miRNAs as therapeutic agents in neurodegeneration, a pilot study

Thèse

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RÉSUMÉ

L’échec des différents essais cliniques souligne la nécessité de développer des nouvelles thérapies pour la maladie d'Alzheimer (MA), la cause la plus commune de démence. Les microARNs (miARNs) sont les ARNs non-codants les plus étudiés et ils jouent un rôle important dans la modulation de l'expression des gènes et de multiples voies de signalisation. Des études antérieures, dont celles de mon laboratoire d’accueil, ont permis de développer l’hypothèse que certains membres de la famille miR-15/107 (c.-à-d. miR-15ab, miR-16, miR-195, miR-424, and miR-497) pourraient être utilisés comme agents thérapeutiques dans MA. En effet, cette famille avait le potentiel de réguler de multiples gènes associés à MA, tels que la protéine précurseur de l'amyloïde (APP), la β-secrétase (BACE1), et la protéine Tau.

Tel que démontré dans ce projet de thèse, j’ai choisi miR-16 comme cible thérapeutique potentielle pour MA parmi tous les membres de la famille. L’essai luciférase dans ce projet confirme que miR-16 peut réguler simultanément APP et BACE1, directement par une interaction avec la région non-codante en 3’ de l’ARNm). Notamment, nous observons aussi une réduction de la production des peptides amyloïdes et de la phosphorylation de Tau après une augmentation de miR-16 en cellule. J’ai ensuite validé mes résultats *in vivo* dans la souris en utilisant une méthode de livraison de miR-16 via une pompe osmotique implanté dans le cerveau. Dans ce cas, l'expression des protéines d’intérêts (APP, BACE1, Tau) a été mesurée par immunobuvardage et PCR à temps réel. Après validation, ces résultats ont été complémentés par une étude protéomique (iTRAQ) du tronc cérébral et de l'hippocampe, deux régions associées à la maladie. Ces données m’ont permis d’identifier d'autres protéines régulées par miR-16 *in vivo*, incluant α-Synucléine, Transferrine receptor1, et SRm300. Une autre observation intéressante : les voies régulées par miR-16 i*n vivo* sont directement en lien avec le stress oxydatif et la neurodégénération.

En résumé, ce travail démontre l’efficacité et la faisabilité d’utiliser un miARN comme outil thérapeutique pour la maladie d’Alzheimer. Ces résultats rentrent dans un cadre plus vaste de découvrir de nouvelles cibles pour MA, et en

particulier la forme sporadique de la maladie qui représente plus de 95% de tous les cas. Évidemment, la découverte d'une molécule pouvant cibler simultanément les deux pathologies de la maladie (plaques amyloïdes et hyper phosphorylation de tau) est nouvelle et intéressante, et ce domaine de recherche ouvre la porte aux autres petits ARNs non-codants dans MA et les maladies neurodégénératives connexes.

ABSTRACT

Failure at different clinical trials emphasizes the need for developing new therapeutics for Alzheimer disease (AD) as the most common cause of dementia. MicroRNAs (miRNA) are the most studied groups of non-coding RNAs and have a critical role in modulating multiple signaling pathways and fine-tuning gene expression. Supporting evidence from other studies, including host lab, suggest that multiple members of the miR-15/107 family (miR-15ab, miR-16, miR-195, miR-424, and miR-497) could be used as therapeutic agents in AD. The potential ability of this miRNA family to modify disease pathway by multiple targeting of AD-associated genes such as Amyloid precursor protein (APP), β-site amyloid-β precursor protein cleaving enzyme (BACE1) and microtubule-associated protein Tau is of attention.

Based on documented results in this study I chose miR-16 as candidate therapeutic miRNA in AD. This choice is based on data obtain from cells and *in vitro* luciferase assay indicating the role of this miRNA in the simultaneous regulation of APP, BACE1 (directly by targeting 3'UTR of these genes). Decrease in Tau phosphorylation and amyloid beta peptides were further observed following increased miR-16 levels. Furthermore, I validated these results *in vivo* by delivering miR-16 oligos using Osmotic pumps implanted subcutaneously to deliver oligos to lateral ventricles of mouse brain also providing a wide distribution of these oligos. Expression of desired protein targets was measured by western blot and qPCR in different brain regions. Results demonstrated a context-dependent action of delivered miR-16 increase on the potential AD involved targets in mouse brain. These results were complemented by proteomics study of Brainstem and Hippocampus regions. Data indicated the potential regulation of other proteins by miR-16 *in vivo* such as α-Synuclein in Brainstem and Transferrin receptor1 and SRm300 in Hippocampus. The increase in miR-16 levels *in vivo* and in vitro was sufficient to downregulate the protein product of these genes confirmed by western blot. Enrichment study predicted oxidative stress and neurodegeneration as top terms in close connection with miR-16.

This work provided a proof-of-principle for possibility and efficiency of miRNA replacement based therapeutics delivered to CNS using miR-16 a member of the miR-15/107 family. Understanding the molecular mechanisms involved in the regulation of AD-related genes could have important implications for sporadic AD, which accounts for more than 95% of all cases with no effective therapy available. Multi-target therapy by non-coding RNA in AD is an emerging concept that would have the potential to change the way that therapeutics is developed for AD and other neurodegenerative diseases with complex nature and no effective therapy available.

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ABBREVIATIONS

AChE Acetylcholinesterase
AGO Argonaute protein
ALS Amyotrophic lateral sclerosis
AD Alzheimer's disease
Aph1 Anterior pharynx-defective 1 protein
ATF6 Activating transcription factor 6
Aβ Amyloid-β
BACE β-site amyloid-β precursor protein cleaving enzyme
CB Calbindin
CamKII Calcium/Calmodulin-dependent kinase II
CD86 T-Lymphocyte Activation Antigen
cDNA Complementary DNA
CDS Coding sequence
CLL Chronic lymphocytic leukemia
CSF Cerebrospinal fluid
CTFs C-terminal fragments
DGCR8 DiGeorge critical region 8
ELISA Enzyme-linked immunosorbent assay
ERK Extracellular signal-regulated kinase
FAD Familial Alzheimer disease
FBS Fetal bovine serum
GAPVD1 GTPase Activating Protein and VPS9 Domains 1
GFAP Glial fibrillary acidic protein
GSK-3β Glycogen Synthase Kinase-3
LOAD Late-onset Alzheimer disease
MAPT microtubule-associated protein tau
miRNA microRNA
NMDA N-methyl-D-aspartate receptor
NFT Neurofibrillary tangles
ORF Open reading frame
PARIS Protein and RNA isolation system
PHF Paired helical filament
PS Presenilin
RNU48 Small Nucleolar RNA, C/D Box 48
RT-qPCR Reverse transcription quantitative polymerase chain reaction
SAD Sporadic Alzheimer disease
SAMP8 Senescence-accelerated prone mouse
Scr Scramble
SNCA α-synuclein
SNPs Single nucleotide polymorphisms
UPR Unfolded protein response
UTR Untranslated region

To my beloved parents those are always inspirational to me and to memory of my dear grandmother with whom I had great memories of my childhood

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PREFACE

The present thesis is written in four main chapters. Chapter I provides an introduction to the work, in which a general review of main therapeutic targets in AD such as APP, BACE1, and Tau is presented. The literature review on miRNA topic continues with a focused concentration on miR-15/107 family and introducing the concept of miRNA-based therapeutics at the end of the chapter.

Chapter II delivers the main results of the current thesis which are already presented as a research article entitled: “Preclinical Evaluation of miR-15/107 Family Members as Multifactorial Drug Targets for Alzheimer’s Disease” published in the peer-reviewed journal of Molecular Therapy-Nucleic Acids (2015) 4, e256 in which I am the first author. The main goal of the project was an assessment of an AD-related miRNA in the context of noncoding RNA therapeutics. To achieve this aim, miR-16, as a candidate, was chosen from multiple members of miR-15/107 according to the *in vitro* result presented in this work. The following *in vivo* experiments tested miR-16 mimics action compared to the controls in mice. This paper was written and executed by me and majorly corrected by my supervisor Dr. Hébert. The first preliminary result indeed was obtained from the study of increased miR-16 levels and the reduction of amyloid beta *in vitro* and in mutant HEK cells performed by Pascal Smith. Claudia Goupil as our expert technician had the main role in optimization and performing osmotic pump implantation and Véronique Dorval contributed to this work by her data obtained from Rip experiment. Reviewer’s comments were also fundamental to shape the final work before its publication. The main revision was adding more supplementary data and information that was not included in the initial submission. The complete supplementary is presented at the end of chapter II of this work.

Chapter III presents the results obtained through parallel investigations of miR-16 upregulation *in vitro* and investigating the possible role of miR-16 itself in cellular stress. The findings in this chapter complement the results of chapter II. The highlighted results in this chapter indicate that loss of miR-16 expression could potentially affect the cellular stress in neurodegenerative context.

Chapter IV conveys the general discussion of the work and attempts to cover the topics that have been less explored in previous chapters. Specifically, a more straightforward hypothesis of miR-16 in neurodegenerative context is presented.

1. CHAPTER I: NTRODUCTION

1.1. Alzheimer's Disease (AD)

"A peculiar severe disease process of the cerebral cortex" appears as the first report of neuropathological findings of AD in the brain by Alois Alzheimer in 1907. He described a 50 years old woman whom he had followed for paranoia, progressive sleep, memory disturbance, aggression, and confusion, until her death 5 years later (Hippius & Neundörfer, 2003). AD is the most common cause of dementia in the elderly and is characterized by the progressive memory loss leading to a gradual and irreversible deterioration of cognitive function. Two major hallmarks of this disease are senile plaques and neurofibrillary tangles (NFT) (Figure 1). Senile plaques are extracellular depositions of β-amyloid (Aβ) peptide while NFT are intraneuronal aggregations composed of hyperphosphorylated TAU (Hardy & Selkoe, 2002).

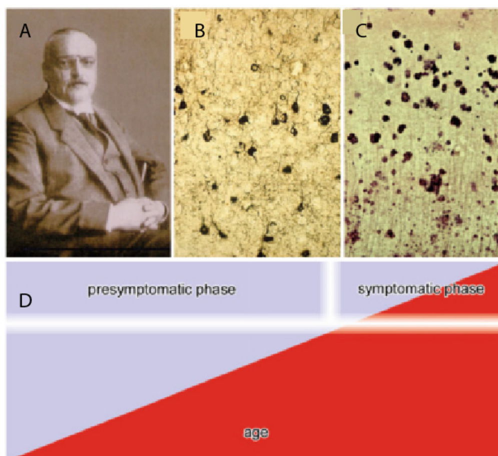


Figure 1 ***(A) Alois Alzheimer. (B) Neurofibrillary lesions shown by Gallyas silver-iodide staining technique. (C) Aβ plaques visualized by Campbell-Switzer silver-pyridine staining technique.***

(D) *The lesions (red) increase in severity and extent without remission during a long presymptomatic disease phase that reaches a shorter and final symptomatic phase. ''With Permission of Springer''(Heiko Braak & Del Tredici, 2015) Advances in Anatomy, Embryology, and Cell Biology V 215 2015 p4 © Springer International Publishing Switzerland 2015*

It is expected that by 2050, one new case of AD is to develop every 33 seconds or nearly a million new cases per year, and the total estimated prevalence is expected to be 13.8 million (Alzheimer's association, 2014)(Prince et al., 2013). According to Alzheimer Society of Canada in 2011 only, 747,000 Canadians were living with cognitive impairment, including dementia -including 14.9 percent of Canadians 65 and older.

1.2. Diagnosis

Early diagnosis of AD has come to attention because the disease manifests its earliest pathology years or decades prior to the onset of dementia. The diagnosis of AD generally resides in observation of neuropsychiatric features such as cognitive impairment that manifests itself at least by minimum two of these symptoms: inability of patients in new learning, disturbances of language function, impairment in reasoning and handling of complex tasks and changes in personality and behaviour (McKhann et al., 2011). Ante-mortem diagnosis still remains a challenge today due to lack of strong biomarkers. For instance, the CSF elevation of Tau could also be implicated pathologically in other diseases such as Parkinson disease (PD), Progressive supranuclear palsy (PSP), and Corticobasal degeneration (CBD) (Maruyama et al., 2013). With advancement in imaging techniques such as Magnetic resonance imaging (MRI), it has been proposed that gray matter atrophy patterns are capable of differentiating between subtypes of mild cognitive impairment (MCI) (H. Zhang et al., 2012). MCI is a syndrome characterized by cognitive impairments that are distinct compared to the decline in cognition by the normal aging process. MCI is often considered as a precursor to dementia or a transitional state between healthy cognitive aging and dementia (Sperling et al., 2011). The imaging study also implies a decrease in Hippocampal volume preceding the transition to AD can also be detected by Structural MRI scans (McEvoy & Brewer, 2010; van der Flier, 2005).

1.3. Microscopic features

1.3.1. Plaques

In mild AD cases, pathological lesions are typically restricted to the medial temporal lobe, including the hippocampus, with a spread to the temporal, the parietal, and eventually the occipital and frontal lobes in severe cases (H Braak & Braak, 1991). Aβ derived from APP is the main component of plaques found in AD brain. Aβ plaques are different in shapes and sizes and with the advancement of the disease, become dense and sensitive to silver staining (argyrophilic). Aβ deposits in AD rarely develop in the white substance; instead, they mainly occur in the gray matter. Neurite plaques (NPs) appear in later stages of the disease.

NPs consist of abnormal astrocytes, microglial cells, dystrophic neuronal processes and Tau aggregates (Heiko Braak & Del Tredici, 2015).

1.3.2. Neurofibrillary tangles (NFT)

Ultra-structurally, NFTs are composed of dense accumulations of structures known as paired helical filaments (PHFs) (KIDD, 1963), which are mainly distributed in the perinuclear area of the neuron and in proximal processes. Neurofibrillary degeneration and phosphorylated tau accumulation are closely aligned with neurological signs, neuropathological stage, and clinical and pathological disease severity as compared to accumulations of Aβ (H Braak & Braak, 1991). Braak staging of AD pathology is based upon the level of progression of NFT pathology, which spans from stage1 or mild to stage 6 that is very late or severe dementia in which patients are not able to do functions of daily living and need constant support. Braak method applies immunoreaction (AT8) for hyperphosphorylated tau protein (Heiko Braak, Alafuzoff, Arzberger, Kretzschmar, & Tredici, 2006) by which six stages of disease propagation can be distinguished with respect to the location of the tangle-bearing neurons and the severity of changes (Heiko Braak et al., 2006). Abnormal tau aggregates appear for the first time in the cerebral cortex generally in the transentorhinal region progressing to the limbic area and neocortical as well as primary sensory areas. Gradually, NFTs fill large portions of the soma of neurons (Figure 2) (Heiko Braak & Del Tredici, 2015).

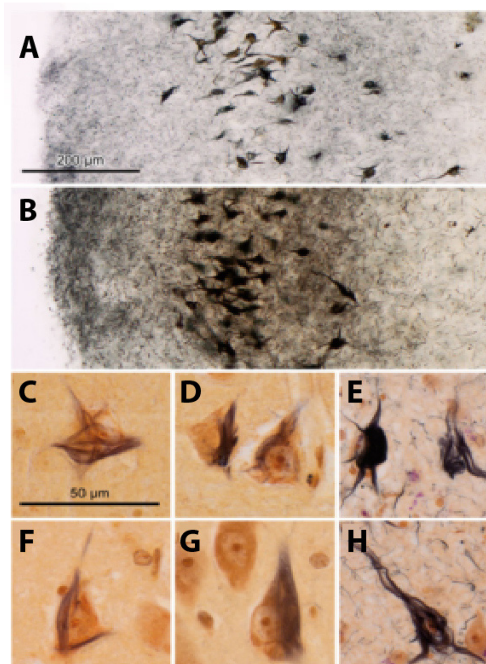


Figure 2 ***Neurofibrillary pathology in the entorhinal layer of cortex***

(A) *The mildly involved entorhinal layer at NFT stage II.* ***(B)*** *A dense network of NTs at NFT stage V.* ***(C–H)*** *Development of neurofibrillary changes using the Gallyas silver-iodide technique combined with Pigment-Nissl staining.* ***(C, D)*** *NFTs stage I) (e, f) Consolidation of a sturdy NFT. A comparison with uninvolved neighboring neurons does not reveal obvious reactive changes* ***(G, H)*** *Dying NFT-bearing nerve cell* ***(G)*** *at the left side* ***(H)*** *NFT stage VI in a 60-yrs male AD patient, ''With Permission of Springer''(Heiko Braak & Del Tredici, 2015) Advances in Anatomy, Embryology and Cell Biology V 215 2015 p102 © Springer International Publishing Switzerland 2015*

1.3.3. Neural loss

AD clinical symptoms develop subtly leading to a gradual loss of fundamental functions by cellular impairments that first appear after a given threshold is exceeded (Figure 1) (Heiko Braak & Del Tredici, 2015). In term of neural loss, degeneration of basal forebrain is more prominent in the advancement of the disease. The basal forebrain plays an important role in acetylcholine production and associated loss of cholinergic neurotransmission contribute significantly to the deterioration in cognitive function seen in patients with Alzheimer's disease (Bartus, Dean, Beer, & Lippa, 1982). Acetylcholine (ACh) was the first neurotransmitter discovered with involvement in memory and cognition in central nervous system (Todman, 2008). In advanced AD, the relative loss of cholinergic connections by region shows different pattern; with the temporal lobe showing most loss of neurons contrary to other neuronal populations such as sensory and motor neurons (Geula & Mesulam, 1989). The mechanism behind this selective vulnerability of different neural populations of the brain in AD is not well understood. Interestingly it has been proposed that morphology also comes to attention where the neuronal types involved in AD-process are found among

projecting neurons with a disproportionately long axon (Heiko Braak & Del Tredici, 2015).

1.4. Macroscopic features

The decrease in brain volume is very prominent in AD, rather diffuse and uniform in cerebral white matter. The gray matter of frontal and parietal cortex and striatum is more affected compared to temporal cortex, cerebellar vermis, and hippocampus; and the occipital cortex is least affected (Mrak, Griffin, & Graham, 1997). Other pathologies include increased in lipofuscin content of neurons (insoluble, autofluorescent glycol lipoprotein), the ventricular and subarachnoid space expansion followed by shrinkage of brain and volume loss (Figure 3)(Shankar, 2010).

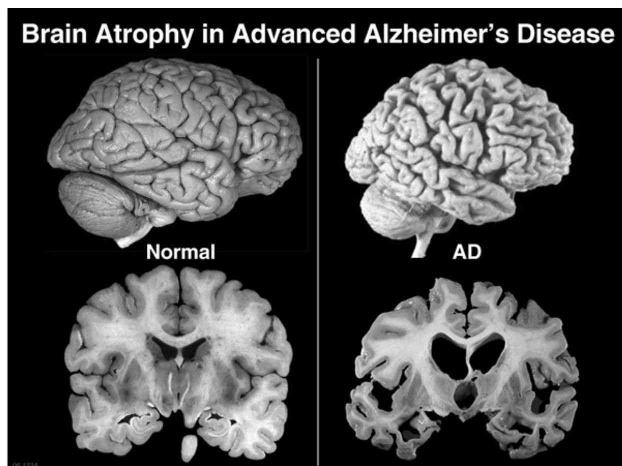


Figure 3 ***A healthy brain contrasted with the effects of Alzheimer's***[1]

1.5. Familial versus sporadic AD

APP coding gene is located on the long arm of chromosome 21 with an extra copy only present in individuals with Down's syndrome or trisomy 21 (Yoshikai, Sasaki, Doh-ura, Furuya, & Sakaki, 1990). APP and its proteolytic signaling play an important role in the onset and progression of familial and non-familial AD (sporadic). Several mutations in APP is reported in hereditary early-onset AD summarized in Figure 4. Mutations mainly located close to the major APP processing sites (β - and γ -secretase sites) or within the Aβ sequence (the α -secretase site). The mechanisms by which mutation causes disease are diverse

[1] http://jungminded.weebly.com/neuropsychology/aphasias

and complex. For example, Swedish mutation (K670NM671NL) (Mullan et al., 1992) makes APP mutant a better BACE-1 substrate, increasing β-secretase cleavage and enhancing Aβ production both Aβ42 and Aβ40 in plasma and fibroblasts from mutation carriers (Citron et al., 1992; Haass et al., 1995). Only one mutation (A673T) has been reported to protect against AD with high penetrance providing provides a proof of principle for the hypothesis that reducing the β-cleavage of APP may protect against the disease (Jonsson et al., 2012).

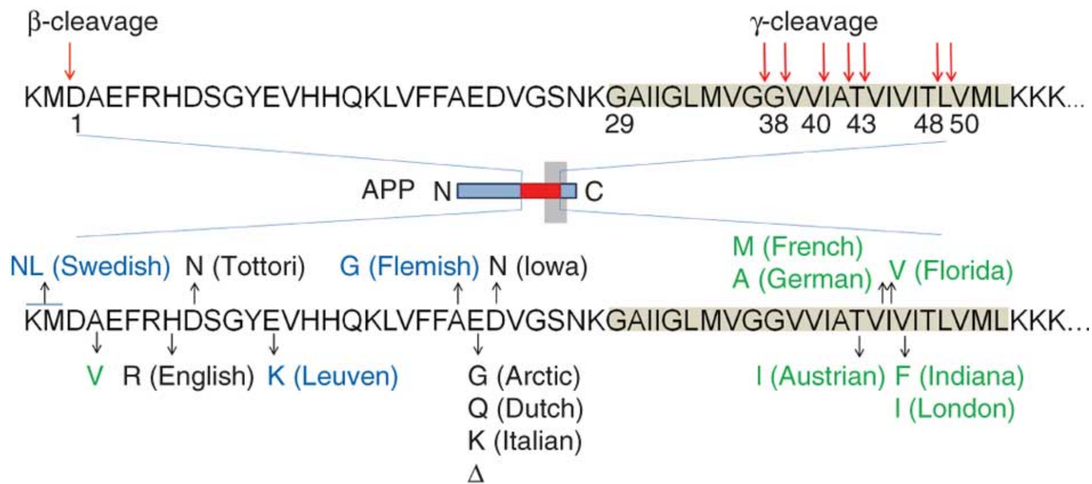


Figure 4 ***Generation of Aβ from amyloid precursor protein***

The sites of β- and γ-secretase–mediated cleavage are indicated by arrows, and the transmembrane domain of APP is highlighted in gray. γ-cleavage produces a pool of Aβ fragments that vary in length and hydrophobicity. The mutations in Aβ region of APP either increase the total Aβ production (marked in blue), alter Aβ biophysical properties (in black) or affect the Aβ spectrum in both quantitative and qualitative ways (in green) Nature Publishing Group Permission (Benilova, Karran, & De Strooper, 2012).

The other important mutation reported is located in the presenilin 1 and presenilin 2 genes (PSEN1, PSEN2) part of γ –secretase components (Strooper et al., 2013). Presenilin cause early-onset (<60 years) autosomal dominant AD (Bertram et al., 2010), which accounts for less than 1% of AD cases (Campion et al., 1999). PS mutations alter PS processing and So far, more than 150 familial AD-causing mutations in *PSEN1* only have been identified, for instance, the Δ9 mutation completely inhibits PS processing (Crook et al., 1998; Mercken et al., 1996). Further studies demonstrated that transgenic mice carrying both mutant

amyloid precursor protein and presenilin 1 transgenes showed accelerated Alzheimer-type phenotype(Holcomb et al., 1998).

Unlike the early-onset familial form of AD as the result of main mutations at least in one of the three amyloid processing genes (APP, PSEN1 or PSEN2), the mechanisms underlying late-onset or sporadic form of the disease (LOAD/sAD) remains unknown. It is important to note that a single pathway does not explain the more common non-familial form (Medway & Morgan, 2014). It has been suggested that advancing age and a family history of dementia may contribute to decreasing threshold of AD appearance; however, it vastly remains to clarify the association of other factors i.e. oxidative stress, diabetes and hypertension with AD and whether they mainly contribute to clinical manifestations or they intervene with amyloidosis and neurofibrillary degeneration processes themselves (David Knopman, 2011).

Studies suggest that both increased Aβ production and decreased Aβ clearance could play an important role in overall Aβ changes in sporadic AD (Carare, Hawkes, Jeffrey, Kalaria, & Weller, 2013). Recently, Aβ accumulation, tau accumulation, and molecules related to Aβ metabolism across 12 brain regions in postmortem tissue from sporadic AD, familial, controls have been studied. This study clearly indicates the difference between the Aβ pattern in both forms of the disease. In sporadic AD, Aβ42 was found to disproportionately accumulate in cortical regions, compared with familial AD, and to strongly correlate with the normal regional distribution of Synaptic markers. Although an increased production of Aβ42 or increased ratio of Aβ42 to Aβ40 is clearly implicated in the pathogenesis of familial type (Karran et al., 2011), it remains unclear how Aβ metabolism is disturbed and thus involved in sporadic AD where there is no autosomal dominant mutation (Shinohara et al., 2014). To clarify how Aβ is involved in sporadic AD some anatomical studies provide the evidence that some nerve cells in the brainstem nuclei are involved in Aβ deposition, those already have undergone cytoskeletal tau changes (Heiko Braak & Del Tredici, 2013).

1.6. Molecular targets in AD Therapeutics

1.6.1. Amyloid Precursor Protein (APP)

APP is a well-conserved member of the greater APP family and the only member with an Aβ peptide domain. First cloned APP isoform is APP695, expressed mainly in the brain without exon 7 and 8 (Kang et al., 1987). An important characteristic of this protein family is having E1 and E2 domains, Kunitz protease inhibitor (KPI) region, and an OX-2 domain (encoded by exons 7 and 8). The latter two do not exist in brain isoform or APP695 (Figure 5) (Coburger et al., 2013) . APP is expressed highly in fetal tissues and has the highest expression levels in brain, kidney, heart and spleen while weak expression in liver. In adult brain, highest expression is found in the frontal cortex specifically (K. Tang et al., 2003).

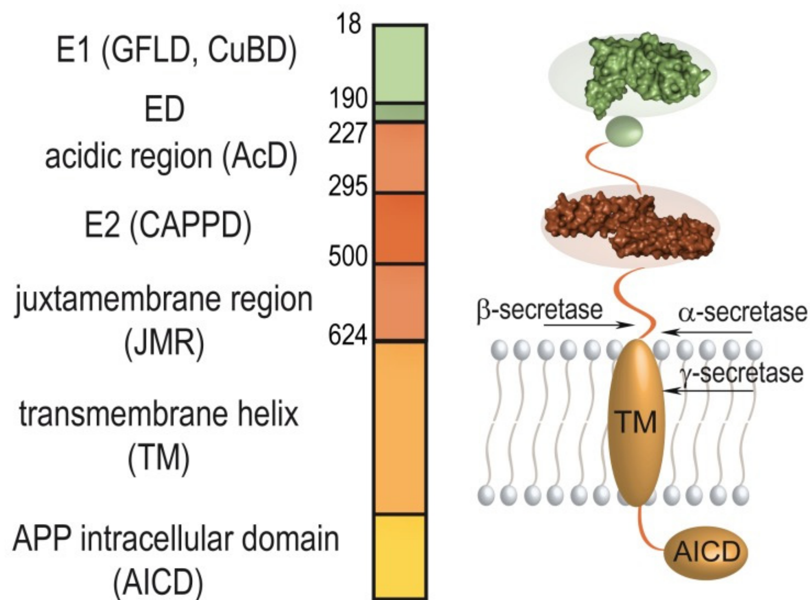


Figure 5 ***Schematic domain structure of APP695***

The different domains of APP brain isoform are schematically demonstrated in the picture. N-terminal growth factor-like domain (GFLD) copper-binding domain (CuBD). central APP domain (CAPPD). and the intracellular domain (AICD) (Coburger et al., 2013).

In neurons, APP can be found in both presynaptic and postsynaptic compartments (Yamazaki, Selkoe, & Koo, 1995). Although the physiological functions of APP are not clear, several possibilities have been proposed (Nakayama, Nagase, Koh, & Ohkawara, 2013). The most considerable functions

are synapse formation and repair (Priller et al., 2006). It has also been suggested that APP acts as a cell adhesion molecule and plays a role in cell-cell interaction (Soba et al., 2005).

1.6.2. Proteolytic processing of APP

The proteolytic process of APP follows the regulated intramembrane proteolysis or simply RIP mechanism. RIP follows a two-step pattern. An initial cut at a site outside the membrane is followed by a second cut inside. Cleavage of APP by α-secretase or β-secretase results in shedding of the entire extracellular domain and α- or β-carboxy terminal fragments (αCTF/C83 or βCTF/C99) (Esch et al., 1990) (Vassar et al., 1999). Several zinc-metalloproteinases, such as TACE and ADAM (Buxbaum et al., 1998; Lammich et al., 1999), and the aspartyl protease BACE2 (Farzan, Schnitzler, Vasilieva, Leung, & Choe, 2000) can cleave APP at the α-site, while BACE1 (β-site APP cleaving enzyme) cleaves APP at the β-site (Figure 6) (Vassar et al., 1999). After α- or β-cleavage, the resulting membrane associated C-terminal fragments are subsequently cleaved in the Transmembrane domain by a γ-secretase complex releasing the P3 or Aβ fragment and an APP intracellular domain (AICD). Generated Aβ peptides might differ in size slightly from 34–50 amino acids since the exact cut generated by the γ-secretase complex varies (Kummer & Heneka, 2014). Most of Aβ fragments are Aβ40, but a smaller fraction is the more prone to oligomerization which is Aβ42 and Aβ43 peptides found in amyloid plaques (Sisodia & St George-Hyslop, 2002). Several enzymes can degrade Aβ: Neprilysin (NEP) and insulin-degrading enzyme (IDE) are expressed in neurons as well as within the vasculature, and the levels of both these enzymes are reduced in AD. Post-translational regulation of NEP and IDE proteins by multiple factors i.e APP intracellular domain AICD leads to increased Aβ clearance (Kerridge, Belyaev, Nalivaeva, & Turner, 2014).

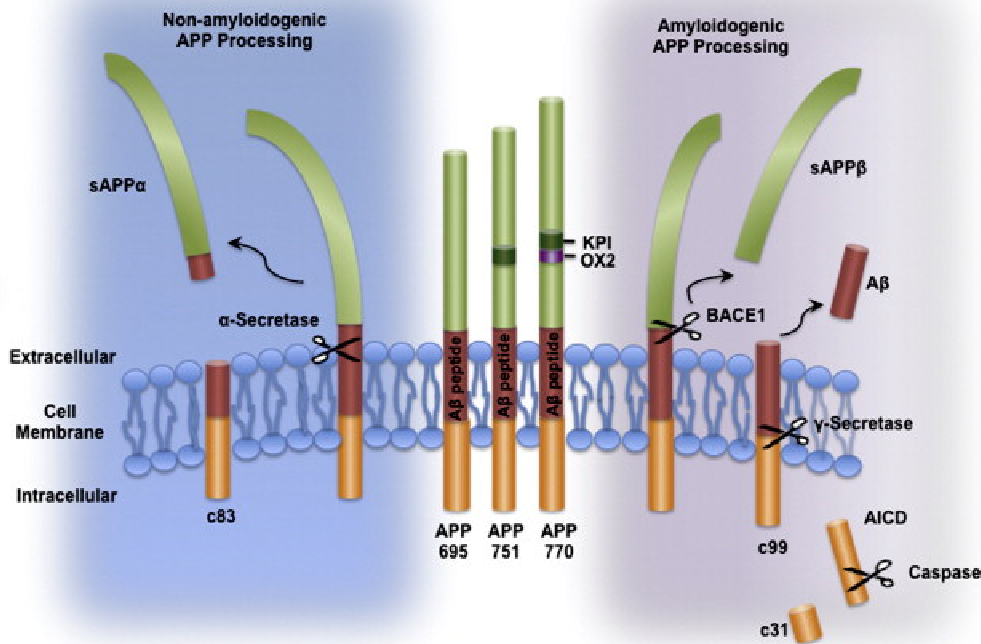


Figure 6 ***Schematic drawing of the Amyloid Precursor Protein (APP) and its processing pathways***

APP can be processed by α-secretase in a non- amyloidogenic pathway, which precludes the formation of Aβ and generates soluble APPα (sAPPα) and a C-terminal fragment (c83). Alternatively, in the amyloidogenic pathway, cleavage by the β-secretase BACE1 generates soluble APPβ (sAPPβ), which is secreted, and a C-terminal fragment (β-CTF or c99). Subsequent cleavage of c99 by the γ-secretase complex generates Aβ and the APP intracellular domain (AICD) which can be further cleaved by caspases to produce a c31 fragment Copyright © 2011 Elsevier Inc. with permission from Elsevier (Schonrock, Matamales, Ittner, & Götz, 2012)

As Aβ plays a role in the pathogenesis of AD the research on targeted therapy focused solely on treatments directed against Aβ. Anti-amyloid approaches intervene with Aβ production by immunotherapy, an increase of amyloid clearance and decrease of its aggregation, modulation of its transport or modulation of secretase (Kumar, Singh, & Ekavali, 2015). The term immunotherapy defies the manipulation of the immune system by inducing, enhancing, or suppressing immune responses *in vivo (Lambracht-Washington & Rosenberg, 2013).* Vaccination was the first treatment to have the significant effect on the AD in animal models. Vaccination of transgenic mice with Aβ prevented Aβ deposition and improved behavioral impairments related to Aβ deposition (Janus et al., 2000; Schenk et al., 1999).

Furthermore, the promising results of vaccination in preclinical and the lack of serious side-effects in transgenic mice encouraged other trials and it was followed by the launch of AN 1792 trial which strongly induces an immune response (T. Li et al., 2007). This trial was eventually stopped since 6% of participants developed symptoms of acute inflammation. Overall, failure of these drugs suggests that targeting Aβ alone might not be enough to prevent or slow AD. One very recent example is the most advanced trial study on monoclonal antibodies Solanezumab and Bapineuzumab, which have not been proven promising in clinical trials phase III (Boutajangout & Wisniewski, 2014).

1.6.3. β Secretase

The β-secretase enzyme that initiates the production of Aβ, BACE1 is a key therapeutic target for AD. The biochemical properties of the β-secretase enzyme had been established well before BACE1 was identified by independent groups as the β-secretase enzyme in 1999 (Vassar et al., 1999). BACE1 is a typeI transmembrane aspartyl protease with optimal protease activity in a low pH environment. As a result, BACE1 tends to localize in acidic compartments, such as endosome and trans-Golgi network (Vassar, Kovacs, Yan, & Wong, 2009). Immunohistochemical studies have indicated BACE1 is expressed both in neuronal and glial cells in the mammalian brain as well (Laird et al., 2005; Rossner, Lange-Dohna, Zeitschel, & Perez-Polo, 2005).

In sporadic AD brain, there is a strong correlation between Aβ loads and BACE elevation and enhanced deposition of amyloid plaques (R. Li et al., 2004) and AD progression (Che et al., 2014; Fukumoto, 2002; H. Harada et al., 2006). To study the role BACE1 in AD, several groups used gene targeting strategies to generate BACE1 knockout mice without gross abnormal phenotype implicating BACE1 inhibition as an interesting drug target (Cole & Vassar, 2007; Roberds et al., 2001). These genetics models further validated BACE1 as the major β secretase in the brain, making BACE1 inhibition a viable treatment strategy for AD (Y. Luo et al., 2003; Ohno et al., 2004). APP Overexpressing mice having a heterozygous BACE1 gene knockout were evaluated for Aβ generation and for the development of pathology. It was demonstrated that Although the 50% reduction in BACE1 enzyme levels caused only a 12% decrease in Aβ levels in young mice, it resulted

in a dramatic reduction in Aβ plaques, neuritic burden, and synaptic deficits in older mice (McConlogue et al., 2007). BACE1 is enriched in neuronal presynaptic terminals suggesting an important role for BACE1 at the synapse as well (Rajapaksha, Eimer, Bozza, & Vassar, 2011). Moreover, the protective A673T APP mutation in humans decreases Aβ generation via reduced β-secretase processing of APP, providing strong proof of concept that BACE1 inhibition should be efficacious for AD (Ohno et al., 2004; Vassar et al., 2009; R. Yan & Vassar, 2014). However, the design of small-molecule BACE1 inhibitors (i.e.LY2811376 a non-peptide molecule) has also shown no success to its extreme side effects in the very recent study (Yan and Vassar, 2014). It would be important to mention that complete suppression of BACE1 as the therapeutic approach is not ideal since recent studies of BACE1−/− mice have shown complex neurological phenotypes (R. Yan & Vassar, 2014). Recently, small-molecule BACE1 inhibitors have been tested that exhibit satisfactory pharmacokinetics and optimal effect on cerebral Aβ reduction in preclinical animal models (R. Yan & Vassar, 2014). Most trials are in early phases and little data about them have been published. The side effects of drugs inhibiting BACE1 still not examined but the possibility exists that BACE1 inhibitor drugs might also cause BACE2 mechanism-based side effects in addition to those of BACE1 (Table 1) (Dominguez et al., 2005).

Table 1 ***Small-molecule BACE1 inhibitors in clinical trials.***

	Phase	NCT trial number
AZD3293	Phase 1	01739647, 01795339
CTS-21166	Phase 1	00621010
E2609	Phase 1	01294540, 01511783, 01600859
HPP854	Phase 1	01482013
LY2886721	Phase 2[a]	01227252, 01534273, 01561430(Terminated because of abnormal liver biochemistry)
MK-8931	Phase 2/3	01496170, 01739348, 01953601
PF-05297909	Phase 1	01462851
RG7129	Phase 1[‡]	Not available (Removed from pipeline)
TAK-070	Phase 1	Not available

with permission from Elsevier (R. Yan & Vassar, 2014)

1.6.4. γ-Secretase

γ-Secretase is a complicated complex composed of Presenilin(PS), Nicastrin (NCT), anterior pharynx defective-1 (Aph-1), and PS enhancer-2 protein (Pen-2) (Figure 7). The first evidence for the role of presenilins in APP processing came from observations that AD-causing mutations in PSEN1 and PSEN2 affect the generation of Aβ peptides, changing the relative amount of Aβ peptide versus the shorter Aβ40 (Borchelt et al., 1996). PSEN1 mutations show the earliest age of onset more commonly affected by seizures and cerebellar signs, whereas PSEN2 mutations have a delayed onset with longest disease duration and significant disorientation (Shea et al., 2015). PSEN1 and PSEN2, catalytic component of γ-Secretase, both synthesized as precursor proteins of 50 kDa processed into a 30kD domain and a 20 kDa fragment during maturation (Thinakaran et al. 1996).

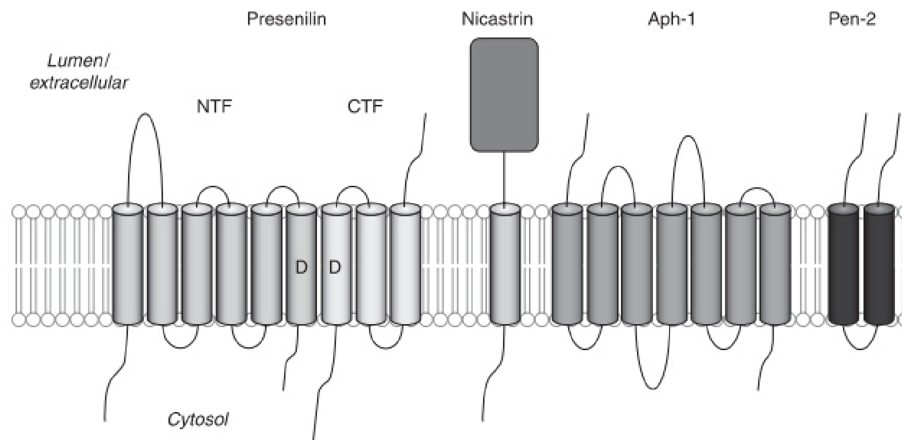


Figure 7 ***Subunits of the γ -secretase complex.***

Presenilin is proteolytically processed into two fragments during maturation of the complex, an amino-terminal fragment (NTF) and a carboxy-terminal fragment (CTF). Other subunits are Nicastrin, APH-1, and PEN-2 Copyright © 2012 Cold Spring Harbor Laboratory Press (Strooper et al., 2013).

Aph-1 act as a scaffold during the process of γ-secretase complex assembly, and Pen-2 as a trigger for the cleavage of PS in order to activate it (Smolarkiewicz, Skrzypczak, & Wojtaszek, 2013). Human Pen-2 overexpression in transgenic mice has been shown to induce all the AD-like phenotypes, including behavioral deficits, motor activity, and feeding behavior dysfunction, Aβ-42 peptide deposition and chronic disease induction (Nam et al., 2011). A direct role of PEN-

2 in cleavage of PS1 and a regulatory function of APH-1, in coordination with PEN-2, in the biogenesis of the PS1 complex has been revealed (W. J. Luo et al., 2003). Unlike PS1, overexpression of APH1, PEN2, and Nicastrin proteins can increase the levels of Aβ, suggested that these proteins are limiting for γ-secretase activity (Marlow et al., 2003).

At 709 amino acids, Nicastrin is the largest component of γ-secretase, with the majority of its mass located to its large, heavily glycosylated ectodomain. Structural studies have been suggested a very comprehensive model of Nicastrin more favoring the hypothesis that Nicastrin may indeed serve as a receptor of γ-secretase substrates (Xie et al., 2014)

Development of therapeutic against γ-secretase faces the specific challenge γ-secretase itself is also an essential component of the Notch signaling (Geling, Steiner, Willem, Bally-Cuif, & Haass, 2002; Godin et al., 2003; Kopan & Ilagan, 2009; Lundkvist & Näslund, 2007; Wolfe, 2008). Strong inhibition of γ-secretase leads to severe adverse effects, as it interferes with signaling by notch proteins and other cell surface receptors. A recent study concluded that a γ-secretase reduction of 30% was sufficient to attenuate Aβ deposition with little or no adverse side effects (Bard et al., 2000).

Heterogeneity of γ-secretase complex suggests that selective targeting of one particular subunit might be a more effective treatment strategy than non-selective γ- secretase inhibition (Serneels et al., 2009). For instance, deletions of APH-1B and APH-1C isoforms in a mouse model of AD decreased Aβ plaque formation and improved behavioral deficits (Serneels et al., 2005). They are current studies on developing more specific inhibitors (D'Onofrio et al., 2012).

1.6.5. Tau

Three types of filaments form the cytoskeleton: microfilaments, intermediate filaments, and microtubules. The cytoskeleton provides a dynamic scaffold to proteins, vesicles, and organelles, essential for cell function and changes in the state of its polymerization, play an important role in the neuronal process such as polarization, axonal transport, maintenance of neuronal extensions, synaptic plasticity and protein sorting (Morris, Maeda, Vossel, & Mucke, 2011). Tau protein

exists as a family of microtubule-associated protein (MAPs) that is found predominantly in axons with an important role in axonal transport (Dixit, Ross, Goldman, & Holzbaur, 2008; Weingarten, Lockwood, Hwo, & Kirschner, 1975; Witman, Cleveland, Weingarten, & Kirschner, 1976). The gene that encodes for tau consists of 16 exons and is located at the chromosomal locus 17q21 (Neve, Harris, Kosik, Kurnit, & Donlon, 1986). Through alternative splicing, six tau isoforms are generated in the CNS-isoforms (Figure 8). These tau isoforms differ according to the presence of 0, 1 or 2 near-amino-terminal inserts (0N, 1N or 2N) and the presence of R2 repeat, yielding 3 or 4 carboxy-terminal repeat domain (3R or 4R) tau species. The expression of human tau is developmentally regulated. In the adult brain, six isoforms of tau are expressed, whereas in the fetal brain only the shortest tau is expressed. In the adult human brain, levels of the 3R and 4R forms are roughly equal and the 2N isoform is underrepresented compared with the others: the 0N, 1N, and 2N tau isoforms comprise ~37%, ~54% and ~9% of total tau (Y. Wang & Mandelkow, 2015).

Through repeated domains located at the c-terminal of the protein and provides stability to the microtubule. This process can be regulated through a balance in the phosphorylation and dephosphorylation process of tau protein (Luna-Muñoz & Harrington, 2013).

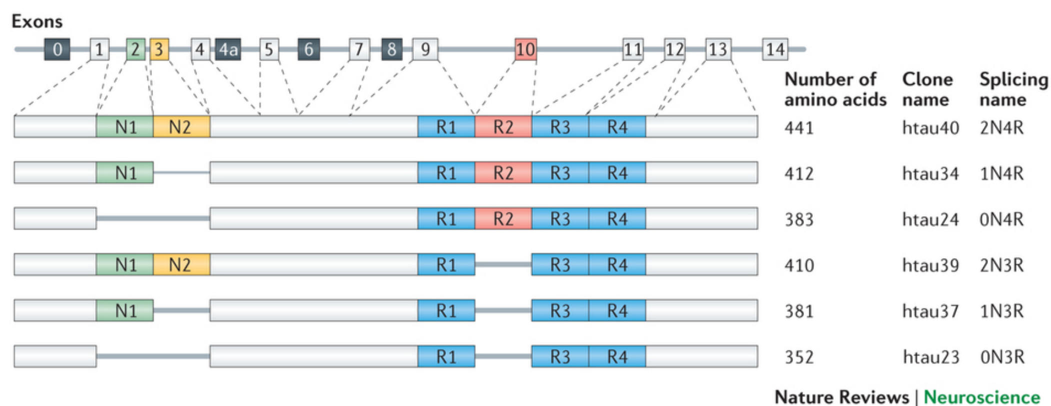


Figure 8 ***MAPT, the gene encoding human tau, contains 16 exons***

Exon 1 (E1), E4, E5, E7, E9, E11, E12, and E13 are constitutive, whereas the others are subject to alternative splicing. E0 and E1 encode the 5' untranslated sequences of MAPT mRNA, whereas E14 is part of the 3' untranslated region. E0 is part of the promoter, which is transcribed but not translated. The translation initiation codon ATG is in E1. E4a, E6, and E8 are transcribed only in peripheral tissue. The six human brain tau isoforms are generated through alternative splicing of

E2, E3, and E10.For more detail refer to the original Ref. Nature Publishing Group's permission (Y. Wang & Mandelkow, 2015).

1.6.6. Phosphorylation of Tau

Phosphorylation of Tau is the most studied modification of this protein to date. Protein phosphorylation is the addition of a phosphate group, by esterification, to one of three different amino acids: serine, threonine, and tyrosine. An increased tau phosphorylation reduces its affinity for microtubules leading to cytoskeletal destabilization. Phosphorylation is thought to be a critical event in both normal regulations of tau function and the pathogenesis of tau-related neuronal degeneration (Kawakami & Ichikawa, 2015).

In AD, PHFs are characterized with abnormally phosphorylated Tau. Eighty-five putative phosphorylation sites on tau protein have been described in AD brain tissue (Hanger, Anderton, & Noble, 2009) (Figure 9). The phosphorylation of tau protein affects its solubility, localization, function, interaction with partners and susceptibility to other posttranslational modifications. However, the role of specific sites of tau phosphorylation in early neurodegenerative mechanisms is yet not fully understood(Luna-Muñoz & Harrington, 2013).

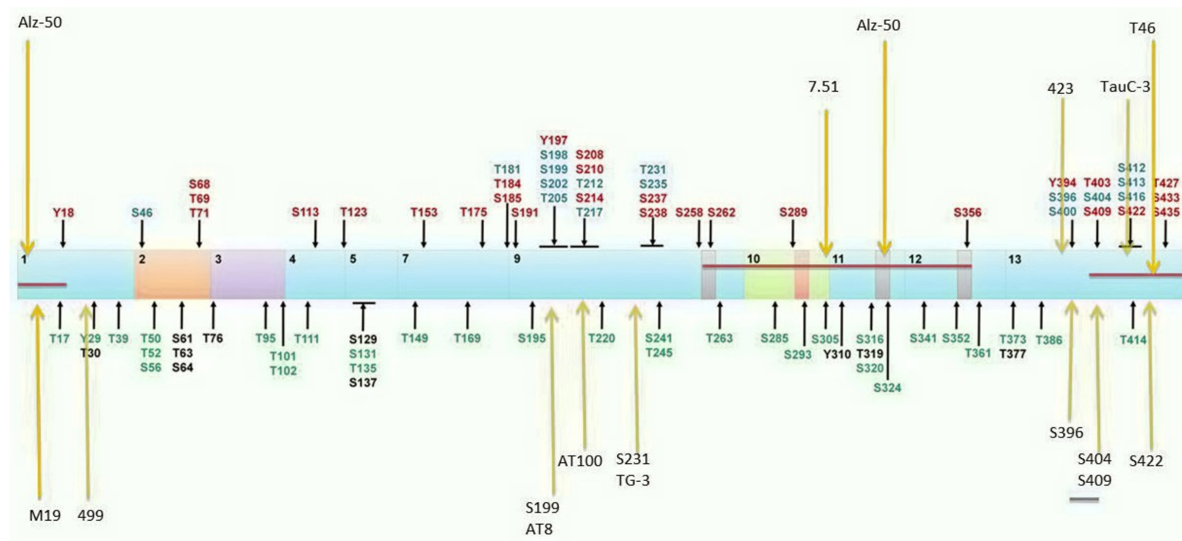


Figure 9 ***Location of tau phosphorylation sites and epitopes for tau antibodies***

Multiple amino acids are phosphorylated with some those observed in AD brain, normal brain (green) and both normal and AD brains (blue)(Luna-Muñoz & Harrington, 2013).

Phosphatases and kinases act in a concert to balance Tau phosphorylation. The imbalance between kinase and phosphatase acting on tau contributes to its abnormal phosphorylation and aggregation (Martin, Latypova, & Terro, 2011). The most implicated kinases for tau phosphorylation are Glycogen synthase kinase (GSK)- 3β, cyclin-dependent kinase 5 (cdk5), camp-dependent protein kinase (PKA), and calcium/calmodulin-dependent kinase II (CaMK-II). (Gong and Iqbal, 2008). Other tau kinases studied later like extracellular signal-regulated kinases 1 and 2- (ERKs 1 and 2) has been also marked in tau phosphorylation (Hébert et al., 2010). ERK1/2 is reported over activated in degenerating neurons in Alzheimer's disease (Pei et al., 2002). Protein phosphatase 1 (PP1), PP2A, PP2B, PP2C, and PP5 have all been implicated in the dephosphorylation of tau (Hanger et al., 2009). Phosphatases downregulation plays an important role in the abnormal tau phosphorylation and aggregation linked to AD (Iqbal et al., 2005). In AD brains, total phosphatase activity is reduced around 50% (F. Liu, Grundke-Iqbal, Iqbal, & Gong, 2005). It has been recently studied this crosstalk between PP2A and Glycogen synthase kinase-3β (GSK-3β). Reduction of PP2A has been suggested to increase Ser9 phosphorylation of GSK-3β leading to its activity decrease, which may further promote a decrease PP2A activity, favoring PP2A against GSK-3β as a therapeutic target (Y. Y. Wang et al., 2014).

1.6.7. Pathology of Tau in AD

Hyperphosphorylation of Tau may also be a connecting bridge between other pathological events found in AD-like synaptic failure, mitochondrial dysfunction and oxidative stress (Mondragón-Rodríguez et al., 2013). Loss of function of Tau, induced by its hyperphosphorylation not only affects interaction with microtubules but also affects synaptic function and neural signaling (Bramblett et al., 1993; Pooler et al., 2012; Souter & Lee, 2010). Synaptic dysfunction is induced by tau missorting from axons to the somatodendritic compartment (Hoover et al., 2010; Thies & Mandelkow, 2007). It remains to clarify how Tau phosphorylation contributes to aggregation of this protein in AD (Y. Wang & Mandelkow, 2015). It is also hypothesized that Tau hyperphosphorylation also could be a contributor factor to cell cycle re-entry of post-mitotic neurons in AD (Jian Zhi Wang, Wang, & Tian, 2014). Tau is also subject to other post-translational modifications(Martin

et al., 2011). For instance, In human AD brains, but not in normal brains, tau is modified by N-glycosylation, which is proposed to help to maintain and stabilize PHF structure (J Z Wang, Grundke-Iqbal, & Iqbal, 1996).

1.6.8. Therapeutic targeting of Tau

The interaction of Tau and Aβ is a determining factor in the pathogenesis of AD and the therapeutics failures focusing solely on one component of disease strengthen this idea; However, the mechanisms linking Aβ toxicity and Tau hyperphosphorylation remains still obscure (Lloret, Fuchsberger, Giraldo, & Viña, 2015). Considering the important role of Tau, blocking or reducing the pathological effects of this protein may be protective against amyloid pathology (Giacobini & Gold, 2013) different common approaches reducing tau pathology are tau-targeted immunotherapy, modulating axonal transport and microtubule function and promotion of tau degradation (Figure 10).

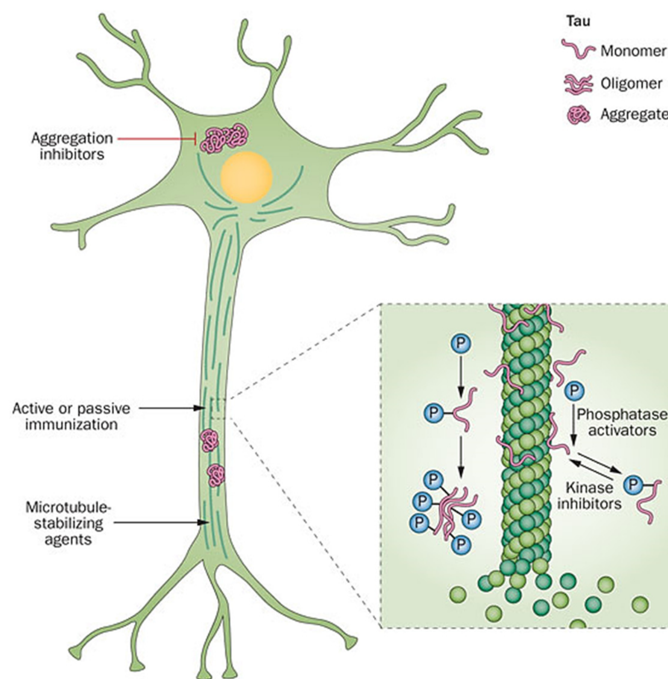


Figure 10 ***Schematic representation of tau-related processes that are potential targets for therapeutic intervention***.

Tau protein exists in neurons in soluble monomeric and oligomeric forms, and as insoluble aggregates (neurofibrillary tangles). Inhibitors of kinases and activators of the phosphatases that mediate this process, therefore, represent possible targets for therapeutic interventions for the treatment of dementia. In addition, anti-aggregation agents could reduce tau aggregation, which is an important step in tau-mediated neuronal damage. Microtubule-stabilizing agents might also have therapeutic potential. Nature Publishing Group Permission (Giacobini & Gold, 2013)

Anti-tau antibodies might block the spread of tau pathology from one neuron to another. As the mechanism of intercellular transmission of tau pathology is not yet fully understood; however, consideration of the process as a valid target for therapy is unanticipated (Giacobini & Gold, 2013).

Findings in transgenic animals carrying tau mutations (LaFerla & Green, 2012) supporting the concept that tau might be a stronger target for therapy tau-targeted interventions particularly anti-tau immunotherapy could reduce tau pathology, facilitate clearance of p-tau, improve cognitive deficits, and delay disease progression (Giacobini & Gold, 2013). TAU knock-out mice established by Harada et al in 1994 does not manifest macroscopic changes (A. Harada et al., 1994). It seems that presence of other MAPs that share several biological roles compensate Tau absence (Dehmelt & Halpain, 2005; Sontag, Nunbhakdi-Craig, White, Halpain, & Sontag, 2012). In term of a therapeutic advantage, it does not seem a complete reduction of Tau be considered as a therapeutic approach because of the compensatory effect of other MAPs (Götz, Xia, Leinenga, Chew, & Nicholas, 2013). Instead, reducing only TAU expression after the development of a pathology seems sufficient to alleviate the pathology (Hochgräfe, Sydow, & Mandelkow, 2013; Van Der Jeugd et al., 2012). Most recent pharmaceutics in test phase is aimed to reduce tau pathology by inhibition of GSK-3 (Tideglusib in phase II) and tau aggregation (TAURx phase II)(Del Ser et al., 2013) (Wischik & Wischik, 2012). It is yet to determine how these therapeutic strategies will work in later clinical phases. It is logical to assume that phosphatase can be more potent targets as it already mentioned earlier, but their activation is yet unlikely to become a therapeutic target for pharmacological intervention due to insufficient data available (Hanger et al., 2009).

1.7. microRNA

MicroRNAs (miRNAs) are short endogenous single-stranded RNA molecules that regulate gene expression (Bartel, 2004). Mature miRNAs and Argonaute (Ago) proteins form the RNA-induced silencing complex or RISC, a ribonucleoprotein complex mediating post-transcriptional gene silencing. Complementary base-

pairing of the miRNA guides RISC to target messenger RNAs, which are degraded, destabilized or translationally inhibited by the Ago protein (Filipowicz, Bhattacharyya, & Sonenberg, 2008).

The first miRNA, lin-4, was reported by Ambros and Ruvkun's labs in 1993. In the nematode, Caenorhabditis elegans lin-4 activity is required for proper larva development and mutations in lin-14 gene causes an opposite phenotype of the null-lin-4 mutations indicating a negative regulation of lin-14 by lin-4 (R. Lee, Feinbaum, & Ambros, 2004). Ambros found two very small lin-4 transcripts of only 61 nt and 22 nt in length (R. C. Lee, Feinbaum, & Ambros, 1993). Another group at the same time found that lin-14 was downregulated at a posttranscriptional level and that the lin-14 3'UTR region was sufficient for the temporal regulation (Wightman, Ha, & Ruvkun, 1993) which finally resulted in the discovery that lin-4 interacts with 3'UTR of lin-14 transcripts. Since the discovery of these small RNAs thousands of them have been identified in different organisms reported in mirbase database (Griffiths-Jones, Grocock, van Dongen, Bateman, & Enright, 2006).

1.7.1. Biogenesis

Mainly, three categories of small RNAs have been previously described microRNAs (miRNAs), siRNAs and PIWI-interacting RNAs (piRNAs) (Aravin et al., 2006; Friedman, Farh, Burge, & Bartel, 2009; Ghildiyal & Zamore, 2009; Huntzinger & Izaurralde, 2011; Ishizu, Siomi, & Siomi, 2012)

miRNA are generated from a short hairpin RNA by the sequential action of two RNase III-type proteins called Drosha and Dicer (Bernstein, Caudy, Hammond, & Hannon, 2001) piRNA production is not RNaseIII-dependent and is shown to be produced from single-stranded precursors by an endonuclease called Zucchini (mitochondrial cardiolipin hydrolase in humans) and as yet uncharacterized trimming enzymes (M. C. Siomi, Sato, Pezic, & Aravin, 2011). siRNA has 21–23 nucleotides with 3′ two nucleotide overhangs.

The siRNA activates the RNA-induced silencing complex (RISC). The AGO2 component of the RISC cleaves the passenger strand while the guide strand remains associated with the RISC. Subsequently, the guide strand guides the active RISC to its target mRNA for cleavage by AGO2. As the guide strand only

binds to mRNA that is fully complementary to it, siRNA causes specific gene silencing (Agrawal et al., 2003; Pecot et al., 2011). The gene silencing effect of siRNAs and miRNAs is distinct. siRNA inhibit the expression of one specific target mRNA but miRNAs regulate the expression of multiple mRNAs (Figure 11) (Lam et al., 2015).

	siRNA	miRNA
Prior to Dicer processing	Double-stranded RNA that contains 30 to over 100 nucleotides	Precursor miRNA (pre-miRNA) that contains 70–100 nucleotides with interspersed mismatches and hairpin structure
Structure	21–23 nucleotide RNA duplex with 2 nucleotides 3'overhang	19–25 nucleotide RNA duplex with 2 nucleotides 3'overhang
Complementary	Fully complementary to mRNA	Partially complementary to mRNA, typically targeting the 3' untranslated region of mRNA
mRNA target	One	Multiple (could be over 100 at the same time)
Mechanism of gene regulation	Endonucleolytic cleavage of mRNA	Translational repression
		Degradation of mRNA
		Endonucleolytic cleavage of mRNA (rare, only when there is a high level of complementary between miRNA and mRNA)
Clinical applications	Therapeutic agent	Drug target
		Therapeutic agent
		Diagnostic and biomarker tool

Figure 11 ***Comparison of general properties between siRNA and miRNA*** (Lam et al., 2015)

Currently, several thousands of miRNA genes have been characterized in different species available from the miRbase database. miRNA sequences are located within various genomic regions (Figure 12), in general, they can be found clustered or not in both coding and non-coding areas of genome; However, the majority of described miRNAs to date are encoded by introns of noncoding or coding transcripts (Y. Lee, Jeon, Lee, Kim, & Kim, 2002; Olena & Patton, 2010). miRNA genes are transcribed by RNA polymerase II (Pol II)(X. Cai, Hagedorn, & Cullen, 2004), and the long primary transcript has a local hairpin structure where miRNA sequences are embedded. Some endogenous miRNA-like small RNAs are derived from tRNAs that are transcribed by RNA Pol III exceptionally (Babiarz, Ruby, Wang, Bartel, & Blelloch, 2008). In a majority of intronic miRNA cases, the promoter of host genes derives the miRNA expression, but it has been demonstrated in some cases they have multiple transcription start sites and that the promoters of intronic miRNAs are sometimes distinct from the promoters of their host genes (Marsico et al., 2013; Monteys et al., 2010).

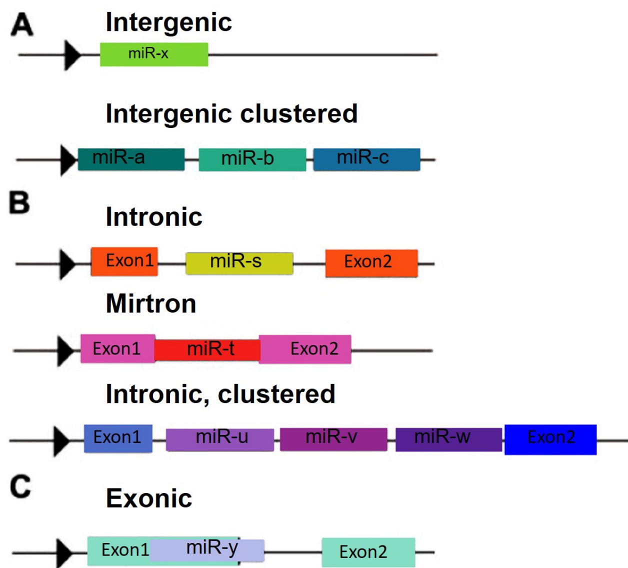


Figure 12 ***Genomic location of miRNAs***

*(**A)** Intergenic miRNA. These miRNAs can be monocistronic (top part) with their own promoters (black arrowhead), or polycistronic, where several miRNAs are transcribed as a cluster of primary transcripts (bottom part) with a shared promoter (black arrowhead). **(B)** Intronic miRNAs are found in the introns of annotated genes, both protein-coding and noncoding. These miRNAs can be present as a single miRNA (top part) or as a cluster of miRNAs (bottom part). Intronic miRNAs are thought to be transcribed from the same promoter as their host genes (black arrowhead, all parts) and processed from the introns of host gene transcripts. In the special case of mirtrons (middle part), the intron is the exact sequence of the pre-miRNA with splicing sites on either side (denoted by white asterisks). **(C)** Exonic miRNAs are far rarer than either of the types above and often overlaps an exon and an intron of a noncoding gene. Copyright © 2009 Wiley-Liss, Inc, with permission from Wiley.(Olena & Patton, 2010).*

Drosha a nuclear protein of ~160kDa, belongs to a family of RNase III-type endonucleases that act specifically on double-stranded RNA (dsRNA) and initiate the first step of miRNA processing (Y. Lee et al., 2003; Provost et al., 2002). Several labs discovered that the double-stranded RNA-binding protein known as Pasha in flies, or its ortholog DGCR8 in *Caenorhabditis elegans* and mammals, acts together with Drosha to convert pri-miRNA to pre-miRNA (Han et al., 2006; Yeom, Lee, Han, Suh, & Kim, 2006) these two proteins form microprocessor complex. miRNA stem–loop is cut by Drosha to release a small hairpin structure of ~65 nucleotides in length (pre-miRNA). Additionally, sequence elements such as motifs of UG motif and the CNNC in pri-miRNA sequence affect the processing of human pri-miRNAs (Auyeung, Ulitsky, McGeary, & Bartel, 2013). CNNC motif is required for DEAD-box RNA helicase p72 (also known as DDX17) binding, which increases processing done by Drosha (Mori et al., 2014). After exportation

to the cytoplasm by protein exportin 5 (EXP5; encoded by XPO5) which is GTP-hydrolysis-dependent, Pre-miRNA is cleaved by Dicer (an RNase IIItype endonuclease of ~200kDa) near the terminal loop, liberating a small RNA duplex (Hutvágner et al., 2001). Although most alternative miRNA pathways depend on Dicer, biogenesis of miR-451 does not require Dicer and instead involves the catalytic activity of AGO2 (Cheloufi, Dos Santos, Chong, & Hannon, 2010).

1.7.2. Gene regulation

A small RNA duplex generated by Dicer is loaded onto an AGO protein to form RISC(Kawamata & Tomari, 2010). RNA duplex subsequently is unwinded with the involvement of AGO proteins then the passenger strand is removed. The less abundant passenger strand (or miRNA*) could be also active in silencing. For instance, miR-142-5p is a dominant isoform in ovaries, testes, and the brain, whereas miR-142-3p was found more frequently in embryonic and newborn tissue samples (Chiang et al., 2010).

miRNAs finely regulate translation by binding not only the 3′UTR but also the 5′UTR or both coding regions of target mRNA. While the binding with 3′UTR has been thoroughly investigated in the last decade, the binding mechanism to 5′UTR and the specificity of such a regulation is still not completely understood (da Sacco & Masotti, 2013). A miRNA can inhibit the initiation of translation by limiting the access of mRNA to the translation machine. At the level of post-initiation, miRNA can intervene by premature termination of translation and degradation of the incomplete forming protein and ribosome drop-off (Pillai, Bhattacharyya, & Filipowicz, 2007). mRNA can be consequent, degraded by deadenylation, 5′-terminal cap removal by decapping enzymes DCP1/2 and hydrolysis of the remaining portion of mRNA by 5′→3′ exonucleases (Parker & Song, 2004).

The binding of miRNAs to the 5′UTR of target genes has been reported to repress or activate translation (I. Lee et al., 2009; Lytle, Yario, & Steitz, 2007). One classical example of this category is miR-10a that can alleviate the translational repression induced upon amino acid starvation (Meyuhas, 2000). Numerous miRNA’s targets also have been identified being regulated by the interaction of miRNA-coding sequence. One example of this is *let-7* miRNA, which directly

targets the miRNA-processing enzyme Dicer within its coding sequence (Forman, Legesse-Miller, & Coller, 2008). The impact of miRNA on coding sequences is not yet fully understood. Multiple pieces of evidence suggest from literature that coding regions of genes can contain additional information besides the amino acid sequence of the encoded protein, including functional microRNA binding sites (Forman & Coller, 2010). miRNAs can target extensively the amino acid coding region of animal mRNAs at locations not necessarily conserved across organisms (Rigoutsos, 2009). A recent study also suggests that mRNA and protein expression of genes containing target sites both in coding regions and 3′UTRs are in general mildly but significantly more regulated than those containing target sites in 3′UTRs only (Fang & Rajewsky, 2011; Schnall-Levin et al., 2011).

1.7.3. miRNA stability and turnover

There are various mechanisms recognized that influence miRNA stability that is reviewed very briefly here but as a general principle, miRNAs is highly stable once they enter RISC because both ends are protected by AGO proteins (Ha & Kim, 2014). For miRNA decay to occur it has been proposed that miRNAs may need to be unloaded first so that exonucleases can access their termini. It remains unclear if and how miRNA unloading takes place or what controls the specificity (Ha & Kim, 2014)

One of the important aspects of miRNA stability resides in the secondary structure of hairpin that may explain variations in the stability of miRNAs (Belter et al., 2014). It has been shown that bases 2-7 of 5′ end of the miRNA are crucial to initiate mRNA binding. For example, 5'-end of miR-296 is knotted in the hairpin stem may explain the high stability of this miRNA (Belter et al., 2014).

Another factor important in miRNA stability is nucleotide polymorphisms that affect miRNA production. Single nucleotide polymorphisms (SNPs) are found in miRNA genes and sometimes affect their biogenesis or change their target specificity (Ryan, Robles, & Harris, 2010). For example, reduced miR-16 expression has been associated with chronic lymphocytic leukemia (CLL due to deletions spanning the intron-containing miR-15a and miR-16-1 (Calin et al.,

2002). Later, a germline C>T single-nucleotide polymorphism (SNP) downstream of the miR-16-1 hairpin was identified that lowered the expression of this miRNA cluster *in vitro*. This SNP lowers miR-16 accumulation by affecting Drosha-mediated processing and SRp20 splicing factor recruitment (Calin et al., 2005a). There are also numerous processing mechanisms recognized that regulate miRNA biogenesis in nuclear and cytoplasmic level by influencing the miRNA production. The binding of RNA-binding proteins for instance to miRNA precursors, particularly to the stem-loop structures, blocks or enhances further processing by competing with or recruiting miRNA processing complexes (H. Siomi & Siomi, 2010). In Table 2 some of the known protein factors that affect miRNA biogenesis have been summarized. The protein factors affect the miRNA at different levels of transcription, processing, maturation and as well direct modulation of Dicer-DGCR8.

Adenylation is another type of RNA tailing that affects the stability of miRNAs (e.g miR-122 in the liver) and occurs mainly after Dicer processing (Katoh et al., 2009). For example, the let-7 family members are post-transcriptionally suppressed in embryonic stages by Uridylation that recruits terminal uridylyl transferases (Heo et al., 2008, 2009). Multiple nucleases have been proposed to cleave and degrade miRNAs. Active degradation of miRNA was initially reported in Arabidopsis thaliana by exoribonucleases that are known as small-RNA-degrading nucleases removes miRNAs (Ramachandran & Chen, 2008). In C. elegans XRN-1 and XRN-2 were shown to degrade mature miRNAs with their 5' to 3' RNA degradation (Chatterjee & Grosshans, 2009). In mammals, rapid turnover of several neural miRNAs (miR-183, miR-96, miR-192, miR-204 and miR-211) in the retina has been reported but the mechanism remains unknown (Krol et al., 2010). Recent studies also suggest that highly complementary targets induce miRNA degradation accompanied by tailing and trimming (Ameres et al., 2010).

Table 2 ***Highlighted protein factors affecting miRNA biogenesis***

p53, MYC and myoblast determination protein 1 (MYOD1)	Regulation of the miR-34, miR-17 and miR-1 clusters, respectively. miR-15a cluster. Suppression by myc.	(Yamakuchi & Lowenstein, 2009)(X. Zhang et al., 2012)
ZEB1-2	miR-200 cluster suppression	(Brabletz & Brabletz, 2010)
p68, p72, KH-type splicing regulatory protein (KSRP), heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) and LIN28	Regulation of pri-miRNA processing	(Fukuda et al., 2007)
TAR DNA-binding protein 43 (TDP43)	Promote Drosha and Dicer processing	(Di Carlo et al., 2013)(Kawahara & Mieda-Sato, 2012)
ADAR1-2	RNA editing, which interferes with Drosha processing	(Kawahara, Zinshteyn, Chendrimada, Shiekhattar, & Nishikura, 2007)
GSK3β	Phosphorylation of Drosha and nuclear localization of Drosha	(X. Tang, Zhang, Tucker, & Ramratnam, 2010)
ERK	Phosphorylation of DGCR8 and increase in its stability	(Herbert, Pimienta, DeGregorio, Alexandrov, & Steitz, 2013)
MECP2	Dephosphorylation of MECP2 releases DGCR8, which in turn leads to miRNA production and dendritic growth	(Cheng et al., 2014)

SMADs and p53	Promote Microprocessor activity through their interaction with p68.	(Davis, Hilyard, Nguyen, Lagna, & Hata, 2010)(Suzuki et al., 2009)
Heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) and KSRP	Bind to the terminal loop of pri-mir-18a and pri-let-7, respectively, and facilitate Drosha-mediated processing	(Guil & Cáceres, 2007)(Michlewski et al 2008)(Gherzi et al., 2007)

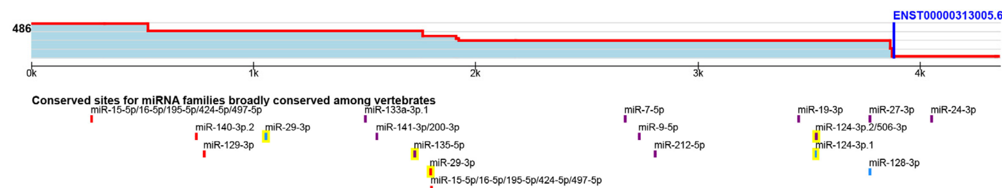
1.7.4. miRNA deregulation in AD

miRNA profiling of human cortical tissue identified thirteen miRNAs that are down-regulated in human AD brain (Cogswell et al., 2008; Hébert et al., 2008). The subsequent generation of mice with a neuronal knockout of Dicer is a proof-of-principle that miRNAs could play a role in neurodegeneration (Hébert et al., 2010). This mouse model expresses Cre recombinase mainly in excitatory neurons of the forebrain and demonstrates a neurodegeneration and inflammation with highlighted Tau hyperphosphorylation. Gene expression study in this experiment pinpointed elevated levels of one of the kinases acting on Tau- mitogen-activated protein kinase 3 (MAPK3/ERK1)- which further could be regulated by miR15/16. Importantly, the expression levels of these miRNAs were significantly reduced in 9-week-old Dicer ko (Hébert et al., 2010).

The miRNA network surrounding APP has both upstream and downstream components which directly or indirectly affect APP (Schonrock et al., 2012). A summary of some examples of miRNAs implication in AD has been summarized in Table 3. Several miRNAs have been identified *in vitro* to directly regulate APP. They include miR-106a, -520c (Patel et al., 2008) as well as members of the miR-20a family (Hebert et al., 2009), miR-16 and -101 (Long and Lahiri, 2011; Vilardo et al., 2010) and miR-147, -655, -323-3p and -153 (Delay et al., 2011). While miR-106b and miR-101 have been shown to be down-regulated in AD brain, therefore potentially contributing to increased APP expression and Aβ generation (Hebert

et al., 2008; Nunez- Iglesias et al., 2010), it remains to be determined which of these miRNAs actually regulate APP *in vivo.* It is important to note that the APP 5′UTR plays fundamental roles in APP regulation and biogenesis as well (Lahiri et al., 2005; Maloney et al., 2004; Rogers et al., 2002); however, the regulation of 5'UTR by miRNA is still poorly understood.

Changes in neuronal APP isoform expression are also associated with an increase in Aβ production and increases of exon 7 and/or 8 containing APP isoforms have been reported in various regions of AD brain (Rockenstein et al., 1995). Another study reveals a contribution of miRNAs to yet another level of APP regulation, alternative splicing (P. Smith, Al Hashimi, Girard, Delay, & Hébert, 2011). The neuron-enriched APP695 form lacking both domains encoded by exon 7 and 8. Lack of miRNAs in post-mitotic neurons *in vivo* in Dicer knockout model was associated with exon 7 and 8 inclusion (P. Smith et al., 2011). Ectopic expression of miR-124 reversed the above effects on APP splicing in cultured neurons. Interestingly, miR-124 levels are reduced in AD (Lukiw, 2007; Smith et al., 2011). BACE1 may also be regulated at the posttranscriptional level. Current research is mostly focused on the regulation of the 3’UTR of BACE1 by miRNAs.

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          .....230........240.......250.......260.........270.......280.....1790......1800.......1810....
   Human  CGGGAAUACUCUUGG-UCACCUCAAAUUUAAGUCGGGAAA--UUCUGCUGCUUGAAACUU----CACUUGGUGCUGCUUUGG-CUGACUGGG--
   Chimp  CGGGAAUACUCUUGG-UCACCUCAAAUUUAAGUCGGGAAA--UUCUGCUGCUUGAAACUU----CACUUGGUGCUGCUUUGG-CUGACUGGG--
  Rhesus  CGGGAAUACUCUUGG-UCACCUCAAAUUUAAGUUGGGAAA--UUCUGCUGCUUGAAACUU----CACUUGGUGCUGCUUUGG-CUGACUGGG--
Squirrel  CAGGAAUACCCUUGG-UCACCUAAAACUCGAGAUGGGAAA--UUCUGCUGCUUGAAACUU----CACUUGGUGCUGCUUUGG-CUGACUGGG--
   Mouse  CAGGAAUAUCCUUAG-ACACCACAAACUUGAG-UUGGAAA--UUUUGCUGCUUGAAGCUU----CACU-GGUGCUGCUCUGG-CUGAGUAGG--
     Rat  CAGGA-UACCCUUAG-GCACCACAAACUUGAG-UUGGAAA--UGUUGCUGCUU-AAGCUU----CACU-GGUGCUGCUCUGG-CUGACAAGG--
  Rabbit  CGGGAAUACUCUUGG-UCACCCCACAGUUGAGUUGGAAAA--UUCUGCUGCUUGAAACUU----CACGUGGUGCUGCCU-GA-CCGACUGGA--
     Pig  CGGGAAUACUCUUGG-UCACCUCCAACUUGAGUUGGGAAA--UUCUGCUGCUUGAAACUU----CACUUGGUGCUGCUUUGG-CUGACUGGG--
     Cow  UGGGAAUACUCUUGG-UCACCUCAAACUUGAGUUGGGAAA--UCCUGCUGCUUGAAACUU----CACUUGAUGCUGCUCUGG-CUGGCUGGG--
     Cat  CGGGAUUAUUCUCGG-UCACCUCGAGCCCGAGUUGGGAAA--UUCUGCUGCUUGACACUU----CACUUGGUGCUGCUUUGG-CUGACUGGG--
     Dog  CAGGAAUAUUCUUGG-UCACCUCAAGCCUGAGUUGGGAAA--UUCUGCUGCUUGACACUU----CACUUGGUGCUGCUCUGG-CUGACUGGG--
Brown bat CAGGAGUAC-----G-UCACCUCAAACUUGAGUUGGGAAA--UUCUGCUGCUUGAAACGU----CACCUGGUGCUGCUU-GG-CUGAC-GGG--
Elephant  UAGGAAUACUCUUGA-UCACCAUAAACUUGAGUUGGGAAA--UUCUGCUGCUUAAAAUUU----CACUUGGUGCUGCUUUGG-UUGACUGGG--
```

Figure 13 ***TargetScan analysis of human BACE1 UTR*** *In upper panel the TargetScan analysis of hBACE1 3'UTR is shown. In lower panel the double target highly conserved site of miR-15/107 is detailed.*

Loss of the miRNA cluster containing miR-29a, -29b1 and -9 in sporadic AD has been found to correlate with an increased BACE1 expression and increased Aβ levels (Hébert et al., 2008). More recently, miR-29c was shown to lower BACE1 protein levels *in vitro*, and miR-29c-overexpressing mice down-regulated BACE1 levels suggesting that miR-29c might be an endogenous BACE1 regulator (Zong et al., 2011).

Another study in an APP/PS1 mutant mouse model of AD revealed an inverse correlation between BACE1 protein levels and two miRNAs (miR-298 and -328) and showed these two miRNAs directly interacted with the BACE1 3′UTR in mouse cell lines (Boissonneault et al., 2009). Bioinformatics approaches predict different sites in the 3′UTR of BACE1 may be targeted by miR-15/ 107 as well (Figure 13) (W.-X. Wang et al., 2008).The overexpression of miR-485-5p caused a reduction in BACE1 protein expression. These results indicate that miR-485-5p binding sites are in the coding region and that miR-485- 5p can regulate BACE1 expression (Faghihi et al., 2010). Furthermore, these results suggest the possibility of *in vivo* interactions between miR-485-5p and BACE1 mRNA (Yanyao Deng, Ding, & Hou, 2013). Very recently, miR-339-5p also has been confirmed to regulate BACE1 in cells and its levels were found to be significantly reduced in brain specimens isolated from AD patients (Long, Ray, & Lahiri, 2014).

Table 3 ***Main miRNAs identified to be associated with AD***

miR-9	The addition of Aβ peptides to primary neuronal cell cultures has been shown to downregulate this small RNA. Targets include FGFR1, NFkB, and SIRT1	(Saunders et al., 2010)
miR-107	Negative correlation with BACE1 and neuritic plaque density; targets BACE1, CDK5, and the metalloproteinase ADAM10	(Finnerty et al., 2010);(Goodall, Heath, Bandmann, Kirby, & Shaw, 2013)
miR-29	Inversely correlated with BACE1	(Hébert et al., 2008)(Zong et al., 2011)(Lei, Lei, Zhang, Zhang, & Cheng, 2015)
miR-106	Directly bind to APP mRNA; can also regulate the expression of the transporter ABCA1, which is involved in ApoE production. miR-106b dramatically increased levels of secreted Aβ by increasing Aβ production and preventing Aβ clearance	(Kim et al., 2012)(H. Wang et al., 2010)
miR-132/212	Inhibition of miR-132 function attenuates neuronal outgrowth, while its dysregulation is associated with several neurological disorders, such as AD and related tauopathies Learning deficits in knockout mice for this miRNA. Tau expression phosphorylation and aggregation	(Wanet, Tacheny, Arnould, & Renard, 2012)(Wong et al., 2013)(Hernandez-Rapp et al., 2015)(P. Y. Smith, Hernandez-rapp, Jolivette, & Lecours, 2015)(Hansen et al., 2016)

miR-15a/16	Potential Regulation of ERK, BACE1 with implication in Tau hyperphosphorylation *in vitro*	(Hébert et al., 2010)(W.-X. Wang, Huang, Hu, Stromberg, & Nelson, 2011)

With modifications (Femminella, Ferrara, & Rengo, 2015)

It has been revealed that numerous miRNA work together or multiple miRNAs could target the same mRNA (Peter, 2010) and miRNA potential as a therapeutic target is an emerging subject. In next section, the effort has been solely focused on the introduction and reviewing the function of miR-15/107 in detail that is also the subject of this thesis. This introduction will discuss the potential of this family on the regulation of APP, and BACE1 mainly and other pathological pathways implicated in AD. This family has been exhaustively studied in the context of cancer and some concepts explained here rely on already published works on neurodegeneration and to some extent cancer.

1.7.5. miR-15/107 family members

Members of the miR-15/107 miRNA gene group[2] expressed in humans are shown in Figure 14A . Most of the miRNAs highly expressed in humans incorporate a 7-nt common sequence AGCAGCA. The seed sequence start in miR103/107 is different with other members; however, they show different expression levels but with common targets with other members of the family (Figure 14B, C)

[2] miR-15a, miR-15b, miR-16, miR-103,miR-107, miR-195, miR-424, miR-497, miR-503 and miR-646

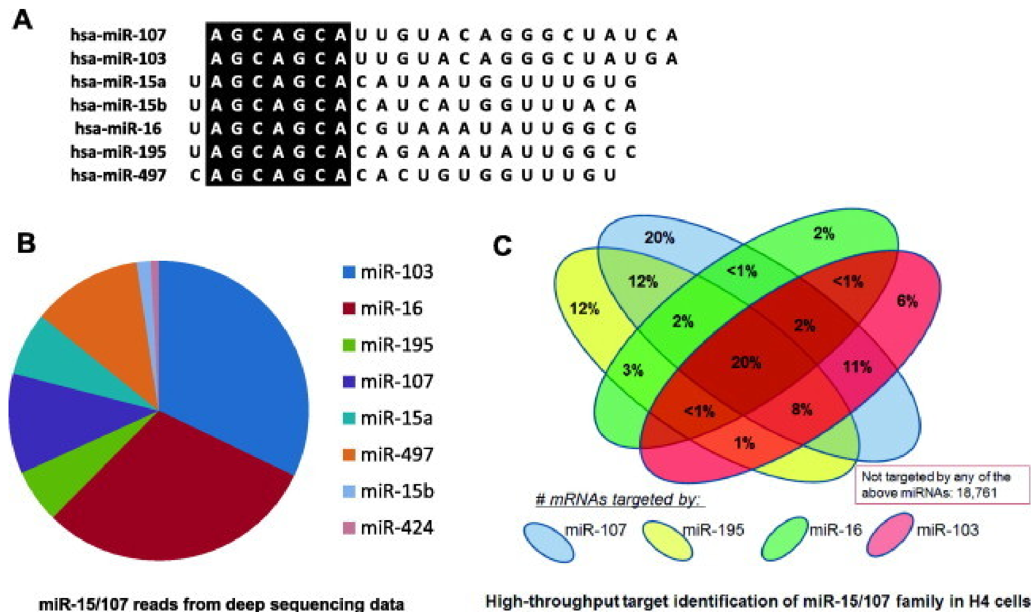

Figure 14 ***Highly expressed miR-15/107 miRNAs with overlapping targets***

(A) *Members of miR-15/107 share a homologous 5' sequence. Shown here are the miR-15/107 miRNAs that are highly expressed in human tissues.* ***(B)*** *Normalized miRNA reads for the miR-15/107 family from deep sequencing RNA-seq data.* ***(C)*** *Biochemical identification of miR-16, miR-103, miR-107 and miR-497 targets in H4 cells[3] (adapted from original ref)* (W.-X. Wang et al., 2014).

There is not a strict procedure for defining miRNA gene family whereas Nelson group describes the above-mentioned members in a gene group others consider each miRNA as distinct miR-15ab, miR-16, miR-103, and miR-107 families (Finnerty et al., 2010; Linsley et al., 2007).

A miR-15 superfamily, comprising miR-15a, miR-15b, miR-16, miR-195, miR-322, miR-424, miR-457, and miR-497 are hypothesized to have evolved in the common ancestor of vertebrates (Finnerty et al., 2010). There are two miR-15/16 clusters in mammals. Many vertebrate species have three or more paralogs of miR-15 and miR-16 (Heimberg, Sempere, Moy, Donoghue, & Peterson, 2008; Peterson, Dietrich, & McPeek, 2009). While all vertebrates examined to date express miR-15a, miR-15b, miR-16, miR-103 and miR-107, only mammals are known to express miR-195, miR-424, miR-497, miR-503, with the exception of miR-646 that appears to be human-specific (Finnerty et al., 2010). The expression of members of this family in non-brain organs has indicated a higher

[3] Homo sapiens brain neuroglioma

expression of miR-15a, miR-15b and miR-16 in the spleen, miR-424 in kidney liver and skeletal muscle (W.-X. Wang et al., 2014).

Altered expressions of miR-15/107 group members in certain stages of mammalian development are also documented. For example, miR-15 and miR-16 expression correlates to particular stages in erythropoiesis (Choong et al., 2007). Multiple miR-15/107 group members have increased expression levels during early mouse brain development (Miska et al., 2004). It has been also demonstrated that miR-16 expression is highly expressed in early human brain development (Nelson et al., 2006).

1.7.6. Expression and regulation of miR-15a/16-1: a cluster in a loop with cell cycle proteins

The miR-15a/16-1cluster is located in an intron of the DLEU2 gene and miR-15b-16-2 genes located within an intron of structural maintenance of chromosomes 4 or (smc4) (Figure 15), DLEU2 is frequently deleted or downregulated in human tumors (Aqeilan, Calin, & Croce, 2010). A knock-out of DLEU2 or the miR-15a/16-bearing intron in mice revealed that loss of miR-15a/16-1 expression is sufficient to cause chronic lymphatic leukemia (CLL) highlighting the role of this miRNA family in cell cycle regulation as potential tumor suppressor miRNAs by discovering BCL2 as one of its targets (Klein et al., 2010; Liu et al., 2008). Chronic lymphocytic leukemia (CLL) is the most common human leukemia that accounts for one-third of all leukemia cases (Mano, 2009). It has been shown that expression of both miR-15 and miR-16 is downregulated in two-thirds of CLL cases (Calin et al., 2002)

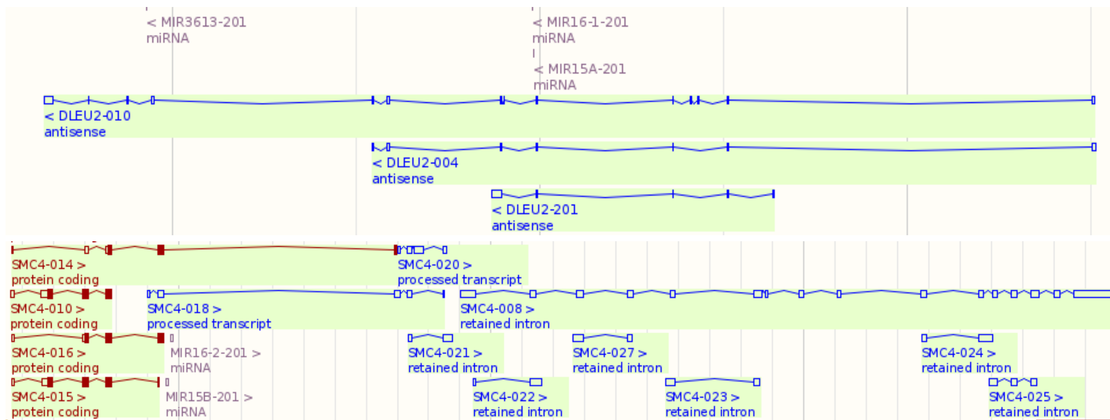

Figure 15 ***Location of dleu2- miR-15a/16 and smc4-miR-15b/16 genes***

Not all isoforms of host gene are shown (From Ensemble databank)

How the expression of the miR-15a/16 cluster is dampened or increased is still not yet fully understood but recent studies have proposed a mechanism that DLEU2 host gene of miR-15a/16-1 can be directly repressed by MYC and Histone deacetylase (HDAC) (Hermeking, 2012; Sampath et al., 2012). HDACs silence miR-15a, miR-16, and miR-29b in 30%-35% of CLL samples accounting for the disproportionately low levels of these miRs. Oncogene c-MYC is a transcription factor that regulates a broad range of cellular processes, which may contribute to the initiation and progression of tumors. Briefly, Myc induces a transcription factor called AP4 that represses DLEU2 expression. As AP4 can also a target for miR-15a/16 cluster it promotes the idea that an important regulatory loop at least in cancer context do exists (Shi et al., 2014). In the same context, p53 was shown to enhance the post-transcriptional maturation of miR-15a/16-1 by directly interacting with DROSHA via the DEAD-box RNA helicase DDX5 and thereby influencing the processing from pri-miRNAs to pre-miRNAs (Suzuki et al., 2009). Later, the DLEU2 gene was shown to be a transcriptional target of p53 in B-cells (Fabbri et al., 2011).

It is interesting to mention that this wildly recognized transcription factor is also studied in Alzheimer disease context (Ferrer, Blanco, Carmona, & Puig, 2001; H. P. Lee, Kudo, Zhu, Smith, & Lee, 2011). It has been demonstrated that activation of MYC in forebrain neurons in a CaMKII-MYC mouse model leads to cell cycle re-entry, neurodegeneration, gliosis, and cognitive deficits (Hyoung-gon Lee et al., 2009). Activation of the cell cycle machinery in neurons has emerged as a

potential pathogenic mechanism of neuronal dysfunction and death in many neurodegenerative diseases, including AD as well (Arendt, Holzer, Grossmann, Zedlick, & Brückner, 1995; Nagy, Esiri, & Smith, 1997; Vincent, Jicha, Rosado, & Dickson, 1997).

This failure of completing cell cycle has been described first back in 2001 (Raina et al., 2001). Differentiated neurons once fully differentiated enter G0 phase so cell cycle re-entry is blocked whereas, in AD, reports indicate a cell cycle-related protein expression in differentiated neurons of AD (van Leeuwen & Hoozemans, 2015). The cell division cycle of eukaryotic cells can be divided into four phases. During S phase, DNA synthesis takes place and during M phase, mitosis and cytokinesis occur. G 1 and G 2 are gap phases, which separate S phase and M phase. Cells like Neurons can enter a permanent resting state, referred to as G 0 phase. From post-mortem studies, it seems that post-mitotic neurons can progress as far as G2/M phase (van Leeuwen & Hoozemans, 2015).

To date, there is no study available about the direct impact of decreased miR-15a/16 on phenomena of cell cycle re-entry in neurodegenerative diseases. The study published in molecular cancer in 2011 discusses how this miRNAs could contribute to regulation of E2Fs transcription factors best known for their involvement in the timely regulation of gene expression required for cell-cycle progression (Ofir, Hacohen, & Ginsberg, 2011; Polager & Ginsberg, 2008). Sequential phosphorylation of product of the retinoblastoma tumor suppressor (RB) by the CDK (cyclin-dependent kinase) complexes cyclin D–CDK4/6 and cyclin E–CDK2 lead to release of E2F from RB and activation of E2F-responsive genes that promote cell-cycle progression (Ohtani, DeGregori, & Nevins, 1995); Interestingly, cyclin E itself one of the pivotal E2F targets, can be inhibited by miR15ab. Inhibition of both miR-- and miR-16 enhances E2F1-induced G1/S transition (Ofir et al., 2011).

It has been shown that other members of this family like miR-103 and 107 also regulate the important cyclin-dependent kinases such as Cyclin-dependent kinase 5 (CDK5) by targeting 3′UTR of CDK5R1 (Cyclin-dependent kinase 5, regulatory subunit 1or P35 (Moncini et al., 2007, 2011). It is also important to

mention that cell cycle deregulation ranks top among networks predicted to be affected in Alzheimer's disease (Satoh, 2012). It is important to note that expression of this family of miRNA is also regulated by β-catenin (cadherin-associated protein) through an unknown protein complex which implicates strongly the role of at least miR-15a/16 in early phases of development (Martello et al., 2007; Schier, 2003).

1.7.7. miR-15/107 family potential regulator of APP and BACE1 in AD

miR-15a is shown to be down-regulated in sporadic AD brain (Nunez-Iglesias et al., 2010) (Hébert et al., 2008 b). miR-16 expression decreases with the age in rats in both hippocampus and cortex and specifically targets APP *in vivo* in the senescence-accelerated prone mouse SAMP8 (Figure 16) (Che et al., 2014; W. Liu et al., 2012). Recently, researchers have also implicated downregulation of miR-16 in the hippocampus in later stages of AD (Müller, Kuiperij, Claassen, Küsters, & Verbeek, 2014). The senescence accelerated mouse (SAMP8) is a spontaneous animal model of overproduction of amyloid precursor protein (APP) and oxidative damage (Takeda, Hosokawa, & Higuchi, 1997). It develops early memory disturbances and changes in the blood-brain barrier resulting in decreased efflux of amyloid-β protein from the brain. It also has a marked increase in oxidative stress in the brain (Morley, Armbrecht, Farr, & Kumar, 2012).

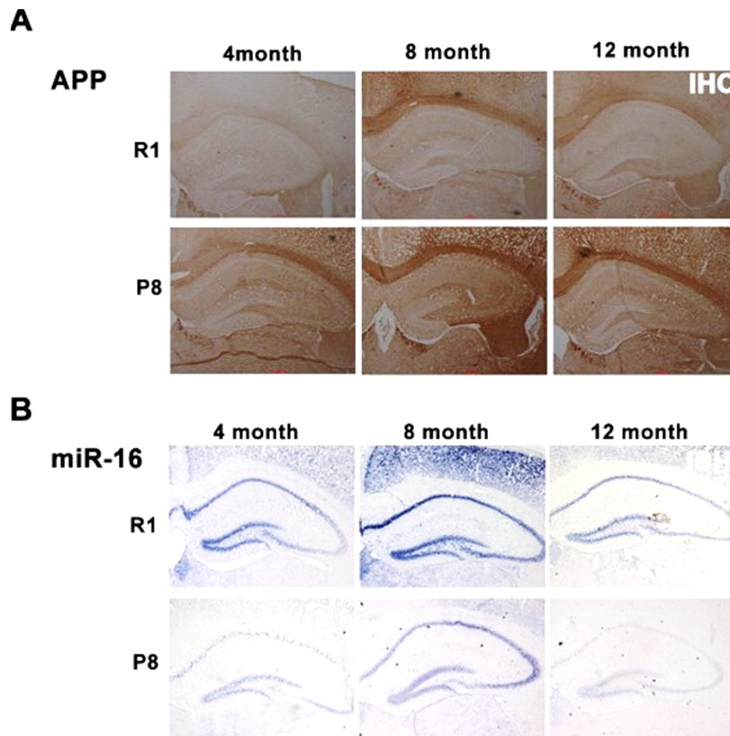

Figure 16 ***miR-16 inhibits amyloid protein precursor (APP) in SAM mice in vivo***

(A) *Immunohistochemistry (IHC) of APP protein in 4-, 8-, and 12-month-old SAMP8 and SAMR1 hippocampus.* ***(B)*** *In situ hybridization (ISH) of miR-16 in 4-, 8-, and 12-month-old SAMP8 and SAMR1 hippocampus by locked nucleic acid-based (LNA)-modified DNA probe of miR-16 adapted from*("MicroRNA-16 targets amyloid precursor protein to potentially modulate Alzheimer's-associated pathogenesis in SAMP8 mice.," 2012) *Copyright © 2012 Elsevier Inc. with permission from Elsevier*

Another member of this family, miR-195, downregulates amyloid-β production by targeting APP and BACE1 (Ai et al., 2013). It has been studied that in Neuro2a cells, overexpression of miR-195 reduces BACE1 protein levels. Conversely, inhibition of miR-195 increases BACE1 levels (Zhu et al., 2012). Recent studies highlight the miR-195 in chronic brain hypoperfusion (CBH) which is accompanied by cognitive impairment (Ai et al., 2013). Authors of this report suggesting an exogenous complement of miR-195 may be a potentially valuable anti-dementia approach. Compelling evidence of this family involvement in Tau phosphorylation comes from studies in Dicer knockout mice in which endogenous Tau is hyperphosphorylated (Hébert et al., 2010). In the same study, ERK1 has been identified as one of the responsible kinase acting on Tau in Dicer KO model. Interestingly, miR-15/107 members (miR- 15, -16, -195, -497) are shown to regulate ERK1 by binding directly to 3′UTR of ERK *in vitro* and decreased miR-

15/16 levels could participate in neuronal Tau hyperphosphorylation in vivo (Hébert et al., 2010)

1.8. Multitarget therapy in AD

Current medications do not delay AD progression from mild cognitive impairment and do not significantly hinder with the functional capability to dementia (Farlow, Miller, & Pejovic, 2008; Pasic et al., 2011; Raschetti, Albanese, Vanacore, & Maggini, 2007). Very first approved treatments by US Food and Drug Administration (FDA), mainly include five drugs: AChEIs–rivastigmine (Exelon), galantamine (Razadyne, Reminyl), tacrine (Cognex), and more recently donepezil (Aricept) and NMDA receptor antagonist–memantine (Namenda) to alleviate symptoms of AD (Auld, Kornecook, Bastianetto, & Quirion, 2002)(Howard et al., 2012).

Over 20 years ago, Tacrine (Cognex®) was approved by the U.S. FDA for the treatment of AD As an acetylcholinesterase (AChE) inhibitor but Due to its hepatotoxicity, Tacrine was soon withdrawn from the pharmaceutical market (Guzior, Wi, Panek, & Malawska, 2015). This symptomatic treatment inhibited AChEs enzyme by decreasing the breakdown of Acetylcholine (Ach) (Kumar et al., 2015). Many different neurotransmitters are deregulated in AD but mainly glutamatergic and cholinergic systems are implicated in the progression of AD (Sultana & Butterfield, 2008) . Memantine as an example is a NMDA antagonist blocking its receptor with following improved spatial learning in animal models of AD with apoptosis decrease (Miguel-Hidalgo, Paul, Wanzo, & Banerjee, 2012).

Most efforts at developing effective strategies aimed at preventing abnormal protein aggregation, reducing Aβ/tau levels, or removing the protein accumulations have been not promising (Bulic, Pickhardt, Mandelkow, & Mandelkow, 2010; Iqbal & Grundke-Iqbal, 2011). It has been suggested that the ideal therapy could approach to reduce pathological conformers of tau and Aβ oligomers together (Boutajangout & Wisniewski, 2014).

Considering the multifactorial etiology of AD, and the numerous and complex pathological mechanisms involved in the progression of the disease, it is quite reasonable that treatments targeting a single causal or modifying factor have had

limited benefits (Aso & Ferrer, 2013). Therefore, growing interest is focused on therapeutic agents with pleiotropic activity to target, in parallel, several processes affected in AD (Cavalli et al., 2008; Frautschy & Cole, 2010)

One-molecule, one-target paradigm has led to the discovery of many successful drugs but that may not be a case specifically in neurodegenerative diseases with difficult challenges ahead and multiple pathogenic factors involved (Cavalli et al., 2008). The ideal strategy may impact multiple impairments (Figure 17) including protein misfolding and aggregation (APP, Tau), oxidative stress and free radical formation, metal dyshomeostasis, mitochondrial dysfunction, and phosphorylation impairment, all occurring concurrently (Jellinger, 2003). Towards this goal identification of novel multi-target-directed drug candidates for the treatment of AD is gaining more attention (Mao et al., 2014)(Agis-Torres, Sölhuber, Fernandez, & Sanchez-Montero, 2014).

With this concept in mind, it would be intriguing to assume that endogenous regulators of these commonly targeted proteins in pharmacology may also be applied as therapeutics. One widely studied category is small non-coding RNAs that have the ability to modulate the expression of a multitude of targets in the physiological context of living organisms. The nature of these molecules and the ability to modify a pathway is emerging in recent research as another potential strategy for neurodegenerative diseases with multifactorial nature such as Alzheimer. In the next section, a specific focus has been made on how pharmaceutical companies have approached miRNA-based strategies.

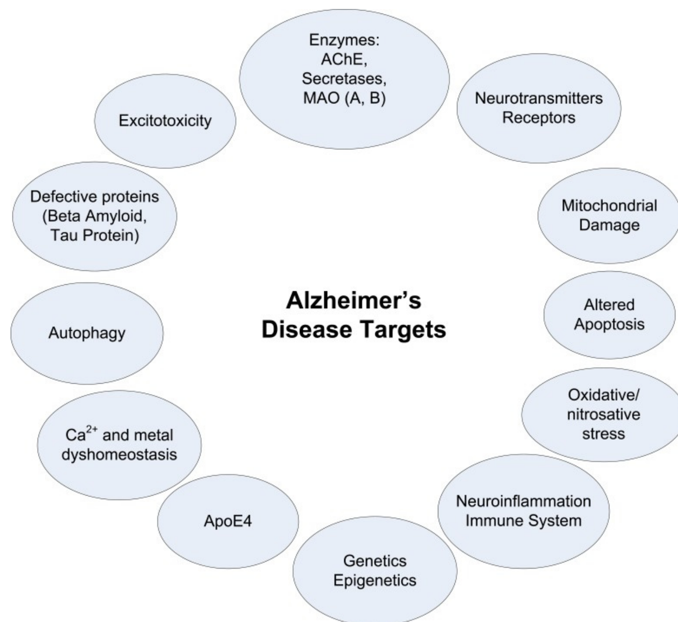

Figure 17 ***The main current targets in AD research***

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1.9. Non-coding RNA-based Therapeutics

The discovery of siRNA and miRNA opened up a whole new therapeutic approach for the treatment of diseases by targeting genes that are involved in the pathological process (Lam, Chow, Zhang, & Leung, 2015). RNAi is a natural cellular process that silences gene expression by driving the degradation of mRNA. It plays an important role in gene regulation and innate defense against invading viruses. RNAi was first described by Fire and Mello winning Nobel Prize for investigating the mechanisms of gene inhibition by exogenous RNA (Elbashir et al., 2001; Fire et al., 1998). Generally, RNAi-based gene silencing is proposed more beneficial than other therapeutic approaches in the treatment of neurological disorders. Diseases such as Huntington's disease, frontotemporal dementia with Parkinsonism; central nervous system (CNS) tumors; chronic pain; prion diseases etc will benefit from non-coding RNA-based therapeutics (Jana, Chakraborty, Nandi, & Deb, 2004; Pecot, Calin, Coleman, Lopez-Berestein, & Sood, 2011). Recent studies have shown that specific manipulation of a miRNA in cell or animal models can significantly alter phenotypes linked with neurological disease (Chan & Kocerha, 2012). Contrary to siRNA for which numerous clinical

trials have started in various neurodegenerative diseases (Table 4) miRNA-based therapeutics is still an unexplored domain and most of the clinical trials focused on cancer (Table 5).

Potentially miRNAs have advantages over siRNAs in the context of pathologies that multitude of pathway genes and their protein products are deregulated. Therefore, having many targets within the cell, a miRNA can modulate a disease pathway including several disease candidates. It has been proposed that the issue of siRNA off-targeting, where a siRNA non-specifically downregulates a non-target gene, is not as much of a concern with miRNA mimics because evolutionary speaking, the miRNA has been evolved for optimal targeting. It remains to be confirmed experimentally whether higher levels of a miRNA mimic has unexpected effects because of its ability to target multiple genes at once (Seto, 2010). It has been hypothesized by others that since miRNAs appear to be programmed to exert global modulation; therefore, miRNAs based therapeutics may be less prone to true off-target effects (Ishida & Selaru, 2013). The mature miRNA sequences are short and often well conserved across multiple vertebrate species. These characteristics make miRNAs relatively easy to target therapeutically and allows for using the same miRNA-modulating compound in preclinical efficacy and safety studies as well as in clinical trials (van Rooij & Kauppinen, 2014).

Basically, there are two main approaches toward this category of therapeutics: Restoring function of a lost miRNA by synthetic double-stranded miRNAs (miRNA mimics) or inhibition of an aberrantly expressed miRNA by antimiRs (van Rooij & Kauppinen, 2014)

miRNA replacement is based on either vector for the expression of a miRNA gene or on synthetic mimics, which are double-stranded RNAs (Iddo Magen & Hornstein, 2014). Systemic delivery of such miRNA mimics could result in uptake by non-target tissues that normally do not express the miRNA of interest, resulting in potential off-target effects. Targeted delivery of miRNA mimics to the appropriate cell or tissue type is considered an important step (van Rooij & Kauppinen, 2014; van Rooij, Purcell, & Levin, 2012). One classic example is

inhibition of miR-122, has reached clinical trials (Phase II) and is being evaluated for its long-term safety and efficacy in patients with chronic hepatitis C virus (HCV) infection (Hildebrandt-Eriksen et al., 2012; Vidigal & Ventura, 2014). Miravirsen is the first miRNA-targeted drug to receive acceptance as an investigational new drug by the US Food and Drug Administration, paving the way for phase II trials of the treatment of hepatitis C (http:// www.santaris.com).

Activation of the innate immune system via TLR might be an issue after *in vivo* administration of miRNAs leading to undesirable effects (Fabbri, Paone, Calore, Galli, & Croce, 2013). Chemical modification is the major approach to tackle this problem. Furthermore, therapeutic miRNAs also face the barriers of poor stability and inefficient delivery. The chemistry of synthetic miRNA mimics has been less studied than inhibitors. Modifications such as 2 -O-methyl (2 -OMe), 2 -O-methyoxyethyl (2 -MOE), and locked nucleic acid (LNA), improve the oligonucleotide’s resistance to exonucleases. For miRNA-targeting strategies, LNA modifications have the additional advantage of increasing the affinity of the oligo to its complementary miRNA, leading to more efficient inhibition (Figure 18) (Vidigal & Ventura, 2015).

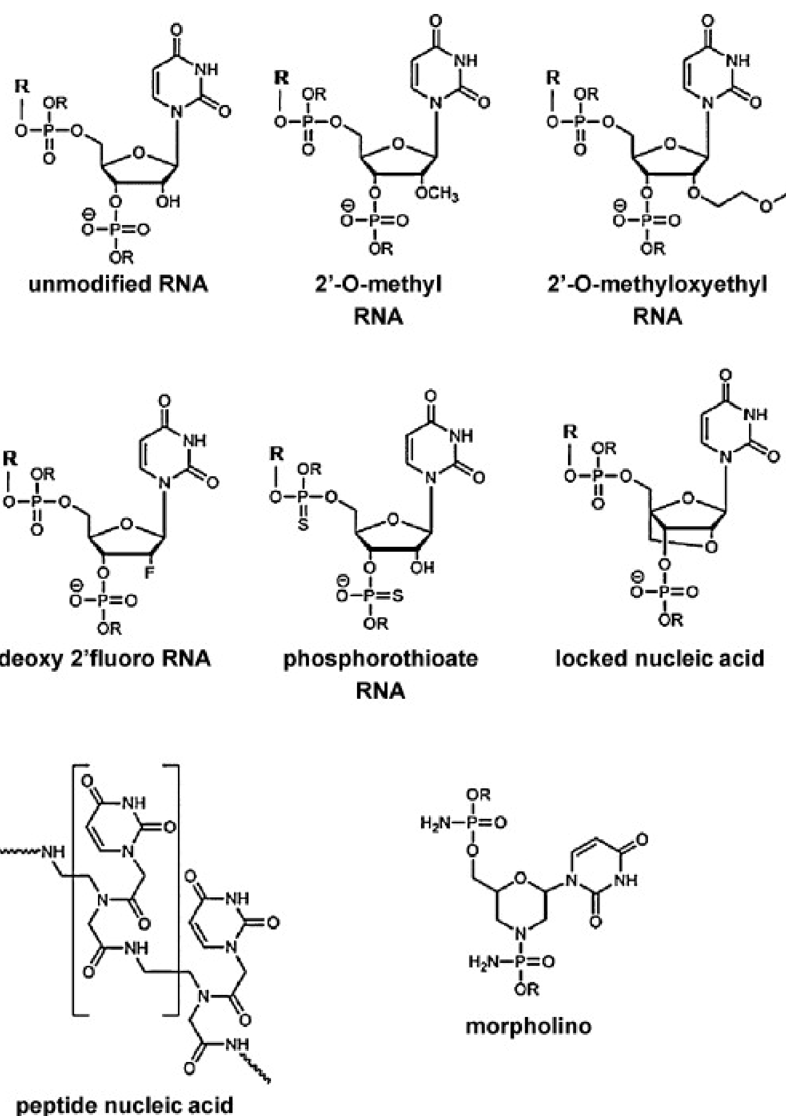

Figure 18 ***Structures of nucleic acid analogs and modifications***

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Table 4 ***Selected clinical trials with RNA-based therapy in neurodegenerative diseases***

Disease	Target	Delivery system	Company	ClinicalTrials.gov identifier (s)
Age-related macular degeneration (AMD)	DNA damage-inducible transcript 4	Naked siRNA	Quark Pharmaceuticals (PF-04523655)	NCT00725686 NCT01445899 NCT00713518 NCT00701181
Amyotrophic lateral sclerosis (ALS)	SOD1	Naked ASO[4]	Isis Pharmaceuticals (SOD1Rx)	NCT01041222
Duchenne muscular dystrophy (DMD)	Dystrophin, exon 51	Naked ASO (morpholino)	Sarepta therapeutics (AVI-4658/PMO)	NCT00159250, NCT00844597,NCT01396239mNCT01540409
	Dystrophin, exon 51	Naked ASO	GlaxoSmithKline (PRO051/GSK2402968)	NCT01128855NCT01910649,NCT01254019,NCT01153932,NCT01480245,NCT01462292
	Dystrophin, exons 44, 45, 53	Naked ASO	Prosensa therapeutics (PRO044, PRO045, PRO053)	NCT01037309,NCT01826474,NCT01957059
Familial amyloid polyneuropathy	TTR	Naked ASO	Isis Pharmaceuticals (ISIS-TTRRx)	NCT01737398
	TTR	LNP-formulated GalNac-conjugated siRNA	Alnylam Pharmaceuticals (Patisaran ALN-TTR02)	NCT01960348
Multiple sclerosis	Integrin alpha (4) beta1	Naked ASO	Isis Pharmaceuticals (ATL/TV-1102)	–
Optic atrophy, non-arteritic anterior ischaemic optic neuropathy	CASP2	Naked siRNA	Quark Pharmaceuticals (QPI-1007)	NCT01064505
Spinal muscular atrophy (SMA)	SMN2	Naked ASO	Isis Pharmaceuticals (ISIS-SMN$_{Rx}$)	NCT01839656 NCT01780246 NCT02052791 NCT01703988 NCT01494701

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Table 5 ***A summary of miRNA therapeutics in clinical trials***

Name	Indications	miRNA	Phase	Delivery system	Route of administration	Trial ID
MRX34	Primary liver cancer or liver metastasis from other cancers	miRNA-34a	1, ongoing	Liposomes (SMARTICLES)	Intravenous	NCT01829971
TargomiRs	Malignant pleural mesothelioma; non–small-cell lung cancer	miRNA-16	1, ongoing	Nanoparticles (nonliving bacterial minicells)	Intravenous	NCT02369198

(Registered with clinicaltrials.gov, last accessed 13 June 2015)(Lam et al., 2015)

[4] Antisense oligonucleotides

1.9.1. Oligonucleotide therapies from bench to Clinique

The FDA approval of RNA-based therapy for CNS is still a vision for the future. Nonetheless, oligonucleotide therapy holds a great promise as a treatment for neurodegenerative diseases since new platforms for enhanced delivery are continuously being developed (Iddo Magen & Hornstein, 2014). It is important to note that advancement in the development of siRNA-based therapeutics for delivery to CNS has been significant and whether the same delivery strategies could also be proven for miRNA-based therapeutics remains to be examined further. For instance, different viral based *in vivo* delivery of shRNA to the nervous system has been investigated in normal mice and in animal models of spinocerebellar ataxia, Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis (Y. Chen et al., 2006; Harper et al., 2005; Ralph et al., 2005; Xia et al., 2004), and abnormal disease phenotypes were improved significantly (Khatri, Rathi, Baradia, Trehan, & Misra, 2012). Examples of basic studies on miRNA-based therapeutics are those of anti-miR-34c delivery that improved memory in a double transgenic model of AD AP/PPS1-21[5](Zovoilis et al., 2011). More recently a study on the mouse model of APPswe/PS1dE9 indicates that Injection of agomiR-299-5p into the cerebral ventricles of AD mice inhibited both autophagy and apoptosis and improved the cognitive performance of mice suggesting the potential of miRNA-299 as a neuroprotective factor in AD (Y. Zhang et al., 2016).

CNS drug delivery remains a big challenge specifically regarding oligonucleotide-base therapeutics. The transition from bench to bedside of RNA-based therapy also depends on the availability of a safe, clinically relevant delivery system that can facilitate cellular uptake of the RNA into target tissues/cells and offer protection against nuclease degradation (Lam et al., 2015).

Localized delivery reduces loss and toxicity to other organs. The cerebrospinal fluid (CSF produced by the choroid plexus circulates throughout the CNS makes

[5] APP/PS1-21 mice coexpress human APP carrying the K670N/M671L "Swedish" double mutation and hPS1 L166P with a 3-fold overexpression of human APP over endogenous mouse APP. Mice express the transgene under the control of a neuron-specific mThy-1 promoter element and generated on a C57Bl6 background (Radde et al., 2006).

it an interesting delivery site. In Figure 19 schematically widely used delivery method in the human and rodents is shown. Osmotic pumps by ALZET® have been widely used in the animal research to-date. The ALZET® osmotic pumps were developed in the seventies is a reliable method for the continuous delivery of agents controlled by osmosis. Water entering the osmotic layer generates a pressure inside the reservoir and displaces the stored drug volume. (Herrlich, Spieth, Messner, & Zengerle, 2012). It has been proposed that delivery of the RNA into the cerebrospinal fluid (CSF) via the lateral ventricles results in an efficient distribution of the infused RNA throughout the CNS (Kordasiewicz et al., 2012; Passini et al., 2011). Specifically, in human, the intrathecal space (Watson et al., 2006) is favored for drug delivery into the CSF, which results in efficient distribution at therapeutic doses throughout the CNS. Intrathecal delivery can be employed in smaller animal models as well but is considered more accessible in larger animal species (Morel et al., 2013).

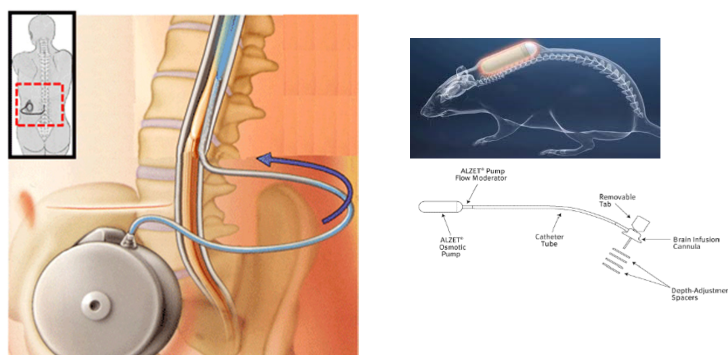

Figure 19 ***Implantable infusion's pump for a continuous intrathecal administration***

A diagram of implantable pumps in human(left) rodents(right) (from http://www.alzet.com/resources) (http://www.neuros.net/). Osmotic pumps consist of an inner core containing drug and osmogens, coated with a semipermeable membrane. As the core absorbs water, it expands in volume, which pushes the drug solution out through the delivery ports. Osmotic pumps release drug at a rate that is independent of the pH and hydrodynamics of the dissolution medium.

Overall, development of tools for the delivery and controlled modulation of miRNA *in vivo* will be valuable in enhancing the therapeutic value of miRNAs. This goal would be achieved not only by increased fundamental knowledge about miRNA but only by drug delivery advancement (Roshan, Ghosh, Scaria, & Pillai, 2009).

1.10. OBJECTIVES OF RESEARCH:

Psychiatric deficits and neurodegeneration may be ideally suited for therapeutic targeting by pleiotropic miRNAs (Chan & Kocerha, 2012). The concept of miRNAs as therapeutics has been fairly advanced in the field of cancer while its potential as therapeutics in neurodegeneration remains unstudied. Previous studies overall, suggest that a multi-therapeutic targeting of APP-BACE1-Tau towards the reduction of toxic species of Aβ might be more advantageous compared to approaches that target only one of these proteins. My lab and others have previously shown that miRNA-15/107 family is a potential candidate regulator of APP, BACE1, and Tau phosphorylation by regulating ERK kinase with lower expression observed in AD. The miRNA-15/107 family could be a therapeutic agent in case of AD by regulating APP, BACE1 and TAU phosphorylation *in vivo*. This project would pursue following objectives to test a miRNA as a candidate in this family for a miRNA-based replacement strategy aiming to restore the expression of a miRNA:

- *Evaluating potential candidate miRNA effect on APP, Bace1 and Tau phosphorylation in vitro.*
- *Delivery of synthetic miRNA mimics in mouse brain*
- *Evaluating the effect of candidate miRNA on AD-related proteins in vivo.*
- *Investigating the candidate miRNA in an induced cellular stress model in vitro*

1- Evaluating potential candidate miRNA effect on APP, Bace1 and Tau phosphorylation *in vitro*

The rationale behind selecting a candidate for mimicking miRNA replacement in the mouse is based on *in vitro* studies. Several molecular techniques would be applied to study the first interaction of all members of the miRNA-15/107 family on APP and BACE1.Only one miRNA for in-depth studies would be chosen based on efficiency on Aβ reduction as well. To study the direct interaction of candidate

miRNA with 3'UTR of APP and BACE1 luciferase assay would be followed by overexpression studies of miRNA in different neural and non-neural cells.

2- • Delivery of synthetic miRNA mimics in mouse brain mouse with C57BL/6 background

Upon introduction of synthetic miRNA (miRNA mimic) into the mouse brain, I should be able to see the down-regulatory effect on APP and BACE1 as its targets *in vivo*. To evaluate miRNA mimics introduction into the brain and the effect on potential targets, wild-type mouse is essential for these experiments, as this will allow studying the regulation of AD-related genes in their natural context. Indeed, most transgenic AD mouse models commonly used in pre-clinical studies express only the coding region (open reading frame) of the gene of interest (e.g., human APP, BACE1) therefore excluding the 3′UTR.

3- Evaluating the effect of candidate miRNA on AD-related proteins *in vivo*

The introduction of one selected candidate from this family will also allow me to perform more in-depth analysis of protein networks affected by that specific miRNA and identifying the potential new targets involved in pathology by proteomics that would follow. This project aims to provide a proof-of-concept for miRNA replacement therapy in AD.

4- Investigating the candidate miRNA in an induced cellular stress model *in vitro*

Considering the data obtained in previous sections this goal allows me to further investigate the overexpressed candidate miRNA *in vitro* and in a cellular context under two different cellular insults of Aβ and unfolded protein induces stress.

1.10.1. Technical note

The strategy to achieve the aforementioned goals is summarized in Supplementary data figure 1. Three main steps would be screening and identification of candidate miRNA, validation and functional studies and study of top candidates regulated by miRNA. The first step mainly relies on available tools for identification of miRNA target relationship Bioinformatically. The typical target

site of miRNA interaction with UTR is through base pairing, with 6–8 nucleotides of the miRNA's 5′ terminus. These characteristics are exploited by effective target site prediction algorithms. Although these algorithms have their own limitations but they speed up the procedure of miRNA-target validation. In this project, two of these tools (mirWalk and TargetScan) are frequently addressed especially in the first step of my research. The web interface of miRWalk2.0, contains a predicted target module and a validated target module, hosts a multilayered view of data and offering information on miRNAs, genes, epigenomics, pathways, ontologies, etc (Dweep & Gretz, 2015). TargetScan predicts biological targets of miRNAs by searching for the presence of conserved 8mer, 7mer, and 6mer sites that match the seed region of each miRNA (Lewis, Burge, & Bartel, 2005).

There are several methods to characterize the miRNA-target relationship *in vitro* which relies on whether potential targets are downregulated when a miRNA is overexpressed or vice versa, or mRNAs that precipitate with miRISC-associated proteins (Baek et al., 2008; Codogno, 2014; Hafner et al., 2010; Lim et al., 2005). Furthermore, High-throughput sequencing of Ago-immunoprecipitated RNAs after crosslinking also provides a way to identify miRNA-target pairings (Beitzinger, Peters, Zhu, Kremmer, & Meister, 2007). Overexpression (*in vitro* and *in vivo*) and downregulation of candidate miRNA (*in vitro*) was the main focus of my study. The expression study of mRNA and miRNA by qPCR was chosen in my study provides a powerful tool to quantify gene expression. The quantitative endpoint for real-time PCR is the threshold cycle (CT). The CT is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold (Schmittgen & Livak, 2008).

Oligonucleotide delivery of miR-16 are supposed to mimic the endogenous miR-16 function in the brain and this upregulation of miR-16 expression can be confirmed by miRNA expression study, immunoprecipitation and proteomics. The proteomics used in my study is an isobaric tag for relative and absolute quantitation (iTRAQ) which is a technique based on having the isobaric mass design of the reagents, so differentially labeled proteins do not differ in mass; accordingly, their corresponding proteolytic peptides appear as single peaks in Mass spectrometry scans.

Given the potential of a miRNA to regulate a large number of genes, it can be challenging to identify key miRNA targets and functions from the long lists of putative targets generated by the experimental methods (Thomas, Lieberman, & Lal, 2010). Multiple tools are used in my study to explore the meaningfulness of data obtained from proteomics and bioinformatical tools. For example, The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 was released in 2003 addresses various aspects of the challenge of functionally analyzing large gene lists (Dennis Jr et al., 2003; Huang, Sherman, & Lempicki, 2009). The other tool that will help me to study the relationships between potential miR-16 targets at the protein level is Genemania (Warde-Farley et al., 2010) a powerful online tool to identify relationships between a set of selected proteins.

2. CHAPTER II: Preclinical evaluation of miR-15/107 family members as multifactorial drug targets for Alzheimer's disease

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Résumé

La maladie d'Alzheimer (MA) est une maladie neurodégénérative mortelle multifactorielle caractérisée par l'accumulation anormale de Aß et Tau dans le cerveau. Le fait qu'il ne semble pas avoir de thérapies efficaces pour traiter MA, et qu'il y a présence d'échecs dans différents essais cliniques, met l'emphase sur la nécessité de développer de nouveaux traitements. Ces dernières années, des progrès significatifs ont été accomplis dans le développement de thérapies à base de miRNA pour les troubles humains. Cette étude a été conçue pour évaluer l'efficacité et la sécurité potentiel de la thérapie de remplacement miARN dans MA, en utilisant miR-15/107 comme candidats de médicaments. Nous avons identifié miR-16 comme un inhibiteur puissant de APP et BACE1 l'expression, la production de peptides Aß et de la phosphorylation de Tau dans les cellules. La livraison de miR-16 dans le cerveau chez la souris a entraîné une réduction des gènes qui sont reliés à la MA (e.g. APP, BACE1 et Tau) où ses effets dépendent de la région du cerveau. Nous avons aussi identifié Nicastrin, un composant γ-sécrétase impliqué dans la production de Aß, comme une cible de miR-16. L'analyse protéomique a identifié un certain nombre de miR-16 cibles prédit *in vivo*, y compris α-synucléine et le récepteur de la transferrine 1. Il y a un haut rang de réseaux biologiques qui sont associés à la livraison de miR-16, incluant MA et le stress oxydatif. En conclusion, nos données suggèrent que miR-16 est un bon candidat pour le futur développement de médicaments en ciblant les régulateurs de MA biomarqueurs (à savoir, Aß et Tau), l'inflammation et le stress oxydatif.

Abstract

Alzheimer's disease (AD) is a multifactorial, fatal neurodegenerative disorder characterized by the abnormal accumulation of Aβ and Tau deposits in the brain. There is no cure for AD, and failure at different clinical trials emphasizes the need for new treatments. In recent years, significant progress has been made towards the development of miRNA-based therapeutics for human disorders. This study was designed to evaluate the efficiency and potential safety of miRNA replacement therapy in AD, using miR-15/107 paralogues as candidate drug targets. We identified miR-16 as a potent inhibitor of APP and BACE1 expression, Aβ peptide production, and Tau phosphorylation in cells. Brain delivery of miR-16 mimics in mice resulted in a reduction of AD-related genes APP, BACE1 and Tau in a region-dependent manner. We further identified Nicastrin, a γ-secretase component involved in Aβ generation, as a target of miR-16. Proteomics analysis identified a number of additional putative miR-16 targets *in vivo,* including α-Synuclein and Transferrin receptor 1. Top-ranking biological networks associated with miR-16 delivery included AD and oxidative stress. Collectively, our data suggest that miR-16 is a good candidate for future drug development by targeting simultaneously endogenous regulators of AD biomarkers (i.e., Aβ and Tau), inflammation, and oxidative stress.

Keywords: microRNA; therapy; Alzheimer's disease; miR-16; brain delivery

2.1. Introduction

AD is the most common form of dementia characterized by the progressive decline of cognitive and behavioral abilities (Prince et al., 2013). Most (>95%) AD cases are of sporadic origin with unknown cause (Campion et al., 1999; Sperling et al., 2011). The two major hallmarks of the disease are senile plaques and neurofibrillary tangles (NFTs) in the brain. Plaques are composed of depositions of β-amyloid (Aβ) peptides, generated by the cleavage of the Amyloid precursor protein (APP) by BACE1/β-secretase and γ-secretase (a multiprotein complex composed of Presenilin, Nicastrin, PEN2, and Aph-1) (Strooper et al., 2013). NFTs are composed of intraneuronal inclusions of hyperphosphorylated and aggregated Tau protein (Hardy and Selkoe, 2002).

Up to now, most therapeutic efforts in AD have been concentrated towards the development of drugs against Aβ (e.g., APP, BACE1, and γ-secretase) or Tau independently. AD pathology is complex and likely multifactorial, where it has been suggested that the conventional "one protein, one drug, one disease" theory for AD would not be effective (Jia et al., 2014). Failure of recent clinical trials is in line with this hypothesis (Boutajangout and Wisniewski, 2014; Jia et al., 2014; Rosenblum, 2014). Strategies that target simultaneously multiple disease components and/or pathways could, therefore, address this issue (Carmo Carreiras et al., 2013; Jaturapatporn et al., 2012).

MiRNAs comprise the largest group of small (~22 nt) endogenous noncoding RNAs driving gene silencing in cells, predicted to regulate more than 60% of protein-coding genes (Ambros, 2004; He and Hannon, 2004). Once incorporated into the RNA-induced silencing complex (RISC), miRNAs function to repress translation and/or promote RNA degradation through imperfect base-pairing with specific mRNA sequences, generally located in the 3' untranslated region (3′UTR) (Bartel, 2004). The aberrant expression of miRNAs in many human diseases and involvement in key biological pathways has made them attractive drug targets (Ling et al., 2013; van Rooij and Kauppinen, 2014; van Rooij et al., 2012). This is well recognized in the cancer field, where miRNAs can function as oncogenes or tumor suppressors (Hammond, 2007). The interest for miRNA replacement therapy is rapidly growing (Bader, 2012; Bader et al., 2010;

Simonson and Das, 2015), which involves the "reintroduction" of a missing miRNA into cells to compensate for a loss-of-function. miRNA mimics used in replacement therapy have the same sequence and structure as the depleted, endogenously expressed miRNA. Thus, off-target effects are less likely to occur as the mimics behave like their natural counterparts by fine-tuning the expression of targets through conserved miRNA: mRNA interactions (Bader et al., 2010). In contrast to conventional gene therapy that involves relatively large DNA plasmids or viral vectors, miRNA mimics are substantially smaller in size, and they merely need to enter the cytoplasm of target cells to be active. Another strong rational to use miRNAs in replacement therapy is based on the fact that a single miRNA can regulate multiple genes simultaneously, therefore acting on "disease pathways" (Pasquinelli, 2012).

The miR-15/107 superfamily controls a number of fundamental processes including metabolism, cell cycle regulation, inflammation, and the stress response (Finnerty et al., 2010; Linsley et al., 2007). Interestingly, several members of this family have been documented to be deregulated in AD brain, including miR-16 (Liu et al., 2012; Müller et al., 2014), miR-15ab (Hébert et al., 2010, 2008; Nunez-Iglesias et al., 2010; Shioya et al., 2010; Wang et al., 2011), miR-195(Ai et al., 2013; Cogswell et al., 2008), and miR-103/107 (Nelson and Wang, 2010; Smith et al., 2011; Wang et al., 2008). Studies in vitro have implicated miR-16, miR-15a, miR-195 in the regulation of BACE1 and APP expression, Aβ production, and Tau phosphorylation (Ai et al., 2013; Hébert et al., 2010; Zhu et al., 2012). More recent studies in vivo have implicated miR-195 in memory formation (Ai et al., 2013). Collectively, these studies point to the potential therapeutic use of miRNAs in AD by targeting genes involved in both Aβ production and Tau metabolism. To date, however, a detailed comparative analysis of miR-15/107 superfamily members has not yet been conducted.

A defined protocol has been proposed before entering miRNAs into the clinic, including the optimization of suitable candidates (van Rooij et al., 2012). However, these procedures have been developed mainly for peripheral disorders (e.g., inhibition of liver-specific miR-122) (Jopling, 2010), leaving neurodegenerative diseases largely unexploited. Several critical questions

remain to be addressed: Is widespread delivery to the brain possible? Are miRNAs functional in the brain, and particularly in neurons? What are potential side effects? In an attempt to address these issues, we sought to evaluate the therapeutic applicability of miRNAs in AD, using miR-15/107 family members as candidate drug targets. Specifically, we wanted to determine the efficiency and potential safety of miRNA mimics towards the regulation of AD-related genes in vivo with a focus on endogenous APP, BACE1, and Tau.

2.2. Results

2.2.1. Comparative analysis of miR-15/107 family members *in vitro* and in cells

Our experimental strategy is presented in supplementary Figure 1. We first evaluated the effects of miR-15a, -15b, -16, -195, -424, -497, and -103 mimics on human APP and BACE1 expression in luciferase-based assays. In contrast to previous studies (Absalon et al., 2013; Ai et al., 2013; Patel et al., 2008), we used the full-length 3′UTR of tested genes to better mimic physiological conditions. As shown in Figure 1A, the predicted miRNA binding sites in APP and BACE1 are highly conserved. We co-transfected the wild-type 3′UTR reporter constructs (Figure 1B) with candidate miRNA mimics into native HEK293 cells. Compared to a scrambled control (SCR), most miRNAs significantly reduced luciferase signal (expression) of both APP and BACE1 (Figure 1C, E). Among tested miRNAs, miR-16 showed the strongest negative effects on both APP and BACE1. To validate the specificity of these results, we generated mutant APP (CTG546-548AAA) and BACE1 (CTG269-271AAA and CTG1798-1800AAA) reporter constructs. As expected, disruption of miR-16 binding sites partly rescued the effects on luciferase activity (Figure 1D, F).

We next performed functional studies in HEK293 cells overexpressing the APP Swedish (KM670/671NL) mutation (hereinafter referred to as HEK293-APPSwe), with as purpose to measure human Aβ levels (Hébert et al., 2008). As before, we introduced equal concentrations of miR-15a, -15b, -16, -195, -424, -497 and -103 mimics in this cell line. These experiments showed that miR-16, -15, and -195 mimics similarly suppressed Aβ production (Figure 1G). This effect was unrelated to overall miRNA levels in the cells (Supplementary Figure 2). APP β-CTF levels,

the direct products of BACE1, were significantly downregulated in these conditions. Inversely, APP -CTF levels were increased. Endogenous BACE1 protein was below detection levels in this cell line. Mutant APP levels remained unchanged following mimic overexpression (Figure 1H), which was expected since expressed ~5 fold over endogenous APP ((Campion et al., 1999) and data not shown) and it does not contain a 3′UTR. On the other hand, all members of this family could downregulate endogenous APP in native HEK293 cells (Supplementary Figure 3).

Given its strong regulatory effects on APP, BACE1, APP β-CTFs and Aβ (both direct substrates of BACE1), we focused our studies on miR-16. We investigated the effects of miR-16 mimics on endogenous APP and BACE1 in neuronal cells. We observed a concomitant reduction of APP and BACE1 protein levels following miR-16 overexpression in native Neuro2a cells (Figure 2A, B). Notably, the introduction of miR-16 induced also a significant decrease in total Tau phosphorylation (as measured using the Tau1 epitope, which specifically labels non-phosphorylated Tau) (Planel et al., 2008) (Figure 2A, B). We also validated the effects of miR-16 on human Aβ in Neuro2a cells expressing APPSwe (hereinafter referred to as Neuro2a-APPSwe) (Supplementary Figure 2). Finally, we observed lower levels of endogenous APP and BACE1 in miR-16-expressing native HT22 cells, an independent neuronal cell line (Figure 2c, d). Unfortunately, Tau protein was below detection levels in this cell type. Taken together, these results identified miR-16 as an endogenous regulator of both Aβ production and Tau phosphorylation.

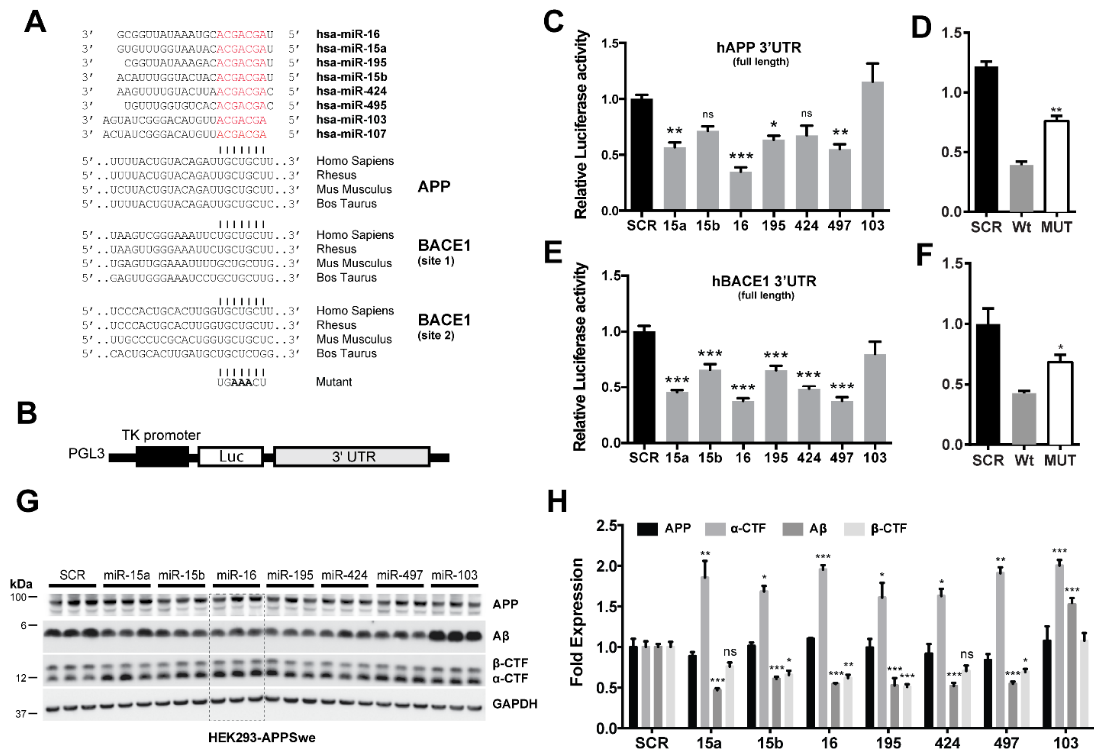

Figure 1 ***Comparative analysis of miR-15/107 family members in vitro****.*

(A) *Mature miRNA sequences are shown. Seed sequences are shown in red. The corresponding binding sites within the APP and BACE1 3'UTRs are shown in gray.* ***(B)*** *Schematic representation (not to scale) of the luciferase reporter construct. TK; thymidine kinase promoter, Luc; luciferase gene.* ***(C)*** *APP 3'UTR regulation by selected miR-15/107 family members. HEK293 cells were transfected with 50nM final concentration of candidate mimics. Twenty-four hours post-transfection luciferase signal was measured. Signals were normalized for transfection efficiency, and the graph represents the relative luciferase signals compared to the scrambled control (SCR). Statistical significance was assessed by one-way ANOVA with Bonferroni multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.* ***(D)*** *Luciferase assays were performed using a mutant 3'UTR construct for APP. Here, cells were treated with and 12.5nM of miR-16 mimics. A significant difference was observed between wild-type and mutant constructs (**$p < 0.01$).* ***(E)*** *BACE1 3'UTR regulation by selected miR-15/107 family members. HEK293 cells were transfected with 50nM final concentration of candidate miRNA mimics. Twenty-four hours post-transfection luciferase signal was measured. The graph represents the relative luciferase signals compared to the scrambled control (SCR). Statistical significance was assessed by one-way ANOVA with Bonferroni multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.* ***(F)*** *Luciferase assay using a 3'UTR BACE1 double mutant construct. Cells were treated with 25nM of miR-16 oligos. Statistical significance was calculated by one-way ANOVA with Bonferroni multiple comparison test.* ***(G, H)*** *HEK293-APPSwe cells were treated with candidate mimics at a final concentration of 50 nM. A strong effect on soluble Aβ levels (measured in cell medium) was observed after 24h treatment. This is in agreement with the down-regulation of APP β-CTFs (the direct BACE1 substrates) and a concomitant increase in APP α-CTFs.*

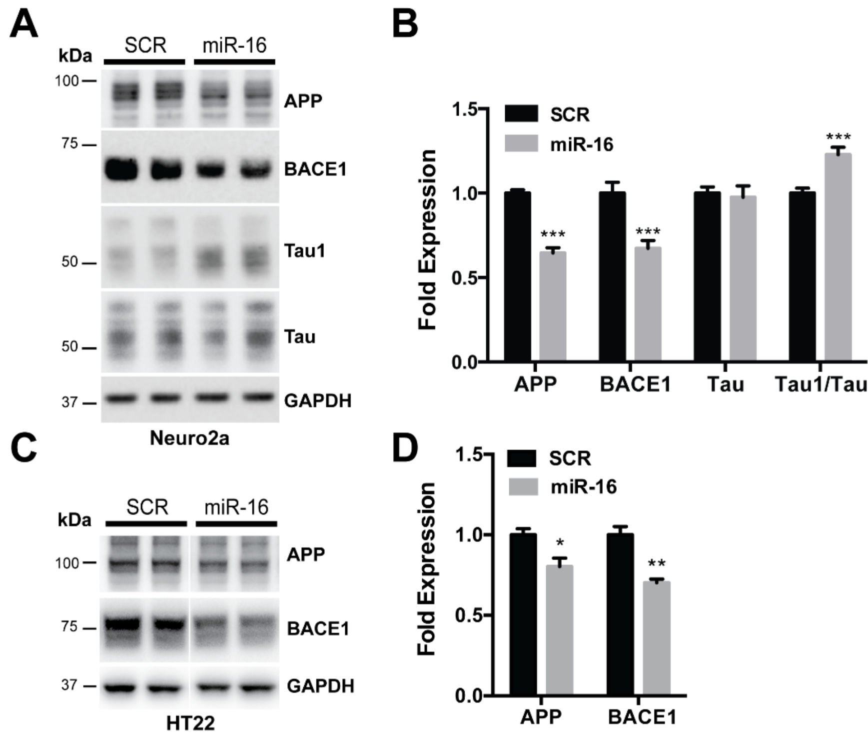

Figure 2 ***Effects of miR-16 overexpression on APP, BACE1, and Tau in neuronal cells***

(A, B) *Representative western blot analysis of Neuro2a cells treated with miR-16 mimics (50nM final concentration). Results are shown 24hrs post-transfection. Shown here is a combined regulatory effect of miR-16 on APP, BACE1, and Tau.* ***(C, D)*** *Western analysis of BACE1 following miR-16 overexpression in HT22 cells. Here, results are shown 48hrs post-transfection. A scrambled oligonucleotide sequence (SCR) was used as negative control in all experiments. Error bars represent standard errors derived from three or more independent experiments performed in triplicate. Statistical significance between SCR- and miRNA-treated cells was determined using unpaired t-test with Bonferroni multiple comparison test as a post-test. Data are shown as mean± SEM.*

2.2.2. Loss of the DLEU2/miR-15a/16-1 in sporadic AD

The above-mentioned results prompted us to re-evaluate the expression levels of miR-16 in AD. The miR-16 and miR-15a cluster is encoded within the DLEU2 noncoding gene on chromosome 13 (Calin et al., 2002). To exclude any bias towards one of the two miRNAs, we measured the DLEU2 transcript in human brain tissues. By qRT-PCR, we observed a significant downregulation of DLEU2 mRNA in AD patients when compared to non-demented controls in both the temporal and frontal cortex (Supplementary Figure 4). These results strengthen the notion that miR-16 (and miR-15a) is lower in sporadic AD, and further validate the use of miR-16 in therapeutic applications.

2.2.3. Effective delivery of miR-16 mimics into the mammalian brain

Oligonucleotide delivery using osmotic pumps is a recognized technique with potential therapeutic applications in mammals including humans (Koval et al., 2013; Stiles et al., 2012; Wilkes, 2014) (Wesemann et al., 2014). We, therefore, used this strategy to deliver miR-16 mimics into the mouse brain. We chose wildtype mice –instead of AD mice– for these in vivo preclinical studies since harboring all physiological regulatory elements (e.g., 3′UTR) of genes of interest. We first treated mice with increasing doses of miR-16 mimics for 7 days (n=3/group). As a control, we used vehicle alone (saline 0.9%). Following delivery, the mice were sacrificed and the hippocampi were isolated for functional analyses. In these conditions, we observed a dose-dependent decrease in endogenous BACE1 and Tau (Figure 3A). Tau1 epitope was significantly increased (mirroring lower Tau phosphorylation), consistent with our cell-based studies. We used the previously recognized miR-16 target ERK1 as an internal control (Hébert et al., 2010). These effects were specific, as they were not reproduced using a chemically-modified non-functional miR-16 mimic (Supplementary Figure 5).

Based on the aforementioned observations, we chose a dose of 50ug/day to pursue our in vivo studies. An independent group of mice received miR-16 mimics for 7 days (n=10/group). We first evaluated the levels of miR-16 mimics in the treated mice. By qRT-PCR, we observed a strong increase of miR-16 in the hippocampus (106 fold), cortex (34 fold), striatum (27 fold), and brainstem (27 fold) (Supplementary Figure 6). Such increases were independent of miR-16 baseline levels (Supplementary Figure 6). We also performed RIP-Chip (i.e., anti-Ago2) assays to determine the enrichment of miR-16 directly in the RISC complex following its overexpression. These experiments showed a 3.63 fold and 2.66-fold enrichment of miR-16 in the cortex and brainstem, respectively (Supplementary Figure 6). Thus, the absolute increase of (functional) miR-16 is physiologically relevant.

Western blot analysis on APP, BACE1, Tau, ERK1 was next performed on 4 different brain regions, including the hippocampus, cortex, striatum, and brainstem (Figure 3). Interestingly, the effects of miR-16 mimics on BACE1, APP,

Tau and ERK1 were region-dependent. For instance, APP protein levels were downregulated in the cortex, brainstem, and striatum but not in the hippocampus. BACE1 protein levels were reduced in the hippocampus, brainstem, and striatum. We also marked a significant downregulation of total Tau in the hippocampus, brainstem and striatum followed by a modest increase in non-phosphorylated Tau in the hippocampus and striatum. We confirmed an overall decrease of phosphorylated Tau (PHF13 epitope) in the hippocampus of treated mice (Supplementary Figure 7). ERK1 was downregulated mainly in the hippocampus and cortex. We also investigated mRNA levels for APP, BACE1, and Tau in these regions. These experiments showed that only APP and Tau mRNA expression was significantly lower in the cortex and brainstem respectively, but not in other regions.

In the course of these studies, we also observed a significant downregulation of Nicastrin in the treated mice (Figure 3C, D). These effects were not observed on other members of the -secretase complex, including Presenilin-1 and PEN2. Using the miRWalk algorithm (Dweep et al., 2011), we identified one putative miR-16 binding site located in 3′UTR of Nicastrin (Supplementary Figure 8). We, therefore, repeated the luciferase-based experiments using the full-length Nicastrin 3′UTR as before (Delay et al., 2014). As hypothesized, miR-16 could significantly downregulate luciferase activity (Nicastrin expression) in these conditions (Supplementary Figure 8).

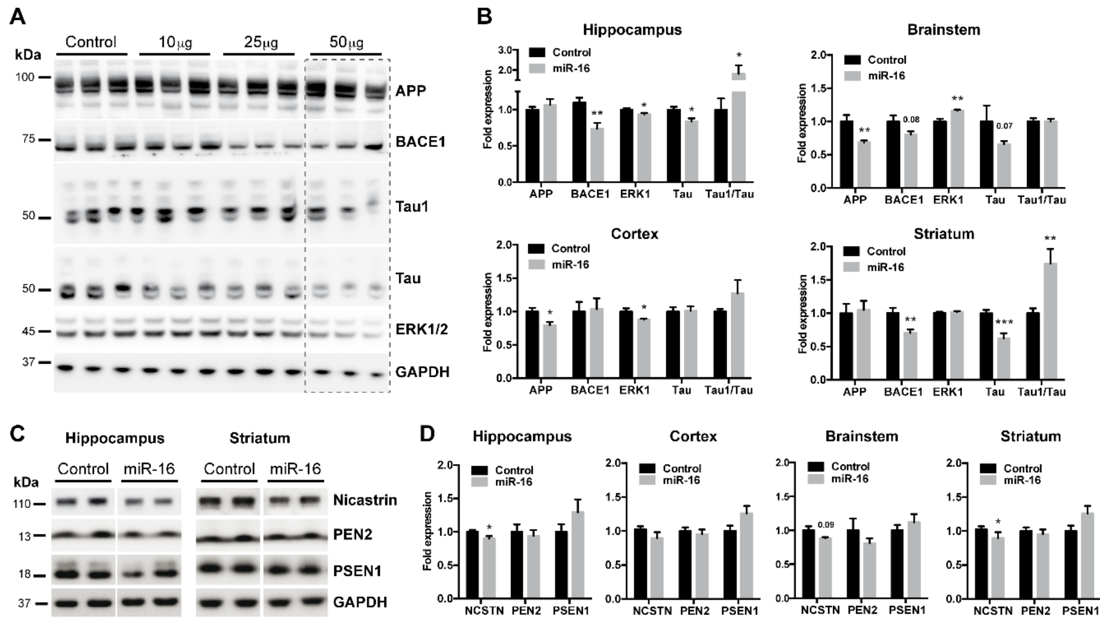

Figure 3 ***In vivo regulation of AD genes by miR-16 mimics.***

(A) *Dose-dependent effects of miR-16 mimics on BACE1, Tau, and ERK1/2 in the hippocampus. As a control, we used vehicle alone (saline 0.9%). APP remained unaffected in this region.* ***(B)*** *Region-dependent effects of miR-16 mimics on AD-related genes. Data are shown as mean ± SEM. GAPDH served as a normalizing control.* ***(C, D)*** *Representative western analysis and quantification of γ-secretase complex members. Overall changes in protein levels were calculated by parametric unpaired t-test with Welch's correction, where *p < 0.05, **p < 0.01, ***p < 0.001.*

2.2.4. Assessment of potential side effects related to miR-16 mimic brain delivery

Previous studies have associated miR-16 with the inflammation response (Zhou et al., 2012). We, therefore, wanted to determine whether miR-16 overexpression was associated with an increase (or decrease) in inflammation markers. Compared to controls, treated mice displayed a significant downregulation of Glial fibrillary acidic protein (Gfap) in the hippocampus and striatum (Figure 4). A tendency for reduced Allograft inflammatory factor 1(Aif1) was also observed in these regions. Other inflammation markers including Toll-like receptor 2 (Tlr2), Bcl-2-associated death promoter (Bad), and T-Lymphocyte Activation Antigen (CD86) remained unchanged in these conditions, with an overall non-statistical trend for lower levels. These results strongly suggest that miR-16 delivery to the brain per se is not associated with overt inflammation.

Given no or little effects of miR-16 mimics on mRNA levels of candidate genes (Supplementary Figure 7), we next thought to perform proteomics studies. The purpose here was twofold: 1) to identify additional miR-16 mimic targets in the brain, and 2) to assess potential indirect effects associated with miR-16 mimic delivery. For these studies, we chose the hippocampus and brainstem, two functionally and temporally distinct regions related to AD (Paduraru et al., 2012; Simic et al., 2009). The itraq (isobaric tags for relative and absolute quantification) analysis identified a total of 4058 proteins in the adult mouse brain (data not shown) (Wiese et al., 2007). Compared to the control group, a total of 16 proteins were significantly misregulated in the hippocampus, including 5 upregulated and 11 downregulated proteins (fold change <0.8 and >1.2, P <0.05) (Supplementary Table S1). In the brainstem, a total of 102 proteins were changed using similar cut-off values, including 47 upregulated and 55 downregulated proteins. Using the miRWalk algorithm, we identified 7/11 (64%) and 31/55 (56%) of downregulated proteins with at least one predicted miR-16 target site in their 3′UTR (Supplementary Table S1). Furthermore, 14/55 (25%) of proteins misregulated in the brainstem had at least one miR-16 site within the coding sequence (open reading frame).

We selected four proteins for further validation, including α-Synuclein (α-Syn) (fold 0.766, P=0.001), serine/arginine repetitive matrix protein 2 (Srrm2) (fold 0.798, P=0.023), GTPase-activating protein, VPS9 domain-containing protein 1 (GAPVD1) (fold 0.771, P=0.038), and Transferrin receptor protein 1 (TfR1) (fold 0.666, P=0.037). By Western blot, we could confirm the downregulation of α-Syn, Srrm2, GAPVD1 and TfR1 in mimic-treated mice when compared to controls (Figure 5). To determine whether these effects were a direct consequence of miR-16 overexpression, we performed gain-of-function studies in HT22 cells. These experiments confirmed the regulation of identified genes by miR-16 in neuronal cells. We next explored the functional relationship of misregulated proteins (up- and down-regulated) using the Genemania online tool (Warde-Farley et al., 2010). This analysis identified a significant (physical) interaction map between α-Syn and various other affected proteins in the brainstem (Supplementary Figure 9). Unfortunately, the relatively low number of affected

proteins in the hippocampus made similar predictions impossible. We also performed an enrichment analysis of genes encoding all top-ranked proteins in the brainstem using the DAVID software (Huang et al., 2009). The highest-ranking network associated with miR-16 delivery was AD (p=5.1E-10). Other relevant networks and pathways included Parkinson's disease (PD) (P=6.3E-9), oxidative phosphorylation (P=7.5E-9), and cytoskeleton protein binding (P=3.6E-7) (Supplementary Table S2).

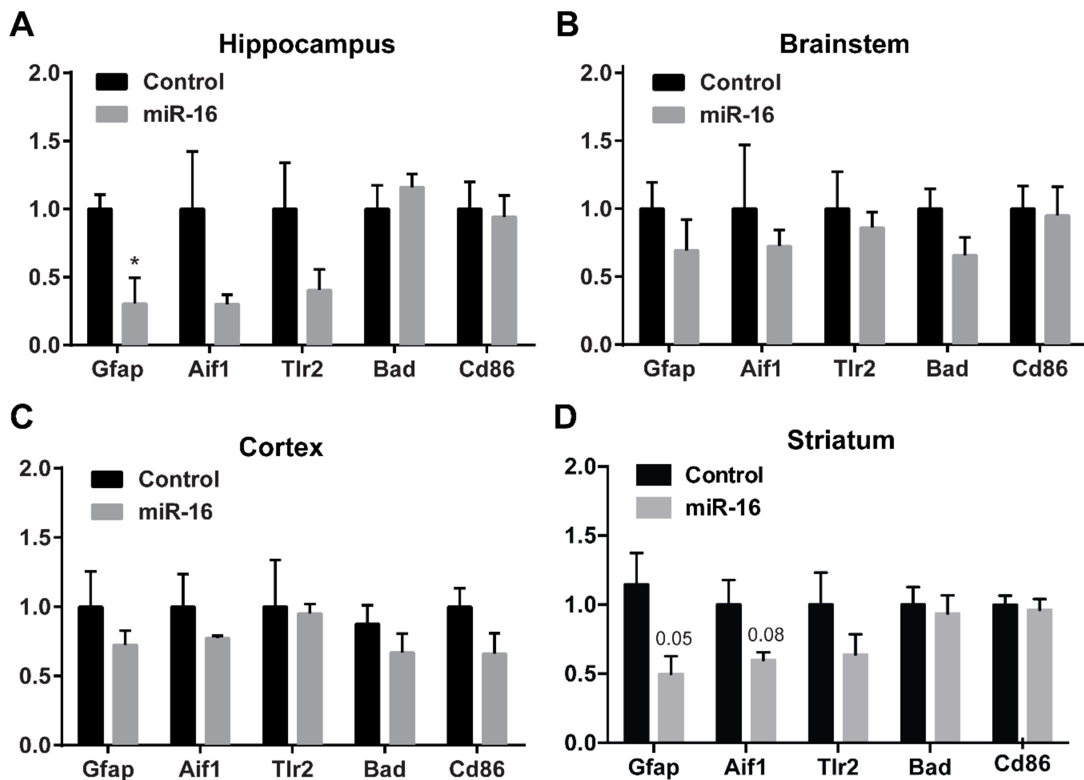

Figure 4 ***Analysis of inflammation markers following miR-16 mimic treatment***.

*Seven days after delivery, mRNA levels of inflammatory markers were measured by qRT-PCR in **(A)** hippocampus, **(B)** brainstem, **(C)** cortex, and **(D)** striatum. Overall changes were calculated by parametric unpaired t-test with Welch's correction, where P<0.05 is considered as statistically significant. Data are shown as mean± SEM. GAPDH served as a normalization control.*

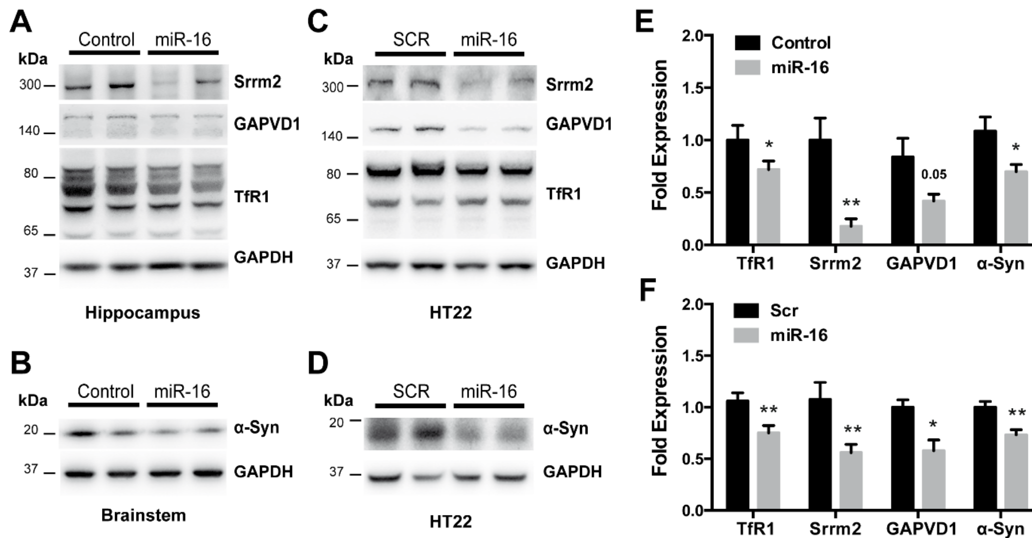

Figure 5 ***Proteomics validation in vivo and in neuronal cells.***

(A, B) *Representative western blot analysis of selected proteins in the hippocampus and brainstem of miR-16 mimic-treated mice.* ***(C, D)*** *Results on HT22 cells treated with 50nM final concentration of miR-16 mimics (n=3 in triplicate). Statistical significance between control- and miRNA-treated mice was determined using unpaired t-test with Bonferroni multiple comparison test as a post-test. Data are shown as mean± SEM.* ***(E, F)*** *Quantification of protein levels. Statistical significance was calculated by parametric unpaired t-test with Welch's correction, where *p < 0.05, **p < 0.01, ***p < 0.001. Data are shown as mean± SEM. GAPDH served as a normalization control.*

2.3. Discussion

This study examines the potential application of miRNA mimics as therapeutic agents in AD and provides new information about the role of miR-16 in the brain. Overexpression studies in vitro, in cells and in mice suggest that miR-16 could target simultaneously (or a combination of) endogenous regulators of Aβ, Tau, inflammation and oxidative stress. Together, these results suggest that selected miR-15/107 family members can function as promising multifactorial drug targets for AD. The results presented herein support the notion that miR-16 and its homologs are involved in the physiological regulation of AD genes across species. It's noteworthy that miR-16 itself is highly conserved (Finnerty et al., 2010). The observation that miR-103 did not regulate BACE1 in most conditions tested herein might reflect a different mode of regulation of miR-103/107 close homologs (i.e., via coding sequence or other regulatory elements) (Wang et al., 2008). Our overexpression paradigms cannot discriminate if identified miR-16

targets in vivo are regulated mainly during development, adult maintenance, and/or disease conditions. Also, this study does not address the question of whether miR-16 regulates these genes simultaneously, and in the same cell populations. Obviously, these questions are biologically meaningful, and should be addressed in future experiments, for instance using loss-of-function paradigms. The overexpression conditions used herein remain, however, a method of choice for therapeutic applications, particularly in miRNA replacement therapy. It is interesting to note that the mimics could effectively target genes expressed mainly in neurons (i.e., APP, BACE1, and Tau), consistent with recent observations (although some non-neuronal genes may also be regulated – see below) (Koval et al., 2013). The fact that central nervous system (CNS)-delivered mimics can distribute throughout the brain can be viewed as an advantage in targeting widespread diseases such as AD; however, further preclinical studies are required to ascertain this hypothesis. The delivery of mimics to specific brain regions and/or cell types is also feasible, for instance using receptor-specific peptides (Wang et al., 2010b). As shown here, brain cells adapt well to "high" levels of exogenous miRNA mimics, thus opening an interesting therapeutic window (Di Martino et al., 2014; Thomson et al., 2013; Trang et al., 2011; Zhang et al., 2013).

Most AD mouse models used in preclinical studies express only the coding sequence of mutant genes of interest (e.g., APP, BACE1, PSEN), therefore excluding partially or entirely the 3′UTR. The use of wildtype mice is, therefore, essential to address our questions, since harboring all physiological regulatory elements. For instance, the use of wild-type mice allowed us to investigate in more detail the role of miR-16 in the regulation of endogenous Tau phosphorylation. In addition, and importantly, we identified protein networks downstream of miR-16 overexpression in the adult brain. These effects are independent of mutant transgenes, known to have pleiotropic effects (Robakis, 2014), and thus, provide a more accurate view of regulated pathways. We hypothesize that miRNA-based therapies will benefit most patients with sporadic AD, thus with no causative mutations in APP or PSEN genes (Magen and Hornstein, 2014).

Continuous delivery of miR-16 mimics in the brain did not induce overt inflammation, another significant advantage when developing CNS-based drugs. We actually noticed a downregulation of Gfap and Aif1 in the treated mice. All members of the miR-15/107 family are predicted to target the human GFAP 3′UTR (targetscan.org). Whether this phenomenon is conserved in mice remains an interesting possibility, and would indicate that a pool of miR-16 mimics could effectively target non-neuronal genes. Interestingly, previous studies have shown that Gfap deficiency in mice protects neurons against metabolic and excitotoxicity (Hanbury et al., 2003), whereas interference with glial activation in AD mice results in improved cognitive and synaptic function (Furman et al., 2012). In AD brain, a large number of GFAP-positive astrocytes are co-localized with amyloid plaques (Hol et al., 2003). While the role of inflammation in AD remains under debate (Meraz-Ríos et al., 2013), our results suggest a potential neuroprotective role for miR-16 upregulation in AD. The underlying mechanisms involved in this process remain to be determined, but might involve previously identified miR-16 downstream effectors (e.g., TNF, IL-8) (Zhou et al., 2012). Of course, longer treatments are required to determine if these effects are maintained over time.

Our quantitative proteomics analyses identified various putative miR-16 targets In vivo with potentially important functions in AD and neurodegeneration. For instance, α-Synuclein, the major component of Lewy bodies in PD (Spillantini et al., 1997), can induce the fibrillation of Tau (Nonaka et al., 2010). TfR1 is a major iron-binding protein, with high affinity for transferrin. Recent evidence suggests that ferritin iron accumulation in the hippocampus of AD patients concurs with decreased tissue integrity (Raven et al., 2013). The reduction of TfR1 in the hippocampus is also thought to be protective against oxidative stress in AD (Huang et al., 2014). GAPVD1, also known as RAP6, is a regulator of endocytosis and regulates Glut4 trafficking mainly in adipocytes (Hunker et al., 2006; Leto and Saltiel, 2012). Although the role of GAPVD1 in the brain is unknown, profiling studies suggest the GAPVD1 mRNA is upregulated in AD patients (NextBio databank: http://www.nextbio.com). Srrm2/SRm300 plays an important role in pre-mRNA splicing as a spliceosome component (Blencowe et al., 1998), and is a candidate gene for PD (Shehadeh et al., 2010). Again, mRNA expression

studies suggest an upregulation of Srrm2 in AD patients when compared to non-demented controls (NextBio databank, datasets GSE48350 and GSE5281) (Blair et al., 2013; Liang et al., 2007). Interestingly, miR-16 mimics also induced a downregulation of various mitochondrial respiration components, including Ndufb5, Ndufb9, ATP5j, ATP6V0, COX4i, Cox5a in the brainstem. In the rat brain, it has been suggested that aging elicits elevates metabolic activity by regulating in part these genes (Baskerville et al., 2008). Whether miR-16 introduction (or re-introduction) could prevent or attenuate oxidative stress associated with aging and/or disease is an interesting possibility.

Considering that a single miRNA can modulate a large number of genes, miRNA-based therapeutics have their own challenges that must be overcome before assessing their efficacy in humans, like stability, delivery, and safety (Junn and Mouradian, 2012). One should keep in mind that certain miRNAs can, however, function through specific “master switches”, thus limiting the number of affected genes (Park et al., 2010; Vidigal and Ventura, 2014). In this context, our study provides important new information with regard to the efficiency of miRNA mimics for AD therapy, by showing the combined action of miR-16 on APP, BACE1, and Tau. Although bioinformatics predicts a large number of miR-16 targets (e.g., >1000 using miRWalk), in vivo study herein the brain shows that relatively few genes (~100 in total) are affected by miR-16 overexpression and in a region-specific manner. These observations suggest that miRNA replacement therapy could be safe with minimal side effects in humans. While previous studies have linked miR-16/15a misregulation to cancer (Aqeilan et al., 2010; Calin et al., 2008; Chen et al., 2013; Yang et al., 2014), it is not expected that proposed target genes (e.g., Bcl-2, Mcl1, Ccnd1, and Wnt3a) are regulated in postmitotic neurons. Such cell and tissue specificity is well documented, for instance, with transcription factors. Consistent with this notion, Bcl-2 protein levels remained unchanged following miR-16 mimic overexpression (data not shown), consistent with our proteomics analysis. In addition, there is no clear indication that major cancer-related networks and pathways are affected in the treated mice.

Considering that miR-16 dysregulation is associated with various other neurodegenerative and psychiatric diseases, these results set the stage to

explore in more detail the role of this superfamily in brain disorders in general. Future experiments include testing the effects of mimics in animal models of neurodegeneration (taking into account their limitations), as well as performing detailed pharmacokinetics analyses of mimics in the brain. Finally, our research suggests that miR-16 replacement therapy can specifically be used for AD and possibly PD.

2.4. Materials and Methods

2.4.1. Cell culture

Mouse neuroblastoma Neuro2a cells, mouse Neuro2a cells expressing the Swedish mutant of APP and Δ9 mutant of PSEN1 (Neuro2a APPSwe/Δ9) (Dr. Gopal Thinakaran, University of Chicago, USA), mouse hippocampal-derived HT22 cells (Dr. Schubert, Salk institute, USA), human HEK293T cells, and human HEK293 cells expressing the Swedish mutant of APP (HEK293-APPSwe) were cultured in DMEM supplemented with 10% fetal calf serum (ThermoFischer Scientific Inc., USA).

2.4.2. Cell transfection

Cells were seeded into six-well plates at the concentration of 1.5×10^5 cells per well the day before transfection. All miRNA mimics used for *in vitro* studies were purchased from Ambion (Life Technologies, USA). These were transfected at various concentrations (see text) using Lipofectamine 2000 (Invitrogen™, Life Technologies USA) according to the manufacturer's instructions.

2.4.3. Western blotting

Total proteins from cells were extracted from cells using RIPA buffer (25mM Tris-HCl (pH 7.6), 150mM NaCl, 1% NP-40, 1% sodium deoxycholate) supplemented with 0.01% Protease and phosphatase inhibitor cocktail and 0.025% Na-deoxycholate10%. Brain proteins and total RNA was extracted using the mirVana PARIS kit (Ambion, Life Technologies USA). Protein lysates were separated by electrophoresis using 4-12% NuPAGE® precast gels and Tris-Acetate3-8% for protein more than 200kD (Life Technologies USA) and wet transferred onto a 0.45μm Nitrocellulose membrane (Bio-Rad, USA). GAPVD1 (k-22, cat#sc-133607) and SRM300 (H111, cat#sc-292291) antibodies were purchased from Santa Cruz Biotechnology (USA). Phospho-ERK1/2 (cat#9101), ERK1/2 (cat #9102), Nicastrin (D38F9, cat#5665), BACE1 (D10E5, cat#5606), and PEN2 (D2G6, cat#8502) antibodies were purchased from Cell signaling (USA). TAU1 (cat#MAB3420), GOAT ANTI-RAT IgG-HRP (#catAP136P), PRESENILIN-1 (PS1-loop, cat#MAB5232), AMYLOID-β (WO-2, cat#MABN10), and GAPDH

(cat#MAB374) antibodies were purchased from Millipore (USA). Other antibodies included: TAU total (cat#A0024, Dako, Denmark), TAU PHF13 (cat# ab24716, Abcam, UK), APP (cat#A8717, Sigma, USA), ALPHA-SYNUCLEIN (cat#PA1-18264, Thermo Fisher Scientifics, USA), and PEROXIDASE-CONJUGATED AFFINIPURE GOAT-ANTI MOUSE IgG (Jackson immuno research, USA). Images were acquired using Immobilon Western Chemiluminescent HRP Substrate (cat#WBKLS0050, Millipore, USA) and Fusion FX (Vilber Lourmat, Germany) imaging system.

2.4.4. qRT-PCR

Total RNA was reverse-transcribed to cDNA using iScript™ Reverse Transcription Supermix (Bio-Rad, Canada) according to the manufacturer's instructions. cDNA was used as template in qRT-PCR reaction performed using soFast EvaGreen Supermix (Bio-Rad, Canada). Primer sequences used were: Aif1 Forward: ATCAACAAGCAATTCCTCGATGA, Aif1 Reverse: CAGCATTCGCTTCAAGGACATA (Primer Bank ID 9506379a1); Tlr2 Forward: ACAACTTACCGAAACCTCAGAC, Tlr2 Reverse: ACCCCAGAAGCATCACATG; Bad Forward: TGAGCCGAGTGAGCAGGAA, Bad Reverse: GCCTCCATGATGACTGTTGGT; Gfap Forward: AGAGGGACAACTTTGCACAG (Primer Bank ID 6671610a1), Gfap Reverse: TCCAAATCCACACGAGCC; CD86 Forward: CTGGACTCTACGACTTCACAATG, CD86 Reverse: AGTTGGCGATCACTGACAGTT; Tau Forward: TGACACGGACGCTGGCCTGAA, Tau Reverse: CACTTGGAGGTCACCTTGCTC; APP Forward: CGAGAGAGAATGTCCCAGGT, APP Reverse: AGTTCTTGGCTTGACGCTCT; Bace1 Forward: CGTGTGGAAATCAATGGTCAAG, Bace1 Reverse: GACGGCAGCTTCAAATACTTTC; Ncstn Forward: TCCGTGGTACTGGCAGGATT Ncstn Reverse: CCCCTGTATCCCCACTAATTGA (Primer Bank ID 31981205a1). Relative expression was calculated by using the ΔΔCt method using a LightCycler 480 II (Roche). GAPDH was used as normalization control. For miRNA quantification, TaqMan® miRNA assays (Applied Biosystem, Canada) for miR-16 was used,

and relative levels were calculated using the ΔΔCt method against RNU19 as reference control [90].

2.4.5. Luciferase assays

The full-length hAPP, hBACE1 and hNicastrin 3'UTR luciferase constructs were described previously (Hebert et al., 2009; Shioya et al., 2010). Mutagenesis was performed by TOP gene Technologies Inc. (Montréal, Canada) and validated by sequencing. miRNA mimics (pre-miRs) with concentrations between 0-25nM, pRL Renilla (10ng) and pGL3 plasmids harboring 3′UTR of interest (500ng) were co-transfected using LipofectAMINE 2000 into HEK293 cells. Twenty-four hours after co-transfection, luciferase activities were measured by using a Dual-Glo Luciferase Assay System (Promega, USA) according to the manufacturer's protocol. Firefly luciferase activity was normalized to Renilla luciferase activity.

2.4.6. ELISA

Supernatants of Neuro2a-APPSwe/Δ9 cells were collected 48hrs post-transfection with miR-16 mimics or SCR control. Soluble (secreted) human Aβ1-40 and Aβ1-42 levels were measured by ELISA (cat #KHB3481 and KHB3441, Invitrogen, USA) following the manufacture's protocol.

2.4.7. *In vivo* administration of mimics

Mouse miR-16 mimics (CONmir® mimics) used for *in vivo* studies were purchased from Riboxx (Germany). All animal protocols were approved by the animal protection committee of the CHU de Québec. Wildtype mice (C57BL/6, female, 2 months old) were used in all experiments. Mice were maintained in a 12-hour light/12-hour dark cycle and received routine veterinary monitoring. The mini-pumps (ALZET® model 1007D) and brain infusion kits (cat#8663) were purchased from Direct (Denmark). Preoperative procedure included 30μl of Anafen (1 mg/ml), 100μl Marcaine (5.0 mg/ml), and 500μl saline (0.9%). ALZET® mini-osmotic pumps were implanted subcutaneously. Mimics were administrated into the brain using coordinates: ventricle A/P=-0.22 M/L=0.5 D/v=3.5) (12hrs per day) (0.5μl per hour with a concentration of 4.2μg/μl). During the post-operative procedure, mice were treated with 50μl Anafen (1mg/ml) and 500μl saline (0.9%).

2.4.8. Proteomics

Proteomics was performed by the proteomics platform of the Centre de recherche du CHU de Québec on two selected regions of brainstem and hippocampus with n=4 mice/region. Frozen tissues were disrupted using a mortar and pestle. Samples were kept frozen on dry-ice, and grind to fine powder. Then lysis buffer (50mM ammonium bicarbonate, 50mM DTT, 0.5% sodium deoxycholate) containing protease inhibitors cocktail (Roche Diagnostics GmbH, Germany) was added, and the sample preparation was homogenized on ice by sonication with a Sonic Dismembrator (Fisher) with 1 sec. pulse (20 times). Samples were centrifuged 10 min at 16000g. The supernatants were mixed with 5 volumes of acetone (stored at -20°C) and incubated overnight at -20°C. Precipitated proteins were centrifuged 15 min at 16000g. Protein pellets were air dry, and the resuspended in 0.5M triethylammonium bicarbonate (TEAB) – 0.5% sodium deoxycholate. Fifteen micrograms of protein for each group was used for iTRAQ labeling. Triethylammonium bicarbonate and sodium deoxycholate were added to each sample to reach a final concentration of 0.5 M and 0.5 %, respectively. Proteins were then reduced and alkylated according to the iTRAQ kit manufacturer's instruction (Applied Biosystems). Samples were digested with trypsin (Sequence grade Modified, Promega) using 1:30 ratio overnight at 37°C. After digestion, peptides were acidified to precipitate deoxycholate, and then purified with an oasis HLB cartridge (1cc, 10mg, Waters Corp USA.) and lyophilized. Dried peptides were dissolved in 30µl 0.5M TEAB and labeled with iTRAQ label reagent (Applied Biosystems). Four-plex labeling was performed for 2 h at room temperature in the dark. Labeled peptides were combined in one tube and dried with the SpeedVac. Samples were cleaned up using HLB cartridge (Waters Corp. USA). Samples were dried and reconstituted 200µl HPLC H_2O and 1/100 ampholytes pH 3-10 (Biorad). Then peptides were fractionated with 7cm IPG strips pH 3-10 using an isoelectric focusing method, and performed for 10,000 V h. IPG strips were cut in 14 fractions and peptides were extracted in 2% ACN -0.1%FA solution followed by 50% ACN-1% FA. Finally, fractions were dried with the SpeedVac. The proteins listed were the one considered to be differentially expressed and they were identified at a false discovery rate (FDR) less than 1% as estimated by a Protein Pilot tool using reverse database search

strategy and their iTRAQ ratios were <0.8 and >1.2 with a p-value lower than 0.05 as calculated by Protein Pilot based on two-tailed t-tests where the degree of freedom is equal to the number of distinct peptides minus one.

2.4.9. RNA Immunoprecipitation (RIP-Chip)

RIP immunoprecipitations were performed as described previously(Dorval et al., 2014; W.-X. Wang, Wilfred, Hu, Stromberg, & Nelson, 2010). Briefly, anti-AGO2 (2A8, cat# MABE56, Millipore (USA) and Control Mouse IgGs were coupled to Protein G Sepharose (GE Healthcare Bioscience). Brainstem and cortex tissues were homogenized in a lysis buffer (25 mM Tris-HCl pH8, 150 mM NaCl, 2 mM MgCl2, 0.5% Triton X-100, 5 mM DTT, 250 U/ml RNAsin and protease inhibitors). Proteins were transferred to a clean tube after high-speed centrifugation. Total lysate was pre-cleared by incubating with protein G alone and then separated into two fractions. These were incubated with either the antibody (AGO2) or Control IgG-coupled beads. Following washes (high salt buffer = lysis buffer at 900 mM NaCl and low Triton X-100 buffer = lysis buffer at 0,05% Triton X-100), proteins, including RNA-binding proteins, were eluted with sample buffer. Immunoprecipitated total RNA was extracted directly from the beads using Trizol (Invitrogen). MiR-16 was subjected to qRT-PCR analysis. Following the immunoprecipitation, the protein fraction was subjected to Western blot analysis (anti-AGO2 C34C6, cat#2897, Cell Signalling (USA) in order to validate the efficiency of Ago2 immunoprecipitation (data not shown).

2.4.10. Statistics

Statistical significance and normality were calculated using GraphPad version 6.0d software. Western blot images were analyzed by ImageJ V1.47 software (USA). Statistical significance was calculated by parametric unpaired t-test with Welch’s correction ($p<0.05$ considered as significant) and multiple comparisons was done using the Bonferroni method.

Conflict of Interest

The authors declare no conflict of interest.

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2.6. Supplementary data

Preclinical evaluation of miR-15/107 family members as multifactorial drug targets for Alzheimer's disease

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1) Supplementary Methods

2) Supplementary Figures and legends

3) Supplementary References

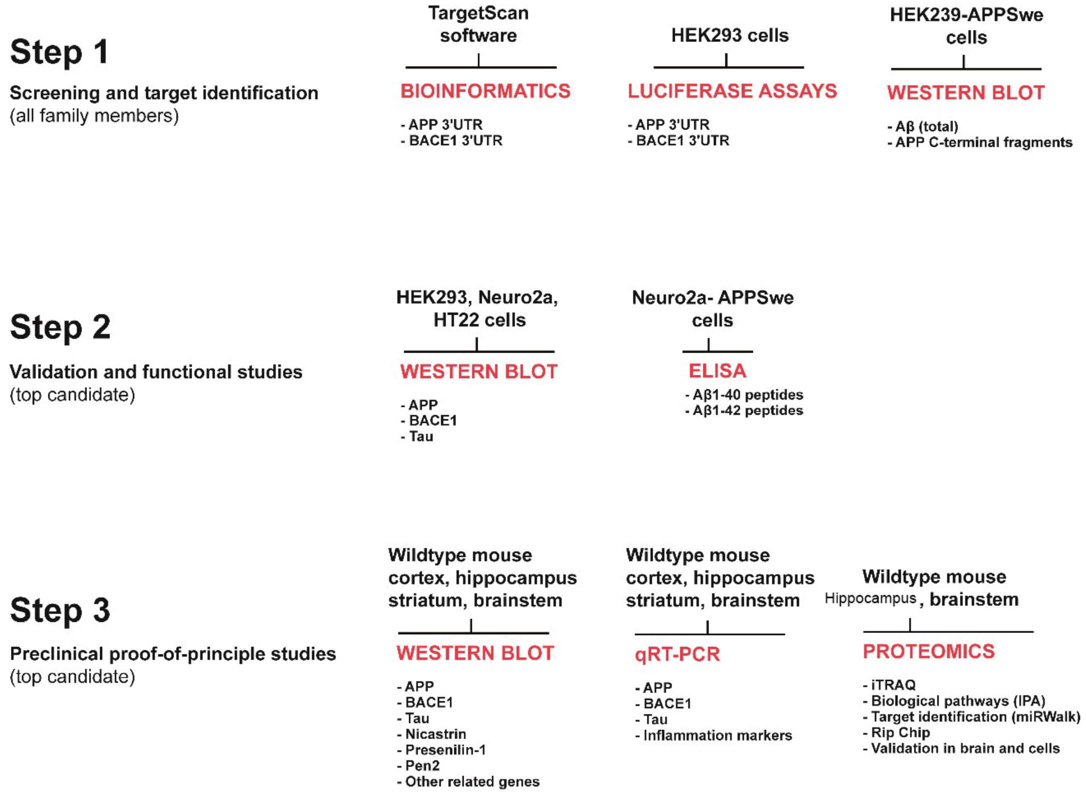
4) Supplementary Tables and legends

2.7. Supplementary Methods

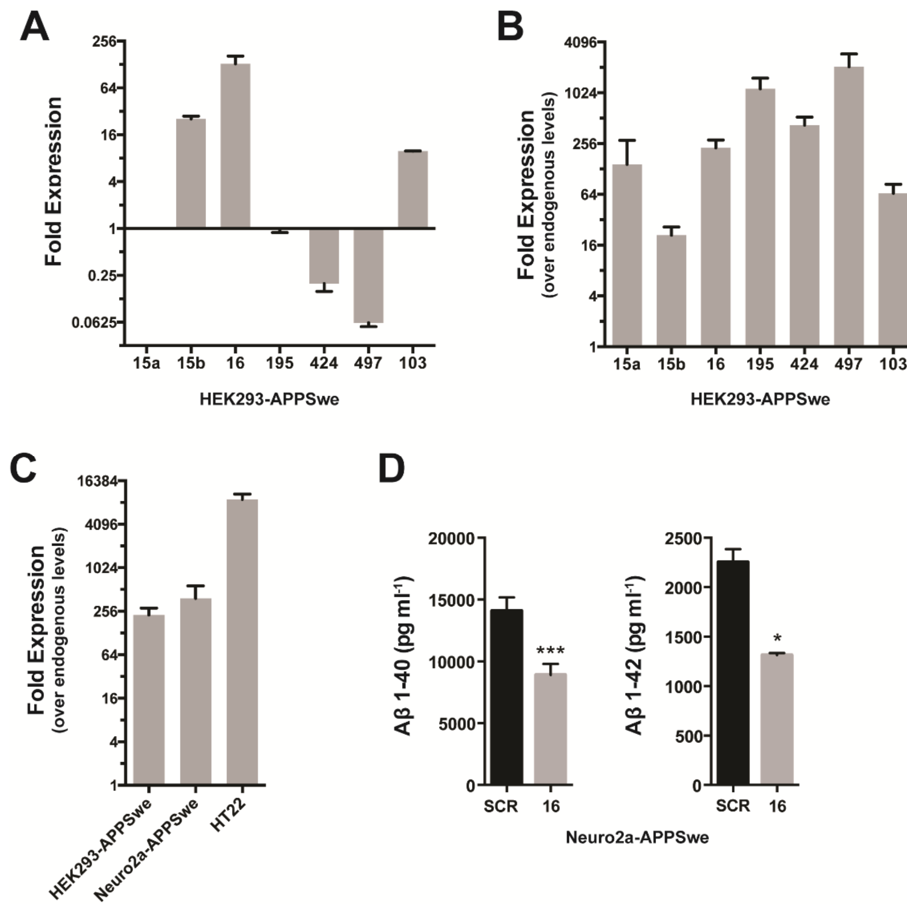
Patient information

All human and mouse studies were approved by the national ethical committee protocols and in agreement with the Université Laval ethical committee. Brain tissue from patients came from the Douglas-Bell Canada Brain Bank, Montreal, Canada, and included non-dementia controls and AD cases, based on neuropathological diagnosis. Patient information is available elsewhere [1, 2]. Blocks of tissue from the temporal cortex, prefrontal cortex and hippocampus were dissected and snap frozen in liquid nitrogen until use.

2.8. Supplementary Figures

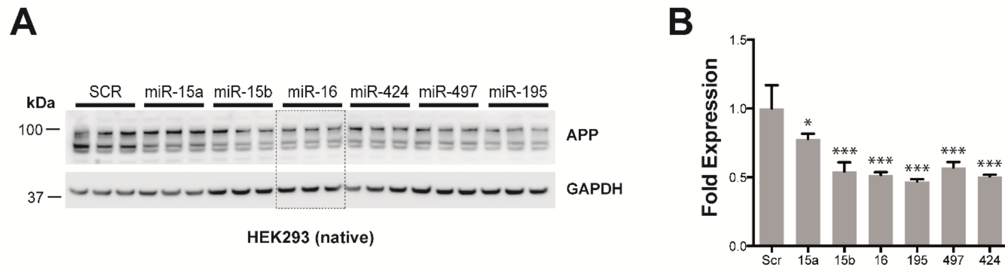


Supplementary Figure 1 ***Experimental overview of current study***



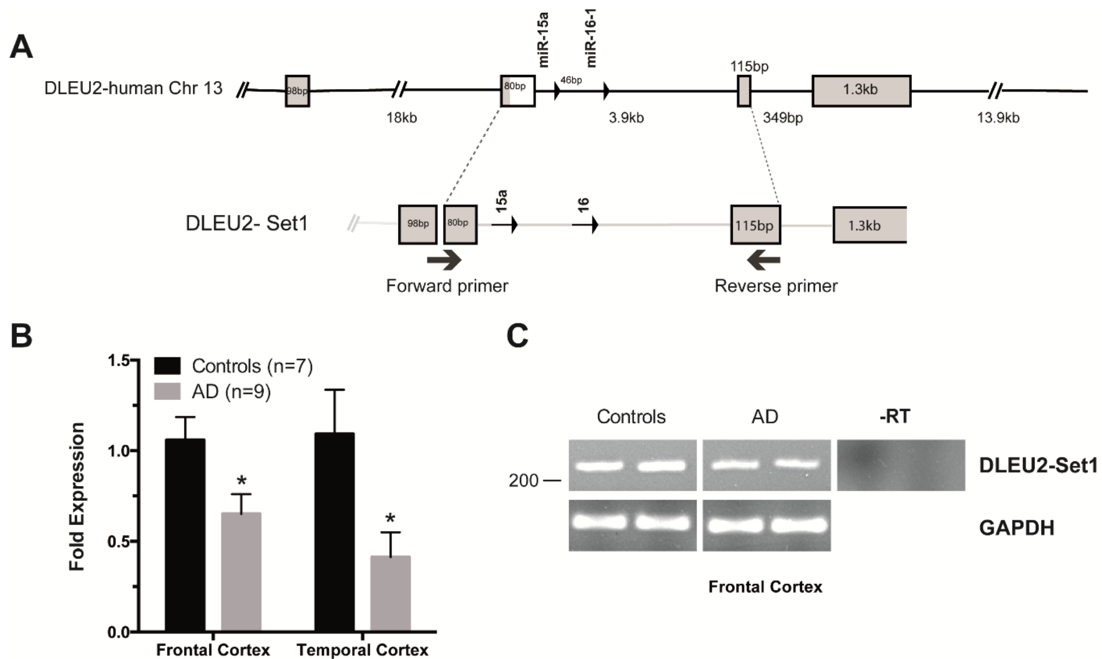
Supplementary Figure 2 ***miR-15a, miR-15b, miR-16, miR-195, miR-424, miR-497 and miR-103 expression level measurement post transfection in vitro compared to endogenous levels***

(A) *Endogenous miR-15a, miR-15b, miR-16, miR-195, miR-424, miR-497 and miR-103 levels were quantified by qRT-PCR in HEK293-APPSwe cells. U48 small nucleolar RNA (RNU48) was used as a normalizing control. The relative expression was calculated using the $\Delta\Delta$Ct method (using miR-15a as 1 fold).* ***(B)*** *qRT-PCR analysis of ectopic miR-16 family members following transfection in HEK293-APPSwe cells. Relative expression is shown (using endogenous miRNAs as 1 fold). RNU48 was used as normalization control.* ***(C)*** *qRT-PCR analysis of transfected miR-16 in various cells lines used in this study. Relative quantifications are shown (using endogenous miR-16 as 1 fold). RNU48 was used as normalization control.* ***(D)*** *ELISA of soluble Aβ40 and Aβ42 in Neuro2a-APPSwe cells transfected with miR-16 or SCR mimics. Measurements were done 48h post-transfection. Statistical significance was determined by a Student paired t-test (* = $p<0.05$, *** = $p<0.001$). All data are shown as mean± SEM from two or more independent experiments in triplicate.*



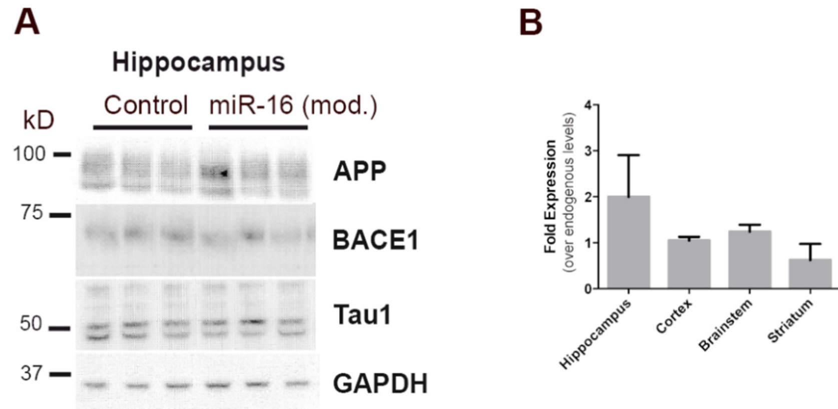
Supplementary Figure 3 ***miR-16 overexpression reduces APP levels in native HEK293 cells***

(A, B) *Western blot analysis of endogenous full-length APP in native HEK293 cells following mimic overexpression at 50 nM final concentration. Shown here are results at 48h post-transfection. Statistical significance was determined by a Student paired t-test (* = $p<0.05$, *** = $p<0.001$). Data are shown as mean± SEM from two experiments performed in triplicate. Quantifications are shown using Gapdh as a normalization control.*



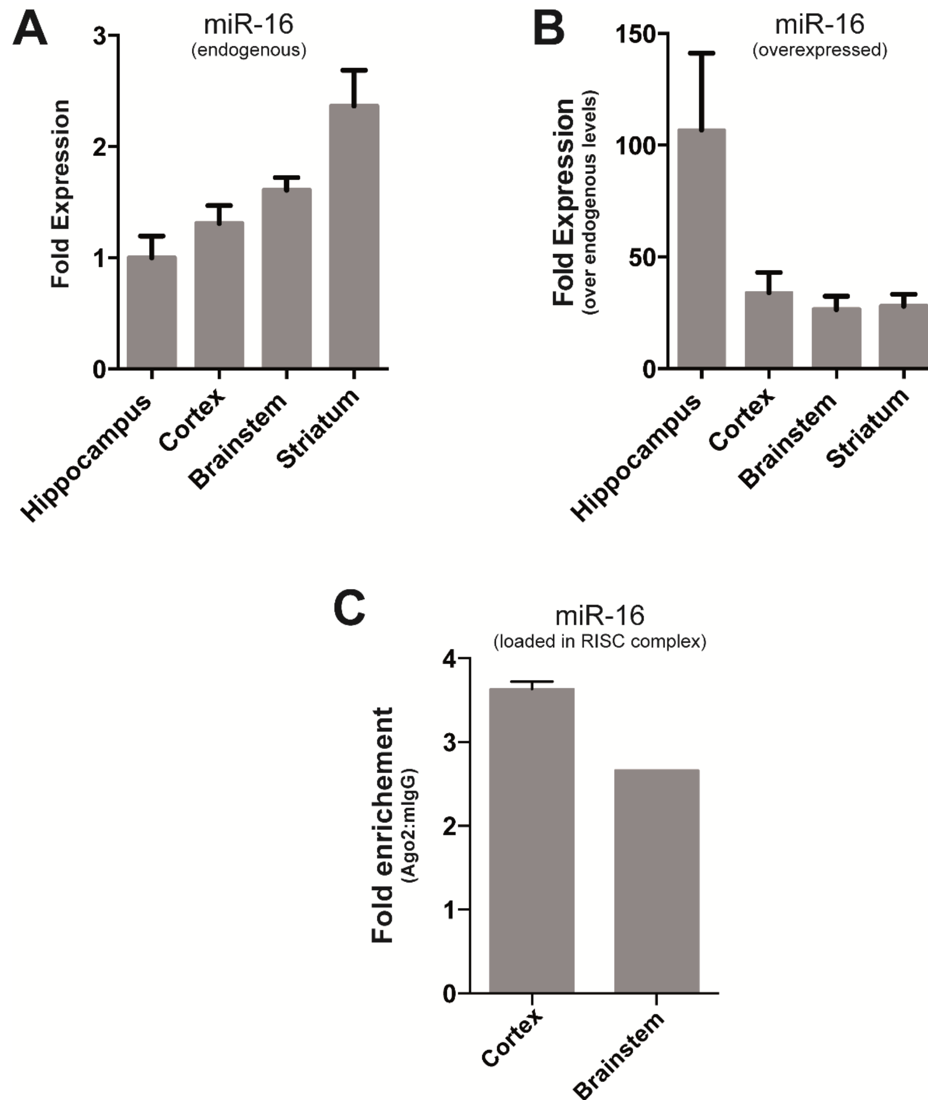
Supplementary Figure 4 **Downregulation of the miR-15a/16 cluster in AD.**

__(A)__ Schematic representation (not to scale) of the DLEU2 transcript encoding miR-15a and miR-16-1 (upper panel). Close-up of the amplified region (lower panel). Primer sequences are Forward: CTCAGCAATTCTTACCTTTCTTAC; Reverse: TTCCTGGATACTCTCCTGTAGTC. __(B)__ qRT-PCR of DLEU2 mRNA from non-demented Controls (N=7) and AD individuals (N=9). Relative expression is shown (using Controls as 1 fold). RNU48 was used as normalization control. All samples were measured in triplicate. Statistical significance was determined by a Mann-Whitney U test (= p<0.05). __(C)__ Validation by conventional PCR in frontal cortex tissue. Shown here are two control and two AD individuals. Minus (-) RT was used as PCR negative control.*



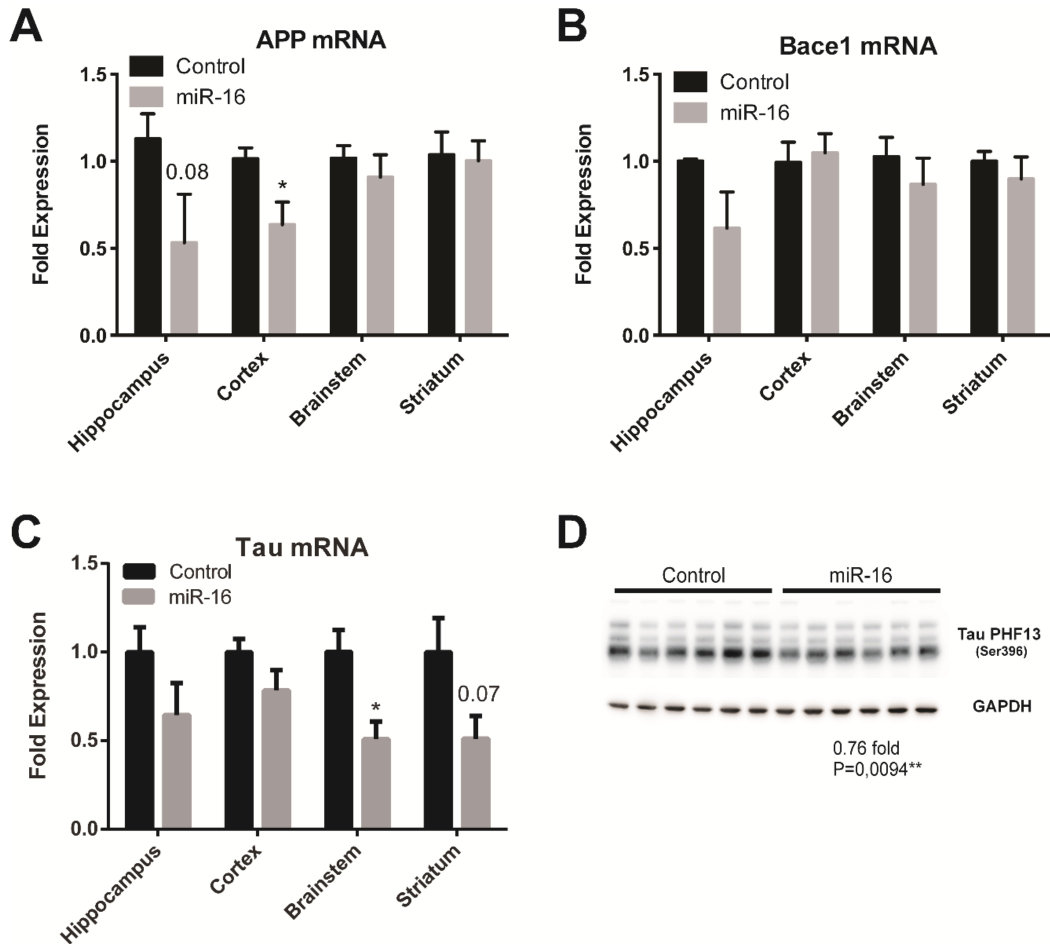
Supplementary Figure 5 ***Validation of miR-16 mimic specificity in vivo***

(A) *Representative western blot analysis of mice treated with chemically modified miR-16 mimics (miR-16 mod.), harboring 2O-Me modifications on both sense and antisense strands (50μg/day for 7 days, n=8 mice/group). Shown here is the hippocampus. Similar negative results were obtained in the cortex, brainstem, and striatum. Control mice received vehicle alone (0.9% saline)* ***(B)*** *qRT-PCR analysis of miR-16 mod. -treated mice. These results indicate that miR-16 expression levels are not significantly increased following treatment (n=8/group), consistent with the notion that modified mimics are not functional. Data are shown as mean± SEM.*



Supplementary Figure 6 ***miR-16 Levels in vivo-Ctrls vs injected***

(A) *Levels of endogenous miR-16 in the different brain regions of control mice (i.e., baseline levels). These experiments were performed from control (saline) treated mice.* ***(B)*** *qRT-PCR analysis showing ectopic miR-16 expression and distribution following mimic delivery. These results indicated a strong increase in miR-16 levels (over endogenous levels) in the hippocampus (106 fold), cortex (34 fold), striatum (27 fold), and brainstem (27 fold).* ***(C)*** *RIP-Chip was performed on cortex and brainstem of miR-16 mimic-treated mice (n=3/group). We observed a 3.63 and 2.66-fold enrichment in the cortex and brainstem, respectively, compared to controls. Control mouse IgGs served as a normalization control. Data are shown as mean± SEM.*



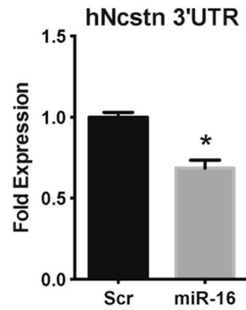
Supplementary Figure 7 ***Endogenous APP, BACE1, and Tau mRNA levels following miR-16 mimic treatment***

(A-C) *mRNA levels were measured by qRT-PCR (n=6/group). Statistical significance was assessed by parametric unpaired t-test with Welch's correction, P<0.05 considered as statistically significant. GAPDH served as a normalization control. Data are shown as mean± SEM.* ***(D)*** *Representative western blot of endogenous Tau (PHF1 epitope) following miR-16 mimic treatment (N=8/group). Blots were normalized to Gapdh. Statistical significance was determined by a Student paired t test.*

A

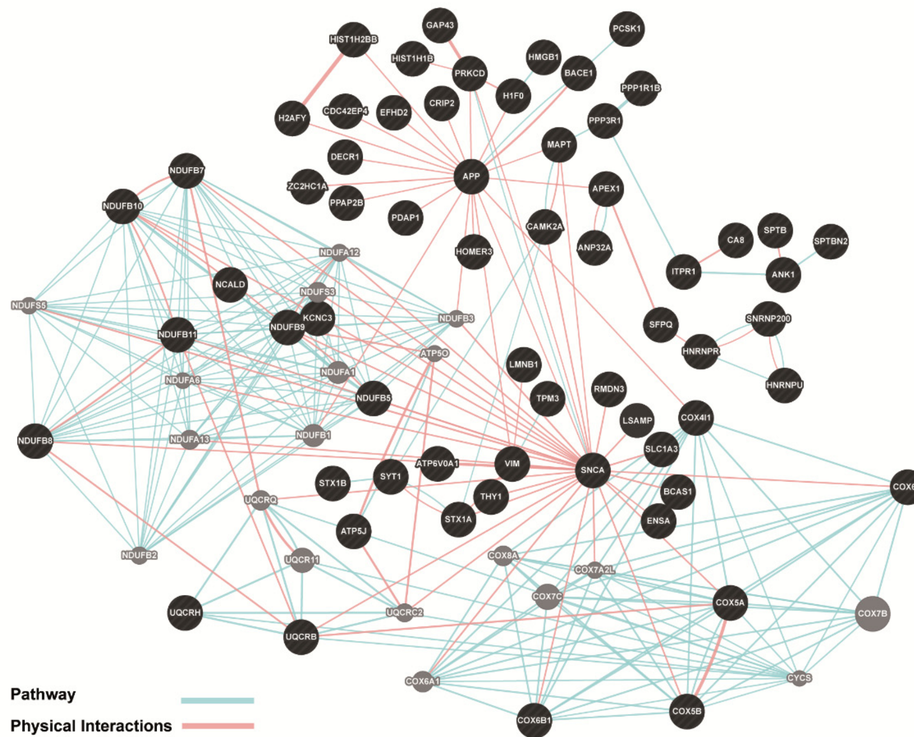
Gene	MicroRNA	DIANAmT	miRanda	miRDB	miRWalk	RNAhybrid	PICTAR5	PITA	RNA22	Targetscan	SUM
NCSTN	hsa-miR-16	1	0	0	0	0	0	0	1	1	3
Ncstn	mmu-miR-16	1	1	0	1	0	1	0	0	1	5

B



Supplementary Figure 8 ***Nicastrin is directly regulated by miR-16***

(A) *Comparative bioinformatics analysis of putative miR-16 binding sites within the mouse or human Nicastrin 3'UTR. Results were taken from the miRWalk program.* ***(B)*** *Luciferase assay on wildtype human Nicastrin 3'UTR co-transfected with 50nM mimics (SCR or miR-16) in HEK293T cells. The cells were lysed 24hrs post-transfection and luciferase signal was measured (n=2 in triplicate). Statistical significance was assessed by one-way ANOVA with Bonferroni post-test (*p < 0.05).*



Supplementary Figure 9 ***Physical interaction networks between putative miR-16 targets in vivo***

The analysis was performed using the Germania software. Both up- and down-regulated proteins were used in these analyses. Black nodes indicate proteins identified in our proteomics analysis. Grey nodes indicate additional putative binding partners in these pathways based on bioinformatics.

Supplementary References

1. Smith, P, Al Hashimi, A, Girard, J, Delay, C, and Hebert, SS (2011). In vivo regulation of amyloid precursor protein neuronal splicing by microRNAs. *Journal of neurochemistry* 116: 240-247.

2. Hebert, SS, Papadopoulou, AS, Smith, P, Galas, MC, Planel, E, Silahtaroglu, AN*, et al.* (2010). Genetic ablation of Dicer in adult forebrain neurons results in abnormal tau hyperphosphorylation and neurodegeneration. *Human molecular genetics* 19: 3959-3969.

2.10. Supplementary Tables

Supplementary Table 1 ***Protein changes in the brainstem and hippocampus of treated mice versus controls.***

A total of 103 proteins were significantly changed in the brainstem, including 47 upregulated (in red) and 55 downregulated (in green) proteins. A total of 16 proteins were misregulated in the hippocampus, including 5 upregulated (in red) and 11 downregulated (in green) proteins (fold change <0.8 and >1.2, P <0.05). Bioinformatics predictions were done by miRWalk.

Brainstem								Predicted miR-16 target site	
No Accession	Gene name	Description	Score	95% Coverage	Number of peptides	RATIO mimics vs controls (brainstem)	p-value	3'UTR	Coding region
Q91XV3	**BASP1**	BASP1_MOUSE Brain acid soluble protein 1	82.29	93%	106	0.501	1.65E-07		
O35526	**STX1A**	STX1A_MOUSE Syntaxin-1A	24.07	56%	19	0.586	8.92E-06		
P19536	**COX5B**	COX5B_MOUSE Cytochrome c oxidase subunit 5B, mitochondrial	21.66	53%	16	0.603	0.00121697		
P12787	**COX5A**	COX5A_MOUSE Cytochrome c oxidase subunit 5A, mitochondrial	39.45	69%	33	0.614	0.00016062		
Q53YX2	**Thy1**	Q53YX2_MOUSE CD90.1	12.01	33%	19	0.618	0.00277828		
Q1MX42	**Prkcd**	Q1MX42_MOUSE Protein kinase C delta type	7.59	9%	5	0.65	0.0326449		
Q3TPT3	**Syt1**	Q3TPT3_MOUSE Putative uncharacterized protein	53.45	58%	48	0.653	9.97E-05		
A2AQ25-4	**Skt**	A2AQ25-4 Isoform 4 of Sickle tail protein	11.19	7%	8	0.673	0.00476251		
Q9D6J5	**Ndufb8**	NDUB8_MOUSE NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial	10	38%	9	0.674	0.00526244		
Q4VBC9	**Ndufb11**	Q4VBC9_MOUSE Ndufb11 protein (Fragment)	10.71	41%	6	0.679	0.0095815		
Q9CPQ1	**COX6C**	COX6C_MOUSE Cytochrome c oxidase subunit 6C	17.85	63%	11	0.685	0.00020333		
P56391	**Cox6b1**	CX6B1_MOUSE Cytochrome c oxidase subunit 6B1	13.62	70%	20	0.685	0.00171406		
Q9D855	**QCR7**	QCR7_MOUSE Cytochrome b-c1 complex subunit 7	17.77	57%	15	0.695	0.00015351		
P31650	**Slc6a11**	S6A11_MOUSE Sodium- and chloride-dependent GABA transporter 3	20.84	16%	12	0.698	0.00502416		
Q9JKC6	**Cend1**	CEND_MOUSE Cell cycle exit and neuronal differentiation protein 1	15.54	57%	19	0.7	0.00042347		
Q60829	**Ppp1r1b**	PPR1B_MOUSE Protein phosphatase 1 regulatory subunit 1B	18	64%	12	0.702	0.004223		
P19783	**Cox4i1**	COX41_MOUSE Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	14.86	43%	19	0.706	0.0155639		
P06837	**Gap43**	NEUM_MOUSE Neuromodulin	36.36	71%	32	0.708	5.42E-07		
Q9CQH3	**Ndufb5**	NDUB5_MOUSE NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5, mitochondrial	10	21%	6	0.711	0.0077551		
Q6ZWX2	**Tmsb4x**	Q6ZWX2_MOUSE Thymosin, beta 4, X chromosome	12.85	77%	11	0.712	0.0218587		
Q9CR61	**Ndufb7**	NDUB7_MOUSE NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7	14.31	58%	8	0.718	0.0129444		

Q8BGZ1	**Hpcal4**	HPCL4_MOUSE Hippocalcin-like protein 4	18.91	81%	23	0.722	0.00098537		
Q9DCT8	**CRIP2**	CRIP2_MOUSE Cysteine-rich protein 2	10.28	39%	7	0.723	0.0114881		
Q8BLF7	**Slc2a3**	Q8BLF7_MOUSE Putative uncharacterized protein	16.87	13%	11	0.731	0.00105785		
P11798	**Camk2a**	KCC2A_MOUSE Calcium/calmodulin-dependent protein kinase type II subunit alpha	59.38	50%	75	0.731	0.00178941		
Q9QXV0	**PCSK1**	PCSK1_MOUSE ProSAAS	16.38	47%	12	0.732	0.0007289		
Q91X97	**NCALD**	NCALD_MOUSE Neurocalcin-delta	14.01	74%	25	0.735	0.0215997		
Q99JY8	**LPP3**	LPP3_MOUSE Lipid phosphate phosphohydrolase 3	11.31	17%	8	0.74	0.0151303		
Q80YN3	**BCAS1**	BCAS1_MOUSE Breast carcinoma-amplified sequence 1 homolog	30.42	27%	23	0.743	1.08E-05		
Q3TYE5	**Lsamp**	Q3TYE5_MOUSE Limbic system-associated membrane protein	20.73	36%	13	0.749	0.0426762		
P60840	**ENSA**	ENSA_MOUSE Alpha-endosulfine	13.51	70%	12	0.749	0.00174027		
P99028	**QCR6**	QCR6_MOUSE Cytochrome b-c1 complex subunit 6, mitochondrial	12.35	60%	8	0.756	0.0278268		
Q3UHX2	**Pdap1**	HAP28_MOUSE 28 kDa heat- and acid-stable phosphoprotein	12.59	34%	9	0.757	0.00129728		
P51830	**ADCY9**	ADCY9_MOUSE Adenylate cyclase type 9	6.95	4%	5	0.759	0.0321568		
O54983	**CRYM**	CRYM_MOUSE Thiomorpholine-carboxylate dehydrogenase	27.14	51%	23	0.761	0.0168747		
P97450	**ATP5J**	ATP5J_MOUSE ATP synthase-coupling factor 6, mitochondrial	13.72	56%	12	0.763	0.00373859		
P63321	**RALA**	RALA_MOUSE Ras-related protein Ral-A	16	43%	11	0.765	0.0349347		
Q8BLE7	**Slc17a6**	VGLU2_MOUSE Vesicular glutamate transporter 2	12.05	17%	8	0.765	0.00936656		
Q9CQZ1	**HSBP1**	HSBP1_MOUSE Heat shock factor-binding protein 1	8	80%	6	0.765	0.0386879		
O55042	**Snca**	SYUA_MOUSE Alpha-synuclein	26.02	91%	31	0.766	0.0013887		
Q63810-2	**Ppp3r1**	Q63810-2 Isoform 2 of Calcineurin subunit B type 1	26.06	77%	28	0.769	0.00459909		
Q9D8B3	**Chmp4b**	CHM4B_MOUSE Charged multivesicular body protein 4b	10.14	29%	9	0.77	0.0217823		
Q03517	**SCG2**	SCG2_MOUSE Secretogranin-2	46.68	48%	26	0.775	2.77E-07		
Q9DCS9	**Ndufb10**	NDUBA_MOUSE NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	18.01	57%	11	0.777	0.0128557		
Q9Z1G4-3	**Atp6v0a1**	Q9Z1G4-3 Isoform A1-III of V-type proton ATPase 116 kDa subunit a isoform 1	47.89	30%	42	0.783	5.00E-07		
B1AS06	**Dlgap3**	B1AS06_MOUSE Disks large-associated protein 3	5.9	5%	5	0.785	0.0338111		
Q8C845	**Efhd2**	Q8C845_MOUSE EF-hand domain-containing protein D2	24.4	51%	18	0.788	0.00030761		
P31324	**KAP3**	KAP3_MOUSE cAMP-dependent protein kinase type II-beta regulatory subunit	35.3	52%	18	0.788	0.00215375		
Q8BJH1	**Zc2hc1a**	ZC21A_MOUSE Zinc finger C2HC domain-containing protein 1A	12.01	19%	7	0.79	0.0404972		
Q9ER00	**STX12**	STX12_MOUSE Syntaxin-12	15.15	35%	11	0.79	0.0119414		
P61264	**STX1B**	STX1B_MOUSE Syntaxin-1B	43.3	56%	38	0.79	1.78E-05		

Q9CQJ8	**Ndufb9**	NDUB9_MOUSE NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9	16.19	48%	10	0.792	0.0217948		
Q3U2J2	**Slc2a1**	Q3U2J2_MOUSE Putative uncharacterized protein	10.01	11%	5	0.793	0.0463827		
Q58E70	**Tpm3**	Q58E70_MOUSE Tpm3 protein	18	49%	22	0.795	0.0242466		
A2A7R6	**Hpca**	A2A7R6_MOUSE Neuron-specific calcium-binding protein hippocalcin (Fragment)	36.76	79%	31	0.795	0.0258137		
Q8BTS0	**Ddx5**	Q8BTS0_MOUSE DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	45.74	44%	29	1.199	0.0023451		
Q9Z1N5	**Ddx39b**	DX39B_MOUSE Spliceosome RNA helicase Ddx39b	27.42	35%	17	1.2	0.00233304		
Q8VIJ6	**Sfpq**	SFPQ_MOUSE Splicing factor, proline- and glutamine-rich	49.54	42%	35	1.202	0.00010903		
Q8VDN2	**Atp1a1**	AT1A1_MOUSE Sodium/potassium-transporting ATPase subunit alpha-1	66.56	52%	159	1.203	9.79E-07		
Q9CT37	**Hnrnpr**	Q9CT37_MOUSE Putative uncharacterized protein (Fragment)	12.01	31%	10	1.211	0.0458658		
Q00PI9	**Hnrnpul2**	HNRL2_MOUSE Heterogeneous nuclear ribonucleoprotein U-like protein 2	27.12	26%	17	1.213	0.00892075		
Q02357-5	**Ank1**	Q02357-5 Isoform 5 of Ankyrin-1	35.26	19%	29	1.223	2.29E-05		
Q9QZQ8-2	**H2afy**	Q9QZQ8-2 Isoform 1 of Core histone macro-H2A.1	26.07	38%	15	1.226	0.00200346		
Q3UTJ2	**Sorbs2**	SRBS2_MOUSE Sorbin and SH3 domain-containing protein 2	15.78	8%	10	1.231	0.0271742		
Q497Z6	**Hmgb1**	Q497Z6_MOUSE High mobility group box 1	15.14	37%	14	1.233	0.0157343		
Q8K0S0	**Phyhip**	PHYIP_MOUSE Phytanoyl-CoA hydroxylase-interacting protein	19.24	42%	15	1.233	0.00591662		
Q8BTM8	**Flna**	FLNA_MOUSE Filamin-A	31.45	12%	23	1.24	0.0234162		
Q8R384	**Myh11**	Q8R384_MOUSE Myh11 protein	32.51	21%	38	1.24	0.00372456		
Q8BH44	**Coro2b**	COR2B_MOUSE Coronin-2B	23.59	31%	13	1.241	0.00286535		
Q6P4T2	**Snrnp200**	Q6P4T2_MOUSE Activating signal cointegrator 1 complex subunit 3-like 1	16.42	7%	13	1.242	0.0415583		
O35381	**Anp32a**	AN32A_MOUSE Acidic leucine-rich nuclear phosphoprotein 32 family member A	20.06	31%	15	1.242	0.0113832		
Q9JMF3	**Gng13**	GBG13_MOUSE Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-13	6.61	46%	5	1.252	0.0364961		
Q9CQ62	**Decr1**	DECR_MOUSE 2,4-dienoyl-CoA reductase, mitochondrial	14.11	24%	7	1.255	0.0164508		
Q3UJU9	**Rmdn3**	RMD3_MOUSE Regulator of microtubule dynamics protein 3	13.61	22%	9	1.258	0.0129001		
Q3UGY4	**Sptb**	Q3UGY4_MOUSE Putative uncharacterized protein	71.35	29%	57	1.262	6.69E-06		
A2A5R2	**Arfgef2**	BIG2_MOUSE Brefeldin A-inhibited guanine nucleotide-exchange protein 2	8.74	4%	6	1.264	0.0346823		
G3XA10	**Hnrnpu**	G3XA10_MOUSE Heterogeneous nuclear ribonucleoprotein U	36.21	31%	25	1.264	5.20E-05		
D3YZV4	**Kcnc3**	D3YZV4_MOUSE Potassium voltage-gated channel subfamily C member 3	11.6	13%	6	1.28	0.0275812		
P14733	**Lmnb1**	LMNB1_MOUSE Lamin-B1	46.05	44%	28	1.285	2.81E-06		
Q68FG2	**sptbn2**	Q68FG2_MOUSE Protein Sptbn2	171.9	58%	139	1.286	1.17E-16		
E0CXN5	**Gpd1**	E0CXN5_MOUSE Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	44.7	68%	25	1.29	0.00073079		
Q3UHH0	**Atp2b2**	Q3UHH0_MOUSE Putative uncharacterized protein	80.6	39%	64	1.292	3.26E-07		
Q80VD1	**Fam98b**	FA98B_MOUSE Protein FAM98B	9.85	17%	5	1.297	0.0259779		
Q3U4Y0	**H1f0**	Q3U4Y0_MOUSE Putative uncharacterized protein	12	27%	9	1.297	0.041291		
P20152	**Vim**	VIME_MOUSE Vimentin	68.44	75%	60	1.316	5.50E-06		
Q9WVK4	**Ehd1**	EHD1_MOUSE EH domain-containing protein 1	33.52	62%	29	1.333	0.000633		
Q8C413	**Dgkg**	Q8C413_MOUSE Diacylglycerol kinase gamma	18.4	19%	10	1.347	0.00102144		
P28352	**Apex1**	APEX1_MOUSE DNA-(apurinic or apyrimidinic site) lyase	16.05	38%	10	1.466	0.0323024		
UPI00000E8E77		unknown [Mus musculus [10090]]	12.65	25%	8	1.512	0.00056015		
P32848	**Pvalb**	PRVA_MOUSE Parvalbumin alpha	20	77%	12	1.518	0.00019895		
Q99NG1	**Slc1a3**	Q99NG1_MOUSE Glutamate/aspartate transporter (Fragment)	21.1	22%	36	1.532	0.0213031		
Q9JM96	**Cdc42ep4**	BORG4_MOUSE Cdc42 effector protein 4	10	16%	5	1.549	0.0279819		
P43276	**Hist1h1b**	H15_MOUSE Histone H1.5	8.21	19%	5	1.714	0.02542		
Q64475	**Hist1h2bb**	H2B1B_MOUSE Histone H2B type 1-B	16.08	43%	34	1.722	0.0243785		
Q6ZQK5-2	**Acap2**	Q6ZQK5-2 Isoform 2 of Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 2	6.39	7%	6	1.731	0.0222356		
Q8BQ28	**Nck2**	Q8BQ28_MOUSE Putative uncharacterized protein	4.96	11%	3	1.735	0.0211423		
P37804	**Tagln**	TAGL_MOUSE Transgelin	18.77	52%	11	1.751	0.00025513		
P12658	**Calb1**	CALB1_MOUSE Calbindin	25.76	67%	24	2.202	4.99E-08		
P11881-8	**Itpr1**	P11881-8 Isoform 8 of Inositol 1,4,5-trisphosphate receptor type 1	71.39	19%	48	3.208	6.43E-19		
Q99JP6-2	**Homer3**	Q99JP6-2 Isoform 2 of Homer protein homolog 3	9.84	22%	6	3.396	0.00010987		
Q3TXM3	**Slc1a6**	Q3TXM3_MOUSE Putative uncharacterized protein	8	17%	10	3.784	0.00077531		
P28651	**Ca8**	CAH8_MOUSE Carbonic anhydrase-related protein	12.02	33%	6	4.709	6.54E-05		

Hippocampus								**Predicted miR-16 target site**	
No Accession	**gene name**	**Description**	**Score**	**95% Converage**	**Number of peptides**	**RATIO mimics vs. control (hippocampus)**	**p-value**	**3'UTR**	**Coding region**
P31725	**S100a9**	S10A9_MOUSE Protein S100-A9	6	30%	4	0.471	0.0039529		
Q8C872	**Tfrc**	Q8C872_MOUSE Transferrin receptor protein 1	4.39	7%	3	0.666	0.0373557		
Q8K0E8	**Fgb**	FIBB_MOUSE Fibrinogen beta chain	15	19%	8	0.671	0.0414647		
Q8C8M3	**Stat1**	Q8C8M3_MOUSE Putative uncharacterized protein	10.44	13%	7	0.714	0.00461793		
Q99K47	**Fga**	Q99K47_MOUSE Fibrinogen, alpha polypeptide	10.34	11%	5	0.736	0.00496785		
F6X7Z3	**Cpeb1**	F6X7Z3_MOUSE Cytoplasmic polyadenylation element-binding protein 1 (Fragment)	5.69	13%	3	0.747	0.0253883		
Q9QYT9	**Prnpb**	Q9QYT9_MOUSE Major prion protein	8.41	22%	7	0.768	0.030742		
Q6PAR5-6	**Gapvd1**	Q6PAR5-6 Isoform 6 of GTPase-activating protein and VPS9 domain-containing protein 1	8.01	3%	5	0.771	0.0382554		
Q91X72	**Hpx**	HEMO_MOUSE Hemopexin	14.38	21%	7	0.78	0.00303796		
Q3UVL4	**Vps51**	VPS51_MOUSE Vacuolar protein sorting-associated protein 51 homolog	13.57	13%	8	0.785	0.0111839		
Q8BTI8	**Srrm2**	SRRM2_MOUSE Serine/arginine repetitive matrix protein 2	3.29	1%	4	0.798	0.0231391		
Q80TQ3	**Nefh**	Q80TQ3_MOUSE MKIAA0845 protein (Fragment)	68.1	34%	61	1.306	0.00336826		
P07309	**Ttr**	TTHY_MOUSE Transthyretin	8	30%	5	1.411	0.0244789		
P28651	**Ca8**	CAH8_MOUSE Carbonic anhydrase-related protein	12.02	33%	6	1.469	0.0400057		
Q925J6	**Ptprn**	Q925J6_MOUSE Protein tyrosine phosphatase receptor type N (Fragment)	5.24	4%	3	1.525	0.0386948		
Q3TCQ7	**Tsc2**	Q3TCQ7_MOUSE Putative uncharacterized protein	3.49	2%	4	1.799	0.0208798		

Supplementary Table 2 ***DAVID gene enrichment analysis of top ranked targets identified in brainstem using Homo sapiens background.***

Category	Term	Count	%	P-Value	Benjamini
KEGG_PATHWAY	Alzheimer's disease	14	15.1	5.1E-10	3.0E-8
	Parkinson's disease	12	12.9	6.3E-9	1.9E-7
	Oxidative phosphorylation	12	12.9	7.5E-9	1.5E-7
	Huntington's disease	12	12.9	2.2E-7	3.3E-6
	Cardiac muscle contraction	7	7.5	4.8E-5	5.6E-4
	SNARE interactions in vesicular transport	3	3.2	4.2E-2	3.5E-1
	GnRH signaling pathway	4	4.3	5.2E-2	3.6E-1
	Calcium signaling pathway	5	5.4	6.4E-2	3.9E-1
	Oocyte meiosis	4	4.3	6.9E-2	3.7E-1
	Vascular smooth muscle contraction	4	4.3	7.2E-2	3.6E-1

Category	Term	Count	%	P-Value	Benjamini
GOTERM_BP_FAT	oxidative phosphorylation	8	8.6	1.7E-6	1.7E-3
	respiratory electron transport chain	7	7.5	2.0E-6	9.8E-4
	generation of precursor metabolites and energy	12	12.9	2.2E-6	7.0E-4
	electron transport chain	8	8.6	4.8E-6	1.2E-3
	mitochondrial ATP synthesis coupled electron transport	6	6.5	1.9E-5	3.7E-3
	ATP synthesis coupled electron transport	6	6.5	1.9E-5	3.7E-3
	cellular respiration	7	7.5	2.3E-5	3.7E-3
	mitochondrial electron transport, NADH to ubiquinone	5	5.4	1.1E-4	1.5E-2
	neurotransmitter transport	6	6.5	1.3E-4	1.5E-2
	regulation of cellular component biogenesis	7	7.5	1.9E-4	2.0E-2
	energy derivation by oxidation of organic compounds	7	7.5	2.1E-4	2.0E-2
	regulation of synaptic transmission	6	6.5	1.3E-3	1.0E-1
	oxidation reduction	12	12.9	1.3E-3	9.9E-2
	negative regulation of cellular component organization	6	6.5	1.5E-3	1.1E-1
	regulation of transmission of nerve impulse	6	6.5	1.8E-3	1.2E-1
	regulation of protein complex assembly	5	5.4	2.0E-3	1.2E-1
	regulation of neurological system process	6	6.5	2.1E-3	1.2E-1
	regulation of system process	8	8.6	2.3E-3	1.2E-1
	cellular macromolecular complex assembly	8	8.6	2.7E-3	1.4E-1
	regulation of cellular localization	7	7.5	3.5E-3	1.6E-1
	regulation of actin filament polymerization	4	4.3	4.0E-3	1.8E-1
	cellular macromolecular complex subunit organization	8	8.6	5.1E-3	2.1E-1
	regulation of actin polymerization or depolymerization	4	4.3	5.6E-3	2.2E-1
	regulation of actin filament length	4	4.3	6.1E-3	2.3E-1
	regulation of synaptic plasticity	4	4.3	6.4E-3	2.3E-1
	regulation of neurotransmitter secretion	3	3.2	6.7E-3	2.3E-1
	vesicle-mediated transport	10	10.8	6.8E-3	2.2E-1
	regulation of secretion	6	6.5	6.9E-3	2.2E-1
	regulation of neurotransmitter levels	4	4.3	7.3E-3	2.2E-1
	regulation of protein polymerization	4	4.3	7.6E-3	2.2E-1
	synaptic transmission	7	7.5	8.4E-3	2.4E-1
	regulation of neurotransmitter transport	3	3.2	1.0E-2	2.7E-1
	positive regulation of transport	6	6.5	1.0E-2	2.7E-1
	actin cytoskeleton organization	6	6.5	1.1E-2	2.7E-1
	purine nucleotide biosynthetic process	5	5.4	1.1E-2	2.8E-1
	negative regulation of actin filament polymerization	3	3.2	1.2E-2	2.8E-1
	protein stabilization	3	3.2	1.2E-2	2.8E-1
	nitrogen compound biosynthetic process	7	7.5	1.2E-2	2.9E-1
	negative regulation of organelle organization	4	4.3	1.3E-2	2.8E-1
	negative regulation of protein polymerization	3	3.2	1.3E-2	2.8E-1
	nucleosome assembly	4	4.3	1.3E-2	2.9E-1
	actin filament-based process	6	6.5	1.4E-2	2.9E-1
	cytoskeleton organization	8	8.6	1.5E-2	2.9E-1
	chromatin assembly	4	4.3	1.5E-2	2.9E-1
	ATP biosynthetic process	4	4.3	1.6E-2	3.0E-1
	regulation of actin cytoskeleton organization	4	4.3	1.6E-2	3.0E-1
	macromolecular complex assembly	10	10.8	1.6E-2	3.0E-1
	protein-DNA complex assembly	4	4.3	1.7E-2	3.0E-1
	regulation of exocytosis	3	3.2	1.7E-2	3.0E-1
	negative regulation of protein complex assembly	3	3.2	1.7E-2	3.0E-1
	regulation of actin filament-based process	4	4.3	1.7E-2	3.0E-1
	transmission of nerve impulse	7	7.5	1.7E-2	3.0E-1
	nucleosome organization	4	4.3	1.8E-2	3.0E-1
	regulation of vesicle-mediated transport	4	4.3	1.9E-2	3.1E-1
	sensory perception of sound	4	4.3	2.0E-2	3.1E-1
	purine ribonucleoside triphosphate biosynthetic process	4	4.3	2.0E-2	3.2E-1
	purine nucleoside triphosphate biosynthetic process	4	4.3	2.1E-2	3.2E-1
	ribonucleoside triphosphate biosynthetic process	4	4.3	2.1E-2	3.2E-1
	regulation of cellular component size	6	6.5	2.2E-2	3.3E-1
	nucleoside triphosphate biosynthetic process	4	4.3	2.2E-2	3.3E-1
	sensory perception of mechanical stimulus	4	4.3	2.3E-2	3.3E-1
	positive regulation of neurotransmitter secretion	2	2.2	2.3E-2	3.3E-1
	macromolecular complex subunit organization	10	10.8	2.4E-2	3.3E-1
	ATP metabolic process	4	4.3	2.4E-2	3.3E-1
	purine nucleotide metabolic process	5	5.4	2.4E-2	3.3E-1
	nucleotide biosynthetic process	5	5.4	2.4E-2	3.3E-1
	cell-cell signaling	9	9.7	2.5E-2	3.3E-1
	regulation of protein stability	3	3.2	2.6E-2	3.3E-1
	nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic	5	5.4	2.7E-2	3.5E-1
	nucleobase, nucleoside and nucleotide biosynthetic process	5	5.4	2.7E-2	3.5E-1
	purine ribonucleotide biosynthetic process	4	4.3	3.2E-2	3.9E-1
	DNA packaging	4	4.3	3.2E-2	3.9E-1
	purine ribonucleoside triphosphate metabolic process	4	4.3	3.2E-2	3.9E-1
	ribonucleoside triphosphate metabolic process	4	4.3	3.3E-2	3.9E-1
	secretion by cell	5	5.4	3.4E-2	4.0E-1
	positive regulation of neurotransmitter transport	2	2.2	3.5E-2	4.0E-1
	purine nucleoside triphosphate metabolic process	4	4.3	3.6E-2	4.0E-1
	posttranscriptional regulation of gene expression	5	5.4	3.6E-2	4.0E-1
	ion transport	10	10.8	3.7E-2	4.1E-1
	ribonucleotide biosynthetic process	4	4.3	3.7E-2	4.0E-1
	chromatin assembly or disassembly	4	4.3	3.9E-2	4.2E-1
	regulation of organelle organization	5	5.4	4.0E-2	4.1E-1
	aspartate transport	2	2.2	4.1E-2	4.2E-1
	negative regulation of cytoskeleton organization	3	3.2	4.2E-2	4.2E-1
	nucleoside triphosphate metabolic process	4	4.3	4.3E-2	4.2E-1
	cation transport	8	8.6	4.5E-2	4.4E-1
	phosphorylation	10	10.8	4.6E-2	4.4E-1
	regulation of cytoskeleton organization	4	4.3	4.7E-2	4.4E-1
	purine ribonucleotide metabolic process	4	4.3	4.8E-2	4.5E-1
	ribonucleotide metabolic process	4	4.3	5.6E-2	5.0E-1
	neurotransmitter uptake	2	2.2	5.8E-2	5.0E-1
	behavior	7	7.5	5.9E-2	5.1E-1
	phosphorus metabolic process	11	11.8	6.0E-2	5.1E-1
	phosphate metabolic process	11	11.8	6.0E-2	5.1E-1
	regulation of binding	4	4.3	6.2E-2	5.2E-1
	locomotory behavior	5	5.4	7.9E-2	6.0E-1

Category	Term	Count	%	P-Value	Benjamini
GOTERM_MF_FAT	inorganic cation transmembrane transporter activity	11	11.8	2.8E-8	8.1E-6
	cytoskeletal protein binding	16	17.2	3.6E-7	5.3E-5
	monovalent inorganic cation transmembrane transporter activity	8	8.6	3.4E-6	3.3E-4
	hydrogen ion transmembrane transporter activity	7	7.5	1.9E-5	1.4E-3
	heme-copper terminal oxidase activity	5	5.4	2.4E-5	1.4E-3
	oxidoreductase activity, acting on heme group of donors, oxygen	5	5.4	2.4E-5	1.4E-3
	oxidoreductase activity, acting on heme group of donors	5	5.4	2.4E-5	1.4E-3
	cytochrome-c oxidase activity	5	5.4	2.4E-5	1.4E-3
	NADH dehydrogenase (quinone) activity	5	5.4	1.4E-4	6.7E-3
	NADH dehydrogenase activity	5	5.4	1.4E-4	6.7E-3
	NADH dehydrogenase (ubiquinone) activity	5	5.4	1.4E-4	6.7E-3
	actin binding	10	10.8	1.7E-4	7.2E-3
	oxidoreductase activity, acting on NADH or NADPH, quinone or s	5	5.4	2.3E-4	8.4E-3
	structural constituent of cytoskeleton	5	5.4	1.1E-3	3.6E-2
	calcium ion binding	15	16.1	1.3E-3	3.9E-2
	symporter activity	6	6.5	1.6E-3	4.1E-2
	L-amino acid transmembrane transporter activity	4	4.3	1.6E-3	3.9E-2
	oxidoreductase activity, acting on NADH or NADPH	5	5.4	1.7E-3	3.8E-2
	calmodulin binding	6	6.5	1.7E-3	3.5E-2
	L-glutamate transmembrane transporter activity	3	3.2	2.0E-3	3.8E-2
	acidic amino acid transmembrane transporter activity	3	3.2	2.4E-3	4.3E-2
	solute:cation symporter activity	5	5.4	2.8E-3	4.7E-2
	cytoskeletal adaptor activity	3	3.2	2.8E-3	4.5E-2
	amino acid transmembrane transporter activity	4	4.3	5.7E-3	8.5E-2
	SNAP receptor activity	3	3.2	7.3E-3	1.0E-1
	organic acid:sodium symporter activity	3	3.2	1.0E-2	1.3E-1
	amine transmembrane transporter activity	4	4.3	1.1E-2	1.3E-1
	di-, tri-valent inorganic cation transmembrane transporter activit	3	3.2	2.4E-2	2.7E-1
	P-P-bond-hydrolysis-driven transmembrane transporter activity	4	4.3	3.9E-2	3.9E-1
	primary active transmembrane transporter activity	4	4.3	3.9E-2	3.9E-1
	solute:sodium symporter activity	3	3.2	4.1E-2	3.9E-1
	enzyme binding	8	8.6	4.2E-2	3.8E-1
	channel regulator activity	3	3.2	5.1E-2	4.4E-1
	protein C-terminus binding	4	4.3	5.6E-2	4.6E-1
	lipid binding	7	7.5	5.9E-2	4.6E-1
	sodium:dicarboxylate symporter activity	2	2.2	6.0E-2	4.5E-1
	molecular adaptor activity	3	3.2	6.4E-2	4.7E-1
	calcium ion transmembrane transporter activity	2	2.2	7.2E-2	4.9E-1
	ATPase activity, coupled to transmembrane movement of ions	3	3.2	7.6E-2	5.1E-1
	ATPase activity, coupled	5	5.4	8.7E-2	5.4E-1
	glucose transmembrane transporter activity	2	2.2	8.9E-2	5.4E-1
	sugar:hydrogen symporter activity	2	2.2	9.4E-2	5.5E-1
	cation:sugar symporter activity	2	2.2	9.4E-2	5.5E-1
	ion channel inhibitor activity	2	2.2	9.4E-2	5.5E-1
	dicarboxylic acid transmembrane transporter activity	2	2.2	9.4E-2	5.5E-1
	structural molecule activity	8	8.6	9.5E-2	5.5E-1
	channel inhibitor activity	2	2.2	1.0E-1	5.6E-1
	calcium channel regulator activity	2	2.2	1.0E-1	5.6E-1

Category	Term	Count	%	P-Value	Benjamini
GOTERM_CC_FAT	organelle inner membrane	15	16.1	8.3E-9	1.8E-6
	mitochondrial inner membrane	13	14.0	2.6E-7	2.8E-5
	organelle membrane	22	23.7	1.2E-6	8.8E-5
	mitochondrial membrane	13	14.0	3.7E-6	2.0E-4
	mitochondrial envelope	13	14.0	7.0E-6	3.0E-4
	organelle envelope	15	16.1	1.7E-5	6.1E-4
	envelope	15	16.1	1.8E-5	5.4E-4
	membrane-bounded vesicle	14	15.1	3.1E-5	8.2E-4
	neuron projection	11	11.8	3.7E-5	8.7E-4
	respiratory chain	6	6.5	8.6E-5	1.8E-3
	cytoplasmic membrane-bounded vesicle	13	14.0	1.0E-4	2.0E-3
	NADH dehydrogenase complex	5	5.4	1.1E-4	2.0E-3
	respiratory chain complex I	5	5.4	1.1E-4	2.0E-3
	mitochondrial respiratory chain complex I	5	5.4	1.1E-4	2.0E-3
	vesicle	14	15.1	1.7E-4	2.7E-3
	actin cytoskeleton	9	9.7	2.1E-4	3.2E-3
	mitochondrial part	13	14.0	2.1E-4	3.0E-3
	vesicle membrane	7	7.5	2.9E-4	3.9E-3
	cytoskeleton	20	21.5	4.2E-4	5.3E-3
	cytoplasmic vesicle	13	14.0	4.3E-4	5.0E-3
	mitochondrial respiratory chain	5	5.4	5.9E-4	6.6E-3
	mitochondrial membrane part	6	6.5	9.3E-4	9.9E-3
	synapse	9	9.7	1.3E-3	1.3E-2
	cytoplasmic vesicle membrane	6	6.5	1.5E-3	1.4E-2
	synapse part	7	7.5	3.6E-3	3.3E-2
	clathrin coated vesicle membrane	4	4.3	4.0E-3	3.5E-2
	cytoplasmic vesicle part	6	6.5	5.4E-3	4.5E-2
	endocytic vesicle	4	4.3	5.4E-3	4.3E-2
	nucleosome	4	4.3	6.4E-3	5.0E-2
	synaptic vesicle membrane	3	3.2	8.3E-3	6.2E-2
	cell projection	11	11.8	8.8E-3	6.3E-2
	coated vesicle membrane	4	4.3	9.7E-3	6.7E-2
	mitochondrion	14	15.1	1.2E-2	8.3E-2
	protein-DNA complex	4	4.3	1.5E-2	9.6E-2
	axon	5	5.4	1.5E-2	9.6E-2
	intracellular non-membrane-bounded organelle	25	26.9	1.6E-2	9.6E-2
	non-membrane-bounded organelle	25	26.9	1.6E-2	9.6E-2
	pigment granule	4	4.3	1.6E-2	9.7E-2
	melanosome	4	4.3	1.6E-2	9.7E-2
	dendrite	5	5.4	1.7E-2	9.6E-2
	membrane fraction	11	11.8	2.3E-2	1.3E-1
	insoluble fraction	11	11.8	2.8E-2	1.5E-1
	calcineurin complex	2	2.2	3.0E-2	1.5E-1
	spectrin-associated cytoskeleton	2	2.2	3.0E-2	1.5E-1
	contractile fiber part	4	4.3	3.1E-2	1.5E-1
	trans-Golgi network	3	3.2	3.1E-2	1.5E-1
	plasma membrane part	21	22.6	3.4E-2	1.6E-1
	contractile fiber	4	4.3	3.6E-2	1.7E-1
	growth cone	3	3.2	4.2E-2	1.9E-1
	site of polarized growth	3	3.2	4.3E-2	1.9E-1
	clathrin-coated vesicle	4	4.3	4.5E-2	1.9E-1
	spliceosome	4	4.3	4.5E-2	1.9E-1
	spectrin	2	2.2	5.3E-2	2.2E-1
	cytosol	14	15.1	5.3E-2	2.2E-1
	membrane raft	4	4.3	5.5E-2	2.2E-1
	cell cortex	4	4.3	5.8E-2	2.3E-1
	cytoskeletal part	11	11.8	5.9E-2	2.2E-1
	cell fraction	12	12.9	5.9E-2	2.2E-1
	postsynaptic density	3	3.2	6.8E-2	2.5E-1
	coated vesicle	4	4.3	7.1E-2	2.5E-1
	synaptic vesicle	3	3.2	7.7E-2	2.7E-1
	plasma membrane	30	32.3	7.7E-2	2.6E-1
	cell junction	7	7.5	9.2E-2	3.0E-1
	synaptosome	3	3.2	9.3E-2	3.0E-1
	secretory granule	4	4.3	9.5E-2	3.0E-1
	endomembrane system	9	9.7	9.8E-2	3.1E-1
	heterogeneous nuclear ribonucleoprotein complex	2	2.2	9.8E-2	3.0E-1

3. CHAPTER III: MiR-16 and cellular stress

3.1. Introduction

The cellular response to stressful stimuli is modulated by miRNAs in many different ways. 1) miRNA activity reduces the cellular stress cascade activity through a negative feedback 2) may also contribute to pathway activation via the inhibition of negative regulators or 3) The relative levels of miRNAs and their cognate target mRNAs determine how much target protein is effectively produced (Byrd & Brewer, 2013; Maurel & Chevet, 2013).

In cultured cells, miR-16 expression increases after ultraviolet light damage and then seems to participate in regulating the DNA-damage response and cell division machinery (Pothof et al., 2009), which may be an adaptive response in that context (Finnerty et al., 2010). The miR-15/107 group may also help regulate cells' response to ischemic stress: miR-107 and miR-424 are downregulated in hypoxia (Kulshreshtha et al., 2007)(Donker, Mouillet, Nelson, & Sadovsky, 2007).

Recently, it has been shown that 3′UTRs of IL-6 and TNF-α mRNAs contain the miR-16-binding sites, and over-expression of miR-16 could significantly down-regulate TNF-α and IL-6 expression level in A549 cells (Z. Cai et al., 2012). In a previous study, it was suggested that miR-144* and miR-16 might be one of the negative regulators for inflammatory cytokine responses after exposure to naturalistic stressors (Honda et al., 2013; Katsuura et al., 2012). In chronically stressed mice, infusion of miR-16 into raphe or injection of antimiR-16 into the locus coeruleus exert beneficial effects that are similar to that obtained with fluoxetine (Baudry, Mouillet-Richard, Schneider, Launay, & Kellermann, 2010, 2011). miR-16 was shown to regulate negatively the expression of the serotonin transporter (SERT), the major target for the selective serotonin reuptake inhibitor (SSRI) class of antidepressant drugs (Baudry et al., 2010).

Another important cellular stress mechanism that has very important influence on AD and other neurodegenerative diseases is unfolded protein response (UPR). Activation of UPR is an early event in Alzheimer's and Parkinson's disease and is closely associated with the first stages of accumulation and aggregation of the

toxic proteins (Hoozemans, Van Haastert, Nijholt, Rozemuller, & Scheper, 2012). Endoplasmic reticulum (ER) is important cellular compartment which assures the correct folding conformations of proteins from where they later are secreted or become transmembrane proteins whereas misfolded are targeted for degradation. Restoring or increasing the capacity of the ER to fold and process proteins is an important and well-studied role of UPR. Activation of the UPR depends on three ER stress sensor proteins inositol-requiring protein (IRE1), PERK, and Activating transcription factor 6 (ATF6) Figure 1 (Hetz & Mollereau, 2014) . Upon ER stress, excessive unfolded proteins accumulate in the ER lumen, resulting in the activation of the UPR branches and promotes cell survival (Bertolotti, Zhang, Hendershot, Harding, & Ron, 2000). Indeed, what happens in neurodegeneration with an increased load of unfolded proteins protective process of UPR prolongs and fails to restore the normal function leading to apoptosis cascade in cells (Ron & Walter, 2007)(Todd, Lee, & Glimcher, 2008).

The relationship between UPR targeting miRNAs and AD has not yet been clearly investigated Since most of the example studies are those investigating the role of miRNA in other organs than brain but it is interesting to mention as an example of proven ATF-6 targeting miRNA is miR-455 (Belmont, Chen, Thuerauf, & Glembotski, 2012) (not miR-15/107 related miRNA) that has been already investigated in AD context. miR-455 playing important role in AD can cause a decrease of Aβ secretion by targeting Nicastrin from gamma-secretase complex (Delay et al., 2014).

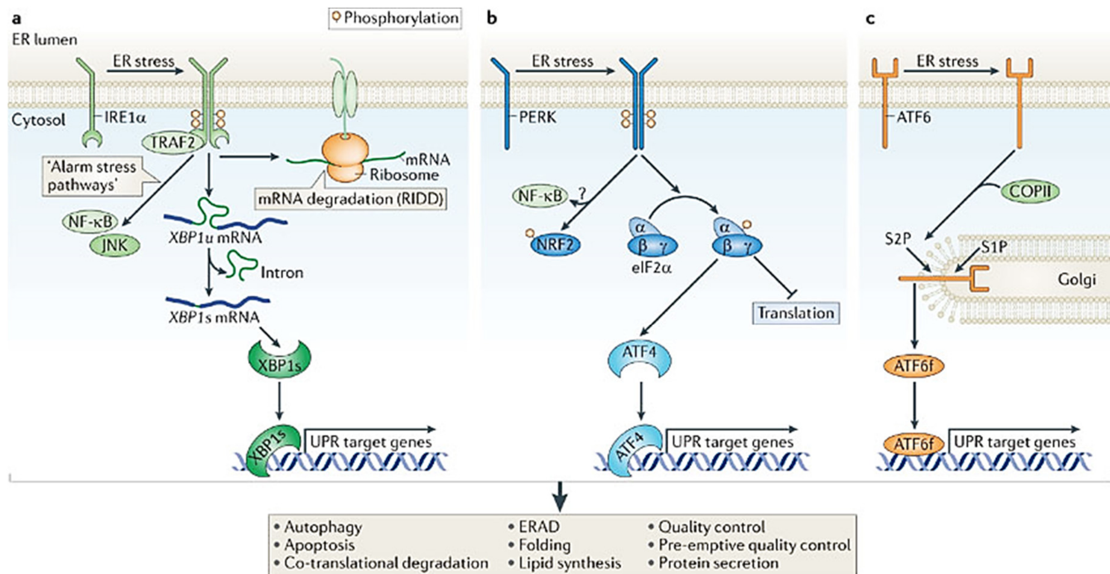


Figure 1 ***The unfolded protein response (UPR) stress sensors***

Inositol-requiring protein 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum (ER) kinase (PERK) and activating transcription factor 6 (ATF6), transduce information about the folding status of the ER to the cytosol and nucleus to restore protein-folding capacity (Hetz, 2012)

miRNA involvement in cellular stress is a relatively new subject and recent studies suggest that miRNA can indeed interact with cellular stress pathways like ER stress followed by unfolded protein response (Bartoszewska et al., 2013). Two members of the miR-15/107 family have been predicted to affect UPR signaling potentially through regulation of ATF6α: miR-16 and miR-424 (X. Yan et al., 2013)(Bartoszewska et al., 2013).

Following the results obtained in the previous section, it was suggested that miR-16 could be involved in the reduction of cellular stress and possibly having a neuroprotective role. Consistently this miRNA introduction also has had a beneficial effect for decreasing soluble amyloid beta in mutant cells. Moreover, there is a prediction of this miRNA involvement in ER stress as mentioned briefly. This idea was tested in the cellular model using Tunicamycin in next section, a recognized UPR inducer. Although this model is an exaggerated form of stress and acute one but would give a general idea about the regulation of this signaling pathway by miR-16.

3.2. Results

miR-15/107 could be involved in modulation of cellular stress specifically the stress induction upon unfolded protein response. To test this idea and bring it more in experimental aspect an *in vitro* test was performed in HT22 cells. Post 12hrs of artificial ER stress induction by Tunicamycin in HT22 hippocampal-derived mouse cells, the level of endogenous miR-16 was determined by Taqman qPCR. A total time dependent reduction of miR-16 endogenous levels was observed post 12hrs of acute stress induction. The stress level was confirmed by overall expression analysis of ER stress genes by qPCR as well as XbP1 splicing confirmation by PCR indicating that ER stress was induced in the cells (Figure 2A, B). Interestingly the specific inhibition of miR-16 in this cell line is not tolerated as confirmed also by colorimetric cell viability assay XTT. Cells transfected with a miR-16 inhibitor plasmid alone (miR-16 Zip) (Systembio) in wild-type HT22 cells show a reduction of viability by reduced colorimetric reaction of XTT reagent (Figure 2C).

This procedure of ER stress induction worked well on HT22 cells as it allowed me to study better the RNA expression of these cells. To examine miR-16 upregulation effect on ER stress genes, the expression of stress genes was evaluated post transfection of HT22 with scrambled control and miR16 mimics as previously described in material methods

In wild-type HT22 cells the overexpression of miR16 post transfection (confirmed by qPCR) did not show any significant effect on ER stress genes compared to scramble controls (Figure 3A). DMSO concentration below 1% was used in all conditions to eliminate the effect of Tunicamycin solvent and prevent toxicity induced by DMSO itself.

Cells transfected with miR-16 oligos were utilized the next day for stress induction and the expression of stress genes was measured post 3.5 hrs. This time period allowed having enough RNA for subsequent measurements. In HT22 cells under acute stress by Tunicamycin and transfected with scramble control, the expression of stress genes was compared with the same condition but transfected with miR16 oligos. Comparing these two aforementioned conditions,

it was observed the expression of Ddi3 and ATF6a were significantly lower in cells transfected with miR-16 under acute stress compared to controls (Figure 3B). As it has been already tested in this work the downstream effect of increased miR-16 on reduced soluble Aβ level in mutant Neuro2a expression APP Swedish and PS1 mutation, the same cells RNA was used to examine the effect of miR-16's up-regulation of ER stress genes. Post 48 hrs of transfection with miR-16 compared to Scr control a significant reduction of ATF6a mRNA was observed in this cell line (Figure 3C).

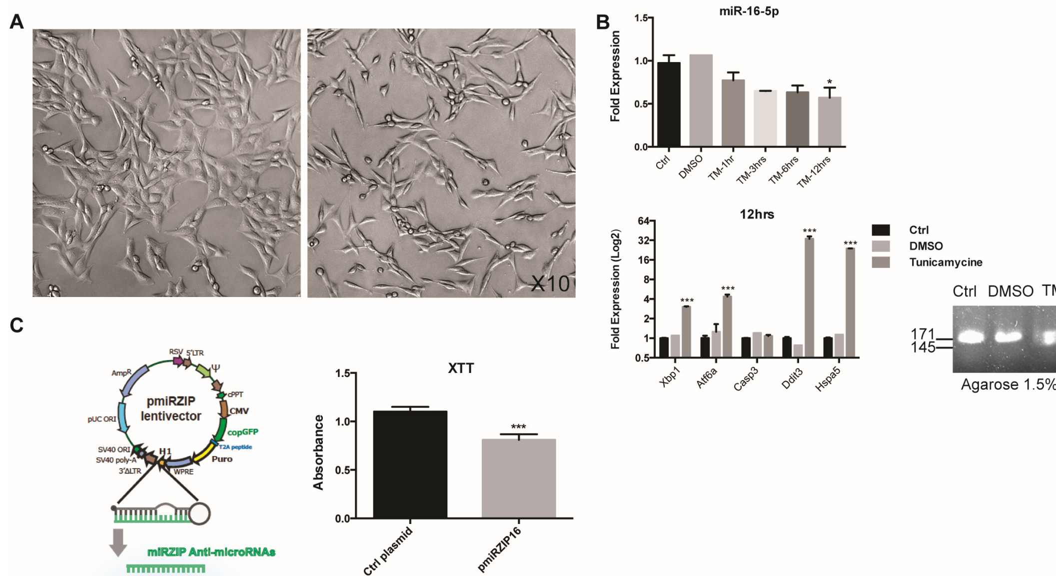


Figure 2 ***Induction of stress in HT22 reduces the expression level of miR-16.*** ***(A)*** *Morphology of HT22 cells post 12hrs of stress induction (magnified 10x)* ***(B)*** *Time-dependent decrease of miR-16 expression compared to controls in Stress condition, lower panel the analysis of expression of stress gene done by real-time PCR. Xbp1 splicing gene test was performed by regular PCR as it has been previously described. qPCR results were normalized to GAPDH as an internal control. Statistics was performed as t-Test *p < 0.05, **p< 0.01, ***p < 0.001* ***(C)*** *Inhibition of miR-16 by miR-zip plasmid transfection reduces viability of cells post 48 hrs compared to cells transfected with control plasmid. Viability measurement was done by XTT colorimetric method and normalized Absorbance (n=6).*

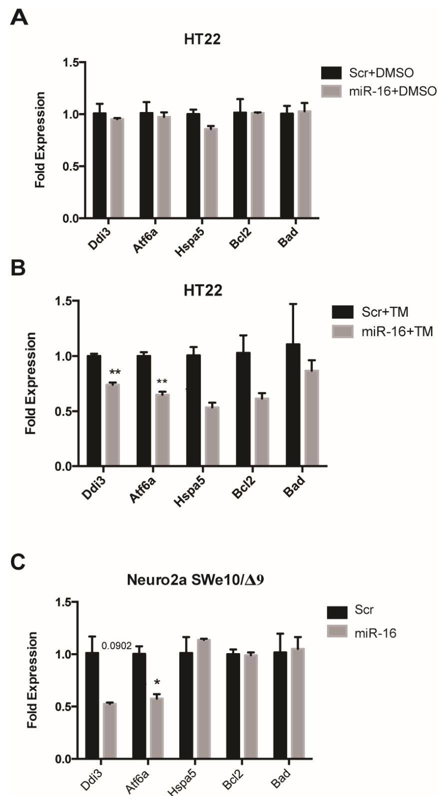


Figure 3 ***miR-16 modifies ATF6a expression in cells under stress***. ***(A)*** *Overexpression of miR-16 does not affect stress gene expression in normal HT22.* ***(B)*** *Stress induction by Tunicamycin in the same transfected cells decreases ATF6 and Ddi3 genes levels. qPCR results were normalized to GAPDH as an internal control.* ***(C)*** *miR-16 up-regulation in mutant Neuro2a cells under the stress of Aβ insult is enough to change ATF6a expression. Statistics was performed as t-Test *p < 0.05, **p< 0.01, ***p < 0.001*

3.3. Discussion

The mechanism regarding the decrease of viability in HT22 cell line following inhibition of miR-16 remains unstudied. It would be interesting to analyze more profoundly the effect of miR-16 inhibition in different cells by more detailed techniques *in vitro*. Very recent *in vitro* studies after the publication of this work suggest that miR-16 Overexpression in the cellular AD model with primary hippocampal neurons decreased apoptosis while inhibition of miR-16 in the same cells increased apoptosis, respectively. After transfection with a miR-16 inhibitor, the cultured cells exhibited a slight increase in apoptosis, even in intact cells without exogenous Aβ insult (B. Zhang, 2015), which is also consistent with presented findings in HT22 cells and reduction of miR-16. These data overall as well as literature suggest that reduction of miR-16 plays a potential role in

hippocampal cell death a very new concept that needs further elucidation. More studies would be needed to decipher the mechanism involved.

The presented findings in this section also represent that miR-16 overexpression only modifies the expression of ER stress genes, ATF6 and CHOP in mutant N2a (under the stress of Aβ insult) and HT22 (Tunicamycin-induced ER stress). Overexpression of this miRNA in normal condition doesn't affect the expression of these genes (Figure 3A). This finding is interesting in a way that the classical miR-16 role in some cancers like leukemia is highlighted by its pro-apoptotic role and decreasing the expression of BCl2, while at least in HT22 cells- the focus of this study- such effect wasn't observed. This observation needs more experiment to be confirmed as well *in vivo.*

Moreover, it has been previously shown in the literature that Neural derived cell lines expressing APP mutation show a more increased expression level of ATF6 and other stress genes. Increased of miR-16 levels was sufficient to downregulate the expression of ATF6 significantly which also supports this idea. The preliminary result in cells presented in the previous section strengthens the regulation of ATF6a and a negative feedback of stress by miR-16 (X. Yan et al., 2013)(Bartoszewska et al., 2013). Targetscan V.7 also predicts regulation of ATF6a by miR-15/107 through interaction with 3'UTR which is very recently confirmed experimentally for one member of this family miR-424 but how this regulation is highlighted in neurodegeneration remains unstudied (Gupta et al., 2015). Importantly, the other predicted miRNA to target 3′UTR of ATF6 gene are reported as well downregulated in AD such as miR-212-5p and miR-140-5p reviewed in detail elsewhere that indicate the importance of regulation of this pathway by miRNA in AD pathogenesis (Satoh, 2012).

3.4. Methods

3.4.1. HT22 cell culture

Mouse hippocampal-derived HT22 cells (Dr. Schubert, Salk institute, USA) were cultured in DMEM supplemented with 10% fetal calf serum (ThermoFischer Scientific Inc., USA).

3.4.2. Viability assay

XTT Cell Proliferation Assay Kit (ATCC) was used for viability assay in 96 well plate. XTT is a colorless or slightly yellow compound that when reduced becomes brightly orange. This color change is accomplished by breaking apart the positively charged quaternary tetrazole ring. The formazan product of XTT reduction is soluble and can be used in real-time assays(Altman, 1976; Berridge, Herst, & Tan, 2005). 50ul of Activated-XTT Solution (ATCC, USA) added to each well and plate was gently shaken before moving cells to 37°C in a 5% CO2 atmosphere incubator for two hours. The specific absorbance of the wells containing the cells and the blank background control wells at a wavelength of 490nm subtracted by the absorbance observed in 630nm (background) using Biorad iMark^{TM} microplate reader. Extra replicates of each condition were kept for Taqman miR-16 expression assay.

3.4.3. UPR stress measurement *in vitro*

ER stress was induced by 0.5 µg/ml Tunicamycin (TM) dissolved in DMSO, and cells were treated for different time points. The equivalent concentration of DMSO as control was used. Total RNA extraction was done immediately after desired time points that were 1, 3, 6 and 12hrs. ER stress induction was confirmed by xbp1 splicing PCR test with primers F-ACACGCTTGGGAATGGACAC R-CCATGGGAAGATGTTCTGGG (Iwakoshi, Lee, & Glimcher, 2003). Other primers sequence that was applied in this study were: ATF6a F-CTTCCTCCAGTTGCTCCATC R-CAACTCCTCAGGAACGTGCT(Cao, Song, & Kaufman, 2012)(Tokutake, Gushima, Miyazaki, Shimosato, & Yonekura, 2015); Ddit3(chop): F-CCACCACACCTGAAAGCAGAA R-GGTGCCCCCAATTTCATCT; F- Hspa5 F-ACGCACTTGGAATGACC R-TTCTTTCCCAAATACGC;

; Caspase 3 F-GCTGACTTCCTGTATGCTTACT R-CGTTGCCACCTTCCTGTTAA(W. G. Zhang et al., 2013) ; Xbp1 F-AGCAGCAAGTGGTGGATTTG R-GAGTTTTCTCCCGTAAAAGCTGA (Spandidos, Wang, Wang, & Seed, 2009)(Kaser et al., 2008)

4. CHAPTER IV: GENERAL DISCUSSION

4.1. miRNA-based Therapeutics challenges

Challenges often observed in the development of RNA-based therapeutics are Instability, Off-target effects, immune response and targeted delivery (Yan Deng et al., 2014). Studies to date have been more focused on RNAi therapeutics than miRNA. In the field of siRNA-based therapeutics, much effort has been made to develop safe and effective non-viral siRNA delivery systems, including direct chemical modification, liposome formulations and nanoparticles (Gao, Liu, & Li, 2011). It seems the same approaches toward siRNA development in Clinique applies to miRNA.

miRNA mimics are used to restore miRNAs that show a loss of function. This approach, also known as miRNA replacement therapy, has attracted much interest as it provides a new opportunity to therapeutically exploit disease pathways (a G. Bader, Brown, Stoudemire, & Lammers, 2011). The first miRNA-based therapy approach, MRX34 (A. G. Bader, 2012) has entered clinical trials in 2013. Using a liposome-based formulation, MRX34 is a synthetic double-stranded RNA oligonucleotide which can substitute depleted miR-34 and restore its activity. Different approaches are available for delivery of the miRNA mimics such as nanoparticles which promote the cellular uptake of miRNA (Wu et al., 2011). An interesting example of developing modified delivery of a miRNA to brain through blood brain barrier was using cationic polymer polyethyleneimine (PEI). This strategy previously used for siRNA (X. Liu et al., 2012) delivered fluorescently tagged miR-124a to mouse brain using rabies peptide RVG modified PEI. no functional study of miRNA was reported in this study(Hwang et al., 2011).

Viral delivery also could be an option. So far, many delivery systems based on virus have been optimized depending on the research aims and cell types, such as retrovirus vectors (RV), lentivirus vectors (LV), adenovirus vectors (AV), and adenovirus-associated vectors (AAV)(Couto & High, 2010). Toxicity still reported while working on siRNA delivery with viral vectors while using a high expression level of shRNAs in CNS neurons (Ehlert, Eggers, Niclou, & Verhaagen, 2010). In

the case of switching to miRNA, it would be still very crucial to assess any strategy of delivery in CNS in term of safety and toxicity.

The approach assessed in my research avoided the complexity of systemic delivery by injecting miRNA mimics to target site (S. H. Chen & Zhaori, 2011). Such strategy could be available by delivering miRNA in CNS by administering oligos in CSF in an intrathecal way (Tan, Yang, Shih, Lan, & Cheng, 2005). Such strategy has been applied to other neurodegenerative diseases such as ALS where researchers were able to deliver antisense oligonucleotide directed against sod1 delivered through Intrathecal in SOD1-Familial ALS Patients in phase I clinical trial (Miller et al., 2013).

4.2. miRNA gain of function study *in vitro*

Following identification of miRNA dysregulated in pathology context, the next step requires functional characterization of the candidate miRNA *in vitro* and *in vivo* through gain or loss of function studies. The gain of function can be accomplished through the introduction of synthetic miRNA (either transfection or viral transduction)(Ishida & Selaru, 2013).The method of choice for miRNA introduction was transient transfection of oligo in cells. Notably, the efficiency of this method of choice was high in my study but there is always a concern about supraphysiological levels of mature miRNAs and non-specific changes in gene expression as suggested as a disadvantage (Jin et al., 2015). The strategy followed was testing different candidates of the miRNA-15/107 family on APP and BACE1 protein reduction in cell lines. For investigation of miRNAs effect on Aβ, the cell lines expressing Swedish mutation of APP was utilized. *In vitro* models based on overexpression of mutant APP have helped scientists to test a hypothesis regarding Aβ pathology in cells. Swedish mutation of APP that was used in this study is one of the first mutations discovered in familial AD. Swedish mutation is indeed a double mutation KM670/671NL found in a Swedish family is located before the Aβ region and results in the increased production and secretion of Aβ (Haass et al., 1995). Neuroblastoma cells overexpressing Swedish mutation don’t go under toxicity induced phenotype change (Thinakaran, Teplow, Siman, Greenberg, & Sisodiat, 1996) that make them suitable for studying the transient effect of miRNA overexpression on RNA and

protein changes within a time period of 0-72hrs. In my study, the effect of candidate miRNA-16 was also tested in not only HEK cells expressing Swedish mutation but also in neuroblastoma cell line expressing APP Swedish mutation with a variant of Presenilin (ΔE9 variant). According to very early findings of authors who developed this cell line ratios of Aβ1–42(43)/Aβ1–40 in media of independent cell lines expressing different PS1 variants (i.e., ΔE9 variant) are uniformly elevated compared with the Aβ1–42(43)/Aβ1–40 ratios in media from cells that express essentially indistinguishable levels of wild-type PS1 (Borchelt et al., 1996). The result obtained on the reduction of Aβ in my work is in line with other studies investigating AD-related miRNA *in vitro.* A similar strategy used herein has been applied for studying other AD-related miRNAs such as miR-298, 328 (Boissonneault, Plante, Rivest, & Provost, 2009), miR-101(Long & Lahiri, 2011), miR-339 (Long et al., 2014).

4.3. MiR-16 non-modified mimics are functional *in vivo*

After the initial confirmation of miR-16 as a potential candidate with effect on APP, Aβ, BACE1 and Tau phosphorylation *in vitro* the efficiency of miRNA-16 mimics delivery in wildtype mice was examined. Different steps of miRNA biogenesis could potentially be exploited *in vivo* such as introduction of double-stranded miRNA gene or overexpression of miRNA precursor or even single-stranded miRNA; however data from other biochemical studies on RNAi pathway suggest that single-stranded small interfering RNA, for example, stimulate immune system more than their double-stranded counterparts (Sioud, 2006) or using a synthetic, single-stranded RNA molecule that contains the same sequence and chemistry as the mature endogenous miRNA tend to be 100–1000-fold less active compared with mimics that feature a second, complementary strand (a G. Bader et al., 2011). Therefore, it has been proposed that double-stranded miRNA mimics are preferred to single-stranded mimics (Martinez, Patkaniowska, Urlaub, Lührmann, & Tuschl, 2002; Schwarz et al., 2003). In my study, the focus was solely on double-stranded mature miRNAs. The previous study in the context of neurodegenerative disease such as Huntington suggests that miRNA-based approaches may provide more appropriate biological tools for expressing inhibitory RNAs in the brain in term of induction of less toxicity (McBride et al.,

2008). As miRNA replacement therapy depends on providing patients with miRNA activities that are identical to those that are reduced or missing in the disease cells (a G. Bader et al., 2011), the modification imposed on oligonucleotides for improving their function *in vivo* is a critical part of designing nucleotide-based therapeutics. Changes in miRNA activity and target specificity are often a result of terminal modifications in the active strand and a high degree of modification in the central regions of both strands (a G. Bader et al., 2011). Therefore, for all experiment done *in vivo* in my thesis preference for simplicity of work was to use non-heavily modified nucleotide version of double miRNA strand (no internal nucleotide modification) provided by Ribbox. Although for an increase in stability of oligos *in vivo* this company conjugates an RNAi-cap® Technology at 3'-end that also reduces off-targets effects and improves the overall stability of these mimics (http://www.riboxx.com/). It is important to mention that modifications placed at the 3'-end of the sense strand have minimal impact on function (IDT technologies). For a miRNA to be functional, it must be incorporated into RISC (Lam et al., 2015) and qPCR amplification alone does not necessarily indicate functionality (Thomson, Bracken, Szubert, & Goodall, 2013). The data from delivery of non-modified mimics *in vivo* suggest that these oligos indeed are recruited by RISC complex and are functional confirmed by RIP-Chip in Supplementary Figure 6c and Western blot on downstream targets in miR-16 mimic-treated mice compared to controls.

In a supplementary experiment in parallel presented in the Supplementary Figure 1, I also repeated the same miRNA delivery using fully modified double-stranded miRNA mimics with 2 -OMe modification. Fully modified mimics are not increased after delivery to mice brain suggesting hypothetically that this full modification indeed has inhibited the proper incorporation of miR-16 in RISC complex in my experiment (Supplementary Figure 5). My observation is aligned with other studies that have reported modifications that affect the functionality of oligos such as RNA duplexes entirely made of 2′OMe bases or 5′-O-methylation that inhibited both RISC loading and function of the modified strand (Collingwood et al., 2008)(Søkilde, Newie, Persson, Borg, & Rovira, 2015).

4.4. MiR-16 loss of expression could play potential key role in AD

Previous results in this work and others refer to a probable loss of the neuroprotective role of miRNA-16 following its decrease of expression in AD. This aspect of miRNA is fewer studied in neurodegenerative diseases.

It is undeniable that Alzheimer is the crossroads of many pathways and culprits but having a simplified model based on our results and proposed importance of tau phosphorylation in neural degeneration proposes a hypothesis regarding the consequence of miR-16 loss which is very simplified in Figure 1.

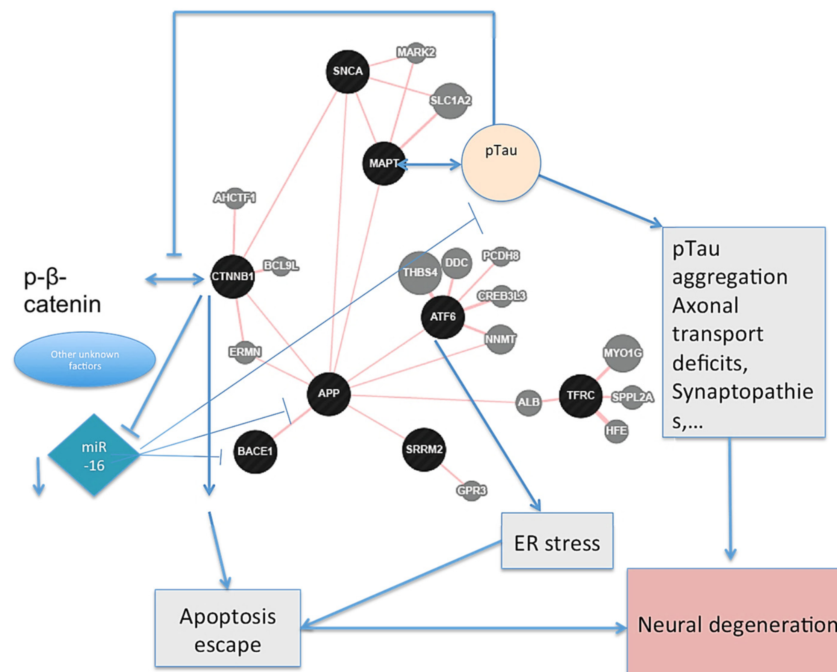


Figure 1 ***miR-16 and AD pathways regulation-a hypothesis****. Tau hyperphosphorylation renders cells more resistant to apoptosis by rescuing nuclear β-catenin upregulating the unfolded protein response system (the ER pathway). Simultaneously, tau hyperphosphorylation results in its pathological actions, inhibiting proteasome activity, damaging axonal transport, and causing synaptopathy, which eventually lead to chronic neurodegeneration. Increase expression of miR-16 can affect APP, BACE1 expression directly and Tau phosphorylation (i.e pSer396) indirectly. The increased expression of miR-16 potentially regulates stress through ATF6 (UPR pathway), Transferrin receptor (TFRC) and through an unknown mechanism by fine tuning of Alpha-Synuclein potential targets of miR-16 have been shown by black circles, predicted physical interaction is shown by pink lines.*

There are possibly unknown factors or known i.e. Catenin (Martello et al., 2007) which can affect the expression of the miR-16 decreasing level of maturation of this miRNA or total level of this miRNA. Finding of the decreased host gene

expression of miR-16-1 or DLEU2 also strengthens this hypothesis (Supplementary Figure 4). However, the mechanism of miR-16 expression regulation in neurodegeneration context needs more examination since the current data in literature mostly relies on the cancer studies and other pathologies that can't extend to AD. As a result as previously also suggested, this network of targets are those mainly up-regulated in AD context are involved in a vicious cycle of Tau hyperphosphorylation, apoptosis escape, stress and unfolded protein response and finally apoptosis and neural degeneration (H. Li et al., 2007; Jian Zhi Wang et al., 2014). It has been suggested that phosphorylation of Tau is the main contributor to the escape of neurons from acute apoptosis and simultaneously impairs the function of neurons (Hyun-pil Lee et al., 2014; McShea, Wahl, & Smith, 1999; Jian Zhi Wang et al., 2014).

In Figure 1 of this chapter, only some of the targets have been indicated. It would be reasonable to suppose that increase of this miRNA can have a protective role by fine-tuning of these pathways specifically Tau phosphorylation in pathological context as our data in previous sections confirmed the *in vitro* and *in vivo* fine tuning of Tau phosphorylation by miR-16. In this context, very few expression studies of miR-16 in post-mortem brains that do observe a biphasic expression of this miRNA with down-regulation of this miRNA by disease progression (Müller et al., 2014) could also be explained. This Biphasic change in miR-16 expression in hippocampus with tendency of loss of miR-16 expression in AD patient has also observed in a non-AD mouse model (prion disease) in which authors suggest that an increase in expression of this miRNA in early disease followed by a decrease with disease progression turning on a switch from neuroprotection towards induction of excitotoxicity (Majer et al., 2012). It remains to confirm these propositions by conditional ablation of miR-16 in animal models or *in vitro* as well.

The interesting finding of the ability of miR-16 to regulate protein levels of new potential targets of α-Syn and SRRM2 following proteomics study opens an opportunity to evaluate the consequence of miR-16 overexpression in PD models for these proteins and whether increase of this miRNA would have any ameliorating effect on the pathology or not. The nature of this regulation remains to be studied since α-Syn UTR is not probable to be targeted by miR-16 by

Targetscan; however, it does not exclude other level of regulations like targeting coding sequence of the protein itself (predicted) or other related protein factors. Though experiments have shown that 3′UTR of other member of Synuclein proteins, γ-Synuclein (γ-Syn) can be also regulated by miR-103/107 and potentially by miR-15a and -15b, -195, -503 and -646. Overexpression of Synuclein in the same experiment *in vitro* causes a response by miR-497 another member of this miRNA family that may play an auto regulatory role, reducing γ-Syn expression (Surgucheva, Gunewardena, Rao, & Surguchov, 2013). Contrary to α-Syn, γ-Syn is poorly studied in neurodegeneration recent studies show γ-Syn positive lesions in human brain (Surgucheva, Newell, Burns, & Surguchov, 2014) and animals expressing high levels of γ-Syn develop severe and fatal neurological disease associated with aggregation of the overexpressed protein and its deposition in cytoplasmic and axonal lesions (Blair et al., 2013). Therapeutic strategies against α-Syn could tackle the caused toxicity by decreasing the synthesis and aggregation or clearing the protein and miRNA or siRNAs based therapeutics has this potential (Lashuel, Overk, Oueslati, & Masliah, 2013). Contrary to PD, no study has been focused on the role of the srrm2 gene in AD. The data about upregulation of this gene in AD as mentioned very briefly in my paper in discussion part was obtained from bioinformatics analysis of raw expression studies from human brain tissues (ctrl vs AD) published in Gene Expression Omnibus (GEO) using GEO2R tool available from NCBI. It remains to identify the role of this protein’s aberrant expression in AD hippocampus.

Another interesting aspect that I observed in proteomics data was the upregulated proteins after miR-16 mimics delivery in the mouse brain. The significance of such relationship though was not the focus of current study, triggers the idea that a miRNA might also exert its neuroprotective effect through increasing the proteins that are frequently downregulated in AD. One interesting example is Calbindin (CB) increased expression in Brainstem after miR-16 delivery in the presented proteomics results. CB is one of the major calcium-binding and buffering proteins, has a critical role in preventing a neuronal death as well as maintaining calcium homeostasis (Christakos & Liu, 2004; Guo,

Christakos, Robinson, & Mattson, 1998). Recent studies suggest that removal of CB from AD transgenic mice aggravates its pathogenesis, suggesting that CB has a critical role in AD pathogenesis(Kook et al., 2014). Progressive loss of Calbindin expression has also been associated in recent studies with the full range of tangle pathology within basal forebrain cholinergic neurons in Alzheimer's disease (Ahmadian et al., 2015)(Stefanits, Wesseling, & Kovacs, 2014). It remains to study the significance and confirmation of such changes after increasing miRNA and whether they are direct or secondary consequences of miRNA upregulation in the brain.

To support the idea that miR-16 loss could affect cellular stress a series of experiments *in vitro* was designed in chapter III of this work. The specific decrease of miR-16 in HT22 cells was followed by a decrease of the viability of this cell line. Interestingly, cells under acute stress induced by Tunicamycin showed a decrease of miR-16 expression. The upregulation of this miRNA *in vitro* also regulates cellular stress genes RNA levels, notably ATF6, in mutant Neuro2a which was discussed in detail in Chapter III of this work. The similar result also reported by an independent group in 2015 which will put more emphasise on the potential role of miR-16 decreased level in hippocampal cell death (B. Zhang, 2015). However, the studies regarding the connection of miRNA with cellular stress specifically ER stress in neurodegenerative context are lacking. In other organs i.e. liver, it has been demonstrated that miRNA indeed play an important role in protecting cells against sustained ER stress. In this case, it has been demonstrated that elevated miR-199-5p disrupts sustained ER stress by targeting ATF6 and prevents hepatocytes cell death (Dai et al., 2013). It would be interesting to investigate whether miRNAs targeting ER stress specifically through ATF6 axis could trigger a neuroprotective response in neural cells under sustained cellular stress in AD or not.

4.5. Perspectives

I studied only one member of this miRNA family but since members have highly overlapping targets it would be important to consider this level of complexity in therapeutics development (W.-X. Wang et al., 2014) and study the combined action of a cocktail of miRNAs on correcting one or multiple pathological

pathways. The challenge in developing the miRNA-based therapeutics in AD could be the lack of ideal lab models. To be closer to clinical testing of miRNA-based therapeutics extending studies to nonhuman primates seems necessary. The ultimate safety and study on the delivery of miRNA-based therapeutics in CSF can be assessed. Importantly, the paradigm that delivery of mimics *in vivo* would increase TLR and immunity responses as was not the case at least at the level of RNA expression in the presented study remains to be also studied in detail in other models as well. To conclude more clearly about the safety of a miRNA-based therapy, it would be essential to study the effect of miR-16 up-regulation in different brain cell populations importantly glial cells and whether any therapeutically targeted delivery should be limited to one specific cell population or not.

Overall, the results described in this study highlight the possibility of miRNA replacement therapy in Alzheimer's. This opens an exciting opportunity to investigate more the therapeutic potential of these regulatory small molecules in neurodegenerative diseases. As this has been already very advanced in Clinique in the context of cancer specifically in term of miR-16 as a well-recognized tumor suppressor in the attention of pharmaceutical companies, it would be interesting to advance the same type of research in neurodegeneration. Furthermore, as this project became focused on miR-16 only it revealed some of the novel potential protein targets regulated by this miRNA in the brain that was not characterized as the target of this miRNA before. The results provided here would help pave the way towards developing more non-coding RNA-based therapeutics or finding novel targets for drug discovery.

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ANNEX

POSTER PRESENTATIONS

(2015) **miRNAs as therapeutic agents for Alzheimer’s disease: a pilot study**. The 12th International Conference on Alzheimer's & Parkinson's. Nice, France

(2014) **miRNAs as therapeutic agents for Alzheimer's disease**. CANAD 2014 symposium

(2013) **miRNAs as therapeutic agents for Alzheimer's disease.** La Journée à la recherche en Neurosciences et Santé mentale. Quebec, Canada (Winning abstract)

COLLABORATIONS

Smith, P. Y., Hernandez-Rapp, J., Jolivette, F., Lecours, C., Bisht, K., Goupil, C., ... & Bennett, D. A. (2015). **miR-132/212 deficiency impairs tau metabolism and promotes pathological aggregation in vivo**. *Human molecular genetics*, *24*(23), 6721-6735.

ABSTRACT: Alzheimer's disease (AD) and related tauopathies comprise a large group of neurodegenerative diseases associated with the pathological aggregation of tau protein. While much effort has focused on understanding the function of tau, little is known about the endogenous mechanisms regulating tau metabolism in vivo and how these contribute to disease. Previously, we have shown that the microRNA (miRNA) cluster miR-132/212 is downregulated in tauopathies such as AD. Here, we report that miR-132/212 deficiency in mice leads to increased tau expression, phosphorylation and aggregation. Using reporter assays and cell-based studies, we demonstrate that miR-132 directly targets tau mRNA to regulate its expression. We identified GSK-3β and PP2B as effectors of abnormal tau phosphorylation in vivo. Deletion of miR-132/212 induced tau aggregation in mice expressing endogenous or human mutant tau, an effect associated with autophagy dysfunction. Conversely, treatment of AD mice with miR-132 mimics restored in part memory function and tau metabolism. Finally, miR-132 and miR-212 levels correlated with insoluble tau and cognitive impairment in humans. These findings support a role for miR-132/212 in the regulation of tau pathology in mice and humans and provide new alternatives for therapeutic development.