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Neuron-to-neuron propagation of neurodegenerative proteins; relation to degradative systems

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Every year is getting shorter, never seem to find the time
Plans that either come to naught or half a page of scribbled lines
Hanging on in quiet desperation is the research way
The time is gone, the thesis over, thought I'd something more to say
mod. from Time, Pink Floyd

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Abstract

Alzheimer's disease (AD) and Parkinson's disease (PD) are defined by neurodegeneration and accumulations of misfolded proteins that spread through the brain in a well characterized manner. In AD these accumulations consist mainly of β -amyloid ($A\beta$) and tau, while in PD, α -synuclein (α -syn) make up the characteristic lewy pathology.

The general aim of this thesis was to investigate mechanisms associated with neurotoxic peptide activity by $A\beta$, tau and α -syn in relation to cellular degradation and transfer with a cell-to-cell transfer model system.

We found that intercellular transfer of oligomeric $A\beta$ occurs independently of isoform. However, the amount of transfer correlates with each isoforms ability to resist degradation or cellular clearance. The $A\beta$ 1-42 isoform showed particular resistance to clearance, which resulted in higher levels of cell-to-cell transfer of the isoform and lysosomal stress caused by accumulation.

As $A\beta$ accumulations can inhibit the proteasomal degradation we investigated how reduced proteasomal degradation affected neuron-like cells. We found increased levels of phosphorylated tau protein, disturbed microtubule stability and impaired neuritic transport after reduced proteasomal activity. These changes was partly linked to c-Jun and ERK 1/2 kinase activity.

We could also show that α -syn transferred from cell-to-cell in our model system, with a higher degree of transfer for the larger oligomer and fibrillar species. Similar to $A\beta$, α -syn mainly colocalized with lysosomes, before and after transfer.

Lastly, we have developed our cell-to-cell transfer system into a model suitable for high throughput screening (HTS). The type of cells have been upgraded from SH-SY5Y cells to induced pluripotent stem cells (iPSCs), with a differentiation profile more similar to mature neurons. The next step will be screening a small molecular library for substances with inhibitory effect on cell-to-cell transfer of $A\beta$ peptides.

The importance of the degradative systems in maintaining protein homeostasis and prevent toxic accumulations in general is well known. Our findings shows the importance of these systems for neurodegenerative diseases and also highlight the link between degradation and cell-to-cell transfer. To restore or enhance the degradative systems would be an interesting avenue to treat neurodegenerative diseases. Another way would be to inhibit the transfer of misfolded protein aggregates. By using the HTS model we developed, a candidate substance with good inhibitory effect on transfer can hopefully be found.

Populärvetenskaplig sammanfattning

Alzheimers sjukdom och Parkinsons sjukdom är två vanligt förekommande sjukdomar som karakteriseras av nervcellsöd i hjärnan samt inlagring av felveckade proteinansamlingar. Dessa proteinansamlingar består främst av proteinen beta-amyloid och tau i Alzheimers, och alfa-synuclein i Parkinsons sjukdom.

Proteinansamlingarna uppkommer initialt i vissa områden i hjärnan, varefter dessa sprider sig till angränsande områden då sjukdomen fortskrider. Ett av målen med den här avhandlingen har varit att undersöka denna spridningsmekanism av beta-amyloid och alfa-synuclein mellan donator- och mottagarceller, som ska simulera interaktionerna mellan hjärnans nervceller.

När vi undersökte varför vissa strukturella varianter av beta-amyloid hade en högre grad av överföring upptäckte vi att det berodde på cellernas förmåga att bryta ner de olika varianterna. De varianter som var mer motståndskraftiga mot nedbrytning överfördes även i större utsträckning. Den cellulära nedbrytningen kan ske via det lysosomala eller proteasomala systemet. Dessa system försämras gradvis vid hög ålder och påverkas negativt av proteinansamlingar av framförallt beta-amyloid. Vi såg att det lysosomala systemet blev mer negativt påverkat av varianten beta-amyloid1-42.

Ansamlingar av β -amyloid i cellen försämrar det proteasomala systemet, därför tittade vi på vilka konsekvenser detta medför i vår cellmodell. Vi såg t.ex. att transporten av material längs cellens utskott försämrades och en ökad nivå av fosforylerat tau ansamlades. Dessa skadliga förändringar kunde till viss del spåras till en ökad aktivitet hos framförallt två proteinkinas.

Därefter frågade vi oss om olika ansamlingar av alfa-synuclein överförs mellan cellerna i vårt modellsystem. Det visade sig att större former av ansamlingar, oligomerer och fibriller, överfördes i större utsträckning än de mindre monomera formerna. Vi kunde även lokalisera majoriteten av detta alfa-synuclein till det lysosomala nedbrytningssystemet inom cellerna, i likhet med beta-amyloid.

Om det går att stoppa överföringen av skadliga proteinansamlingar mellan nervcellerna, är det förmodligen möjligt att bromsa upp sjukdomsutvecklingen av t.ex. Alzheimers sjukdom. Genom att utveckla vårt cellulära modellsystem och anpassa det till ett högkapacitets-screening format, har vi möjliggjort för att testa tiotusentals substanser för att hitta molekyler vilka kan bromsa eller stoppa den cellulära överföringen av beta-amyloid-ansamlingar.

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List of original publications

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- I. **Jakob Domert**, Sahana Bhima Rao, Lotta Agholme, Ann-Christine Brorsson, Jan Marcusson, Martin Hallbeck, Sangeeta Nath. Spreading of amyloid- β peptides via neuritic cell-to-cell transfer is dependent on insufficient cellular clearance. *Neurobiology of Disease*, 2014, 65, 82-92.
- II. Lotta Agholme, Sangeeta Nath, **Jakob Domert**, Jan Marcusson, Katarina Kågedal, Martin Hallbeck. Proteasome inhibition induces stress kinase dependent transport deficits – implications for Alzheimer’s disease. *Molecular and Cellular Neuroscience*, 2014, 58, 29-39.
- III. **Jakob Domert**, Chris Sackmann, Emelie Severinsson, Lotta Agholme, Joakim Bergström, Martin Ingelsson, Martin Hallbeck. Aggregated alpha-synuclein transfer efficiently between cultured human neuron-like cells and localize to lysosomes. Published in *PLoS ONE*, 2016.
- IV. **Jakob Domert**, Emelie Severinsson, Magdalena Otrocka, Juan F. Reyes, Livia Civitelli, Ann-Christine Brorsson, Martin Hallbeck. A new method to screen for inhibitors of cell-to-cell transfer of oligomeric beta amyloid. Manuscript.

Abbreviations

A β	β -amyloid
APP	Amyloid precursor protein
oA β	Oligomeric β -amyloid
AD	Alzheimer's disease
AICD	APP intracellular domain
APOE4	Apolipoprotein E4
CLSM	Confocal laser scanning microscopy
CNS	Central nervous system
CTF α/β	Carboxy-terminal fragment α/β
DMSO	Dimethyl sulphoxide
ECM gel	Extracellular matrix gel
EGFP	Enhanced green fluorescent protein
ENS	Enteric nervous system
ERK 1/2	Extracellular signal-regulated protein kinase 1/2
FAD	Familial Alzheimer's disease
GSK3 β	Glycogen synthase kinase 3 β
HCS	High content screening
HFIP	Hexafluoroisopropanol
HNE	4-hydroxy-2-nonenal
Hsc70	heat shock cognate protein 70
HSPGs	Heparan sulphate proteoglycans
HTS	High throughput screening
iPSCs	Induced pluripotent stem cells
LAG3	Lymphocyte-activation gene 3
LAMP	Lysosomal associated membrane protein
LRRK2	Leucine-rich repeat kinase 2
Lt-NES	Long-term self-renewing neuroepithelial-like stem cells
MEK	MAPK/ERK kinase
MSD	Mesoscale discovery
MTT	Methyl-thiazolyl-tetrazolium
MVBs	Multivesicular bodies
NFTs	Neurofibrillary tangles

NMDA	N-methyl-D-aspartate receptor
NO	Nitric oxide
PD	Parkinson's disease
PHFs	Paired helical filaments
RFP	Red fluorescent protein
SEC	Size exclusion chromatography
SNARE	N-ethylmaleimide-sensitive factor attachment protein receptor
SNCA	Synuclein alpha
α -syn	α -synuclein
TEM	Transmission electron microscopy
TMR	Tetramethylrhodamine
TNT	Tunneling nanotubes

Introduction

Neurodegenerative diseases

Improved living conditions and modern medicine are increasing the human lifespan around the world, however, advanced age unfortunately also correlates with higher risk of developing a neurodegenerative disease. These diseases are characterized by a progressive loss of neurons and cognitive function.

Alzheimer's disease

According to Alzheimer's disease international, 45 million people were suffering from some form of dementia in 2015 with an annual cost of \$818 billion for treatment and care. This number is estimated to increase to \$2 trillion by 2030, which would be a significant economic burden for our societies. Their prognosis predict that the number of people affected will increase to 131.5 million by 2050 (Prince et al. 2015) if no treatment breakthroughs are made.

Alzheimer's disease (AD) is the most common type of dementia and is estimated to account for 50-60 % of all reported cases (Blennow, de Leon, and Zetterberg 2006).

Alzheimer's disease was first described in 1907 by Alois Alzheimer after a long-term case study of his patient Auguste D, who was admitted due to severe cognitive dysfunctions. When he examined the brain histology after her death Alzheimer found the notorious plaques and tangles, which became known as the classical hallmarks of AD (Alzheimer 1907).

The symptoms of AD emerge progressively during the cognitive decline, usually dysfunction in the formation of new memories early on, then followed by impairment of language, semantic knowledge, executive functions and working memory (Weintraub, Wicklund, and Salmon 2012). Apathy and depression are very common neuropsychological symptoms early on, while delusions and hallucinations may develop later in the disease (Lyketsos et al. 2011). As cognition gradually deteriorates, managing everyday tasks becomes increasingly difficult until full-time care is required. Living with AD is agonizing for the patient, but is also a tragedy for their family who is, in later stages, treated as complete strangers, due to the progressive memory loss.

Cholinesterase inhibitors and NMDA-receptor antagonists are used to relieve some of the cognitive symptoms in AD, important to note is that these substances have no effect on slowing the pathological progression (Raina et al. 2008).

Pathology

The advanced neuropathology of AD has been shown to include macroscopic brain atrophy, due to loss of neuritic processes, synapses and neurons. There is also neurovascular dysfunction, an inflammatory component, mitochondrial dysfunction (Luth et al. 2014, Reddy 2007, Serrano-Pozo et al. 2011), oxidative stress (Bonda et al. 2010), and the widespread distributions of plaques, neurofibrillary tangles (NFT) and neuropil threads. These pathological changes are gradual, and it is believed the initial pathology start to develop many years or even decades before any clinical symptoms are apparent.

Due to the abundance of plaques and NFTs observed in AD brains, these structures and their composing proteins has been studied extensively. The protein aggregates constituting the plaques and NFTs were both isolated in the mid 80's, and identified as β -amyloid ($A\beta$), and tau respectively (Glenner and Wong 1984, Grundke-Iqbal et al. 1986). The cognitive decline has been shown to correlate better with NFTs than plaques (Braak and Braak 1991), although intracellular soluble $A\beta$ toxicity plays an important role in driving the disease progression, and precedes the formation of NFTs (Gouras et al. 2010, Hardy and Selkoe 2002, LaFerla, Green, and Oddo 2007).

Etiology

The familial type of AD (FAD) makes up only a few percent of the total cases, it is caused by autosomal dominant inherited mutations in the amyloid precursor protein (APP) gene or in genes coding for enzymes that process APP. The APP gene is located on chromosome 21, and duplications of this gene also causes AD, which is why people with Down syndrome develop the disease. These genetic deviations are responsible for inducing FAD, which often has an early age of onset (30-40 years) compared with the sporadic type (mostly >65 years) (Goate 2006). Even though the development of FAD deviates from the sporadic type, the histology is similar and

much has been learned of the pathological mechanisms regarding APP processing that are present in both types.

The sporadic type accounts for approximately 98-99% of all AD cases and predominantly affects people over 65 years old. In contrast to FAD, the etiology of sporadic AD still remains unclear, but seem to depend on a combination of genetics and environmental risk factors. The most significant risk factor is aging, as the prevalence increases exponentially after 65 years. Environmental risk factors involve an earlier history of cerebrovascular disease, hypertension, type II diabetes, obesity, severe head trauma, smoking and pesticides (Campdelacreu 2014). Carrying the apolipoprotein E4 (APOE4) allele is the biggest genetic risk factor of developing sporadic AD, APOE4 is involved in lipid metabolism and is speculated to be involved in A β clearance. However, there are additional genetic risk factors, and more are emerging, creating a complex picture of the etiology of AD (Mayeux and Stern 2012, Tanzi 2012).

APP processing and generation of β -amyloid

APP is a transmembrane glycoprotein expressed as multiple isoforms throughout the body, where the APP695 variant is the predominant form in neuronal tissue (Ling, Morgan, and Kalsheker 2003). The normal physiological function of APP has been suggested to be a cell surface receptor, reported to bind multiple ligands, that affects the APP processing and downstream signaling. It also has extracellular adhesion properties, interacting with extracellular matrix proteins (Breen, Bruce, and Anderton 1991). APP is essential for development of proper motor neurons and neuronal migration in mice (Herms et al. 2004). Also, the evolutionary conserved intracellular domain of APP has been proposed to bind several kinases, adaptor proteins and affect transcription (Muller and Zheng 2012).

APP can be processed by the non-amyloidogenic or the amyloidogenic pathway (Fig 1). In the non-amyloidogenic pathway, APP is cleaved by α -secretase within the A β -region in the long extracellular domain, creating the sAPP α and carboxy-terminal fragment (CTF α), which are subsequently cleaved by γ -secretase into P3 and APP intracellular domain (AICD) respectively. The sAPP α fragment is neuroprotective, it promotes neurite outgrowth, regulate proliferation of neural stem cells and has

positive cognitive effects in animal models (reviewed in (LaFerla, Green, and Oddo 2007)).

The amyloidogenic pathway is initiated by β -secretase cleavage of APP into sAPP β and CTF β , followed by processing of CTF β by γ -secretase, creating AICD and A β . Several different A β isoforms can be generated by γ -secretase as it recognizes multiple cleavage sites, creating A β fragments ranging between 17-42 amino acids in length. The A β 1-40 variant is most frequently produced, followed by A β 1-42. However, the A β 1-42 isoform is the predominant variant found in A β -plaques, likely due to its notorious ability to misfold and aggregate. (Marcusson et al. 2011, Muller and Zheng 2012).

Figure 1: A schematic of APP processing illustrating the two pathways. In the non-amyloidogenic pathway, APP is cleaved first by α -secretase into sAPP α and CTF α , followed by γ -secretase processing which creates the P3 and AICD fragments. The amyloidogenic pathways generates sAPP β and CTF β , through APP processing by β -secretase, with the subsequent cleavage of γ -secretase which produce A β fragments of various length and AICD.

APP is mainly localized to the plasma membrane, however, it can also be found in the membranes of trans-Golgi network, endoplasmic reticulum, endosomes, lysosomes and mitochondria. Processing of APP at these sites would give rise to both extracellular and intracellular A β (Fig 2). A β can also accumulate intracellularly from extracellular uptake, as it has been shown to bind various receptors with subsequent internalization (Kanekiyo et al. 2011, LaFerla, Green, and Oddo 2007).

Figure 2: APP processing can occur at the plasma membrane or at multiple intracellular locations, including endosomes, lysosomes, autophagosomes, golgi and the ER.

β-amyloid

The normal physiological function of Aβ is not yet fully understood, in picomolar concentrations, it has been shown to promote synaptic plasticity and long-term potentiation in hippocampus (Puzzo et al. 2008, Morley et al. 2010). However, in AD,

there is an imbalanced A β metabolism, resulting in pathological levels which has a negative impact on plasticity and long-term potentiation (Haass and Selkoe 2007).

Accumulation of intracellular A β has been shown to affect multiple cellular functions such as early synaptic dysfunction (Billings et al. 2005), proteasomal inhibition, impaired mitochondria, increased reactive oxygen species, Ca²⁺ dyshomeostasis and tau hyperphosphorylation (reviewed in (LaFerla, Green, and Oddo 2007))

Aggregation of β -amyloid

A β peptides are hydrophobic peptide fragments, generated from APP with a propensity for aggregation. A β can exist as α -helical or β -sheet rich secondary structure protein conformations. The β -sheet conformation is the more hydrophobic and aggregation prone form and it is believed to initiate the assembly of dimers, trimers and small oligomers. These species can then form larger oligomers, protofibrils and large fibrils that can then aggregate to plaques (Fig 3). The mechanisms of A β aggregation is poorly understood, partly because it is dependent on multiple variables and that the created species can be transient. *In vitro* experiments have provided some insight that concentration and pH are important factors. Interestingly, *in vitro* aggregation usually require micromolar concentrations, while physiological concentrations, even elevated in AD, is in the nanomolar range. Also, the rate of aggregation is optimal between pH 5-6, coincidentally the same pH range promote the β -sheet conformation of A β . The aggregation mechanisms *in vivo*, is further complicated by a multitude of possible A β posttranslational modifications and interactions with cellular components (reviewed in (Finder and Glockshuber 2007)).

Figure 3: Misfolding of native monomeric A β into a β -sheet rich structure can result in aggregation into oligomers, protofibrils, fibrils and plaques. These structures are not static but interchangeable.

There is increased levels of soluble oA β in brain tissue from AD patients (Kuo et al. 1996, McLean et al. 1999), A β -depositions precedes the expected onset of symptoms in FAD by 15 years (Bateman et al. 2012) while the formation of oA β is probably earlier.

Tau

Tau belongs to the microtubule-associating protein family and normally modulates microtubule stability by binding or dissociation, mainly in axons. There are six tau

isoforms generated from alternative splicing in the adult human brain (Goedert et al. 1989).

Tau binding affinity to the microtubule is affected by phosphorylation, and during pathological conditions, tau can be heavily phosphorylated resulting in the dissociation from, and destabilization of microtubules. Early events in AD pathology has been shown to include obstructions of axons and impairments of axonal transport caused by hyperphosphorylated tau (Stokin et al. 2005), partly due to interference with kinesin motor protein complex functions (Ittner, Ke, and Gotz 2009).

Hyperphosphorylation of tau also increases its propensity to self-assemble into larger aggregates, resulting in accumulation of tau assemblies in the somatodendritic compartment and formation of paired helical filaments (PHFs). All six isoforms of tau can be found in the PHFs making out the NFTs in AD (Iqbal et al. 2009). Hyperphosphorylation of tau is due to a dysregulated balance between kinases and phosphatases, resulting in an increase in net phosphorylation. Tau has been shown to have at least 45 possible phosphorylation sites, and multiple kinases with the ability to phosphorylate it (Hanger et al. 2007), glycogen synthase kinase (GSK) 3 β is one of the important kinases in AD.

Linking A β and tau pathology in AD

There seems to be a general consensus that A β lies upstream from tau pathology, however, tau seems to play an important role in mediating the A β toxic mechanisms in AD.

A β has been shown to induce hyperphosphorylated tau and initiate microtubule destabilization (King et al. 2006, De Felice et al. 2008). The kinase GSK3 β has been reported in multiple studies to be responsible for tau phosphorylation following extracellular exposure to various A β species, probably through the N-methyl-D-aspartate (NMDA) receptor pathway (reviewed in (Stancu et al. 2014)).

Interestingly, neurons lacking tau or mice with reduced tau expression, has been shown to remove and reduce the cytotoxic effects by A β , proving the importance of tau in AD pathology (Rapoport et al. 2002, Roberson et al. 2007). Extracellular oligomeric A β (oA β) has been shown to induce excitotoxicity by over-activation of the NMDA-receptor pathway, resulting in neuronal damage through Ca²⁺

dysregulation and generation of nitric oxide (NO) at toxic levels (Ittner et al. 2010). Increased NO levels can mediate downstream misfolding of proteins, aggregation and fragmentation of mitochondria. Interestingly, this damaging pathway is further enhanced by tau, which is responsible for guiding the kinase Fyn to dendrites. Fyn then phosphorylates the NMDA receptor subunit NR2B, with the subsequent complex formation with post-synaptic density protein 95 resulting in increased NMDA receptor activation (reviewed in (Gotz et al. 2011)).

Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease and is named after James Parkinson who described the disease as shaking palsy in 1817. PD often manifests between the ages 50 and 70 with a following gradual deterioration that can last 10 to 20 years. Early symptoms include hyposmia and autonomic dysfunctions such as gastrointestinal problems, followed by the characteristic symptoms from impaired motor functions with resting tremors, bradykinesia and rigidity. In the later stages of PD there is a gradual cognitive impairment and dementia (Reid et al. 2011). As with the $\text{oA}\beta$ peptides in AD, it is the oligomeric α -synuclein (α -syn) in PD that is suspected to be the most neurotoxic species and driver of the disease (Winner et al. 2011). The motor symptoms in PD are treated with dopamine receptor agonists and carbidopa/levodopa, MAO-B inhibitors are also used to reduce the breakdown of dopamine. Cholinesterase inhibitors have shown a beneficial effect on the dementia symptoms of PD (Rascol et al. 2011).

Pathology

The PD pathology is characterized by widespread neurodegeneration and neuronal inclusions in cell bodies and their extensions termed Lewy bodies and Lewy neurites respectively (Braak, Del Tredici, Rub, et al. 2003), named after Fritz Lewy who first described them in 1912. In 1997 a familial PD mutation was traced to the synuclein alpha (SNCA) gene encoding the protein α -syn, and the same year α -syn was shown to be the main component of Lewy pathology (Spillantini et al. 1997, Polymeropoulos et al. 1997). The characteristic motordysfunction associated with PD is caused by the loss of neuromelanin-containing dopaminergic neurons in the substantia nigra pars compacta of the midbrain. Lewy pathology is also a component of other

neurodegenerative diseases, including dementia with Lewy bodies, multiple system atrophy and AD with Lewy pathology.

Etiology

The majority of PD cases are sporadic with unknown cause, while less than 10 % have a family history (Thomas and Beal 2007). The disease is multifactorial and the genetic component of PD is complicated, with 28 known loci associated with the disease.

Mutations in six genes with the ability to cause monogenic PD has been identified, of these the SNCA and leucine-rich repeat kinase 2 (LRRK2) genes cause PD in an autosomal dominant way, while Parkin, PINK1, DJ-1 and ATP13A2 are responsible for the disease in an autosomal recessive manner (Klein and Westenberger 2012).

Similar to AD, advanced age is the biggest risk factor for developing PD, with nearly 2% of the population over 80 affected (Pringsheim et al. 2014). Other environmental risk factors include head trauma, depression and pesticide exposure, interestingly tobacco smoking has a protective effect (Noyce et al. 2012).

α -synuclein

The SNCA gene encodes the 140 amino acid α -syn protein, which is highly expressed in the nervous system and can be found in most subcellular compartments, however, it primarily localizes to presynaptic terminals (Kahle et al. 2000). The protein has amphipathic properties and its secondary structure is normally α -helical (Davidson et al. 1998). The N-terminal region is associated with membrane binding, and all familial SNCA mutations are affecting this region. The C-terminal region have phosphorylation sites, which suggest a regulatory role, (Sato, Kato, and Arawaka 2013) and seem to interact with synaptobrevin of the N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, where α -syn is promoting SNARE complex assembly formation (Burre et al. 2010).

Expression of α -syn is taking place during the later stages in the neuronal maturation, and the protein is not required for synapse formation, but seem to be important for maintaining a sizable pool of synaptic vesicles (Murphy et al. 2000). There are multiple modes of α -syn toxicity proposed, as increased copynumber of the SNCA gene can cause PD (Singleton et al. 2003), suggesting a pathological role of increased expression of the α -syn protein. Interestingly, overexpression of α -syn was

seen to inhibit synaptic vesicle reclustering and transmitter release in primary hippocampal neurons (Nemani et al. 2010). α -Syn can also increase the uptake of polyunsaturated fatty acids in the cell which in turn can promote self-aggregation of α -syn (Assayag et al. 2007, Sharon et al. 2003). Accumulated α -syn in the mitochondrial membrane reduced mitochondrial function and increased production of reactive oxygen species (Devi et al. 2008). The familial mutations of SNCA either increase its ability to aggregate (Conway et al. 2000), or reduces binding to lipid rafts which impairs the synaptic localization (Fortin et al. 2004).

Aggregation of α -synuclein

Similar to the A β peptide, the native unstructured form of α -syn can misfold into β -sheet rich conformations, which makes it prone to aggregation. The aggregation-process is characterized by multiple transient forms of oligomers and fibrils that eventually result in the accumulations of Lewy pathology (Celej et al. 2012). The aggregation can be divided into a primary and secondary nucleation phase (Fig 4). In the primary phase, misfolded monomers forms oligomers which forms prefibrillar nuclei which form larger fibrils. During the second phase fibrils can create new fibrils through fragmentation and catalyze the formation of new oligomers at hydrophobic surfaces of the nuclei (Ghosh et al. 2016).

Exposure to certain pesticides has been linked to the development of PD, coincidentally, several pesticides, such as diethyldithiocarbamate, paraquat and rotenone promote aggregation of α -syn *in vitro* (Uversky, Li, and Fink 2001). The familial SNCA mutations A30P and A53T also accelerate aggregation of α -syn *in vitro* (Narhi et al. 1999).

Figure 4: Pathways of aggregation for α -syn.

Protein degradation pathways

There are two primary systems for cellular protein degradation, the ubiquitin-proteasome and the autophagy-lysosomal pathways. Besides recycling damaged cellular components, these machineries affect a wide range of vital cellular functions, such as cell-cycle control, transcription, differentiation, controlled cell death etc. In regard to neurodegenerative diseases, the degradative systems are crucial in preventing the accumulation and aggregation of misfolded proteins.

Ubiquitin-Proteasome degradation pathway

Proteins that are misfolded, damaged or otherwise exposing their degradation signals are inviting attachment of ubiquitin protein chains. This process is initiated by a group of ubiquitin activating enzymes (E1) by binding of an ubiquitin protein and followed by its ATP dependent activation. The activated ubiquitin protein is then transferred to an ubiquitin ligase complex (E2 and E3), there are several hundred E2-E3 complexes in the mammalian cell that recognize different degradation signals,

such as oxidations or hydrophobic patches. The E2-E3 complex bind their recognized target protein and transfer the activated ubiquitin protein to an amine-group on a lysine side chain. The E1 then continues to activate and transfer additional ubiquitins to the E2-E3 complex which in turn add these in a polyubiquitin-chain on the target protein that is recognized by the proteasome for degradation.

The proteasome is a multiprotein degradation complex that constitute nearly 1% of the entire cells protein content (Fig 5). It has a barrel shaped catalytic center unit (the 20S core) which is flanked by two cap units (19S) on either side. The barrel unit is hollow and is made up of multiple proteases with their catalytic sites facing the inside of the barrel. The caps recognize and bind proteins that has been polyubiquitinated, followed by an ATP driven unfolding. The caps will then feed unfolded proteins inside the proteolytic core unit, resulting in protein degradation into short peptide fragments that exits the proteasome and are hydrolyzed by cytosolic proteases (Alberts et al. 2008a). The proteasome plays an important role in managing the levels of short lived proteins such as cyclins, messenger proteins or transcription factors regulating the cell cycle or other dynamic cellular processes.

Due to its availability and efficiency, the ubiquitin-proteasome degradation pathway is an important system in preventing misfolded or damaged proteins from remaining and aggregate. However, if insoluble protein aggregates are allowed to form, the proteasome is ill equipped degrading these because it requires its substrates to be able to unfold for proteolysis (Verhoef et al. 2002).

Autophagy-lysosome degradation pathways

The lysosome is an acidic (pH ~5) membrane enclosed organelle filled with hydrolytic enzymes capable of digesting all manner of macromolecules. They contain about 50-60 different types of hydrolytic enzymes with the ability to digest proteins, lipids and polysaccharides, recycling their basic components. The lysosome is the final destination for several different pathways of autophagy, where the best characterized are macro-, micro- and chaperone-mediated autophagy (Fig 5) (reviewed in (Knecht et al. 2009)).

Macroautophagy is a highly conserved mechanism in eukaryotic cells and is regulated by nutritional stress, to provide recycled material for the cellular needs during starvation. It is initiated by a series of phosphorylation events resulting in the

membrane formation of the autophagosome, where a portion of the cytoplasm is internalized within a double layered membrane vacuole. The autophagosome then docks and fuses with lysosomes, exposing the cytoplasmic contents to the hydrolytic enzymes enabling their degradation. Macroautophagy facilitates the degradation and recycling of large macromolecules and organelles such as mitochondria, the process seems to be able to recognize and favor the recycling of damaged organelles.

Microautophagy is the uptake through lysosomal membrane invaginations of cytosolic components directly by lysosomes, including macromolecules such as organelles. Microautophagy is believed to be important for regular turnover of long-lived proteins under normal conditions.

Chaperone-mediated autophagy is a form of degradation that is dependent on the chaperone heat shock cognate protein 70 (hsc70) which recognize and guide cytosolic proteins, exposing a certain peptide sequence, to the lysosomal membrane. At the membrane hsc70 binds to lysosomal associated membrane protein (LAMP) 2A, which facilitates the internalization of the targeted protein into the lysosomal lumen, resulting in its degradation.

The autophagy-pathways are important modulators of multiple systems through the turnover of cellular components, affected mechanisms include; differentiation, proliferation and programmed cell death (Martinez-Vicente, Sovak, and Cuervo 2005).

Figure 5: Showing the main intracellular degradation pathways.

Degradation in aging and neurodegeneration

During aging, the challenge from environmental and genetic factors gradually impair the cells ability to maintain protein homeostasis. Synthesis of new proteins is altered and degradation pathways become less effective or dysfunctional (Ward 2000,

Toyama and Hetzer 2013). The proteasomal system becomes less effective with age, and even worse in AD patients. The proteasomal degradative function was shown to be reduced in hippocampus by almost 50% in brains from AD patients compared with age matched controls (Keller, Hanni, and Markesbery 2000). The ubiquitin-proteasome system is also implicated in other neurodegenerative diseases such as PD and Huntingtons disease (Ross and Pickart 2004). In PD, a missense mutation in the parkin gene, encoding an E3 ubiquitin ligase, cause PD in an autosomal recessively manner (Rubinsztein 2006).

Macroautophagy is reduced due to decreased autophagosome formation and impaired fusion of autophagosomes with lysosomes. There is also decreased LAMP2A levels in the lysosomal membrane with age, resulting in a reduction of chaperone-mediated autophagy. Degradation of α -syn has been shown to be facilitated by the proteasome but also by chaperone-mediated autophagy, which seems to be impaired by certain familial SNCA mutations in PD (Cuervo et al. 2004). The lysosomes also undergo age related changes, including increased volume, decreased stability, altered hydrolase activity and intralysosomal accumulations of nondegradable material, reducing turnover of long-lived proteins and damaged organelles (Martinez-Vicente, Sovak, and Cuervo 2005). There is also a connection between the lysosomal storage disorder Gaucher disease and PD, where the lysosomal hydrolase β -glucocerebrosidase activity is disturbed (Blanz and Saftig 2016).

Spreading of the pathology

The progression of the pathology in AD and PD is suggested to advance via neuroanatomical connections to additional areas of the brain (Harris et al. 2010, Recasens and Dehay 2014) (Fig 6).

In AD, A β plaques first develop in the basal neocortex then in neighboring areas followed by hippocampus. In later stages of the disease they appear throughout the entire cortex (Braak and Braak 1997). NFTs can be found earliest in locus coeruleus in the pons and in the transentorhinal cortex, followed by lesions in entorhinal cortex and hippocampus. From there the lesions eventually spread through broad areas of the neocortex (Braak et al. 2006). In PD, the lewy pathology can first be observed in the olfactory bulb and dorsal motor nucleus of the glossopharyngeal and vagal

nerves, then ascend through the brainstem eventually reaching the midbrain and affecting substantia nigra followed by lesions in the neocortex (Braak, Del Tredici, Rüb, et al. 2003). There is also Lewy pathology within the enteric nervous system (ENS) early on. The ENS is connected with the central nervous system (CNS) with both afferent and efferent connections and it has been suggested that the ENS might be one starting point where the pathological process is initiated, followed by spreading to the CNS (Lebouvier et al. 2009).

Figure 6: A schematic of the progression of amyloid plaques (yellow) and tau tangles (blue) in AD, and Lewy pathology (purple) in PD. Modified from (Jucker and Walker 2013).

Self-propagation

This propagation of pathology has been proposed to be prion-like, where misfolded aggregate seeds transfer from neuron to neuron, promoting misfolding and aggregation of additional native proteins in the “infected” cells. Due to the lack of disease transmission between individuals, and the slow disease progression, the

prion concept in AD and PD is still somewhat controversial. However, AD and PD share many characteristics with the neurodegenerative disorder Creutzfeldt–Jakob disease, which is caused by the self-propagation of misfolded conformations of the prion protein. Similar to the prion protein, A β and α -syn adopts β -sheet rich conformations and have the ability to form different strains, with varying self-propagating potential (Woerman et al. 2015, Stohr et al. 2014). Numerous studies have also confirmed self-propagation of A β and α -syn pathology in cell models and transgenic mouse models with either synthetic proteins or purified brain homogenates (reviewed here (Walker and Jucker 2015)). This mode of propagation is supported by the findings of α -syn Lewy pathology in transplanted dopaminergic neurons in PD patients (Li et al. 2008).

Mechanisms of transfer

Cells can release and transfer material to each other in several different pathways (Fig 7). Exocytosis through the constitutive secretory pathway is probably the most common form, where budding vesicles containing various cargo from the trans-golgi network relocate to the plasma membrane where they fuse, and resulting in the subsequent extracellular release of its content (Alberts et al. 2008b). The receiving cell can then internalize the extracellular molecules by different modes of endocytosis. One pathway is pinocytosis, where the cell samples the extracellular constituents by small invaginations of the plasma membrane (100nm) that are internalized as pinocytotic vesicles. This process is achieved through clathrin-dependent endocytosis or the formation of caveolae within lipid rafts of the plasma membrane. The forming caveolae vesicles are pinched off by the constricting protein dynamin.

While pinocytosis seems to randomly internalize extracellular material, there is a more specific internalization pathway known as receptor mediated endocytosis. The receptor mediated endocytosis is a means for the cell to internalize more specific targets from the extracellular space. The target molecule binds to a transmembrane receptor on the plasma membrane, which initiates the internalization process with the recruitment of clathrin molecules (Alberts et al. 2008c). Soluble A β has been shown to bind several plasma membrane receptors such as, the α 7 nicotinic acetylcholine receptor, Heparan sulphate proteoglycans (HSPGs) and the low-density

lipoprotein receptor-related protein 1, facilitating the A β internalization (Kanekiyo et al. 2011, Yang et al. 2014). The HSPGs seems to mediate internalization of α -synuclein fibrils as well.

Multivesicular bodies (MVBs) are a subset of endosomes that contain small (30-150 nm) intraluminal vesicles. These vesicles are known as exosomes, and can contain various cargo, such as proteins, mRNAs and lipids. When MVBs fuse with the plasma membrane, exosomes are released extracellularly, allowing them to be taken up by neighboring cells through fusion, a form of intercellular communication. Small and large vesicles (1 μ m) can also be released extracellularly by direct budding from the plasma membrane itself (Colombo, Raposo, and Thery 2014). Both A β and α -syn has been shown to transfer between cells via exosomes (Danzer et al. 2012, Malm, Loppi, and Kanninen 2016).

Figure 7: A schematic of possible pathways involved in cell-to-cell transfer of neurodegenerative proteins.

Another means of cell-to-cell transfer is through tunneling nanotubes (TNTs). These TNTs form direct cytoplasmic contacts between cells through membrane tunnels, containing F-actin, allowing cytoplasmic material to pass between the connected

cells. A recent study has shown TNT transfer of α -syn fibrils in lysosomes, *in vitro*, between neuron-like cells and primary neurons (Abounit et al. 2016).

To summarize, we hypothesize that cellular impairments in protein homeostasis results in the accumulation of misfolded species that can aggregate, and be transported to neighboring cells through various intercellular transfer mechanisms (Fig 8). The misfolded aggregates can then recruit additional proteins from the recipient cell and influence these to misfold as well, acting in a seed like manner.

Figure 8: A schematic summary of pathological spread in AD. Initial A β misfolding that escapes degradation can aggregate and accumulate. Aggregates are transported along axons to synaptic terminals, where the aggregates can utilize several transfer pathways to reach neighboring cells. Later in the pathological process, there is hyperphosphorylation of tau which detaches from the microtubule and aggregates, resulting in impaired axonal transport.

High throughput screening

High throughput screening (HTS) is a method that is based on miniaturized model systems and automation which enables the user to run large numbers of experiments in a short time frame. HTS is usually used to screen large compound libraries of small organic molecules in order to identify molecules with particular biological properties and, depending on the assay, tens or hundreds of thousands of substances can be screened. The assays can be biochemical target based, e.g. a binding study of a certain protein, or cell-based phenotypic, where cell models are investigated, usually for multiple parameters, and these are referred to as high content screening (HCS).

Therapeutic strategies in clinical trials

Successful treatment of AD and PD will probably rely on early diagnosis, so that therapeutic strategies can be administered before too much permanent neuronal damage has occurred. Novel probes with the ability to reveal early tau tangles or A β -aggregates are being developed for positron emission tomography, these probes and the characterization of CSF biomarkers are the main tools currently available for making an early diagnosis (de Souza et al. 2014, Watanabe, Ono, and Saji 2015).

A mutation that reduced β -secretase cleavage by 40% was shown to improve cognition and also protected against developing AD (Jonsson et al. 2012). This proves that the concept of reducing activity of the amyloidogenic pathway can be beneficial to target therapeutically.

Verubecestat is a selective β -secretase inhibitor that significantly reduces A β processing *in vivo*, and seems to be well tolerated by humans in early clinical trials (Kennedy et al. 2016). It remains to be seen if verubecestat will be able to halt the disease progression and improve cognition in AD patients enrolled in a phase 3 clinical trial that is completed in the summer of 2017.

Another therapeutic avenue in AD is passive immunization, where monoclonal antibodies with affinity for A β species and aggregates are administered. In theory, the antibodies would bind and neutralize toxic A β species and promote the removal of aggregates by immune cells. Several different antibodies have failed to show any significant improvement, however Aducanumab, which only bind A β aggregates, have shown reduced cortical A β accumulations and some cognitive benefits in a dose dependent manner. Two phase 3 clinical trials were started in 2015 which are scheduled to continue until 2021.

There is also active immunization therapies underway in phase 2/3 clinical trials that has been well tolerated, targeting A β or modified forms of tau. Their effect on the disease progression remains to be seen in the coming years.

All these therapeutic strategies seem promising, perhaps some can even be combined with greater effect, only the future will tell.

Aims of the thesis

The general aim of this thesis was to investigate mechanisms associated with neurotoxic peptide activity in relation to cellular degradation and transfer in neurodegenerative diseases.

Paper I

The aim of paper I was to investigate the pathological mechanisms of cell-to-cell transfer of different isoforms of A β peptides and their cellular clearance.

Paper II

The aim of paper II was to investigate the effects of reduced proteasomal degradation on tau phosphorylation, microtubule stability and neuritic transport.

Paper III

In paper III we aimed to investigate the transfer and intracellular colocalization of α -syn species with our established co-culture system.

Paper IV

The aim in paper IV was to develop a co-culture model capable to study and quantify cell-to-cell transfer of oA β in a high throughput format.

Methods

Many different methods were employed to investigate the aims of this thesis, in this section the key methods are summarized and briefly explained. For further details please refer to the method sections of each paper.

Cell models and culturing

SH-SY5Y

The SH-SY5Y cell line was used in all four papers and originates from the cell line SK-N-SH. The SK-N-SH cell line was established from a neuroblastoma metastasis taken from a bone marrow biopsy from a 4 year old girl in the 1970s. The SK-N-SH has since been subcloned three times to finally generate the SH-SY5Y cell line, which express better neuronal characteristics. The disadvantage with this cell line is that it has tumor origin, but the advantages are that the cells are human and have an inherent potential for differentiation into neuron like cells. This group previously developed a differentiation protocol where the SH-SY5Y cells were pre-treated with retinoic acid for 7 days, followed by 10 days of differentiation suspended in extracellular matrix (ECM) gel with the addition of brain derived neurotrophic factor, neuregulin β 1, nerve growth factor and vitamin D₃ (Agholme et al. 2010). This resulted in cells with a morphology very similar to primary neurons, expressing neuritic processes and forming extensive networks. These cells also expressed neuronal markers, e.g. the nuclear protein NeuN and synaptic vesicle protein 2. In addition, all six human isoforms of tau were expressed in levels comparable with the brain, making it a potent *in vitro* model for neuroscience, particularly tau-pathies such as AD.

AF22

The AF22 cells originates from human adult dermal fibroblasts, which has been virally induced to express the transcription factors Oct4, Klf4 and Sox2 in order to achieve pluripotency, hence the induced pluripotent stem cells (iPSCs) characterization. Long-term self-renewing neuroepithelial-like stem cells (It-NES) were then derived by blocking bone morphogenic signals with noggin, selection of neural rosettes and subsequent disassociation into single cells. These It-NES cells have stable neuronal differentiation competence and the capacity to generate functionally mature human

neurons (Falk et al. 2012). AF22 cells are proliferated with the growth factors epidermal growth factor and fibroblast growth factor 2, while neuronal differentiation is initiated by the withdrawal of these factors. AF22 cells are undergoing 40 days of differentiation and develop a mature neuron phenotype with extensive neuritic networks. Most cells are GABAergic, and a small subset are expressing the astrocyte marker GFAP. The mature neuronal markers β III-tubulin and MAP2 are expressed and electrophysiological characteristics were better than differentiated SH-SY5Y cells.

Co-culturing and fluorescent labeling of cells

Prior to co-culture of SH-SY5Y cells, the acceptor cell population is differentiated within an ECM gel for 10 days, while the donor cell population is differentiated without, due to impracticalities at co-culture (fig 9). However, at the moment of coculturing the two cell populations, the donor cells are seeded within a thin layer of ECM gel on top of the acceptor cells. This may be considered a drawback compared with the AF22 cells, where there is no alteration between donor and acceptor cell differentiation.

Acceptor cells were transfected with the BacMam systems baculoviral agent, in order to express enhanced green fluorescent protein (EGFP), allowing us to distinguish them from the unlabeled or red fluorescent protein (RFP) expressing donor cell population. The EGFP was expressed together with a specific protein, allowing for labeling of specific cellular structures, such as endosomes, lysosomes or tubulin.

Protein labeling and preparation of aggregates

A β

Labeling of A β has been done in papers I, II and IV with amine reactive tetramethylrhodamine (TMR) or alexafluor700 N-hydroxysuccinimide-esters. Initially TMR was used, which worked well with uptake and transfer assays on confocal microscopy. However, when the same assays were measured with flow cytometry, laser frequencies available to us was less than optimal. As we were planning on RFP transfecting donor cells in paper IV, we decided to change A β -labeling fluorophore to the far-red spectrum and selected alexafluor700, which also surpassed TMR in brightness. Labeling was performed according to the manufacturer's instruction, theoretically covalently linking one fluorophore to the amine group of each A β -peptide. The sample was then separated with size exclusion chromatography (SEC) to remove unbound fluorophores. The labeled-A β was then spectrophotometrically quantified and lyophilized

Synthetic labeled or unlabeled A β was suspended in hexafluoroisopropanol (HFIP) with subsequent lyophilization in a vacuum centrifuge, followed by resuspension in dimethyl sulfoxide (DMSO) and HEPES buffer and then sonicated to break up eventual aggregates. HFIP prevents immediate aggregation and DMSO has regulative effect on the rate of aggregation. The sample is then incubated 24 h at 4°C to form oligomers (Nath et al. 2012).

Pre-fibrillary A β was prepared with a characterized protocol (Klingstedt et al. 2011), where synthetic A β was dissolved in NaOH, followed dilution in PBS and finally incubated 7 h at 37°C.

These protocols have been verified with non-denaturing PAGE and SDS-PAGE western blot, transmission electron microscopy (TEM), oligomer specific antibodies and SEC. However, manipulating A β is very tricky due to its inherent ability to aggregate or dissociate, depending on multiple factors such as concentration, pH, ionic strength, temperature and time in solution (Stine et al. 2003). This dynamic makes it hard to predict how long the aggregates remain stable after cellular internalization. However, if stable aggregates were made this might cause other unforeseen effects on the intracellular dynamics of A β .

The effects of labeling had no significant effect on oligomerization with our protocol according to SEC and TEM, interestingly, a different group noticed altered aggregation products with fluorophore labeled A β (Jungbauer et al. 2009). A potential issue with fluorescently labeling proteins is that cellular processing might cleave away the fluorophore, thus mislead us into tracking the fluorophore instead of the fluorophore protein complex. However, according to our unpublished data (Chris Sackmann personal communication), western blot experiments have shown that oligomeric A β is still attached to the fluorophore 24 h after uptake in iPSCs.

α -syn

In paper III the α -syn oligomers and fibrills were prepared as previously described by (Nässtrom et al. 2011). Monomeric α -syn was incubated with an excess of 4-hydroxy-2-nonenal (HNE) in sodium carbonate buffer for 18 h at 37°C, while during fibrillary formation incubation was 4-5 days at 37°C on a shaker. The HNE is a naturally occurring lipid peroxidation metabolite found in the cell, which has been shown to induce β -sheet rich α -syn and covalently link monomers together, creating a more stable aggregate.

Size exclusion chromatography

Size exclusion chromatography (SEC) is a separation method used for molecules in solution, usually proteins. It is based on sephadex gel filtration through a column, where larger size results in a shorter distance to travel through the pores, resulting in separation and fractioning of the sample by size. The system operates with high pressure, which facilitates fast separation through a long column, which increase the resolution. The separated fractions are then detected spectrophotometrically, which allow each fraction to be collected separately afterward. We used SEC to separate fluorophore-labeled A β from unbound fluorophores, acquiring fractions of labeled monomers and oligomers.

Protein expression and phosphorylation

Protein concentration determination

The DC protein assay (Bio-Rad) was mainly used to determine protein concentrations of cell lysates in papers I, II and III. It's a colorimetric assay performed in two steps,

first by the addition of an alkaline copper tartrate solution, followed by the addition of Folin reagent which is reduced into a blue color by the copper treated proteins. The absorbance is measured of the protein samples at 750 nm and quantified by comparison with a standard curve of known protein dilutions. It is mainly tyrosine and tryptophan that are responsible for the color development, which may produce a less reliable concentration if proteins are low or lacking those amino acids, such as A β , if it were to be measured. Instead A β were measured by quantifying peptide bonds at 215 nm (Ciccotosto et al. 2011).

Western blot

Western blot is a commonly used method to separate, detect and quantify different proteins, such as the lysosomal protein LAMP-2 in paper I or proteins with post-transcriptional modifications, such as phosphorylated tau protein in paper II. Proteins are denaturated with sodium dodecyl sulphate (SDS) and loaded into a porous polyacrylamide gel, by adding current the negatively charged proteins are separated with electrophoresis depending on charge and size. The proteins are then transferred (blotted) onto a nitrocellulose membrane, followed by blocking unspecific binding sites, the membranes are incubated with primary antibodies against the protein of interest followed by secondary antibodies conjugated with horseradish peroxidase (HRP). A substrate is added and converted by the HRP enzyme into a chemiluminescent product where the protein bands are located, and the emitted light is collected and developed on films followed by densitometric quantification.

Immunocytochemistry

Immunocytochemical methods were used in all four papers to visualize cellular targets with specific antibodies. Cells are fixed, permeabilized and blocked, followed by incubation with primary antibodies specific for the target of interest. Secondary antibodies specific for the primary antibodies are subsequently added. In our papers, these antibodies are labeled with fluorophores, but can be linked with other molecules, such as enzymes, depending on the assay.

After completing secondary antibody incubation the samples was imbedded in antifade reagent, intended to decrease photobleaching and sample degradation.

Electrochemiluminescence linked immunoassay

In paper II the Mesoscale discovery (MSD) system was used to quantify tau and phosphorylated tau proteins. MSD is similar to a sandwich enzyme linked immunosorbent assay (ELISA), with coated capture antibodies and detection antibodies. The detection antibodies are conjugated with SULFO-TAG molecules, instead of an enzymes. After an electrical current is applied, the SULFO-TAGs will generate light emission, and the light intensity will correlate with the amount of protein. The detection system can distinguish the location of the emitted light which enables the coating of several different caption antibodies within the same well. The method has high sensitivity, because signal amplification can be achieved with multiple excitation cycles.

Cell viability assays

MTT-based

Methyl-thiazolyl-tetrazolium (MTT) based colorimetric assays are widely used to measure cytotoxicity or cell viability in cell cultures. The assay measures the cells combined conversion of the yellow MTT substrate into an insoluble violet-blue formazan product. This reaction is mediated by dehydrogenases and reducing agents and is considered to be a measure of the cells metabolic activity. A decrease in conversion between treated cells compared with untreated controls, is considered a decrease in viability. A weakness with this assay is that cytotoxic mechanisms that do not affect the cells metabolism is not detected, and that certain cellular stress conditions can instead lead to increased metabolism. MTT assays were used in paper II and XTT assays, which is similar but results in a water soluble product, was used in paper I and III.

JC-1 assay

This assay was used in paper I and is another method used to measure cytotoxicity or cell viability. The method is based on the fluorescent properties of the JC-1 probe, a cationic molecule that accumulates in mitochondria. The accumulation of the JC-1 probe in the mitochondria is proportional to the mitochondrial membrane potential, which is a measure on the cells ability to generate energy. The fluorescence of the

JC-1 probe shifts from green (525 nm) to red (590nm) when it aggregates within the mitochondria, which facilitates the spectrophotometric quantification of the membrane potential with flow cytometry.

Flow cytometry

Flow cytometry was used in papers I and IV. It is an excellent method for evaluating large numbers of cells in suspension. In principle, cells are forced into a single file, where they are individually subjected to lasers, resulting in the acquisition of multiple parameters, such as cell size, granularity and fluorescence emission. In paper I flow cytometry was used to measure changes in emission spectra of the endocytosed JC-1 probe, which can be used to determine the viability parameter mitochondrial potential. Cell-to-cell transfer inhibition was investigated with flow cytometry in paper IV. There is a huge time saving aspect of using flow cytometry compared with confocal microscopy in regard of quantification of transfer. A more accurate value of transfer is also acquired due to the number of cells possible to measure, and the quantification is less biased. Drawbacks with this method is that you cannot actually see your cells or determine intracellular location of your fluorescent targets, such as colocalization assays (unless using an imaging cytometer). Similar to confocal microscopy autofluorescence can be an issue, flow cytometry requires a lot of controls and gating to verify the parameter of interest is actually what is measured.

Confocal microscopy

Confocal laser scanning microscopy (CLSM) is a versatile and useful optical image technique used in all four papers to examine fixed immunocytochemical samples, live cell imaging or cell-to-cell transfer samples. Confocal microscopy enables the acquisition of thin optical sections of the sample, allowing a look within different levels of the sample. The principle behind CLSM is exposing the sample to a focused laser beam, the emitted light from the sample is then collected and refocused through a pinhole aperture. The size of the pinhole then determines the thickness of the optical section by blocking out light outside the desired focal plane.

The CLSM system used were equipped with four lasers (405 nm, 488 nm, 555 nm and 639 nm) allowing the excitation and emission acquisition of multiple

fluorophores within a single sample, and optical filters allowed simultaneous scanning with different lasers. When using multiple fluorophores within the same sample it is important to consider and avoiding spectral overlapping, which can be avoided with optical filters, or/and scanning the lasers successively with multiple designated channels, resulting in increased acquisition time. Another important factor when working with fluorescence is the potential autofluorescence, often generated by biological samples such as cells. Interference from autofluorescence can be reduced by using high intensity fluorophores, and more importantly, by setting an emission detector sensitivity threshold with negative controls.

Differential interference contrast (DIC), which work on the principle of separating polarized light, were used to acquire contrast, 3D-like, images of the cell bodies and their extensions.

In paper III intracellular colocalization of CY3-labeled α -syn and different subcellular components were investigated with confocal microscopy. However, a limitation with this technique is that objects closer than 200 nm cannot be distinguished between, which may produce false positive colocalization.

Image acquisition and analysis of cell-to-cell transfer with A β or α -syn

Transfer of A β and α -syn was investigated in papers I and IV and in paper III respectively. The 488 nm laser were used to detect EGFP expressing acceptor cells, while the 555 nm or 633 nm lasers detected TMR/CY3 and AlexaFluor700 respectively. Transfer was established by counting all acceptor cells (green) which were positive for either A β / α -syn (red granules), finding a proportion when comparing them with total number of acceptor cells.

High-content imaging

High-content imaging was used in paper IV, which is a great method to capture large quantities of image data from microtiter plates, enabling high throughput screening. It is basically a fluorescent microscope plate reader, capable of automatically scanning each wells surface with the objective of our choosing. We used 96-well and 384-well microtiter plates designed for imaging, in which we co-cultured our cell transfer model. The high-content imaging device then takes multiple images of each well and stitches them together in the software. The software uses different masking

criteria, which utilizes the fluorescent markers of the co-culture system, to determine how much transfer has occurred. The high-content imaging device is connected to an automatic loading robot, with the ability to switch between microtiter plates and load additional into the imaging chamber.

Summary of papers

In this section the main findings are presented and discussed.

Paper I

The slow progressive nature of AD pathology is most likely related to the spreading of misfolded A β and tau proteins throughout the brain in a characteristic manner. The aim of paper I was therefore to further investigate the pathologic mechanisms of cell-to-cell transfer of different isoforms of A β and their cellular clearance.

Our group has previously shown cell-to-cell transfer with oligomeric amyloid beta (A β) residues 1-42 (oA β 1-42) using our donor-acceptor 3D co-culture model. However, the amyloid precursor protein (APP) can be postrtranslationally processed into several other isoforms of A β , which has been shown to exhibit varying degrees of toxicity. In this study we examine oligomeric aggregates of fluorescently labeled A β 1-42, 3pyroglutamate (pE) A β 1-40, A β 1-40 and A β 11-42 regarding their abilities to transfer between cells, resist degradation and induce toxicity.

First we categorized our different A β species using transmission electron microscopy. Then propensity for cell-to-cell transfer of the different species were compared with the same donor-acceptor co-culture model previously used (Nath et al., 2012). Confocal images were acquired and subsequently analyzed with the software Volocity. All isoforms of oA β investigated showed positive transfer in our model, however, the net amount of A β transferred were shown to differ between the isoforms (Fig 10).

Figure 10: The proportion of acceptor cells with positive transferred oA β isoforms over time (24, 48 and 72h).

By measuring intracellular oA β in donor cells at different time points (0 h, 24 h, 48 h and 72 h) after internalization, the differences in stability became apparent between the isoforms. Quantification of internalized oA β revealed that oA β 1-42 were significantly more resilient to degradation/clearance in comparison to the other isoforms, o3(pE)A β 1-40 was the second most stable isoform, while oA β 1-40 and A β 11-42 were more easily cleared by the cells (Fig 11).

The percentage of intracellular accumulations of the different oA β isoforms correlated significantly with cell-to-cell transfer of the respective isoforms over time. This finding suggests that cellular inability to degrade or clear A β accumulations can result in enhanced transfer of the more resistant peptides.

Figure 11: Stability of oA β isoforms over time in donor cells.

By inducing autophagy with rapamycin, oA β 1-42 accumulations could be reduced after 72 h in donor cells, and a trend of reduced transfer was observed indicating that lysosomal involvement probably can affect the net amount of cell-to-cell transfer.

Colocalization of the oA β isoforms and lysosomes was investigated with live cell confocal microscopy and lysotracker 3 h after oA β -TMR incubation. No significant difference in colocalization between the different isoforms and lysosomes was detected.

Image analysis of lysosomal vesicle size in acceptor cells after oA β transfer revealed a significant increase in abnormally large lysosomes subsequent to oA β 1-42 transfer, which increased with time (Fig 12A and B). In addition, the LAMP2 protein was significantly increased in donor cells after 72 h only with oA β 1-42 (Fig 12C), indicating

an upregulation of lysosomal activity, probably in an effort to degrade the resistant oAβ1-42 isoform.

Figure 12: oAβ1-42 induced lysosomal stress-like behavior. (A) Abnormally large lysosomes were observed in acceptor cells after oAβ1-42 transfer (white arrow) and oAβ3(pE)-40 (black arrow). (B) Lysosomes shifted towards increased mean size with time after transfer in acceptor cells. (C) The lysosomal membrane protein LAMP2 was increased in donor cells 72 h after oAβ1-42 uptake.

XTT assays and JC-1 assays were employed to investigate cytotoxicity and mitochondrial potential respectively, after oAβ internalization. No cytotoxicity and no change in mitochondrial potential was observed in cells treated with either isoforms in our experimental window (24-72 h), which would indicate that cell-to-cell transfer is an earlier event than cytotoxicity.

Figure 13: A graphical representation of our major findings in paper I.

All isoforms investigated are able to transfer from one cell to another, thus cell-to-cell transfer is not dependent on a particular A β isoform. However, the net amount of any oA β transferred seem to be dependent on the resistance to degradation/clearance of each isoform. Out of the isoforms investigated, oA β 1-42 was shown to be more resilient to degradation/clearance as well resulting in a greater level of transfer (Fig 13). Transfer of oA β seems to occur prior to cellular cytotoxic events, suggesting that cell-to-cell transfer might be an early stress response to prevent cytotoxic protein accumulations.

Paper II

In paper I we found that intracellular clearance mechanisms affect the level of A β transfer, this is relevant because the effectiveness of the two major cellular degradation systems, the autophagic-lysosomal and proteasomal systems, has reduced function in the aging brain. Also, intracellular A β accumulation has been shown to induce proteasome inhibition (Almeida, Takahashi, and Gouras 2006)

In paper II our aim was to investigate the effect of reduced proteasomal degradation on tau phosphorylation, microtubule stability and neuritic transport. The reversible proteasome inhibitor MG-115 was used in varying concentrations to reduce proteasome activity in differentiated neuroblastoma cells. Axonal transport was investigated because it is believed to be impaired as an early event in AD pathology. Time-lapse imaging revealed that even the lowest proteasome inhibitor concentration used, significantly decreased neuritic vesicle transport and increased vesicle size (Fig 14A, B and C). In addition, mitochondria was displaced from neurites after proteasomal inhibition, which further supports the important role of proteasomal degradation in maintaining functional neuritic transport (Fig 14D, E). Destabilization of the microtubules was detected with immunocytochemistry and immunoblotting as tubulin beading (Fig 14F) and tubulin disruption as an increased proportion of tyrosinated α -tubulin.

Figure 14: (A) Live cell imaging of untreated control and proteasome inhibited cells. (B) Vesicle transport velocity along neurites decreased (C) and size increased upon proteasome inhibition. (D) Untreated cells with normal mitochondria distributions along neurites. (E) Mitochondria located in the neurites were decreased after proteasomal inhibition and (F) increased tubulin beading was observed (green).

Tau is a microtubule stabilizing protein, however, it detaches when hyperphosphorylated, probably resulting in destabilization of microtubules. Relevant phosphorylation sites on tau was quantified upon proteasomal inhibition with western blot and electrochemiluminescence. 4 out of 5 epitopes showed increased phosphorylation after proteasomal inhibition (Fig 15).

Epitope	proteasome inhibition		
	-	+	-
S202	-	+	-
AT8 (S202/S205)	-	+	+
T231	+	+	-
PHF1 (S396/S404)	-	-	-
S422	+	++	+++

Figure 15. An increase in phosphorylation of Tau-epitopes were observed after proteasome inhibition.

Several kinases have been implicated in the hyperphosphorylation of tau, the most frequently investigated are GSK3 β and CDK5. However, many other kinases have also been linked to the phosphorylation of tau. To determine which kinases are affected by proteasome inhibition, a proteome profiler phospho-kinase array was used to quantify kinase activation compared with controls. Extracellular signal-regulated protein kinase (ERK) 1/2 and c-Jun especially stood out with increased activation which was confirmed with western blot.

To determine if these kinases were responsible for the MG-115 induced vesicle transport impairment and tau phosphorylation, inhibitors of their upstream activators, MEK for ERK 1/2, and JNK for c-Jun, were employed. Inhibition of ERK 1/2 rescued the impaired vesicle transport (Fig 16A) and a combination of MEK and JNK inhibitors significantly reduced tau phosphorylation of the investigated epitope 231 (Fig 16B).

Figure 16: (A) Vesicle velocity was rescued with a MEK inhibitor and (B) phosphorylated tau at epitope 231 decreased with JNK/MEK inhibitor combination after proteasome inhibition.

These findings suggests that a decreased proteasome activity, observed in the old, and especially in the AD brain, can through activation of the kinases ERK 1/2 and c-Jun lead to impaired neuritic vesicle transport and tau hyperphosphorylation. Perhaps making these kinases relevant in potential therapeutic strategies together with restoring proteasome activity in AD.

Paper III

In papers I and II we have studied mechanisms relevant to Alzheimer's disease, we believe that similar mechanisms, especially regarding cell-to-cell transfer, might be relevant in other neurodegenerative diseases as well, such as the role of α -synuclein (α -syn) in Parkinson's disease. By using our co-culture model, immunocytochemistry and image analysis we aimed to investigate the uptake and transfer of different α -syn species between human neuronal-like cells.

Fluorescently labeled α -syn monomers, oligomers and fibrils were successfully endocytosed by donor cells. Oligomers and especially fibrils were more readily taken up by the cells (Fig 17A and B).

All forms of α -syn showed positive transfer from donor- to acceptor cells in our model after 24 h. However, the proportion of acceptor cells receiving α -syn varied between the species (Fig 17C), where fibrillary transfer was most extensive (35 % of total acceptor cells), followed by oligomers (23 %) and monomers (14 %) (Fig 17D). The transfer capacity is probably dependent on variables such as donor cell uptake and resistance to degradation/clearance. Fibrillary endocytosis has been shown to be mediated by lymphocyte-activation gene (LAG3) 3 (Mao et al. 2016), which could explain the higher degree of transfer.

Figure 17. (A) Confocal images showing uptake of α -syn species (red) in donor cells after 24h. (B) Proportions of donor cells with α -syn uptake. (C) Confocal images showing positive transfer of α -syn species (red) from donor to acceptor cells (green) after 24h. (D) Proportion of acceptor cells with positive transfer of respective α -syn species.

It is also of importance to know what intracellular subsystems α -syn localizes to in order to better understand the processing and transfer of the protein, therefore the subcellular localization of the internalized α -syn species was investigated with confocal microscopy and the colocalization was quantified.

The different α -syn species mainly colocalized with the lysosomal/endosomal compartment but were also found to interact to a lesser degree with synaptic vesicles, SNARE related vesicles, the ER and golgi network and multivesicular bodies both before transfer, in donor cells (Fig 18A, B, C and D), and after transfer in acceptor cells (Fig 18E, F, G and H).

Figure 18. (A) Confocal image showing oligomeric α -syn (red), lysosomes (green) and their colocalization (yellow) in donor cells. (B) Proportion of monomeric, (C) oligomeric and (D) fibrillar α -syn with respective investigated marker. (E) Confocal image showing oligomeric α -syn (red), lysosomes (blue) and their colocalization (purple) in acceptor cells. (F) Proportion of monomeric, (G) oligomeric and (H) fibrillar α -syn with respective investigated marker.

These findings show that monomeric, oligomeric and fibrillary forms of α -syn can transfer between human neuronal-like cells, the differences in the degree of transfer are most likely due to differences in uptake and processing of the α -syn species. α -Syn mainly localized to compartments related to the lysosomal system which possibly reflects a cellular attempt at degradation.

Paper IV

In papers I and III we have observed that A β and α -syn are readily transferred from donor to acceptor cells in our co-culture model system. They seem to be interacting with similar intracellular compartments, mostly lysosomes, thus they may be transferred by similar mechanisms. In paper IV we aimed to modify and optimize our transfer co-culture model, to allow for high throughput screening (HTS) of small molecular substance libraries in order to find substances that inhibit A β transfer.

To adapt for the HTS format, the co-culture model required extensive modifications and optimization, such as miniaturizing it to 384-well microplates and the reduction of unnecessary steps in the established protocol to better accommodate robotic assistance and automatic image acquisition. Optimization for seeding density, ECM

coating, positive controls and improved fluorescent labeling of SH-SY5Y cells was carried out. However, by comparing advantages and drawbacks of SH-SY5Y and the long-term self-renewing neuroepithelial-like stem cell line AF22, we predicted that using the AF22 cells would decrease eventual confounding factors. Factors such as the tumor heritage of the SH-SY5Y cell line. It would also increase the translatability of the screening outcome to adult human neurons with better neuronal characteristics.

Cell densities for both cell types in 96- and 384-well plates were established (Fig 19A and B). Different options for fluorescent labeling of the cells was evaluated. The aim was to label as many acceptor and donor cells as possible, with similar even fluorescent intensity and visible neuritic extensions.

Figure 19. Model optimization of differentiated AF22 cells. (A and B) Fluorescently labeled donor (red) and acceptor (green) cells co-cultured in a 384-well plate. (C) Transfer of oA β 1-42 (red) between donor (blue) and acceptor cells (green). (D) Differentiated AF22 cells immunostained for β III-tubulin (red) and (E) MAP2 (red) and DAPI (blue). (F) Pharmacological inhibition of transfer from donor to acceptor cells, measured with flow cytometry.

These factors are important for the scanning microscopy based high-content analysis system intended for this method, as automatic quantification of transfer is dependent on reliable masking during image analysis. In this assay we decided to use tubulin-GFP and -RFP, due to its even distribution within the cell and its extensions.

Transfer of oA β 1-42 labeled with alexafluor 700 was confirmed with the intended high-content screening microscopy equipment (Fig 19C).

Differentiated AF22 cells expressed adult neuronal markers, such as β III-tubulin and MAP2 (Fig 19D and E), in addition AF22 displayed better electrophysiological characteristics than the SH-SY5Y cells.

Unexpected events and variation may occur between plates during screening, and to compensate controls are very important. The negative controls would be normal uninterrupted transfer, while the positive controls would inhibit the transfer. In this model a pharmacological agent would be most beneficial, several were tested targeting endocytic mechanisms and cytoskeletal movement, however, the substance with most potent inhibitory effect on oA β 1-42 transfer was heparin. Heparin has been shown to affect cellular A β uptake by competitive binding to heparan sulfate proteoglycans on the cell surface (Kanekiyo et al. 2011). The effects of heparin significantly decreased transfer of oA β 1-42 from donor to acceptor cells, when measured with flow cytometry (Fig 19 F), providing a useful substance for positive controls.

AD, PD and other neurodegenerative diseases seem to share slow spread of neurotoxic proteins throughout the brain, acting in a prion-like manner, from cell to cell. This HTS adapted model can now be used to screen small molecular libraries for substances that inhibit these spreading mechanisms. Using human iPSC derived AF22 cell line, with its many neuronal characteristics, improves the chances that substance effects have a similar effect in the human central nervous system.

There are many different approaches currently under development aiming to find working treatments for AD and other neurodegenerative diseases. Halting the spreading of neurotoxic proteins would be a novel approach to treat neurodegenerative diseases and might work well in combination with other therapeutic strategies.

Conclusion

The neurodegenerative diseases are heterogeneous and have multifactorial etiology which complicates the development of effective therapeutic strategies. The cellular degradative systems play an important role in preventing damaged and misfolded

proteins to remain, aggregate and accumulate. Unfortunately these systems effectiveness are reduced with advanced age which is a variable that increases the risk of developing a neurodegenerative disease.

Neurons communicate not only through neurotransmitter release, but also employ various other pathways such as the constitutive secretory pathway, exosomes or tunneling nanotubes. When misfolded and aggregated proteins fail to be degraded these proteins hijack the cell-to-cell transfer pathways and are able to propagate to additional neurons. In a prion-like manner the misfolded protein aggregates can then “infect” nearby cells and recruit native proteins into a misfolded state which promote further aggregation.

Our findings in paper I shows that degradation resistant proteins, such as A β 1-42, result in increased cell-to-cell transfer and lysosomal accumulation, supporting the important preventive role of the degradative systems in proteinopathies such as AD and PD. Intracellular A β accumulations have been reported to inhibit the proteasomal degradation pathway. In paper II we investigated proteasomal inhibition which resulted in c-jun and ERK1/2 kinase activation followed by impaired neuritic vesicle transport and tau hyperphosphorylation. These pathological changes are most likely secondary to intracellular A β accumulations and cell-to-cell transfer. Similar to A β in paper I, α -syn in paper III were shown to transfer from cell-to-cell and localize mainly with lysosomes, which may indicate similar means of intercellular transfer and processing. In paper IV we developed a HTS-protocol which can be used to screen for substances that reduce/prevent cell-to-cell transfer of A β . To inhibit the cell-to-cell transfer of A β or α -syn, would be an interesting therapeutic avenue. It would not cure the underlying pathology, but may arrest the disease progression enough to give the patients more time with an improved quality of life.

Future perspectives

In the future we would like to utilize our HTS modified model to screen for substances that could inhibit the transfer of oA β . We would also like to map which intracellular mechanisms are involved in this transfer, with targeted down regulation of particular key proteins through siRNA.

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