Analysis of $A\beta_{x-42}/A\beta_{x-40}$ and Tau upon siRNA-mediated downregulation of APOE, BIN1, PICALM, CLU, PRNP and CST3 in wildtype mouse primary neurons

Gabriele Siegel, Lawrence Rajendran
Systems and Cell Biology of Neurodegeneration, IREM, University of Zurich, Schlieren Campus, 8952 Schlieren (Zurich), Switzerland

Abstract
Alzheimer’s disease (AD) is the most common form of dementia in the elderly. It is a progressive neurodegenerative disorder that is characterized by the abundant presence of cerebral β-amyloid (Aβ) plaques and neurofibrillary Tau tangles. The rare, early-onset AD is caused by mutations mainly within either the amyloid precursor protein (APP) or Presenilins 1 or 2 (PS1 or PS2), the catalytic subunits of the γ-secretase complex. These mutations either increase overall Aβ production or specifically alter γ-secretase mediated processing towards an increased production ratio of $A\beta_{42}/A\beta_{40}$. For late-onset AD, however, which accounts for the vast majority of AD cases, the exact mechanisms by which the disease is caused are not known. While genome-wide association studies (GWAS) have identified certain genetic risk factors associated with late-onset AD, the mechanisms through which they contribute to the pathogenesis are still elusive. Previously, using a HeLa cell model of Aβ production, it was shown that, in contrast to the early-onset AD causing mutations, knockdown of late-onset AD susceptibility genes did not specifically affect the $A\beta_{42}/A\beta_{40}$ ratio [1]. To validate these findings in a neuronal setting without any overexpression of APP, here we re-addressed the role of six late-onset AD risk genes (APOE, BIN1, PICALM, CLU, PRNP and CST3) in the regulation of γ-secretase mediated APP processing in wild-type mouse primary neurons by analyzing $A\beta_{x-40}$ and $A\beta_{x-42}$. In addition, we extended the analysis by also including measurements of total Tau protein and phosphorylation of Tau at Threonine 231, a non-physiological phosphorylation site that is associated with AD. The siRNA-mediated knockdown of the studied LOAD risk genes neither affected the $A\beta_{42}/A\beta_{40}$ ratio nor altered the levels of total Tau or phospho(Thr231)-Tau. Our results thus show that acute downregulation of these genes in wild-type mouse primary neurons does not significantly impact on γ-secretase mediated APP processing nor Tau homeostasis or Tau phosphorylation.

Introduction
The brains of Alzheimer’s disease (AD) patients show widespread formation of extracellular senile plaques composed of aggregated β-amyloid (Aβ) peptides as well as intraneuronal aggregates of misfolded Tau protein, so-called neurofibrillary tangles. According to the onset of disease one can distinguish two types of AD: a rare, early-onset AD (EOAD) with an onset before 65 years of age, and the common, late-onset AD (LOAD) with an onset after 65 years of age. EOAD is caused by a specific set of highly penetrant mutations, which affect almost exclusively either the amyloid precursor protein (APP) or the catalytically active Presenilin subunit of the Aβ-producing γ-secretase complex. These mutations enhance the overall production of Aβ peptides and/or increase the ratio of the aggregation prone and neurotoxic $A\beta_{12}$ over the common, shorter $A\beta_{40}$. The genetic contribution to LOAD, on the other hand, is not very well understood. Inheritance patterns within families point to multifactorial inheritance of LOAD, involving both genetic and environmental factors. Initial linkage analysis studies and GWAS analyses have identified a number of susceptibility genes for LOAD (Alzgenes), the majority of which having only small effect sizes on disease risk [2]. The only exception is the APOE ι4 locus which increases LOAD risk 2- to 4-fold in individuals with one copy of the allele and 8-to 15-fold in individuals carrying two copies [3] [4] [5] [6] [7].
While an increased production of Aβ and/or an increased Aβ 42:40 ratio have been acknowledged as the driving pathogenic mechanism in EOAD, the etiology of LOAD appears to be much more complex and it is still not absolutely clear if Aβ accumulation plays an active causative role in the initial pathogenesis of the disease. Also the mechanisms that drive Aβ accumulation appear to be different in LOAD and EOAD. While some studies showed effects of LOAD risk genes on Aβ production, most of the data suggest that Aβ accumulation in the brains of LOAD patients results from impaired Aβ clearance rather than an increased production as seen in EOAD [8] [9]. For example APOE, which is the strongest genetic risk factor for LOAD, was shown to bind to Aβ and mediate its clearance across the blood brain barrier (BBB) or its endocytosis into brain cells for lysosomal degradation [10] [11]. Similarly, also CLU and PICALM had been shown to enhance the transport of Aβ across the blood-brain barrier and the blood-cerebrospinal fluid barrier [12] [13] [14] [15]. In line with the notion that LOAD genes do not specifically affect Aβ production, a recent study did not detect specific effects of the knockdowns of several LOAD susceptibility genes on Aβ levels or the 42:40 ratio in a HeLa cell APP overexpression system [1].

**Objective**

We wanted to study the effect of acute downregulation of selected LOAD risk genes on γ-secretase mediated processing of endogenous APP and on levels of endogenous total Tau and phospho-Tau (pThr231) in wild-type neurons. The selected risk genes comprised four high ranking Alzgenes (Apolipoprotein E [APOE], Bridging integrator 1 [BIN1], Phosphatidylinositol binding clathrin assembly protein [PICALM], Clusterin [CLU] and two LOAD risk genes with weaker association (Prion protein [PRNP] and Cystatin C [CST3]).
Figure 1. Knockdown of APOE, BIN1, CLU, PICALM, PRNP or CST3 in wildtype mouse primary neurons does not alter levels of secreted Aβx-40 and Aβx-42 nor levels of total Tau and phospho(Thr231)-Tau.

4DIV wild-type mouse primary neurons were transfected with the indicated siRNAs and cultured until 8DIV. Levels of secreted Aβx-40, and Aβx-42 and levels of total Tau and phosphoThr231-Tau were measured by an electrochemiluminescence (ECL) assay.

(A) Left panel: Analysis of relative Aβx-40 and Aβx-42 levels in 24 h conditioned medium that was collected from 7DIV until 8DIV. Results are displayed as average ± STDEV (n=3). Statistical significance was calculated by student’s t-test for comparison of each experimental condition to the control siRNA condition (*** p <0.001). Right panel: mRNA knockdown efficiencies as analyzed by real-time PCR.

(B) Left panel: Analysis of relative levels of total Tau and phosphoThr231-Tau in the protein fraction of whole cell lysates from 8DIV neuronal cultures. Results are displayed as average ± STDEV (n=3). Statistical significance was calculated by student’s t-test for comparison of each experimental condition to the control siRNA condition (*** p <0.001). Right panel: mRNA knockdown efficiencies as analyzed by real-time PCR.

(C) Representative Western Blots for analysis of knockdown efficiency on protein level for the tested LOAD risk genes. APOE, BIN1, CLU, PICALM and PRNP levels were analyzed from cell lysates. CST3 levels were analyzed from 24h conditioned medium.

Results & Discussion
In order to study the role of selected LOAD risk genes on γ-secretase mediated processing of endogenous APP and on levels of endogenous total Tau and phospho-Tau in neurons, we performed individual siRNA-mediated gene knockdowns in primary neuronal cultures prepared from embryonic wildtype mice. We chose a set of four high ranking Alzgenes (APOE, BIN1, CLU, PICALM) and two risk genes with weaker association (PRNP and CST3) which were readily detectable at mRNA level in the primary cultures and amenable to gene knockdown by RNAi. As negative control, we transfected a pool of non-targeting siRNAs. In addition, we chose four non-AD control genes (TARDBP: TAR-DNA-binding protein 43, RELN: Reelin, KAT5: Lysine acetyltransferase 5 and SNCA: α-synuclein) that are implicated in other neurodegenerative diseases and neurological conditions including amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTD), schizophrenia, autism, multiple sclerosis (MS) and Parkinson’s disease (PD). As positive controls, we transfected siRNAs that target either APP or MAPT. Knockdown-efficiencies of all candidate and control genes were assessed at the mRNA level by real-time PCR in each experimental round. In addition, knockdown of candidate genes at the protein level were confirmed by Western Blot analysis. For the simultaneous detection of both Aβx-40 and Aβx-42 or phospho(Thr231)-Tau and total Tau, we used specific multiplex electrochemiluminescence assays for analysis of the
conditioned neuronal culture medium or the recovered protein fraction of the whole cell lysate respectively. Knockdown of the positive control APP led to the expected reduction of both $\alpha_\beta_{40}$ and $\alpha_\beta_{42}$ without altering Tau levels (Fig.1A) while transfection of MAPT siRNA significantly lowered the signal of total and phospho (Thr231)-Tau without affecting $\alpha_\beta$ (Fig.1B), thus proving the functionality of the RNAi approach and the validity of the assays.

Silencing any of the tested A/genes did not affect secreted $\beta_{40}$ and $\beta_{42}$ or the $\beta_{42}$/$\beta_{40}$ ratio (Fig.1A) nor did it alter total Tau and phospho(Thr231)-Tau levels (Fig.1B). Our results suggest that, in wildtype mouse primary cortical/hippocampal neurons, acute knockdown of the studied LOAD risk genes does not crucially impact on $\gamma$-secretase mediated APP processing nor on total Tau levels or Tau(Thr231)-phosphorylation. Susceptibility to AD through these genes might involve effects on APP processing or Tau that build up only over a longer time or only at a later stage, when neurons have already undergone substantial aging. Alternatively, these genes might play a role in mediating $\alpha_\beta$ toxicity to neurons, or alter $\alpha_\beta$ clearance pathways by non-neuronal cell types, or modulate $\alpha_\beta$ aggregation. Indeed, experimental evidence for multiple of these possible mechanisms have been provided by previous studies for several of the herein studied susceptibility genes. For example, in addition to the aforementioned roles of APOE, CLU and PICALM in facilitating $\alpha_\beta$ clearance, APOE lipoproteins were shown to regulate the association of oligomeric $\alpha_\beta$ with synapses [16], CLU and CST3 were shown to inhibit $\alpha_\beta$ aggregation [17] [18] [19] [20] and PRNP was shown to play a role in mediating the deleterious effects of $\alpha_\beta$ on synaptic transmission and learning and memory [21] [22] [23] [24] [25] [26] [27], though this finding was challenged by at least three other groups [28] [29] [30]. Importantly, while in our study the knockdown of the tested genes in mouse primary neurons altered neither $\beta_{40}$ and $\beta_{42}$ nor total Tau and phospho(Thr231)-Tau levels, a number of previous studies using different systems reported effects on $\alpha_\beta$ or Tau pathology for some of the tested genes. APOE, for example, was shown to regulate neuronal $\alpha_\beta$ production by modulating APP recycling [31] or APP transcription [32] when applied extracellularly. It is conceivable that the reason why we did not observe any effects of APOE knockdown on $\beta_{40}$ and $\beta_{42}$ is because it is expressed mainly by astrocytes and produced only at low amounts by primary neuronal cultures as used for our study. For BIN1, conflicting results have been reported on its role in $\alpha_\beta$ regulation: while depletion of BIN1 in neurons resulted in a slight decrease in secreted $\beta_{40}$ with a concomitant increase in intracellular $\beta_{40}$ and $\beta_{42}$ in one study [33], increased levels of secreted $\alpha_\beta$ were observed by another group [34] and no effects were detected in neuroblastoma cells in a third study [35]. Similarly, also for PICALM the observed effects on $\alpha_\beta$ are diverging: in one study, PICALM was reported to promote $\alpha_\beta$ generation in cells and to accelerate $\alpha_\beta$ pathology in APP transgenic mice [36] whereas another study observed no effect on cellular $\alpha_\beta$ production [37]. As for PRNP, a direct role was proposed in the regulation of $\alpha_\beta$ production through inhibition of $\beta$-secretase-mediated cleavage of APP [38]. In our study, neither $\beta_{40}$ nor $\beta_{42}$ levels changed upon knockdown of BIN1, PICALM or PRNP in mouse primary neurons. Our results thus suggest that the overall APP processing by $\gamma$-secretase is not altered by downregulation of either of these genes in young cultured wild-type neurons. Since the assay used in our analysis was chosen so as to detect all $\beta_{40}$ and $\beta_{42}$ species produced by $\gamma$-secretase, comprising full-length $\alpha_\beta$ and the shorter $\alpha_\beta$ and $\beta_3$ peptides [39], our data do not necessarily challenge earlier findings on altered full-length $\alpha_\beta$ levels. Regarding Tau pathology, only for BIN1 a link has been reported previously: BIN1 expression was shown to correlate with the amount of neurofibrillary tangles (NFT) or total-Tau/phospho-Tau in AD patients in two studies [40] [41] and a direct interaction between BIN1 and Tau was suggested by another [42]. However, no significant overlap between BIN1 and neurofibrillary tangles was seen in a more recent study [43]. Previously reported effects of experimentally altered BIN1 expression on Tau pathology have been controversial too. While loss of BIN1 was shown to reduce Tau mediated neurodegeneration in Drosophila [42], it reduced the propagation of Tau pathology in an in vitro system [44]. We did not observe a significant effect of BIN1s downregulation on wildtype total Tau or pTau(Thr231) levels in our study. Effects on Tau pathology progression as seen in the earlier studies might possibly
require a disease context as provided by the presence of Tau mutations in these studies.

Conclusions
Our results show that acute downregulation of the studied LOAD risk genes in mouse primary neurons does not significantly alter γ-secretase mediated APP processing, Tau levels or Tau(Thr231)-phosphorylation. Susceptibility to AD through these genes might be conferred through other mechanisms, for example modulation of Aβ clearance or Aβ aggregation/toxicity as it has been proposed by some of the studies discussed above and/or Aβ and Tau-independent signaling pathways that directly affect neuronal function.

Limitations
There are three important limitations to our study:
1.) The in vitro character of the study: The simple intercellular connections that are formed in a primary neuronal culture do not allow to model the complex interactions that occur in an intact brain. Any effects that depend on the network activity within or between certain brain areas can therefore not be assessed by our model.
2.) The limited time frame during which we assessed the role of the studied Alzgenes: Knockdowns were performed for about 72 h. Any effects on APP processing or Tau that build up only over a longer period, as it might be the case in AD, would not be detected in our system.
3.) The age of the primary neurons: Primary neuronal cultures were prepared from embryonic neurons, thus neurons in their very early developmental stage. LOAD, however, is a disease of the aged brain. The expression profiles of the tested Alzgenes and their interaction partners might be very different in young and old neurons.
We are now planning to study the role of the respective risk gene variants in neurons derived from human induced pluripotent stem cells (iPSCs) which have been modified using CRISPR/Cas9 technology for targeted genome editing to introduce the respective LOAD associated genetic polymorphisms. This will not only allow us to assess long-term effects of the respective risk variants on APP processing and Tau homeostasis but also to test whether these genes would have any roles in neuronal physiology and function in an Aβ and Tau independent manner.

Additional Information

Methods and Supplementary Material
Please see https://sciencematters.io/articles/201803000014.

Funding Statement
L.R. was supported by the Swiss National Science Foundation grants (Sinergia and Core Interdisciplinary grants), by the Velux Foundation, the Novartis Foundation grant, the Cure Alzheimer Foundation, the Hurka Stiftung, the Bangerter Stiftung, the Baugarten Stiftung and the Synapsis Foundation. G.S. was supported by an EMBO long-term fellowship (ALTF 668-2011) and the University of Zurich’s Forschungskredit (K-82033-02-01).

Acknowledgements
We thank the lab members for their stimulating discussions.

Ethics Statement
All animal experiments were done according to the guidelines of and approved by the veterinary office of the Canton of Zürich, Switzerland.


