Linköping University Medical Dissertation No. 1702

Investigation of the intercellular transmission of α -synuclein, amyloid- β and TDP-43

Christopher Sackmann

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ABSTRACT

Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), frontotemporal lobar dementia (FTLD) and amyotrophic lateral sclerosis (ALS) are disorders characterized by the progressive deposition of proteinaceous inclusions throughout the brain in a predictable manner. Each disease is described by the involvement of different misfolded and aggregated proteins (AD, amyloid- β and tau; PD, α -synuclein; ALS and FTLD, TDP-43) that spread between anatomically connected brain regions, causing cell death in previously healthy regions. Disease progresses as these aggregated proteins spread throughout the brain in a prion-like fashion. Oligomeric forms of these proteins (aggregates comprising of ≈ 3 -30 individual proteins) are thought to be the most relevant to disease progression, as they are capable of prion-like propagation and can cause cellular toxicity. The work in this thesis aims to elucidate the mechanisms by which different neurodegenerative disease related proteins (amyloid- β , α -synuclein and TDP-43) are taken up and transferred between cells, and the effects exerted by these proteins on downstream cells.

Paper I examined the uptake and cell to cell transmission of α -synuclein (α -syn). Using a 3D co-culture model, we determined that α -syn (monomeric, oligomeric and fibrillar assemblies) were readily taken up and transferred between neuron-like cells, and that this transfer was mediated by an endosomal/lysosomal mechanism. It was also determined that larger α -syn assemblies (oligomers and fibrils) were found in donor and acceptor cells more frequently than monomeric α -syn, which we speculate is due to the larger aggregates' resistance to cellular proteases.

In Paper II, we identified a novel mechanism for the preferential uptake of oligomeric proteins through the gap junction channel protein connexin 32. Gap junction proteins act as a means of communication between adjacent cells, forming a transmembrane pore to facilitate the passage of small molecules. Here, we determined that connexin 32 drives the preferential uptake of oligomeric α -syn relative to monomeric and fibrillar α -syn. This system was not exclusive to α -syn however, as the preferential uptake of oligomeric amyloid- β (A β) was also observed. In addition to the uptake of oligomers, we observed that increased α -syn expression elicited the increased expression of connexin 32, in a positive feedback mechanism. When connexin 32 was inhibited pharmacologically or knocked out using CRISPR/Cas9, the preferential uptake of oligomers was abolished. These phenomena were also observed in oligodendrocytes (the accumulation of oligomeric α -syn in oligodendrocytes is a hallmark of Multiple Systems Atrophy), three different mouse models of α -syn overexpression, as well as in post-mortem human tissues.

Paper III undertook the investigation of cell to cell transfer of TDP-43. Although it was recently confirmed that TDP-43 propagates throughout the brain in a prion-like fashion, it remains unclear how post-translational modifications of TDP-43 affect its propensity to be transferred between cells. This has left a gap in the understanding of how TDP-43 proteinopathies progress, as post-translationally modified TDP-43 is understood to be critical to pathogenesis. To study this, we generated several TDP-43 cell lines, expressing full-length TDP-43 or C-/N-terminally truncated fragments, known contributors to TDP-43 proteinopathies. Using the 3D co-culture model, we

determined that preservation of the N-terminus of TDP-43 enhanced its ability to transmit between cells, whereas an intact C-terminus reduced transfer. Additionally, since we have previously shown that both oligomeric A β and α -syn are incorporated into extracellular vesicles (EVs) such as exosomes, and that these EVs can mediate the transfer of protein oligomers to downstream cells, we investigated whether this was also true for TDP-43. We demonstrated that full-length TDP-43 and TDP-43 fragments could be found within EVs generated by these cells, but that these EVs were unable to propagate the protein to downstream cells. Instead, the transmission of TDP-43 occurs in a manner dependent upon physical proximity between cells, possibly across the synaptic cleft itself.

Next, we studied the acute effects exerted by oligomeric A β upon healthy neurons in order to understand the earliest effects of oligomeric A β challenge. In Paper IV, we used iPSC-derived neurons generated from human donors expressing different amyloid precursor protein (APP) genes: one harbouring the familial AD-causing V717I London mutation, the other expressing wild type APP. After differentiating these cells into functional neurons, the neurons were challenged with acute exposure to exogenous oligomeric A β and analyzed by LC-MS/MS to observe the early effects. By analyzing the proteome and phosphoproteome of these cells, we identified many proteins and phosphoproteins that were up- or down-regulated in response to oligomeric A β at this early timepoint. Among these changes, oligomeric A β caused the downregulation of TDP-43, heterogeneous nuclear ribonucleoproteins, and coatomer complex I proteins. Conversely, increases were observed in 20S proteasome subunits and vesicle associated proteins VAMP1/2. We also observed the differential phosphorylation of tau at serine 208, indicating that phosphorylation at this residue might be an important early event in tauopathy.

Altogether, the work described in this thesis has provided new understanding as to how different neurodegenerative disease related proteins are taken up and transferred between cells. In doing so, we have identified some of the mechanisms by which this spreading occurs, and that the changes elicited by these toxic oligomeric proteins are rapid and widespread. By learning about these processes, we have identified novel targets that could be used in the development of disease modifying therapeutics.

POPULAR SCIENTIFIC SUMMARY

Incidence of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), frontotemporal lobar dementia (FTLD) and amyotrophic lateral sclerosis (ALS) are increasing along with the aging global population. Neurodegenerative disorders advance progressively, where initial symptoms are relatively mild but become increasingly severe, leading to dementia and eventually causing the death of those afflicted. The World Health Organization estimates that 50 million people currently suffer from dementia and expect this number to grow to 152 million by 2050. The most prominent form of dementia, AD, accounts for 60-70% of these cases. Further, dementias are an enormous economic strain on society, where costs associated with the care of AD patients alone estimated to exceed USD\$1 trillion per year, globally. Despite the high human and financial costs of dementia to individuals and society alike, there are currently no treatments that are effective at stopping or delaying any neurodegenerative disease. Part of the reason why no treatments currently exist is that we do not fully understand how these diseases begin and progress. In AD, PD, FTLD and ALS, the incidence and progression of each respective disorder is characterized by the misfolding of proteins in the brain. These aberrantly folded proteins readily clump together into protein aggregates of various sizes, which themselves can cause different brain cells (e.g. neurons, oligodendrocytes, astrocytes, etc.) to become sick and die. These aggregates encapsulate many copies of the misfolded proteins, but it is thought that oligomers (comprising of $\approx 3-30$ individual proteins) are the most toxic form and may also propagate disease in a prion-like fashion. The prion-like spreading hypothesis states that these oligomeric proteins themselves can cause previously healthy, correctly folded protein to take on the disease-causing misfolded conformation, where it then aggregates, causes cellular damage and propagates these effects to nearby healthy cells. The work in this thesis aims to understand at the molecular level how cells pass these disease-causing proteins to one another, thereby spreading disease throughout the brain.

In Paper I we investigated how the PD-associated protein α -synuclein is taken up by cells, and how it is transferred between cells. Using a 3-dimensional cell model and oligomeric α -synuclein labeled with a fluorescent probe, we were able to determine that these α -synuclein oligomers were transferred readily between cells. We then labeled subcellular compartments of interest using specific antibodies and observed colocalization of the α -synuclein with these labeled compartments. In doing so, we found that the transfer of α -synuclein between cells occurred in a way that favoured the endosomal/lysosomal system, a protein uptake and degradation pathway.

Paper II examined the uptake of oligomeric α -synuclein from outside the cell. Using different brain cell models, we made these cells generate excessive amounts of the gap junction protein connexin 32. Gap junction proteins, under normal circumstances, form a pore between adjacent cells, which allows the exchange of small molecules including proteins. Here, we observed that connexin 32 expressing cells took up oligomeric α -synuclein more readily than large α -synuclein aggregates or individual α -synuclein proteins. When we inhibited connexin 32 with drugs or genetically using CRISPR/Cas9, the preferential uptake of oligomeric α -synuclein was eliminated. We also saw that, in the post-mortem tissues of human PD patients and PD mouse models, that the amount of

connexin 32 was increased in concert with the increase of α -synuclein. This means that during PD, there seems to be an increase in connexin 32, which causes an increase in the accumulation of toxic α -synuclein oligomers.

Paper III looked at the transfer of the ALS and FTLT-causing protein, TDP-43, between cells. Here, we made several different cells, each expressing a different portion of the TDP-43 protein, along with a fluorescent marker attached to it. In this way, we were able to track the passage of the TDP-43 proteins between cells. We found that this passage occurred regardless of which piece of TDP-43 was used, but that the expression of the N-terminus of TDP-43 (the piece at the beginning of the protein) enhanced its ability to transfer between cells. Furthermore, we investigated whether this transfer could occur through extracellular vesicles (EVs). EVs are small, spherical entities that can contain cargo (such as proteins), which cells release in order to communicate with distant cells. We found that TDP-43 was unable to be transferred between cells via EVs, instead requiring physical proximity between one another in order to facilitate protein transfer.

Lastly, we looked at the AD-related protein amyloid- β . In Paper IV, we used human inducible pluripotent stem cells (iPSCs) to study the effects that amyloid- β oligomers exert upon these cells. By carefully culturing these iPSCs for 60 days, they become almost indistinguishable from bona fide human brain cells (neurons). These neurons were subjected to the addition of amyloid- β oligomers, followed by analysis by mass spectrometry. In this way, we were able to study the effects that amyloid- β oligomers caused to these cells in a holistic way, as mass spectrometry enables the analysis of all proteins within the cell. By doing this, we discovered some of the early changes that amyloid- β oligomers cause to cells, allowing us to learn about factors contributing to the onset of AD at the cellular level.

Through this work, we have identified some of the ways by which α -synuclein and TDP-43 spread between cells at the molecular level. We also learned which cellular systems were among those first affected by oligomeric proteins, shedding light on how these misfolded proteins cause cell damage. Overall, these findings contribute new understanding of how proteins spread throughout the brain and progress neurodegeneration. These data may be useful in identifying molecular targets for the development of disease-inhibiting drugs.

POPULÄRVETENSKAPLIG SAMMANFATTNING

I takt med att den åldrande befolkningen ökar globalt, så ökar också antalet drabbade av neurodegenerativa sjukdomar. Exempel på sådana sjukdomar är Alzheimers sjukdom (AS), Parkinsons sjukdom (PS), frontallobsdegeneration (FTLD) och amyotrofisk lateral skleros (ALS). Neurodegenerativa sjukdomar fortskrider progressivt, där de tidiga symptomen är relativt milda men sedan tilltar gradvis, den drabbade blir alltmer beroende av hjälp och dör i förtid. Olika kognitiva symptom, inklusive demens, är vanliga. Sjukdomarna drabbar inte bara individen utan även de anhöriga. Världshälsoorganisationen, WHO, uppskattar idag att runt 50 miljoner människor lider av demenssjukdom och antalet förväntas öka till 150 miljoner år 2050. AS är den vanligaste demenssjukdomen, den står för 60-70% av alla fall. Demenssjukdomar medför utöver lidande en stor ekonomisk utmaning för samhället, där de globala kostnaderna enbart i samband med vård av AS patienter uppskattas till mer än 1 miljard USD. Tyvärr saknar vi behandlingar som kan bota eller hämma dessa sjukdomar. Detta beror fram för allt på att vi bara delvis förstår orsaken och utvecklingen av sjukdomarna.

Förekomsten och utvecklingen av AS, PS, FTLD och ALS karakteriseras av felveckade proteiner i hjärnan. De felveckade proteinerna klumpar lätt ihop sig i proteinansamlingar, som orsakar skador på olika typer av hjärnceller (t.ex. nervceller och oligodendrocyter) som gradvis blir sjuka och dör. Dessa proteinansamlingar kan innehålla många kopior av de felveckade proteinerna, där oligomerer (uppbbyggda av $\approx 3-30$ enskilda proteiner) tros vara den mest skadliga storleken och de tycks även kunna sprida sjukdomsförloppet på ett prionliknande sätt. Det innebär att de oligomera proteinansamlingarna själva verkar kunna orsaka tidigare friska och korrekt veckade proteiner att anamma sjukdomsframkallande felveckade konformationer, vilka sedan klumpas ihop, orsakar cellskador och sprider dessa effekter till friska celler. Arbetet i denna avhandling syftar till att på molekylär nivå förstå hur överföringen av dessa sjukdomsframkallande proteiner sker mellan celler och därigenom till nya delar av hjärnan så att patienten gradvis blir allt sämre.

I Artikel I undersökte vi hur det PS-associerade proteinet α -synuklein tas upp av celler och hur det förs över mellan celler. Vi använde oss av en 3-dimensionell cellmodell och oligomert α -synuklein märkt med en fluorescerande markör, och kunde visa att dessa α -synukleinoligomerer enkelt överfördes mellan cellerna. Vi färgade också olika cellulära organeller. Det visade att överföring av α -synuklein mellan cellerna är nära sammankopplat med det endosomala/lysosomala systemet, ett av cellens system för upptag och nedbrytning

I Artikel II undersökte vi hur upptaget av oligomert α -synuklein från utsidan av cellen sker. Vi undersökte en typ av proteiner (connexiner) som bildar små kanaler i cellmembranet och mellan angränsande celler, så kallade gap-junctions. Med genetiska metoder fick vi olika typer av hjärnceller att producera extra mycket av sådana kanalproteiner. Vi observerade då att celler som uttryckte connexin 32 tog upp mer α -synuklein, särskilt de oligomera formerna. Vi kunde visa att oligomert α -synuklein binder till connexin 32, något vi visade sker även i PS-musmodeller och i hjärnvävnad från avlidna patienter med PS. När vi hämmade connexin 32 med läkemedel eller genetiskt med CRISPR/Cas9, hindrades cellernas upptag av oligomert α -synuklein. Vi såg

även att mängden connexin 32 ökade i samband med ökningen av α -synuklein, både i celler och i PS-musmodeller. Detta betyder att connexin 32 bidrar till upptaget och spridningen av toxiska α -synukleinoligomerer vid PS och andra synukleinsjukdomar, det leder i sin tur till en ökning av mängden av connexin 32 i en ond spiral.

Artikel III fokuserade på överföringen mellan celler av proteinet TDP-43 som orsakar ALS- och FTLD. Med genetiska metoder modifierade vi odlade celler så att de uttryckte olika delar av TDP-43-proteinet, märkta med en fluorescerande markör. På så sätt kunde vi spåra förflyttningen av TDP-43-proteiner mellan cellerna. Vi fann att överföringen skedde oavsett vilken del av TDP-43-proteinet som cellerna producerade, men att förekomsten av proteinets inledande ände, N-terminalen, ökade förmågan hos TDP-43 att överföras mellan cellerna. Vi undersökte också om denna överföring sker med hjälp av extracellulära vesiklar (EV). EV frisätts av celler för transport och kommunikation mellan celler, de kan innehålla olika typer av material som proteiner och RNA. Trots att vi kunde påvisa att det finns TDP-43 i EV fann vi att EV inte används för överföringen av TDP-43 mellan celler. Istället sker det med någon mekanism som kräver fysisk närhet mellan celler för att främja proteinöverföring.

Slutligen studerade vi det AD-relaterade proteinet amyloid- β i artikel IV. Vi använde humana inducerade pluripotenta stamceller (iPSC) för att studera de effekter som amyloid- β -oligomerer har på dessa celler. Genom omsorgsfull odling av iPSC i 60 dagar blir de nästan oskiljbara från nervceller i den mänskliga hjärnan. Dessa odlade nervceller utsatte vi för tillsats av amyloid- β -oligomerer, följt av analys av deras proteinuttryck med masspektrometri. Det här tillvägagångssättet gav oss möjligheten att studera effekterna av amyloid- β -oligomerer på ett holistiskt sätt, då man samtidigt kan analysera alla proteiner som befinner sig i cellen med masspektrometri. Genom detta förfarande kunde vi upptäcka tidiga förändringar som amyloid- β -oligomerer orsakar i celler, vilket i sin tur låter oss förstå vilka faktorer som kan bidra till utvecklingen av AD på cellnivå.

Genom arbetena i denna avhandling har vi på cellulär och molekylär nivå identifierat några av de sätt på vilka α -synuklein och TDP-43 sprids mellan cellerna. Vi har även visat vilka cellulära system som först påverkas av oligomera proteiner, vilket sprider ljus över hur dessa felveckade proteiner orsakar cellskador. Sammanfattat bidrar våra resultat till ny förståelse om hur proteiner sprider sig i hjärnan och utvecklar neurodegeneration. Resultaten är viktiga då de påvisar molekylära mål som kan bli viktiga för framtida utveckling av sjukdomsinhiberande läkemedel.

LIST OF INCLUDED PAPERS

Paper I

Aggregated alpha-synuclein transfer efficiently between cultured human neuron-like cells and localize to lysosomes.

Jakob Domert[‡], Christopher Sackmann[‡], Emelie Severinsson, Lotta Agholme, Joakim Bergström, Martin Ingelsson, Martin Hallbeck.
PLoS ONE, 2016. DOI: 10.1371/journal.pone.0168700

[‡]Authors contributed equally

Paper II

Binding of α -synuclein oligomers to Cx32 facilitates protein uptake and transfer in neurons and oligodendrocytes.

Juan F. Reyes, Christopher Sackmann, Alana Hoffmann, Per Svenningsson, Jürgen Winkler, Martin Ingelsson, Martin Hallbeck.
Acta Neuropathologica, 2019. DOI: 10.1007/s00401-019-02007-x

Paper III

Truncated and full-length TDP-43 proteins are efficiently transferred between neuron-like cells in a manner enhanced by preservation of its N-terminus.

Christopher Sackmann, Valerie Sackmann, Emelie Severinsson, Martin Hallbeck.
Submitted manuscript, 2019.

Paper IV

Oligomeric amyloid- β induces early and widespread changes to the proteome in human iPSC-derived neurons.

Christopher Sackmann, Martin Hallbeck.
Submitted manuscript, 2019.

LIST OF PAPERS NOT INCLUDED

Anti-inflammatory (M2) macrophage media reduce transmission of oligomeric amyloid-beta in SH-SY5Y cells.

Valerie Sackmann, Anna Ansell, **Christopher Sackmann**, Harald Lund, Robert A Harris, Martin Hallbeck, Camilla Nilsberth.

Neurobiology of Aging, 2017. DOI: 10.1016/j.neurobiolaging.2017.08.022

Inhibition of nSMase2 reduces the transfer of oligomeric α -synuclein irrespective of hypoxia.

Valerie Sackmann, Maitrayee Sardar Sinha, **Christopher Sackmann**, Anna Ansell-Schultz, Livia Civitelli, Joakim Bergström, Martin Hallbeck.

Frontiers in Molecular Neuroscience, 2019. DOI: 10.3389/FNMOL.2019.00200

ABBREVIATIONS

4-hydroxy-2-nonenal	HNE
Acetylcholinesterase Inhibitor	AChEI
Alpha-synuclein	α -syn
Alzheimer's disease	AD
Amino Acid	AA
Amyloid- β	A β
Amyloid Precursor Protein	APP
Amyotrophic Lateral Sclerosis	ALS
Argyrophilic Grain Disease	AGD
Brain-Derived Neurotrophic Factor	BDNF
Clustered Regularly Interspaced Short Palindromic Repeats	CRISPR
Corticobasal Degeneration	CBD
Dementia with Lewy Bodies	DLB
Early Onset Alzheimer's Disease	EOAD
Epidermal Growth Factor	EGF
Extracellular Matrix	ECM
Extracellular Vesicle	EV
familial Alzheimer's Disease	fAD
familial Parkinson's Disease	fPD
Fibroblast Growth Factor	FGF
Fluorescence Activated Cell Sorting	FACS
Frontotemporal Lobar Degeneration	FTLD

Glycine Rich Domain	GRD
Knockout	KO
Label-Free Quantification	LFQ
Late Onset Alzheimer's Disease	LOAD
Levodopa	L-DOPA
Lewy Bodies	LBs
Lewy Neurites	LNs
Lysosome-Associated Membrane Protein	LAMP
long-term Neuroepithelial Stem Cells	ltNES
Immunocytochemistry	ICC
Immunohistochemistry	IHC
inducible Pluripotent Stem Cell	iPSC
Microtubule Associated Protein Tau	Tau
Microtubule Binding Region	MTBR
Mild Cognitive Impairment	MCI
Mini-Mental State Exam	MMSE
Multiple Systems Atrophy	MSA
Multivesicular Body	MVB
Myelin Basic Protein	MBP
nano Liquid Chromatography Tandem Mass Spectrometry	nLC-MS/MS
Neuregulin	NRG
Neurodegenerative disease	ND
Neurofibrillary Tangles	NFT
Oligodendrocyte Precursor Cell	OPC
Paired Helical Filament	PHF
Parkinson's disease	PD
Pick's disease	PiD
Post-Translational Modification	PTM
Pre-Formed Fibrils	PFF
Presenilin	PSEN
Progressive Supranuclear Palsy	PSP
Reverse Transcriptase Polymerase Chain Reaction	RT-PCR
RNA Recognition Motif	RRM
Single Nucleotide Polymorphism	SNP
Size Exclusion Chromatography	SEC
sporadic Alzheimer's Disease	sAD
sporadic Parkinson's Disease	sPD
Substantia Nigra pars compacta	SNpc
Transmission Electron Microscopy	TEM
Transactive Response DNA Binding Protein 43 kDa	TDP-43
Vesicle-Associated Membrane Protein	VAMP

INTRODUCTION

Neurodegenerative diseases

The incidence of neurodegenerative disease (ND) is increasing globally along with the aging population. In 2017, the World Health Organization estimated that there were currently 50 million people suffering from dementias worldwide and expect that number to grow to 152 million by 2050¹. Of these dementias, Alzheimer's disease (AD) accounts for 60-70% and carries a substantive financial impact, with Alzheimer's Disease International estimating the global societal costs of AD alone to be USD\$1 trillion in 2018². While the etiology of each ND differs, they all exhibit the progressive loss of neurons, prompting impairment of the functions executed by the damaged brain region (Figure 1), leading to cognitive decline and ultimately death.

Figure 1. Primary proteinaceous pathologies hallmark different NDs. Misfolded proteins spread between interconnected brain regions, from initial areas of pathogenesis (darker colours) to regions implicated in later stages of disease (lighter colours). In Alzheimer's disease, tau aggregates are first observed in the locus coeruleus, then in the entorhinal cortex, hippocampus and neocortex. Also in Alzheimer's disease, amyloid- β is first found in the neocortex, then spreads to the allocortex, diencephalon and brainstem. In Parkinson's disease, α -synuclein containing Lewy bodies and Lewy neurites are first observed in the olfactory bulb and dorsal motor nucleus of the vagus nerve, later spreading through the pons and midbrain into the basal forebrain and neocortex. Amyotrophic Lateral Sclerosis displays the progressive involvement of TDP-43 pathology, beginning in the agranular motor cortex and in the brainstem motor nuclei, spreading to the prefrontal cortex, brainstem reticular formation and pre-cerebellar nuclei. In the later stages of Amyotrophic Lateral Sclerosis, TDP-43 pathology is observed in the striatum, temporal lobe and hippocampus. Image was adapted with permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Neuroscience, Spreading of pathology in neurodegenerative diseases: a focus on human studies. Johannes Brettschneider, Kelly Del Tredici, Virginia M.-Y. Lee, John Q. Trojanowski³, ©2015.

Alzheimer's disease

AD was first described by the German psychiatrist Alois Alzheimer in 1907, who identified amyloid plaques and neurofibrillary tangles (NFTs) in the brain of a 51-year old female patient experiencing short-term memory impairment, which he called “presenile dementia”^{4,5}, later renamed to “Alzheimer’s disease”.

AD symptoms develop progressively, beginning years before diagnosis. Symptoms often commence with mild memory impairment and difficulties completing familiar tasks, advancing to difficulties with language, executive functions, semantic knowledge and working memory. In the late stages of AD patients require 24 hour care, losing awareness of personal experiences, the ability to communicate and developing an inability perform basic tasks such as walking, sitting and swallowing⁶. Late stage AD patients can also develop delusions and hallucinations, and eventually succumb to complications from AD such as dysphagia or pneumonia⁷.

There are currently no disease-modifying treatments available for AD, with current interventions relying upon NMDA-receptor antagonism and acetylcholinesterase (AChEI) inhibition (reviewed in⁸), both of which result in very minor improvements relative to placebo. In fact, after 6 months of treatment with the NMDAR antagonist memantine, patients experience an improvement of only 0.36 points on the mini mental state examination (MMSE) relative to those untreated, while AChEI’s resulted in only a 0.91-point improvement on the 30 point exam⁹. Given the suggested annual rate of decline on this scale of 4-5 MMSE points per year, these treatments represent a delay in cognitive decline of approximately 3 months^{9,10}.

Disease modifying therapeutics have been long sought-after for AD, with much of the focus over the previous 30 years surrounding amyloid- β ($A\beta$) and the amyloid cascade hypothesis. This idea surmises that, because $A\beta$ is a large component of senile plaques, that $A\beta$ “*is the causative agent of Alzheimer’s pathology and that the neurofibrillary tangles, cell loss, vascular damage, and dementia follow as a direct result of this deposition*”¹¹. This hypothesis suggested a seemingly intuitive target molecule for the development of $A\beta$ reducing drugs, such as anti- $A\beta$ immunotherapies and γ -secretase inhibitors, among others. Unfortunately, these drugs have all failed in clinical trials^{12,13}. It remains a possibility that these interventions would have some benefit if provided at an earlier time point before symptoms arise, or perhaps in combination with drugs addressing other mediators of AD. For instance, a multifactorial approach targeting $A\beta$, tau and inflammation simultaneously may be worthy of investigation. However, the failures of these monotherapies in the clinic show very clearly that there is insufficient knowledge about the etiology of AD.

AD can be subcategorized into two main groups. The first, early-onset AD (EOAD, also known as familial AD (fAD)), which accounts for approximately 5% of patients, and has a strong genetic component such as a mutation in presenilin 1/2 (PSEN1/PSEN2) or amyloid precursor protein (APP). The second form, late-onset AD (LOAD, also known as sporadic AD (sAD)), accounts for the remaining 95% of cases, and while certain alleles enhance the risk of developing LOAD (e.g. APOE4, TREM2), the underlying causes of LOAD pathogenesis remain unknown (reviewed in¹⁴).

While A β is not the sole mediator responsible for AD pathogenesis as previously proposed¹¹, it still remains an important target protein along with the microtubule associated protein tau (hereon tau). In AD, A β , TDP-43 and tau pathologies are observed in predictable brain structures, spreading among interconnected, previously unaffected brain regions, in a stereotypical manner (Figure 1)^{15–20}. In the case of tau propagation, symptomatic impairments correlating to damage in these newly affected brain regions can be observed, providing compelling evidence for tau as an important mediator of AD pathogenesis. In recent years, a great deal of evidence has shown that each of these proteins (A β , tau and TDP-43) all spread in a prion-like fashion^{21–27}. This discovery means that the misfolded proteins themselves can act as disease-causing “seeds”, whereby their interaction with naïve, natively folded proteins causes them to take on the pathogenic conformation and propagate further protein pathology in nearby cells. Further to this notion, there is also evidence to suggest that seeds of one protein (e.g. A β) have the capacity to induce the misfolding of other ND-related proteins (e.g. tau), so-called “cross-seeding” or heterotypic seeding^{28–30}. How these proteins are transmitted between cells remains unknown and is the basis of much of the work in this thesis (Figure 2). It is likely that successful, heretofore undiscovered treatments will approach this problem with a more holistic approach, targeting A β , tau, TDP-43, chronic inflammation and metabolic disturbances, and/or other currently unknown systems. By learning which cellular mechanisms are involved in the transmission of these protein aggregates (and whether there is a common mechanism for multiple ND-related proteins), we aim to identify novel therapeutic targets that could be used to inhibit the transmission of ND proteins, conceivably limiting disease progression.

Figure 2. Depiction of how oligomerization of ND-related proteins occurs and possible mechanisms by which they might transfer between cells. Monomers of ND-related proteins (shown here as α -syn, but similar processes occur for tau, $A\beta$, TDP-43, etc.) undergo conformational changes (often secondary structure changes and/or post-translational modifications) that promote its self-aggregation into oligomers and fibrils. These aggregates act as “seeds” that can recruit natively folded proteins to take on the aggregate-prone alterations and initiate an aggregation cascade. Oligomers of many of these proteins can initiate this process in non-self proteins as well, a process called heterotypic seeding or “cross-seeding”. Monomeric, oligomeric and fibrillar forms of these proteins can be transferred between nearby cells according to several possible mechanisms including exosomes, exocytosis/endocytosis, or in a specific receptor mediated fashion. Tunneling nanotube mediated transfer has been suggested but has not been demonstrated for ND proteins.

A β

A β is generated from the sequential cleavage of APP, a 770 amino acid (AA) transmembrane glycoprotein. The native function of APP is not completely understood, but it is thought to play a role in the function and repair of synapses, as well as neuronal plasticity and long-term potentiation^{31,32}. The processing of APP can occur by one of two different pathways: amyloidogenic and non-amyloidogenic (Figure 3). In the non-amyloidogenic pathway, α -secretase cleaves the extracellular domain of APP, generating a C-terminal fragment (CTF α) and a soluble APP fragment (sAPP α). Thereon, the CTF α can be further cleaved by γ -secretase, creating two by-products: the peptide P3 (AKA A β _{17-40/42}) and the AICD fragment (A β peptide and the intracellular domain). In the amyloidogenic pathway, APP is first cleaved by β -secretase, generating sAPP β and CTF β subunits, where CTF β can then be further processed by γ -secretase into the AICD and A β (reviewed in³³). Due to the ability of γ -secretase to recognize multiple possible cleavage sites within the CTF β , A β can be produced in a range of lengths, from 17 to 49 AAs^{34,35}. The modulation of these enzymatic cascades (e.g. γ -secretase and β -secretase inhibitors, α -secretase enhancers) was thought to be a promising target for potential treatments, under the hypothesis that inhibiting the amyloidogenic pathway would lead to a reduction in the amount of A β generated. Unfortunately, to date, these have all been unsuccessful (reviewed in³⁶).

In the AD brain, A β ₁₋₃₈, A β ₁₋₄₀ and A β ₁₋₄₂ are thought to be the most important to pathogenesis, where the hydrophobic A β ₁₋₄₂ isoform is most prominently associated with senile plaques (likely relating to its high propensity for fibrillization³⁷) relative to the hydrophilic A β ₁₋₃₈ and A β ₁₋₄₀^{38,39}. In the cerebrospinal fluid (CSF) of AD patients, A β ₁₋₃₈ and particularly A β ₁₋₄₀ are elevated relative to healthy controls, whereas A β ₁₋₄₂ is decreased³⁹⁻⁴¹. Currently, the CSF A β _{42/40} ratio is one of the most widely used diagnostic marker for mild cognitive impairment (MCI) and AD patients, and may be used to predict further cognitive decline^{39,40,42,43}.

During amyloid pathology, accumulations of A β are thought to cause damage to cells by impairing mitochondria, promoting Ca²⁺ dysregulation, increasing reactive oxygen species, contributing to synaptic dysfunction and cross-seeding other ND-related proteins such as tau and TDP-43^{30,44,45}. The fibrillization-prone A β ₁₋₄₂ fragment (and to a lesser extent, other A β isoforms) can self-assemble into higher ordered structures known as oligomers. This oligomerization occurs when natively folded α -helix structures take on an aberrant β -sheet conformation. These β -sheet structures, which are commonplace in amyloidogenic proteins, are highly hydrophobic and aggregation prone, and under the appropriate environmental conditions (pH, concentration), can self-aggregate (reviewed in⁴⁶). These aggregated A β oligomers are widely considered to be the basic subunit of A β amyloidopathies, eliciting the detrimental cellular effects noted above (reviewed in^{45,47}), and harbouring the ability to self-propagate in a prion-like manner^{21,24}.

Figure 3. APP processing can occur according to two main pathways: the amyloidogenic pathway and the non-amyloidogenic pathway. In the amyloidogenic pathway, sequential enzymatic cleavage of APP by β -secretase and γ -secretase generates $A\beta$ fragments, which may then self-aggregate into oligomers. In the non-amyloidogenic pathway, sequential cleavage by α -secretase and γ -secretase generates the non-aggregating P3 fragment.

Parkinson's disease and other Synucleinopathies

Parkinson's disease (PD) was first described by James Parkinson in 1817 in his manuscript "An Essay on the Shaking Palsy"^{48,49}, in which he described many of the symptoms of PD: tremor, rigidity and shuffling gait, which he suggested was related to the degradation of brain tissue. PD is the second most common neurodegenerative disorder globally, with patients experiencing rigidity, bradykinesia, tremor and gait disturbances, progressing to cognitive and behavioural complications as the disease advances^{50,51}. In the late stages of PD, patients commonly experience dementia, and much like AD patients, succumb to complications related to PD including falling, aspiration pneumonia and dysphagia^{52,53}.

In the brain, PD is characterized by intraneuronal deposits of aggregated proteins, known as Lewy bodies (LBs) and Lewy neurites (LNs) (when found in neuritic compartments), consisting primarily of misfolded α -synuclein (α -syn)⁵⁴. This α -syn deposition in dopaminergic neurons of the substantia nigra pars compacta (SNpc) contributes to the death of the affected cells, leading to striatal dopamine depletion and contributes to the movement symptoms seen in PD. Much like AD and other NDs, this progressive loss of neurons occurs in a predictable, progressive manner, beginning in the brainstem and spreading throughout interconnected brain regions (Figure 1)^{20,55}. The loss of neurons in the previously unaffected brain regions correlates with the progressive

symptoms related to each region's function (e.g. early brainstem damage is related to autonomic complications, while late-stage cortical degeneration is related to dementia)^{20,55,56}.

While PD pathogenesis is conclusively tied to α -syn, like other NDs, it involves considerable secondary contributors as well. These include, but are not limited to, mitochondrial dysfunction, deficiencies in the proteasomal degradation systems, inflammation and genetics⁵⁶⁻⁶⁰. In PD, 90-95% of cases have no known inherited cause (sporadic PD; sPD), while the remainder have known mutations influencing the onset of PD (familial PD; fPD), including genetic abnormalities in α -syn, Parkin and LRRK2 among others⁶¹. To date, there have been 23 identified loci which either increase risk for sPD, or contribute to fPD, named chronologically by date of discovery, PARK1-PARK23⁵⁶.

Current treatment for PD involves the pharmacological replacement of the depleted dopamine resulting from the loss of dopaminergic neurons in the SNpc. The use of levodopa (L-DOPA) as an antiparkinsonian prodrug was pioneered by Arvid Carlsson and George Cotzias (and colleagues) in the 1950's and 60's^{62,63}. The principles outlined in this study form the basis for L-DOPA treatment that are still in use today. Patients ingest L-DOPA, which crosses the blood-brain barrier, and is enzymatically processed into dopamine by DOPA decarboxylase, replenishing the previously depleted dopamine. While this treatment represented a substantial breakthrough, it is not without problems. As PD progresses, L-DOPA treatment becomes less effective, where administration can result in serious motor complications such as dyskinesias and dystonias (reviewed in⁶⁴). Because the motor symptoms of PD are related to the loss of neurons in the midbrain, Lindvall et al. grafted fetal stem cells into the midbrains of PD patients, under the assumption that the grafted cells could replace the lost neurons in the host⁶⁵. This procedure provided symptomatic relief to the patients for years, but ultimately the symptoms returned^{66,67}. Upon post-mortem analysis of the patients, they discovered that the grafted tissue had developed synuclein pathology, despite the tissue being from healthy donors. Further, the synucleinopathy observed in these individuals was unlikely to have developed *de novo*, as the tissues were analyzed only 12-16 years post-transplantation⁶⁸. This was a compelling demonstration of α -syn's ability to propagate from diseased tissue to healthy tissue, and was strong evidence for the prion-like protein propagation of α -syn.

The prion-like templating of α -syn occurs much like A β described previously, where misfolded α -syn acts as a seed to initiate further α -syn misfolding in previously natively folded α -syn^{28,69-73}. This misfolded α -syn also displays the cross-seeding phenomenon, whereby α -syn seeds can induce the misfolding of tau^{28,74}. In PD, α -syn deposition occurs primarily in the neurons, which leads to neuronal cell death. Beyond PD, there is a family of closely related NDs involving α -syn, termed synucleinopathies, and which also experience the deposition of misfolded α -syn. Two synucleinopathies with similarity in symptoms and disease progression with PD are multiple system atrophy (MSA) and dementia with Lewy bodies (DLB). PD, DLB and MSA all exhibit neuronal deposition of α -syn, but MSA is further characterized by α -syn pathology in the glia (particularly oligodendrocytes), which is thought to be generated in the neurons and transmitted in some fashion between neurons and oligodendrocytes⁷⁵ (reviewed in^{76,77}). The differential vulnerability of cell populations to α -syn (spatially, temporally and among different CNS cell

types) in different synucleinopathies is extremely complex, with the pathogenesis of each of these diseases involving many factors outside of α -syn itself. This hypothesis is referred to as selective vulnerability (reviewed in⁷⁸), and involves the consideration of inflammation, α -syn induced cytotoxicity, immune modulation, cellular energy demands, among others. This selective vulnerability is thought to contribute to the differentially affected brain cells and regions implicated in each disease, despite sharing α -syn as the primary disease mediator.

The discovery of the prion-like phenomenon involving α -syn provided important understanding with respect to disease etiology but proposes new challenges for disease-modifying therapeutics. We aimed to further understand how misfolded, aggregated α -syn is transmitted between cells, and in doing so hope to identify new targets which could be used to inhibit or halt the spreading of α -syn throughout the brain, thus diminishing disease progression. It is foreseeable that a therapy which successfully inhibits α -syn propagation within the brain could be use in a multimodal approach, in combination with additional therapies directed at the other mediators of synucleinopathies.

α -synuclein

α -syn is encoded by the *SNCA* gene, generating a 140 AA protein that, in healthy CNS tissue, localizes to synaptic terminals. α -syn is thought to stabilize SNARE complexes by interacting with vesicle-associated membrane protein 2 (VAMP2), a process implicated in vesicle recycling⁷⁹. The α -syn protein has three putative domains: the N-terminal domain, the core (AKA the non-amyloid- β component of AD plaques; NAC), and the C-terminal domain. The N-terminal domain (AA 1-60) carries a positive charge and contains several instances of the KTKEGV motif, a commonly expressed sequence in proteins with α -helical secondary structure. The NAC (AA 61-95) is thought to be most involved in the fibrillization of α -syn, as this domain has been shown to be necessary for α -syn fibrillization, but can also elicit fibrilization alone⁸⁰. Although the NAC region retains the moniker of the fibrillization domain, studies indicate that the residues extending into both N- and C-termini (between AA ~30 to ~110) modulate the effectiveness of NAC driven self-aggregation^{81,82}. Finally, the hydrophobic C-terminal domain (AA 96-140) lacks defined secondary structure and carries a negative charge, which under normal circumstances, is inhibitory to α -syn aggregation⁸³. The most clinically relevant region of the C-terminal domain is serine 129, which is highly phosphorylated in α -syn found in LBs relative to healthy tissue (>90% versus <4%, respectively)^{84,85}. While the phosphorylation of α -syn at Ser129 plays an important role in the physiology of α -syn (influencing α -syn protein/ion interactions, degradation, subcellular localization and membrane binding), it remains highly contentious as to whether pSer129 enhances or suppress α -syn aggregation and toxicity (reviewed in^{86,87}).

In the PD brain, intracellular inclusions consist predominantly of α -syn fibrils, but also involve other proteins such as ubiquitin and tau. These proteinaceous inclusions form the synucleinopathy hallmark LBs and LNs. In concert with *bona fide* prion proteins and A β oligomers, during α -synucleinopathy, the natively folded α -syn acquires β -sheet repeats, particularly within the expanded NAC region⁸². This α -helix to β -sheet morphological switch is likely an important moment in pathogenesis, as physiological α -syn exists as aggregation resistant tetramers^{88,89}, but when dissociated into monomers (resulting from secondary structure changes or single nucleotide

polymorphisms (SNPs), for instance), becomes highly aggregation prone^{90,91}. These monomers aggregate into higher ordered structures, oligomers (AKA protofibrils), which are widely considered to be the disease propagating subunit (reviewed in⁹²). These α -syn oligomers have the ability to initiate the misfolding and aggregation of natively folded α -syn, recruiting further α -syn aggregation and thus propagating α -synucleinopathy in a prion-like manner^{25,71–73,93}. Further, α -syn oligomers display the ability to cross-seed other ND proteins (especially tau), implicating α -syn in the pathogenesis of other NDs^{28,74,94}.

TDP-43 proteinopathies

Transactive response-DNA Binding Protein 43 (TDP-43) is known to play a key role in the pathogenesis of canonical TDP-43 proteinopathies such as frontotemporal lobar degeneration (FTLD) and Amyotrophic Lateral Sclerosis (ALS). FTLDs are progressive neurodegenerative disorders, and are the third most common form of dementia, including the second most common in adults under 65⁹⁵. Historically, FTLD was indistinguishable from Pick's disease, with the delineation of these disorders occurring as recently as the 1990's^{96–98}. FTLD is further subcategorized into two subtypes, behavioural variant FTLD (bvFTLD) and progressive aphasia subtypes (PPA). FTLD symptoms vary by subtype, but symptoms are related to the loss of brain matter in the affected regions, which can include behavioural disinhibition, apathy, loss of empathy, compulsive behaviour and deficits to executive functioning. The PPA subtype is more associated with language deficiencies such as comprehension, impaired speech production and dysphagia (reviewed in^{99,100}). As the disease progresses, dementia and parkinsonian motor symptoms can occur, with patients succumbing to complications such as asphyxiation, dysphagia and pneumonia in the same manner as other NDs¹⁰¹.

Similarly, in ALS, motor neurons in the brain (upper motor neurons; UMN) and brainstem/spinal cord (lower motor neurons; LMN) are increasingly degenerated in concert with symptoms relating to the affected CNS regions. First described by Charles Bell in 1824 and in 1874 coined ALS by Jean-Martin Charcot, both Bell and Charcot detailed the motor disturbances that are now understood to be associated with the disease^{102,103}. The initial symptoms of the disease include muscle weakness and atrophy, slurred speech and difficulty breathing or swallowing, eventually progressing to widespread motor deficits with patients losing the ability to use their hands and legs and inability to walk, speak and swallow (reviewed in¹⁰⁴). Much like with other NDs, ALS patients often succumb to dysphagia, pneumonia or respiratory failure¹⁰⁵. Unlike the other NDs discussed here, however, classical ALS patients rarely develop dementia, which is seen in patients with FTLD/ALS comorbidity (reviewed in^{104,106}). There is significant overlap in the symptoms and pathology of ALS and FTLD, leading to speculation that both of these disorders exist on a spectrum, differing mainly by the regions of the CNS that are afflicted¹⁰⁷.

Pathologically, both FTLD and ALS exhibit aggregated protein deposition in neurons. Although the brain regions afflicted are different in pure FTLD and ALS, both display nuclear clearance of TDP-43 into the cytoplasm, an indicator of TDP-43 pathology as TDP-43 is localized almost exclusively to the nucleus in healthy cells. During pathogenesis, TDP-43 manifests several post-translational modifications (PTMs) that contribute to its dysfunction, primarily phosphorylation, ubiquitination and truncations¹⁰⁸. These PTMs affect the localization of TDP-43 and lead to its

translocation to the cytoplasm, where it can no longer perform its native RNA-splicing functions. In FTLT, TDP-43 deposition begins in the cortex, while in ALS protein deposits are first seen in the spinal cord, brain stem and agranular motor cortex (reviewed in³). As the disease progresses, the TDP-43 pathology spreads to previously unaffected regions of the brain, and symptoms relating to affected regions arise along with loss of neurons in these areas (Figure 1). While TDP-43 has historically been associated with FTLT and ALS, recent evidence has shown that TDP-43 pathology also exists in more than 50% of AD patients, and those AD patients with TDP-43 pathology are ten-times more likely to suffer from cognitive impairment than those without^{18,19,109–111}. In addition to TDP-43 (also known as ALS10), several other proteins contribute to ALS etiology, such as Cu/Zn superoxide dismutase (SOD1; ALS1), fused in sarcoma (FUS; ALS6) and chromosome 9 open reading frame 72 (C9orf72) among others. In fact, to date, 23 ALS associated genes have been identified, deemed ALS1-ALS23^{112,113}. Pathological involvement in FTLT, however, may involve one or more of: TDP-43, tau, progranulin, C9orf72 and FUS (and others; reviewed in¹¹⁴).

In concert with other NDs, the large majority of those afflicted with ALS and FTLT have no obvious genetic cause for disease onset. Sporadic forms of ALS (sALS) account for approximately 90% of cases, with onset occurring around 60 years of age, while the remaining 10% of ALS patients have genetically influenced disease (fALS) and begin experiencing symptoms before 45 (reviewed in^{104,115}). Similarly, familial FTLT (fFTLT) accounts for about 10-30% of FTLT cases, where symptoms emerge at 50-55 years of age, while the remainder of cases occur with no obvious genetic basis (sFTLT) and manifest several years later (reviewed in^{114,116}). Currently, treatment of ALS is primarily restricted to the management of symptoms, where the anti-glutamatergic drug riluzole represents the only currently approved ALS treatment. Riluzole is thought to interact directly with glutamate receptors and delay glutamate excitotoxicity, which may extend patient survival for 2-3 months. Previous attempts to modulate oxidation, inflammation and neurotrophic factors have thus far proven unsuccessful (reviewed in¹¹⁷). There is currently no approved disease-modifying treatments available for FTLT, with symptom management being the only currently available option (reviewed in¹⁰⁰). In fact, attempts to implement the AD therapeutics memantine and AChEI's in FTLT patients proved to accelerate cognitive decline¹¹⁸.

While pathology of TDP-43 is known to spread throughout the brain in FTLT and ALS, it is poorly understood as to whether this is due to propagation of the TDP-43 protein itself. Previous studies have attempted to investigate the spread of TDP-43 and the possibility of prion-like propagation^{27,119,120}, however these phenomena are considerably less established compared to other ND proteins such as A β , α -syn and tau. Further, these previous studies did not consider whether cell to cell transfer is possible with truncated TDP-43, an important disease mediating PTM in TDP-43 proteinopathies. In a 2017 review on this topic by Hanspal et al., they concluded that *"It is still unclear whether TDP-43 consistently undergoes cell-to-cell transfer"*¹²¹. In Paper III, we demonstrate the ability of not only full-length TDP-43 to transfer between cells, but also N- and C-terminally truncated fragments. Considering our findings along with the current understanding of FTLT and ALS, it is reasonable to speculate that effective treatment of these diseases will involve mitigating cell to cell transmission of TDP-43 (and possibly other disease-relevant proteins), while also managing secondary mediators such as neuroinflammation.

TDP-43

TDP-43 is a 414 AA nuclear protein that is constitutively expressed in all tissues and is evolutionarily conserved, where TDP-43 orthologs are required for normal development in *C. elegans* and *D. melanogaster*^{122,123}. In higher ordered mammals such as mice, knockout of TDP-43 is embryonically lethal at E3.5-E8.5¹²⁴. The native functions of TDP-43 are multifaceted, but its most understood function is its transcriptional regulation through alternative splicing of pre-mRNA, where TDP-43 has been shown to interact with more than 4000 RNA targets¹²⁵. In TDP-43 proteinopathies, TDP-43 dysfunction is characterized at the molecular level by its inability to repress cryptic exons (reviewed in¹²⁶), leading to incorporation of irregular sequences into mRNA and ultimately culminating in the generation of aberrant proteins (e.g. incorporation of intronic sequences, missense/nonsense frameshifts). In two recent studies, it was shown that all examined ALS, FTLN and AD post-mortem tissues with TDP-43 inclusion pathology exhibited cryptic exon incorporation, as did those with nuclear depletion of TDP-43 without inclusions, implicating cryptic exon incorporation as an early pathogenic event in TDP-43 pathologies^{111,127}. TDP-43 has also been shown to regulate the 3'UTR of tau, which impacts the overall expression levels of tau, but TDP-43 is also responsible for splicing of exon 10 in tau pre-mRNA, influencing the 3R:4R tau ratio that is implicated in tauopathies (Figure 4)^{128,129}.

The TDP-43 protein has 4 distinct domains, the N-terminus (AA 1-105), RNA recognition motif 1 (RRM1; AA 106-192), RRM2 (AA 193-274) and the glycine-rich domain (GRD; AA 275-414). The functions afforded to each domain are highly contested, with many studies suggesting the importance of contrasting regions in their capacity to drive aggregation¹³⁰⁻¹³⁵. The Petrucelli group surmises that the extreme N-terminus (AA 1-10) of TDP-43 is necessary for homodimerization of TDP-43, which is required for its natural RNA-splicing activities, but also that this region is responsible for its aberrant aggregation¹³⁴. Conversely, Yang et al. describe RRM2 as the aggregation dependent domain (specifically AA 216-257)¹³⁵, while Hasegawa and colleagues indicate that sections of the GRD in the C-terminus are responsible for aggregation¹³¹. It is conceivable that each of these domains contribute to the pathogenesis of TDP-43 proteinopathies and that any one region of the protein is not solely responsible for aggregation. Nevertheless, most studies investigating TDP-43 proteinopathies examine the C-terminus, with particular focus given to the C-terminally truncated fragments. The presence of C-terminal TDP-43 fragments in proteinaceous cytoplasmic inclusions of ALS and FTLN were identified in 2006, the first report tying TDP-43 to ALS and FTLN¹⁰⁸. Since then, the C-terminal GRD has been shown to harbour the loci of the majority of disease causing TDP-43 mutations, but also important residue(s) for phosphorylation, specifically AAs 409/410. The serine 409/410 residues promote TDP-43 aggregation, cytotoxicity and mislocalization to the cytoplasm¹³⁶, to the extent that pSer409/410 is used in the staging of TDP-43 pathologies^{18,19,137}. Along with C-terminal truncations and phosphorylation at Ser409/410, there is the possibility of many other TDP-43 PTMs that contribute to pathology, such as N-terminal and protein core truncation events¹³², ubiquitination¹⁰⁸, acetylation, oxidation and others, although these are less understood (reviewed in^{138,139}). These PTMs ultimately culminate in the nuclear clearance of TDP-43 into cytoplasmic aggregated inclusions, the basic hallmark of TDP-43 proteinopathies.

Much like the previously described ND-related proteins A β and α -syn, amyloidogenesis of TDP-43 likely relies upon aggregation of β -sheet structures, possibly involving the conformational switching of α -helices to β -sheets. TDP-43 differs from A β and α -syn however, in that its native secondary structure is rich in β -sheets, particularly at the N-terminus¹⁴⁰. At the N-terminus, β -sheets are required for the formation of TDP-43 homodimers, therefore regulating its ability to recruit proteins necessary for its native RNA splicing function¹³⁴. This homodimerization mechanism may also be a prerequisite for the recruitment of other proteins during the establishment of protease-resistant aggregates¹⁴¹. In addition to the β -sheets observed in the N-terminus, TDP-43 also demonstrates the acquisition of further β -sheet structures during cellular stress, a common motif among amyloidopathies. The C-terminal domain of TDP-43 comprises of a prion-like Q/N repeat domain, which is prone to the switching of its natively disordered conformation to take on β -sheet rich conformations which are capable of spontaneous oligomerization^{142–144} and seeding^{3,26,27,120,131,144,145}. Lastly, these TDP-43 oligomeric seeds also retain the ability to induce the cross-seeding of A β ^{146,147}, and vice versa^{30,44}.

Figure 4. TDP-43 native functions and disease mechanisms. Under normal circumstances, TDP-43 is localized almost exclusively to the nucleus, where it performs transcriptional regulation through RNA splicing. In TDP-43 pathologies such as AD and FTL, TDP-43 acquires PTMs including truncations and phosphorylations (and others) that remit TDP-43 to the cytoplasm, inhibiting its ability to perform its native functions. The resulting cytoplasmic localization of TDP-43 alters the 3R:4R tau ratio by relative suppression of tau exon 10¹²⁹, enhances incorporation of cryptic exons leading to protein frameshifts and nonsense mediated decay^{111,127}, and increases overall tau expression through its inability to bind and process UG repeats in the 3' UTR of tau¹²⁸.

Tauopathies

In many of the NDs discussed previously, accumulation of tau contributes to disease in some capacity, with AD and FTLN variants (FTLD-17) implicating tau as a primary driver of disease^{15,16,148}. In addition to AD and FTLN, several other NDs fall under the tauopathy classification: Pick's disease (PiD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), argyrophilic grain disease (AGD) and others (reviewed in¹⁴⁹). These tauopathies can be further subcategorized by their expression of tau repeat domains, known as 3R tau and 4R tau, where the 3R isoforms arise from the omission of exon 10 of the tau gene (*MAPT*), while 4R isoforms include exon 10 (see Figure 4). The deposition of 3R or 4R tau into inclusions can be used to classify tauopathies, where PiD is characterized primarily by 3R tau pathology, while tau inclusions in AGD, PSP and CBD consist of mostly 4R tau. Further complicating matters, AD and FTLN contain mixtures of 3R and 4R, the ratio of which (i.e. 3R tau:4R tau) is thought to play a role in their respective pathologies^{150,151}. In each of these disorders, misfolded, aggregated and post-translationally modified tau are deposited into protease-resistant intracellular NFTs. The involvement of specific domains of tau in different tauopathies has led to the speculation that specific tau conformations or "strains" necessitate the involvement of disease specific isoforms for self-assembly and propagation in downstream cells^{25,152–154}.

Like the NDs discussed previously, tau demonstrates the ability to propagate throughout the brain along anatomical connections, from affected regions to naïve regions (Figure 1)^{3,155,156}. There is significant evidence to suggest that this occurs in a prion-like manner, whereby misfolded tau or specific tau PTMs effectively seed naïve tau, which then takes on the pathogenic conformation^{25,157,158}. Another commonality that tauopathies share with other NDs is that the large majority of cases occur sporadically, that is, with no dominantly inherited cause. While there have been more than one-hundred identified tau mutations, the overwhelming majority of these SNPs evoke FTLN, although a small number of SNPs are known to lead to PSP and CBD as well^{159–163}. Nevertheless, heritable tau mutations account for the minority of tauopathy incidence: FTLN, 13%; CBD, 19%; PiD, 8% and PSP, 6%¹⁶⁴.

There are still no disease-modifying treatments for tauopathies, although there are currently many ongoing clinical trials (reviewed in¹⁶⁵). An attractive idea currently being explored is the use of anti-tau immunotherapy. Investigators should be cautious in this approach, however, carefully considering the antigen used to generate antibodies. The prion-like templating in tau is well described, and is proven to propagate conserved tau phenotypes over several generations of seeding^{25,154}. Commonly occurring tau mutations (e.g. P301L in FTLN) likely give rise to similar tau strains, so an immunotherapy generated from this strain carries some probability of being effective among patients harbouring this SNP. However, sporadically arising tauopathies presumably express different strain phenotypes, relating to their secondary, tertiary and quaternary structure, which are influenced by phosphorylation, truncation, ubiquitination, etc. It is possible that immunotherapy directed against a single tau epitope proves effective across tauopathies, but it is reasonable to expect that disease specific (or even patient specific) anti-tau therapies will need to be produced.

Tau

In the human brain, alternative splicing of the *MAPT* leads to six canonical tau isoforms, each expressing either 0, 1 or 2 N-terminal inserts and 3 or 4 microtubule binding repeats (3R, 4R). The 3R:4R ratio is approximately equal in the cortex of healthy adult brains (3R:4R), and deviations from normal 3R:4R ratios are hallmarks of different tauopathies^{150,166}. The tau protein has four distinct domains, the N-terminal acidic domain, which encompasses 0, 1 or 2 inserts (AA 1-150; residue numbering according to the 2N4R 441 AA isoform), the proline-rich domain (AA 151-243), the microtubule binding region (MTBR), which encompasses the repeat domains (AA 244-370) and the C-terminal domain (AA 371-441). The MTBR is the region most involved with tau's native microtubule stabilization function. Under normal physiological conditions, tau exists as a soluble protein, taking on the so-called "paperclip" conformation, wherein the C-terminus folds over the MTBR while the N-terminus folds back over the C-terminus¹⁶⁷. The paperclip conformation is lost upon binding of the MTBR to microtubules, but can also be disrupted in solution via site-specific phosphorylations (reviewed in¹⁶⁸). During binding of the MTBR to microtubules, the N-terminal domain projects away from the microtubules, regulating interactions between the microtubules and other cell components¹⁶⁹.

During tauopathy, tau takes on an amyloidogenic, insoluble conformation containing numerous PTMs that have been widely described¹⁷⁰⁻¹⁷². This abnormal conformational state is driven predominantly by site-specific phosphorylation events within the proline-rich region of the molecule (e.g. AT8 (Ser202/Thr205), AT270 (Thr181), AT100 (Thr212/Thr214)), but also within the MTBR (e.g. Ser262) and C-terminal domains (e.g. PHF-1 (Ser396/Ser404))¹⁷³. In addition to the 85 possible phosphorylation sites, there are numerous other tau PTMs that may occur including glycosylation, glycation, truncation, nitration, ubiquitination, SUMOylation and oxidation, among others, however compared to phosphorylation, very little is known about these PTMs' contributions to pathology (reviewed in^{168,174}). Tau phosphorylation is thought to reduce the ability of tau to bind and stabilize microtubules, becoming prone to self-aggregation and further PTMs (especially truncation, further phosphorylation and conformational changes)^{170,175}. These initial PTMs incite a cascade of events that eventually leave pathogenic tau fragments harbouring the core of the MTBR, which are responsible for the self-assembly and transmissibility of tau *in vitro* and *in vivo*^{25,155}. The specific regions where aggregation begins is likely within the second and third MTBR domains (AA 275-280 (AKA PHF6*) and AA 306-311 (AKA PHF6), respectively), which harbour hexapeptide motifs that display high propensity to adopt β -sheet conformations capable of self-assembly¹⁷⁵⁻¹⁷⁷. It is hypothesized that tau dimerization occurs between two PHF6 regions, two PHF6* or one of each PHF6 and PHF6*, and that this dimer can recruit further tau monomers and dimers, eventually developing into a self-perpetuating trimer, the minimal unit of tau propagation, which then drives oligomerization and fibrillization (Figure 5)¹⁷⁸⁻¹⁸⁰.

Figure 5. Native function and pathogenesis of tau protein. Under normal circumstances, soluble tau exists in a paperclip conformation. The paperclip conformation is loosened upon tau's binding to microtubules, where this interaction acts to stabilize the microtubules. During tau pathology, the paperclip conformation is lost through the addition of PTMs, making tau unable to bind microtubules and perform its native function. After the acquisition of β -sheet conformation in R2 and R3 of the MTBR, tau can self-assemble into trimers and eventually higher ordered aggregates capable of prion-like propagation.

Perhaps more so than any other ND-related protein, the prion-like propagation of tau is well established and appears to rely upon highly stable prion-like tau "strains"^{25,152–154,158,181}. These tau strains recapitulate a consistent phenotype through several generations of seeding both *in vitro* and *in vivo*^{25,158}. This notion of strains is further exemplified throughout recent *in vivo* studies. For instance, tau fibrils originating from human AD brain induced less severe tau pathology in a tau mutant mouse model (P301L-GFP) than tau pre-formed fibrils (PFF) generated from synthetic P301L¹⁸¹. In contrast, tau PFF isolated from AD brain successfully induced robust tau pathology in wild type mice after 3 months (post-injection), whereas synthetically generated PFF required 24 months to induce miniscule pathology^{153,154}. While previous studies have demonstrated the potent seeding capability of 100% recombinant tau, this phenomenon likely requires virulent strains of tau (e.g. P301L), transgenic humanized tau expression, or both^{182–184}. Indeed, Falcon and colleagues have shown that recombinant, un-templated tau has a significantly diminished capacity to induce tau pathology, relating to the different physical properties of synthetic PFF versus brain derived seeds (conformation, PTMs, sequence, isoform, 3R:4R ratio)¹⁸⁵. Like previously discussed ND proteins, tau exhibits the ability to self-perpetuate, but it has also been shown that tau misfolding and aggregation can be initiated by other proteins (cross-seeding), although it is less clear whether tau aggregates themselves are capable of cross-seeding other ND-related proteins^{30,94}.

Tau phosphorylation plays a vital role in tau pathogenesis, but the temporality of these phosphorylations is poorly understood. Detection of the tau AT8 phosphoepitope is used clinically to stage AD disease progression, implying that tau is differentially phosphorylated at specific epitopes during different stages of disease^{15,16,171,172}. In Paper IV, we investigated tau phosphorylation (among other aspects of the proteome and phosphoproteome) after an acute challenge with oligomeric A β to model early AD. In this way, we aimed to identify which tau phosphorylation site(s) were implicated in the earliest stages of A β induced tauopathy.

Gap junction proteins

Gap junction proteins are a family of proteins that mediate cellular functions relating predominantly to intercellular communication. These proteins form porous channels between adjacent cells and facilitate the passage of small molecules such as ions, RNAs and peptides between cells. Each gap junction consists of two membrane spanning hemichannels, which are, in turn, made up of six individual proteins called connexins (reviewed in¹⁸⁶). Gap junction proteins are highly conserved evolutionarily, are expressed in nearly all mammalian cell types, and defects in the encoding genes lead to embryonic lethality or other serious disorders (reviewed in¹⁸⁷). In humans, there are twenty-one different connexin proteins, named according to their predicted molecular weight (e.g. connexin 43; 43 kDa) that are differentially expressed in different tissues. Adding further complexity to gap junctions, each hemichannel can be comprised of a single connexin subtype (homomeric) or a combination of different connexins (heteromeric), and the hemichannels may form junctions with alike hemichannels (homotypic) or unlike hemichannels (heterotypic) (Figure 6) (reviewed in¹⁸⁶). The number of possible combinations of connexins making up each gap junction channel is tempered by tissue-specific expression and the incompatibility of certain connexin subtypes during the formation of heteromeric hemichannels¹⁸⁸. Even so, there is a vast possibility for diversity among gap junction channels, relating to its connexin subunit makeup, which in turn modulates the function of the gap junction channel itself, altering cellular communication and ultimately function (reviewed in^{186,187}).

In the brain, several different connexin subtypes are expressed, with preference shown to specific isoforms in different cell lineages. For example, neurons are canonically associated with connexins 36 and 50 (although connexin 32 has been described in neurons), oligodendrocytes favour connexins 29, 32 and 47 while microglia mainly express connexins 32, 36 and 43^{189,190}. In Paper II, we investigated the possibility that oligomeric α -syn could be taken up by neurons and oligodendrocytes in a manner mediated by connexin 32.

Figure 6. Depiction of gap junction protein structure and function. Connexin 32 is made up of several distinct regions: four transmembrane domains, two extracellular loops, one intracellular loop, and intracellular N- and C-terminals. In Paper II, specific peptide mimetic inhibitors were used, which bind and occlude specific regions of connexin 32, shown here in red at their respective binding locations. Gap junctions consist of two hemichannels, which themselves consist of six individual connexin proteins. Hemichannels made of a single connexin are known as homomeric hemichannels, whereas those made up of more than one type of connexin are referred to as heteromeric hemichannels. Likewise, gap junctions made of different hemichannels are regarded as heterotypic gap junctions while those made up of like hemichannels are termed homotypic gap junctions. Gap junctions facilitate the passage of molecules such as ions, proteins, RNA and others between adjacent cells.

Connexin 32

Connexin 32, encoded by the *GJB1* gene, is a 283 AA transmembrane protein that, when assembled with other connexins (into two hemichannels), creates a gap junction between adjacent cells, facilitating intercellular communication. The connexin 32 protein encompasses four membrane spanning domains, two extracellular loops, one intracellular loop and intracellular N- and C-terminal tails (Figure 6)¹⁹¹. While connexins are canonically associated with intercellular communication through the formation of gap junctions, they also permit the direct exchange of material between cells and the extracellular environment through the formation of so-called “free hemichannels” (reviewed in¹⁹²). The specific function of each gap junction channel is tied to its makeup of connexin subunits, with different connexins being preferentially expressed in different tissues, promoting the specific functions necessitated by each cell type. Connexin proteins are constitutively expressed, with a rapid turnover rate (<2 hours) regulated by phosphorylation^{193,194}. Connexin 32 is typically associated with hepatocytes, oligodendrocytes, Schwann cells, and microglia, but has also been described in neurons^{186,187,189,190}.

Connexin 32 is also implicated in the pathology of X-linked Charcot-Marie-Tooth disease (CMT1X), a demyelinating CNS and peripheral nervous system (PNS) disease. There are >400 described mutations of the *GJB1* gene that cause CMT1X, characterized by symptoms including muscular spasticity, dysarthria and progressive cerebellar ataxia (reviewed in¹⁹⁵). In CMT1X, loss

of functional connexin 32 leads to aberrant neurofilament phosphorylation and distribution, causing disruptions in axonal transport (reviewed in¹⁹⁵).

Neurodegenerative disease as a continuum of protein misfolding disorders

Over the past decade, understanding of the factors underlying pathogenesis of NDs has greatly improved. The notion that ND-related proteins propagate in a prion-like fashion is well described, and there is a growing understanding that these different proteins can induce the heterotypic seeding of non-self proteins (Figure 7A). Further, there is an expanding appreciation for the involvement of non-canonically associated proteins to the disease process across NDs. For example, there is an increasing understanding that TDP-43 influences the prognosis of AD, while in ALS (generally considered a TDP-43 proteinopathy) shows contributions from A β and/or α -syn in almost half of ALS diagnosed cases^{18,19,137,148,196}.

The idea that pathologically similar NDs exist on a spectrum is not new. In 2010, Geser, Lee and Trojanowski surmised that FTLTDP and ALS “*should be refined in favor of the concept that TDP-43 proteinopathies represent a broad clinico-pathological spectrum of a single disorder involving multiple systems and sharing similar disease mechanisms*”¹⁹⁷. In a similar proposal, Günter Höglinger has advocated for removal of the delineation between PSP and CBD, instead proposing the classification of these disorders simply as 4R tauopathies, at least until such a time where more specific diagnostic criteria can be established¹⁹⁸. Recently, a meta-genome-wide association study was published wherein the authors analyzed the brains of 124,876 ND and control cases (ALS, AD, PD, CBD, PSP, FTLTDP and controls), examining whether ALS-related SNPs were associated with other NDs. Here, they identified that these SNPs (in genes *MOBP*, *C9orf72*, *NSF*, *UNC13A*) were associated with increased risk for sFTLTD, FTLTDP, PSP and CBD¹⁹⁹. This provided further evidence that these pathologies share a significant overlap in disease mechanisms.

Given the overwhelming similarities among NDs, but particularly FTLTDP, ALS, PSP and CBD, it is conceivable that these NDs might occur as points along a spectrum, stratified by the regional and temporal involvement of disease-causing agents. These include the primary misfolded proteins in addition to the involvement of secondary pathology (non-canonically associated misfolded proteins, but also other mediators such as inflammation, cellular dyshomeostasis, trauma, etc.). Considering that the vast majority of NDs occur sporadically, this hypothesis relies heavily upon the selective vulnerability of different CNS regions, especially during the onset of the initial pathogenic event, but also during disease progression. That is, the development of a protease-resistant, self-templating, propagation competent protein seed must occur in the microregion especially vulnerable to this specific pathogenic insult in order to successfully initiate pathology. For instance, if an A β protein were to accomplish the α -helix to β -sheet conformational switch and initiate self-aggregation in a region that is inherently (either genetically or through the accumulation of preceding insults) vulnerable to A β aggregation (such as the orbitofrontal neocortex or basal temporal lobe), this may be sufficient to initiate A β pathology. Conversely, if this same event were to occur in a region without inherent vulnerability to A β aggregates (e.g. cerebellar cortex), this singular misfolded A β aggregate might be digested effectively, preventing the onset of pathogenesis. This pathogenesis within the selectively vulnerable might also require

a “perfect storm”, where additional priming within the susceptible microregion (e.g. inflammation, high cellular energy requirements, cellular stress) is necessary during the initial pathogenic event. This is thought to underlie the vulnerability of the SNpc in PD, where the intense energy requirements of dopaminergic neurons within this region (relating to their long and highly dendritically branched axons) make these neurons particularly vulnerable to α -syn induced mitochondrial dysfunction (reviewed in^{200,201}).

As the disease progresses, cell damage and dyshomeostasis influences the recruitment of additional disease mediators, further establishing the vulnerable microenvironment for secondary events like the heterotypic seeding of misfolded proteins. The ND that develops in this scenario therefore arises due to the proportion of the involved ND proteins and other mediators in a specific brain region as influenced by its microenvironment. According to this hypothesis, many, if not all NDs, exist along a regionally and temporally defined continuum, classified by their proportional involvement of different ND proteins (tau, α -syn, A β , TDP-43, FUS, SOD1, C9orf72, etc.), emanating from sporadically acquired protein misfolds that act to initiate a progressive degenerative cascade reliant upon vulnerable microenvironments.

In the future, it may be useful to classify NDs as we know them today according to this type of paradigm, where the regional involvement of pathological proteins defines the clinical diagnosis rather than adjusting the ridged definitions of NDs with asterisks. For example, current definitions of AD assume pure A β and tau pathology, but we are becoming increasingly aware that most of these patients also exhibit comorbidity of TDP-43, α -syn or both (Figure 77B)¹⁴⁸. The current definitions of NDs are too rigid to accommodate the nuanced influences of the heterogeneous misfolded protein mixture that contribute to an individual’s disease. Instead, it would be beneficial to consider these disorders according to their unique proteinopathies and spatial distribution (e.g. Braak IV tauopathy, Josephs III TDP-43 proteinopathy, Braak I α -synucleinopathy). This holistic approach to NDs might encourage the scientific community to accelerate research into these heterogeneous diseases, where successful future treatments will likely address patients by their involvement of specific pathologies (e.g. tauopathy with A β and α -syn comorbidity) rather than approaching each disease as separate, monolithic disorders.

Figure 7. Visualizations of the cross-seeding phenomenon and NDs as a continuum hypothesis. Misfolded, aggregated proteins can recruit naïve proteins to become pathogenic in a cross-seeding manner (A). In this way, the canonical pathological hallmarks in a given ND rarely display “pure” pathology. Consequently, this cross-seeding mechanism may influence the secondary pathologies observed in NDs (B). Each line transects each protein at a position relating to the percentage of patients positive for each protein pathology^{148,202}. These two related ideas form the basis behind this proposal; that each proteinopathy may initiate the misfolding of non-self proteins (cross-seeding), and that each ND is a heterogeneous mixture of protein pathologies with more or less contribution from specific proteins in a given brain region.

AIMS

The aim of the thesis work was to elucidate some of the mechanisms by which neurodegenerative disease related proteins (A β , α -syn, TDP-43) propagate between neurons, thereby promoting pathogenesis. Further, once this protein transmission had occurred, understanding the effects mediated by the protein on naïve cells.

Paper I

In Paper I, we aimed to investigate the transmission and subcellular localization of different (monomer, oligomer, fibrillar) α -syn assemblies.

Paper II

The aim of Paper II was to explore the role that gap-junction proteins play in the uptake and propagation of oligomeric α -syn.

Paper III

In Paper III, we aimed to determine whether TDP-43 and truncated fragments of TDP-43 transfer directly between cells, and if so, which fragments were transmitted most readily.

Paper IV

The aim of Paper IV was to investigate the early effects elicited by oligomeric A β upon naïve neurons, examining the proteome and phosphoproteome.

METHODOLOGY

The following section briefly describes materials and methods used in the thesis work. For further details, refer to the method sections of each paper.

Models and sample material used

Cell models

SH-SY5Y cells

The SH-SY5Y cell line was used in Papers I-III. These cells were sub-cloned from the SK-N-SH cell line, which itself originates from the bone marrow biopsy of a four year old female, by selecting for neuroblast-like phenotypes^{203,204}. In 2010, a study was published by the Hallbeck lab which detailed the differentiation of these cells into neuron-like cells, which display several mature neuron markers, such as the neuronal nucleus marker NeuN and synaptic vesicle protein 2 (SV2)²⁰⁵. Briefly, SH-SY5Y cells are pre-differentiated with retinoic acid for 7 days, followed by a 10 day differentiation in extracellular matrix gel (ECM) with four growth factors: brain-derived neurotrophic factor (BDNF), neuregulin β 1 (NRG β 1), nerve growth factor (NGF), and vitamin D₃^{205,206}, resulting in neuron-like cells with extensive neuritic networks.

3-dimensional co-cultures of SH-SY5Y cells

In order to study the propagation of ND-related proteins between neuron-like cells, we use the 3D co-culture model previously established in the Hallbeck lab²⁰⁶. This protein transfer model is used in Papers I-III. The co-culture model consists of fully differentiated (ECM gel and growth factor cocktail as described above) SH-SY5Y “acceptor cells”, and so-called “donor cells”, which are SH-SY5Y cells differentiated in retinoic acid for 7 days. In Paper I, donor cells were treated with exogenous α -syn conjugated to Cy3, a red fluorophore, while acceptor cells were transfected with a baculovirus (BacMam), to induce the expression of Rab5-GFP. The differential fluorescence allows the discernment of the donor and acceptor cells populations.

In Papers II and III, cells were transfected and selected to stably express their respective constructs, rather than using transient transfection. This approach provides two main advantages: all cells will express the desired construct, whereas BacMam transduction results in only a fraction of construct expressing cells. Further, by having stable expression of the desired ND-related protein conjugated to a fluorescent reporter, we eliminate the need for the exogenous addition of protein, negating batch to batch variance of protein preparations, but also ensuring that within-cell protein concentrations are more uniform between experiments.

Long-term Neuroepithelial Stem Cells

Long-term Neuroepithelial Stem Cells (ltNES) were used in Paper IV. ltNES cells are well-described stem cells derived from iPSCs, retaining pluripotency and proliferative potential^{207–212}. Their ability to proliferate *in vitro* allows these cell lines to be particularly useful for the study of neurodegeneration, in that one can easily obtain the necessary numbers of cells needed for material intensive studies. Furthermore, upon differentiation these cells exhibit mature neuronal phenotypes, including functional electrophysiology, and can be differentiated into different neurotransmitter-releasing neuronal cell types²⁰⁷. The cells lines used in Paper IV, AF22 and ADP2, spontaneously generate primarily GABAergic neurons in a heterogeneous mixture along with glia,

ensuring a significantly more physiologically relevant model of a CNS-like environment compared to immortalized cell lines or primary cultures. In ItNES cells, proliferation is achieved by supplementing the growth medium with fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF), whereas differentiation is initiated by removal of these growth factors. ItNES cells were differentiated for 60 days in differentiation media: 50% DMEM/F12 with GlutaMax, 50% Neurobasal, supplemented with B27 and N2, where half of the media is replaced every day. ADP2 ItNES cells are derived from a donor harbouring the V717I (London) APP mutation, while the AF22 cells are derived from a healthy donor.

Other cell lines

In Papers I-III, the primary cellular model used was the differentiated SH-SY5Y model described above. However, in Paper II, several other cell models were used to confirm the initial findings from the SH-SY5Y model. These experiments included the use of HEK-293, rat OLN-93, oligodendrocyte progenitor cells (OPCs), and mouse primary cortical neurons. Differentiation of OPCs into myelin-basic protein (MBP) expressing oligodendrocytes were accomplished by 30 days differentiation in 50% DMEM/F12 with GlutaMax, 50% Neurobasal supplemented with B27, L-glutamine, ascorbic acid, BDNF, cAMP, non-essential amino acids, biotin, and thyroid hormone Triiodo-L-Thyronine. Mouse primary cortical neurons were obtained from mouse cortices on embryonic day 16 and cultured in Neurobasal supplemented with B27 and L-glutamine.

Human and animal tissues

Mouse Tissues

In Paper II, tissue from several different mouse lines were used to confirm the findings from the *in vitro* experiments. Wild-type mouse brains, as well as those overexpressing α -syn, were purchased from QPS Neuro, while A30P α -syn mutant and MBP29 humanized α -syn brains were provided by Professor Philipp Kahle²¹³ and Professor Eliezer Masliah²¹⁴, respectively. Cortices and corpus callosa were micro-dissected, homogenized, and used for Western blotting (see section on Western blotting for more information). All experiments were performed in accordance with approved ethical permits (see Paper II for detailed information).

Human Tissues

In Papers II and III, human CNS tissues were used to confirm *in vitro* findings. For Paper II, human MSA samples and age/sex-matched controls were obtained from the Netherlands Brain Bank, while PD brain tissue was obtained from the brain bank at Karolinska Institute (KI). Putamen, SNpc, and cortices from these sources were homogenized and subjected to Western blotting (see section on Western blotting for more information).

For Paper III, we used human brain and CSF tissues to verify and validate the extracellular vesicles (EVs) that were isolated from the TDP-43 cell lines. Here, temporal neocortices from healthy (non-demented) donors were provided by Professor Martin Ingelsson at Uppsala University. After tissue dissociation with papain, crude EVs were isolated from the tissues by differential centrifugation (see section on EV isolation). All experiments were performed in accordance with approved ethical permits (see Paper II and III for detailed information).

Generation of cell lines

Conventional molecular cloning

In order to obtain cells with stable expression of genes of interest tagged to fluorescent proteins (e.g. GFP, tdTomato, mCherry), we generated several cell lines using plasmid-based approaches. Most of the plasmids used in Papers II and IV were obtained from the non-profit plasmid depository AddGene. Where necessary, additional plasmids were purchased from commercial sources. Genetic alterations to these plasmids were done by conventional means, such as restriction enzyme cloning. Site-directed mutagenesis was used to make minor changes to plasmids (such as deletions to TDP-43 in Paper IV), and TSS chemically competent Stbl3 *E. coli* were used for transformation and production of plasmids²¹⁵. In Papers II and IV, plasmids containing neomycin resistance cassettes were transfected into cells using Lipofectamine LTX. Plasmid containing cells were then selected using G418 over several generations to establish stable expression of the desired construct.

CRISPR/Cas9

In Paper II, we designed and created clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 constructs in order to create a connexin 32 knockout (KO) SH-SY5Y cell line. Evolutionarily, CRISPR/Cas9 is part of the prokaryotic innate immune system that acts to excise virally introduced DNA from its genome. This system consists of two main parts: the scaffold, which contains the guide RNA (gRNA) followed immediately (3') by a protospacer adjacent motif (PAM), and the endonuclease Cas9. The PAM (in this case NGG) serves as the binding sequence and is immediately downstream of the sequence-specific gRNA. The scaffold forms a complex with Cas9, which induces a double-strand break (DSB) in the targeted DNA sequence. Next, the cell will attempt to repair the DSB and in a small percentage of cells, will make an error during repair, inserting or deleting (indel) nucleotide(s). These indels can cause frameshift mutations leading to premature stop codons in the open reading frame of the targeted gene. Since most DNA DSBs are repaired successfully, but also considering the wide variety of possible mutations that can occur during DSB repair, extensive screening (genetic, functional) of CRISPR/Cas9 generated clones is necessary.

In Paper II, gRNAs were designed against the connexin 32 gene *GJB1* using software (Benchling). In order to generate an effective KO, we designed the gRNAs to target exons 1 and 2 of *GJB1* to generate indels as close as possible to the N-terminus of the connexin 32 protein. Candidate gRNAs were inserted into the pSpCas9(BB)-2A-GFP plasmid²¹⁶, and transfected into SH-SY5Y cells, where GFP-positive cells could be selected by FACS for further screening.

Generation of aggregated proteins

α -syn

For Paper I, different assemblies of α -syn were generated: monomers, oligomers, and fibrils as previously described²¹⁷. In this study, oligomerization of monomeric α -syn was induced with the oxidative stress related aldehyde 4-hydroxy-2-nonenal (HNE). Recombinant α -syn was incubated with HNE and incubated at 37°C for different durations (0h, 18h and 120h to generate monomers, oligomers and fibrils, respectively). For fibrillar preparations, preparations were agitated during incubation. Next, each preparation was labeled with the Cy3 dye labeling kit according to

manufacturer's instructions. Each preparation of these Cy3-HNE- α -syn monomer, oligomer and fibril assemblies were subjected to size exclusion chromatography (SEC; see separate section on SEC) to collect appropriately sized assemblies, and to remove unbounded reagents/dyes. Characterization of the resulting α -syn assemblies was previously described by Näsström et al, using transmission electron microscopy (TEM)²¹⁷.

In Paper II, we again generated monomeric, oligomeric and fibrillar α -syn, however the oligomerization process here was induced by agitation alone. First, recombinant α -syn monomers were fluorescently labeled with ATTO-488 or ATTO-550 according to manufacturer's instructions. Thereon, labeled α -syn were either stored directly (monomers), or agitated at 37°C for 120h; 350 RPM (3mm orbit) to generate oligomers, and 1000 RPM to generate fibrils. As with Paper I, SEC was performed to confirm and select appropriately sized α -syn assemblies, and to remove unbounded reagents and dyes. Morphological characterization of the preparations was performed with TEM.

A β

Oligomeric A β was used in Papers II and IV. The oligomerization and characterization of A β oligomers generated by this method have been previously described^{206,218,219}. Recombinant A β 1-42 peptides are dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), lyophilized and resuspended in HEPES, vortexed, sonicated and incubated overnight at 4°C to generate oligomers. Generation of A β oligomers were confirmed and collected using SEC.

Size exclusion chromatography (SEC)

SEC was used in Papers I, II and IV. Since the outcomes of the studies outlined in this work are highly dependent upon aggregated A β and α -syn assemblies, it was important to ensure consistency between batches of aggregated protein preparations with respect to their aggregated state and size (monomer, oligomer and fibril). Additionally, it is important to remove any unwanted precursors from the preparations, such as unbound dyes, or unlabeled proteins. To accomplish this, we subjected the protein preparations to SEC. In this process, the aqueous protein preparations are loaded into a separation column packed with porous beads. As the solution passes through the column, small molecules enter the pores, while large molecules cannot, and consequently transverse the column faster. The elution time is therefore directly related to the size of the molecules, which are collected in different fractions and analyzed spectrophotometrically.

Transmission electron microscopy (TEM)

TEM was used to morphologically characterize the oligomeric α -syn in Paper II. This was not explicitly shown for α -syn and A β preparations in Papers I and IV, as TEM characterization of aggregates generated by these established methods have been well described previously^{206,217-219}. Here, monomeric, oligomeric and fibrillar preparations were added directly to carbon-coated nickel grids and negatively stained with uranyl acetate. Images were acquired with a JEOL 1400 TEM coupled to a CCD camera.

Extracellular vesicle isolation

Differential centrifugation

The non-specific term extracellular vesicles (EVs) encompasses many small vesicles, including exosomes, ectosomes and microvesicles, among others. EVs play an important role in cell to cell communication, as well as the uptake, recycling and release pathways of many types of small molecules. Some of the small molecule cargos mediated by EVs include DNA, mRNA, miRNA, small proteins, and even aggregated proteins in the case of A β and α -syn^{219–221}. In Paper III, we looked at whether TDP-43 could be found in EVs, and if so, whether they could propagate TDP-43 pathology. To isolate crude EV populations from each of the TDP-43 expressing cell lines, we followed the previously established differential centrifugation method²²⁰. After differentiation of the clonal cell lines, serum-free conditioned media was collected and centrifuged at 10,000 xg to remove cellular debris, and subsequently ultracentrifuged 100,000 xg. The pellet is resuspended in PBS and spun again at 100,000 xg. This pellet is again resuspended in PBS and used for downstream experimentation. All EV preparations were characterized by Western blot, probing for EV markers such as Flotillin-1.

To validate and verify the findings from our *in vitro* EV isolations, we also isolated EVs from freshly frozen human brain tissue and CSF. Here, the procedure was the same, with the exception of an added enzymatic (papain) tissue dissociation step prior to the centrifugation and confirmation of EV markers by Western blot.

Western blot

Cell and EV preparations

Papers II-IV utilized Western blotting of lysates from cell lines. Here, sufficient cell lysis and protein solubilization could be achieved by simply resuspending pelleted cells in 2% SDS lysis buffer supplemented with proteinase and phosphatase inhibitors. When preparing EVs for Western blotting as in Paper III, a gentler lysis buffer provided more reproducible results. In this case, we solubilized the EVs in a RIPA buffer containing 1% Triton X-100, and 0.1% SDS.

Tissue preparations

In Paper II, mouse and human brain tissues were analyzed by Western blot to validate findings from the *in vitro* work. Using tissues involves separate challenges compared to the straightforward process of lysing and solubilizing cell, arising from the heterogeneous mixture of tissue components: blood, interstitial fluid, connective tissues, fats, etc. These components make it difficult to fully homogenize the tissue and sufficiently solubilize protein, and so require different lysis buffers and homogenization techniques. In paper II, frozen brain tissues were mechanically homogenized in Qiagen QIAzol or RLT buffer (when also preparing mRNA from the same sample), using a Polytron PT 2100 hand-held homogenizer, followed by sonication to further dissociate any lysis resistant protein aggregates. Lysis buffers were supplemented with proteinase and phosphatase inhibitors.

Immunoblotting

In order to measure the levels of protein in cells and tissues, we used the common techniques of SDS-PAGE and Western blot in Papers II-IV. In the SDS-PAGE procedure, cells (or other biological material) are lysed in an SDS/DTT solution. This detergent results in the proteins being

negatively charged, and in combination with heating, denatures the proteins in the lysate. The DTT acts to reduce disulfide bonds within the protein, resulting in a negatively charged, linearized proteins. This solution is then centrifuged to pellet the SDS-resistant material, and the solubilized supernatant is then used for analysis. After loading this sample onto a polyacrylamide gel, a voltage is applied, which allows the separation of proteins according to their molecular weight. After transferring the proteins from the gel to a nitrocellulose membrane, proteins can be detected by the addition of specific antibodies conjugated directly (or indirectly, with animal immunoglobulin isotype-specific secondary antibodies) to reporter molecules, such as horseradish peroxidase (HRP). In the case of HRP, this enzyme acts to convert a substrate into a chemiluminescent product, which can be analyzed by an imaging system. Following image acquisition, specialized software, such as ImageJ, is used to measure the density of a given protein band, which correlates to its relative abundance in the sample.

Immunocytochemistry and Immunohistochemistry (ICC and IHC)

Immunocytochemistry (ICC) and immunohistochemistry (IHC) are common techniques used to visualize the localization of molecules within cells and tissues, respectively. In ICC and IHC, cellular structure and protein localization are first preserved with a cross-linking fixative such as paraformaldehyde. Next, in order to disrupt the cellular membranes to allow translocation of antibodies into the cells, the samples are permeabilized using detergents such as Triton X-100, TWEEN-20 or saponin. Samples are then blocked in a solution containing proteins such as bovine serum albumin (BSA) to reduce non-specific binding of antibodies, followed by the addition of the antibodies themselves. Here, primary antibodies specific to antigens are incubated with the samples, which bind to the protein of interest, and are subsequently treated with (animal Ig isotype specific) secondary antibodies. The secondary antibodies then bind to the primary antibody and are conjugated to reporter molecules such as fluorophores or enzymes that can then be used for microscopy. In IHC, following fixation, tissues must be sectioned thinly enough to allow the passage of light through the tissues in order to be useable for microscopy. This can be done by cryosectioning or by paraffin embedding and sectioning, the latter of which requires additional rehydration in xylene and ethanol before antibody conjugation can begin. After the sufficiently thin slices have been obtained, the processes of permeabilization, blocking, and antibody conjugation are the same as in ICC.

ICC was used in Papers I-II while IHC was used in Paper II. In Paper I, ICC was a major component of the study. Here, we probed donor and acceptor cells for proteins of interest in order to determine which of the targets (KDEL, SV2, TSG101, VAMP2 and LAMP2) colocalized with the added α -syn most readily. In Paper II, ICC was used for the identification of different cell lineages, such as β III-tubulin to denote neurons, and MBP which is specific to mature oligodendrocytes. This work also utilized IHC, primarily to show the colocalization of connexin 32 with α -syn.

Confocal Microscopy

Laser confocal scanning microscopy (hereon confocal microscopy) is a commonly used, versatile optical imaging technique that allows the acquisition of two-dimensional images at specific depths within a sample. This allows image acquisition at specific focal planes in the z-dimension (known

as optical sectioning), but also permits reconstruction of three-dimensional images from the compilation of the two-dimensional micrographs. Optical sectioning is achieved by exposure of the sample to a focused laser, whereby the emitted light is collected and refocused through a pinhole aperture, eliminating out-of-focus light. The diameter of the pinhole is therefore related to the “thickness” of the optical section and can be adjusted to allow more or less light to pass to detectors.

Confocal microscopy was used in Papers I-III. In Paper I, confocal microscopy was used extensively to characterize the colocalization of Cy3-labeled α -syn with several fluorescently labeled (by ICC) intracellular proteins of interest, before and after α -syn was transferred between cells. In Paper II, confocal microscopy was used to analyze the uptake and colocalization of ATTO-488 or ATTO-550 labeled α -syn with different connexin proteins. Paper III utilized confocal microscopy to determine the subcellular localization of GFP or tdTomato tagged TDP-43 and TDP-43 fragments, following collection by FACS. Here, we collected three-dimensional z-stack images of the collected cells to determine where the TDP-43 could be found in acceptor cells after protein transfer from donor cells, as translocation of TDP-43 from the nucleus to the cytoplasm is a measure of TDP-43 pathology.

Fluorescence-activated cell sorting (FACS)

Flow cytometry is a multi-parametric analysis tool that allows the evaluation of thousands of cells per second. In conventional flow cytometry, cells in suspension are analyzed with lasers, wherein the resulting light scatter is directly related to the cells' size and granularity. Furthermore, these lasers can excite fluorophores that are within, or bound to the cells, allowing the detection of different fluorophore containing populations of cells. In fluorescence-activated cell sorting (FACS), the underlying principles are the same, with the added ability to direct individual cell-containing droplets into collection tubes, therefore allowing the collection of specific fluorescently labeled cells. Droplets that meet the desired sorting criteria are electrically charged after breaking off from the stream, which then pass through two strongly charged deflection plates. These deflection plates electrostatically attract or repulse the charged droplets to direct them to the desired collection tubes.

In Papers II and III, flow cytometry was used to quantify the efficiency of protein transfer between donor and acceptor cell populations (see section on SH-SY5Y co-cultures) for α -syn and TDP-43, respectively. In Paper III, FACS was used to collect specific populations of donor and/or acceptor cells with detected transfer of TDP-43, allowing the downstream analysis of these populations with confocal microscopy.

Semi-quantitative reverse-transcriptase PCR (RT-PCR)

To measure mRNA expression of genes of interest, we used the semi-quantitative RT-PCR method. RNA was isolated from cell lysates using an RNA isolation kit, and RNA was converted to cDNA by reverse transcription. The conversion of RNA to cDNA is necessary due to the inherent instability of RNA, but also because the downstream PCR amplification requires both sense and antisense strands in order to successfully double with each cycle. The cDNA is mixed with a Taq polymerase containing master mix, along with a TaqMan primer/probe, whose sequence is specific to the gene of interest. Each TaqMan probe consists of three parts: a

fluorophore, a quencher and a sequence specific oligonucleotide. The fluorescence of the fluorophore is quenched by FRET (fluorescence resonance energy transfer) when in proximity with the quencher. During each PCR cycle, the Taq polymerase extends the primer, then the 5' to 3' exonuclease activity of the Taq polymerase displaces and degrades the probe, releasing the fluorophore. This fluorescence can then be measured and is directly proportional to the amount of cDNA template present in the sample, which doubles every PCR cycle. The cycle at which the fluorescent signal becomes discernable from background is known as the Ct value. The relative expression of each gene can then be calculated using the comparative $\Delta\Delta C_t$ method, which normalizes the expression of the gene of interest to housekeeping genes in control cells.

In Paper II, we used RT-PCR to determine whether there was any correlation between gene expression and protein expression for several genes/proteins of interest, including connexin 32 and α -syn. Using this method, we investigated whether mRNA expression levels changed in response to oligomeric α -syn, connexin inhibiting drugs, and in α -syn overexpressing brain tissues.

Cell viability assays

Two methods were used to measure cell viability: Sytox and lactate dehydrogenase (LDH) assays. In Paper II, the flow cytometry optimized Sytox assay was used to discriminate live from dead cell populations. Sytox dyes are a family of fluorescent nucleic acid stains able to penetrate compromised cell plasma membranes, such as those in dead or dying cells, where they bind to and dye DNA. Conversely, these dyes cannot penetrate the membranes of healthy cells. In this way, it is possible to fluorescently label dead cells for quantification by flow cytometry. In Paper III, the cytotoxicity of co-cultures was measured by LDH assay. When the cell membrane is damaged (as in the case of cell toxicity), otherwise intracellular molecules such as LDH are released into the media, which can be collected and analyzed. During analysis, the LDH catalyzes a series of enzymatic reactions to form a red formazan product that can be measured at specific wavelengths in a plate reader. The amount of formazan produced is proportional to the amount of released LDH in the medium, which is in turn, proportional to the number of dead cells.

Nano-liquid chromatography tandem mass spectrometry (nLC-MS/MS)

Principles of nLC-MS/MS

nLC-MS/MS is a powerful proteomics method that allows for the identification and quantification of unknown peptides in a given biological sample. In the preparation of samples for nLC-MS/MS, proteins need to be digested into peptide fragments. First, SDS is used to solubilize/denature proteins from cell or tissue lysates, then reduced with DTT and alkylated with iodoacetamide, which act to break disulfide bonds and prevent cysteine from reforming these bonds, respectively. Next, the proteins are digested with a proteinase. In this work, trypsin was used, which cleaves proteins at the carboxyl side of lysine and arginine residues, except when immediately followed by proline. This digestion generates peptide fragments, which are lyophilized and reconstituted in 0.1% formic acid (in water) to enhance peptide ionization. The peptides in formic acid solution are then loaded into a C18 analytical column, where they are separated according to their size and hydrophobicity with an increasing concentration of the organic solvent acetonitrile.

Peptides are then ionized by electrospray ionization (ESI), a process where a voltage is applied between the tip of the capillary and the mass spectrometer, leading to a spray of the ionized peptide solution. This spray causes the droplets containing charged ions to break apart in the air from solvent evaporation, releasing the charged ions into the mass spectrometer²²².

In our studies, we used a Thermo Scientific LTQ Orbitrap Velos Pro system, a hybrid instrument with two detectors: a linear ion trap and an orbitrap. Incoming precursor ions are first scanned in the linear ion trap, where the most intense ions are selected and dissociated into smaller fragments by colliding them with inert gas molecules in a process called collision-induced dissociation (CID). The fragmented ions enter the high-resolution orbitrap, where they rotate around and migrate along an electrode, which directly corresponds to each fragment's mass-to-charge (m/z) ratio. This system allows for high resolution, accuracy, sensitivity and speed.

Data processing after nLC-MS/MS

Each sample analyzed by nLC-MS/MS produces a large amount of spectral and chromatographic data. Precursor and product ion spectra obtained from the linear ion trap and orbitrap are used to sequence peptides. Software (Proteome Discoverer 1.4) was used to analyze the acquired spectra, which compares the m/z of the fragments against a proteome database; in this case the manually curated human UniProtKB/Swiss-Prot. By comparing the weight of the fragmented ions to these known peptide sequences (and therefore known weights), it is possible to determine from which protein (or protein family) each peptide fragment originated. It is also possible to identify PTMs in the dataset, since each PTM corresponds to a known weight shift.

Label-free quantification (LFQ)

Protein quantification in mass spectrometry is challenging, however there are several techniques available to accomplish this. Isotopic and isobaric labeling, which involve the utilization of stable isotopes that correspond to expected mass-shifts of peptides, allow the user to determine which sample a given ion originated from in a multiplexed assay. This allows for a fairly robust relative quantification, however, also involves significant downsides, including additional preparation time and substantial costs. In recent years, great strides have been taken to improve LFQ methods for mass spectrometry, now allowing for reliable quantification of MS data through spectral counting and precursor ion intensity, among other approaches (reviewed in^{223–225}). In Paper IV, we used software (Scaffold 4), to perform the LFQ of our acquired data using the normalized total TIC^{226,227}.

Phosphoenrichment and LFQ

In Paper IV, we examined the changes to the phosphoproteome and proteome of AF22 and ADP2 iPSC-derived neurons in response to acute oligomeric A β challenge. Detection of phosphorylated proteins within a complex proteome sample is challenging due to their high hydrophilicity, poor ionization and fragmentation difficulty. Further complicating matters, the low relative abundance of phosphorylated proteins in complex protein samples makes their detection difficult. Therefore, enrichment of phosphopeptides is essential to effectively study the phosphoproteome. We tried several methods to accomplish this, including enrichment with FeNTA and TiO₂, and fragmentation by CID, electron-transfer dissociation (ETD) and high-energy collisional dissociation (HCD), as ETD and HCD are reported to be more appropriate for studying

phosphopeptides (reviewed in²²⁸). However, since we were most interested in the phosphorylation of the ND-associated protein tau, with a minor interest in other phosphoproteins, we selected the combination of TiO₂ enrichment and CID fragmentation, as this resulted in the most reliable detection of phospho-tau, with the greatest degree of protein coverage in its disease related regions.

RESULTS & DISCUSSION

Paper I

Previously in the Hallbeck lab, it was shown that A β could be transferred directly between neuron-like cells using the established 3D co-culture cell model²⁰⁶. Given that AD and PD have similarly predictable progression, in that proteins are transmitted to predictable brain regions, we investigated whether similar protein transmission occurred for α -syn.

First, we examined whether the fluorescently labeled α -syn monomers, oligomers and fibrils could be taken up by donor cells, and found that fibrils and oligomers were taken up more readily than monomers (Figure 8A and 8B). Similarly, each of these different α -syn assemblies could be found in the acceptor cells after transfer. Fibrils were found to be transferred most readily (35% of acceptors), whereas oligomers and monomers transferred less efficiently (23% and 14%, respectively) (Figure 8C and 8D). Given these data, the uptake and transfer of aggregated α -syn appears to be related to the degree by which the protein itself is aggregated. This may be explained by the larger assemblies' resistance to proteasomal degradation, whereas less aggregated α -syn (monomeric) can be readily degraded. Further, there appears to be specific mechanisms that mediate the uptake of α -syn fibrils, including lymphocyte-activation gene 3 (LAG3) and heparan sulfate proteoglycans (HSPG)^{229,230}. While mechanisms for uptake of lower order α -syn assemblies also exist (see Paper II), the combination of degradation resistance and the greater receptor-mediated uptake might explain the disparity observed here.

Figure 8. α -syn fibrils are taken up and transferred more readily than oligomers and monomers. Representative images of the uptake of monomeric, oligomeric and fibrillar α -syn after 24h (A). Quantification of α -syn uptake as a percentage of total cells (B). Representative images of donor/acceptor cell co-cultures demonstrating colocalization (arrows) (C). Quantification of α -syn transferred for each of the different α -syn assemblies, presented as the percentage of total acceptor cells (D). Results presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. not significant.

To gain a further understanding of how the transfer of α -syn occurs and whether this was different for the different α -syn assemblies, we also examined its subcellular localization before (Figure 9A-C) and after transfer (Figure 9D-F). We quantified the colocalization of each of the α -syn assemblies with synaptic vesicles (SV2), multivesicular bodies (MVBs; TSG101), SNARE proteins (VAMP2), endoplasmic reticulum (ER)/Golgi derived proteins (KDEL) and lysosomes (LAMP2). From these data, we found that α -syn colocalized with lysosomes most readily in all scenarios (monomer, oligomer, fibril, before and after transfer), indicating that the lysosomal system is highly involved in the uptake and transfer of α -syn, perhaps signifying an attempt to degrade the excess protein.

Altogether, Paper I showed that all forms of α -syn (monomer, oligomer, fibrillar) are transferred readily between neuron-like cells in a manner involving the endosomal/lysosomal system. These findings have been supported by later publications^{231,232}.

Figure 9. α -syn colocalizes with LAMP2-positive lysosomes before and after transfer. In donor cells, α -syn monomers (A), oligomers (B) and fibrils (C) localize preferentially to LAMP2 structures relative to other intracellular compartments. Similarly, after protein transfer in acceptor cells, α -syn monomers (D), oligomers (E) and fibrils (F) colocalized with LAMP2 relative to the other markers investigated. In all cases, data are presented as percentages of total donor (A-C) or acceptor (D-F) cells examined. Results presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. not significant.

Paper II

Following the discovery of the α -syn fibril specific uptake mechanisms LAG3 and HSPGs^{229,230}, we investigated whether there could be a similar mechanism specific to the uptake of the oligomeric form of α -syn. In doing so, we examined the gap junction proteins connexin 26, 32 and 43 to explore their possible roles in facilitating the uptake of α -syn oligomers.

Using cell lines expressing different connexins, we observed that connexin 32 expression significantly increased the uptake of α -syn oligomers, and to a lesser degree, fibrils (Figure 10A-F). Further supporting this notion, this increase of oligomeric α -syn uptake was abolished when connexin 32 was knocked-out using CRISPR/Cas9 (Figure 10A-D). While connexin 32 is found in neurons¹⁸⁹, it is canonically associated with oligodendrocytes^{189,233,234}. It has also been shown that α -syn can be transferred between neurons and oligodendrocytes, hallmarks of PD and MSA, respectively^{75,235,236}. Because of the importance of oligodendrocytes to synucleinopathies and connexin 32, we repeated these oligomeric α -syn uptake studies in an oligodendrocyte cell line (OLN-93) and differentiated oligodendrocyte precursor cells (OPCs), and found that oligomeric α -syn was taken up more readily in these cells when overexpressing connexin 32 (Figure 10C and E). Similar to our observations in SH-SY5Y cells, we observed that this increase was abolished when connexin 32 was knocked-out of OLN-93 cells (Figure 10C).

To confirm these data by non-genetic means, we also inhibited connexin 32 pharmacologically using two approaches: the first, peptide mimetic Cx3211, which binds and occludes the first extracellular loop of connexin 32, thus inhibiting binding of proteins with connexin 32 specifically (see Figure 6). The second approach was treatment with 2-aminoethoxydiphenyl borate (2-APB), which inhibits connexins 26 and 32 by blocking capacitive current transients²³⁷. Because we had specific interest in connexin 32, neurons, and α -syn oligomers, we also examined another mimetic, Cx2409, which binds specifically to the intracellular loop of connexin 32, shown previously to inhibit the uptake of dyes and ions (see Figure 6)²³⁸. SH-SY5Y cells displayed a marked, dose-dependent decrease in the uptake of oligomeric and fibrillar α -syn in response to 2-APB, whereas Cx3211 treatment resulted in a significant decrease in α -syn oligomer uptake, but only a minor decrease in α -syn fibril uptake (Figure 11A-D). The trend observed in SH-SY5Y cells was also observed in the OLN-93 oligodendrocyte cell line (Figure 11E-H), while in primary neurons all treatments (2-APB, Cx3211 and Cx2409) displayed significant, dose-dependent decreases in both oligomeric and fibrillar α -syn uptake (Figure 11I-L). The difference observed between 2-APB (connexins 26 and 32) and Cx3211 (connexin 32 only) in the uptake of fibrillar α -syn may indicate a role for connexin 26 in the uptake of α -syn fibrils.

Figure 10. Uptake of α -syn oligomers is enhanced by the expression of connexin 32. Overexpression of connexin 32 increases the uptake of α -syn oligomers in neuron-like SH-SY5Y cells (A) and oligodendrocyte-like cells (C, E). Overexpression of connexin 32 increased uptake of fibrillar α -syn in SH-SY5Y cells (B), but not OLN-93 or OPCs (D, F). Knockout of connexin 32 (Cx32 KO) by CRISPR/Cas9 abolishes the enhanced uptake of α -syn oligomers and fibrils in SH-SY5Y and OLN-93 cells (A-D). Results presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. not significant.

In addition to the uptake experiments, we also observed that overexpression of α -syn led to higher rates of connexin 32 expression in SH-SY5Y cells, and that this increased connexin 32 expression resulted in the further uptake of exogenously added oligomeric α -syn, in a positive-feedback manner (Figure 12A-C). In tissues from α -syn overexpressing mice (Line 61 (humanized WT α -syn), A30P α -syn and MBP29 (humanized WT α -syn expressed in oligodendrocytes)), we detected significant increases in connexin 32 expression relative to WT mice (Figure 12D-F). In human tissues, connexin 32 levels were not significantly different in PD putamen or cortices but were decreased in PD SNpc and MSA putamen (Figure 12G-J). We speculate that this may be due to

the overall loss of neurons (and oligodendrocytes) at late disease stages in these regions, which could alter the ratio of connexin 32 expressing cells relative to control tissue. Finally, we also observed that connexin 32 overexpression was associated with increased uptake of oligomeric A β , leading to speculation that this uptake mechanism may be specific to oligomeric proteins, rather than oligomeric α -syn itself (Figure 13A and B). Altogether, Paper II provides strong evidence for a previously unknown mechanism for the uptake of oligomeric α -syn, but possibly other oligomeric proteins as well.

Figure 12. Increased α -syn expression leads to greater connexin 32 expression in a positive-feedback manner. In SH-SY5Y cells, overexpression of α -syn led to the increased uptake of exogenously added oligomeric α -syn (A) and increases to connexin 32 expression at the protein (B) and mRNA level (C). In mouse tissues, α -syn overexpression resulted in increased connexin 32 expression in three different lines: Line 61 (humanized WT α -syn overexpression) (D), A30P α -syn (E) and MBP29 (humanized WT α -syn expressed in oligodendrocytes) (F). In the PD brain, connexin 32 levels were unchanged in the putamen and cortices of PD patients relative to controls (G and H, respectively), while connexin 32 was found to be decreased in the SNpc of PD patients (I). In the putamen of MSA patients, connexin 32 expression was found to be decreased relative to control patients (J). Results presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. not significant.

Figure 13. Connexin 32 expression increases the uptake of oligomeric proteins other than α -syn. Oligomeric A β is taken up more readily in SH-SY5Y cells overexpressing connexin 32 compared to controls with baseline connexin 32 expression levels, as determined by Western blotting (A) and total image fluorescence (B). Results presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. not significant.

Paper III

TDP-43 is known to be implicated in canonical TDP-43 pathologies such as ALS and FTLN for over a decade, however there has been recent interest in TDP-43's contribution to the pathology of other NDs such as synucleinopathies and tauopathies including AD, where up to 57% of AD patients display TDP-43 pathology^{18,109}. TDP-43 shares several similarities with other NDs: TDP-43 PTMs greatly affect its cytotoxicity^{131,134,135}, TDP-43 spreads throughout predictable brain regions²³⁹, and misfolded TDP-43 can induce native TDP-43 to take on a misfolded morphology, demonstrating prion-like propagation^{26,27}. Despite this, very little is known about how TDP-43 is transferred directly between neurons, or if it occurs at all (reviewed in¹²¹). Given our lab's expertise in studying the cell to cell transfer of proteins, we adapted our 3D co-culture model (Figure 14A) to investigate whether TDP-43 is transferred between cells, and if so, which TDP-43 truncation events influence its transmissibility. Further, we also aimed to determine which cellular mechanism(s) might be involved in the protein transfer.

To study the transfer of TDP-43 fragments, we generated a number of cell lines stably expressing different TDP-43 constructs tagged to fluorescent proteins, encompassing different regions of the protein (Figure 14B). Using the 3D SH-SY5Y co-culture model, we quantified the amount of transfer for each of these TDP-43 fragments and saw that those with an intact N-terminus (Clone 6 and the full-length WT-TDP-GFP) demonstrated significantly more protein transfer compared to the other fragments (Figure 14C). As an RNA-binding molecule, TDP-43 is natively found in the nucleus, while pathogenic TDP-43 can be found in the cytoplasm¹⁰⁸, so we examined where each of the TDP-43 fragments localized before and after transfer. We found that localization was similar both pre- and post-synaptically and was strongly influenced by the presence or absence of nuclear localization and nuclear export sequences (NLS and NES, respectively). For example, Clone 5, which lacks both the NLS and NES was highly localized to the nucleus (Figure 15A). Conversely, Clone 6 displayed some degree of abnormal localization, in that cytoplasmic punctate structures were visible in addition to the expected diffuse nuclear labeling, indicative of TDP-43 pathology (Figure 15B). When examining the full-length TDP-43 clones, WT-TDP-GFP and tdTomatoTDP43, it was possible to observe healthy, diffuse nuclear localization, but also diffuse (WT-TDP-GFP) and punctate (tdTomatoTDP43) cytoplasmic localization (Figure 15C).

Figure 14. Preservation of the N-terminus of TDP-43 promotes its transmissibility between cells. A visualization of the co-culture model is provided in (A). Experiments involved the use of full-length TDP-43 and TDP-43 truncated fragments, all of which are fused to fluorescent proteins at the C-terminus (B). All fragments of TDP-43 transferred directly from donor cells (GFP) to td-tomatoTDP43 acceptor cells, where Clone 6 was transferred most readily among the different TDP-43 fragments, indicating that preservation of the N-terminus (and/or loss of the extreme C-terminus) of TDP-43 is influential to its transmissibility between cells (C). Results presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. not significant.

While performing these studies, we noticed that the EV marker CD63 appeared to be particularly relevant in regard to its protein transfer and colocalization with TDP-43. CD63-GFP experiments displayed among the highest amount of transfer (Figure 14C) and exhibited a high degree of colocalization with full-length TDP-43 (Figure 15D). Considering these findings along with our groups' previous publications that have demonstrated the transfer of oligomeric A β and α -syn via EVs^{219,221}, we aimed to investigate whether TDP-43 could be transferred by EVs. From our stably expressing TDP-43 cell lines, we collected and isolated EVs from conditioned media and treated naïve cells with these preparations, but were unable to observe any appreciable transfer of TDP-

43, even using 5-fold higher concentration of EVs than described previously²¹⁹ (Figure 16A). To allow for the possibility that other extracellular mediators (e.g. apoptotic bodies, free protein) might facilitate the transfer of TDP-43 and its fragments, we also performed co-culture experiments using physical separation with transwell inserts, but again saw no protein transfer under these circumstances (Figure 16B and C).

Figure 15. Representative images depicting cellular localization of GFP-tagged TDP-43 fragments (donor cells; green) after transfer to full-length td-tomatoTDP43 acceptor cells (acceptor cells; red). Expression of NLS and NES were important to the compartmentalization, where Clone 5 lacking the NLS and NES is found exclusively in the nucleus (A). Clone 6, which demonstrated the greatest propensity for protein transfer, and exhibits an intact N-terminus, displayed a mixture of cytoplasmic and nuclear localized puncta (B), whereas the full-length WT-TDP-43 clone was found in the cytoplasm (C), indicative of TDP-43 pathology. Substantial colocalization of full-length TDP-43 from acceptor cells was observed with CD63 (D), indicating that EVs might play a role in transfer of TDP-43, which was further examined.

Paper III shows that fragmented TDP-43 and full-length TDP-43 are transferred efficiently between neuron-like cells. While full-length TDP-43 and all N- and C-terminally truncated fragments displayed some degree of transfer between cells, those with intact N-termini were transferred more readily. This cell to cell protein transfer is mediated by a mechanism dependent on physical proximity, possibly occurring across the synaptic cleft itself, as suggested previously²⁴⁰. These findings provide novel insights into the disease propagation mechanisms of TDP-43 proteinopathies, which could be important in the search for disease-modifying therapeutics for TDP-43 proteinopathies.

Figure 16. Transfer of TDP-43 and TDP-43 fragments requires physical connectivity between cells. Isolated EVs were added directly onto WT SH-SY5Y cells but failed to achieve demonstrable protein transfer (A). Further, donor and acceptor cells were physically separated during co-culture using transwell inserts, allowing the distribution of cell products within a shared medium while creating a physical barrier between the cells (B and C), but proteins also failed to transfer by this method. Results presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. not significant.

Paper IV

In our previous studies, we have concentrated on protein transfer between the cells themselves, with less emphasis paid to the downstream effects. In much of the previous and ongoing research in the AD and ND fields, researchers examine chronic exposure to A β (animal models) or short-term exposure to A β using mouse/rat neurons or cancer cell lines. While this has provided valuable information towards the etiology of AD and NDs, there remains an absence in the understanding of the first stages in the disease cascade induced by A β challenge, particularly with human neurons. In Paper IV, we aimed to investigate the changes elicited by acute oligomeric A β challenge upon human neurons, using iPSC-derived cells. In this way, we recreate a scenario in which naïve neurons are acutely exposed to oligomeric A β , mimicking the spread of A β to previously unaffected brain regions. Using this early exposure model, together with nLC-MS/MS and label-free quantification (LFQ), we were able to detect changes to the proteome and phosphoproteome elicited by this acute exposure to oligomeric A β .

In this study, we used iPSC-derived neurons from donors of two different APP lineages; AF22 cells with WT APP, and the ADP2 line which harbours the London APP mutation (V717I), one of the most common fAD mutations, which results in clinical dementia at an average age of 59^{241,242}. This mutation results in an increased A β ₁₋₄₂:A β ₁₋₄₀ ratio in the AD brain and in patient derived iPSCs through the relative increase in production of A β ₁₋₄₂^{241,243-245}. After 60 days of differentiation, iPSC-derived ItNES cells display mature neuronal phenotypes such as mature electrophysiology and expression of neuronal markers, including those for neuronal nuclei (NeuN) and neurons (β -III-tubulin, MAP2 and GABA), among others²⁰⁷. The neurons were then treated with oligomeric A β over 24h and collected for LFQ by nLC-MS/MS.

We found 42 proteins that were significantly up- or downregulated in response to this treatment in AF22 neurons, while ADP2 neurons only exhibited significant changes in 11 proteins. The differences in the amount of changes elicited by the oligomeric A β between neurons from each genotype was presumably related to the substantial baseline expression of A β ₁₋₄₂ in ADP2 neurons. This hypothesis presumes that during the proliferation and differentiation processes, there is a selection pressure among the developing ADP2 neurons, favouring those with somewhat of a resistance to A β ₁₋₄₂'s cytotoxic effects that ultimately results in a relatively muted response to the exogenously added A β ₁₋₄₂. Nevertheless, we found oligomeric A β induced both ADP2 and AF22 neurons to significantly downregulate coatomer complex proteins (COPG1 in ADP2 (fold change = 0.2) and COPD in AF22 (fold change = 0.2)) (Table 1). COPD and COPG1 are subunits of the larger protein complex COPI, where COPI-coated vesicles facilitate retrograde transport of protein cargo from the Golgi to the ER (reviewed in²⁴⁶). COPI function is important in AD pathogenesis, as it regulates APP trafficking, maturation and therefore production of A β peptides²⁴⁷. Furthermore, SNPs in the COPI subunits gamma-1 and delta have been identified as risk factors for the development of sAD, causing intracellular protein accumulations, ER stress, and promoting deposition of NFTs^{248,249}. Conversely, COPI downregulation, as we observed here, may be protective, leading to accumulation of APP at the Golgi and therefore hindering its maturation into A β ^{247,248}. The identification of COPI in both the WT and APP mutant neurons, along with its known role as a sAD risk factor, provides strong evidence for its role in the early A β response, and should be studied further.

Table 1. Selected proteins of interest demonstrating fold change and p-value (T-test, Benjamini-Hochberg correction) in response to 24h oligomeric A β treatment. The table depicts proteins with changes in expression relative to untreated cells (i.e. AF22 + oligomeric A β relative to AF22 untreated). Full data tables available in Paper IV.

APP genotype	Identified Protein	Protein ID	T-Test (p-value)	Fold Change	PTM
WT (AF22)	Coatomer subunit delta	COPD_HUMAN	0.026	0.2	/
WT (AF22)	TAR DNA-binding protein 43	TADBP_HUMAN	0.038	0.4	/
WT (AF22)	Microtubule-associated protein tau	TAU_HUMAN	0.25	1.8	/
WT (AF22)	Microtubule-associated protein tau	TAU_HUMAN	0.100	1.3	Phosphorylation (TiO ₂ enrichment)
V717I (ADP2)	Coatomer subunit gamma-1	COPG1_HUMAN	0.028	0.2	/
V717I (ADP2)	TAR DNA-binding protein 43	TADBP_HUMAN	0.64	0.8	/
V717I (ADP2)	Microtubule-associated protein tau	TAU_HUMAN	0.33	1.8	/

TDP-43 was significantly downregulated in response to A β in AF22 neurons (fold change = 0.4), but not in ADP2 neurons (Table 1). In animal models of AD, TDP-43 levels are initially indistinguishable from the WT but increase over time during chronic oligomeric A β exposure²⁵⁰. We suspect that there is an initial reduction in TDP-43 expression during initial oligomeric A β exposure, which gradually increases over time as the cell is exposed to A β chronically^{241,250}. This may explain why TDP-43 reduction was not as robust in the ADP2 neurons, owing to their chronic exposure to endogenously generated A β ₁₋₄₂ during differentiation²⁴¹. It is possible that the difference in TDP-43 regulation between AF22 and ADP2 neurons in response to A β observed here is a contributing factor toward the differences in sAD versus fAD pathogenesis.

Next, we examined the phosphoproteome in AF22 neurons using TiO₂ enrichment prior to nLC-MS/MS. Phosphorylation is one of the most common PTMs and is involved in the pathogenesis of AD, especially the phosphorylation of tau. We found that global phosphorylated tau expression was elevated in response to oligomeric A β , but this elevation did not achieve statistical significance (fold change 1.3, p=0.10) (Table 1). However, we did observe that at residue Ser208 of tau (numbered according to the 2N4R, 441 AA isoform of tau), 75% of the oligomeric A β treated samples displayed phosphorylation, whereas only 27% of the control samples experienced phosphorylation at the same site. Other canonical tau phosphorylation sites including early (Thr181, Ser202/Ser205 and Ser262), intermediate (Thr231 and Thr212/Ser214) and late residues (Ser396/Ser404 and Ser422)^{15,171} did not display any marked differences in phosphorylation. This finding indicates that Ser208 should be further investigated as a potential early marker of tauopathy in A β related NDs.

Paper IV identifies several proteins and phosphoproteins that are up- and downregulated in response to acute oligomeric A β challenge, including TDP-43, COPI- δ/γ and phosphorylation of tau at Ser208, among others. The proteins and phosphoproteins identified here should be further studied to understand their contributions to the early pathogenesis of A β induced pathology in NDs.

CONCLUDING REMARKS

Neurodegenerative diseases are becoming increasingly prevalent in society as the global population ages, with an estimated 50 million people currently suffering from dementia worldwide. Despite this epidemic, there are still no disease-modifying therapeutics available to patients. Many attempts have been made to develop disease-modifying therapies for neurodegenerative diseases, which have targeted amyloid- β plaques, tau tangles, neuroinflammation, and other mediators, but none have been successful. These failures indicate that as a scientific community, we currently do not have sufficient understanding of the factors that cause these disorders. Until we learn how these diseases begin and progress, it will remain difficult to design or discover effective pharmaceutical interventions.

The work in this thesis aimed to expand the understanding about how misfolded, aggregated proteins are internalized and transmitted, but also how these proteins initiate damage to cells. The proteins investigated in this work (α -synuclein, amyloid- β , TDP-43) all exhibit the ability to propagate pathology throughout the CNS, doing so in a prion-like manner. While it has been shown that this phenomenon occurs in the CNS, there is relatively little known about the cellular and molecular mechanisms that facilitate this propagation and ensuing disease progression.

Using *in vitro* models, we have shown that α -synuclein can be internalized via the endosomal/lysosomal system but have also identified a previously undescribed mechanism of oligomeric protein uptake mediated by the gap junction protein connexin 32. The identification of these mechanisms of protein uptake provide new understanding at the molecular level about how these neurodegenerative disorders propagate. In this study, we demonstrated that the occlusion or removal of connexin 32 was an effective way to mitigate the uptake of α -synuclein oligomers, which should be further investigated for its role as a therapeutic target among α -synucleinopathies.

We have also performed studies to understand the contribution of PTMs toward the disease process of TDP-43 proteinopathies. By expressing different portions of the TDP-43 protein in cells, we were able to determine that the N-terminus of TDP-43 enhanced its propensity to transfer between cells, which demonstrates this region's importance to disease progression. The N-terminus has been historically overlooked, as the C-terminal fragments are often considered to be more pathogenic. We hope that this work will spark an interest in the further study of the TDP-43 N-terminus as an important contributor to the disease progression of TDP-43 proteinopathies.

Finally, we investigated the early effects that oligomeric amyloid- β exerts upon human neurons using an iPSC-derived neuron model, coupled with LC-MS/MS and label-free quantification. By using these neuronally differentiated cells, we were able to show that amyloid- β elicits widespread, robust changes to the cells within 24h of internalization. We showed that TDP-43, retrograde transport COPI proteins and tau phosphorylation at serine 208 were among the earliest detectable changes experienced by neurons under amyloid- β induced duress. This work provides new understanding about how the amyloid- β initiates cellular damage in newly challenged neurons.

Altogether, the studies outlined in this thesis were aimed to elucidate some of the cellular mechanisms by which neurodegenerative disease-causing proteins are taken up and propagating between cells, modeling disease progression at the cellular level. The discovery of connexin 32 as an oligomer-specific uptake mechanism warrants intensive future studies for its potential importance to the progression of Parkinsonian disorders. The further identification of the importance of the TDP-43 N-terminus toward transmissibility and the early responding factors to amyloid- β challenge should also be further studied for their roles in their respective diseases' progression.

It is my belief that any successful, yet to be developed therapeutics are likely to involve the mitigation of processes outlined in this thesis: protein uptake, protein transmission and protein propagation. Previous clinical trials have relied upon removal of proteinaceous deposits from mid to late-stage patients who have already experienced irreparable amounts of neurodegeneration. It is conceivable to envision a multi-faceted, combinatorial intervention targeting several aspects of neurodegeneration: protein-specific immunotherapies, blockage of protein uptake, inhibition of protein oligomerization, anti-inflammatory treatment, etc. However, the successful treatment of these patients likely requires earlier screening and diagnosis than what is available with current technologies. Until it is possible to accurately diagnose patients with neurodegenerative disorders at an early stage (i.e. before the widespread loss of neurons), the effectiveness of therapeutics is likely to be marginal at best.

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