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AN RNAi THERAPEUTIC TARGETING APP REDUCED BETA-CTF AND CORRECTED ENDOSOMAL ABNORMALITIES IN MULTIPLE HUMAN ALZHEIMER'S DISEASE iPSC LINES

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Abstract

Objectives: Endosomal-lysosomal abnormalities are hallmark of Alzheimer's disease (AD). Endolysosomal dysfunction contributes to abnormal amyloid precursor protein (APP) clearance; concurrently, peptides resulting from dysregulated APP metabolism can also impair endolysosomal function, creating a cycle of endolysosomal dysfunction and APP metabolism dysregulation. An RNAi therapeutic targeting *APP* mRNA is intended to reduce APP production. To explore the impact of APP lowering on endolysosomal dysfunction, we evaluated the effect of an siRNA targeting *APP* mRNA on downstream APP cleavage products, including beta-secretase-derived APP C-terminal fragment (beta-CTF), and on endosomal defects in multiple human AD induced pluripotent stem cell (iPSC) lines.

Methods: Early-onset AD patient-derived iPSCs carrying pathogenic mutations in PSEN1 and CRISPR/Cas9-edited iPSCs carrying APP homozygous Swedish mutation (swe/swe) lines were differentiated to neurons and astrocyte cocultures, transfected with siRNA targeting *APP* mRNA or control siRNA on Day 7. Beta-CTF, soluble APP-alpha, soluble APP-beta protein analysis by Meso Scale Discovery enzyme-linked immunosorbent assay, and high content imaging for Rab5+ early endosome and Rab7+ late endosome size were performed on Day 30.

Results: An RNAi therapeutic targeting *APP* mRNA significantly reduced *APP* mRNA and levels of downstream APP cleavage products in human AD patient-derived iPSC lines with pathogenic mutations in PSEN1. Reducing APP expression reduced early endosomal enlargement defects. Immunohistochemistry showed high accumulation of intracellular beta-CTF in both APP and PSEN1 mutation lines, which was significantly reduced in cells treated with *APP* siRNA.

Conclusions: By lowering both intracellular and extracellular drivers of AD pathology, an RNAi therapeutic targeting *APP* may potentially alter the cascade of pathological events that result in neurodegeneration. The Phase 1 first-in-human study of ALN-APP, an investigational RNAi therapeutic targeting *APP* mRNA, is ongoing in patients with early-onset AD (NCT05231785).

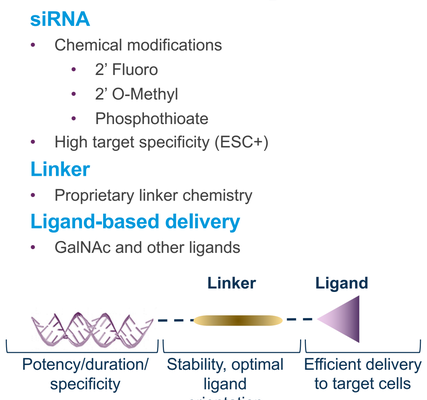
Introduction

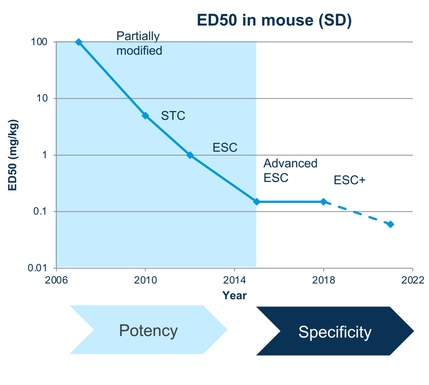
Advances in siRNA design make CNS delivery possible

siRNA
- Chemical modifications
  - 2' Fluoro
  - 2' O-Methyl
  - Phosphothioate
- High target specificity (ESC+)

Linker
- Proprietary linker chemistry

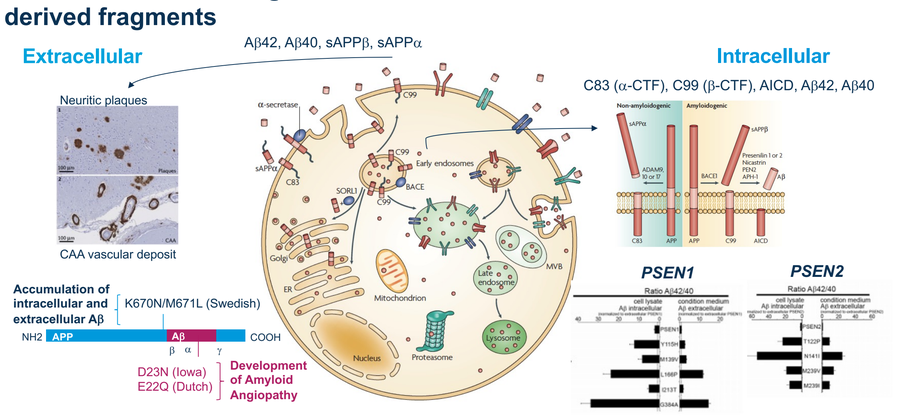
Ligand-based delivery
- GalNAc and other ligands





The successful clinical translation of GalNAc-siRNA conjugates for a wide variety of disease targets in the liver has prompted us to explore our advanced siRNA designs combined with alternative conjugation strategies to address disorders of the CNS. siRNA conjugates targeting APP mRNA, for example, can be delivered to the CNS via intrathecal administration in pre-clinical species such as rodents and NHPs, with silencing of the target transcript detected throughout the brain and the spinal cord following a single intrathecal bolus administration of siRNA. Silencing is robust and durable, showing sustained duration in some regions for at least three months following a single dose with concomitant drug levels detectable over the same time course. The Phase 1 first-in-human study of ALN-APP, an investigational RNAi therapeutic targeting *APP* mRNA, is ongoing in patients with early-onset AD (NCT05231785).

Knockdown of APP targets both Intracellular and Extracellular APP derived fragments



La Ferla et al (2007) *Nat Rev Neurosci*; Greenberg et al (2020) *Nature Reviews Neurology*; Sannerud et al (2016) *Cell*;

Alzheimer's disease (AD) is characterized by the accumulation of extracellular and intracellular amyloid β (Aβ) aggregates, intracellular tangles, and age-dependent neuronal loss, and is the most common type of dementia worldwide. Both genetic and molecular evidence suggest changes to the amyloid precursor protein (APP) metabolism as a putative cause of AD. However, the distribution of Aβ deposits in the brains of AD patients do not correlate well with disease severity, neuronal loss of function or cognitive decline. Although much of the earlier works in AD focused on the secreted Aβ fragment and extracellular Aβ deposits, multiple recent studies have suggested a role for intracellular APP metabolites, such as the β C-terminal fragment (βCTF). βCTF is generated by the cleavage of APP by β-secretase and has been shown in AD post-mortem samples to be accumulated in neurons that are prone to degeneration, and thus highly correlated with the degree of cognitive impairments in patients.

Summary

Here, we show that an RNAi therapeutic targeting *APP* mRNA, is able to reduce APP protein expression and thereby reduce both the extracellular Aβ as well as intracellular βCTF. Using human AD patient-derived iPSCs that harbor mutations in PSEN1, we show that reducing APP expression alleviates not only the intracellular accumulation of βCTF, but also the early endosomal defects which are characteristic of these mutant cells. *APP* siRNA may offer a novel approach for the treatment of AD, with non-clinical data suggesting the potential to address unmet medical need by potentially slowing or preventing the progression of disease. The *APP*-targeting siRNA is intended to comprehensively lower all intracellular and extracellular amyloid protein species, including Aβ40, Aβ42, as well as AICD and βCTF.

Key References

- Brown et al (2022) *Nature Biotechnology* **40**:1500-1508
- Kwart et al (2019) *Neuron* **104**(2):256-270
- Lee et al (2022) *Nature Neuroscience* **25**:688-701

Results

PSEN1 patient iPSC-derived cortical neuron models treated with *APP* siRNA show a reduction in APP cleavage products

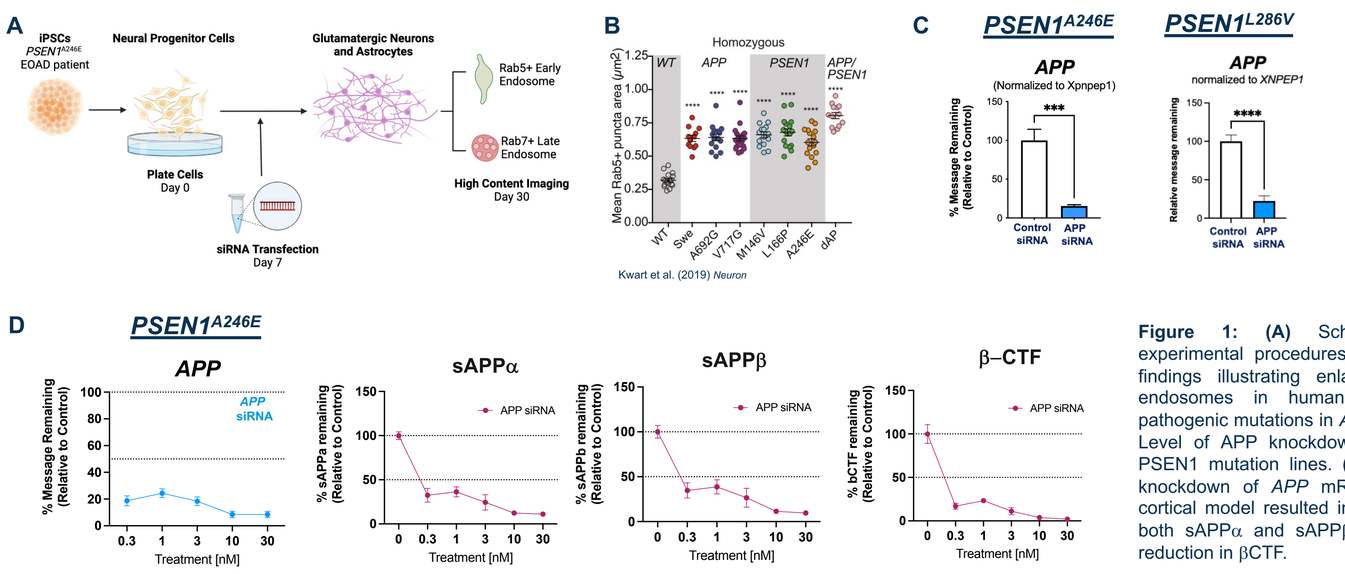
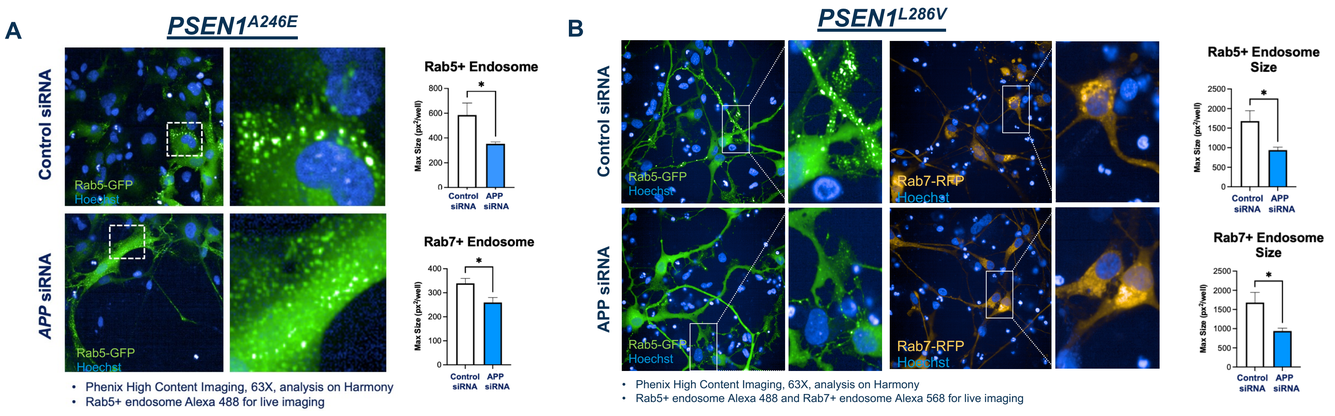


Figure 1: (A) Schematic illustrating experimental procedures and **(B)** published findings illustrating enlarged Rab5+ early endosomes in human iPSCs harboring pathogenic mutations in *APP* and *PSEN1*. **(C)** Level of APP knockdown observed in two PSEN1 mutation lines. **(D)** Dose-dependent knockdown of *APP* mRNA in the in vitro cortical model resulted in >60% reduction in both sAPPα and sAPPβ as well as >75% reduction in βCTF.

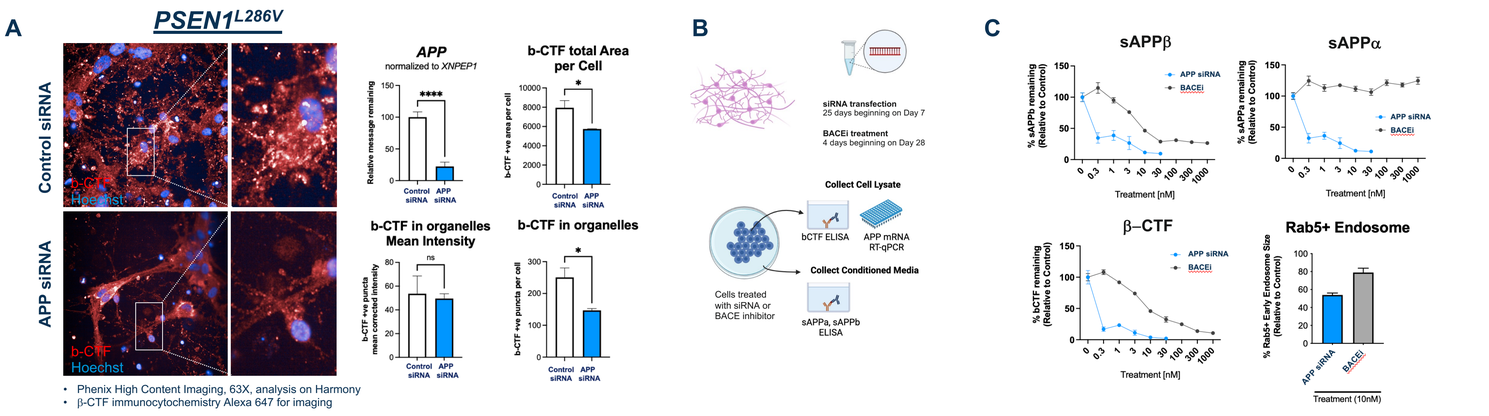
PSEN1 patient iPSC-derived cortical neuron models treated with *APP* siRNA show a reduction Rab5+ and Rab7+ endosome size



- Phenix High Content Imaging, 63X, analysis on Harmony
- Rab5+ endosome Alexa 488 for live imaging
- Phenix High Content Imaging, 63X, analysis on Harmony
- Rab5+ endosome Alexa 488 and Rab7+ endosome Alexa 568 for live imaging

Figure 2: (A) Representative images of the control siRNA or the *APP* siRNA treated cortical neuron cultures harboring the *PSEN1*^{A246E} mutation, depicting Rab5-GFP early endosome size and quantification. N=6/group. 20-36 images/well. In addition, APP siRNA treatment similarly reduced Rab7-RFP late endosome size quantification (representative images not shown). **(B)** Representative images of the control siRNA or the APP siRNA treated cortical neuron cultures harboring the L186V Early Onset AD mutation in *PSEN1*, depicting Rab5-GFP early endosome size and quantification. N=6/group. 36 images/well. Similarly to the *PSEN1*^{A246E} mutation, cells harboring the *PSEN1*^{L286V} mutation treated with APP siRNA, showed a reduction in Rab7-RFP late endosome size. N=6/group. 36 images/well.

PSEN1^{L286V} EOAD patient iPSC-derived cortical neuron model showed a reduction of intracellular β-CTF as well as number of β-CTF positive organelles following *APP* siRNA treatment



- Phenix High Content Imaging, 63X, analysis on Harmony
- β-CTF immunocytochemistry Alexa 647 for imaging

Figure 3: (A) Representative images of the control siRNA or the *APP* siRNA treated cortical neuron cultures harboring the *PSEN1*^{L286V} mutation, depicting intracellular β-CTF immunocytochemistry and quantification. N=3-6/group. 36 images/well **(B)** Schematic illustrating experimental procedures and (C) extracellular sAPPβ, sAPPα and intracellular β-CTF measurements in EOAD patient derived cortical neuron cultures treated with APP siRNA or a BACE inhibitor. At equimolar concentrations, APP siRNA treatment reduced ~50% of Rab5+ early endosome size compared to ~20% early endosome size reduction with the BACE inhibitor. In addition sAPPα is unaltered with BACE inhibition.

PSEN1^{L286V} EOAD patient iPSC-derived cortical neuron models treated with *APP* siRNA show a reduction of Rab5+ and Rab7+ endosomes in neurites as well as increased neuronal synchronicity in *APP*$^{Swe/Swe}$ EOAD CRISPR derived neurons

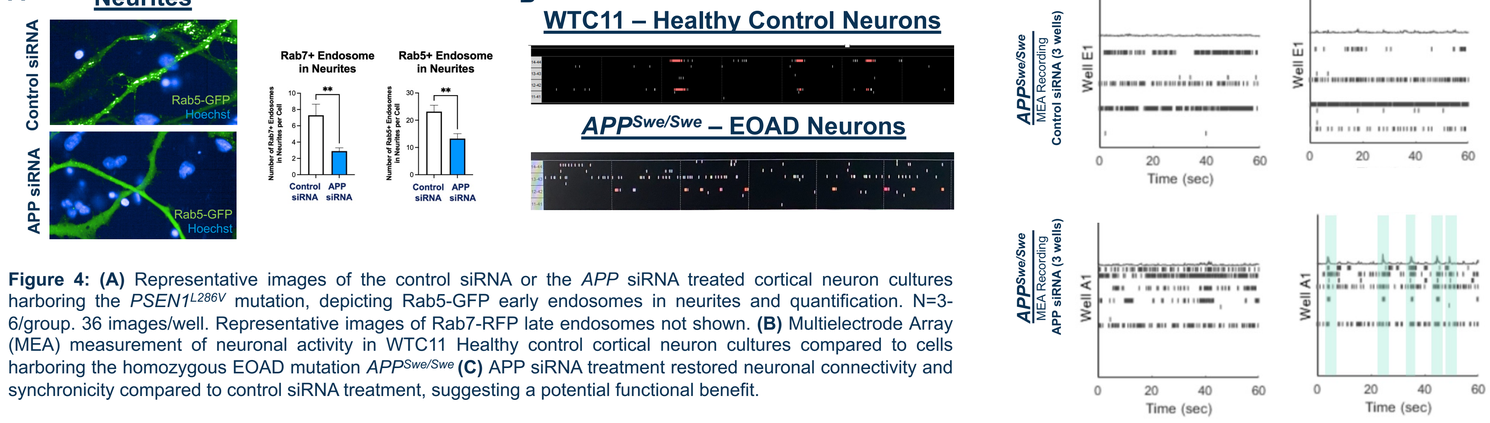


Figure 4: (A) Representative images of the control siRNA or the *APP* siRNA treated cortical neuron cultures harboring the *PSEN1*^{L286V} mutation, depicting Rab5-GFP early endosomes in neurites and quantification. N=3-6/group. 36 images/well. Representative images of Rab7-RFP late endosomes not shown. **(B)** Multielectrode Array (MEA) measurement of neuronal activity in WTC11 Healthy control cortical neuron cultures compared to cells harboring the homozygous EOAD mutation *APP*$^{Swe/Swe}$ **(C)** APP siRNA treatment restored neuronal connectivity and synchronicity compared to control siRNA treatment, suggesting a potential functional benefit.

Highly durable *APP* knockdown with a single intrathecal dose of ALN-APP in NHPs supports bi-annual or less frequent administration in humans

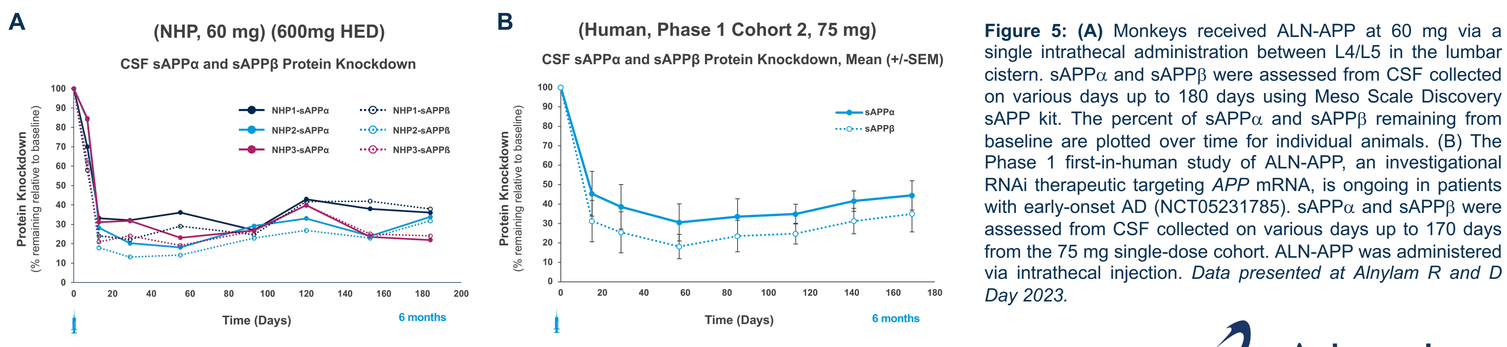


Figure 5: (A) Monkeys received ALN-APP at 60 mg via a single intrathecal administration between L4/L5 in the lumbar cistern. sAPPα and sAPPβ were assessed from CSF collected on various days up to 180 days using Meso Scale Discovery sAPP kit. The percent of sAPPα and sAPPβ remaining from baseline are plotted over time for individual animals. (B) The Phase 1 first-in-human study of ALN-APP, an investigational RNAi therapeutic targeting *APP* mRNA, is ongoing in patients with early-onset AD (NCT05231785). sAPPα and sAPPβ were assessed from CSF collected on various days up to 170 days from the 75 mg single-dose cohort. ALN-APP was administered via intrathecal injection. *Data presented at Alnylam R and D Day 2023.*

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