side effects. In another example D2R has been shown to interact with the NMDA receptor (NMDAR), a glutamate receptor, in striatal synapses[32]. Here D2R activation reduced NMDAR signaling in the indirect pathway, easing the brake on motor initiation (see Sect. 1.2.2 for introduction to the dopaminergic networks). D2R has also been suggested to interact with GPR37 *in vitro*, an interaction further evaluated in this Thesis[33, 34]. Protein-protein interactions

reveal new potential treatment opportunities and expand the possibilities to modulate one of our most pharmacologically targeted GPCRs.

1.1.3.4 Function of dopamine receptors in health and disease

Since its discovery in the 1960s' the dopaminergic system is one of the most studied neurotransmitter systems due to its involvement in fundamental human functions as well as common diseases such as PD, schizophrenia, and addiction. Dopamine signaling is essential in voluntary movement, reward, learning, and cognition[25]. In voluntary movement D1, D2 and D3 are primarily involved. D1 alters a pro-kinetic response, whereas the effect of D2 and D3 activation is more complex due to the dual localization, both pre- and postsynaptic. D1 and D2 are also involved in the evolutionary essential function feelings of reward. However, these are a major issue in drug abuse. Due to the critical roles of dopamine, dopamine receptors are common targets in clinical treatment[25]. PD is caused by a progressive loss of dopamine producing cells causing motor deficits. The standard care includes a precursor of dopamine, L-DOPA, as well as dopamine receptor agonists to alleviate symptoms (please see Sect. 1.4.1). Furthermore, D2R antagonists were the first generation of antipsychotic medications. Those have been refined to reduce the accompanying motor function related side-effects, e.g., via activation of biased signaling[35].

1.1.4 GPR37

G-protein-coupled receptor 37 (GPR37) is an orphan receptor with mainly unknown physiological function. In the literature the receptor has been extensively linked to PD pathogenesis due to its tendency to misfold in the ER causing protein aggregates. However, during recent years the receptor has been proposed to have neuroprotective functions if correctly folded. Due to its bidirectional role, it has been suggested as a potential pharmacological target in PD[36].

1.1.4.1 Structure and expression profile of GPR37

GPR37 is a rhodopsine-like GPCR first recognized in 1997[37, 38]. GPR37 and its closest relative GPR37L1 were identified through their homology with the endothelin (ET_A and ET_B) and bombesin (BB_1, BB_2 and BB_3) receptors. Apart from its classical 7-transmembrane structure, GPR37 have a large extracellular N-terminal domain. This structure is similar to adhesion receptors where the extracellular domain is cleaved by autocatalysis[39]. Indeed, the N-terminal domain of GPR37, GPR37L1 and ET_B are all cleaved but through metalloprotease activity[40-42]. The physiological effect of the cleavage is still elusive for GPR37. The receptor is almost exclusively expressed in the CNS, especially the corpus callosum and midbrain[37, 38]. Expression is mapped predominately to oligodendrocytes but also reported to specific neuronal populations in the hippocampus and the dopaminergic neurons in the SN[43-45].

1.1.4.2 Signaling pathways of GPR37 and suggested ligands

In order to understand the characteristics of GPCR, a ligand which activates or deactivates the receptor is critical. GPR37 is still considered an orphan receptor and different dominating

downstream signaling cascades are reported. GPR37, although identified through close homology to endothelin and bombesin receptors, does not respond to either group of ligands. Several endogenous ligands have been suggested. First was head activator (HA), an 11 amino acid neuropeptide isolated from the Hydra. HA was reported to co-localize with the receptor and cause internalization. GPR37 was suggested to be $G\alpha_i$-coupled and induce Ca^{2+} influx[46]. These results have been difficult to reproduce. Dunham et al. report lack of internalization, cAMP alteration and ERK phosphorylation and in a ligand screen, HA did not induce β-arrestin recruitment in GPR37-expressing cells[33, 47]. Furthermore, there is no evidence of an HA analog in the human genome and hence it is unlikely that HA is an endogenous ligand[48].

In 2013, prosaposin (PSAP, sphingolipid activator protein-1 or sulfated glycoprotein-1) was reported as the endogenous ligand for GPR37 and GPR37L1[49]. Prosaposin is a well known neuroprotective and neurotropic protein when secreted extracellularly in its full length form[50]. Intracellularly, it is cleaved in the lysosome by cathepsin D into saposin A-D which all facilitate sphingolipid degradation[51]. Prosaposin, as well as the synthetic peptide of the neurotrophic sequence TX14(A), was reported to activate GPR37/GPR37L1 at nanomolar concentrations in a pertussis toxin-sensitive manner. This is specific for $G\alpha_i$-coupled receptors. Two separate groups have reported that PSAP confer protection against oxidative stress in GPR37/GPR37L1-dependent models[49, 52]. As presented in Paper I of this Thesis, PSAP has been detected to diffuse through the plasma membrane together with GPR37, indicative of a functional interaction. However, the pairing of GPR37/GPR37L1 and PSAP is still challenged as the changes detected in the signaling cascades are unusually small and not reproduced in other assays[47, 48, 53]. Moreover, REG4 which is a small proliferative protein has been suggested to enhance metastasis through GPR37[54]. Finally, neuroprotectin D1 (NPD1), a derivative of an omega-3 essential fatty acid member, was shown to increase intracellular Ca^{2+} in GPR37-expressing macrophages. The same effect was seen with TX14(A) treatment and would indicate $G\alpha_q$-coupling of GPR37[55]. NPD1 has previously been shown to have neuroprotective effects in ischemic as well as oxidative stress models possibly by downregulation of pro-inflammatory and pro-apoptotic genes[56]. In order to circumvent the lack of a ligand, Zheng et al. elegantly generated a channelrhodopsin-GPR37 chimeric protein which when activated by light caused decreased cAMP levels and IP_3-signaling concurrent with increased locomotion in mice[57]. As no consensus is achieved, GPR37 has regained its orphan receptor status and the initial steps of the induced signaling remains to be fully determined.

1.1.4.3 Molecular interactions of GPR37

In the absence of known ligands, the receptor's interactome can aid advances in understanding the function of GPCRs. Molecular studies have identified numerous binding partners for GPR37 with various functional consequences. In a systematic protein-protein interaction study using a modified membrane yeast two hybrid screen assay for 48 human receptors, GPR37 was proposed to interact with several GPCRs including $A_{2A}R$, D2R and the serotonin receptor, $5\text{-}HT4_D$[34]. The most studied interaction is between $A_{2A}R$ and GPR37 in the striatum where GPR37 act as chaperone of $A_{2A}R$, facilitating trafficking of $A_{2A}R$ to the plasma membrane[58].

Thereby GPR37 directly affects adenosine signaling. In turn, this also alters $A_{2A}R$'s modulatory functions, e.g. its regulation of D2R signaling described above. Through co-immunoprecipitation GPR37 has also been shown to interact directly with D2R and dual expression led to enhanced plasma membrane expression, facilitating assessment of functional studies of classical GPCRs[33]. Furthermore, GPR37 has been reported to interact with the dopamine transporter (DAT) and thereby modulate dopamine reuptake[59]. The number of proposed interactions, indicative of physiological function, is increasing and suggests a role of GPR37 modifying dopamine transmission.

1.1.4.4 GPR37 in health and disease

The physiological function of GPR37 is still mainly unknown with multiple lines of current investigations. Linked to its expression in oligodendrocytes it has been proposed as a negative regulator of myelination and when knocked out (KO)[60], mice exhibit increased susceptibility of demyelination[61]. GPR37 has also been proposed to regulate inflammation and enhance macrophage phagocytosis in mice after ischemic stroke. KO mice show augmented damage, both in size of ischemic injury and functional impairment[55]. However, GPR37 has gained most research attention due to its connection to PD pathogenesis. GPR37 is substrate of Parkin, an E3 ubiquitin ligase which should ubiquintinate misfolded proteins to induce degradation[44]. Mutations in Parkin is one of the most common causes in early onset PD, increasing the risk of accumulation of misfolded proteins[62, 63]. In Parkin-deficient mice, over expression of GPR37 caused a progressive loss of catecholaminergic neurons. However, this effect was not seen in mice with a normal Parkin-gene[64]. In cellular studies, GPR37 is suggested to misfold and cause endoplasmic reticulum stress instead of being trafficked to the plasma membrane[44]. In human postmortem tissues from Parkin-deficient and sporadic PD cases, GPR37 is accumulated in the core of Lewy bodies (LB), the pathological hallmark of PD[65]. Furthermore, in an epigenome-wide DNA methylation analysis GPR37 was the only gene differing in methylation level between monozygotic twins which are discordant for PD[66]. This indicates an acquired alternation possibly due to environmental factors. Taken together, GPR37 was suggested to easily misfold, aggregate and induce neurodegeneration. However in a cellular model, when GPR37 is expressed at lower, more physiological, levels and trafficked to the plasma membrane, the receptor was protective against neurotoxins[67]. The suggested ligands, PSAP and NPD1, are both known to exert neuroprotective functions[49, 56]. Hence, there is a hypothesis of a bidirectional role of GPR37 in neurodegeneration depending on cellular localization[36]. The physiological role, as well as GPR37's role in neurodegeneration, still remains to be fully elucidated.

1.1.5 GPR37L1

GPR37L1, the receptor with 68% sequence similarity to GPR37, was identified a year after GPR37[48, 68]. Structure wise, GPR37L1 has a relatively large N-terminal domain similar to GPR37. As GPR37, GPR37L1 is highly expressed in the brain, especially the cerebellum, and the receptor is enriched in astrocytes. GPR37L1 is less studied than GPR37 and its physiological role is largely unknown. KO mice show precocious glia, Purkinje neuron

maturation and improved motor skills[69]. Furthermore, KO mice has an increased risk of seizures, like GPR37. Interestingly, a mutation in GPR37L1 has been found in a family with progressive myoclonus epilepsy[70]. Regarding ligand and signaling cascade, the studies proposing PSAP as the ligand for GPR37 claims that PSAP is also the endogenous ligand of GPR37L1 and that the receptor is $G\alpha_{i/o}$-coupled, possibly constituently active[70]. There are also reports of $G\alpha_s$-coupling for GPR37L1[70]. In several papers, GPR37L1 is studied in parallel with GPR37 in order to compare the results of those, relatively unknown receptors[49, 52, 71].

1.2 PARKINSON'S DISEASE

1.2.1 The clinical picture

The first description of Parkinson's disease (PD) was six illustrative cases in *An Essay on The Shaking Palsy* by James Parkinson in 1817[72]. PD is now known to be the second most common neurodegenerative disorder after Alzheimer's disease (AD), affecting 2-3% of people over 65 years of age[73]. Even though 200 years have passed since the first description the PD diagnosis is still clinical based on the features of parkinsonism defined as bradykinesia in combination with resting tremor and/or rigidity[74]. Gait impairment is common but is not an essential diagnostic sign today. In addition to progressive motor symptoms, patients experience a wide range of non-motor symptoms (NMS) including rapid eye movement (REM) sleep behavior disorder (RBD), loss of smell, obstipation, depression/anxiety and cognitive decline[75]. NMS often precede the onset of motor symptoms, disclosing disease progression before the characteristic motor symptoms are evident (Fig. 2). The combination of motor- and NMS unfortunately result in low quality of life for a large proportion of PD patients[76, 77]. The progression rate is highly individual and the combination of symptoms results in a heterogeneous disease where prognosis for the individual is difficult to predict[78].

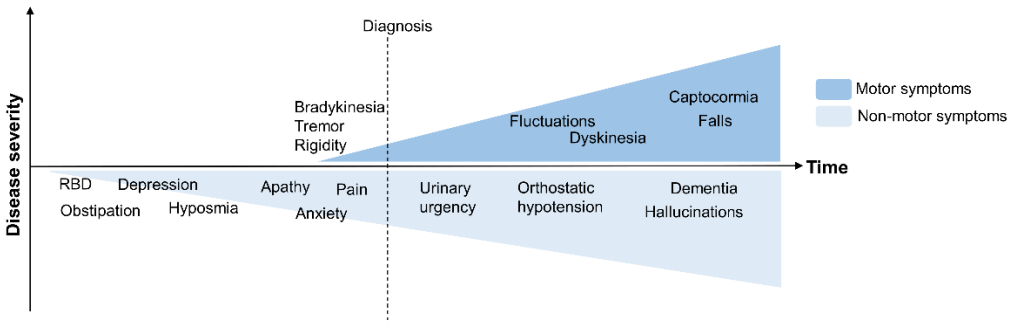

Figure 2. Clinical symptoms associated with PD progression. Schematic of when common clinical symptoms usually occur. PD is diagnosed after the onset of motor symptoms. Patients may have experienced NMS years prior to diagnosis.

The cause of the motor symptoms is a progressive loss of mostly dopaminergic neurons in the substantia nigra pars compacta (SNpc) causing striatal dopamine deficiency. Several other neurotransmitter systems are affected as the dopaminergic system receives modulatory input from serotonergic, cholinergic and glutamatergic neurons. Possibly, these other neurotransmitter networks are responsible for some of the non-motor features of PD[73]. In the

remaining neurons there are protein aggregates known as Lewy bodies (LB). The main constituent of LB is the presynaptic protein α-synuclein (α-syn) but they also include numerous other proteins, defect organelles and membrane lipids[79,80]. During disease progression, LB is suggested to spread in a topological predetermined pattern through the nervous system. According to the Braak neuropathological staging theory, inclusions are initially detected in the dorsal nucleus of the vagus nerve in the brain stem and in the olfactory bulb (stage 1). At stage 2 inclusion bodies are found in the medulla oblongata and at stage 3 the SN is reached which coincide with the onset of motor symptoms. In stage 4, LB pathology is spread to temporal cortex and when reaching the neocortex, stage 5 and 6, the cognitive decline can develop[81].

There are various diagnostic criteria of PD, all depending on the clinical signs of parkinsonism[74]. Studies indicate that up to 25% of PD diagnoses have another neuropathological disorder confirmed post mortem including atypical parkinsonism, vascular perturbations or AD-like pathology[82]. DaTSCAN is a single-photon emission computed tomography, visualizing striatal DAT. It has a high sensitivity for dopaminergic cell loss and can advice in uncertain cases of Parkinsonism but cannot distinguish between different forms[78]. The need of imaging methods or biomarkers are therefore immense to increase diagnostic precision. Despite intense research, there are no biomarkers in clinical use to date, neither for diagnosis, progression or treatment response[83]. Neurofilament light (NfL), a general marker of neurodegeneration, in cerebrospinal fluid (CSF) or blood can distinguish PD from atypical forms of Parkinsonism, were NfL is elevated[84, 85]. A recent study reports that blood NfL modestly correlate with clinical motor score and can be used as a marker of progression also in idiopathic PD[86]. This remains to be established especially since the PD cases included in the study were not pathologically confirmed. The precision of clinical PD diagnosis could be greatly improved by a biomarker. In addition, a biomarker could possible clarify etiological subtypes of PD giving a molecular understanding for the heterogeneity of the disease[87]. A new potential biomarker is presented in Paper IV of this Thesis.

1.2.2 Overview of the dopaminergic system and structures important in PD

The catecholamines are monoamine neurotransmittors derived from the amino acid tyrosine. In 1957, noradrenaline and dopamine was first suggested as specific neurotransmittors[88, 89]. A few years later the catecholaminergic system in the rat brain was mapped, initially localizing 12 cell body groups (A1-A12)[90]. Later, five more groups were added (A13-A17). With improved biochemical techniques, our understanding of the anatomy of the catecholaminergic system advanced and the groups were separated into distinct groups of adrenaline, noradrenaline and dopamine producing neurons respectively. For humans, the majority of the dopaminergic neurons are located in the midbrain, in the SNpc (A9) and the VTA (A10)[91].

The most common projection site of the dopaminergic groups are the basal ganglia. The basal ganglia are a group of anatomically defined subcortical nuclei which integrate signals from different brain areas. They are key in controlling voluntary movement, learning and emotion. The main nuclei are SN, globus pallidus (GP), subthalamic nucleus (STN) and the striatum,

consisting of putamen, nucleus caudatus and nucleus accumbens. The dopaminergic neurons in the SNpc predominately project to the dorsal ('motor') striatum and neurons in VTA primarily project to the ventromedial ('limbic') striatum. This is an simplified view and there is an extensive overlap in projections from SN and VTA[91, 92]. The striatum is the main output nuclei, receiving input from cortex, thalamus and SNpc. 90% of the neurons in the striatum are GABAergic medium spiny neurons (MSN) projecting via two distinct pathways. The direct pathway directly innervates output nuclei whereas the indirect pathway relay via STN and GP externa. The MSN of the direct pathway express D1, increasing excitability in response to dopamine, whereas the indirect pathway express D2, decreasing excitability of the MSN. Due to this, the direct pathway is generally accepted to be pro-kinetic, while the indirect pathway is anti-kinetic and the balance between the two is essential for correct voluntary movement[93].

1.3 MOLECULAR MECHANISMS IN PARKINSON'S DISEASE

The cause of dopaminergic cell death and accumulation of α-syn in PD is still elusive. Numerous cellular mechanisms are discussed as initiating and/or contributive to α-syn aggregation and disease progression. These include dysregulation of proteostasis, neuroinflammation, lipid alterations, mitochondrial damage, and axonal transport[73]. Historically, PD was perceived to mainly be caused by environmental factors. Now, a more multifaceted picture is accepted where genetic risk factors interact with the environmental factors[94]. A hereditary contribution, which can initiate the pathological cellular phenomena, is estimated in at least 30% of all cases[95]. Hence, there is likely to be several risk factors leading to cell death in each individual case. Therefore, a fundamental question today is if PD is one disease or the representative of a diverse set of biological processes leading to dopaminergic cell loss[96]. Below are the etiological theories relevant to the Thesis presented, beginning with presenting the essential protein α-syn.

1.3.1 α-synuclein

The central protein in PD pathogenesis is α-synuclein (α-syn) which misfolds into amyloid fibrils in LB. The link between PD and α-syn was first recognized in families with dominant autosomal PD where mutations where detected in SNCA, the gene encoding α-syn[97]. The same year, aggregated α-syn was acknowledged as the major component of LB[79]. α-syn is a 140 amino acid small presynaptic protein which affects membrane curvature and synaptic homeostatis[98]. However, its full physiological mechanism is not completely understood. Monomeric α-syn is intrinsically disordered[99-101] and exist in an equilibrium between an unfolded soluble state and a membrane-bound α-helical confirmation[102, 103]. Structurally the protein has three large domains. The central non amyloid component (NAC) domain is required, and sufficient, for aggregation[104]. Both increased levels of α-syn, induced by gene duplications or decreased degradation, and specific alterations, such as SNCA point mutations or posttranslational phosphorylation, increase the propensity to aggregate[102,105]. The misfolding process includes both metastable on-pathway oligomers, transforming into highly organized β-sheet structured fibrils, and non-fibrillary off-pathway species[98]. The debate whether oligomers or fibrils are more toxic is still ongoing[98, 106, 107] and since the oligomeric species are transient

by nature, they are difficult to study *in vivo*. These aggregates impair mitochondrial function, protein degradation and affects biological lipid bilayers which in turn is thought to cause neurodegeneration[73]. Even if there is strong evidence of α-syn's role in PD, there are a number of unresolved fundamental questions[108]. Upon neuropathological examination of the oldest surviving individuals (90+), 25% show LB pathology without parkinsonism[109] and a subgroup of PD cases associated with LRRK2-mutation show neuronal loss in SN but lack of LB[110]. This complicates the apparent direct connection between α-syn and neurodegeneration and highlights the need for a better molecular understanding.

Evidence accumulates that misfolded α-syn spread from cell to cell and drive normal α-syn into pathological structures in a prion-like way[111]. In human induced pluripotent stem cells (iPSC) exogenous α-syn induce α-syn pathology which is later trafficked between cells[112]. In patients, striatal grafted neurons show LB-like inclusions, indicating a spread from the surrounding diseased brain[113]. In addition, the Braak staging of PD suggests a spatial spread where some data support initial aggregation in the gut's autonomic plexi which extends into the CNS[114]. Since α-syn is central in PD pathogenesis and can be detected in the periphery, numerous efforts to measure α-syn as a clinical biomarker has been made. The results have been contradictory but most studies report decreased total level of α-syn in CSF and increased oligomeric α-syn[83,115]. The discrepancies is partly due to that α-syn is produced by erythrocytes and hence it is difficult to detect only the brain-derived portion in the periphery. To circumvent this, methods assessing α-syn derived from neuronal exosomes or aggregated α-syn have emerged[83,116]. In Paper IV of this Thesis a method for detecting nanoamyloids, potentially consisting of small α-syn aggregates, is evaluated as a biomarker.

1.3.2 Dysfunctional protein folding and aggregation

Protein misfolding and accumulation is a common theme in neurodegenerative disorders, including PD. The folding of proteins into their functional three dimensional configuration is vital. Cells therefore have an elaborative cellular network to maintain proteostasis, which includes protein synthesis, folding and degradation[117]. To date, 1000-2000 factors are estimated to play a role in the system, demonstrating the complexity[118]. Under physiological conditions, proteins are folded to the thermodynamically most favorable configuration, usually with hydrophobic amino acids in the core shielded from forming β-sheets and aggregate. However, during folding, proteins are only metastable and therefore convert between different configurations, making the folding process error prone[117]. A number of internal or external factors, such as genetic mutations, ageing, environmental stressors or overload of the cellular machinery, can overturn the protein homeostasis. Most of the folding process and quality control take place in the endoplasmatic reticulum (ER). Increased levels of misfolded proteins cause so called ER stress which in turn trigger the unfolded protein response (UPR). UPR includes halting translation, increasing protein folding factors and increasing ER-associated as well as proteosomal degradation, all aiming to lower the protein load. In the case of long-term activation of the UPR, apoptotic signals are initiated and the stress response leads to cell death[119,120].

In PD, the neuropathological hallmark is intracellular deposits of misfolded proteins indicative of disturbed proteostasis in the cell. If the phenotype is a cause or a consequence is unknown. Activation of ER stress and UPR is reported in a number of models as well as in patients. In iPSCs derived from PD patients with triplication in SNCA, increased α-syn expression induce a phenotype with increased ER stress[121]. Furthermore, decreasing ER stress alleviates dopaminergic cell death in a PD-mouse model[122]. In patients, several factors involved in UPR are up regulated in the SN[123,124]. The crucial role of ER protein control is also evident in genetic forms of PD. Mutations in Parkin, causing autosomal recessive PD, impede how misfolded proteins are ubiquitinated and marked for proteosomal degradation[63]. Thereby, Parkin mutations induce ER stress and subsequent neurodegeneration. Several of the substrates of Parkin are aggregation prone and found in LB[125]. As explained above, GPR37 is a substrate of Parkin reported to cause ER stress if overexpressed and therefore gained attention in the Parkinson's field[44]. Even though the ER stress and subsequent UPR response aims to restore proteostasis, the chronic stress state is deleterious for the cell as evident in both patients and model systems.

1.3.3 Protein degradation

Protein aggregation is closely linked to disturbances in the cell's protein degradation system. When misfolded proteins can be refolded by chaperones, targeted with ubiquitin for degradation in the proteasome (ubiquitin–proteasome system, UPS) or degraded in the autophagy-lysosome pathway (ALP). There is extensive crosstalk between the systems[126]. If degradation fails, protein form amyloids in order to reduce the hydrophobic areas which can cause toxic interactions. Both impaired proteosomal and lysosomal degradation is detected in brains from PD patients[127,128]. α-syn has been shown to be degraded by both UPS and ALP[129-131]. Both wild-type, mutated and oligomers of α-syn, are reported to cause UPS impairment in dopaminergic neurons[132-134]. Moreover, common toxin models with pesticides and MPP+ show reduced proteasome activity both *in vitro* and *in vivo*[135-137]. The involvement of ALP is evident by the more than 40 ALP-associated genes linked to PD[138,139]. These include the most common genetic risk factor known today, heterozygotic mutations in GBA1[140]. GBA1 encodes a lysosomal enzyme, glucocerebrosidase, which hydrolyzes glucocerebroside into ceramide and glucose when the essential activator saposin C is present. Mutations in GBA1 are preset in 5-20% of PD patients depending on population[138]. Mutations causing severe perturbation on enzyme activity have increased risk of PD compared to mild mutations, indicating a dose effect[141]. The mechanism by which mutations in GBA1 leads to PD is not fully understood. Hypothesis include alterations in lipid composition (see sect. 1.3.4), ER stress caused by misfolded of GCase and direct interaction with α-syn decreasing GCase activity resulting in a pathogenic positive feedback loop[142]. In summary, there are multiple evidence that perturbations in the cellular protein degradation machinery, leading to a vicious cycle where disrupted proteostasis leads to accumulated α-syn which further exacerbates the deficiencies.

1.3.4 Lipids in neurodegeneration

Apart from the theory of protein misfolding and perturbed degradation as key features of PD, the role of lipids in the pathogenesis has emerged. The brain has a particularly high expression of lipids and most abundant are cholesterol, sphingolipids and glycerosphingolipids[143]. Lipids are essential both for lateral organization of plasma membranes and as regulatory role of neuronal physiological function including synaptic activity[144]. Lipid homeostasis is more prone to alteration with increased age which is the greatest risk factor PD[145]. In neurodegenerative disorders specific changes in lipid homeostasis are reported[146-149]. In PD, the ganglioside GM1 is reduced specifically in the SN suggested to contribute to dopaminergic vulnerability[149]. GM1, and other sphingolipids, are responsible for the structural stability of lipid rafts. As described above, lipid rafts regulate GPCR's interactions and signaling[17]. Abnormalities in lipid rafts content are reported in early stages of PD possibly affecting raft function[146]. Much remains to be discovered but since protein-lipid interactions are crucial for GPCR physiology, these abnormalities are suggested to have judgmental effects.

Furthermore, the cellular distribution and aggregation of α-syn is highly dependent on properties of the lipid bilayer[103,150]. α-syn is known to interact with several types of lipids including fatty acids, gangliosides and glycosphigolipids[151-154]. SNCA mutations are clustered in the central region of the protein which includes the lipid binding domain as well as the NAC-domain[103,155]. Disruption of lipid homeostasis is reported in iPSC after overexpression of α-syn[156]. In addition, in experimental models, MPTP- treated mice have distinct changes in several lipids paralleled with α-syn oligomerization[105]. Specifically, the glycosphingolipid GM1 is suggested to inhibit α-syn fibrillation *in vitro* posing a potential mechanism[152]. Taken together, alteration in lipid composition may tip the balance of α-syn confirmations and act as driving force of oligomerization and fibrillation[157].

Genetic evidence also points towards a role of lipids in neurodegeneration. Sphingolipidoses are a group of inherited disorders caused by disturbances in lysosomal lipid metabolism with subsequent tissue storage of sphingolipids. Almost all sphingolipidoses, otherwise clinically heterogeneous, show neuronal engagement and neurodegeneration[158]. As explained above, GCase, the enzyme translated from the risk gene GBA1, is responsible for degradation of glucosylceramide and is hence part of the lipid homeostasis network. PD patients have therefore been investigated for substrate accumulation, similarly to the case of sphingolipidoses, but this have led to conflicting results[159-162]. A recent study, however, shows reduction of multiple lysosomal hydrolases and concomitant glycosphingolipid substrate accumulation[163].

Lipid alterations, either causing general membrane reorganization or specific levels increasing α-syn's tendency to form fibrils, now have strong evidence as a mechanistic factor in PD development. Furthermore, treatments aiming to normalize lipid levels is under evaluation as described in Sect.1.4.2.

1.3.5 Mitochondria dysfunction and oxidative stress

Mitochondrial dysfunction has a distinct role in PD pathogenesis both in experimental models and from genetic evidence. Mitochondria are responsible for producing energy in the form of adenosine 5' triphosphate (ATP) in the respiratory chain. In addition, mitochondria regulate Ca^{2+} homeostasis, lipid metabolism and apoptosis[164]. Mitochondria are the most important source of reactive oxygen species (ROS), especially from complex I and III of the respiratory chain[165]. Consequently, mitochondria also have high antioxidant capacity[166]. If the balance between the two is disturbed the cell experience oxidative stress with toxic effects on nucleic acids, proteins and lipids. This is judgmental in dopaminergic neurons that have high metabolism and are therefore highly dependent on efficient mitochondria[164,167].

The pivotal role of mitochondrial respiration for dopaminergic neuronal survival was evident from injections of 1-methyl-4-phenyl-1,2,3,6 tetrahydrodropyridine (MPTP) which caused severe parkinsonism in four cases[168]. Today a MPTP's derivative, MPP+, is a common experimental model in PD research. MPP+ enters the cell through DAT, NET or SERT causing a selective cell death[169]. MPP+ inhibit the mitochondrial complex I and thereby increase ROS, leading to cell death[170]. In idiopathic PD patients, complex I activity is compromised in SN[171] and there is evidence of misassembled complex I, enforcing clinical relevance of complex I inhibition[172]. In addition, several of the genetic causes of PD are directly or indirectly linked to mitochondrial function. DJ-1 encodes a chaperone which scavenger mitochondrial ROS and PINK detects mitochondrial dysfunction and recruits Parkin to induce autophagy of the damaged mitochondria[173]. Due to both clinical and preclinical evidence, disruption of mitochondrial homeostasis is today often used to model neurotoxicity as in Paper III of this Thesis.

1.3.6 Summary of pathogenic mechanisms

In summary, all the cellular insults described can initiate or support progression of dopaminergic cell loss in PD. Therefore, one can argue if clinically diagnosed PD is a collection of biological entities, each one needed to be addressed separately[96]. In addition, there is substantial cross talk between the pathways and it is therefore difficult to disentangle in what is causative and which cellular responses are reactive, triggered by initial events. The large number of molecular signals result in individual combinations responsible for disease. Understanding an individual's combination of biological signals as well recognizing the timeline of the molecular proceedings would considerably aid development of disease-modifying treatments.

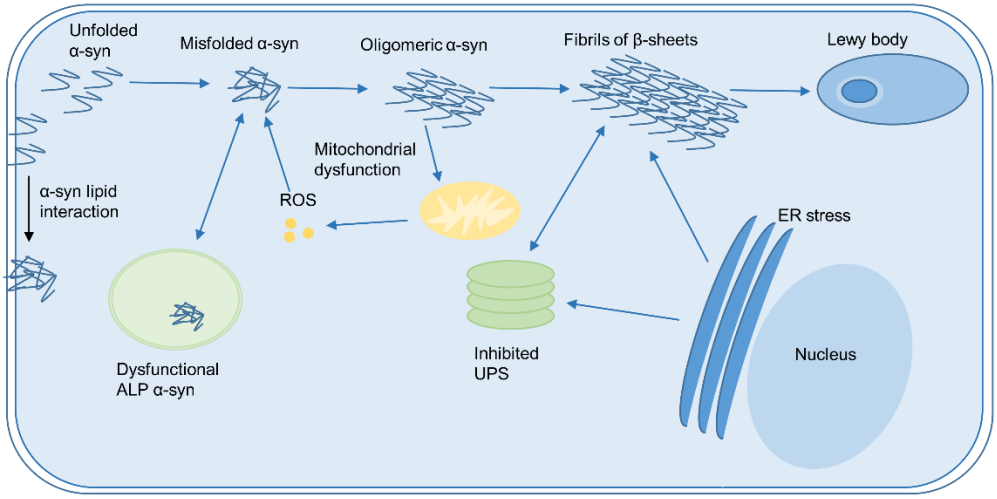

Figure 3. Summary of pathological mechanisms in PD relevant to this Thesis. Graphic abstract of how α-syn initially misfold, form oligomer species and later fibrils. Oligomeric α-syn and fibrils have toxic effects on a number of organelles.

1.4 TREATMENT OF PARKINSON'S DISEASE

1.4.1 Treatment today

To date, we can only offer our PD patients symptomatic treatment which aims to elevate the reduced dopamine signaling[78]. The gold standard treatment is the dopamine precursor L-DOPA, which is converted to dopamine in neurons. In addition, monoamine oxidase B (MAO-B) or catechol-O-methyltransferase (COMT) inhibitors which reduce the degradation pace of dopamine are used[174]. Early in the disease, or as a supplement at later stages, dopamine agonists which activate the D2-class receptors are commonly used. Dopamine agonists are associated with lower dyskinesia risk and is efficacious in treating motor fluctuations but is associated with an overall higher risk of adverse events than L-DOPA[78,174,175]. One severe side effect is impulse control disorder (ICD), including gambling, compulsive spending and binge eating. ICD affects 15-40% of patients treated with oral dopamine agonists and is a common cause of discontinuation of medication[176,177]. Mechanistically ICD is likely linked to a hyperdopaminergic state, either due to increased sensitivity of striatal D3 receptors after dopamine denervation or over dosing of dopamine to the relatively preserved ventral striatum. The latter theory would result in overstimulation of postsynaptic dopamine D2-like receptors causing an imbalance in the reward-related cortico-striatal-thalamocortical circuit, stimulating impulsivity[178]. This promiscuity in the ligand-receptor interaction between closely related receptors or unbiased activation might be reasons for the disabling side-effects. The side-effects have encouraged development of selective D2-agonists and biased D2-ligands which mainly activate non-canonical pathways. In preclinical studies biased ligands has been suggested to be beneficial in treatment of dyskinesias as well as offering antipsychotic effects without motoric inhibition[35,179]. These advancements point out the possibilities of pharmacological tools and also the gaps of knowledge of how the treatments used today affect receptors at the molecular level. This is partly addressed in Paper II.

In addition to dopaminergic treatments aiming to mitigate motor symptoms, symptomatic relief for specific NMS such as selective serotonin reuptake inhibitors (SSRI) for depression or acetylcholinesterase inhibitors in cognitive decline are used[78]. This highlights the need to modulate several neurotransmitters to successfully treat all aspects of PD. Besides pharmacological treatment, some PD patients are suitable deep brain stimulation. Here an electrode is surgically implanted in brain nuclei controlling movement which suppress symptoms[73]. None of the available treatments slow down the progressive neuronal death and all can cause severe side effects both in short- and long-term[78]. Therefore, there is a need both to understand how today's treatments affect the remaining dopaminergic neurons as well as to disentangle the causative steps in the pathogenesis to identify suitable targets which can halt or delay the progression of the disease.

1.4.2 Potential future treatment

Since PD, per definition, is progressive the clinical need for disease-modifying treatment is evident. As explained, PD is caused by a complex interplay between hereditary and environmental factors causing perturbation on multiple cellular pathways to an individual extent. This results in a clinical heterogeneity and likely a need for personalized medicine in order to slow down disease progression in the individual case[180].

Due to the complex pathogenesis, a long list of potential treatment has been examined. Common current treatments such as the MAO-B inhibitor selegiline, the dopamine agonist pramipexole[181,182] and L-DOPA[183], have been suggested to have neuroprotective effects. However, all have failed in clinical trials or, the decreased progression rate detected might be due to symptomatic relief rather than neuroprotection[184-187].

Large efforts have focused on modulating α-syn toxicity due to its pivotal role in PD pathogenesis. Targeting α-syn's toxicity through reduced protein synthesis, inhibition of fibril formation or increased degradation are all potential treatments[184]. To date there are clinical trials with passive or active immunization for α-syn to reduce α-syn production. On target binding and reduction of free α-syn in serum, but not in CSF, has been reported with monoclonal antibodies although the effect on disease remains to be investigated[188]. Different methods interrupting α-syn aggregation, possibly the key step, have also been evaluated. Since phosphorylated α-syn has higher tendency to aggregate, treatment with phosphokinase inhibitors has been evaluated[189]. The poor penetrance through the blood-brain-barrier remains a challenge as well as risk of off-targets effects. Another line of investigation is to modulate lipid levels to reduce α-syn's capacity to aggregate. GM1 binds α-syn and inhibit fibrillation *in vitro*[152, 190] . *In vivo* GM1 treatment reduces α-syn aggregate size and reverse behavioral deficits in rats overexpressing α-syn[191]. In addition, two small clinical studies have reported slowing of symptom progression in GM1 treated patients compared to placebo treated controls[192, 193]. Despite the clinical and preclinical findings, the mechanisms through which GM1 acts are uncertain, why this is investigated in Paper III. Finally, methods to strengthen lysosomal degradation of α-syn through its reciprocal relation with GCase activity is in current

clinical trials. Recently, a small open labeled non-controlled clinical study of ambroxol, a chaperone guiding GCase to the lysosome, showed CNS penetration and increased total α-syn levels. MDS-UPDRS III, motor symptom subscore, is reported to decrease by 7 points, a clinically relevant change. However, this should be interpreted with care due to the study design[194]. Furthermore, a multi-center, placebo controlled phase II trial using a glucosylceramide synthase inhibitor is ongoing in PD patients carrying GBA1 mutations aiming to reduce potential substrate accumulation. Although extensive research has focused on α-syn toxicity there are still unmet fundamental questions since α-syn aggregation does not correlate well with neurodegeneration and neurodegeneration does not require α-syn aggregation[110, 195]. Hence, more mechanisms are need to be investigated in order to succeed. Orphan GPCRs are often suggested an untapped source of druggable targets due to their specific distribution and functional selectivity[196]. This Thesis focus on one of them, GPR37.

1.5 SUMMARY

The heterogeneous PD has been a disease entity for 200 years and a plethora of cellular mechanisms have been linked to the disease. The link to α-syn has partly increased our understanding but not developed into a better set of diagnostic criteria. Dopamine receptors are the primary target in present treatment and despite being extensively studied there is lack of understanding how to fine-tune the system. The diversity of GPCRs gives a unique feature to induce cell specific responses but we need to understand how the dopaminergic receptors interact with other GPCRs and lipids to advance to better pharmacological tools. This will require a deeper understanding of molecular interactions as well as increased granularity in our clinical phenotyping to know which patient will gain from which treatment.

2 AIMS

The overall aim of this thesis is twofold. Our first aim was to understand how molecular interactions modulate the function of GPR37, a Parkinson's disease associated receptor (Papers I, II and III). Here we study GPR37 in live cells using advanced biophysical and pharmacological methods. The second aim was to detect structured protein aggregates in blood serum of PD patients (Paper IV). Here we take advantage of the extreme sensitivity of one of the methods, Fluorescence Correlation Spectroscopy. The specific aims of our studies were to:

I. Elucidate factors contributing to GPR37 membrane trafficking in catecholaminergic cells.
II. Investigate GPR37 interactions with dopamine 2 receptor and functional lipids.
III. Determine potential cytoprotective effects of GM1 in a GPR37-dependent model.
IV. Investigate if detection of nanoaggregates in serum can be used as a biomarker in PD and correlate with clinical progression.

3 METHODOLOGICAL CONSIDERATIONS

Methods used in this Thesis are summarized in the table below and detailed descriptions are found in the papers. Below selected methods will be discussed more in depth.

Method	**Paper**
Cell culture	I, II, III
Cell transfection	II, III
Generation of stable cell line	II
Fluorescence correlation spectroscopy	I, IV
Fluorescence cross-correlation spectroscopy	I, II
Confocal laser scanning microscopy	I, II, III
qPCR	III
Dot blot	III
Cell toxicity assays	I, III
HTRF signaling assay	III
Clinical evaluation of PD	IV

3.1 FLUORESCENCE CORRELATION AND CROSS-CORRELATION SPECTROSCOPY (FCS/FCCS)

Fluorescence Correlation Spectroscopy (FCS) is a quantitative analytical method based on monitoring with sub-microsecond temporal resolution the spontaneous fluctuations in fluorescence intensity that are generated when fluorescent molecules pass through a minute observation volume by Brownian diffusion. The technique can detect single molecules and be used to measure the concentration of molecules as well as their diffusion in solution and in live cells. It can also characterize protein-protein interactions, both in a single-channel mode but in particular using a two-channel variant called Fluorescence Cross-Correlation Spectroscopy (FCCS). In this Thesis, the methods were used to study GPR37 interactions in Papers I and II, and to detect structured amyloid aggregates in the serum from PD patients in Paper IV.

3.1.1 Principles

Figure 4A shows an FCS arrangement. The method is extensively described in, e.g., Ref. [197-201]. In brief, a high-numerical aperture microscope objective sharply focuses the incident laser light into the sample and light from the sample is collected by the same objective. In order to reduce the volume from which light is being detected, a confocal pinhole is placed in an image plane of the optics, thereby limiting the collected light in the z-direction. In this way, light is collected from a tiny observation volume element (OVE), which in modern instruments is about $(0.2 – 1)\times10^{-15}$ l. The OVE size depends on the wavelength of light and properties of the optical elements. A small size of the OVE is essential to achieve single-molecule sensitivity since the background is reduced, resulting in a higher signal-to-noise-ratio. In conventional FCS instruments the OVE size cannot be arbitrarily small due to diffraction of light, explaining why these instruments are called “diffraction-limited”.

While the incident laser light is monochromatic, the light collected from the sample is not. It consists of elastically scattered incident light, fluorescence from the analyzed fluorophore, and

autofluorescence from other molecules present in the sample. Elastically scattered light is separated from the emitted fluorescent light by a main dichroic beam splitter. The fluorescence is directed through optical filters to spectrally narrow the signal before reaching the detector. Avalanche photodiodes are used as detectors since they have single-photon sensitivity. For FCCS measurements, a secondary dichroic beam splitter is used to spectrally separate fluorescence originating from different fluorophores, and two detectors are used to independently detect the signals (see Sect. 3.1.2).

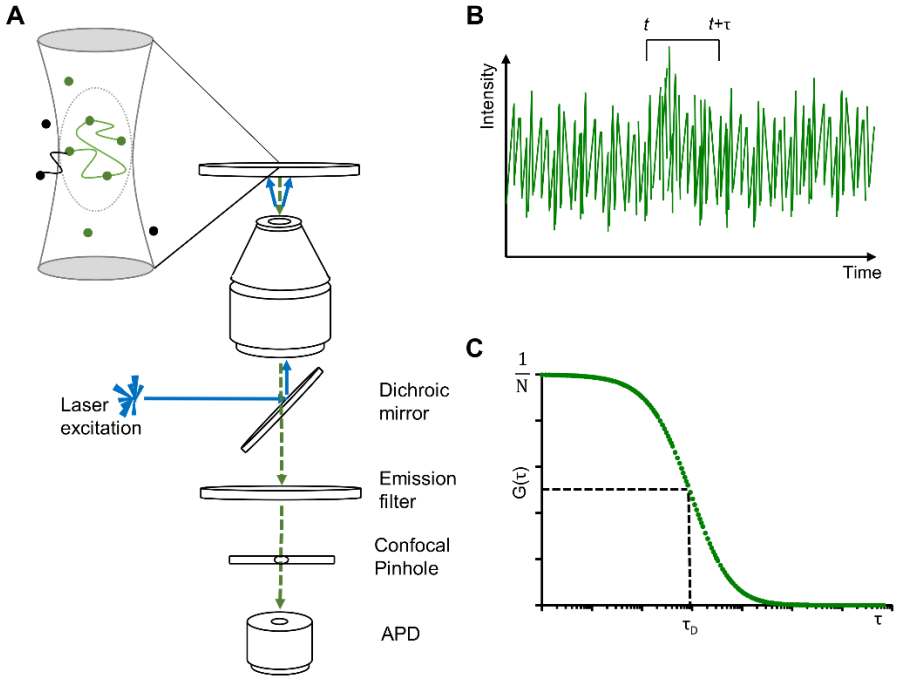

Figure 4. **Schematic FCS arrangement, fluctuation detection, and temporal autocorrelation analysis.** A) A confocal microscope arrangement illuminating fluorescent molecules diffusing in the sample. Emitted photons are guided through filters and a pinhole to reduce background before reaching the APD detector. B) Fluorescent intensity fluctuations are recorded over time. C) Fluctuations are analyzed for self-similarity by temporal autocorrelation analysis to give an autocorrelation curve G(τ). From the autocorrelation curve the number of molecules N and characteristic decay time (τ_D) can be deducted.

The measured fluorescence intensity depends on the laser intensity, brightness of the fluorophore, number of fluorescent molecules in the OVE, detector characteristics, and the background from the sample. Due to the spontaneous diffusion of fluorescent molecules, the number of fluorescent molecules in the OVE will not be constant. Hence, the recorded fluorescent signal will fluctuate around the mean fluorescence intensity (Fig. 4B). The lower the average fluorescence intensity, i.e., low concentration of fluorophores, the larger is the relative intensity variation due to each fluorescent photon. Figure 5 depicts time-series of fluorescent fluctuations in high-concentration (A) and low-concentration systems (B) visualizing the difference in impact of one photon on fluctuation variability. Thus, unlike other analytical techniques, FCS is especially suitable for low-concentration systems such as

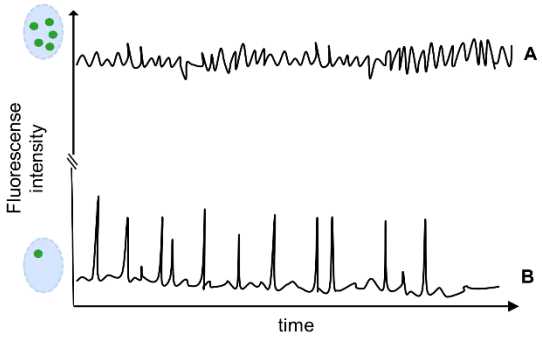

Figure 5. The effect of concentration on fluorescence intensity fluctuation. A) At high concentrations, the mean intensity is high and the relative amplitude of the fluctuations is small. B) At low concentrations, the mean intensity is low, and the relative amplitude of the fluorescence intensity fluctuations is comparatively high.

fluorescently tagged proteins which move randomly in live cells. The time-course of the fluctuation depends on the diffusion rate of the molecule and the size of the OVE. Thus, the OVE size needs to be determined using standard solutions of fluorescent molecules with known diffusion properties. In this work, Rhodamine 6G was used as a standard for instrument calibration and OVE size determination.

In order to extract relevant information from the fluorescence intensity fluctuation time series, several different methods are being used. The most commonly adopted is to analyze the variations over time using so-called temporal autocorrelation analysis. Here the time-dependent fluorescence fluctuation δF(t) is defined as the difference between the fluorescent signal F(*t*) at time point *t*, and the average fluorescent signal over the whole time series ⟨F(*t*)⟩

$$\delta F(t) = F(t) - \langle F(t) \rangle, \quad (1)$$

where the chevron brackets denote average values over time.

The normalized (temporal) autocorrelation function G(τ) is the correlation of a time series with itself shifted by a lag time τ, as a function of the lag time τ. The lag time τ is varied to identify whether there is a characteristic value after which the correlation is being lost. The normalized autocorrelation function is calculated as

$$G(\tau) = 1 + \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}. \quad (2)$$

Figure 4C shows an example of G(τ), also called the autocorrelation curve. The average number of molecules in the OVE can be determined from the amplitude of G(τ) at lag time zero, and the diffusion time from the characteristic decay time (τ_D). In order to derive these values, the autocorrelation function is fitted to theoretically derived model functions for 2D or 3D diffusion, depending on the system investigated. For example, when analyzing receptor diffusion and interactions in the plasma membrane of live cells in Paper I, a 2D model was used. In Paper IV, where measurements were made in blood serum, a 3D model was used.

3.1.2 Fluorescence cross-correlation spectroscopy (FCCS)

FCS may also be applicable for investigations of interaction between two molecules, e.g., a small ligand binding to a membrane. However, the diffusion coefficient in solution depends on molecular weight, MW, as $MW^{-1/3}$. Hence, interaction between two molecules of similar

molecular weight does not give an easily detectable change in diffusion time[202, 203] . To enable studies of interactions between molecules of similar size, fluorescence cross-correlation spectroscopy (FCCS) was developed. The principles are shown in Fig. 6 and the theory is reviewed in Ref. [200]. In FCCS, fluorescence intensity fluctuations are recorded for two spectrally distinct fluorophores simultaneously, hereafter called green and red, using a two-channel instrument. The OVEs generated by two lasers overlap, enabling simultaneous excitation of both fluorophores, but the detection pathways are separated (Fig. 6A). For each fluorophore, temporal autocorrelation analysis is used to derive the autocorrelation function but the intensity fluctuations of each fluorophore is also compared to each other. If the two fluorophores move together through the observation volume, the intensity variations in the two channels will co-variate as a sign of complex formation (Fig. 6B). This generates two autocorrelation curves and one cross-correlation curve (Fig. 6C). Assuming optimal conditions, the normalized cross-correlation function is described as

$$G_{CC}(\tau) = 1 + \frac{\langle \delta F_{green}(t) \delta F_{red}(t+\tau) \rangle}{\langle F_{green}(t) \rangle \langle F_{red}(t) \rangle}. \quad (3)$$

The amplitude of the cross-correlation curve is proportional to the degree of dually labeled molecules[200]. In order to normalize between cells, the cross-correlation amplitude is divided by either of the autocorrelation amplitudes at lag time zero, to have a measure of the degree of binding. This generates a dimensionless "relative cross-correlation amplitude" (RCCA), in Paper II described as

$$RCCA = \frac{G_{CC}(\tau)-1}{G_{AC}(\tau)-1}, \quad (4)$$

where $G_{AC}(\tau)$ is the autocorrelation of green fluorophore.

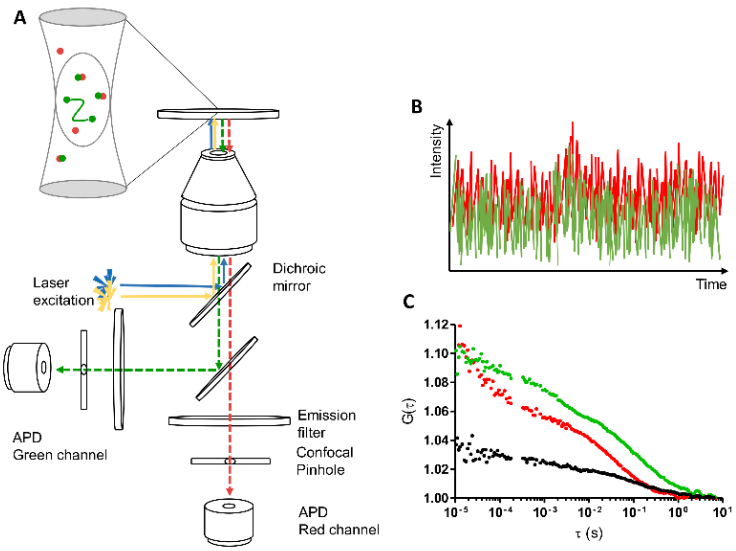

Figure 6. **Schematic FCCS arrangement**. A) FCCS arrangement where two lasers illuminate two spectrally separated fluorophores. Emitted light is separated to two detectors. B) Intensity fluctuations are recorded from the two detectors separately. C) The corresponding temporal autocorrelation curves G(τ) (green and red) and cross-correlation curve (black) indicating simultaneous intensity fluctuations in the separate channels.

3.1.3 Applications

In Papers I and II, fluorescence intensity fluctuations where recorded at the apical plasma membrane of live N2a cells in order to evaluate GPR37 movement in the plasma membrane as well as molecular interactions. In Paper I, FCS measurements analyzed with temporal autocorrelation analysis was applied to evaluate diffusion of GPR37 and FCCS was applied to elucidate GPR37 interaction with GM1-enriched lipid rafts as well as the proposed ligand PSAP. In Paper II, FCCS was applied to study interaction between GPR37/GPR37L1 and D2R.

In Paper IV, FCS was applied to detect Thioflavin T (ThT)-positive β-sheet enriched structures, hereafter called nanoamyloids/nanoplaques, in the blood serum of PD patients. The FCS detected fluctuations were analyzed by an automated program detecting fluorescent "peaks", indicating the passage of a ThT-positive amyloid structure through the observation volume. The number of peaks was then normalized to the measurement time to determine the frequency of single events occurrence (f_{SEO}) in the OVE. The f_{SEO} is given in units per hour (h^{-1}). The average size of the nanoamyloids was determined by temporal autocorrelation analysis as explained above. The individual autocorrelation curves were normalized to the same amplitude at τ = 10 μs, and the autocorrelation curves from the patient and the control groups, were combined to an average autocorrelation curve for each group. To identify whether nanoamyloids of different size are present in the serum, without having to specify the number of components in advance, the average autocorrelation curves were fitted using the Maximum Entropy Method (MEMFCS) that is specifically developed for bias-free fitting of FCS measurements in highly heterogeneous biological systems[204]. The validation experiments of the assay can be found in Ref. [205].

3.1.4 Methodological considerations

The number of methods studying molecular interactions are now numerous. FCCS has proven to be well suited for live cell experiments since the method is almost non-invasive, measurements do not perturb the system's dynamics, and the method has a single-molecule detection sensitivity. Other common methods to study molecular interactions, such as Förster/fluorescence resonance energy transfer (FRET) or Proximity Ligation Assay (PLA), differ fundamentally from FCCS in that they measure spatial proximity. In order for non-radiative energy transfer to occur in FRET or ligation in PLA, the two molecules have to be in close proximity, maximum 10 nm apart. The close proximity, however, does not necessarily prove that the proteins interact. FCCS, on the other hand, can detect simultaneous movement which indicates functional complex formation.

As all methods, FCS/FCCS have their limitations. Ref. [199, 200, 203] provide details. In brief, fluorescence variations within the observation volume can be caused by other processes than molecular diffusion. This includes photophysical and photochemical processes such as blinking and bleaching which can change the amplitude, shape and characteristic decay time of the autocorrelation curve. In cell measurements, an autofluorescent background may decrease the signal-to-noise-ratio. In FCCS, the situation is further complicated by bleed-

through between channels which can give false positive cross-correlations. Due to the single-molecule-detection limit, FCCS is very sensitive to photon noise and fluorophores with high photostability and quantum yield are needed. This limits the number of available fluorophores since most of the fluorophores in the far red part of the spectrum, desirable to limit cross-talk, do not fulfill the brightness requirement. There are several strategies to asses bleed-through. In Paper I and II, most essential FCCS results were validated using sequential illumination, which separates the spectrally distinct fluorescence signals temporally, in order to confirm a true cross-correlation. In addition, one should examine cross-correlation at the time lag of the triplet state which indicate that one fluorophore is detected in both channels. To perform cross-talk free FCCS measurements is not always possible. Under such conditions, one can compare the RCCA to the RCCA value measured in a negative control experiment where the same fluorophores are used, as in Paper II. Finally, bleed-through vary with brightness, which is not uniform in live cells due to local environmental differences and/or due to oligomerization-inducing treatments. These differences are more difficult to account for. Therefore, findings by FCCS should be interpreted carefully and, whenever possible, be validated with other methods.

3.2 CELL TOXICITY ASSAYS

Determining cell viability is crucial in several cell culture studies and there is a plethora of different methods. The methods assess various cellular functions such as cell membrane permeability, metabolic activity, ATP production, or DNA synthesis[206]. The most appropriate method depends on cell type, culture conditions and which hypothesis to be tested[207].

Metabolic activity is usually assessed via detection of oxidation-reduction reactions. MTT assay is one of the most widely used cell viability assays in which soluble yellow MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) is converted into insoluble purple formazan crystals. The exact cellular mechanism is not completely understood but likely involves transfer of electrons to MTT from reducing molecules. Under most culture conditions, the amount converted is proportional to the number of viable cells. The crystals are solubilized and the absorbance is analyzed. The analysis is robust and widely acknowledged[207]. Rezasurin is an alternative to MTT. Rezasurin is a dark blue non-fluorescent compound which upon entering the cells is reduced irreversibly to resorufin, a pink and fluorescent compound. Since rezasurin is fluorescent, the assay is considered slightly more sensitive than MTT. It is also less toxic to cells and requires less laboratory steps. However, under certain conditions interference of fluorescence can lead to false results at higher cell density and resorufin can be further reduced to non-fluorescent hydroresorufin, giving false read out[207]. In Paper I, MTT is used, whereas in Paper III rezasurin was chosen due to increased specificity as well as the reduced number of steps, reducing technical variation. Both these assays, however, measure cellular metabolism by oxidation-reduction reactions in the mitochondria and are appropriate when assessing adherent cells with several treatment groups where the throughput is essential.

3.3 CELL SIGNALING ASSAYS

Cell transduction from GPCR is one of the most extensively studied topics in biology. Since the network consist of numerous molecules and are highly entwined, there are countless of assays designed to study cell transduction at different stages of the cascade. Depending on the question asked, different targets should be investigated. Signals far downstream of the ligand-receptor interaction event can be generated by more than one signaling pathway[7]. Detecting those are therefore more difficult to directly link to the GPCR activation but potentially indicate the functional outcome.

Changes in cAMP levels are relatively upstream in the signaling cascade and hence, commonly measured directly or indirectly as a marker of GPCR activation. Homogeneous time-resolved fluorescence (HTRF) assays combines FRET technology with time-resolved measurement in order to reduce background noise. It has been applied to examine kinase activity, protein-protein interactions and GPCR signaling via determination of cAMP levels[208]. In principle, we used is a competitive immunoassay using d2-labeled cAMP and $Eu3^+$ cryptate-labeled anti-cAMP antibody. The close proximity of the $Eu3^+$ cryptate donor and the d2-acceptor upon binding of the antibody to labeled cAMP will allow FRET from the long-lived fluorescent donor to short-lived fluorescent acceptor. Unlabeled cAMP in cells compete for d2-antibody binding and FRET signal decreases (Fig. 7). $Eu3^+$ cryptate has a large Stokes shift and long fluorescent lifetime which introduces a time delay, 50-150 µs, between excitation and fluorescence emission. Together those properties reduce non-specific fluorescent signal and hence increase sensitivity. In addition, in each well HTRF emissions are measured at two different wavelengths, 620nm (donor) and 665nm (acceptor) which allows the emission at 620 nm to be used as an internal reference to reduce well-to-well variations.

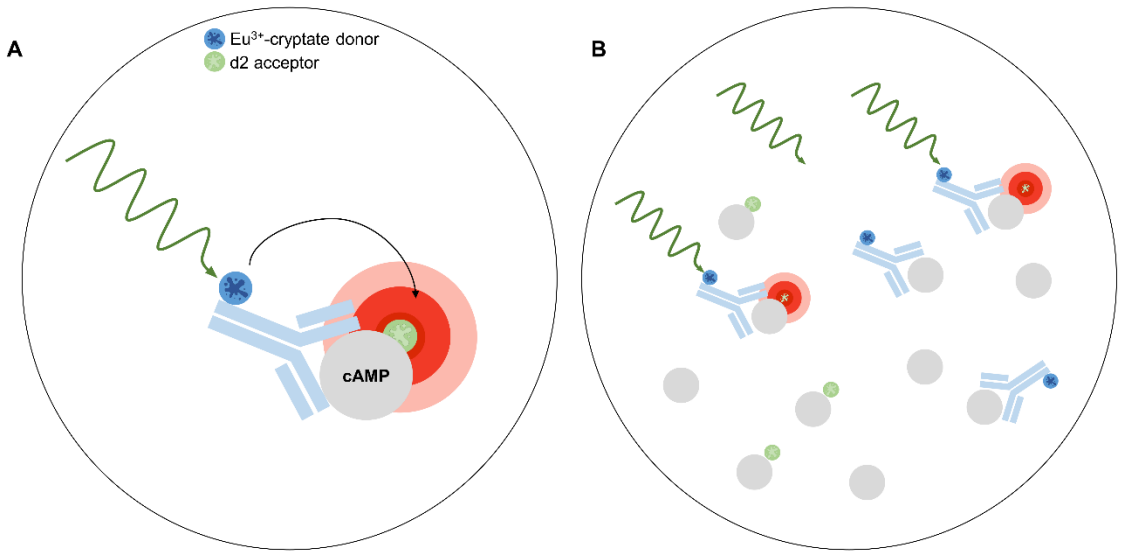

Figure 7. Schematic drawing of operation of HTRF. A) When $Eu3^+$ cryptate-labeled anti-cAMP binds d2-labeled cAMP the FRET pair is in close proximity and energy transfer occur. B) Cellular cAMP will compete for binding anti-cAMP and the FRET signal will decrease with higher concentration of endogenous cAMP.

In Paper III this assay was used to detect change in cAMP levels in HEK cells at baseline and with GM1 treatment. Since the G protein-coupling of GPR37 and downstream signaling targets are still partly undetermined, an assay to quantify second messenger was chosen. HTRF assays are highly sensitive, optimized for homogenous assays and well established in high throughput assays. In addition, they have previously been used to characterize orphan receptors[209, 210].

3.4 CLINICAL EVALUATION OF PARKINSON'S DISEASE

Since PD is a highly heterogeneous disease, the clinical evaluation of disease burden needs to include several aspects. In addition, since the pathophysiological understanding is insufficient, detailed clinical evaluation can reveal if certain symptoms are linked to prognosis, treatment response or specific biomarkers. The scales used to correlate nanoplaques with clinical aspects in Paper IV are discussed below. All the patients included in the study were included at the Academic Center of Neurology in Stockholm. The study was approved by the Regional Ethics Committee in Stockholm.

3.4.1 Parkinson symptoms

To assess the full clinical spectrum of PD symptoms the most widely used scales are Unified Parkinson's disease rating scale (UPDRS), and the more elaborative Movement Disorder Society-UPDRS (MDS-UPDRS). Both scales consist of four parts, non-motor and motor experiences in daily life (part I and II respectively), motor examination (part III) and motor complications (part IV)[211]. Prior to the development of UPDRS, various scales were used in different centers making inter-center comparisons difficult. Today, the scales are the gold standard in clinical practice, as well as clinical research. Both has been evaluated with low inter-rater variability. As UPDRS is shorter than MDS-UPDRS, it is plausible to use in daily clinical work. However, it has also been criticized to lack questions regarding important non-motor symptoms (NMS)[211]. This highlight the continuous consideration between optimal and feasible clinical characterization in daily clinical work. To circumvent this there are formulas to convert UPDRS score to MDS-UPDRS, either for day-to-day evaluation and for research properties[212, 213]. Both these studies report significant concordance for part II and III, while part I and IV are structured differently and hence not appropriate to convert.

The clinical evaluation of patients in Paper IV was assessed at the end of their doctor's consultation. A vast majority of the patients are evaluated by the same rater which decrease variability between observations. However, due to the discomfort, patients were not instructed to avoid dopaminergic medication prior to the visit, which affects the motor score ratings. Only the motor examination (UPDRS III) was converted using Goetz et al formula for research purposes as these show excellent correlation between UPDRS and MDS-UPDRS[213].

3.4.2 Disease severity

The Hoehn and Yahr (H&Y) scale is the reference standard to assess PD severity and progression[214]. First published in 1967, it classifies PD patients from stage 1-5, from unilateral mild disease (stage 1) to bilateral disease without postural stability (stage 2), to disease

affecting balance (stage 3) and further to severe disability (stage 4) and confinement in bed or wheelchair (stage 5)[215]. Since each stage is broad, the scale was modified to include 0.5 increments to increase granularity. It was designed give an overview of functional disability and objective impairment. It is fast to complete and easy to use, also for non-movement disorder specialists. The stages are highly clinically relevant but the combination of disability and impairment leads to ambiguity and due to the broad stages the scale is less sensitive to detect clinical change. In addition, even though the modified scale is more commonly used today than the original, there is a lack of clinimetric studies[214]. Even with those flaws, H&Y is still a standard scale which is highly useful in PD research, partly due to that it is well recognized among colleagues.

3.4.3 Non-motor symptoms scales

As described above, the gold standard for evaluation of motor symptoms in PD is UPDRS III. For specific NMS there are numerous scales used with various levels of validation. In 2006 the NMS screening questionnaire (NMSQuest) was developed due to the lack of scales assessing the wide range of NMS in PD in a simple 30-item, yes/no, self-reporting form[216]. NMSQuest is today widely used and validated at all stages of PD and it correlates with disease burden[216,217]. Since our understanding of NMS is increasing the preliminary MDS-NMS, a comprehensive clinician reported outcome scale, was launched in 2015. The pilot version was evaluated and after additional modifications a finalized version was published 2019 and is under current validation[218]. The scale is estimated to take 15-40 min to complete which is significantly longer than the 5-7 min for NMSQuest. In a healthcare system highly pressed with time, NMSQuest has a strong an advantage in order to achieve complete data sets in general PD patients, not only patients included at academic centers or included in clinical research.

3.4.4 Cognitive decline

After 20 years, up to 80% of patients experience cognitive decline related to PD[219]. Two subtypes of cognitive dysfunction have been suggested, executive dysfunction and visuospatial/semantic fluency dysfunction. Detailed neuropsychological testing covers all domains although it is time consuming and not readily available why screening scales are developed. MDS recommends three scales; Parkinson's Disease-Cognitive Rating Scale (PD-CRS), Mattis Dementia Rating Scale Second Edition (DRS-2) and Montreal Cognitive Assessment (MoCa)[220], where the latter is used in this Thesis. The strengths of MoCa is that it, in comparably short administration time, assess global cognitive function including executive dysfunction. For general dementia screening in Sweden, Mini Mental State Examination (MMSE) is commonly used. In MMSE, the coverage of executive abilities, visuospatial and constructional praxis is limited. Therefore, the scale is less appropriate for PD dementia screening. MMSE has also been shown to be less sensitive to early cognitive impairment than MoCa[221].

3.4.5 Depression

In cross sectional studies 30-40% of PD patients have depressive like symptoms[222]. The gold standard for depression is the diagnosis criteria in Diagnostic and Statistical Manual of mental disorders (DSM). Since many symptoms of PD overlap with symptoms of depression there are diagnostic difficulties. In addition, patients with cognitive decline are difficult to evaluate properly regarding emotional symptoms and, on the contrary, depression can be perceived as cognitive decline due to psychomotor changes. In general, observer-rated scales are preferred and are therefore in focus in recommendations. Hamilton Depression Rating Scale (Ham-D), Montgomery-Asberg Depression Rating Scale (MADRS) and Beck Depression Inventory (BDI) are generally recommended[223]. MADRS is originally designed to evaluate depression depth and not for diagnostic screening purposes. However, there are reports suggesting cut offs and it is today recommended for screening as used in this Thesis[224]. Compared to other scales MADRS has relatively few somatic items which is suitable for PD patients. It is specifically recommended for correlation studies with biological markers and hence applicable in Paper IV[223].

4 RESULTS AND DISCUSSION

Molecular interactions can predict a protein's function and indicate physiologically relevant networks. However, interactions can also be a sign of pathological incidents in a cell, as in the case of protein aggregation. The work summarized in this Thesis focuses on two aspects that are important for Parkinson's disease (PD) pathogenesis. First, molecular interactions of disease-related GPCRs with subsequent signaling alterations and secondly, the formation of α-synuclein (α-syn) amyloid aggregates which is the pathological hallmark of PD. Regarding GPCRs, we have specifically studied GPR37, its protein-protein interactions important for subcellular localization as well as effects of protein-lipid interactions. Regarding amyloid formation, we have detected β-enriched protein aggregates, nanoplaques, in blood serum from PD patients and correlated them with clinical parameters. These different interactions are studied by a range of methods of which fluorescence correlation spectroscopy (FCS) play a key role. In the following section a summary of the results in Paper I-IV are discussed in a thematic approach. For a comprehensive description, the reader is referred to the full papers.

4.1 INTERACTIONS AFFECTING TRAFFICKING OF GPR37 (PAPER I AND II)

In heterologous systems GPR37 is primarily expressed intracellularly[33, 44]. According to the literature, this is due to disrupted trafficking which enhances the risk of toxic protein accumulation potentially leading to neurodegeneration. Finding methods to traffic GPR37 to the plasma membrane is therefore potentially of pharmacological relevance. Different methods to increase GPR37's association with the plasma membrane are therefore applied and further evaluated in this Thesis. In general, membrane expression depends on a number of intra- and extracellular variables including ligand concentration, chaperone availability, and β-arrestin recruitment. In particular, GPR37 membrane localization is enhanced by inducing molecular chaperones, cell differentiation, and co-expression of GPCRs, including D2R[33, 67, 225] . The GPR37-D2R interaction further studied in Paper II.

In Paper I, prosaposin (PSAP) was evaluated as a factor determining GPR37 subcellular localization in N2a cells. At the time when we commenced our studies, PSAP was recently published to be the endogenous ligand for GPR37 and GPR37L1 and, as such, a potential regulator of membrane trafficking[49]. Since PSAP is expressed by most mammalian cells and is secreted extracellularly, conditioned media from N2a cells was tested for the presence of PSAP using Western blot. The concentration of PSAP in the media was several times higher than the suggested EC_{50} of 7 nM for PSAP at the receptor[49]. A substantial fraction of GPR37 was therefore suggested to be bound to PSAP in the model system used. In order to block extracellular PSAP from binding to the cell surface, an antibody targeting the neurotrophic region of PSAP was added to the cell media. After the antibody addition a pronounced decrease in surface density of GPR37 was noted and Western blot showed a sharp decrease of free PSAP in the media (Fig. 8). In addition, a positive cross-correlation between GPR37-tGFP and the PSAP-derived peptide TX14(A) was identified indicating a complex formation between GPR37- TX14(A). This suggested a role of PSAP-induced regulation of GPR37 subcellular localization.

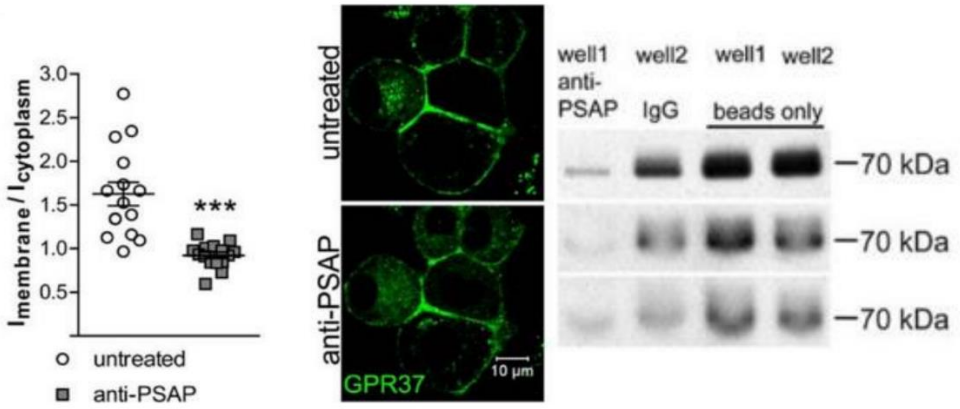

Figure 8: Immunoabsorption of extracellular PSAP in cell media. Quantification and representative images of GPR37 density at the plasma membrane expression before and after anti-PSAP treatment and the corresponding immunoprecipitation of free PSAP in the media.

The traditional view of ligand-regulated membrane association is that agonists induce internalization. Reduced agonist availability, as in the case of immunoabsorption, would therefore increase membrane density. GPCR trafficking is probably more complex. There are reports of increased surface density of receptors by agonist treatment, e.g., for the delta opioid receptor[226]. There is also an increasing group of GPCRs with primarily intracellular function of which activation and trafficking is less well characterized. Furthermore, PSAP can have a modulatory role of GPR37 trafficking without having all the properties of an agonist. All of these alternatives are possible explanations of the observed phenomena.

In Paper II, the interaction between GPR37 and D2R was further studied. In *in vitro* studies, the two receptors interact[33, 34]. Co-expression of the receptors was also reported to increase GPR37 trafficking to the plasma membrane indicating an interaction important for its function. However, the interaction had not been studied in live cells. Stable N2a-GPR37-eGFP cells, transiently transfected with D2R-TdTomato of either the postsynaptic or the presynaptic splice form (D2RL-TdTomato and D2RS-TdTomato, respectively), were studied using FCCS to determine if GPR37-eGFP and D2R-TdTomato diffuse as a complex in the plasma membrane (Fig. 9). At baseline, we detected a cross-correlation between N2a-GPR37-eGFP and D2RL-TdTomato, indicating complex formation. The relative cross-correlation amplitude (RCCA) was low, indicating that only a small fraction the receptors formed heterodimers. RCCA between GPR37-eGFP and D2RS-TdTomato was at the same range as a previously published negative control. Possibly, the difference between the D2R splice forms can be explained by that D2RL, under these experimental conditions, has a slightly better trafficking capacity of GPR37 than D2RS.

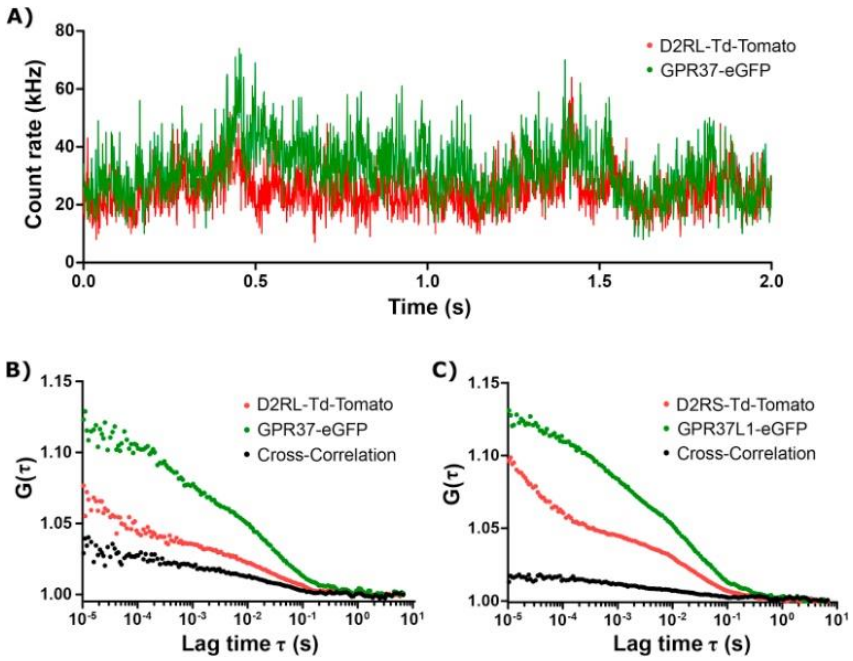

Figure 9. FCCS measurements in live cells. A) Fluorescent intensity fluctuations recorded at the apical membrane of N2a cells expressing GPR37-eGFP and D2RL-TdTomato. B) Corresponding temporal autocorrelation curves (red and green) and cross-correlation curve (black) between GPR37 and D2RL. C) Temporal autocorrelation curves (red and green) and cross-correlation curve (black) between GPR37L1 and D2RS.

However, from imaging it was evident that a large fraction of GPR37 was located intracellularly, despite D2R co-expression. To evaluate if increased trafficking of GPR37 to the plasma membrane could increase the fraction of GPR37-D2R heterodimer receptor complexes, we used 4-phenylbutyrate (4-PBA). 4-PBA is a chemical chaperone which facilitates correct protein folding and has previously been used to augment GPR37 expression at the plasma membrane[225]. Treatment with 4-PBA increased the fraction of interacting GPR37 with both splice variants of D2R (Fig. 10).

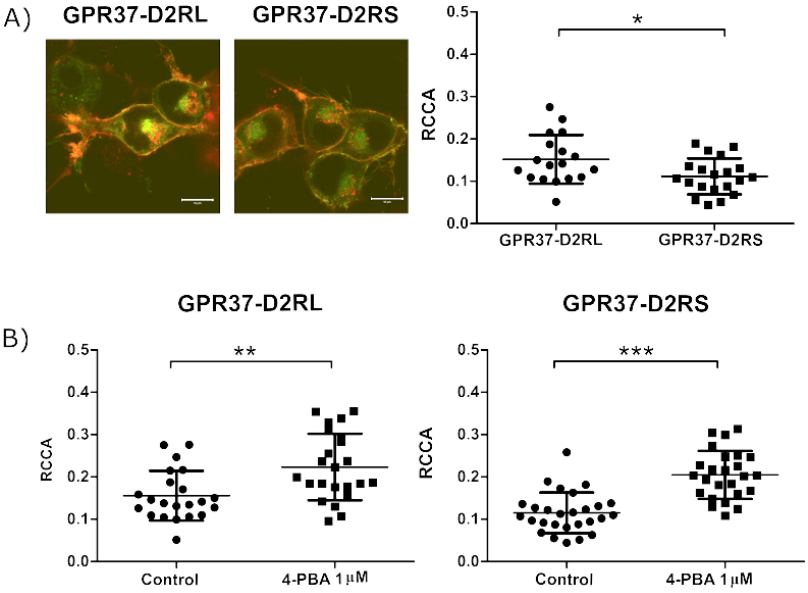

Figure 10. Complex formation of GPR37 and D2R in live cells A) Representative images of N2a cells expressing GPR37 and the two isoforms of D2R. GPR37 and D2RL interact under baseline conditions. B) Induction of GPR37 trafficking to the plasma membrane by 4-PBA treatment increased the fraction of GPR37 interacting molecules both with D2RL and D2RS.

Heterodimerization can also be affected by pharmacological tools. In order to investigate if this was the case for the GPR37-D2R complex we studied the fraction of interacting molecules during pramipexole treatment, a clinically commonly used D3/D2 dopamine agonist. Pramipexole increased the fraction of heterodimers with both alternative splice forms (Fig. 11).

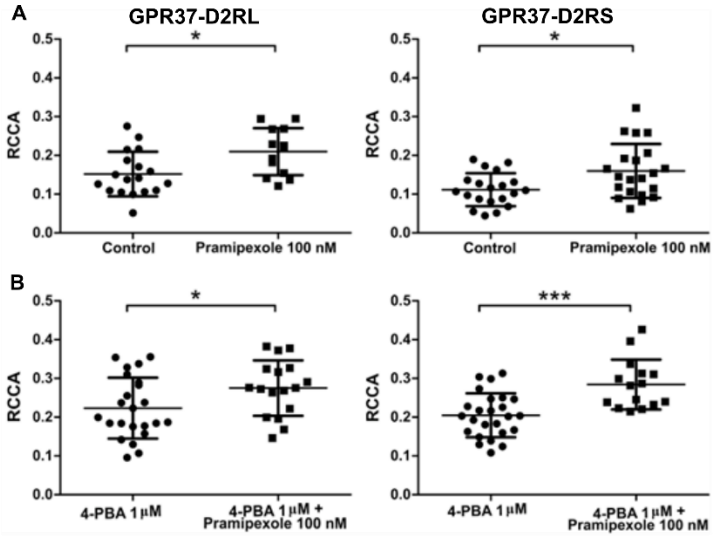

Figure 11. Dopamine agonist treatment increases fraction of GPR37-D2R complexes. A) Pramipexole treatment increases heterodimerization between GPR37 and both splice forms of D2R. B) After 4-PBA treatment, dopamine agonist treatment further enhanced complex formation between GPR37 and both splice forms of D2R.

GPR37L1 is often examined in parallel with GPR37 to compare their functions and the receptors are often reported to have similar effects[49, 52, 70]. In our live cell studies, the RCCA at baseline was higher for transiently transfected GPR37L1-D2RS than for GPR37L1-D2RL, opposite of the result of GPR37, and indicating heterodimerization. In contrast to GPR37, GPR37L1 is in most cellular models primarily located in the plasma membrane, without any need of interaction partners or chemical chaperones. This was also evident in our model and 4-PBA treatment did not increase the RCCA levels detected for GPR37L1-D2R. Furthermore, GPR37L1-D2R binding was not changed with pramipexole treatment, indicating a specific impact on the GPR37-D2R heterodimerization. This finding also excludes most possibilities of that 4-PBA or pramipexole treatment changed experimental factors which could influence the fluorescent properties leading to misinterpretations. However, for all the experimental settings examined GPR37L1-D2R the RCCA is low and could not be increased with pharmacological treatments, risking misinterpretation due to artifacts such as bleed-through. Hence, the results for GPR37L1 should be interpreted with care.

Paper II was the first to suggest GPR37-D2R heterodimerization in live cells. In addition, a clinically relevant dopamine agonist was shown to increase the fraction of interacting molecules, which can be of importance for clinical practice. D2R interacts with a large number of other GPCRs and dimerization alters its function. D2R dimerization with $A_{2A}R$, a receptor also interacting with GPR37, shifts the preferred D2R coupling from $G\alpha_i$ to β-arrestin signaling[30]. How the complex formation between GPR37 and D2R affects downstream signaling and physiological outcome remains to be investigated. From a clinical perspective

the molecular pathways need to be further investigated, especially since the interaction is augmented by pramipexole treatment. Potentially, the shift in equilibrium towards heterodimerization and its role on trafficking is a drug target by itself.

The results obtained in this cellular model require further studies in biologically more relevant systems due to the study's limitations. First, increased protein levels at the plasma membrane, as in a plasmid-transfected cell line, can induce interactions not naturally occurring. In order to reduce the risk, a stable cell line was used for GPR37 since those generally express lower protein levels. In addition, the structurally similar GPR37 and GPR37L1 were analyzed in comparable manners resulting in different outcomes which indicates a biological reason for the differences. Secondly, bleed-through can cause false positive cross-correlations which needs to be accounted for. The GPR37-D2R interaction and the increased cross-correlation amplitude after pramipexole treatment have been detected using sequential illumination which lower the risk of bleed-through being a major component of the cross-correlation detected. Relative changes can be interpreted but the lowest RCCA values in this study need further validation to exclude are methodological artifacts with high certainly. The data in Paper II support previous *in vitro* data and add one more part of the puzzle of GPR37's interactome.

4.2 GPR37 INTERACTION WITH GM1 (PAPER I AND III)

Lateral organization of the plasma membrane into ganglioside GM1-enriched lipid rafts are central in coordinating GPCR function[17]. In addition, GM1 has gained researchers' attention for decades due to its neuroprotective and neurotropic effects seen *in vitro* and *in vivo*. A number of pathways including potentiation of neurotrophins' effects[227] are suggested. As explained, expression levels of GM1 decrease specifically in PD[149] and GM1 inhibits α-syn from forming β-sheets[152]. Therefore GM1 is suggested to be key to avoid neurodegeneration[228]. As such, the interaction between GM1 on GPR37 were investigated in Paper I and III.

In Paper I we sought to investigate if lipid rafts sorted GPR37 at the plasma membrane. To study lipid rafts, Cholera toxin β-subunit (CtxB), which binds GM1, is often used as a marker for the nanodomains in cellular studies. In differentiated N2a cells, GPR37-tGFP was shown to colocalize with CtxB-Alexa594 in the presence of PSAP (Fig. 12). Using FCCS a positive cross-correlation between the GM1-marker and GPR37-tGFP was detected indicating that GPR37 forms a complex with GM1. Indeed, treatment with cholesterol depleting agents which

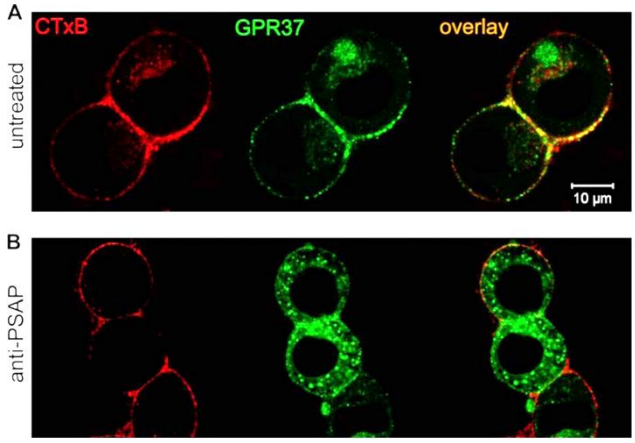

Figure 12. GPR37 colocalize with GM1
A) Representative images of GPR37 colocalizing with the GM1 marker CTxB in the presence of PSAP indicating a sorting of GPR37 into lipid rafts in N2a cells. B) PSAP-immunoabsorption reduced co-localization between GPR37 and GM1.

disrupts the lipid rafts resulted in a loss of cross-correlation between GPR37-GM1 and GPR37-TX14(A). This indicates that the three molecules have a functional connectivity, either by direct physical interaction or as members of a larger signaling domain.

Due to the interaction between GPR37 and GM1 studied in Paper I, we aimed to identify the functional outcome of the interaction in Paper III. In a toxin model using MPP+, we report a GPR37-specific rescue with exogenous GM1 in non-differentiated N2a cells compared to N2a-eGFP (Fig. 13). This effect was detected in both stably overexpressing cells as well as transiently transfected. In transient transfections, we included D2RL since overexpression of a receptor could alter the lateral organization of the plasma membrane inducing the differences. However, this was not the case and D2R-N2a cells were not rescued by GM1 treatment, indicating a specific effect of GPR37. The two models have different characteristics. Transiently transfected cells are in general under high cellular stress due to the recent transfecting event and express high levels of protein, potentially overwhelming the protein quality system. Stable cell lines express lower protein levels which allows for long-term survival. Results from stable cell lines are usually more reproducible as the variability and toxicity induced by the transfection event is diminished. On the other hand, generation and propagation of stable cell lines can induce differences due to long-term adaptation or cytogenetic instability which has been reported for a number of cell lines[229, 230]. Hence, the two models are complimentary and help to exclude model-induced errors.

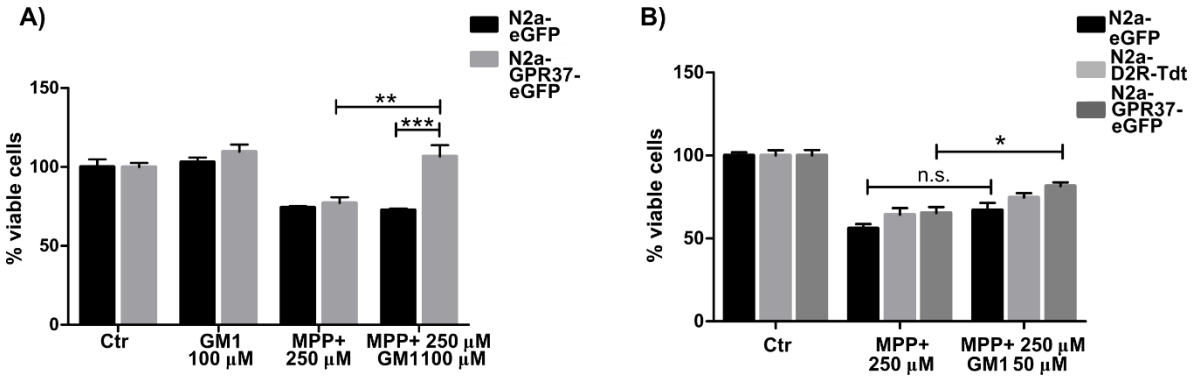

Figure 13. GM1 treatment is cytoprotective in a GPR37-dependent model. A) Stable GPR37-eGFP-N2a cells are specifically rescued with GM1 treatment towards MPP+ treatment. B) Transiently transfected N2a cells overexpressing GPR37 are rescued by GM1 treatment. This effect is not seen for eGFP or D2R-expressing cells.

Studying signaling events are crucial in understanding GPCR function. Without a confirmed ligand, studies are more complex and the literature for the preferred G protein-coupling of GPR37 is partly contradictory. During baseline conditions, HEK-GPR37-eGFP cells show decreased cAMP levels compared to HEK-eGFP, interpreted as increased $G\alpha_i$- activity. Since the endogenous ligand of GPR37 is undetermined we cannot differentiate if the receptor is constituently active or if the media contain the endogenous ligand. After 2 hours of GM1 treatment the reduced cAMP levels are normalized, in indicating an altered effect on GPR37-signaling (Fig. 14). GM1 treatment did not affect cAMP levels in D2R transfected cells but

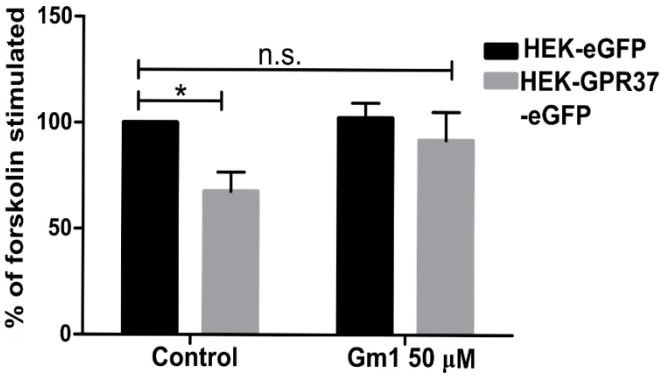

Figure 14. GM1 alters GPR37 signaling. Under baseline conditions HEK-GPR37 contain lower levels of cAMP, indicative of a G_i-coupled receptor which is either constituently active or presence of endogenous ligand in the media. After two hours of GM1 treatment the cAMP levels are normalized.

D2R does not exhibit constituent activity and the absence of change is therefore more difficult to interpret. The reduced $G\alpha_i$-signaling, initially counterintuitive of the positive effect of GPR37 on cell viability, can be explained by a shift in G protein-coupling upon GM1 treatment responsible for the effect. The fact that we detect a direct effect of GPR37 signaling strengthen the conclusion that cytoprotective effects of GM1 treatment is partly dependent on the function of GPR37.

The reduced $G\alpha_i$-signaling intensity can be due to both rearrangement in the micro-environment by addition of exogenous GM1 as well as direct protein-lipid interaction. GM1 is reported to directly interact with the GPCR serotonin 1A receptor (5-HT_{1A}) via a sphingolipid binding domain (SBD)[231]. The SBD consist of a conserved combination of aromatic, basic and turn-inducing amino acid residue. Interestingly, we identify a motif in an extracellular loop of GPR37 which has high similarity to the known SBD in 5-HT_{1A}. Modeling suggested similar loop configuration. The effects reported could hence be due to a direct protein-lipid interaction and the positive cross-correlation detected in Paper I would not only be due to GPR37 localization in lipid rafts.

Apart from the direct effect on GPR37 signaling, other possible mechanisms of GM1 effects was investigated in Paper III. GM1 is reported to interact with chromatin to regulate gene expression[232]. GPR37 can, under certain conditions, confer a cytoprotective effect why increased expression of GPR37 could explain the toxin-resistant effect detected. However, GPR37-expression was not changed; neither in endogenously expressed GPR37 in N2a-eGFP or in the stably overexpressing N2a-GPR37-eGFP measured by qPCR. Furthermore, our stable N2a-GPR37-eGFP cell model was found to express lower levels of endogenous GM1. This phenomena, induced by knocking down the GM1-producing enzyme B3GALT4, has been reported to cause increased vulnerability to MPP+[233]. In our model, there is no increased susceptibility to MPP+ paralleled to the GM1 decrease and no change in B3GALT4 levels evaluated by qPCR. This indicates that two different mechanisms are studied or that GPR37 expression rescues the more susceptible phenotype.

As the proposed functions of GM1 are plentiful, it is difficult to separate the distinct functions. To mutate the key amino acids of the SBD into amino acids with contrasting properties is one alternative to disentangle lipid-raft formation from ligand-like interaction. However, this assumes that GPR37 folds, inserts into the plasma membrane, and binds other potential ligands

in the media correctly, despite having another primary structure. Furthermore, even indications of a direct interaction can be interpreted differently. GM1 interaction can either directly inhibit GPR37 signaling, analogous to an inverse agonist, or block binding of an endogenous ligand in the media. The lack of pharmacological tools is a major issue when studying orphan receptors and hinders progress.

4.3 DETECTION OF NANOAMYLOIDS IN PD PATIENTS (PAPER IV)

Paper IV focuses on detecting improper protein-protein interactions as a sign of disease. Brain protein aggregates is the characteristic of neurodegenerative diseases. In PD, amyloid fibrils of α-synuclein (α-syn) is the main constituent and has therefore been appreciated as a feasible biomarker. Research efforts have investigated α-syn levels mainly in CSF and blood[234, 235]. CSF is directly reflecting processes in CNS but from a patient perspective and applicability to use in everyday clinic, a serum marker is preferable. In Paper IV, the first aim was to investigate if it is possible to detect amyloid aggregates in serum of PD patients using FCS. To detect structured aggregates exclusively, without interference from the non-toxic monomers present in excess, the fluorescent dye Thioflavin T (ThT) was used as it binds specifically to amyloid structures. The main findings are that the detected frequency of nanoamyloids (f_{SEO}) is increased in PD patients compared to controls and preliminary data indicate that nanoamyloids in the patients and controls have a different size distribution (Fig. 15A).

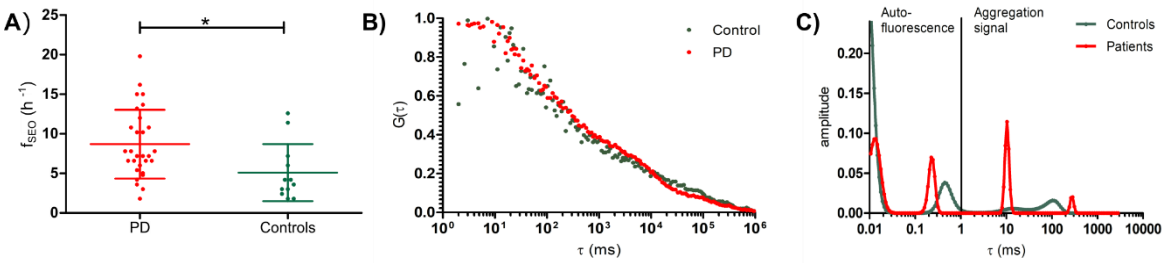

Figure 15. f_{SEO} of nanoamyloids is increased in PD patients A) Serum samples from PD patients have a higher frequency of detected ThT-positive nanoplaques compared to healthy controls. B) Average temporal autocorrelation curves for each group. C) Characteristic decay times for each group representing distinct sizes of nanoamyloids revealed by MEMFCS.

Since α-syn aggregation into amyloids is key in PD pathogenesis, the detected nanoplaques are potentially toxic aggregates, which have been organized into a structured β-sheet conformation. One of the strengths of the method is that it only detects structured amyloid aggregates without interference from the non-toxic monomeric α-syn. Since α-syn is extensively expressed in erythrocytes, variations in hemolysis easily affects total α-syn levels. This has been suggested to contribute to discrepancies between biomarker studies and has been drawback for blood-based measurements[235]. Control experiments show that our method shows no increase of f_{SEO} when applied to whole erythrocytes or lysed erythrocytes in plasma.

A limitation is that ThT does not discriminate between different amyloid-structured proteins, reflected by the fact that also patients with Alzheimer's disease (AD) have increased f_{SEO} of nanoamyloids[205]. Hence, increased number of nanoplaques might be a sign of proteinopathy.

A large proportion of PD patients exhibit, in addition to Lewy bodies (LB), AD-like pathology upon neuropathological examination[236, 237]. Mechanistically, there is extensive data suggesting toxic association between α-syn and amyloid-β, the protein linked to AD[238, 239]. Methods detecting protein aggregates, such as the nanoaggregates, have therefore been suggested to be clinically relevant to demonstrate the common pathogenesis[108].

In addition to measuring the frequency of encountering the structured nanoaggregates (f_{SEO}), the ThT-based FCS method enables simultaneous examination of the aggregates' size distribution. In the data set analyzed in Paper IV, the MEMFCS analysis suggests that there are differences in aggregate size distribution between PD and controls (Fig. 15C). In particular, the fraction of smaller aggregates seems to be enriched in PD, which is in contrast to data for AD patients[205]. The size distribution of the ThT-responsive nanoaggregates may therefore be a specific disease-related biomarker.

While the study in Paper IV provides a proof of principle, there are several important issues to be further investigated before the method can be considered for clinical use. Most notably, the number of individuals included in the study is very low and the results therefore needs to be replicated in a larger cohort. Of special importance is to understand if the measured bimodal size distribution is a characteristic for PD patients. If so, it should be possible to find a better clinical biomarker than the present f_{SEO}, which does not correspond directly to nanoaggregate concentration due to the difference in diffusion speed between large and small aggregates. With known size distributions the f_{SEO} could be directly correlated to concentration, which is preferable to a clinician. Finally the statistical power needs to be improved, either by prolonging the data acquisition time, or by increasing the sampling volumes, as in in massively paralleled FCS[240], as the number of detected amyloids per individual are few.

A quantitative biomarker can aid in several clinical situations. Detecting a disease is the most common application but indication of progression and treatment response is valuable information, especially in pharmacological studies. The first aim of Paper IV was to determine if PD patients had an increased number of detected amyloid formed protein in serum, which we concluded and the result is discussed above. However, while recruiting patients to studies there are several factors to acknowledge. The clinical diagnosis of PD is in 25% of the cases not consistent with the neuropathological confirmed diagnosis[82, 241] Apart from retrospective studies using neuropathological confirmed cases, this is a methodological issue for all clinical studies. In order to only include genuine PD and not atypical parkinsonism all cases diagnosed in less than 5 years were evaluated according to the MDS criteria for PD as those are designed to use in research settings[74]. Only patients fulfilling "clinically established PD" were included. Furthermore, included patients were regularly examined at an academic center focused on movement disorders, previously reported to increase accuracy of correct parkinsonian diagnosis[242]. There is a large difference in age between patients and controls in the study. Even if we, to date, have no data suggesting a correlation between age and nanoaggregates, this is a potential bias. There is a decline in the proteostatis network with age and loss of proteostasis

has even been suggested as a hallmark of aging why this should be taken into consideration[117, 243].

	Patients	*Controls*	*p=*
Age (years)	73,8±8,6	46,5±11,4	<0,001
Gender (M:F)	1:1	1:1	1
Age of onset (years)	64,6±8,2	NA	
Disease duration (years)	8,1±5,2	NA	
MDS-UPDRS	56,8±29,4	NA	
UPDRS III	28,7±16,1	NA	
MoCa	23,9±6,1	NA	
MADRS-S	10,2±5,6	NA	

Table 1. Demographic data of patients presented in Paper IV.

The second aim of the study was to evaluate the applicability of this biomarker to mirror disease progression or severity. The frequency of detected amyloid plaques (f_{SEO}) were therefore correlated to clinical data and symptom scores. Unfortunately, just like previous effort for PD biomarkers, there was no correlation with age of onset, disease duration or H&Y stage, representing disease severity (Fig. 16). There can be a number of reasons for this apparent divergence. First, the sample size is small and the disease is highly heterogeneous possibly requiring larger cohort to find correlations. Second, this biomarker might detect a subset of PD patients with more pronounced protein aggregation disturbances. Finally, there is poor correlation even between symptom severity and α-syn load in the brain[195, 244]. This reveals that the apparent connection between LB load and disease is more complex and likely a reason why we, to date, do not have any peripheral biomarker for progression in clinical use.

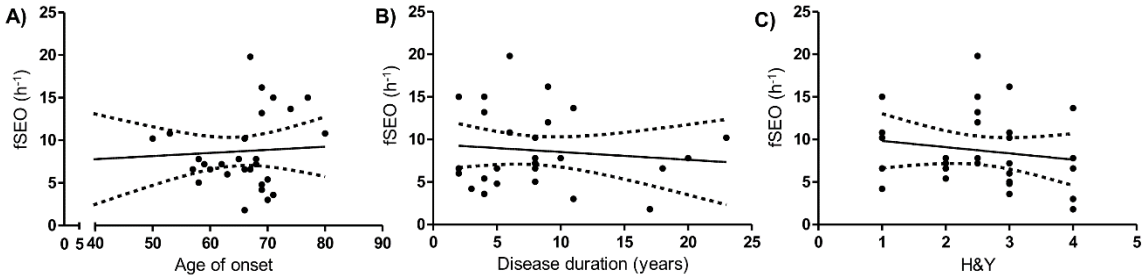

Figure 16 f_{SEO} does not correlate with disease severity or progression. No correlation between age of onset (A), disease duration (B) or the severity scale Hoehn and Yahr (C). All data set are evaluated by linear regression.

Both motor- and non-motor symptoms (NMS) were correlated to the f_{SEO} of nanoplaques. Motor symptoms, evaluated by UPDRS III, did not correlate with detected frequency of nanoplaques (Fig. 17A). UPDRS III varies with treatment response and time since medication. In theory, this could easily be standardized. However, for severely diseased patients it is challenging, if not impossible, to come to an appointment without medication. Since we aimed to determine if the nanoplaques correlated with disease progression, we needed to include also the most severely affected patients who require regular medication. Apart from a significant,

however, weak inverse correlation with depression depth score we could not detect correlation with other NMS examined (Fig. 17B and C). The very weak inverse correlation with MADRS-S score needs to be replicated in a larger study including subgroup analysis with treatment included as a variable before any conclusion can be made.

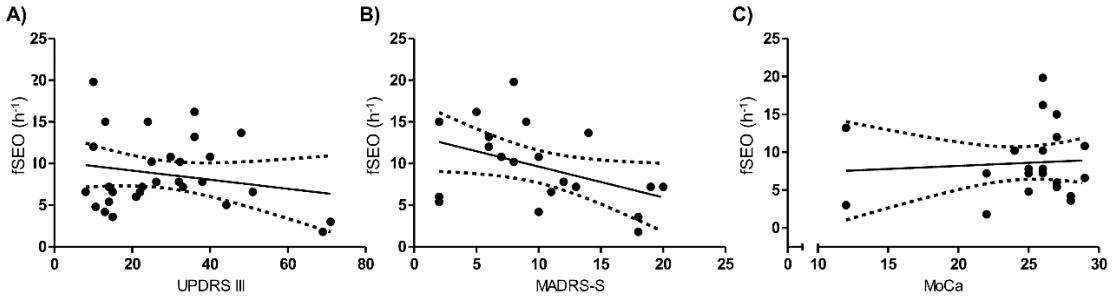

Figure 17. Correlation examination of f_{SEO} with symptoms of PD. A) No correlation between motor symptom score and f_{SEO}. B) A slight inverse correlation (r^2=0,19, p=0,046) between depression depth and f_{SEO} but as evident from the plot only accounts for a small part of the variability within the patient group and needs further validation. C) No correlation between cognitive decline determined by MoCa score and f_{SEO}. All data set are evaluated by linear regression.

In summary, the study concludes an increased number detected nanoplaques in PD compared to controls but the correlation to clinical measures remains to be determined. As is evident from the introduction of the Thesis, there are a number of pathological mechanisms contributing to PD development. Most likely, different mechanisms have various impact on disease progress in each individual. Also in the small cohort in Paper IV, there is a large inter-individual variation in the f_{SEO} of nanoaggregates. Potentially, increased nanoaggregates reflect the subgroup of PD patients with major dysfunction in protein aggregation and/or protein degradation system. To increase diagnostic precision, multiple biomarkers reflecting different pathological events might be needed where the nanoamyloids possibly can be one of them. To validate this, the method needs to be applied to an increased sample size and long-term follow up in patients to identify if there is a subgroup where this biomarker can represent clinical progression.

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5 CONCLUSIONS AND OUTLOOK

Moving from molecular understanding to explanations of human physiology is complicated. This Thesis investigates, in a translational attempt, a number of molecular interaction between proteins linked to PD to elucidate physiological function, sign of disease, and possible pharmacological interventions. We report functional connectivity between GPR37 with a proposed ligand, PSAP, the lipid GM1 and the receptor D2R and try to elucidate functional outcomes of each interaction. The heterodimerization between GPR37 and D2R is affected by pramipexole and is therefore of direct clinical relevance. GPR37 partitions with endogenous GM1-enriched lipid rafts, common for GPCRs. In addition, exogenous GM1 elicit cytoprotection via a GPR37-dependent mechanism. Finally, we describe increased detection of nanoamyloids in serum from PD patients, a potential molecular sign of disease. This paper reflects how methods, usually only applied in preclinical research, has potential improve clinical research investigations.

To achieve translationally relevant results, the molecular conclusions of this Thesis needs to be extended from the simple systems to more complex. With the limitations that arise with cell lines as model systems future studies can hopefully use the results as one step towards clinical application. For the interaction studies, future experiments should include validation in primary systems and elucidate the physiological outcome of GPR37-D2R interaction *in vivo*. Since heterodimerization is reported to change with pramipexole treatment, both the physiological outcome as well as the molecular details of the interaction need to be explained further. Long term, this can potentially help in designing more specific dopamine agonists with less side-effects, a daily clinical issue. Furthermore, it should be deciphered if GM1's role on GPR37 function is via direct interaction or changes in membrane composition. However, more important is to confirm the cytoprotective mechanism *in vivo* since the translation of GM1 from experimental treatment into clinical standard care has been hampered. Future studies regarding the nanoamyloids in PD patients would benefit from molecular characterization of the nanoplaques and hence, should move in the opposite direction from complex to simple. Characterization of the proteins in the nanoplaques is needed to identify the biological signal which might give us a hint of the cellular pathological events. Translational medicine is usually described as "bench to bedside". Maybe we should try to transfer bidirectionally.

6 SUMMARY OF PAPERS

This Thesis is based on the four papers listed below. The papers are related to molecular interactions in the dopaminergic system and specifically related to Parkinson's disease. Paper I-III include biomolecular studies to elucidate the function of GPR37. In Paper I and II, GPR37 is studied using FCS and FCCS in live cells. FCS is then applied in Paper IV to investigate nanoamyloids in serum from PD patients. The author has been main responsible for Paper II and III including study design, preparation and execution of experiments, analyzing data and writing the papers. Modeling of GPR37 in Paper III was done by M. Saarinen who also optimized the HTRF assay. In Paper I, the author did revision experiments regarding free PSAP levels after immunoabsorption, analyzed the data and edited the manuscript thereafter. The other results including the FCS measurements were done by the first author, E. Gregorsson Lundius. Regarding Paper IV, the main responsibility is shared. The author planned and executed the clinical evaluations, analyzed the data for correlations and wrote the paper. The FCS experiments and analysis were done by A. Tiiman.

Paper I: GPR37 Protein Trafficking to the Plasma Membrane Regulated by Prosaposin and GM1 Gangliosides Promotes Cell Viability.

This paper investigates factors affecting GPR37 trafficking which in turn affects N2a cell viability. Using FCCS, GPR37 is shown to form a complex with both PSAP and GM1. PSAP immunoabsorption reduced GPR37 localization at the plasma membrane, reduced colocalization with GM1, and decreased cell viability, both in WT and GPR37-overexpressing cells.

Paper II: GPR37 and GPR37L1 Differently Interact With Dopamine 2 Receptors in Live Cells

This paper studies the interaction between GPR37 and splice variants of D2R and compare the results with GPR37L1, using FCCS. At baseline conditions, GPR37 and D2RL show higher complex-formation than GPR37 and D2RS. The level of complex formation can be increased by treating cells with a dopamine agonist or a chemical chaperone increasing GPR37 expression at the plasma membrane. GPR37L1, on the other hand, show higher complex formation with D2RS than D2RL. The level of complex formation is very low, cannot be increased with treatments as above and is hence difficult to distinguish with certainty from background noise.

Paper III: GM1 is Cytoprotective in GPR37-expressing Cells and Downregulates Signaling

This paper investigates the functional effects of the interaction between GM1 and GPR37 detected in Paper I. GPR37-overexpressing N2a cells express decreased levels of GM1 and are specifically rescued with exogenous GM1 in a toxin-induced cellular model. GM1 treatment directly inhibit GPR37-induced decreased cAMP levels in HEK cells, suggesting a hypothesis of the mechanism.

Paper IV: Increased Amyloid Nanoplaques in Serum from Patients with Parkinson's Disease.

This paper evaluates ThT-positive nanoamyloids in serum from patients with PD and controls as a biomarker for PD. The nanoaggregates are investigated both as a marker for disease as well as in correlation with clinical parameters and symptoms to measure disease progression. We detect increased number of nanoaggregates in PD patients compared to controls and the size of the aggregates are partly different, both from controls and AD patients. There is presently no correlation with demographic data, progression or motor symptoms. The weak inverse correlation between depression symptoms and number of nanoaggregates reported needs to be replicated in a larger cohort.

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