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# CRISPR/Cas9-based therapies and the role of astrocytes in Alzheimer's disease and Parkinson's disease

EVANGELOS KONSTANTINIDIS



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### Abstract

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Alzheimer's disease (AD) and Parkinson's disease (PD) are the two most common neurodegenerative disorders. Whereas the AD brain features plaques of amyloid-beta ( $A\beta$ ) and neurofibrillary tangles of tau, the PD brain is characterized by Lewy bodies and Lewy neurites containing  $\alpha$ -synuclein ( $\alpha$ Syn). Rare familial disease forms have illustrated a central involvement of these proteins in the respective pathogeneses. Mutations in the genes for the presenilins (*PSEN1*, *PSEN2*) result in AD by an increased generation of the more aggregation prone  $A\beta$ 42 peptide, whereas mutations in the  $\alpha$ Syn gene (*SNCA*) cause PD by affecting aggregation of  $\alpha$ Syn.

This thesis has investigated the gene editing tool CRISPR/Cas9 as a potential treatment strategy against AD and PD. When targeting *PSEN1* M146L in patient fibroblasts, the increased  $A\beta$ 42/ $A\beta$ 40 ratio was partially restored and the treatment typically normalized the mutation-induced conformation of presenilin 1. Moreover, the treatment did not cause any major off-target effects across the genome. For *SNCA*, both the wild-type form and the A53T mutant were targeted. Lentivirus-mediated delivery of CRISPR/Cas9 to patient fibroblasts and HEK293T cells led to a targeting efficiency of up to 87%. However, treatment of A53T mutant patient fibroblasts only resulted in low and inconsistent targeting efficiencies.

During the course of AD, progressive cellular dysfunction and degeneration cause widespread neuronal death. Apart from neurons, also glial cells are affected by the disease process. Astrocytes, the most abundant glial cell type, play a key role in maintaining brain homeostasis. However, in a neurodegenerative environment, astrocytes enter a reactive and inflammatory state that can potentially harm nearby neurons.

To further investigate the role of astrocytes in AD, we generated a co-culture system of human induced pluripotent stem cell-derived neurons and astrocytes. We observed a differential effect of direct and remote astrocytic control on neuronal viability and functionality. Physical astrocytic contact combined with the presence of  $A\beta$  resulted in increased phagocytosis and clearance of dead cells as well as a reduced neuronal activity. However, indirect contact via conditioned media from control astrocytes improved the viability of neurons, whereas addition of  $A\beta$  led to hyperactivity. Analyses of long-term astrocytic cultures revealed a persistent reactive state accompanied by a limited  $A\beta$  degradation capacity and severe cellular stress.

Overall, this thesis has explored novel gene therapeutic strategies for AD and PD as well as contributed with knowledge regarding the role of astrocytes in AD progression.

*Keywords:* Alzheimer's disease, Parkinson's disease, gene editing, CRISPR/Cas9, amyloid-beta, astrocytes, neurons, iPSCs, electrophysiology

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*To the family we're born with  
and to the family we make along the way...*



# List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. **Konstantinidis E**, Molisak A, Perrin F, Streubel-Gallasch L, Fayad S, Kim DY, Petri K, Aryee MJ, Aguilar X, György B, Giedraitis V, Joung JK, Pattanayak V, Essand M, Erlandsson A, Berezovska O, Ingelsson M. (2022) CRISPR-Cas9 treatment partially restores amyloid- $\beta$  42/40 in human fibroblasts with the Alzheimer's disease *PSEN1* M146L mutation. *Molecular Therapy Nucleic Acids*, 28:450-461.
- II. Molisak A, **Konstantinidis E**, Aguilar X, Giedraitis V, Stefanis L, Vekrellis K, Ramachandran M, Sarén T, Erlandsson A, Mary B, György B, Essand M, Ingelsson M. CRISPR/Cas9 as a tool to disrupt wild-type and A53T *SNCA* in sporadic and familial Parkinson's disease. *Manuscript*.
- III. **Konstantinidis E**, Portal B, Mothes T, Beretta C, Lindskog M, Erlandsson A. Amyloid-beta accumulation in astrocytes affects their impact on neuronal function in a human iPSC-based model of Alzheimer's disease. *Submitted Translational Neurodegeneration*.
- IV. **Konstantinidis E**, Dakhel A, Beretta C, Erlandsson A. Long-term effects of amyloid-beta accumulation in human iPSC-derived astrocytes. *Manuscript*.

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# Abbreviations

AAV	Adeno-associated virus
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AICD	APP intracellular domain
ALP	Autophagy-lysosomal pathway
APH	Anterior pharynx defective
<i>APOE</i> /ApoE	Apolipoprotein E (gene/protein)
APP	Amyloid precursor protein
AQP	Aquaporin
ATP	Adenosine triphosphate
A $\beta$	Amyloid-beta
BACE	Beta-site APP cleaving enzyme
BBB	Blood-brain barrier
Cas	CRISPR-associated protein
CMA	Chaperone-mediated autophagy
CNTF	Ciliary neurotrophic factor
CNS	Central nervous system
CRISPR	Clustered regularly interspaced short palindromic repeats
CTF	C-terminal fragment
ddNTPs	Dideoxynucleoside triphosphates
DNA	Deoxyribonucleic acid
EEs	Early endosomes
ELISA	Enzyme-linked immunosorbent assay
EPSCs	Excitatory post synaptic currents
ER	Endoplasmic reticulum
EVs	Extracellular vesicles
FDA	United States Food and Drug Administration
FLIM	Fluorescence lifetime imaging microscopy
FRET	Fluorescence resonance energy transfer
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
gRNA	Guide RNA
GS	Gamma-secretase
HDR	Homology directed repair
HEK	Human embryonic kidney

hiPSC	Human induced pluripotent stem cell
ICC	Immunocytochemistry
LAMP	Lysosomal-associated membrane protein
LC3B	Microtubule-associated protein 1B-light chain 3
LEs	Late endosomes
ltNES	Long-term neural epithelial stem
NAC	Non-amyloid component
NCT	Nicastrin
NFTs	Neurofibrillary tangles
NGS	Next generation sequencing
NHEJ	Nonhomologous end joining
NMDA	N-methyl-d-aspartate
NTF	N-terminal fragment
PAM	Protospacer adjacent motif
PCR	Polymerase chain reaction
PD	Parkinson's disease
PEI	Polyethylenimine
PEN	Presenilin enhancer
<i>PSEN/PS</i>	Presenilin (gene/protein)
PVDF	Polyvinylidene fluoride
RMP	Resting membrane potential
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SaCas9	<i>Staphylococcus aureus</i> Cas9
SDS	Sodium dodecyl sulphate
SNC	Substantia nigra pars compacta
<i>SNCA/αSyn</i>	Alpha-synuclein (gene/protein)
SpCas9	<i>Streptococcus pyogenes</i> Cas9
TALEN	Transcription activator-like nuclease
TEM	Transmission electron microscopy
TM	Transmembrane
TNT	Tunnelling nanotube
TTx	Tetrodotoxin
WB	Western blot
ZFN	Zinc finger nuclease

# Introduction

## Alzheimer's disease

Advancements in medicine and technology over the past century have led to a prolonged human life expectancy. Albeit an extraordinary accomplishment, these developments are also entailed with an increase in age-related diseases. Alzheimer's disease (AD) is the most common neurodegenerative disorder, currently affecting approximately 50 million people worldwide, a number that is estimated to have tripled by 2050<sup>1</sup>. The main clinical feature of AD is dementia, caused by degeneration of neurons and synapses in the cerebral cortex and subcortical regions of the brain<sup>2</sup>. Dementia in AD is characterised by compromised short-term memory in combination with impairment in at least one other cognitive functions and difficulties in performing activities of daily living<sup>3</sup>.

Alzheimer's disease is a slowly progressive disease, whose onset is estimated to date 15-20 years before the first clinical symptoms emerge<sup>4</sup>. The pathological hallmarks of AD are accumulation of extracellular amyloid-beta ( $A\beta$ ) as plaques and intracellular inclusions of the microtubule associated protein tau as neurofibrillary tangles (NFTs) (Figure 1)<sup>5</sup>.

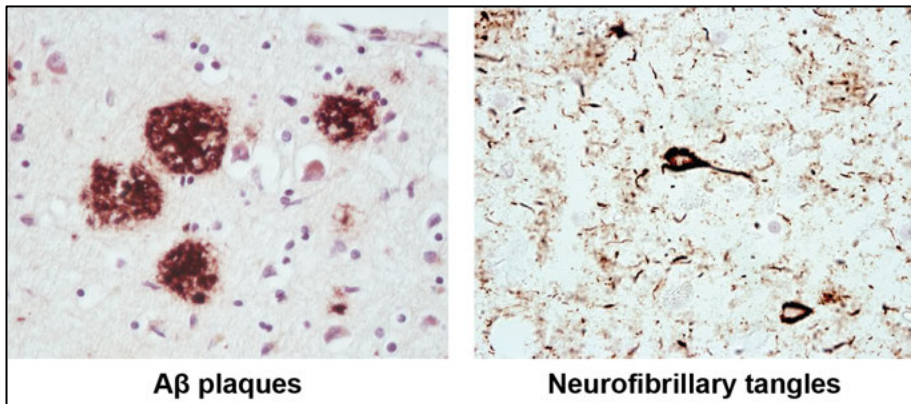


Figure 1. Pathological hallmarks of the AD brain. Amyloid-beta plaques (left) and neurofibrillary tangles (right) in the brain of an AD patient as detected with antibodies against  $A\beta_{42}$  and tau, respectively (20x magnification). Images kindly provided by Maria Pagnon and Anish Behere, Uppsala University.

## A $\beta$ and its precursor protein

The A $\beta$  peptide is part of a larger protein, known as amyloid precursor protein (APP), which is a conserved type I transmembrane protein that can be found in most tissues, including the brain. The human *APP* gene (*APP*) is located on chromosome 21 and its alternative splicing can generate mRNA transcripts ranging from 365 to 770 amino acids. The most common isoforms are APP695, which is predominantly expressed in the CNS, as well as APP751 and APP770, which are more ubiquitously expressed<sup>6,7</sup>.

Sequential cleavage of APP occurs via two distinct pathways, the amyloidogenic and non-amyloidogenic pathway (Figure 2). In the amyloidogenic pathway, full length APP is first cleaved by  $\beta$ -secretase (BACE1) to produce extracellular soluble sAPP $\beta$  and membrane tethered  $\beta$ -CTF (C99), which is then cleaved by  $\gamma$ -secretase, resulting in the formation of A $\beta$  peptides of varying lengths (37-43 amino acids) and the APP intracellular domain (AICD). In the non-amyloidogenic pathway, APP is first cleaved by  $\alpha$ -secretase to produce extracellular soluble sAPP $\alpha$  and membrane associated  $\alpha$ -CTF (C83), which is then cleaved by  $\gamma$ -secretase, resulting in the generation of P3 and a longer AICD<sup>8</sup>. Amyloidogenic processing appears to be the favoured pathway of APP metabolism in neurons, largely because of the greater presence of BACE1, whereas the non-amyloidogenic pathway is predominant in all other cell types<sup>9</sup>.

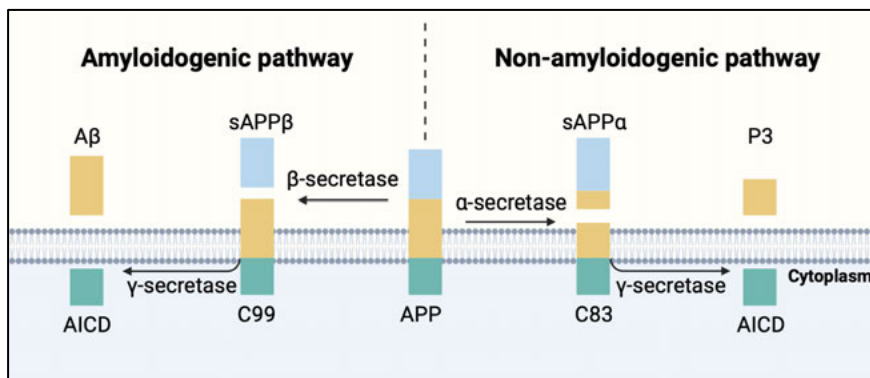


Figure 2. Pathways of APP processing. Amyloid-beta peptides are released under the amyloidogenic pathway (left) while sequential cleavage of APP by  $\alpha$ - and  $\gamma$ -secretase leads to secretion of soluble P3 fragments under the non-amyloidogenic pathway (right). Created with BioRender.com.

Full-length or truncated A $\beta$  peptides, especially A $\beta$ 42, are the main components of A $\beta$  plaques found in AD brains<sup>10</sup>. In their monomeric form, the A $\beta$  peptides are considered intrinsically unstructured. However, they tend to aggregate into larger soluble forms, such as oligomers and protofibrils. These aggregated structures will eventually give rise to insoluble fibrils that

form the A $\beta$  plaques<sup>11</sup>. Protofibrils are considered the most toxic of the A $\beta$  species, as their intrinsic properties, such as size, conformation and hydrophobicity, promote the formation of larger insoluble fibrils<sup>12</sup>.

### Gamma( $\gamma$ )-secretase

Gamma( $\gamma$ )-secretase (GS) plays a crucial role in the formation of A $\beta$ . It is composed of four essential subunits, presenilin (PS) (PS1 or PS2), nicastrin (Nct), anterior pharynx defective 1 (APH-1) and presenilin enhancer 2 (Pen-2), that bind together at an 1:1:1:1 ratio to form the GS complex<sup>13</sup>. Nicastrin and APH-1 first combine in the endoplasmic reticulum (ER) to create the scaffold that full length PS can bind to, with Pen-2 being the last component to attach to the complex. Presenilin enhancer 2 subsequently instigates endoproteolysis of full-length PS to produce the N- and C-terminal PS fragments, which together constitute the active form of PS<sup>14</sup>. The complex is not fully active until it is transferred to the Golgi apparatus and become glycosylated<sup>15</sup>. To date, GS is known to cleave more than 90 membrane proteins that have previously undergone ectodomain shedding, including APP and Notch<sup>16</sup>.

As mentioned above, A $\beta$  peptides are produced via the amyloidogenic pathway of APP processing. This process includes several steps that start from cleavage of  $\beta$ -CTF by GS in position  $\epsilon$  to release A $\beta$ 48 and A $\beta$ 49. These long peptides are subsequently processed, at a rate of three amino acids at a time, to produce shorter peptides, with A $\beta$ 42 and A $\beta$ 40 being the two most common forms<sup>17</sup>. Because of its higher fibril-forming capacity, A $\beta$ 42 is considered to be more toxic compared to the other peptides and is the form most commonly associated with AD pathology<sup>18</sup>.

Of the four GS subunits, PS is the one that has been more thoroughly studied, as it provides the catalytic domain of the complex. In humans, there are two PS proteins, PS1 and PS2, encoded by *PSEN1* and *PSEN2* on chromosomes 14 and 1, respectively<sup>19,20</sup>. They are multi-transmembrane proteins with nine transmembrane domains (TMDs) (Figure 3). The two catalytic aspartyl residues, responsible for the cleaving activity of GS, are located in TMDs six and seven<sup>21,22</sup>. Active PS requires endoproteolytic cleavage between TMD6 and TMD7 in order to produce the N- and C-terminal fragments that bind together at a 1:1 stoichiometry to form stable PS heterodimers<sup>14,23</sup>.

Although very similar in function and structure, GS complexes with PS1 seem to have significantly higher specific activity compared to those containing PS2. Accordingly, PS1-deficient mice show severe developmental abnormalities and embryonic lethality, while PS2-deficient mice are viable, have a milder phenotype and display unaltered sAPP $\beta$  processing<sup>24-27</sup>. Further studies suggest that PS1 and PS2 recognize distinct substrates with varied specificity despite sharing a sequence homology of 66%<sup>28-30</sup>. The recognition

of APP by PS1 has been shown to occur via an interaction between the N- and C-terminals of the APP TM domain and specific amino acid residues of PS1<sup>31</sup>. These bonds alter the conformation of both proteins leading to the positioning of the APP TMD in a cut-through channel of PS1 from where the longer A $\beta$  peptides are released<sup>31</sup>. Notably, certain early-onset AD mutations in the interacting amino acids of APP and PS1 are likely to be pathogenic by affecting the complex conformation<sup>32-34</sup>.

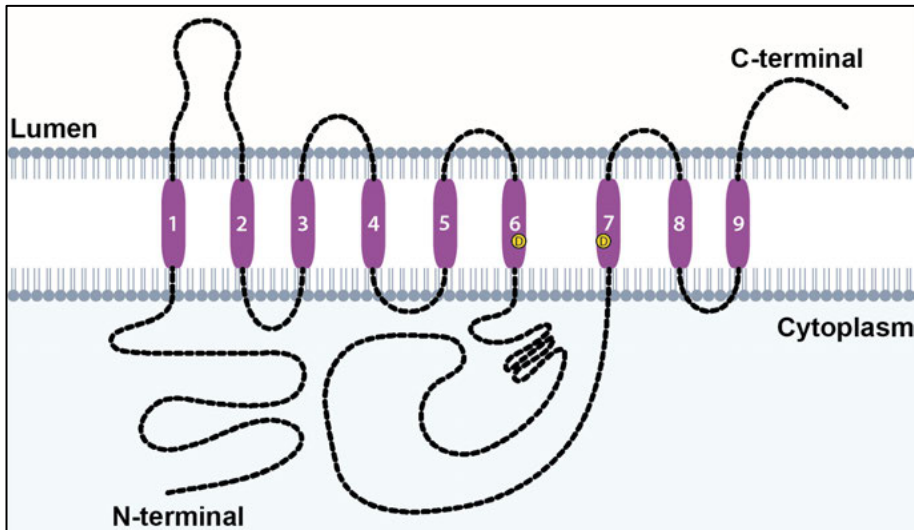


Figure 3. Presenilin transmembrane structure. Both human presenilins share the same number of TMDs. The aspartyl (D) residues in TMD6 and TMD7 are responsible for the catalytic activity of the GS complex.

## Familial Alzheimer's disease

Approximately 95% of AD patients are late-onset (age >65 years) cases, whereas only about 5% are early-onset (age <65 years) cases. Early-onset familial AD results from dominantly inherited mutations in either *APP*, *PSEN1* or *PSEN2*. Although these mutations only represent a small fraction of AD cases, a careful examination of patients, as well as detailed studies on animal and cellular models, help us to better understand the underlying disease mechanisms<sup>35</sup>.

### *PSEN1* and *PSEN2*

Mutations in *PSEN1* and *PSEN2* are the most common causes of familial AD. To date, more than 300 mutations in *PSEN1* and over 50 mutations in *PSEN2* have been described, most of which alter the A $\beta$ 42/40 ratio by shifting the generation from A $\beta$ 40 to the more aggregation prone A $\beta$ 42 without affecting the overall A $\beta$  production<sup>36</sup>. Most of them are autosomal dominant point

mutations that lead to a single amino acid change, while further categorization suggests the existence of mutational hotspots within the substrate recognition/binding area and catalytic domain of the PS1 protein<sup>31</sup>.

The exact mechanisms whereby *PSEN1* and *PSEN2* mutations cause AD are not fully understood. Several studies suggest that the main outcome of most *PSEN1/2* mutations is a partial loss of GS activity, which leads to an insufficient cleavage of  $\beta$ -CTF and results in the production of more A $\beta$ 42 and A $\beta$ 43<sup>37-39</sup>. This proposed mechanism of action further supports the amyloid cascade hypothesis under which the toxic A $\beta$  species, mainly A $\beta$ 42, are assumed to form insoluble amyloid plaques as well as lead to the formation of NFTs and subsequent neurodegeneration<sup>40</sup>. Other studies suggest that certain mutations lead to a complete loss of GS activity with almost undetectable A $\beta$  levels and that they are pathogenic because of a loss of essential presenilin functions in the brain, which in turn triggers the neurodegenerative process<sup>34,41-43</sup>.

### ***APP***

Mutations in *APP* represent 10-15% of genetically confirmed familial AD cases. To date, 69 mutations have been identified, 27 of which are considered pathogenic<sup>44</sup>. Most of them are clustered around the  $\beta$ - and  $\gamma$ -secretase cleavage sites and affect APP processing. The effect on A $\beta$  can vary significantly between the different mutations with some causing an increase in total A $\beta$  production (e.g. *APP* Swedish mutation<sup>45</sup>) or aggregation (e.g. *APP* Arctic mutation<sup>46</sup>), while others increase the A $\beta$ 42/40 ratio (e.g. *APP* London mutation<sup>47</sup>).

### Sporadic Alzheimer's disease

The remaining 95% of AD cases, usually sporadic cases with late-onset manifestation, most likely result from several different genetic risk factors together with environmental factors such as age and brain injury. So far, the only known substantial genetic risk factor for sporadic AD is the gene for apolipoprotein E (*APOE*).

### ***APOE***

The *APOE* gene has three major allelic variants, *APOE*  $\epsilon$ 2, *APOE*  $\epsilon$ 3 and *APOE*  $\epsilon$ 4. The  $\epsilon$ 4 variant is associated with an increased risk and earlier age of onset for AD<sup>48,49</sup>, whereas the  $\epsilon$ 2 variant seems to confer protection against AD<sup>50,51</sup>. Subjects who are heterozygous for *APOE*  $\epsilon$ 4 have a three to four times increased disease risk, while *APOE*  $\epsilon$ 4 homozygotes are 10 to 15 times more likely than non- $\epsilon$ 4 carriers to develop the disease<sup>48,52</sup>. The exact mechanism is not completely understood, but *APOE*  $\epsilon$ 4 has been suggested to promote A $\beta$  accumulation in the brain by affecting clearance and transport of A $\beta$  over the

blood-brain barrier (BBB), as well as altering the transcription levels of *APP*<sup>53-57</sup>.

## Cellular dysfunction in Alzheimer's disease

Neuronal dysfunction, and subsequent degeneration, is assumed to cause the cognitive decline during AD. According to the amyloid cascade hypothesis, accumulation of soluble and insoluble A $\beta$  species in the extracellular space leads to hyperphosphorylation of tau and the formation of intracellular NFTs, followed by synaptic failure and, ultimately, cell death<sup>40,58</sup>. The A $\beta$  and tau pathology affect cells in several ways, including disruption of neuronal connectivity, increased excitability and reduced branching<sup>59,60</sup>. In most of these phenomena of cellular dysfunction, neuroinflammation seems to play a key role.

In affected brain areas of AD patients, inflammatory responses are thought to predate neurodegeneration, suggesting a connection between neuroinflammation and cellular death<sup>61</sup>. Although acute inflammatory responses are mainly beneficial, a prolonged activation of the immune system can cause chronic inflammation, thereby exacerbating the pre-existing pathology<sup>62</sup>. This process involves several molecular mechanisms that lead to activation of microglia and astrocytes, the two main cell types driving neuroinflammation<sup>63</sup>.

Microglia are considered as resident macrophages of the central nervous system (CNS) and are mostly known for their phagocytic characteristics<sup>64</sup>. However, in response to the presence of A $\beta$  aggregates and various forms of tissue damage they can induce inflammatory responses<sup>65</sup>. Furthermore, recent data have highlighted the relevance of microglia-astrocyte crosstalk in the progression of AD-related brain pathology<sup>66,67</sup>.

### **Astrocytes in AD**

The involvement of astrocytes in AD has been neglected and insufficiently researched for many years, but recent data indicate that they may be key players in the disease progression. As the most abundant non-neuronal cells in the human brain, astrocytes play an important role in maintaining the integrity of the BBB<sup>68</sup> and overall brain homeostasis by providing physical, energetic, metabolic and trophic support to neurons and other cell types<sup>69</sup>. Their interaction with the brain vasculature is of great importance as they express several tight junction proteins as well as aquaporin 4 (AQP4) water channels, which regulate the glymphatic system that is responsible for the clearance of proteins and other solutes, including A $\beta$ , from the brain<sup>70,71</sup>. Their characteristic end-feet, in combination with their ability to sense synaptic activity, make them capable of orchestrating the delivery of various molecules throughout the tissue<sup>72,73</sup>.

### The “tripartite” synapse

Astrocytes contribute to synapse formation and maturation by forming the “tripartite” synapse together with pre-synaptic and post-synaptic neuronal terminals (Figure 4)<sup>74-76</sup>. Receptors scattered across astrocytic membranes respond to the presence of neurotransmitters in the synaptic cleft by triggering the release of neuroactive molecules, known as gliotransmitters<sup>77</sup>. These molecules, including glutamate, gamma-aminobutyric acid (GABA), D-serine and adenosine triphosphate (ATP), play an important role in regulating synaptic transmission and neuronal activity<sup>78,79</sup>. Recycling and removal of the gliotransmitters, especially glutamate and GABA, from the tripartite synapse is tightly regulated by astrocytes in order to protect the neurons from excitotoxicity and oxidative stress<sup>70,80,81</sup>.

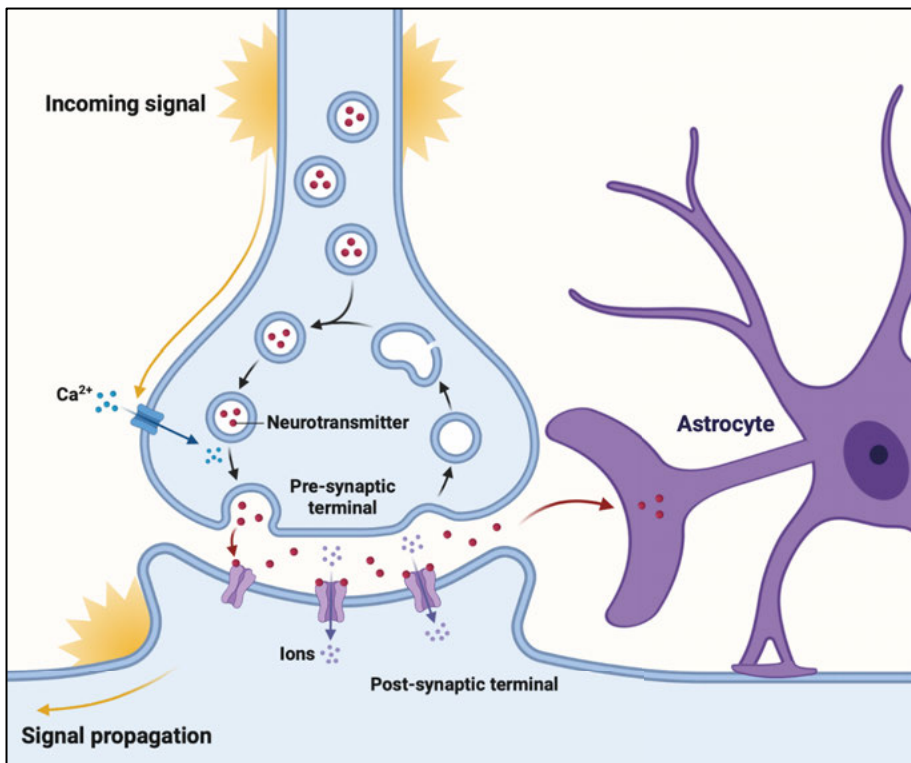


Figure 4. The "tripartite" synapse. Neurons propagate electrical signals via secretion of neurotransmitters within the synaptic cleft that bind to receptors in the post-synaptic terminal. These receptors allow the influx and efflux of ions that regulate signal transmission. Astrocytes modulate synaptic activity by recycling unused neurotransmitters. Created with BioRender.com.

### Astrogliosis

During a neurodegenerative condition, such as AD, astrocytes respond to pathogenic stimuli by entering a reactive state, astrogliosis, which is

characterized by increased size, proliferation rate and expression of cell-type-specific proteins, e.g. glial fibrillary acidic protein (GFAP), S100 $\beta$  and vimentin<sup>82,83</sup>. Already during the very early stages of AD, reactive astrocytes are found in the vicinity of A $\beta$  plaques, thereby further suggesting that A $\beta$  triggers astrogliosis<sup>84,85</sup>. Although this process is considered to occur as a protective measure to minimize the extent of the injury, it may eventually cause more harm to nearby cells<sup>67</sup>.

Astrogliosis is part of the neuroinflammatory reaction that involves the upregulation and secretion of cytokines, chemokines as well as reactive oxygen species (ROS) that are detrimental to surrounding neurons<sup>86-88</sup>. Moreover, stressed astrocytes have been shown to transfer pathological protein aggregates to neighbouring cells via thin protrusions called tunnelling nanotubes (TNTs)<sup>89</sup>. These structures consist mainly of filamentous actin and facilitate the exchange of organelles, including lysosomes and mitochondria as well as vesicles and small proteins between cells<sup>90-92</sup>. Their formation is upregulated under pathological conditions and recent studies have confirmed their participation in spreading of toxic material involved in experimental models of various diseases<sup>89,93,94</sup>. However, astrocytes have numerous ways of communicating with each other, both via direct and indirect mechanisms.

Extracellular vesicles (EVs) are spherical or donut-shaped structures comprised of a lipid bi-layer membrane that vary greatly in size (30 nm-10  $\mu$ m)<sup>95,96</sup>. They can carry numerous cargos, including RNA and DNA molecules as well as lipids and proteins<sup>97</sup>. The EVs express markers that are associated with their origin, thus enabling easier tracing and classification<sup>98</sup>. Astrocytes are secretory cells and astrocyte-secreted EVs have been shown to participate in diverse cellular processes, including transmission of pathological proteins<sup>99-101</sup>.

### *A $\beta$ clearance and degradation pathways*

In familial AD, the increased presence and subsequent spreading of A $\beta$  is attributed to mutations in specific genes. However, in sporadic AD the mechanisms behind increased levels of A $\beta$  are not completely understood. Recently, it was shown that astrocytes can produce A $\beta$  and exhibit BACE1 activity, especially under stress conditions, that further contribute to their role in propagating AD pathology<sup>102,103</sup>. Another proposed explanation for the persisting A $\beta$  levels and accumulation, is the impairment of the clearance machinery. This process is known to be less effective in the aged brain and is regulated by astrocytes and microglia<sup>104,105</sup>.

Astrocytes are the main source of cholesterol and ApoE in the brain, which are both essential for A $\beta$  clearance through the BBB<sup>106</sup>. They also actively participate in the amyloid degradation pathway via secreting peptidases such as neprilysin<sup>107,108</sup>, insulin-degrading enzyme<sup>109</sup> and matrix metalloproteinase-9<sup>110</sup>. Moreover, *in vitro* and *in vivo* studies suggest that astrocytes can take up large amounts of A $\beta$ , but that they have a limited capacity for degradation<sup>99,111-</sup>

<sup>113</sup>. Reactive astrocytes situated around A $\beta$  plaques often attempt to penetrate the dense plaque core and clear A $\beta$ <sup>114</sup>. This process could end up releasing partially digested truncated and potentially neurotoxic A $\beta$  peptides to the extracellular space<sup>99,115</sup>.

Processing of A $\beta$  occurs mainly via the autophagy-lysosomal pathway (ALP) that refers to the cellular mechanisms that enable the delivery of ingested material to lysosomes for degradation<sup>116</sup>. The ALP utilizes the endosomal pathway in order to guide the ingested material through the plasma membrane and generate the first cellular structures in this process, the early endosomes (EEs)<sup>117,118</sup>. Formation of EEs is regulated by the GTPase Rab5 whose conversion to Rab7 leads to the maturation of EEs to late endosomes (LEs)<sup>117,119</sup>. Late endosomes are located around the cell nucleus and display a slightly acidic intraluminal environment with a pH of  $\sim 5.5$ <sup>120</sup>. During that stage, LEs recruit several key proteins that are important for further lowering of the pH, such as the lysosomal-associated membrane proteins (LAMP) 1 and 2a as well as a number of proteolytic enzymes<sup>121,122</sup>. The ingested material is degraded at the last step of this process, where LEs fuse with existing lysosomes to form mature lysosomes (Figure 5A).

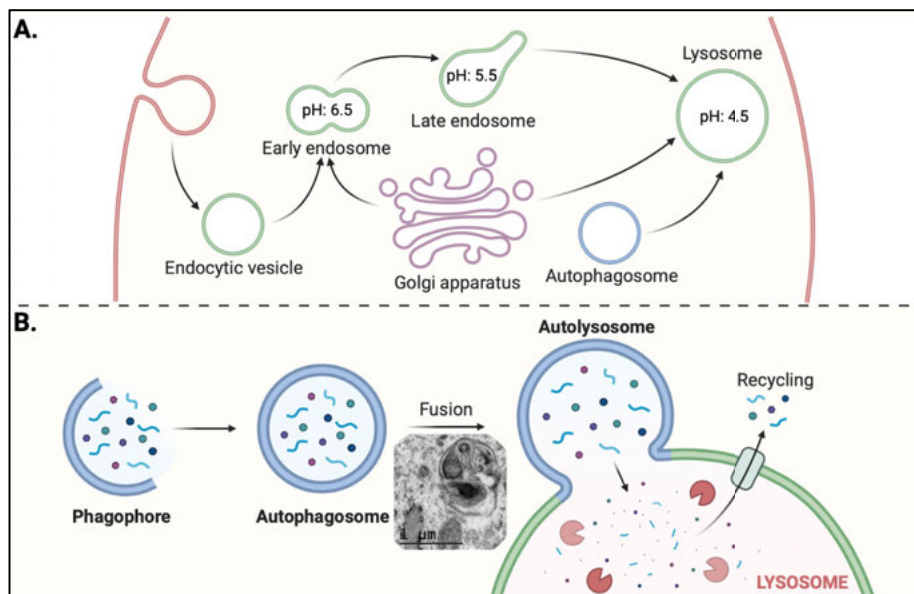


Figure 5. The endosomal and macroautophagy pathways. During the endosomal pathway, ingested material is guided to the lysosomes for degradation through a series of endosomal structures with different pH (A). The macroautophagy pathway relies on the formation of double-membrane vesicles, known as autophagosomes, that carry the enclosed material to lysosomes for degradation (B). Created with BioRender.com.

Autophagy includes three different pathways: macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy. Macroautophagy involves

the formation of distinct double-membrane vesicles, known as autophagosomes (Figure 5B)<sup>123</sup>. These structures are used by the cell in order to degrade large molecules, including aggregated proteins such as A $\beta$ , as well as cellular organelles<sup>123</sup>. Their formation has been closely related to the microtubule-associated protein 1B-light chain 3 (LC3B)<sup>124</sup>. Pre-autophagosomal membranes, known as phagophores, recognize and bind to the internalized substrate. Subsequently, the membrane is elongated in order to encapsulate the substrate and form the autophagosome via a mechanism regulated by LC3BII, a product of lipidation of LC3BI<sup>125</sup>. Recruitment of LC3BII to the autophagosome is mediated by p62 that is also responsible for the ubiquitination and translocation of cellular organelles to the autophagosome<sup>126</sup>. The final step involves the fusion of the autophagosome with a pre-existing lysosome in order to form the autolysosome that will degrade the enclosed content<sup>127</sup>. Impaired autophagy, and degradation in general, are believed to be central for sporadic AD development and are also linked to secretion of harmful A $\beta$  species that participate in disease spreading<sup>128</sup>.

## Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease, currently affecting 7-10 million people worldwide<sup>129</sup>. The number of cases is expected to double until 2040<sup>130</sup>. The clinical features of PD are categorized as motor and non-motor symptoms. Motor symptoms include bradykinesia, muscular rigidity, rest tremor as well as postural and gait impairment<sup>131</sup>. Non-motor symptoms include olfactory dysfunction, cognitive decline, sleep disorders, fatigue and autonomic dysfunction and usually precede the onset of motor symptoms (prodromal phase)<sup>132,133</sup>.

The main pathological hallmarks of PD are loss of dopaminergic neurons within the substantia nigra pars compacta (SNc) and presence of Lewy pathology in this and other brain areas. Parkinson's disease is a slowly progressive disorder and the first symptoms arise only after approximately 30% of the dopaminergic neurons in the SNc have already been lost<sup>134</sup>. Lewy pathology consists of insoluble aggregates of misfolded proteins, mainly  $\alpha$ -synuclein ( $\alpha$ Syn), within the cell body (Lewy bodies) and processes of neurons (Lewy neurites) (Figure 6)<sup>135-137</sup>.

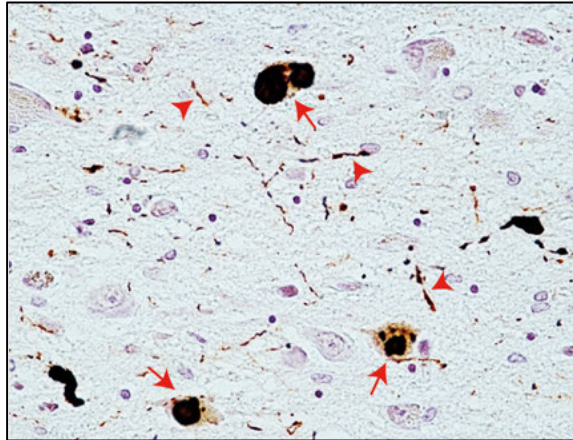


Figure 6. Lewy pathology in the substantia nigra. Lewy bodies (arrows) and Lewy neurites (arrowheads) in the SNc of a PD patient (20× magnification). Image kindly provided by Anish Behere, Uppsala University.

### Risk factors and the *SNCA* gene

The most important risk factor for PD is age, as the prevalence increases significantly in people over 80 years<sup>138</sup>. Furthermore, gender<sup>139</sup> - as well as many environmental factors such as pesticide exposure, prior head injury, the use of beta blockers and even drinking well water<sup>140</sup> - have been proposed to increase the disease risk. Genome wide association studies have identified several genes that are associated with familial PD, the first of which was the *SNCA* gene (that encodes  $\alpha$ Syn)<sup>141</sup>.

To date, two types of mutations in *SNCA* have been identified. These are inherited in an autosomal dominant manner and cause early-onset PD (<50 years of age). The first type consists of point mutations that lead to single amino acid changes (A30P, A53E/T/V, E46K, H50Q, G51D), which in turn cause  $\alpha$ Syn to adopt an incorrect 3-dimensional shape and alter its aggregation propensity and lipid binding features<sup>141-148</sup>. The second type consists of multiplications of the *SNCA* gene that result in an increased production of  $\alpha$ Syn<sup>149,150</sup>.

### Alpha-synuclein

Alpha-synuclein is a 140-amino acid long protein that is encoded by the *SNCA* gene, located on the long arm of chromosome 4<sup>141,151</sup>. It belongs to the family of synucleins, alongside  $\beta$ - and  $\gamma$ -synuclein. All three proteins are abundantly expressed in the brain with preferential localization to the presynaptic terminals<sup>152</sup>. Although  $\alpha$ Syn is a natively unfolded protein, it is composed of three regions (Figure 7)<sup>153,154</sup>. The first is the positively charged N-terminal which can form helical structures and interact with lipids<sup>155</sup>. The second is the

hydrophobic mid-region, unique to  $\alpha$ Syn, which was first discovered in amyloid plaques from AD patients and is therefore called the non-amyloid component (NAC) region<sup>156</sup>. The third is the negatively charged C-terminal that can bind to metals and other proteins<sup>157</sup>.

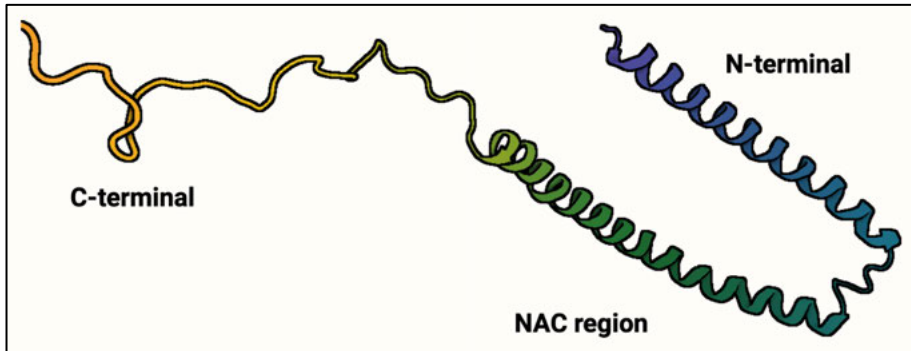


Figure 7. Alpha-synuclein structure. The N- and C-terminals consist of 60 and 45 amino acids respectively, and allow  $\alpha$ Syn to interact with lipids, metals and other proteins. The mid-region (NAC) consists of 35 amino acids and is unique to  $\alpha$ Syn. Created with BioRender.com.

One of the main pathogenic features of  $\alpha$ Syn is aggregation. Unlike its native unfolded state, under pathogenic conditions monomeric  $\alpha$ Syn adopts a partially folded conformation that promotes self-assembly and results in the formation of larger aggregates that eventually become insoluble fibrils<sup>158,159</sup>. The NAC region is crucial for this aggregation step, as the same process cannot be replicated with  $\beta$ - or  $\gamma$ -synucleins (as they lack this region)<sup>160</sup>. The mechanism behind the toxic effects of  $\alpha$ Syn are not yet fully understood. Oligomeric forms have been associated with mitochondrial oxidative stress and dysfunction as well as defects in intracellular protein and membrane trafficking<sup>161,162</sup>.

## Current treatment strategies for Alzheimer's disease and Parkinson's disease

Today, we only have symptomatic therapies for AD and PD. For AD, acetylcholinesterase (AChE) inhibitors and N-methyl-d-aspartate (NMDA)-receptor antagonists have been proven to relieve some of the cognitive symptoms<sup>163</sup> and delay placement in nursing homes<sup>164</sup>. The AChE inhibitors increase acetylcholine levels in the brain, thereby delaying progressive cognitive decline, while NMDA receptor antagonists block glutamate from binding to its receptors, which in turn may prevent excessive excitotoxicity and neuronal cell death<sup>165</sup>. For PD, either L-dopa or dopamine receptor

agonists are effective in reducing the motor-related symptoms during the initial years of the disease but do not slow down the disease process.

Various forms of immunotherapy have been developed for both diseases and are currently undergoing clinical trials, while only one has received approval by the U.S. Food and Drug administration (FDA). Aducanumab (Aduhelm™) is the first immunotherapy available for AD, thereby reigniting the hope for better and more effective treatments for millions of patients. Of particular notice is lecanemab (BAN2401), a monoclonal antibody against A $\beta$  oligomers and protofibrils that is based on an antibody that was developed in our laboratory and which recently received approval for rolling submission to the FDA under the accelerated approval pathway, following a successful outcome of a phase 2b-study<sup>166,167</sup>.

Given the lack of treatment targeting the underlying causes, rather than just the symptoms of AD and PD, genome editing provides an exciting therapeutic alternative that has started to be explored.

## Gene editing

Gene editing techniques have been extensively used during the past decade in order to intentionally modify specific genomic sequences<sup>168</sup>. One way this can be achieved is by targeting genes through means of homology recombination. Genome editing via double strand break repair mechanisms has become possible through the development of genetically engineered nuclease complexes that allow the efficient targeting of specific genomic loci<sup>169</sup>.

These nucleases include systems whose action requires the mediation of DNA-binding proteins, such as zinc finger nucleases (ZFN)<sup>170</sup> and transcription activator-like nucleases (TALENs)<sup>171</sup>. More recently, the clustered regulatory interspaced short palindromic repeats (CRISPR) system, that utilizes the simple base-pairing rule through a modified RNA that can bind directly to the desired DNA sequence, was discovered and adapted for use in eukaryotic cells<sup>172</sup>.

The CRISPR system was originally identified as part of the adaptive immunity of bacteria and archaea<sup>173</sup>. Its role is to protect the host genome from non-self DNA that has successfully penetrated the cell membrane<sup>174</sup>. A fragment of the non-self DNA (spacer) is integrated into the host genome at the CRISPR locus. The spacer sequence is transcribed alongside the CRISPR locus into an RNA molecule that forms a complex with the CRISPR associated (Cas) 9 protein<sup>175,176</sup>. This complex can successfully identify the non-self DNA and subsequently cleave it, leading to its disintegration<sup>177</sup>. The cleaving ability is provided by the Cas9 nuclease, which contains two catalytic domains that can produce double strand breaks in DNA sequences<sup>174</sup>. However, Cas9 is only activated upon recognition of a conserved dinucleotide-containing protospacer adjacent motif (PAM) sequence located directly downstream of

the target DNA sequence<sup>178</sup>. The CRISPR/Cas9 system has been adapted to efficiently target specific genomic sequences through a single guide RNA (gRNA) that, depending on the Cas9 variant, consists of 20-23 nucleotides (Figure 8). The gRNA sequence can be adjusted in order to target the gene of interest<sup>172</sup>.

Double strand breaks activate the cell's innate repair mechanisms that respond via two possible pathways, nonhomologous end joining (NHEJ) and homology directed repair (HDR)<sup>179</sup>. Nonhomologous end joining can lead to insertion or deletion of genomic sequences at the targeted gene locus that could potentially affect its expression either by changing the reading frame of the protein or by disrupting the effective binding of transcription factors<sup>180</sup>. Homology directed repair allows for the introduction of point mutations or even entire genomic sequences via means of recombination between the targeted gene locus and a supplied DNA donor vector<sup>181</sup>.

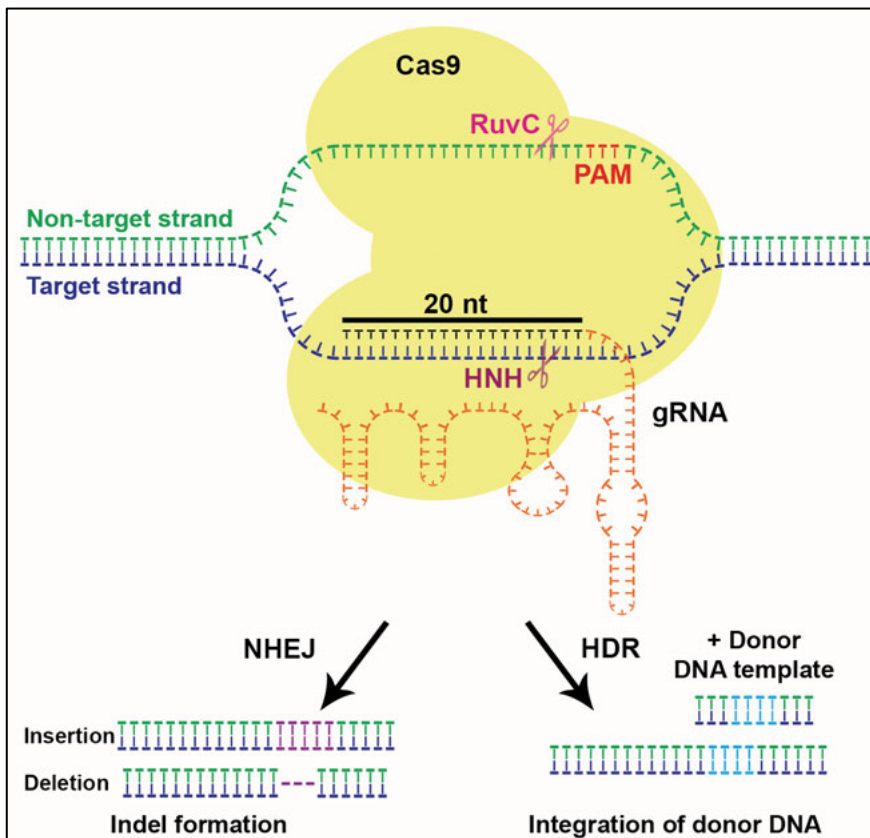


Figure 8. The CRISPR/Cas9 system and DNA repair mechanisms. Upon recognition of the target sequence, the Cas9 forms a complex with the gRNA and target DNA that results in double strand breaks. The most common repair mechanism is the NHEJ, where indels can be formed, while under the HDR pathway integration of donor DNA can occur.

Since the discovery of the CRISPR/Cas9 system in *Streptococcus pyogenes* (SpCas9)<sup>182</sup>, several other Cas variants that recognize novel PAM sites and allow for targeting of more DNA sequences have been either identified in bacteria or genetically engineered<sup>183</sup>. Apart from causing double strand breaks, Cas proteins can be combined with other regulating elements in order to enhance or block gene expression, change methylation patterns, substitute single base pairs or replace entire genomic sequences<sup>184</sup>.

A number of clinical trials based on CRISPR/Cas9 have been initiated over the past five years. Although the majority of them use genetic modification of a patient's cells *ex vivo*, Editas Medicine<sup>185</sup> and Intelia Therapeutics/Regeneron<sup>186</sup> were the first to administer CRISPR components directly to the patient. Editas Medicine developed a product targeting a hereditary eye disorder known as Leber congenital amaurosis, while Intelia Therapeutics/Regeneron aimed to treat patients with transthyretin amyloidosis (ATTR). The latter trial could provide novel insights to the treatment of several diseases that are caused by accumulation of misfolded proteins, such as AD and PD. Further results from both studies are expected within the next year and potential positive news will further solidify CRISPR's status as a candidate for therapies against human disorders.

## Challenges of the CRISPR/Cas9 system

The main requirements of any gene editing tool are accuracy and precision. Accuracy refers to the ratio of on- versus off-target genetic changes, whereas precision relates to the fraction of on-target edits that produce the desired genetic outcome. Off-target effects can occur in genomic areas with DNA sequences very similar to the target sequence<sup>187-189</sup>. To reduce the probability of off-target effects, new Cas9 variants have been developed that have lower tolerance for mismatches within the gRNA sequence while maintaining the same editing efficiency<sup>190</sup>.

Chromatin states can also affect on- and off-target accuracy. Assuming a potential off-target site is in an open chromatin region, that site is more likely to be edited than a similar site located in a closed chromatin region<sup>191</sup>. Furthermore, another crucial factor is the availability of Cas9 within the cells as well the duration of its activity. Lowering the concentration of Cas9 improves the specificity without impeding with the on-target activity<sup>188</sup>. Similarly, a shorter expression time can reduce off-target effects<sup>192,193</sup>.

Currently there are three general categories of off-target prediction methods. The first is a bioinformatics approach that ranks potential off-target sites based on an algorithm that analyses sequence homology. The second utilises *in cellulo* techniques that investigate nuclease activity by measuring the presence of successfully integrated exogenously supplied DNA tags in double strand breaks throughout the genome and subsequently amplifying the affected regions for further analysis (e.g. IDLV<sup>194</sup>, Guide-seq<sup>195</sup>). The third is

represented by *in vitro* techniques that assess nuclease activity on purified cell free DNA as a result of nuclease concentration (e.g. CIRCLE-seq)<sup>196</sup>. These unbiased methods vary in their comprehensiveness and sensitivity, which are defined by their ability to identify the full spectrum of off-target sites spanning high and low mutagenesis frequencies (characteristic of inefficiently cleaved DNA sequences) within the genome. Emerging tools utilise a combination of bioinformatics and *in vitro* techniques. Such an approach provides higher sensitivity and allows for investigation of multiple genomes, thereby enabling the nomination of potential off-target effects on a population wide scale (e.g. ONE-seq)<sup>197</sup>. Although off-target effects are relatively low, validation of editing events via Next Generation Sequencing (NGS) is crucial as it allows for sensitive (high depth) assessment of nominated off-target sites in their actual cellular contexts. New analytical tools emerge with a rapid pace and will make detection of imprecise genomic events easier and more cost effective.

# Aims

The overall objective of this thesis was to investigate the CRISPR/Cas9 system as a potential future treatment strategy for AD and PD and to explore astrocyte-mediated pathogenic mechanisms in AD.

## Specific aims

- I. To selectively target the early-onset AD-associated *PSEN1* M146L mutation in human fibroblasts and assess the restoration of disease phenotypes.
- II. To disrupt the *SNCA* gene by targeting either wild-type *SNCA* (pan-*SNCA*) or the early-onset PD-associated *SNCA* A53T mutation.
- III. To investigate how A $\beta$  pathology affects the crosstalk between hiPSC-derived astrocytes and neurons.
- IV. To evaluate the long-term effects of A $\beta$  inclusions in hiPSC-derived astrocytes.

# Methods

## Cell culture models

The use of appropriate research models was essential for all parts of this thesis. In **Paper I** we used human fibroblasts that were isolated from patient skin biopsies. Although fibroblasts are not brain cells, they express AD-associated proteins and exhibit some of the pathogenic phenotypes that are present in disease-affected neuronal cells. Moreover, they are easy to isolate and culture *ex vivo*, which makes them a suitable model to study our proof-of-concept approach. Similarly, human fibroblasts from PD patients carrying the *SNCA* A53T mutation were used in **Paper II**. In **Paper II** we also used human embryonic kidney (HEK) 293T cells to validate our different gRNAs and transfection protocols. These cells were chosen primarily for their fast multiplication rate and overall ease of culturing.

In **Papers III** and **IV** we wanted to explore the interplay between neurons and astrocytes and how the presence of A $\beta$  could affect their communication. To that end, we made some modifications to an already established protocol for generation of human induced pluripotent stem cell (hiPSC)-derived neurons<sup>198,199</sup> and astrocytes<sup>200</sup>. Both populations originate from neural precursors known as long-term neural epithelial stem (ltNES) cells and require a strict 28-day differentiation protocol (Figure 9). Our modifications include the use of ciliary neurotrophic factor (CNTF) during the astrocytic differentiation that results in mature astrocytes with characteristic morphology<sup>201</sup>.

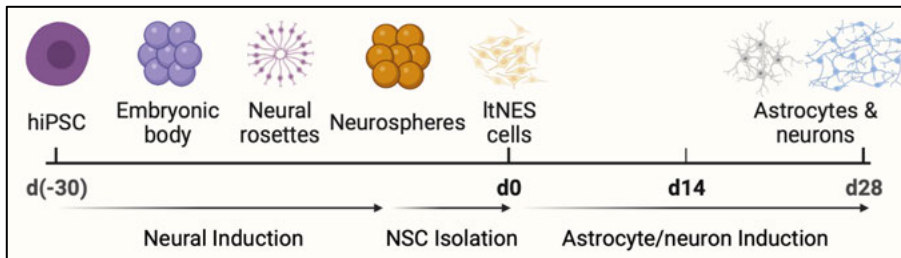


Figure 9. Differentiation timeline. Induction of ltNES cells takes 30 days, while derivation of astrocytes and neurons takes an additional 28 days. Created with BioRender.com.

## Gene editing

The appropriate CRISPR delivery method depends on the experimental model and the research question. In **Papers I and II** we used electroporation and polyethylenimine (PEI) to deliver a single plasmid vector into human fibroblasts and HEK293T cells, respectively. Electroporation is the process through which electric pulses are directed to the cell membrane, resulting in the opening of pores that allow large molecules, such as nucleic acids, to enter the cytoplasm. Polyethylenimine is a polymer that forms complexes with DNA molecules and allows their transportation into the cells via endocytosis<sup>202,203</sup>. These types of transfections allow for transient expression of the CRISPR components that last for a limited number of replication cycles and do not integrate with the cellular DNA.

The plasmid vectors that were used in both papers express Cas9, the appropriate gRNA sequence and a puromycin resistance cassette that enabled selection of positively transfected cells. In **Paper I** we used the Cas9 variant isolated from *Streptococcus pyogenes* (SpCas9) that recognizes a “*NGG*” PAM sequence that was suitable for targeting the *PSEN1* M146L mutation<sup>188,204</sup>. In **Paper II** we used both the SpCas9 as well as the KKHSaCas9 variant from *Staphylococcus aureus* that recognizes a “*NNRRRT*” PAM site to target the *SNCA* gene<sup>205</sup>. Furthermore, in **Paper II** we evaluated constant expression of Cas9 and gRNA via the use of lentiviral vectors that enable integration of the transgenes to the host DNA<sup>206</sup>.

In both **Papers I and II**, a scramble gRNA sequence was used as a control. This sequence is expressed from the plasmid vector and can form a complex with Cas9 but does not recognize any region in the human genome.

## DNA sequencing

Evaluation of gene editing outcomes requires the sequencing of the affected genomic regions. In **Papers I and II** we used Sanger sequencing and the more advanced NGS to determine the efficiency of *PSEN1* and *SNCA* disruption.

In the case of Sanger sequencing, DNA was isolated from the cells and specific regions of the genes of interest were amplified via polymerase chain reaction (PCR). These DNA fragments were of relatively short length (<500 base pairs) and their sequencing was performed using primers that are listed in the Methods section of the respective paper.

Sanger sequencing is in principle a PCR that relies on the incorporation of fluorescently labelled dideoxynucleoside triphosphates (ddNTPs) during the elongation of the DNA strand (Figure 10)<sup>207</sup>. These ddNTPs also block the extension of the DNA strand and are finally sorted according to their size. The order of DNA bases is determined by proper recognition of the emitted fluorescence from each ddNTP. Next generation sequencing follows the same

basic principles as the Sanger method with the added benefit of increased sensitivity, higher depth, and the ability to analyse many more sequences simultaneously.

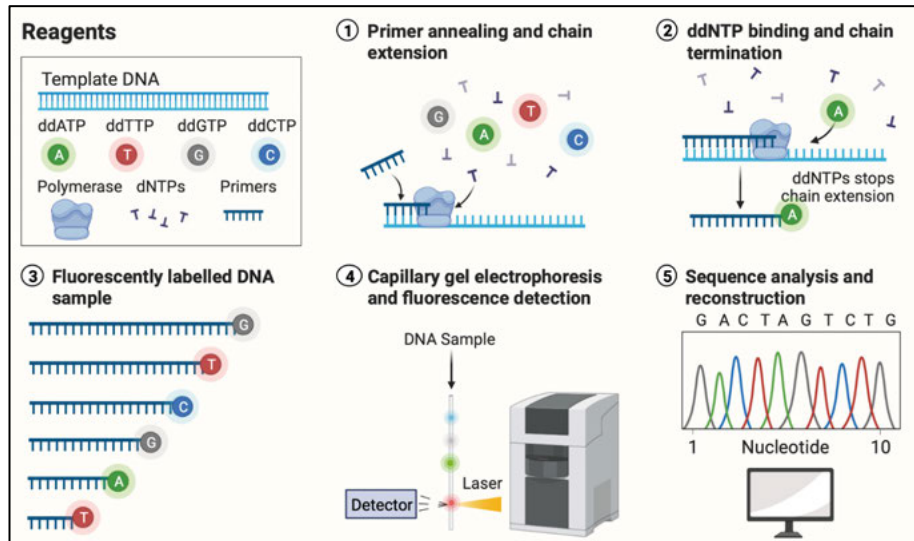


Figure 10. Sanger sequencing. The DNA template is amplified through the incorporation of ddNTPs by a polymerase. Termination of the chain extension together with the fluorescently labelled ddNTPs allow for size separation and reconstruction of the DNA sequence. Created with BioRender.com.

## ELISA

The enzyme-linked immunosorbent assay (ELISA) is a very common method for detection of proteins in samples that are in the form of a solution<sup>208</sup>. The technique relies on the coating of a surface with an antibody suitable for binding the molecule of interest (capture antibody). Subsequently, a second antibody that is conjugated with an appropriate enzyme, is used to recognize the bound antigen. The final step involves the addition of a substrate that the attached enzyme can metabolize and generate a detectable signal that can be measured. This method can also be used to detect several molecules of interest at the same time, by using different capture antibodies.

In **Paper I**, we used highly sensitive ELISA kits to detect A $\beta$ 40 and A $\beta$ 42 peptides in conditioned media from human fibroblasts with or without the *PSEN1* M146L mutation. Quantification of the measured A $\beta$ 40 and A $\beta$ 42 levels was possible via the use of protein standards with known protein concentrations.

## Immunocytochemistry

Immunocytochemistry (ICC) refers to the techniques that enable visual detection of antigens in fixed cell samples. This method involves the binding of primary antibodies to their antigen targets within the specimen. Fluorescently labelled secondary antibodies that bind specifically to the primary antibodies can be visualized under a fluorescent microscope and provide information regarding the antigen's localization and quantity. The technique was widely used in this thesis to detect specific proteins relevant to each paper.

In **Paper I**, we identified the N- and C-terminals of PS1 in control, mutant, and CRISPR-treated samples. In **Papers III and IV** we used ICC to analyse the expression of lineage-specific neuronal and astrocytic markers in hiPSC-derived cells (Figure 11). We also detected intracellular A $\beta$  deposits and quantified the viability of cultures based on the labelling of dead cells via a fluorescent dye (TUNEL). Finally, we checked for markers associated with astrocyte reactivity and explored the localization of LAMP1 and Rab5 positive organelles in relation to the A $\beta$  deposits. All the antibodies used have been listed within the Methods section of the respective paper.

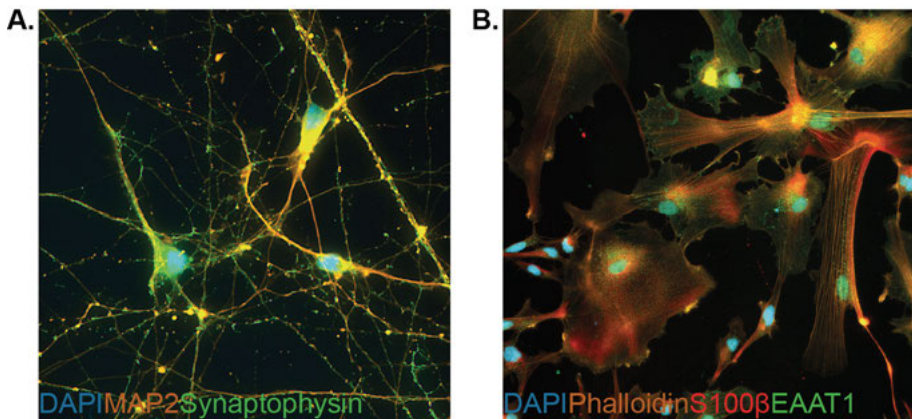


Figure 11. Expression of lineage-specific markers. Representative images from neurons expressing MAP2 and synaptophysin (A) as well as from astrocytes expressing S100 $\beta$  and EAAT1 (B). DAPI and phalloidin staining represent the nucleus and cytoskeleton, respectively.

## FRET/FLIM

Fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM) are two techniques that can be combined to study protein interactions within a cell sample<sup>209</sup>. This method involves the use of two fluorophores that are bound to different proteins or to different terminals

of the same protein. Fluorescence can occur from natively expressed, genetically modified proteins or from the staining of proteins with labelled antibodies. Energy transfer can occur from the donor to the acceptor fluorophore, only if the distance between them is less than 10 nm. The FLIM technique enables the recording of FRET signal by considering only the energy from the donor fluorophore, thereby making comparisons between samples and instruments easier and more reliable<sup>210</sup>.

In **Paper I**, FRET/FLIM was used to quantify the distance between the N- and C-terminals of PS1 expressed in human fibroblasts. The C-terminal was labelled with Alexa 488 and the N-terminal with Alexa 555 secondary antibodies. The signal from the donor fluorophore was translated to %FRET efficiency using the following equation:  $E_{\text{FRET}} = (t_1 - t_2)/t_1$ . Whereas  $t_1$  refers to the lifetime of the donor fluorophore in the absence of an acceptor,  $t_2$  refers to the same measurement in the presence of the acceptor. A lower %FRET represents a tighter PS1 conformation and subsequently a pathological phenotype.

## Off-target effects

The term “off-target effects” refers to the undesirable editing of genomic regions that are similar to the target DNA sequence. These unintentional edits can occur in any part of the genome and interfere with expression of proteins and transcription factors, thereby altering the cell’s behaviour<sup>187–189</sup>.

Initial screening of gRNA candidates minimizes the chances of off-target editing. In **Papers I** and **II**, we used the free online bioinformatic tool “CRISPOR”<sup>211</sup> to evaluate potential gRNA sequences and select the ones with optimal scores for the genes of interest. Furthermore, in **Paper I**, we evaluated the occurrence of off-target effects in human fibroblasts following treatment with CRISPR components. We applied two different methods, one established (CIRCLE-seq<sup>196</sup>) and one novel (ONE-seq<sup>197</sup>) to ensure the selectivity of our gRNA and show its potential safety in future *in vivo* applications. The key difference between these methods is that one uses genomic DNA that has been isolated from actual cells (CIRCLE-seq) while the other relies on high throughput DNA synthesis (ONE-seq). The combination of an *in vitro* and an *in silico* approach resulted in hundreds of potential off-target sites, from which we selected the top10 sites for sequencing and analysis.

## Western blot

Western blot (WB) is a technique used for detection of proteins in cell or tissue samples. The samples are first mixed with sodium dodecyl sulphate (SDS) to remove their secondary structure and obtain a uniform negative charge. They

are then loaded onto gels for electrophoresis and are separated according to their size. The contents of the gels are then transferred to a nitrocellulose or polyvinylidene fluoride (PVDF) membrane where the immunoblotting procedure occurs via the use of primary and secondary antibodies that are specific towards the protein of interest. Quantification of the protein relies on detection of the signal provided by the secondary antibodies either in the form of fluorescence or chemiluminescence.

In **Paper I**, WB was used to measure the levels of N- and C-terminal fragments of PS1 in cell lysates from human fibroblasts to establish the effect of gene disruption on the protein level. In **Paper II**, we quantified Cas9 in fibroblasts treated with lentiviral vectors to troubleshoot the observed low editing efficiency. In **Paper IV**, we evaluated the levels of LAMP1, Rab5 and several astrocyte reactivity markers in cell lysates from astrocytes with or without A $\beta$  inclusions, both at early and late time points.

## Electrophysiology

Electrophysiology represents a group of methods used to characterize the electrical properties of cells and tissues. In our work, it was applied to measure the electrical activity of neurons as determined by action potentials.

In **Paper III**, we used the patch clamp technique to measure the frequency and amplitude of excitatory post synaptic currents (EPSCs) as well as the resting membrane potential (RMP) in hiPSC-derived neurons. That was made possible via the use of a glass pipette directed toward the cell membrane of individual cells that forced a small opening through which the intracellular contents could be reached (Figure 12A). Current injections were used to stimulate the cell in order to determine the RMP. The frequency and amplitude of EPSCs were recorded in either the absence (sEPSCs) or presence (mEPSCs) of 0.5  $\mu$ M of tetrodotoxin (TTx) in the extracellular solution. Tetrodotoxin can block sodium channels that are involved in signal propagation and therefore allow us to measure current at the synaptic level, where one event is represented by one activated synapse (Figure 12B).

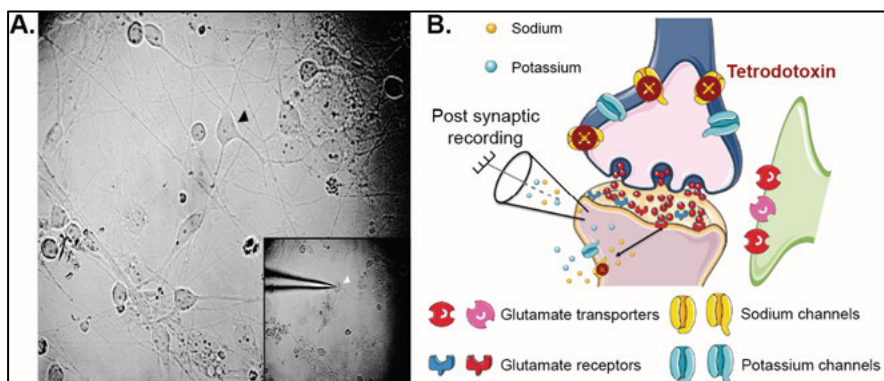


Figure 12. Patch clamp configuration. Representative image of a glass pipette in contact with a neuron in culture (A). The electrode measures activity from the post synaptic terminal, which is regulated by the state of sodium and potassium channels and the release and uptake of glutamate (B). TTx blocks sodium channels and enables measurement of spontaneous events. Images kindly provided by Benjamin Portal, Uppsala University.

## Microscopy and image analysis

Throughout this thesis, we used several image analysis techniques to quantify information and extract data. In **Paper I**, we used FLIM to measure the distance between the terminal of PS1 and thereby evaluate its conformation. In **Papers III** and **IV** we used fluorescent microscopy to detect the expression of lineage-specific markers in differentiated cells, measure cell viability, quantify A $\beta$  load, assess the localization of lysosomes and A $\beta$  inclusions as well as to identify abnormalities in cellular organelles.

Quantification of cell viability required the generation of a pipeline in CellProfiler, a free cell image analysis software<sup>212,213</sup>. Briefly, dead cells were labelled with a green, fluorescent signal (TUNEL) and all cells were identified via DAPI staining that represents the nucleus of a cell. The two different populations were detected separately in each image and the overlay provided the percentage of dead/live cell ratio.

The A $\beta$  load at the different time points of **Paper IV**, required the acquisition of images on different focal planes that were later compiled together using the ImageJ software<sup>214</sup>.

Colocalization of LAMP1 positive organelles and A $\beta$  inclusions was studied under a confocal microscope that allows for higher resolution compared to the typical fluorescent microscope<sup>215</sup>.

Finally, visualization of intracellular organelles was made possible via transmission electron microscopy (TEM) that relies on the capture of electrons passing through the specimen and offers superb resolution<sup>216</sup>.

# Results and discussion

## CRISPR technology for the treatment of familial AD

In **Paper I**, we aimed to evaluate the use of the CRISPR/Cas9 system against the familial AD-causing *PSEN1* M146L (A>C) mutation. Affected patients display an increased A $\beta$ 42/40 ratio that can be detected in neuronal as well as non-neuronal cells, such as fibroblasts<sup>217-219</sup>. For that purpose, we used human fibroblasts from individuals carrying the mutation (*PSEN1*<sup>M146L/WT</sup>) as well as healthy controls (*PSEN1*<sup>WT/WT</sup>) (Figure 13A).

We designed a gRNA that was specific for the mutation site and incorporated the RNA sequence in a plasmid vector that expressed SpCas9 (Figure 13B). Mutant and control cells were treated with equal amounts of the vector. Assessment of the gene editing outcome via NGS demonstrated that the M146L gRNA could successfully distinguish the mutant from the wild-type allele. Allele specific disruption led to indel formation that exceeded 50% of the mutant allele transcripts (Figure 13C).

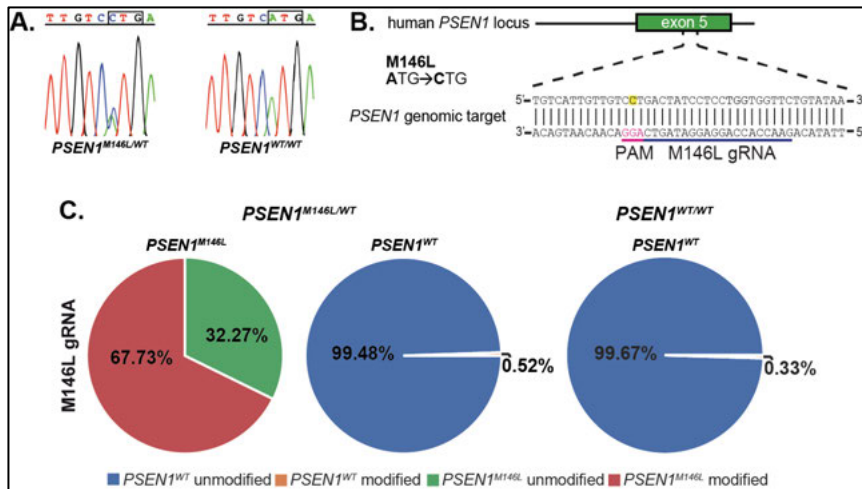


Figure 13. Precise and effective targeting of the *PSEN1* M146L mutation. The *PSEN1* M146L mutation is a single base substitution (A>C) (A). The gRNA was designed against the mutation site (B) and only affected the mutant *PSEN1* M146L allele (C).

In addition, we applied two separate methods to detect potential off-target genomic effects. Sequences within the genome similar to our M146L gRNA could have been mistakenly recognized by our CRISPR components.

Therefore, the top10 genomic sites from both nomination methods were sequenced and analysed via NGS. We did not detect any indel formation in any of the loci, thereby confirming the selectivity of our gRNA towards the *PSEN1* M146L sequence (Figure 14).

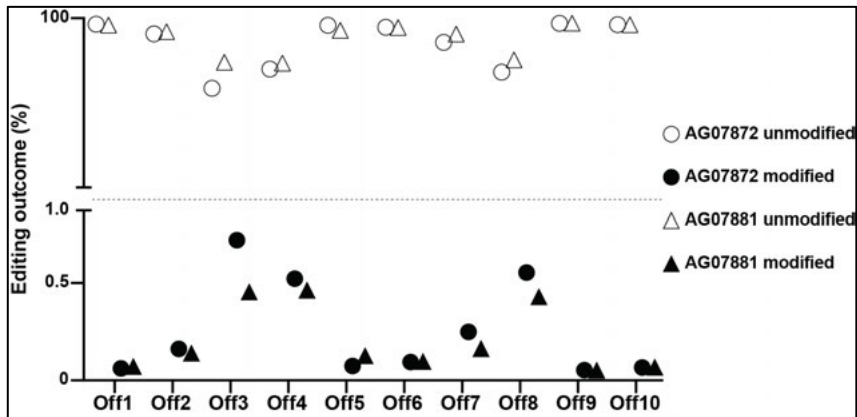


Figure 14. Off-target analysis. DNA from samples AG07872 and AG07881 was analysed for indel formation in the top10 nominated sites. No editing was observed in any of the sites.

## Gene disruption translates to functional alterations in treated *PSEN1*<sup>M146L/WT</sup> cells

Having validated our approach on the genomic level in the first part of **Paper I**, we continued to analyse its effect on other disease-associated aspects. The elevated pathogenic A $\beta$ 42/40 ratio was first confirmed in the *PSEN1* M146L samples (Figure 15A). In comparison, treated cells displayed significantly decreased protein ratios, suggesting a direct effect of the allele-specific gene disruption on the protein level (Figure 15B).

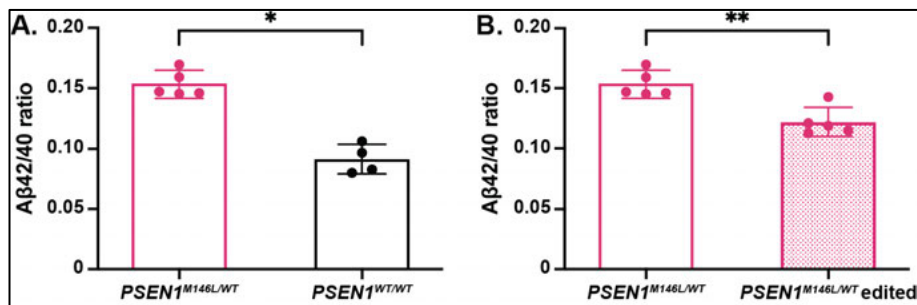


Figure 15. CRISPR/Cas9 treatment partially normalises the A $\beta$ 42/40 ratio. Mutant samples displayed increased A $\beta$ 42/40 ratio compared to wild-type cells (A). The ratio was significantly decreased in treated mutant samples (B).

Apart from the characteristic A $\beta$ 42/40 ratio, cells from individuals carrying the *PSEN1* M146L mutation display a different PS1 conformation compared to their wild-type counterparts<sup>220</sup>. It has been shown that the mutation forces the two terminals of PS1 to come closer to each other, resulting in a tighter conformation that can be measured via FRET/FLIM (Figure 16A)<sup>220</sup>. Analysis of FRET/FLIM measurements revealed that our CRISPR/Cas9 approach was sufficient to decrease %FRET efficiency in a subset of treated cells (Figure 16B). A lower %FRET efficiency translates to a more open PS1 conformation that resembles the wild-type protein. However, due to the wide variability in data from different patient samples, the overall %FRET efficiency did not differ significantly from the non-treated mutant samples.

We hypothesised that the allele-specific indel formation caused by the combined action of Cas9 and the M146L gRNA, would lead to the expression of truncated *PSEN1* transcripts that are degraded before reaching the translation machinery. Indeed, treated samples displayed significantly decreased N- and C-terminal PS1 fragments, thereby confirming our hypothesis (Paper I, Figure S4).

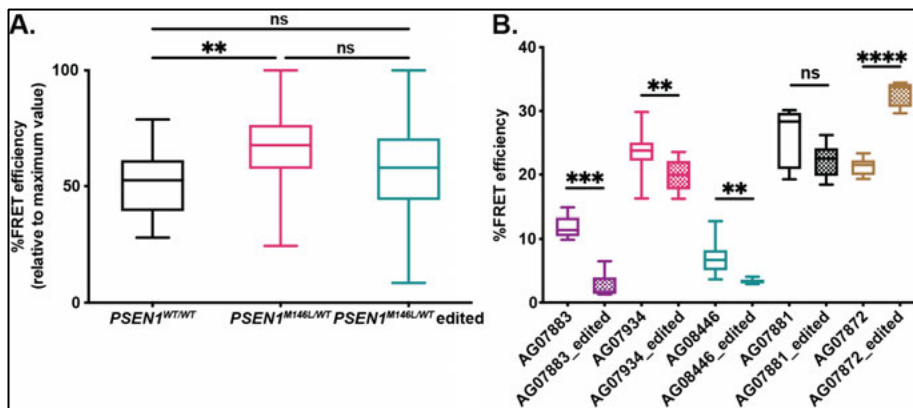


Figure 16. PS1 conformation is altered upon gene disruption. The %FRET efficiency in mutant samples was significantly higher compared to wild-type samples and showed a trend for decrease after treatment (A). Individual patient samples responded differently to the treatment, with most of them displaying a decrease in %FRET efficiency (B).

Overall, in this proof-of-concept study we were able to show that our CRISPR/Cas9 approach was selective towards the *PSEN1* M146L mutation and did not affect the wild-type sequence. Furthermore, we were able to partially restore the pathogenic A $\beta$ 42/40 ratio in patient fibroblasts without causing any detectable off-target effects. In addition, we could propose a potential mechanism for the partial decrease by evaluating the conformation of PS1. We hypothesised that genomic disruption led to the generation of

truncated transcripts that were degraded by the cell, thereby enabling the participation of more wild-type PS1 in GS complexes.

## Exploring the potential of CRISPR as a therapeutic tool against sporadic and familial PD

In **Paper II**, we aimed to apply the experience gained from the AD project in experimental models of sporadic and familial PD. First, we explored a pan-*SNCA* approach, where wild-type human samples were treated with two different Cas9 variants in order to disrupt the *SNCA* gene. Unlike SpCas9, the KKHSaCas9 variant recognises a “NNNRRT” PAM site that allowed us to select gRNAs against different *SNCA* exons. In total, we designed ten gRNAs, five for KKHSaCas9, a smaller Cas9 orthologue, and five for SpCas9, spanning the entire *SNCA* sequence (Table 1).

A. KKHSaCas9			
CATGAATACATCCATGGCTA	ATGAAT	SNCA1 gRNA	Exon 2
AAGCAGCAGGAAAGACAAA	GAGGGT	SNCA2 gRNA	Exon 2
ACACCATGCACCACTCCCTC	CTGGT	SNCA3 gRNA	Exon 3
AAACCAAGGAGGGAGTGGTG	CATGGT	SNCA4 gRNA	Exon 3
TTGACAAAGCCAGTGGCTGC	TGCAAT	SNCA5 gRNA	Exon 4

B. SpCas9			
GCTGCTGAGAAAACCAAACA	GGG	SNCA6 gRNA	Exon 2
CAGGGTGTGGCAGAAGCAGC	AGG	SNCA7 gRNA	Exon 2
ACCAAGGAGGGAGTGGTGCA	TGG	SNCA8 gRNA	Exon 3
GGAGGGAGTGGTGCATGGTG	TGG	SNCA9 gRNA	Exon 3
CTTTGTCAAAAAGGACCAGT	TGG	SNCA10 gRNA	Exon 4

Table 1. Sequences of gRNAs across the *SNCA* gene. Five gRNAs were designed for KKHSaCas9 (A) and five for SpCas9 (B). The PAM sites are depicted in red. The best gRNAs, based on editing efficiency, are highlighted in yellow.

Following a preliminary screening process, the best gRNA candidates for the respective Cas9 variants in terms of editing efficiency were selected and HEK293T cells were transfected with plasmid vectors containing the CRISPR components. Sanger sequencing followed by indel analysis revealed that both Cas9 and gRNA combinations resulted in genomic disruption that reached 42% (Figure 17). However, the extent of indel formation was only quantified in SpCas9+SNCA8 gRNA treated cells, as the online software used for sanger sequencing analysis only recognises SpCas9 related PAM sites.

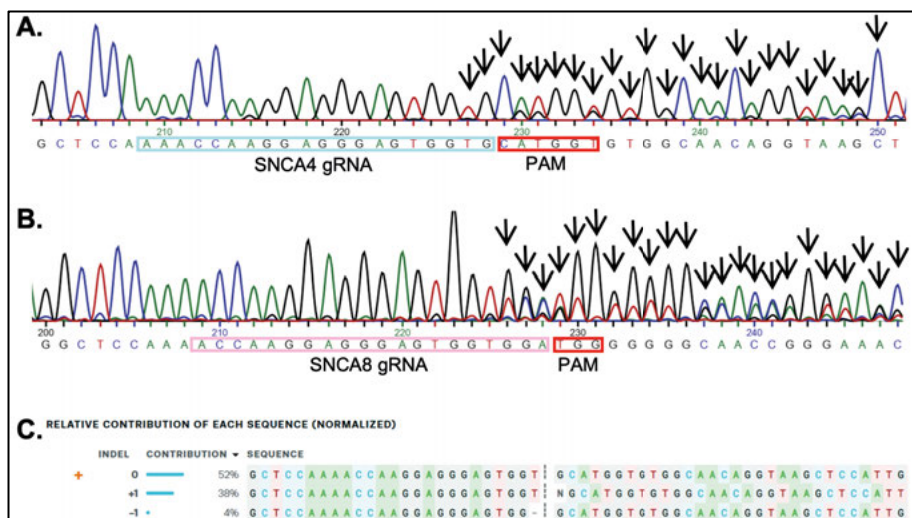


Figure 17. Sequencing traces of CRISPR-treated HEK293T cells. The combination of KKHSaCas9+SNCA4 gRNA led to indel formation in the *SNCA* gene as shown by background peaks in the sequencing trace (A). The SpCas9+SNCA8 combination displayed a more robust editing efficiency that affected almost half of the wild-type alleles (B). Analysis of gene editing events revealed that the majority of indels reflect a single base pair insertion or deletion at the cleavage site (C).

Given the relatively low percentage of editing efficiency, we decided to employ a system that would enable a stable intracellular expression of the CRISPR components. Thus, we designed a lentiviral vector that expressed SpCas9 and SNCA8 gRNA. Apart from targeting wild-type human *SNCA* in HEK293T cells, we included fibroblasts from healthy individuals (F1-4) and evaluated the editing efficiency in both cell types. Lentiviral transduction of CRISPR components led to disruption of up to 87% in HEK293T cells, while the fibroblast samples displayed varying results extending from 5 to 78% (Figure 18A, B). We hypothesise that the variability of editing among the fibroblast samples was due to the heterogeneity of the genetic makeup of each individual.

Next, we modified our approach to target a mutation that leads to early-onset familial PD. The *SNCA* A53T mutation is a single base pair substitution (G>A) in exon 3 of *SNCA*. A gRNA was designed to target the mutant but not the wild-type sequence (Figure 19A). Human fibroblasts from two PD patients and two healthy controls were treated with a plasmid vector expressing SpCas9 and A53T gRNA. Although the specificity of this approach was very high, as shown by the lack of indel formation in the wild-type samples (Figure 19B), the efficiency was relatively low compared to the pan-*SNCA* approach. One of the mutant samples was targeted with an efficiency of 13% and the other one with 9% (Figure 19C).

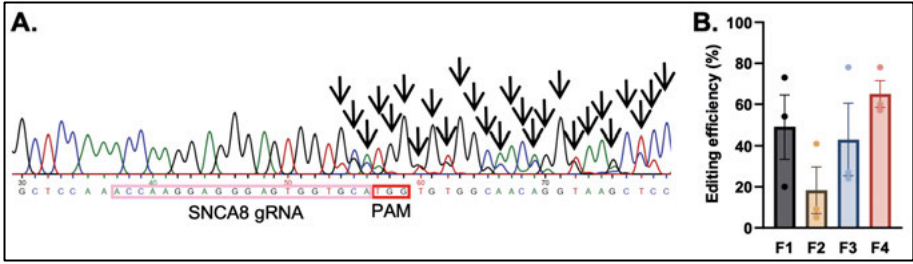


Figure 18. Lentiviral transduction of human fibroblasts with CRISPR components. The stable integration of the lentiviral vector expressing SpCas9+SNCA8 gRNA in human fibroblasts led to formation of indels in the *SNCA* gene as shown by background peaks in the sequencing trace (A). Analysis of gene editing outcomes in fibroblasts from four different individuals (F1-4) revealed editing efficiencies that ranged between 5 and 78% (fibroblasts from each individual were analysed in triplicates) (B).

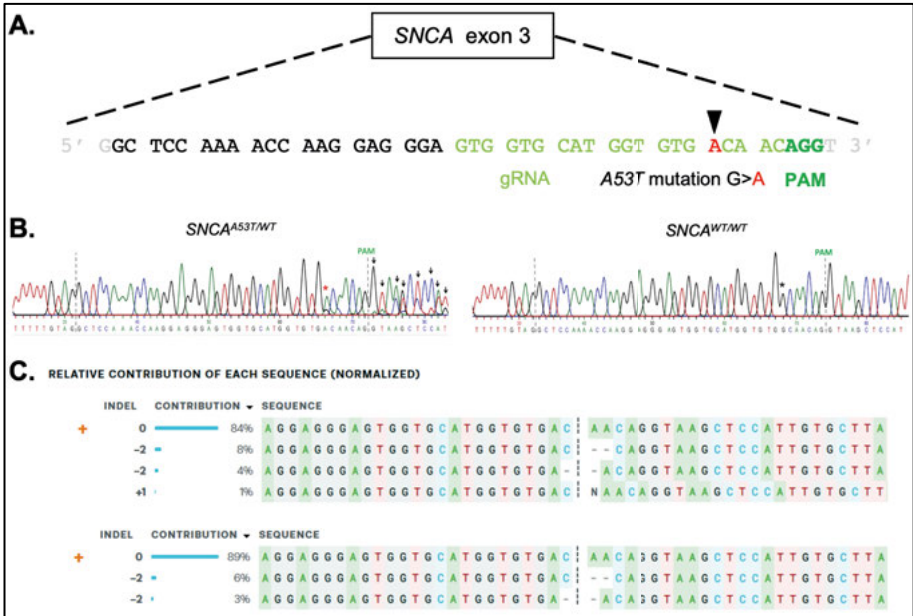


Figure 19. Targeting of the *SNCA* A53T mutation in patient fibroblasts. A gRNA was designed against the single base pair *SNCA* A53T mutation in exon 3 of *SNCA* (A). Sequencing traces of fibroblasts from PD patients, but not healthy controls that were treated with SpCas9+A53T gRNA revealed background peaks that point to genomic disruption (B). Analysis of gene editing outcomes displayed a low percentage of editing efficiency that reached 13% in one of the two PD samples (C).

Taken together, we explored the use of two CRISPR/Cas9 systems and two delivery methods to determine the potential of targeting *SNCA* in immortalised (HEK293T) and non-immortalised (fibroblasts) human cells.

## A $\beta$ pathology affects the interplay between astrocytes and neurons

In the first part of **Paper III**, we sought to investigate the effect of astrocytes on neuronal viability. Several studies have demonstrated that astrocytes are crucial for the viability of neurons in the brain<sup>70</sup>. In our study, we aimed to elucidate how inclusions of A $\beta$  could alter astrocytic behaviour and consequently hamper neuronal function. For this purpose, we designed two experimental setups. The first was a co-culture system of hiPSC-derived neurons and astrocytes with or without A $\beta$  inclusions. The second was based on addition of conditioned media or isolated EVs from astrocytes with or without A $\beta$  inclusions to neuronal monocultures (Figure 20). In both setups, the astrocytes were exposed to sonicated A $\beta$ 42 fibrils for 3-6 days to ensure maximum uptake. Astrocytes or conditioned media/EVs were added to human iPSC-derived neuronal cultures and the cells were analysed after 6 to 12 days.

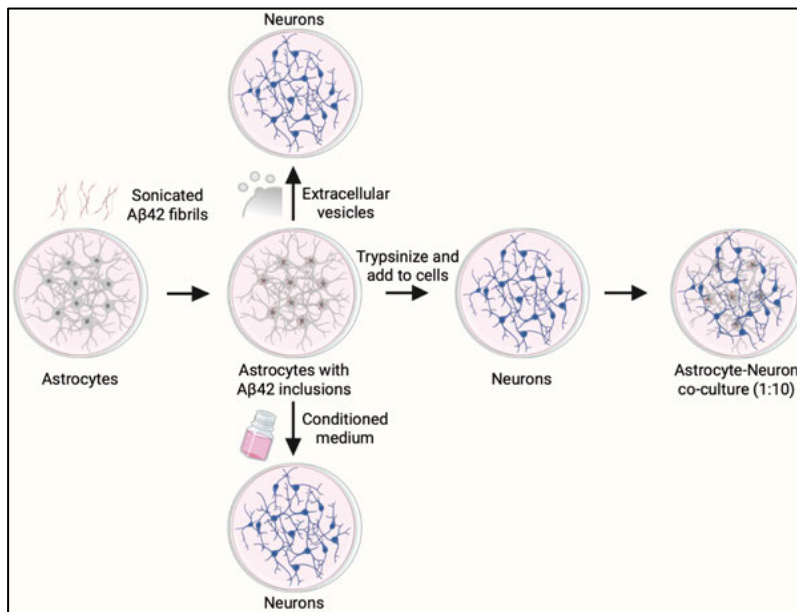


Figure 20. Paper III study design. In the first part, A $\beta$ -exposed hiPSC-derived astrocytes were co-cultured with hiPSC-derived neurons at a 1:10 ratio. In the second part, that aimed at exploring the indirect effect of astrocytes and A $\beta$  in neuronal cultures, conditioned media or extracellular vesicles were isolated from astrocytes and added to neuronal cultures.

During the 28-day neuronal differentiation, we noticed a high degree of cell death that was in line with previously published data<sup>198</sup>. In the absence of glial cells, cell corpses remained within the culture and formed hubs together with

viable neurons. Astrocytes with A $\beta$  inclusions (Figure 21A) appeared to be drawn to these hubs that were fewer in the A $\beta$  co-cultures (Figure 21B).

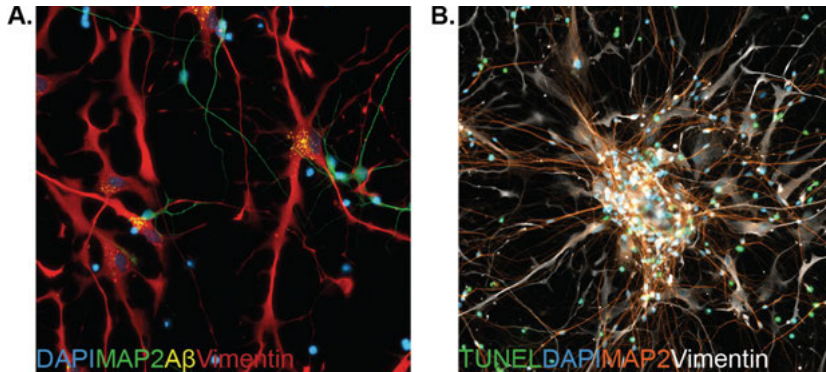


Figure 21. Co-cultures of neurons and astrocytes with A $\beta$  inclusions. Astrocytes are integrated within the neuronal network and continue to carry their A $\beta$  load (A). Hubs of dead cells attracted astrocytes in the A $\beta$  co-cultures (B). DAPI represents nuclear staining, while MAP2 and vimentin represent neurons and astrocytes, respectively. TUNEL refers to the technique used to stain apoptotic nuclei.

We hypothesized that A $\beta$  would promote the astrocytes to shift towards a more reactive state and thereby upregulate their clearance activity. That was evident in the viability analyses that we performed between control monocultures and control or A $\beta$  co-cultures, where we observed a decreased dead/live cell ratio in the A $\beta$  co-cultures (Figure 22A). Interestingly, the indirect effect of astrocytes on neurons, as explored via the addition of conditioned media, produced a very different result. Neuronal monocultures, that received conditioned media from control astrocytes, displayed increased viability compared to untreated monocultures and monocultures exposed to conditioned media from A $\beta$  astrocytes (Figure 22B). However, no difference was evident in the neuronal monocultures that received EVs isolated from control or A $\beta$  astrocytes (Figure 22C).

These results demonstrate the distinct effects that astrocytes exert on neurons depending on their physical or remote presence. Reactive astrocytes are known to engulf dead cells more effectively compared to healthy astrocytes, which would explain the viability measurements from the co-culture analysis<sup>221</sup>. Furthermore, astrocytes secrete a wide array of classic neuroactive molecules and hormones, as well as metabolic, trophic and plastic factors<sup>222</sup>. The presence of A $\beta$  deposits could potentially alter the type of secreted factors, thereby modifying the effect of conditioned media on neuronal monocultures.

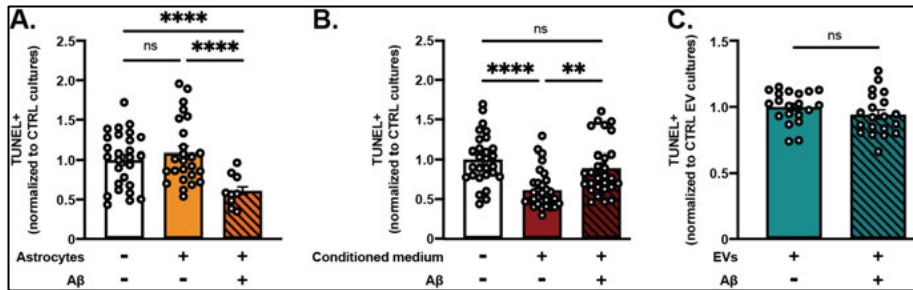


Figure 22. Astrocytes affect neuronal viability. Identification of dead cells using TUNEL staining revealed a lower number of cell corpses in the A $\beta$ -treated co-cultures compared to the monocultures and control co-cultures (A). Addition of conditioned media from control astrocytes to neuronal monocultures resulted in increased viability compared to untreated neuronal monocultures and neuronal monocultures that received A $\beta$ -astrocyte conditioned media (B). No effect in viability was seen in neuronal monocultures treated with EVs isolated from control or A $\beta$ -exposed astrocytes (C).

## Astrocytic A $\beta$ inclusions interfere with neuronal synaptic activity

One of many physiological roles of astrocytes in the human brain is to provide support to neurons. Studies from *ex vivo* and *in vivo* models suggest that synaptic activity is affected during AD progression<sup>223</sup>. In the second part of **Paper III**, we aimed to evaluate the way in which direct or indirect presence of astrocytes with A $\beta$  inclusions can modulate neuronal function and maturation. To that end, we analysed the electrophysiological properties of neurons in our co-culture and conditioned media setups.

Both the physical and remote presence of astrocytes resulted in increased synaptic activity of neurons, as measured by the frequency of excitatory post synaptic currents (EPSCs) (Figure 23A). However, in the situation with astrocytic A $\beta$  inclusions the effects were opposite when comparing direct and indirect astrocytic contact. Neurons in co-cultures containing A $\beta$ -exposed astrocytes displayed significantly decreased EPSC frequency, while neurons that received A $\beta$  conditioned media exhibited an increase, compared to their respective control counterparts (Figure 23B, C). This comparison further supports the differential effect of astrocytic A $\beta$  inclusions on the physical and remote tuning of neuronal activity.

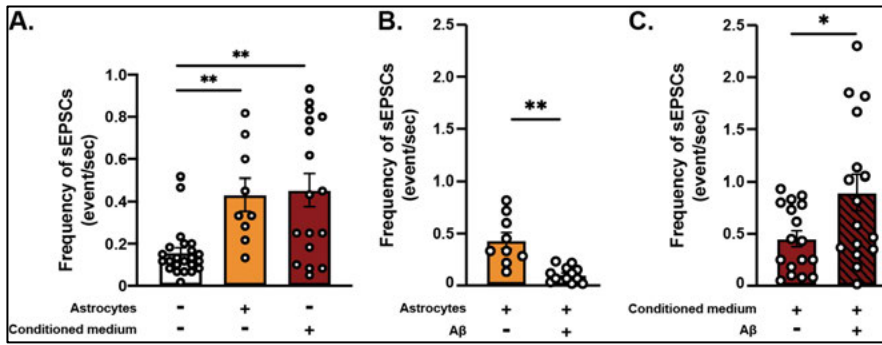


Figure 23. Synaptic activity is affected by A $\beta$  inclusions. Addition of astrocytes or astrocytic conditioned media increased the frequency of sEPSCs (A). Astrocytic A $\beta$  inclusions decreased the frequency of sEPSCs in the co-culture setup (B), while A $\beta$  conditioned media increased the frequency (C).

## Astrocytes can store A $\beta$ inclusions for an extended period of time

Alzheimer's disease is a slowly progressive disease where the first pathological changes in the brain are estimated to occur 15-20 years before the first clinical symptoms emerge<sup>224</sup>. The cellular mechanisms in action during that period are not completely understood, but according to the amyloid hypothesis, A $\beta$  accumulation precedes the neurodegeneration<sup>225</sup>. The interaction of astrocytes with A $\beta$  pathology and their possible involvement in AD progression has recently gained increasing attention<sup>226</sup>. To that end, in **Paper IV** we investigated how astrocytes respond to long-term intracellular storage of A $\beta$ .

Astrocytes with A $\beta$  inclusions were cultured for a total of 10 weeks (T2) and compared with 1-week (T1) A $\beta$ -exposed astrocytes. Quantification of intracellular A $\beta$  load revealed a significant increase in T2 astrocytes (Figure 24A). As the average number of astrocytes was decreased over time, this finding is likely attributed to the phagocytic capacity of astrocytes to engulf dead cells and consequently take over their A $\beta$  deposits (Figure 24B).

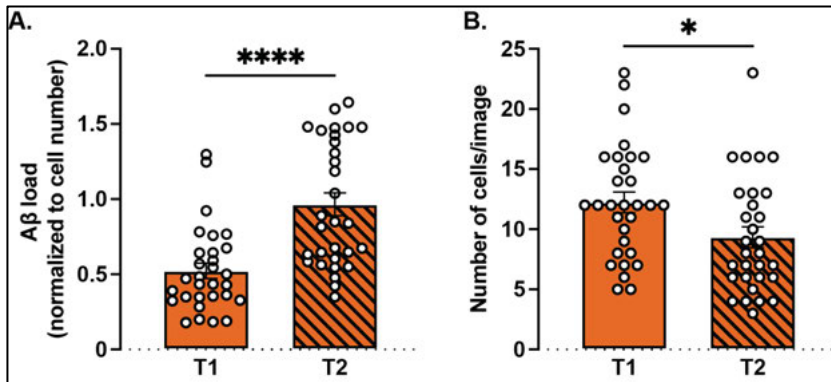


Figure 24. Increased intracellular A $\beta$  levels in T2 astrocytes correlate with decreased cell numbers. Quantification of A $\beta$  load in T1 and T2 astrocytes revealed an increase in the long-term cultures (T2) (A). However, further analyses of the quantified images revealed a decrease in the number of cells included per image in T2 (B) (T1=1 week, T2=10 weeks).

Previous studies suggest that astrocytes release truncated A $\beta$  peptides that are toxic to neurons<sup>99,101</sup>. The degradation process that leads to the secretion of harmful fragments takes place in the lysosomal compartments. To establish whether lysosomal number and cellular localization were affected by the long-term A $\beta$  inclusions, we analysed LAMP1 positive organelles in the astrocytes at the two time points. We found that LAMP1 colocalized with A $\beta$  in both T1 and T2 astrocytes suggesting that, although the astrocytes successfully confined the A $\beta$  within the lysosomal compartments, they failed to degrade the material (Figure 25A).

Notably, we observed an increased number of TNTs in A $\beta$ -exposed T1 and T2 astrocytes, compared to their control counterparts. Our staining revealed an exchange of LAMP1 positive organelles between nearby cells (Figure 25B, B1). However, unlike previous reports confirming the transfer of  $\alpha$ Syn via TNTs, we did not detect any exchange of A $\beta$  via TNTs<sup>89</sup>.

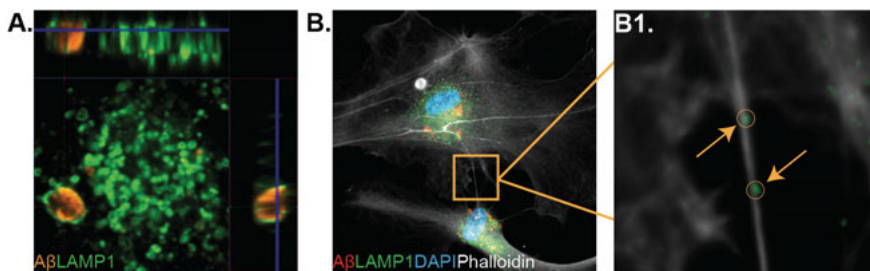


Figure 25. Localization of A $\beta$  and intercellular communication via TNTs. The A $\beta$  inclusions were surrounded by LAMP1 positive organelles, suggesting containment within the cell's degradation units (A). The astrocytes communicated with one another via the exchange of LAMP1 positive organelles through thin actin filaments known as TNTs (B). Magnified TNT containing LAMP1 positive organelles (B1).

Reactive astrocytes are characterized by altered biochemical and morphological features<sup>227</sup>. To better understand the way A $\beta$  inclusions affect the astrocytic reactivity, we quantified the levels of various proteins by WB. The reactivity markers vimentin, GFAP and S100 $\beta$  were equally expressed in control and A $\beta$ -exposed astrocytes at T1. However, after 10 weeks of culture the levels had decreased in the control cells, but not as much in the A $\beta$ -exposed cells (Figure 26).

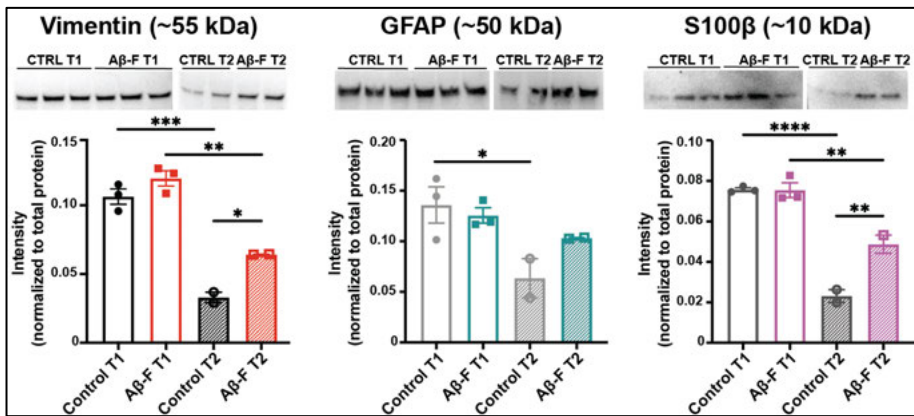


Figure 26. Astrocytes retain reactivity markers at T2. Quantification of WB data from cell lysates isolated at T1 and T2 revealed sustained astrocyte reactivity at T2 as shown by the higher levels of expressed reactivity markers such as vimentin, GFAP and S100 $\beta$  (T1=1 week, T2=10 weeks).

The extended culturing time in combination with the presence of A $\beta$  inclusions led to abnormal mitochondria and swollen ER structures in A $\beta$ -exposed T2 astrocytes (Figure 27). These findings correlate with previous reports that propose A $\beta$  as a strong stressor for cells in general and astrocytes in particular<sup>228</sup>.

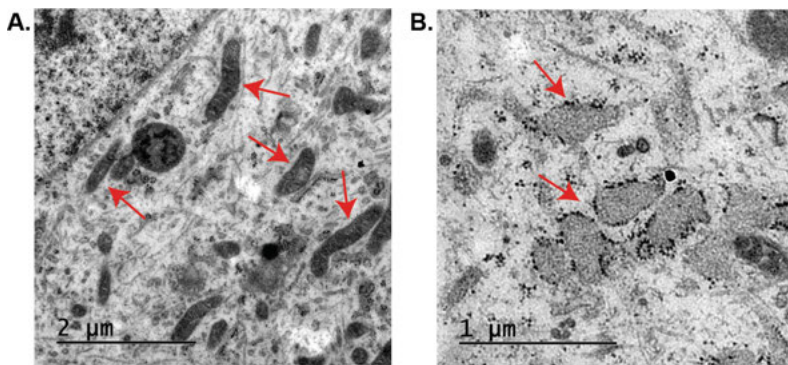


Figure 27. Cellular structures affected by A $\beta$  inclusions. Astrocytes exposed to A $\beta$  display abnormal mitochondria (A) and swollen ER structures (B) that point to an overall impairment of cellular homeostasis.

## Future perspectives

The work presented in this thesis aimed at providing insight into novel therapeutic strategies for neurodegenerative diseases and the interplay between key cellular players in AD progression. Even though we explored as many aspects as time allowed us, there are always more things to be done and more experiments to be run.

In **Papers I** and **II**, we showed how the CRISPR/Cas9 system can be employed to target pathogenic mutations associated with early-onset Alzheimer's disease and Parkinson's disease. The treatment protocol was tested on human fibroblasts and HEK293T cells as a proof-of-principle study, but it will have to be applied to a more relevant cellular type, such as human neurons, to allow us to draw more appropriate conclusions. Neuronal cultures could be established by using hiPSCs generated from individuals with these mutations or from genetically modified, wild-type hiPSCs. In the case of  $\alpha$ Syn and the *SNCA* gene, it would enable us to study the effects of the treatment on a deeper biochemical level as the levels of  $\alpha$ Syn are undetectable in human fibroblasts, both on the protein and the transcription level. Moreover, certain pathological phenotypes, such as mitochondrial dysfunction and the accompanied increase of ROS, are only detected in neuronal cells. Therefore, the use of hiPSC-derived neurons would provide us with additional data to better understand the outcome of genomic disruption.

To further extend our approach on a preclinical level, *in vivo* studies involving mouse models that express human *PSEN1* M146L or *SNCA* would provide conclusive data regarding the effectiveness and specificity of our gRNA design. In addition, it would enable us to evaluate the use of the CRISPR/Cas9 system both as a prevention and a treatment strategy. Animals could be treated either at an early stage, before the onset of disease pathology, as a prevention study or at a later stage, after disease onset, as a treatment study. Delivery of CRISPR components would need to be optimised and different methods to the ones used in **Papers I** and **II** would have to be employed. One such option could be to use adeno-associated viruses (AAVs), which do not integrate to the host genome and allow stable expression of the transgenes. However, AAVs have a limited packaging capacity of  $\sim 4.7$  kilobases, which means that smaller variants of the Cas9 family will have to be explored, such as SaCas9 that was used in **Paper II**. Inclusion of *in vivo* models and successful application of our CRISPR/Cas9 approach can provide a more complete body of work that would allow us to make broader and more

concrete conclusions regarding the potential use of our approach in a clinical trial setting.

In **Paper III** we explored the interplay between astrocytes and neurons and the impact of A $\beta$  pathology in this relationship. We generated a co-culture system that allowed us to study the effects of astrocytes in neuronal synaptic activity and analyse how A $\beta$  deposits in astrocytes interfered with their physiological functions. To evaluate the changes in synaptic activity, it was important to generate hiPSC-derived neurons that were electrically mature and could fire and propagate action potentials. Although our extended culturing time yielded cells that resembled mature neurons, their RMP was still significantly below the physiological value of -65 mV. Therefore, we would have to improve this aspect of our *in vitro* model to resemble the electrophysiological properties of mature human cells more accurately. This might be achieved via the addition of growth factor cocktails in the culture medium or culturing the cells on matrices that allow cellular development within a 3D environment.

Furthermore, given the very different results from the addition of conditioned media in neuronal monocultures, we could analyse the contents of the media in more detail. The use of advanced techniques on these samples, such as mass spectrometry, could provide clues to their mode of action. Moreover, gene expression analysis of both astrocytic and neuronal populations would offer valuable information regarding the way in which A $\beta$  alters cell behaviour and modulates synaptic activity.

Although astrocytes are the most abundant cells in the brain, microglia are responsible for most of the phagocytosis and degradation that occur. Therefore, we are currently working on establishing a tri-culture system of astrocytes, neurons and microglia that will hopefully recapitulate the real-life situation in a more accurate way. In fact, a previous study on this astrocyte-microglia co-culture system from our laboratory suggested that microglia can extract ingested material from the astrocytes that the astrocytes cannot process properly<sup>66</sup>.

In **Paper IV**, we aimed to investigate how astrocytes process A $\beta$  inclusions after extended culturing time. Based on our previous results that astrocytes are poor at degrading oligomeric and fibrillar forms of A $\beta$ , we sought to understand the mechanisms behind their tendency to store A $\beta$  inclusions. Our findings suggest a method of containment within lysosomal compartments that potentially protects the cell from exposure to toxic forms of A $\beta$ . Analysis of the intracellular A $\beta$  deposits via mass spectrometry would provide us with more information regarding the condition of the A $\beta$  peptides within the inclusions, especially in terms of partial degradation and modifications.

A long-term goal with **Papers III** and **IV** is to set up an “AD model in a dish” that mimics sporadic AD, which is suitable for our scientific questions regarding cellular interplay and cell-to-cell disease propagation, instead of focusing on familial mutations that induce pathology by overexpression of A $\beta$

or tau<sup>229-231</sup>. Since *APOE*, one of the major sporadic AD risk factors, is assumed to participate in the intracellular trafficking of A $\beta$ , we plan to evaluate the effects of different *APOE* genotypes using the co-cultures and long-term exposure previously described. Astrocytes and neurons generated from isogenic *APOE* hiPSCs, genetically engineered by the CRISPR/Cas9 system to produce four different genotypes (*APOE* $\epsilon$ 2/2, *APOE* $\epsilon$ 3/3, *APOE* $\epsilon$ 4/4 and *APOE* knockout). Previously published data indicate that *APOE*  $\epsilon$ 4 astrocytes produce more A $\beta$  compared to the other genotypes<sup>53,100</sup>. However, the exact link between ApoE and neurodegeneration is still unclear. Using such isogenic cell lines, we aim to investigate if different *APOE* genotypes affect A $\beta$  accumulation and degradation processes as well as to evaluate their involvement in other AD-related pathological phenotypes.

## Popular science

Alzheimer's disease (AD) and Parkinson's disease (PD) are the two most common neurodegenerative disorders. Over the past decades, advancements in healthcare and medicine have increased the life expectancy of humans. However, this has also increased the cases of AD and PD that currently affect millions of people worldwide. Unfortunately, patients with AD and PD can only be offered treatments that manage a few of the disease symptoms, while a cure or medication that slow down the disease progression remain elusive.

The main clinical feature of AD is cognitive impairment, presumably caused by the death of neuronal cells in the brain. It is thought to be a slowly progressive process that starts decades before the first symptoms emerge. Several theories have been proposed regarding the way in which AD develops, but the most prominent one is known as "the amyloid cascade hypothesis". According to this theory, there are two different proteins that are responsible for the disease. One is the amyloid-beta ( $A\beta$ ) protein and the other is the tau protein, both of which are naturally found within several cell types of the human body. For reasons that are still not very well understood,  $A\beta$  can start to form aggregates that cannot be cleared from the brain, leading to the formation of structures known as plaques. These plaques and smaller  $A\beta$  aggregates affect the behaviour of the tau protein, which is important for the stability of the neuronal network within the brain and leads to the aforementioned cell loss.

One of the key aspects of the amyloid cascade hypothesis relies on the different forms of  $A\beta$  present in the brain and how they are generated. They derive from the processing of the amyloid precursor protein (APP) by an enzyme called gamma-secretase (GS). Gamma-secretase cuts the APP into several smaller proteins, among which is  $A\beta$  consisting of 40 amino acids ( $A\beta_{40}$ ) and 42 amino acids ( $A\beta_{42}$ ). In its physiological state, GS ensures the proper ratio of these two  $A\beta$  forms. The  $A\beta_{42}$  is more prone to form  $A\beta$  plaques and is therefore considered more toxic compared to  $A\beta_{40}$ . The ability of GS to cut the APP in varying lengths comes from one of the four proteins that compose it, namely presenilin 1 (PS1). The action of PS1 regulates the generation of the different  $A\beta$  forms and is crucial to AD progression.

Mutations in the genes that express PS1 (*PSEN1*) and APP (*APP*) lead to severe cases of AD that start at a young age (mainly between 35-60 years) and can be potentially inherited. These early-onset cases only represent about 5% of the total AD cases, while the rest are mainly attributed to old age. The

involvement of environmental and genetic risk factors that may contribute to late-onset AD are still under investigation.

Although AD is closely associated with dementia, there is a lot happening behind the scenes before that occurs. Within the brain and throughout the entire nervous system, neurons coexist with several other cell types that all have a role to play. Astrocytes are one of the most important of these cell types and are responsible for a number of tasks that ensure maintenance of proper brain environment and function. They take their name from the Greek words “ἄστρον, ástron = star” and “κύτος, kútos = cell”, owing to their characteristic starshaped appearance. Astrocytes regulate the communication between various cell types of the brain in general, and neurons in particular. However, in the event of injury or when they sense irregularities in the brain environment, such as the presence of A $\beta$  plaques, they can quickly change their behaviour (in order to protect the brain), by entering a so called “reactive state”. This reactive state is accompanied by changes in the astrocytes’ appearance as well as in their protein expression. Astrocytes send signals to nearby cells, alerting them about the existence of a threat but, at the same time, they also try to contain the damage themselves. In the case of A $\beta$  aggregates, astrocytes sometimes find it difficult to break them up and therefore a lot of A $\beta$  (that they are unable to clear) ends up being stored within the cells. Perhaps their inability to properly dispose the A $\beta$  they consumed could be a key to understanding how the disease moves from one cell to another and finally spreads through the brain.

In a similar manner, PD is characterised by the accumulation of another protein, known as  $\alpha$ -synuclein ( $\alpha$ Syn). This protein is present throughout the brain, but is most commonly found in between communicating neurons. Accumulation of  $\alpha$ Syn aggregates leads to the loss of neurons in a specific brain region. The symptoms of PD can range from mild sleep and smell impairment at the early stages, to uncontrollable tremor and dementia at the final stages. Most PD cases result from a combination of ageing and environmental factors, whereas a small percentage is associated with mutations in the gene that expresses  $\alpha$ Syn (*SNCA*).

Although mutation-associated AD and PD cases only represent a small fraction of the patients, studying them allows us to better understand the mechanisms behind disease onset and progression. So far, treatment strategies for both diseases are far from ideal and, therefore, we need to come up with novel approaches, such as gene editing, to help the patients and their families.

The term “gene editing” refers to any modification that occurs to a specific gene in a living organism. As difficult and futuristic as this may sound, the recent discovery and adaptation of a certain defence system found in bacteria, has provided us with a tool that can do just that. The CRISPR/Cas9 system is in principle a tiny molecular pair of scissors that can be guided to any gene and alter its sequence. The guidance is provided by a small piece of RNA (guideRNA) that acts as a GPS signal, providing very precise coordinates, for

the scissors to cut and modify. This tool is very simple to use and is already being tested in clinical trials against diseases that lack a cure. Nevertheless, its application entails risks that can be minimised by proper experimental design and follow-up monitoring.

To that end, in **Paper I**, we used the CRISPR/Cas9 system to investigate if we could target a specific mutation in the *PSEN1* gene that leads to AD with a very young age of onset, at an average of 43 years. One key characteristic of this mutation is the increased ratio of A $\beta$ 42/40 seen in cells from these individuals compared to healthy ones. Therefore, we obtained skin cells from subjects with the *PSEN1* M146L mutation and treated them with the CRISPR/Cas9 system. Each individual had two copies of the *PSEN1* gene, one with the mutation (mutant) and one without (wild-type). Our goal was to only modify the mutant copy without affecting the wild-type. Indeed, we were successful at selectively targeting the mutant copy without causing any damage to the wild-type. Furthermore, we saw a reduction in the A $\beta$ 42/40 ratio of treated cells that resembled the normal levels in wild-type cells. Finally, we proposed a mechanism of how our treatment led to the reduction of the ratio, suggesting that the CRISPR/Cas9 system practically minimized the expression of the mutant *PSEN1* copy and allowed the wild-type form to perform its normal function.

In **Paper II**, we modified our approach and evaluated the use of the CRISPR/Cas9 system against PD, by targeting the expression of the *SNCA* gene that expresses  $\alpha$ Syn. Our idea was to lower the levels of expressed  $\alpha$ Syn and thereby avoid its accumulation. We utilised two different versions of the CRISPR/Cas9 system as well as two different methods of delivering it inside cells. We were able to successfully modify the *SNCA* gene in two different types of cells from healthy individuals, albeit with a varying degree of consistency. We then moved on to tailor our approach towards the *SNCA* A53T mutation that leads to PD at a young age and can be passed on to future generations. As with *PSEN1*, there are two copies of the *SNCA* gene in human cells. One copy has the mutation, while the other does not. Although our experimental design was able to modify the mutant copy, the efficiency of the approach was not as high, compared to our previous attempts. Nevertheless, we are still working on optimising the system and finding new ways to make the most of this tool.

In **Paper III**, we looked into how neurons and astrocytes communicate with each other by growing cells in a controlled setting in the laboratory. To mimic the characteristics of an AD environment, we added A $\beta$  to the cultures and monitored if and how that affected the interaction between cells. We were able to show that the addition of A $\beta$  altered the behaviour of astrocytes at the point that they neglected some of their normal functions, such as providing support and taking care of the neurons. Moreover, the effect of astrocytes on neurons depended on whether they had direct contact with them or only indirect, through molecules that they secreted. That aspect became clearer

when we looked at neuron-to-neuron communication, conducted through the transmission of electric signals. By measuring the signal exchange between neurons, we could demonstrate that the presence of astrocytes interfered with the exchange and that addition of A $\beta$  further altered this interplay. Taken together, this information could provide us with a better understanding of the way in which astrocytes that carry A $\beta$  inclusions could potentially participate in the progression of AD.

In **Paper IV**, we took our cell cultures one step further and explored the mechanisms behind long-term storage of A $\beta$  by astrocytes. As previously mentioned, AD is a slowly progressive disease and the first symptoms appear only 15-20 years after the process has already started. By culturing our astrocytes with A $\beta$  for an extended period of time, we hoped to get some hints of what happens between the time that the first A $\beta$  plaques appear in the brain and the first symptoms emerge and how astrocytes may be involved in this process. We demonstrated that astrocytes did try to clear A $\beta$  by engulfing it, but were unable to break up the aggregates, even after very long time. Instead, A $\beta$  was stuck within certain cell compartments, called lysosomes, that are meant to digest any material that ends up there. Also, the astrocytes seemed to remain in a reactive state, which could potentially affect their other activities, including interaction with neighbouring brain cells. Furthermore, we found small changes in the molecules that the astrocytes secreted, which also pointed towards an altered behaviour following A $\beta$  exposure that kept them distracted from their ordinary tasks. We believe that this project has given us new insights into the early steps of AD.

Taken together, this thesis aimed to explore novel treatment strategies for AD and PD as well as to contribute with knowledge into the mechanisms that dictate AD progression in the hope of getting one step closer to finding a cure.

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# References

1. Crous-Bou, M., Minguillón, C., Gramunt, N. & Molinuevo, J. L. Alzheimer's disease prevention: From risk factors to early intervention. *Alzheimer's Res. Ther.* **9**, 1–9 (2017).
2. Serrano-Pozo, A., Frosch, M. P., Masliah, E. & Hyman, B. T. Neuropathological alterations in Alzheimer's disease. *Cold Spring Harb. Perspect. Med.* **1** (2011).
3. McKhann, G. *et al.* Clinical diagnosis of Alzheimer's disease. *Neurology* **34**, 939 (1984).
4. Skaper, S. D. Alzheimer's Disease and Amyloid: Culprit or Coincidence? in *New Perspectives of Central Nervous System Injury and Neuroprotection* vol. 102 277–316 (2012).
5. Ingelsson, M. & Hyman, B. T. Disordered proteins in dementia. *Ann. Med.* **34**, 259–71 (2002).
6. Vetrivel, K. S. & Thinakaran, G. Amyloidogenic processing of  $\beta$ -amyloid precursor protein in intracellular compartments. *Neurology* **66**, S69–S73 (2006).
7. Bayer, T. A., Cappai, R., Masters, C. L., Beyreuther, K. & Multhaup, G. It all sticks together—the APP-related family of proteins and Alzheimer's disease. *Mol. Psychiatry* **4**, 524–528 (1999).
8. Walsh, D. M. & Teplow, D. B. Chapter 4 - Alzheimer's Disease and the Amyloid  $\beta$ -Protein. in *Molecular Biology of Neurodegenerative Diseases* vol. 107 101–124 (2012).
9. van der Kant, R. & Goldstein, L. S. B. Cellular Functions of the Amyloid Precursor Protein from Development to Dementia. *Dev. Cell* **32**, 502–515 (2015).
10. Murphy, M. P. & LeVine 3rd, H. Alzheimer's disease and the amyloid-beta peptide. *J. Alzheimers. Dis.* **19**, 311–323 (2010).
11. Chen, G. *et al.* Amyloid beta: structure, biology and structure-based therapeutic development. *Acta Pharmacol. Sin.* **38**, 1205–1235 (2017).
12. Kaye, R. *et al.* Annular protofibrils are a structurally and functionally distinct type of amyloid oligomer. *J. Biol. Chem.* **284**, 4230–4237 (2009).
13. De Strooper, B. Aph-1, Pen-2, and Nicastrin with Presenilin Generate an Active  $\gamma$ -Secretase Complex. *Neuron* **38**, 9–12 (2003).
14. Thinakaran, G. *et al.* Endoproteolysis of Presenilin 1 and Accumulation of Processed Derivatives In Vivo. *Neuron* **17**, 181–190 (1996).
15. Takasugi, N. *et al.* The role of presenilin cofactors in the  $\gamma$ -secretase complex. *Nature* **422**, 438–441 (2003).
16. Haapasalo, A. & Kovacs, D. M. The many substrates of presenilin/ $\gamma$ -secretase. *J. Alzheimer's Dis.* **25**, 3–28 (2011).

17. Takami, M. *et al.*  $\gamma$ -Secretase: Successive Tripeptide and Tetrapeptide Release from the Transmembrane Domain of  $\beta$ -Carboxyl Terminal Fragment. *J. Neurosci.* **29**, 13042 LP – 13052 (2009).
18. Haass, C., Kaether, C., Thinakaran, G. & Sisodia, S. Trafficking and proteolytic processing of APP. *Cold Spring Harb. Perspect. Med.* **2**, 1–26 (2012).
19. Sherr, R. *et al.* Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* **375**, 754–760 (1995).
20. Levy-lahad, A. E. *et al.* Candidate Gene for the Chromosome 1 Familial Alzheimer's Disease Locus. *Science* **18**, 973–7 (1995).
21. Ahn, K. *et al.* Activation and intrinsic  $\gamma$ -secretase activity of presenilin 1. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 21435–21440 (2010).
22. Laudon, H. *et al.* A nine-transmembrane domain topology for presenilin 1. *J. Biol. Chem.* **280**, 35352–35360 (2005).
23. Podlisny, M. B. *et al.* Presenilin Proteins Undergo Heterogeneous Endoproteolysis between Thr291 and Ala299 and Occur as Stable N- and C-Terminal Fragments in Normal and Alzheimer Brain Tissue. *Neurobiol. Dis.* **3**, 325–337 (1997).
24. Lai, M. T. *et al.* Presenilin-1 and presenilin-2 exhibit distinct yet overlapping  $\gamma$ -secretase activities. *J. Biol. Chem.* **278**, 22475–22481 (2003).
25. Mastrangelo, P. *et al.* Dissociated phenotypes in presenilin transgenic mice define functionally distinct gamma-secretases. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 8972–8977 (2005).
26. Herreman, A. *et al.* Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 11872–11877 (1999).
27. Shen, J. *et al.* Skeletal and CNS Defects in Presenilin-1-Deficient Mice. *Cell* **89**, 629–639 (1997).
28. Huysseune, S. *et al.* Epigenetic control of aquaporin 1 expression by the amyloid precursor protein. *FASEB J.* **23**, 4158–4167 (2009).
29. Maetzel, D. *et al.* Nuclear signalling by tumour-associated antigen EpCAM. *Nat. Cell Biol.* **11**, 162–171 (2009).
30. Escamilla-Ayala, A., Wouters, R., Sannerud, R. & Annaert, W. Contribution of the Presenilins in the cell biology, structure and function of  $\gamma$ -secretase. *Semin. Cell Dev. Biol.* **105**, 12–26 (2020).
31. Zhou, R. *et al.* Recognition of the amyloid precursor protein by human  $\gamma$ -secretase. *Science* **363** (2019).
32. Cruts, M. *et al.* Genetic and physical characterization of the early-onset Alzheimer's disease AD3 locus on chromosome 14q24.3. *Hum. Mol. Genet.* **4**, 1355–1364 (1995).
33. Lichtenthaler, S. F. *et al.* Mechanism of the cleavage specificity of Alzheimer's disease  $\gamma$ -secretase identified by phenylalanine-scanning mutagenesis of the transmembrane domain of the amyloid precursor protein. *Proc. Natl. Acad. Sci.* **96**, 3053–3058 (1999).

34. Sun, L., Zhou, R., Yang, G. & Shi, Y. Analysis of 138 pathogenic mutations in presenilin-1 on the in vitro production of A $\beta$ 42 and A $\beta$ 40 peptides by  $\gamma$ -secretase. *Proc. Natl. Acad. Sci.* **114**, E476–E485 (2017).
35. Tanzi, R. E. The genetics of Alzheimer disease. *Cold Spring Harb. Perspect. Med.* **2**, a006296 (2012).
36. Bertram, L. & Tanzi, R. E. The Genetics of Alzheimer’s Disease. *Mol. Biol. Neurodegener. Dis.* **107**, 79–100 (2012).
37. Szaruga, M. *et al.* Qualitative changes in human  $\gamma$ -secretase underlie familial Alzheimer’s disease. *J. Exp. Med.* **212**, 2003–2013 (2015).
38. Chávez-Gutiérrez, L. *et al.* The mechanism of  $\gamma$ -Secretase dysfunction in familial Alzheimer disease. *EMBO J.* **31**, 2261–2274 (2012).
39. Veugelen, S., Saito, T., Saido, T. C., Chávez-Gutiérrez, L. & De Strooper, B. Familial Alzheimer’s Disease Mutations in Presenilin Generate Amyloidogenic A $\beta$  Peptide Seeds. *Neuron* **90**, 410–416 (2016).
40. Hardy, J. A. & Higgins, G. A. Alzheimer’s disease: the amyloid cascade hypothesis. *Science* **256**, 184–5 (1992).
41. Shen, J. & Kelleher, R. J. The presenilin hypothesis of Alzheimer’s disease: Evidence for a loss-of-function pathogenic mechanism. *Proc. Natl. Acad. Sci.* **104**, 403–409 (2007).
42. Xia, D., Kelleher, R. J. & Shen, J. Loss of A $\beta$ 43 Production Caused by Presenilin-1 Mutations in the Knockin Mouse Brain. *Neuron* **90**, 417–422 (2016).
43. Xia, D. *et al.* Presenilin-1 Knockin Mice Reveal Loss-of-Function Mechanism for Familial Alzheimer’s Disease. *Neuron* **85**, 967–981 (2015).
44. Bi, C., Bi, S. & Li, B. Processing of mutant  $\beta$ -amyloid precursor protein and the clinicopathological features of familial alzheimer’s disease. *Aging Dis.* **10**, 383–403 (2019).
45. Mullan, M. *et al.* A pathogenic mutation for probable Alzheimer’s disease in the APP gene at the N-terminus of  $\beta$ -amyloid. *Nat. Genet.* **1**, 345–347 (1992).
46. Nilsberth, C. *et al.* The ‘Arctic’ APP mutation (E693G) causes Alzheimer’s disease by enhanced A $\beta$  protofibril formation. *Nat. Neurosci.* **4**, 887–893 (2001).
47. Goate, A. *et al.* Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer’s disease. *Nature* **349**, 704–706 (1991).
48. Corder, E. H. *et al.* Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer’s disease in late onset families. *Science* **261**, 921–923 (1993).
49. Saunders, A. M. *et al.* Association of apolipoprotein E allele  $\epsilon$ 4 with late-onset familial and sporadic Alzheimer’s disease. *Neurology* **43**, 1467 (1993).
50. Farrer, L. A. *et al.* Effects of Age, Sex, and Ethnicity on the Association Between Apolipoprotein E Genotype and Alzheimer Disease: A Meta-analysis. *JAMA* **278**, 1349–1356 (1997).
51. Corder, E. H. *et al.* Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. *Nat. Genet.* **7**, 180–184 (1994).
52. Neu, S. C. *et al.* Apolipoprotein E Genotype and Sex Risk Factors for Alzheimer Disease: A Meta-analysis. *JAMA Neurol.* **74**, 1178–1189 (2017).

53. Huang, Y.-W. A., Zhou, B., Wernig, M. & Südhof, T. C. ApoE2, ApoE3, and ApoE4 Differentially Stimulate APP Transcription and A $\beta$  Secretion. *Cell* **168**, 427–441 (2017).
54. Schmechel, D. E. *et al.* Increased amyloid beta-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 9649–9653 (1993).
55. Christensen, D. Z., Schneider-Axmann, T., Lucassen, P. J., Bayer, T. A. & Wirths, O. Accumulation of intraneuronal Abeta correlates with ApoE4 genotype. *Acta Neuropathol.* **119**, 555–566 (2010).
56. Robert, J. *et al.* Clearance of beta-amyloid is facilitated by apolipoprotein E and circulating high-density lipoproteins in bioengineered human vessels. *Elife* **6**, e29595 (2017).
57. Castellano, J. M. *et al.* Human apoE isoforms differentially regulate brain amyloid- $\beta$  peptide clearance. *Sci. Transl. Med.* **3**, 89ra57 (2011).
58. Giannakopoulos, P. *et al.* Tangle and neuron numbers, but not amyloid load, predict cognitive status in Alzheimer’s disease. *Neurology* **60**, 1495–1500 (2003).
59. Walsh, D. M. & Selkoe, D. J. Deciphering the Molecular Basis of Memory Failure in Alzheimer’s Disease. *Neuron* **44**, 181–193 (2004).
60. Lyman, M., Lloyd, D. G., Ji, X., Vizcaychipi, M. P. & Ma, D. Neuroinflammation: the role and consequences. *Neurosci. Res.* **79**, 1–12 (2014).
61. Heneka, M. T. *et al.* Neuroinflammation in Alzheimer’s disease. *Lancet Neurol.* **14**, 388–405 (2015).
62. Streit, W. J., Mrak, R. E. & Griffin, W. S. T. Microglia and neuroinflammation: a pathological perspective. *J. Neuroinflammation* **1**, 14 (2004).
63. Webers, A., Heneka, M. T. & Gleeson, P. A. The role of innate immune responses and neuroinflammation in amyloid accumulation and progression of Alzheimer’s disease. *Immunol. Cell Biol.* **98**, 28–41 (2020).
64. Helmut, K., Hanisch, U. K., Noda, M. & Verkhratsky, A. Physiology of microglia. *Physiol. Rev.* **91**, 461–553 (2011).
65. Lee, C. Y. D. & Landreth, G. E. The role of microglia in amyloid clearance from the AD brain. *J. Neural Transm.* **117**, 949–960 (2010).
66. Rostami, J. *et al.* Crosstalk between astrocytes and microglia results in increased degradation of  $\alpha$ -synuclein and amyloid- $\beta$  aggregates. *J. Neuroinflammation* **18**, 124 (2021).
67. Liddelw, S. A. *et al.* Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* **541**, 481–487 (2017).
68. Abbott, N. J., Rönnbäck, L. & Hansson, E. Astrocyte–endothelial interactions at the blood–brain barrier. *Nat. Rev. Neurosci.* **7**, 41–53 (2006).
69. Weber, B. & Barros, L. F. The Astrocyte: Powerhouse and Recycling Center. *Cold Spring Harb. Perspect. Biol.* **7**, a020396 (2015).
70. Sofroniew, M. V. & Vinters, H. V. Astrocytes: biology and pathology. *Acta Neuropathol.* **119**, 7 (2010).

71. J., I. J. *et al.* A Paravascular Pathway Facilitates CSF Flow Through the Brain Parenchyma and the Clearance of Interstitial Solutes, Including Amyloid  $\beta$ . *Sci. Transl. Med.* **4** (2012).
72. Howarth, C. The contribution of astrocytes to the regulation of cerebral blood flow. *Frontiers in Neuroscience* vol. 8 (2014).
73. Bélanger, M., Allaman, I. & Magistretti, P. J. Brain Energy Metabolism: Focus on Astrocyte-Neuron Metabolic Cooperation. *Cell Metab.* **14**, 724–738 (2011).
74. Araque, A., Parpura, V., Sanzgiri, R. P. & Haydon, P. G. Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci.* **22**, 208–215 (1999).
75. Clarke, L. E. & Barres, B. A. Emerging roles of astrocytes in neural circuit development. *Nat. Rev. Neurosci.* **14**, 311–321 (2013).
76. Chung, W.-S., Allen, N. J. & Eroglu, C. Astrocytes Control Synapse Formation, Function, and Elimination. *Cold Spring Harb. Perspect. Biol.* **7**, (2015).
77. Gertrudis, P. & Alfonso, A. Astrocytes Potentiate Transmitter Release at Single Hippocampal Synapses. *Science* **317**, 1083–1086 (2007).
78. Araque, A. *et al.* Gliotransmitters Travel in Time and Space. *Neuron* **81**, 728–739 (2014).
79. Martín, R., Bajo-Grañeras R., Moratalla, R., Perea, G., Araque A. Circuit-specific signaling in astrocyte-neuron networks in basal ganglia pathways. *Science*. **349**, 730–734 (2015).
80. Schousboe, A., Sarup, A., Bak, L. K., Waagepetersen, H. S. & Larsson, O. M. Role of astrocytic transport processes in glutamatergic and GABAergic neurotransmission. *Neurochem. Int.* **45**, 521–527 (2004).
81. Chen, Y. *et al.* Astrocytes protect neurons from nitric oxide toxicity by a glutathione-dependent mechanism. *J. Neurochem.* **77**, 1601–1610 (2001).
82. Burda, J. E. & Sofroniew, M. V. Reactive Gliosis and the Multicellular Response to CNS Damage and Disease. *Neuron* **81**, 229–248 (2014).
83. Verkhratsky, A., Olabarria, M., Noristani, H. N., Yeh, C.-Y. & Rodriguez, J. J. Astrocytes in Alzheimer’s disease. *Neurotherapeutics* **7**, 399–412 (2010).
84. Wisniewski, H. M. & Wegiel, J. Spatial relationships between astrocytes and classical plaque components. *Neurobiol. Aging* **12**, 593–600 (1991).
85. Carter, S. F. *et al.* Evidence for Astrocytosis in Prodromal Alzheimer Disease Provided by <sup>11</sup>C-Deuterium-L-Deprenyl: A Multitracer PET Paradigm Combining <sup>11</sup>C-Pittsburgh Compound B and <sup>18</sup>F-FDG. *J. Nucl. Med.* **53**, 37–46 (2012).
86. Perry, V. H. Contribution of systemic inflammation to chronic neurodegeneration. *Acta Neuropathol.* **120**, 277–286 (2010).
87. Garwood, C. J., Pooler, A. M., Atherton, J., Hanger, D. P. & Noble, W. Astrocytes are important mediators of A $\beta$ -induced neurotoxicity and tau phosphorylation in primary culture. *Cell Death Dis.* **2** (2011).
88. Jana, A. & Pahan, K. Fibrillar amyloid-beta-activated human astroglia kill primary human neurons via neutral sphingomyelinase: implications for Alzheimer’s disease. *J. Neurosci.* **30**, 12676–12689 (2010).
89. Rostami, J. *et al.* Human Astrocytes Transfer Aggregated Alpha-Synuclein via Tunneling Nanotubes. *J. Neurosci.* **37**, 11835–11853 (2017).

90. Önfelt, B., Nedvetzki, S., Yanagi, K. & Davis, D. M. Cutting Edge: Membrane Nanotubes Connect Immune Cells. *J. Immunol.* **173**, 1511–1513 (2004).
91. Amin, R., Rainer, S., Ivanka, M., Paul, W. & Hans-Hermann, G. Nanotubular Highways for Intercellular Organelle Transport. *Science* **303**, 1007–1010 (2004).
92. Gerdes, H. H., Bukoreshtliev, N. V. & Barroso, J. F. V. Tunneling nanotubes: A new route for the exchange of components between animal cells. *FEBS Lett.* **581**, 2194–2201 (2007).
93. Gerdes, H.-H., Rustom, A. & Wang, X. Tunneling nanotubes, an emerging intercellular communication route in development. *Mech. Dev.* **130**, 381–387 (2013).
94. Abounit, S., Wu, J. W., Duff, K., Victoria, G. S. & Zurzolo, C. Tunneling nanotubes: A possible highway in the spreading of tau and other prion-like proteins in neurodegenerative diseases. *Prion* **10**, 344–351 (2016).
95. Johnstone, R. M., Adam, M., Hammond, J. R., Orr, L. & Turbide, C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J. Biol. Chem.* **262**, 9412–9420 (1987).
96. Lötvall, J. *et al.* Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. *J. Extracell. Vesicles* **3**, 26913 (2014).
97. Mathieu, M., Martin-Jaular, L., Lavieue, G. & Théry, C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat. Cell Biol.* **21**, 9–17 (2019).
98. Doyle, L. M. & Wang, M. Z. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. *Cells* vol. 8 (2019).
99. Söllvander, S. *et al.* Accumulation of amyloid- $\beta$  by astrocytes result in enlarged endosomes and microvesicle-induced apoptosis of neurons. *Mol. Neurodegener.* **11**, 38 (2016).
100. Nikitidou, E. *et al.* Increased Release of Apolipoprotein E in Extracellular Vesicles Following Amyloid- $\beta$  Protofibril Exposure of Neuroglial Co-Cultures. *J. Alzheimer's Dis.* **60**, 305–321 (2017).
101. Beretta, C. *et al.* Extracellular vesicles from amyloid- $\beta$  exposed cell cultures induce severe dysfunction in cortical neurons. *Sci. Reports 2020 101* **10**, 1–14 (2020).
102. Roßner, S., Lange-Dohna, C., Zeitschel, U. & Perez-Polo, J. R. Alzheimer's disease  $\beta$ -secretase BACE1 is not a neuron-specific enzyme. *J. Neurochem.* **92**, 226–234 (2005).
103. Zhao, J., O'Connor, T. & Vassar, R. The contribution of activated astrocytes to A $\beta$  production: Implications for Alzheimer's disease pathogenesis. *J. Neuroinflammation* **8**, 150 (2011).
104. Fyfe, I. Brain waste clearance reduced by ageing. *Nat. Rev. Neurol.* **16**, 128 (2020).

105. Thal, D. R. The role of astrocytes in amyloid  $\beta$ -protein toxicity and clearance. *Exp. Neurol.* **236**, 1–5 (2012).
106. Koistinaho, M. *et al.* Apolipoprotein E promotes astrocyte colocalization and degradation of deposited amyloid- $\beta$  peptides. *Nat. Med.* **10**, 719–726 (2004).
107. Carpentier, M., Robitaille, Y., DesGroseillers, L., Boileau, G. & Marcinkiewicz, M. Declining Expression of Neprilysin in Alzheimer Disease Vasculature: Possible Involvement in Cerebral Amyloid Angiopathy. *J. Neuropathol. Exp. Neurol.* **61**, 849–856 (2002).
108. Iwata, N. *et al.* Identification of the major A $\beta$ 1-42-degrading catabolic pathway in brain parenchyma: Suppression leads to biochemical and pathological deposition. *Nat. Med.* **6**, 143–150 (2000).
109. Dorfman, V. B. *et al.* Differential cerebral deposition of IDE and NEP in sporadic and familial Alzheimer’s disease. *Neurobiol. Aging* **31**, 1743–1757 (2010).
110. Yin, K.-J. *et al.* Matrix metalloproteinases expressed by astrocytes mediate extracellular amyloid- $\beta$  peptide catabolism. *J. Neurosci.* **26**, 10939–10948 (2006).
111. Nielsen, H. M., Veerhuis, R., Holmqvist, B. & Janciauskiene, S. Binding and uptake of A $\beta$ 1-42 by primary human astrocytes in vitro. *Glia* **57**, 978–988 (2009).
112. Nielsen, H. M. *et al.* Astrocytic A beta 1-42 uptake is determined by A beta-aggregation state and the presence of amyloid-associated proteins. *Glia* **58**, 1235–1246 (2010).
113. Lööf, C., Mitchell, C. H., Simonsson, M. & Erlandsson, A. Slow degradation in phagocytic astrocytes can be enhanced by lysosomal acidification. *Glia* **63**, 1997–2009 (2015).
114. Guénette, S. Y. Astrocytes: a cellular player in A $\beta$  clearance and degradation. *Trends Mol. Med.* **9**, 279–280 (2003).
115. Thal, D. R. *et al.* Amyloid  $\beta$ -protein (A $\beta$ )-containing astrocytes are located preferentially near N-terminal-truncated A $\beta$  deposits in the human entorhinal cortex. *Acta Neuropathol.* **100**, 608–617 (2000).
116. de Duve, C. & Wattiaux, R. Functions of Lysosomes. *Annu. Rev. Physiol.* **28**, 435–492 (1966).
117. Ao, X., Zou, L. & Wu, Y. Regulation of autophagy by the Rab GTPase network. *Cell Death Differ.* **21**, 348–358 (2014).
118. Lamb, C. A., Dooley, H. C. & Tooze, S. A. Endocytosis and autophagy: Shared machinery for degradation. *BioEssays* **35**, 34–45 (2013).
119. Perera, R. M. & Zoncu, R. The Lysosome as a Regulatory Hub. *Annu. Rev. Cell Dev. Biol.* **32**, 223–253 (2016).
120. Tjelle, T. E., Brech, A., Juvet, L. K., Griffiths, G. & Berg, T. Isolation and characterization of early endosomes, late endosomes and terminal lysosomes: their role in protein degradation. *J. Cell Sci.* **109** ( Pt 12), 2905–2914 (1996).
121. Pillay, C. S., Elliott, E. & Dennison, C. Endolysosomal proteolysis and its regulation. *Biochem. J.* **363**, 417–429 (2002).
122. Ishida, Y., Nayak, S., Mindell, J. A. & Grabe, M. A model of lysosomal pH regulation. *J. Gen. Physiol.* **141**, 705–720 (2013).

123. Gatica, D., Lahiri, V. & Klionsky, D. J. Cargo recognition and degradation by selective autophagy. *Nat. Cell Biol.* **20**, 233–242 (2018).
124. Hurley, J. H. & Young, L. N. Mechanisms of Autophagy Initiation. *Annu. Rev. Biochem.* **86**, 225–244 (2017).
125. Lee, Y. K. & Lee, J. A. Role of the mammalian ATG8/LC3 family in autophagy: differential and compensatory roles in the spatiotemporal regulation of autophagy. *BMB Rep.* **49**, 424–430 (2016).
126. Liu, W. J. *et al.* p62 links the autophagy pathway and the ubiquitin–proteasome system upon ubiquitinated protein degradation. *Cell. Mol. Biol. Lett.* **21**, 29 (2016).
127. Myeku, N. & Figueiredo-Pereira, M. E. Dynamics of the Degradation of Ubiquitinated Proteins by Proteasomes and Autophagy: association with sequestosome 1/p62. *J. Biol. Chem.* **286**, 22426–22440 (2011).
128. Takalo, M., Salminen, A., Soininen, H., Hiltunen, M. & Haapasalo, A. Protein aggregation and degradation mechanisms in neurodegenerative diseases. *Am. J. Neurodegener. Dis.* **2**, 1–14 (2013).
129. Dorsey, E. R., Sherer, T., Okun, M. S. & Bloem, B. R. The Emerging Evidence of the Parkinson Pandemic. *J. Parkinsons. Dis.* **8**, S3–S8 (2018).
130. Dorsey, E. R. & Bloem, B. R. The Parkinson Pandemic—A Call to Action. *JAMA Neurol.* **75**, 9–10 (2018).
131. Gibb, W. R. & Lees, A. J. The relevance of the Lewy body to the pathogenesis of idiopathic Parkinson’s disease. *J. Neurol. Neurosurg. & Psychiatry* **51**, 745–752 (1988).
132. Khoo, T. K. *et al.* The spectrum of nonmotor symptoms in early Parkinson disease. *Neurology* **80**, 276–281 (2013).
133. Postuma, R. B. *et al.* Identifying prodromal Parkinson’s disease: Pre-Motor disorders in Parkinson’s disease. *Mov. Disord.* **27**, 617–626 (2012).
134. Cheng, H.-C., Ulane, C. M. & Burke, R. E. Clinical progression in Parkinson disease and the neurobiology of axons. *Ann. Neurol.* **67**, 715–725 (2010).
135. Grazia Spillantini, M. *et al.* Filamentous  $\alpha$ -synuclein inclusions link multiple system atrophy with Parkinson’s disease and dementia with Lewy bodies. *Neurosci. Lett.* **251**, 205–208 (1998).
136. Spillantini, M. G. *et al.*  $\alpha$ -Synuclein in Lewy bodies. *Nature* **388**, 839–840 (1997).
137. Ingelsson, M. Alpha-Synuclein Oligomers—Neurotoxic Molecules in Parkinson’s Disease and Other Lewy Body Disorders. *Frontiers in Neuroscience* vol. 10 408 (2016).
138. Lee, A. & Gilbert, R. M. Epidemiology of Parkinson Disease. *Neurol. Clin.* **34**, 955–965 (2016).
139. de Lau, L. M. L. & Breteler, M. M. B. Epidemiology of Parkinson’s disease. *Lancet Neurol.* **5**, 525–535 (2006).
140. Noyce, A. J. *et al.* Meta-analysis of early nonmotor features and risk factors for Parkinson disease. *Ann. Neurol.* **72**, 893–901 (2012).
141. Polymeropoulos, M. H. *et al.* Mutation in the  $\alpha$ -Synuclein Gene Identified in Families with Parkinson’s Disease. *Science* **276**, 2045–2047 (1997).
142. Krüger, R. *et al.* AlaSOPro mutation in the gene encoding  $\alpha$ -synuclein in Parkinson’s disease. *Nat. Genet.* **18**, 106–108 (1998).

143. Appel-Cresswell, S. *et al.* Alpha-synuclein p.H50Q, a novel pathogenic mutation for Parkinson's disease. *Mov. Disord.* **28**, 811–813 (2013).
144. Lesage, S. *et al.* G51D  $\alpha$ -synuclein mutation causes a novel Parkinsonian–pyramidal syndrome. *Ann. Neurol.* **73**, 459–471 (2013).
145. Zarranz, J. J. *et al.* The new mutation, E46K, of  $\alpha$ -synuclein causes parkinson and Lewy body dementia. *Ann. Neurol.* **55**, 164–173 (2004).
146. Yoshino, H. *et al.* Homozygous alpha-synuclein p.A53V in familial Parkinson's disease. *Neurobiol. Aging* **57**, 248.e7-248.e12 (2017).
147. Pasanen, P. *et al.* A novel  $\alpha$ -synuclein mutation A53E associated with atypical multiple system atrophy and Parkinson's disease-type pathology. *Neurobiol. Aging* **35**, 2180.e1-2180.e5 (2014).
148. Meade, R. M., Fairlie, D. P. & Mason, J. M. Alpha-synuclein structure and Parkinson's disease – lessons and emerging principles. *Mol. Neurodegener.* **14**, 29 (2019).
149. Ibáñez, P. *et al.* Causal relation between  $\alpha$ -synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet* **364**, 1169–1171 (2004).
150. Singleton, A. B. *et al.* a-Synuclein Locus Triplication Causes Parkinson's Disease. *Science* **302**, 841–841 (2003).
151. Uéda, K. *et al.* Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 11282–11286 (1993).
152. George, J. M. The synucleins. *Genome Biol.* **3**, reviews3002.1 (2001).
153. Stefanis, L.  $\alpha$ -Synuclein in Parkinson's disease. *Cold Spring Harb. Perspect. Med.* **2** (2012).
154. Gonzalez-Horta, A. Fluorescence as a Tool to Study Lipid-Protein Interactions: The Case of  $\alpha$ -Synuclein. *Open J. Biophys.* **3**, 112–119 (2013).
155. Maroteaux, L., Campanelli, J. T. & Scheller, R. H. Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal. *J. Neurosci.* **8**, 2804–2815 (1988).
156. Culvenor, J. G. *et al.* Non-Abeta component of Alzheimer's disease amyloid (NAC) revisited. NAC and alpha-synuclein are not associated with Abeta amyloid. *Am. J. Pathol.* **155**, 1173–1181 (1999).
157. Ly, T. & Julian, R. R. Protein-metal interactions of calmodulin and  $\alpha$ -synuclein monitored by selective noncovalent adduct protein probing mass spectrometry. *J. Am. Soc. Mass Spectrom.* **19**, 1663–1672 (2008).
158. Vilar, M. *et al.* The fold of  $\alpha$ -synuclein fibrils. *Proc. Natl. Acad. Sci.* **105**, 8637–8642 (2008).
159. Uversky, V. N., Li, J. & Fink, A. L. Evidence for a Partially Folded Intermediate in  $\alpha$ -Synuclein Fibril Formation. *J. Biol. Chem.* **276**, 10737–10744 (2001).
160. Periquet, M., Fulga, T., Myllykangas, L., Schlossmacher, M. G. & Feany, M. B. Aggregated  $\alpha$ -Synuclein Mediates Dopaminergic Neurotoxicity. *J. Neurosci.* **27**, 3338–3346 (2007).
161. Hsu, L. J. *et al.*  $\alpha$ -Synuclein Promotes Mitochondrial Deficit and Oxidative Stress. *Am. J. Pathol.* **157**, 401–410 (2000).
162. Alim, M. A. *et al.* Demonstration of a role for  $\alpha$ -synuclein as a functional microtubule-associated protein. *J. Alzheimer's Dis.* **6**, 435–442 (2004).

163. Rogers, S. L., Farlow, M. R., Doody, R. S., Mohs, R. & Friedhoff, L. T. A 24-week, double-blind, placebo-controlled trial of donepezil in patients with Alzheimer's disease. *Neurology* **50**, 136–145 (1998).
164. Geldmacher, D. S., Provenzano, G., McRae, T., Mastey, V. & Ieni, J. R. Donepezil is associated with delayed nursing home placement in patients with Alzheimer's disease. *J. Am. Geriatr. Soc.* **51**, 937–44 (2003).
165. Massoud, F. & Gauthier, S. Update on the pharmacological treatment of Alzheimer's disease. *Curr. Neuropharmacol.* **8**, 69–80 (2010).
166. Englund, H. *et al.* Sensitive ELISA detection of amyloid- $\beta$  protofibrils in biological samples. *J. Neurochem.* **103**, 334–345 (2007).
167. Logovinsky, V. *et al.* Safety and tolerability of BAN2401—a clinical study in Alzheimer's disease with a protofibril selective A $\beta$  antibody. *Alzheimers. Res. Ther.* **8**, 14 (2016).
168. Capecchi, M. R. Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. *Nat. Rev. Genet.* **6**, 507–512 (2005).
169. Gaj, T., Gersbach, C. A. & Barbas, C. F. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol.* **31**, 397–405 (2013).
170. Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S. & Gregory, P. D. Genome editing with engineered zinc finger nucleases. *Nat. Rev. Genet.* **11**, 636–646 (2010).
171. Christian, M. *et al.* Targeting DNA Double-Strand Breaks with TAL Effector Nucleases. *Genetics* **186**, 757–761 (2010).
172. Jinek, M. *et al.* A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science* **337**, 816–821 (2012).
173. Mojica, F. J. M., Díez-Villaseñor, C., Soria, E. & Juez, G. Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. *Mol. Microbiol.* **36**, 244–246 (2000).
174. Barrangou, R. *et al.* CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. *Science* **315**, 1709–1712 (2007).
175. Brouns, S. J. J. *et al.* Small CRISPR RNAs Guide Antiviral Defense in Prokaryotes. *Science* **321**, 960–964 (2008).
176. Marraffini, L. A. & Sontheimer, E. J. CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nat. Rev. Genet.* **11**, 181–190 (2010).
177. Garneau, J. E. *et al.* The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* **468**, 67–71 (2010).
178. Marraffini, L. A. & Sontheimer, E. J. Self versus non-self discrimination during CRISPR RNA-directed immunity. *Nature* **463**, 568–571 (2010).
179. Wyman, C. & Kanaar, R. DNA Double-Strand Break Repair: All's Well that Ends Well. *Annu. Rev. Genet.* **40**, 363–383 (2006).
180. O'Driscoll, M. & Jeggo, P. A. The role of double-strand break repair — insights from human genetics. *Nat. Rev. Genet.* **7**, 45–54 (2006).
181. Wyman, C., Ristic, D. & Kanaar, R. Homologous recombination-mediated double-strand break repair. *DNA Repair (Amst)*. **3**, 827–833 (2004).

182. Sapranaukas, R. *et al.* The *Streptococcus thermophilus* CRISPR/Cas system provides immunity in *Escherichia coli*. *Nucleic Acids Res.* **39**, 9275–9282 (2011).
183. Kleinstiver, B. P. *et al.* Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature* **523**, 481–485 (2015).
184. Doudna, J. A. The promise and challenge of therapeutic genome editing. *Nature* **578**, 229–236 (2020).
185. Maeder, M. L. *et al.* Development of a gene-editing approach to restore vision loss in Leber congenital amaurosis type 10. *Nat. Med.* **25**, 229–233 (2019).
186. Gillmore, J. D. *et al.* CRISPR-Cas9 In Vivo Gene Editing for Transthyretin Amyloidosis. *N. Engl. J. Med.* **385**, 493–502 (2021).
187. Fu, Y. *et al.* High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* **31**, 822–826 (2013).
188. Hsu, P. D. *et al.* DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol.* **31**, 827–832 (2013).
189. Pattanayak, V. *et al.* High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat. Biotechnol.* **31**, 839–843 (2013).
190. Kleinstiver, B. P. *et al.* High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* **529**, 490–495 (2016).
191. Singh, R., Kuscus, C., Quinlan, A., Qi, Y. & Adli, M. Cas9-chromatin binding information enables more accurate CRISPR off-target prediction. *Nucleic Acids Res.* **43**, e118 (2015).
192. Ramakrishna, S. *et al.* Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. *Genome Res.* **24**, 1020–1027 (2014).
193. Kim, S., Kim, D., Cho, S. W., Kim, J. & Kim, J.-S. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res.* **24**, 1012–1019 (2014).
194. Gabriel, R. *et al.* An unbiased genome-wide analysis of zinc-finger nuclease specificity. *Nat. Biotechnol.* **29**, 816–823 (2011).
195. Tsai, S. Q. *et al.* GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat. Biotechnol.* **33**, 187–197 (2015).
196. Tsai, S. Q. *et al.* CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR–Cas9 nuclease off-targets. *Nat. Methods* **14**, 607–614 (2017).
197. Petri, K. *et al.* Global-scale CRISPR gene editor specificity profiling by ONE-seq identifies population-specific, variant off-target effects. *bioRxiv* 2021.04.05.438458 (2021)
198. Falk, A. *et al.* Capture of Neuroepithelial-Like Stem Cells from Pluripotent Stem Cells Provides a Versatile System for In Vitro Production of Human Neurons. *PLoS One* **7**, e29597 (2012).
199. Calvo-Garrido, J. *et al.* Protocol for the derivation, culturing, and differentiation of human iPS-cell-derived neuroepithelial stem cells to study neural differentiation in vitro. *STAR Protoc.* **2**, 100528 (2021).

200. Lundin, A. *et al.* Human iPS-Derived Astroglia from a Stable Neural Precursor State Show Improved Functionality Compared with Conventional Astrocytic Models. *Stem cell reports* **10**, 1030–1045 (2018).
201. Hughes, S. M., Lillien, L. E., Raff, M. C., Rohrer, H. & Sendtner, M. Ciliary neurotrophic factor induces type-2 astrocyte differentiation in culture. *Nature* **335**, 70–73 (1988).
202. Boussif, O. *et al.* A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7297 (1995).
203. Sonawane, N. D., Szoka, F. C. & Verkman, A. S. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *J. Biol. Chem.* **278**, 44826–44831 (2003).
204. Ran, F. A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281–2308 (2013).
205. Kleinstiver, B. P. *et al.* Broadening the targeting range of *Staphylococcus aureus* CRISPR-Cas9 by modifying PAM recognition. *Nat. Biotechnol.* **33**, 1293–1298 (2015).
206. Dull, T. *et al.* A third-generation lentivirus vector with a conditional packaging system. *J. Virol.* **72**, 8463–8471 (1998).
207. Sanger, F., Nicklen, S. & Coulson, A. R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463 (1977).
208. Engvall, E. & Perlmann, P. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry* **8**, 871–874 (1971).
209. Chen, Y., Mills, J. D. & Periasamy, A. Protein localization in living cells and tissues using FRET and FLIM. *Differentiation* **71**, 528–541 (2003).
210. Jares-Erijman, E. A. & Jovin, T. M. Imaging molecular interactions in living cells by FRET microscopy. *Curr. Opin. Chem. Biol.* **10**, 409–416 (2006).
211. Haessler, M. *et al.* Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biol.* **17**, (2016).
212. Carpenter, A. E. *et al.* CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* **7**, R100 (2006).
213. Stirling, D. R., Carpenter, A. E. & Cimini, B. A. CellProfiler Analyst 3.0: accessible data exploration and machine learning for image analysis. *Bioinformatics* **37**, 3992–3994 (2021).
214. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675 (2012).
215. Elliott, A. D. Confocal Microscopy: Principles and Modern Practices. *Curr. Protoc. Cytom.* **92**, (2020).
216. Winey, M., Meehl, J. B., O’Toole, E. T. & Giddings, T. H. Conventional transmission electron microscopy. *Mol. Biol. Cell* **25**, 319–323 (2014).
217. Citron, M. *et al.* Excessive production of amyloid beta-protein by peripheral cells of symptomatic and presymptomatic patients carrying the Swedish familial Alzheimer disease mutation. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 11993–7 (1994).

218. Sherrington, R. *et al.* Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* **375**, 754–760 (1995).
219. Liu, Q. *et al.* Effect of Potent  $\gamma$ -Secretase Modulator in Human Neurons Derived From Multiple Presenilin 1–Induced Pluripotent Stem Cell Mutant Carriers. *JAMA Neurol.* **71**, 1481–1489 (2014).
220. Berezovska, O. *et al.* Familial Alzheimer's disease presenilin 1 mutations cause alterations in the conformation of presenilin and interactions with amyloid precursor protein. *J. Neurosci.* **25**, 3009–3017 (2005).
221. Lee, S. Y. & Chung, W. S. The roles of astrocytic phagocytosis in maintaining homeostasis of brains. *J. Pharmacol. Sci.* **145**, 223–227 (2021).
222. Sofroniew, M. V. Multiple roles for astrocytes as effectors of cytokines and inflammatory mediators. *Neuroscientist* **20**, 160–172 (2014).
223. Kashyap, G. *et al.* Synapse loss and progress of Alzheimer's disease -A network model. *Sci. Rep.* **9**, 6555 (2019).
224. Sperling, R. A. *et al.* Toward defining the preclinical stages of Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's Dement.* **7**, 280–292 (2011).
225. Musiek, E. S. & Holtzman, D. M. Three dimensions of the amyloid hypothesis: time, space and 'wingmen'. *Nat. Neurosci.* **18**, 800–806 (2015).
226. Preman, P., Alfonso-Triguero, M., Alberdi, E., Verkhratsky, A. & Arranz, A. M. Astrocytes in Alzheimer's Disease: Pathological Significance and Molecular Pathways. *Cells* vol. 10 (2021).
227. Anderson, M. A., Ao, Y. & Sofroniew, M. V. Heterogeneity of reactive astrocytes. *Neurosci. Lett.* **565**, 23–29 (2014).
228. González-Reyes, R. E., Nava-Mesa, M. O., Vargas-Sánchez, K., Ariza-Salamanca, D. & Mora-Muñoz, L. Involvement of Astrocytes in Alzheimer's Disease from a Neuroinflammatory and Oxidative Stress Perspective. *Frontiers in Molecular Neuroscience* vol. 10 (2017).
229. Choi, S. H. *et al.* A three-dimensional human neural cell culture model of Alzheimer's disease. *Nature* **515**, 274–278 (2014).
230. Park, J. *et al.* A 3D human triculture system modeling neurodegeneration and neuroinflammation in Alzheimer's disease. *Nat. Neurosci.* **21**, 941–951 (2018).
231. Kwak, S. S. *et al.* Amyloid- $\beta$ 42/40 ratio drives tau pathology in 3D human neural cell culture models of Alzheimer's disease. *Nat. Commun.* **11**, 1377 (2020).



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