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Advances in MicroRNAs and Alzheimer's Disease Research

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1. Introduction

Molecular and cellular neurobiological studies of the miRNA-mediated gene silencing in Alzheimer's disease represent the exploration of a new frontier of miRNAs biology and the potential development of new diagnostic tests and genetic therapies for this neurodegenerative disease. In few years our understanding of microRNA (miRNA) biogenesis, molecular mechanisms by which miRNAs regulate gene expression, and the functional roles of miRNAs has been expanded. MiRNA are double-stranded RNAs (dsRNAs) ≈22 nucleotides in length. These small noncoding RNA molecules operate as guides for RISC (RNA Induced Silencing Complex) to cleave a target mRNA in case of a perfect complementarity (siRNA) or to block the target mRNA translation (miRNA) when there is an imperfect pairing between miRNAs and the targets. In mammalian cells the repression of translation by miRNA is mediated by an imperfect pairing with the 3'UTRs of the mRNA targets. Interestingly, numerous miRNAs are expressed in a spatially and temporally controlled manner in the nervous system, suggesting that mRNA post-transcriptional regulation by microRNAs may be particularly relevant in neural development and function. Individual microRNAs can reduce the production of hundred proteins and miRNAs-mediated post-transcriptional regulation is involved in neuronal differentiation, dendritic spine development and synaptic plasticity. Recently expression profiles of miRNA in Alzheimer's disease brain revealed alterations in many individual miRNAs and several in vitro and in vivo studies aimed to the exploration of functional role of miRNA in Alzheimer's disease pathogenesis.

1.1 MicroRNA-mediated gene silencing

MicroRNAs are non-coding single stranded RNA molecules, 18- to 25 nucleotides in length, encoded in the genomes of invertebrates, plants and vertebrates. MicroRNA genes represent about 1-2% of the known eukaryotic genomes. The first two miRNAs to be discovered were *lin-4* and *let-7* in *Caenorhabditis elegans*, which regulate expression of *lin-14* and *lin-28* mRNAs, respectively, and are required for larval developmental timing (Lee et al., 1993;

Olsen et al., 1999; Reinhart et al., 2000). Since then, miRNAs have been observed in many contexts and their function involved in numerous regulatory pathways, deserving the attention they are now receiving.

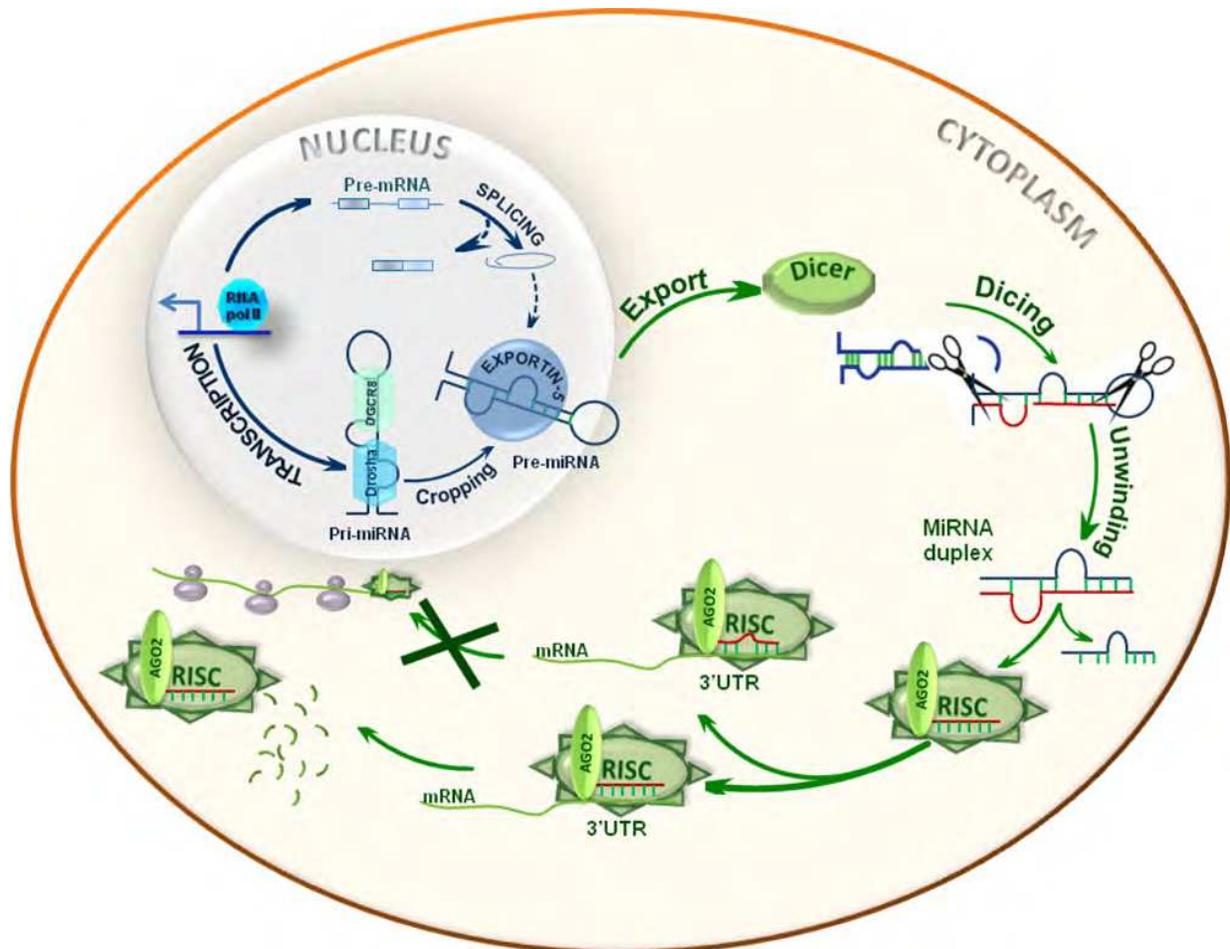


Fig. 1. MicroRNAs biogenesis and activity.

MiRNAs are transcribed by RNA polymerase II (Rodriguez et al., 2004), which mediates the transcription of most miRNA genes to generate an initial transcript, named primary miRNA (pri-miRNA). Pri-miRNAs can be hundreds to thousands nucleotides long, contain several hairpin structures, and undergo capping and polyadenylation (Lee et al., 2003). The first step in miRNA biogenesis occurs in the nucleus and requires the excision of this hairpin structure by a complex containing the RNase III-endonuclease Drosha enzyme and the RNA binding protein DiGeorge syndrome critical region gene 8 (DGCR8) (Gregory et al., 2004). Apart from canonical intronic miRNAs, a group of intronic miRNAs (named mirtrons), present in the introns of flies and mammals, are derived from small introns that resemble pre-miRNAs and can bypass the Drosha-processing step. The excised hairpin, now called pre-miRNA, is exported to the cytoplasm by a protein heterodimer consisting of the transport factor Exportin-5/RanGTP (Bohnsack et al., 2004), and processed by Dicer, a double-stranded RNA (dsRNA)-specific endonuclease RNaseIII, which removes the loop region of the hairpin, releasing the mature miRNA: miRNA duplex. Dicer interacts with several RNA binding proteins, such as TRBP and PACT, which are not essential for its cleavage activity but are important for miRNA

stability and loading of the effector complex RISC (RNA-induced silencing complex) (Chendrimada et al., 2005; Lee et al., 2006). During the assembly of the RISC complex with the miRNA, only one strand of the duplex is loaded, whereas the complementary miRNA* strand is removed and degraded (Kim et al., 2005). The mature miRNA is now ready to direct its activity on a target mRNA, by binding miRNA responsive elements usually located in the 3'UTR of the transcript, which leads to post-transcriptional gene silencing via inhibition of translation initiation or elongation. MicroRNAs associate with Argonaute proteins (Ago1-4, in mammals), which constitute the core of the RISC complex, and mediate post-transcriptional repression of target messenger RNAs. In particular Argonaute proteins are highly basic proteins which contain four domains: the N-terminal, PAZ, Mid and PIWI domains (Hutvagner and Simard, 2008). The PAZ domain binds the single stranded 3' end of miRNAs.

1.2 MicroRNAs in nervous system

The bewildering diversity of neurons, including their distribution in specific functional areas and complex synaptic circuitry, is determined during development and differentiation and is achieved by multiple levels of gene regulation. The transcriptional and post-transcriptional gene regulation mechanisms of development, plasticity and networking might participate in the determination and maintenance of such complexity. Recently, microRNAs are emerging as important players in post-transcriptional regulation in the brain. A major advancement in understanding how miRNAs are involved in this phenomenon comes from studies on miRNAs expression profiles. Several analyses have shown spatially and/or temporally restricted distribution of miRNAs, suggesting that they may control the fine-tuning regulation of neuronal gene expression (Cao et al., 2006; Schratt, 2009).

1.3 MicroRNAs, synaptic plasticity and memory

This section focuses on more relevant works highlighting the relevance of microRNAs post-transcriptional regulation in cellular pathways and functions such as synaptic plasticity and memory. However pathological implications of miRNAs expression associated to cognitive decline in Alzheimer's disease, are still unknown.

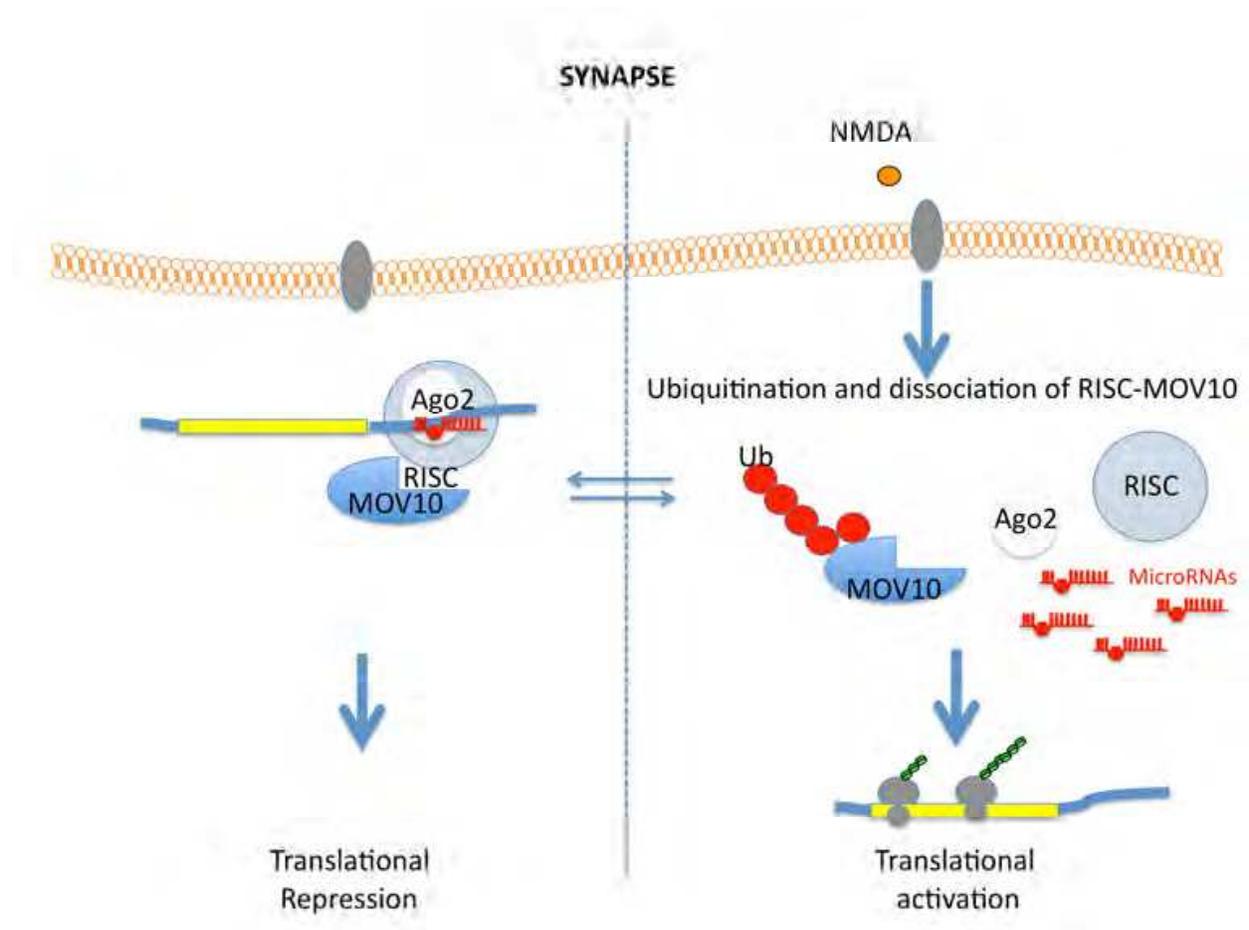
1.3.1 MicroRNAs and the dendritic neuronal compartment

Synaptic loss is the major neurobiological substrate of cognitive decline in Alzheimer's disease. The alteration of synaptic integrity occurs very early in AD, and it can be observed in patients with mild cognitive impairment (MCI) (Scheff et al., 2006). Loss of molecular components of presynaptic and postsynaptic membranes, synaptic vesicles, and proteins contributing to the morphological and architectural characteristic of axons and dendrites, is the main feature associated with an impairment of synaptic plasticity in neuronal cells. Neuronal activity is a critical regulator of several nervous system functions, including long-term memory (Malenka and Nicoll, 1999; Sutton and Schuman, 2006). Beyond the reduction of transcripts related to synaptic vesicle trafficking (Coleman and Yao, 2003), it is questionable if there is a post-transcriptional deregulation of mRNAs functionally associated to axonal and dendritic synaptic remodelling in AD. Little is known about the mechanisms that underlie the regulation of protein synthesis in polarized cells like neurons. The synaptic protein synthesis makes necessary the

corresponding mRNAs to be transported in the dendritic compartment and to be translated upon site-specific activation. It is generally thought that dendritic mRNAs are transported in a translationally silenced state within ribonucleoprotein complexes (Kiebler and Bassell, 2006). The recent discovery of the existence of a group of molecules regulating gene expression at post-transcriptional levels, named microRNAs, opened a new window on the research of molecular mechanisms of plasticity in the nervous system. Specific sites of protein synthesis are the dendritic spines. In neurons there are thousand of spines throughout multiple arborizations. Spine structures are dynamically regulated (Hotulainen and Hoogenraad, 2010), and functional and structural changes at spines and synapses are proposed as the basis of learning and memory (Kasai et al., 2010). MiRNAs, that are expressed in spatially and temporally controlled manner in the brain, are ideal candidates as modulators of dendritic protein synthesis. MicroRNAs modulate dendritic morphology by regulating expression of proteins involved in the actin cytoskeleton (Vo et al., 2005; Schratt et al., 2006; Siegel et al., 2009; Wayman et al., 2008) mRNA transport (Fiore et al., 2009) and neurotransmission (Edbauer et al., 2010). The participation of miRNAs in synaptic expression of mRNAs was first observed in *Drosophila melanogaster*. The *Drosophila* ortholog of mammalian fragile X protein, dFmr1 was described to interact with RISC complex, and in particular with the main component Ago2 (Caudy et al., 2002; Ishizuka et al., 2002). Two years later, Warren and collaborators showed that FMRP associates with endogenous miRNAs and Ago1 (Jin et al., 2004). They performed genetic studies in *Drosophila* to find that Ago1 depletion suppresses dFmr1 overexpression phenotype, and that a trans-heterozygote for both AGO1 and dFmrp1 shows an even more pronounced synaptic overgrowth phenotype than the dFmr1 null mutant. This indicates that AGO1 might be a limiting factor in dFmr1 function in synaptogenesis, and suggests that microRNAs might mediate the FMR1 role in silencing of neuronal mRNAs.

1.3.2 RISC, microRNAs and synaptic plasticity

Evidence of miRNAs involvement in synaptic plasticity was first reported by Kunes' laboratory (Ashraf et al., 2006). The regulated disruption of the silencing complex component Armitage, led to the removal of miRNA-mediated repression of CaMKII, an mRNA involved in synaptic plasticity. Putative miRNA binding sites are present within the 3'UTR of CaMKII, as well as within the 3'UTR of the transcripts coding for Staufen and Kinesin-Heavy Chain, two dendritic granule-associated proteins. To test whether these mRNAs are targets of miRNA-silencing activity, their expression levels were assayed in brains from mutants of the RISC pathway. The results indicate that synaptic translation of CaMKII increases in dicer-, armitage-, and aubergine-mutant brains. Indeed, this work suggests an armitage-driven repression of CaMKII expression in drosophila olfactory system. Synaptic activation induces a decrease in the levels of Armitage protein and a correspondent increase in CaMKII abundance. The decrease in Armitage protein is shown to be due to the activity of the proteasome which is known to act at the synaptic level to contribute in modulating synaptic protein content (Bingol and Schuman 2005; 2006). Overall, Ashraf et al. (2006) propose a novel and intriguing regulatory mechanism whereby CaMKII translational repression is driven by miRNAs and in turn is relieved by activity-dependent proteasome-mediated degradation of Armitage (Fig.2)



The model depicted here summarizes miRNAs modulation of synaptic protein synthesis and plasticity (Ashraf et al., 2006; Banerjee et al., 2009). MiRNAs are required for the translational silencing of several neuronal mRNAs important for synaptic protein synthesis (see text). Patterns of synaptic activity that induce long-term memory trigger localized proteasome-mediated degradation of MOV10 in mammalian (Armitage in *Drosophila* RISC) and consequent release of the translational repression guided by miRNAs at synapse.

Fig. 2. RISC regulation in synaptic plasticity.

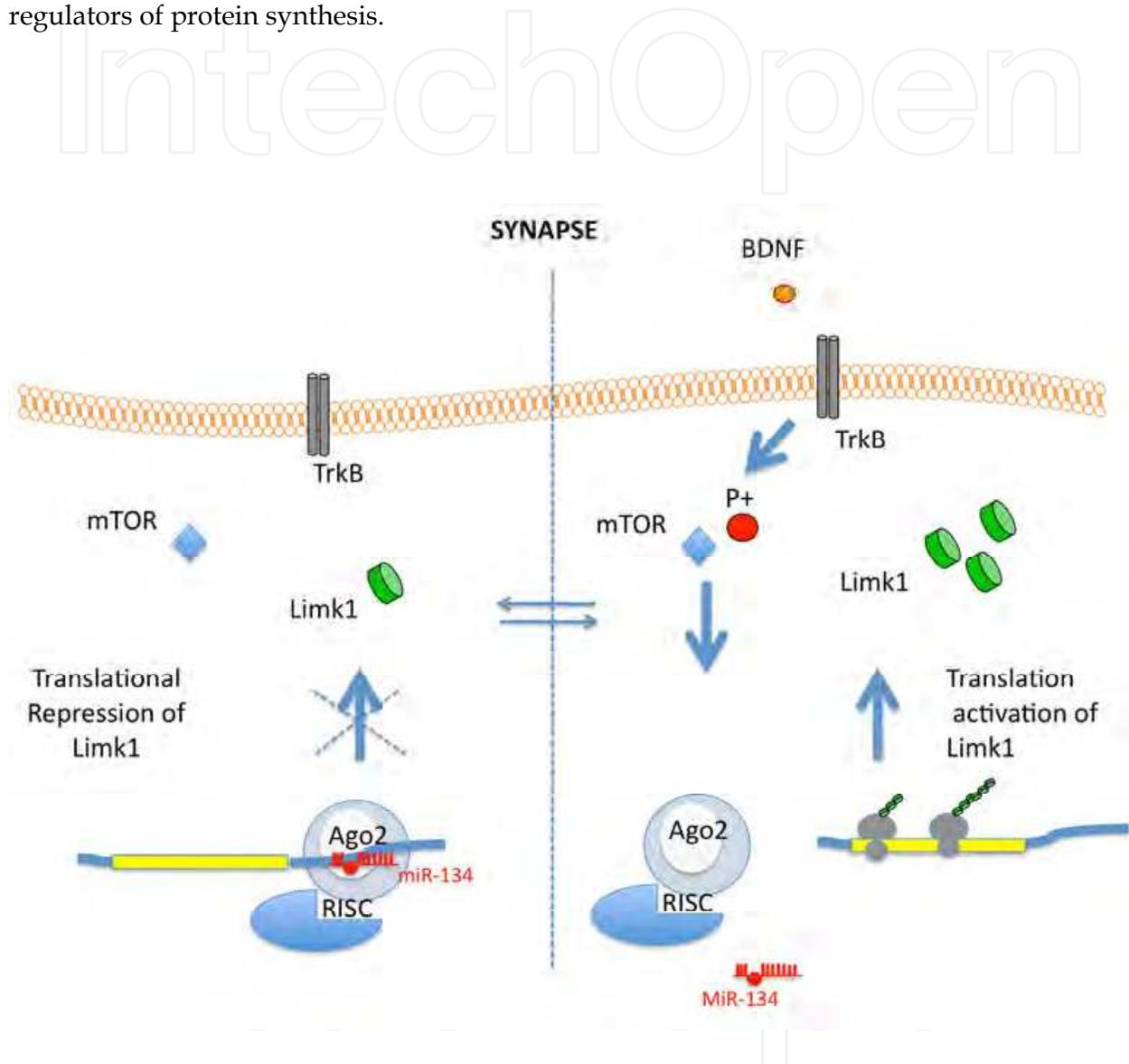
More recently the silencing of the mammalian ortholog of Armitage, Moloney leukemia virus 10 homolog (MOV10), by RNA interference mediated knockdown, showed the relief of translational repression of miRNA targets. Kosik's group, with the aim to identify dendritic mRNAs under RISC's degradative control, individuated several mRNA localized in the dendrites. They trapped both known RISC-regulated mRNAs, as *Limk1* and *alfaCaMKII*, and novel mRNA *Lysophospholipase 1* (*Lypla1*), (also known as *acyl protein thioesterase* (*APT1*), a depalmitoylation enzyme, regulated post-transcriptionally by dendritic miR-138 (Banerjee et al., 2009). Previously Schratt's group showed that in rat hippocampal neurons, miR-138 was enriched at synapses and modulated synaptic development and spine size through the regulation levels of the *APT1* (Siegel et al., 2009), followed by depalmitoylation of *Ga13*, a downstream target of *APT1*, which is an activator of *Rho* downstream of G-protein coupled receptor (Kurose et al., 2003). Another finding which associates miRNA function to the synaptic plasticity in mammalian neurons, was made by Schratt and collaborators, with the brain-specific microRNA-134 (Schratt et al., 2006). The overexpression of miR-134 causes a

significant reduction in dendritic spine size, whereas its inhibition by 2'-O-methyl antisense oligonucleotides induces a slight increase in spine volume. The mRNA target of miR-134 was identified as Lim-domain containing protein kinase 1 (Limk1). Like to Kunes' laboratory findings (Ashraf et al., 2006), in this case neuronal activation also intervenes to put a brake on miRNA-mediated silencing. MiR-134 repression of Limk1 translation is mitigated upon BDNF stimulation of synaptic activity. In cortical neurons, BDNF induced the translation of the 3'UTR Limk1 mRNA luciferase reporter, but not when neurons were transfected with a reporter in which miR-134 responsive sequence was mutated. This suggests that the BDNF/miR-134/Limk1 connection plays a role in synaptic plasticity at synaptodendritic compartment of hippocampal neurons (Fig.3). The studies described above, indicate that miRNAs might contribute to fine-tuning regulation of synaptic protein synthesis and plasticity by modulating expression of dendritic mRNAs.

1.3.3 Memory

Dysregulation in the entorhinal cortex and dentate gyrus during the acquisition of memory is one of the hallmarks that occurs early during Alzheimer's neurodegeneration. The loss of coordination between different pathways orchestrating protein expression at synapses, consists in a loss of control of plasticity. The assumption that synaptic plasticity is considered to underlie memory formation (Morris et al., 2003), and the evidence that forms of long-lasting synaptic plasticity depend on protein synthesis (Manahan-Vaughan et al., 2000), suggest that microRNAs may indeed be important for this phenomenon. What are the downstream effectors that mediate such activity? Several observations suggest that the induction of long-term potentiation (LTP) and long-term depression (LTD), two forms of synaptic plasticity, requires microRNAs. Park and Tang (2009) performed the temporal expression profile of sixty hippocampal microRNAs following induction of chemical LTP (C-LTP) and metabotropic glutamate receptor-dependent LTD (mGluR-LTD) in hippocampal slices. They observed that C-LTP or mGluR-LTD evokes changes of the expression levels of most hippocampal miRNAs, suggesting a role for miRNA-mediated translational repression. MiRNAs regulated in both experimental paradigm, displayed distinct temporal expression dynamics. Further, many miRNAs were upregulated at specific time points of C-LTP and mGluR-LTD induction, like to provide an active mechanism to restore the dormant state of mRNA translation after a transient activation (Park and Tang, 2009). Recent studies demonstrated finely regulation of primary and mature miRNA expression by mGluR and NMDAR signaling during LTP induction (Wibrand et al., 2010). Transgenic mouse strain that expresses miR-132 in forebrain neurons, showed an increase in dendritic spine density and a deficits in novel object recognition, suggesting that dysregulation of miR-132 could contribute to an array of cognitive disorders (Hansen et al., 2010). The first evidence that miRNA expression is specifically altered during an *in vivo* learning paradigm in mammals was carried out by Smalheiser's group, demonstrating that olfactory discrimination training, up-regulates and reorganizes expression of microRNAs in adult mouse hippocampus (Smalheiser et al., 2010). How up-regulation of a subset of miRNAs modulates gene expression pathways during several phases of learning and memory? A new work showing miRNAs as key players in the learning and memory process of mammals, was published by (Konopka et al., 2010). In a mouse model with an inducible disruption of the Dicer1 gene in the adult forebrain, after induction of Dicer1 gene deletion, a progressive loss of a whole set of brain-specific miRNAs was observed. Mice were tested

in a battery of both aversively and appetitively motivated cognitive tasks, such as Morris water maze, IntelliCage system, or trace fear conditioning. An enhancement of memory was recorded twelve weeks after the Dicer1 gene mutation. To date, we can't say a "better memory with less microRNA" or the reverse, but only that microRNAs may modulate memory, that components of long-term potentiation (LTP) require local protein translation, which regulate synaptic plasticity, and that microRNAs have been identified as master regulators of protein synthesis.



The mechanism unveiled by Schrott et al. (2006) in mammalian neurons is illustrated. Limk1 mRNA is subject to miR-134-mediated repression. Upon synaptic activation, induced by BDNF treatment, the authors observe translational derepression of Limk1 mRNA and an increase in spine size. How synaptic activity might release Limk1 mRNA from miR-134 repression is unclear.

Fig. 3. MicroRNA targets in synaptic plasticity and memory.

2. MicroRNA expression profiles in Alzheimer's disease brain

During the last four years, great strides have been made to profile miRNA expression in several regions of the AD brain. To point out whether miRNA expression may be misregulated in AD, Cogswell and coworkers compared the expression of over 300 miRNAs, isolated from hippocampus, medial frontal gyrus and cerebellum from early and late stage AD, to normal age-matched controls (Cogswell et al., 2008). Tissues were grouped by Braak stage and miRNAs were extracted, quantified and amplified by real-time quantitative PCR assay. Experimental data provide a statistically significant number of under- and over-expressed microRNAs. In particular, hippocampus and medial frontal gyrus, which are the earlier regions affected by AD pathology, were characterized by the major modifications. Expression of twenty-one miRNA (miR-200c, -212, -26a, -27a, -30c, -30e-5p, -34a, 381, -422a, -423, -9, -92, 100, -125b, -132, -145, -146b, -148a, -210, -27b, -425) was altered both in early and in end stages of pathology (Cogswell et al., 2008). The pathological cerebrospinal fluids (CSF) were also analyzed. These samples show a different miRNAs expression between AD and non-affected patients. Both miRNA expression profiles represent the first work aimed to identify specific miRNAs as biomarkers of AD (Cogswell et al., 2008). Several efforts have been made to understand whether miRNAs are also altered in sporadic AD. A microRNA expression profile shows that many miRNAs are potentially involved in the regulation of APP and BACE1 (Hebert et al., 2008). In this study the expression of 328 human miRNAs in the anterior temporal cortex and the cerebellum from five AD patients was monitored and compared with age-matched controls. Among all miRNAs analyzed, 13 were significantly altered and at least 7 were predicted to target the 3' UTR of BACE1 (miR-15a, 29b-1, -9, and -19b) or APP (let-7, miR101, miR15a, and miR106b) (Hebert et al., 2008). Nunez-Iglesias (2010) used microarrays for the first joint profiling and analysis of miRNAs and mRNAs expression in brain cortex from AD and age-matched control subjects. These data provided the unique opportunity to study the relationship between miRNA and mRNA expression in normal and AD brains. Starting from genome-wide miRNA and mRNA data expression, analysis was carried out to determine the correlation between levels of miRNAs and their target mRNAs (Nunez-Iglesias et al., 2010). The results reveal that most miRNA-mRNA pairs are actually uncorrelated making more difficult to understand the correlation level between miRNAs with their targets. By an elegant analysis performed taking advantage from gene ontology biological processes, RNAs were grouped together. Among the processes most positively correlated with their regulating miRNA, the authors found some specific processes, including metabolism of both carbohydrates and fatty acid, as well as protein refolding which indicate the importance of these processes in the brain. Among the processes most negatively correlated are oxygen transport, cell adhesion, inflammatory response, cytoskeletal organization and dendrite development (Nunez-Iglesias et al., 2010). The method depicted can find active miRNA-mRNA relationships dependent from the tissue context. These authors demonstrate a relationship between the levels of miRNAs and those of their targets in the brain, identifying a large set of miRNA-mRNA associations that are changed in AD versus control, and AD-specific changes in the miRNA regulatory system. Microarray techniques, based on correlation methods, are proving to be powerful tools for investigation on miRNAs deregulation in AD. The expression of 5-6% of miRNAs was affected by AD at statistical significant level in the studies described above (Hebert et al., 2008; Cogswell et al., 2008; Nunez-Iglesias et al., 2010) suggesting that a substantial number of miRNAs are deregulated in this pathology.

BRAIN AREA	EXPRESSION IN ALZHEIMER'S DISEASE	miRNA	Reference
Gray matter	Upregulated	miR-519e, miR-574-5p, miR-498, miR-518a-5p/miR-527, miR-525-5p, miR-300, miR-576-3p, miR-583, miR-146b-3p, miR-490-3p, miR-549, miR-516a-5p, miR-510, miR-184, miR-516b, miR-298, miR-214, miR-198, miR-451, miR-144, miR-424, let-7e	(Wang et al., 2011)
Gray matter	Downregulated	miR-485-3p, miR-381, miR-124, miR-34a, miR-129-5p, miR-29a, miR-143, miR-36, miR-145, miR-138, miR-129-3p, miR-128, miR-143, miR-136, miR-145, miR-138, miR-129-3p, miR-128, miR-126, miR-411, miR-335, miR-9, miR-378, miR-488, miR-32, miR-127-5p, miR-127-3p, miR-491-5p, miR-376c, miR-377, miR-95, miR-222, miR-29b, miR-329, miR-495, miR-551b, miR-195, miR-125b, miR-30b, miR-221, miR-139-5p, miR-487a, miR-487b, miR-107, miR-146b-5p, miR-29c, miR-30a, miR-582-5p, miR-103, miR-342-3p, miR-331-3p, miR-30c, miR-30d, miR-382, miR-22, miR-125a-5p, miR-425, miR-191, miR-519d, let-7g, miR-98, miR-99a, miR-30e	(Wang et al., 2011)
White matter	Upregulated	miR-509-5p, miR-574-3p, miR-576-5p, miR-302e, miR-220b, miR-208a, miR-215	(Wang et al., 2011)
White matter	Downregulated	miR-491-3p, miR-423-5p, miR-34b, miR-422a, miR-34c-5p, miR-584, miR-219-5p, miR-338-3p, miR-219-2-3p, miR-338-5p, miR-181a, miR-181b, let-7b, miR-151-3p, miR-197, miR-19a, miR-20a, miR-17, miR-106a, miR-32, miR-340, miR-19b, miR-21, miR-151-5p, miR-194, let-7c, miR-330-3p, miR-27b, miR-93, miR-15a, miR-339-5p, miR-193b, miR-106b, miR-16, miR-23b, miR-15b, miR-320d, miR-320b, miR-320c, miR-320a, miR-557, miR-33a, let-7a, miR-374b, miR-140-3p, miR-374a, miR-24, miR-140-5p, miR-26a, miR-513a-5p, miR-212, miR-142-5p, miR-142-3p, miR-26b, miR-520d-5p, miR-193a-3p, miR-92b, miR-330-5p, miR-186, let-7f, miR-223, miR-412, miR-185, miR-148b, miR-101, miR-99b, miR-27a, miR-589, let-7i, miR-361-3p, miR-361-5p, miR-423-3p, miR-190, miR-301a, miR-365, miR-23a, miR-363, miR-326	(Wang et al., 2011)

Hippocampus	Upregulated	miR-26a, miR-27a, miR-30e-5p, miR-34a, miR-92, miR-381, miR-422a, miR-423, miR-27b, miR-125b, miR-145, miR200c	(Cogswell et al., 2008)
		miR-146a	(Cui et al., 2010)
Hippocampus	Downregulated	miR-9, miR-128, miR-125b	(Lukiw, 2007)
		miR-30c, miR-212, miR-132, miR-146b, miR-210, miR-425, miR-9	(Cogswell et al., 2008)
Medial frontal gyrus	Upregulated	miR-27a, miR-30c, miR-30e-5p, miR-34a, miR-92, miR-381, miR-422a, miR-423, miR-27b, miR-100, miR-125b, miR-145, miR-148a, miR-29a, miR-29b, miR-423, miR-145	(Cogswell et al., 2008)
Medial frontal gyrus	Downregulated	miR-26a, miR-200c, miR-212, miR-132, miR-146b, miR-210, miR-425	(Cogswell et al., 2008)

Parietal lobe cortex	Upregulated	miR-30184, miR-617,miR-188,miR-06383, miR-10912,miR-601,miR-23974, miR-10939, miR-19790, miR-35456, miR-134, miR-671, miR-320, miR-575, miR572, miR-45605, miR-765, miR-18895, miR-432, miR-382, miR-185, miR-486, miR-28648	(Nunez-Iglesias et al., 2010)
Parietal lobe cortex	Downregulated	miR-101,miR-20546,miR-29b, miR-181c, miR-08570, miR-42448, miR-44608, miR-02532, miR-12497, miR-582, miR-15a, miR-374, miR-95, miR-05109, miR-30e-5p, miR-148b, miR-130a, miR-598, miR-376a, miR-29c, miR-12504, miR-494, miR-20b, miR-368	(Nunez-Iglesias et al., 2010)
Temporal lobe cortex (Sporadic AD)	Upregulated	miR-197, miR-511, miR-320	(Hebert et al., 2008)
Temporal lobe cortex (Sporadic AD)	Downregulated	miR-210, miR-181c, miR-15a, miR-9, miR-22, miR-101, miR-29b-1, miR-19b, let-7i, miR-106b, miR-26b, miR-363, miR-93	(Hebert et al., 2008)
Temporal lobe cortex	Upregulated	miR-9, miR-125b, miR-146a	(Sethi & Lukiw, 2009)

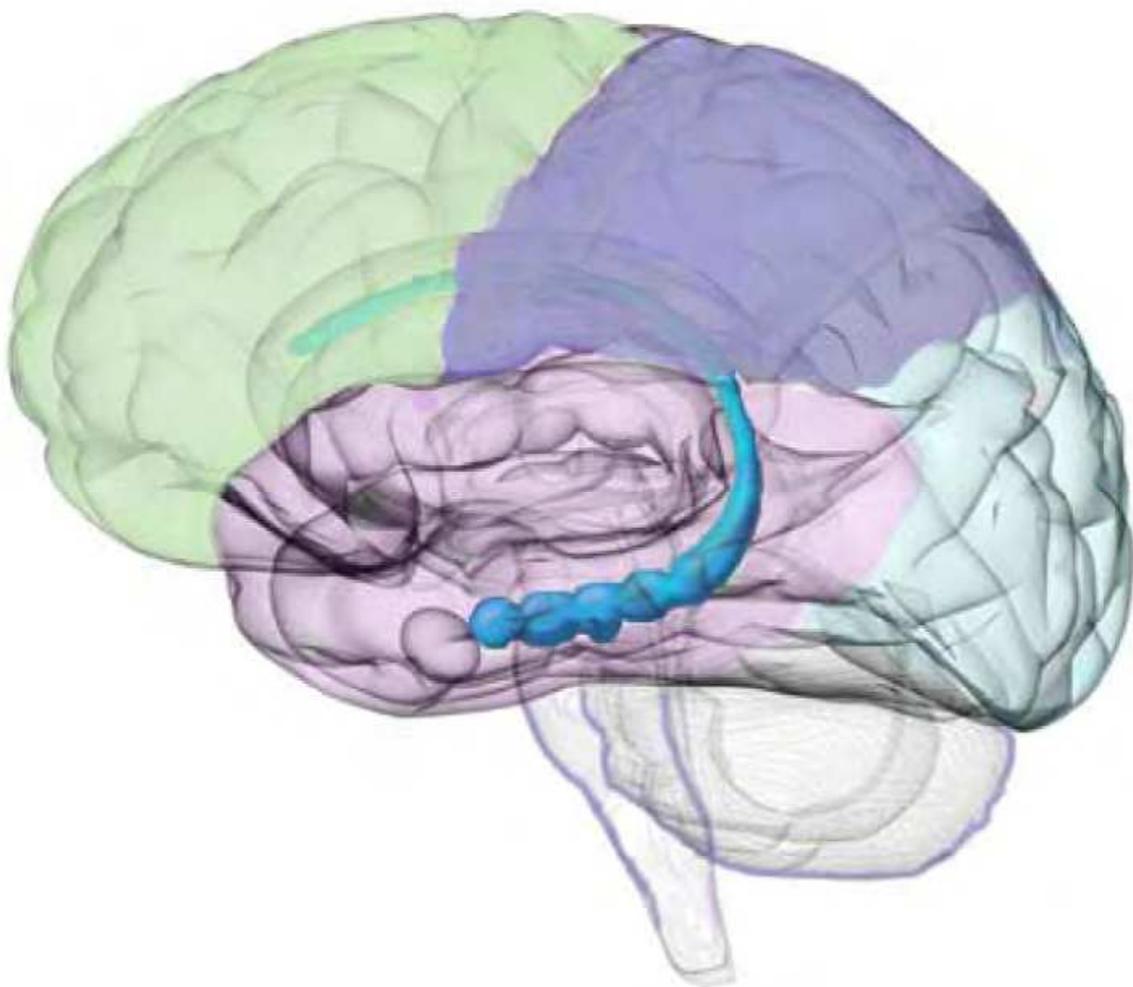


Table 1. Studies reporting changes of miRNAs expression profiles in Alzheimer's disease and in different brain areas.

2.1 Addressing brain tissue complexity

In gene expression studies the heterogeneity of the human cerebral cortex should be considered. For example, former tissue sampling protocols did not segregate white matter from gray matter, increasing variability that reflects differently sample cell population rather than a disease condition (Wang et al., 2011) (Table 1). Nelson's laboratory deals with this question either analyzing different miRNAs expression in human cerebral cortical white matter and gray matter in normal and early AD pathology and using different experimental techniques. MiRNA profiling experiments were performed using a locked nucleic-acid-microarray (LNA-microarray) which highlights a subset of miRNAs that appeared to be strongly expressed and did not appear to be conventional miRNAs (Wang et al., 2011). Using clustering analysis they demonstrated a different miRNA expression pattern between white matter and gray matter and between normal and pathological condition. Further, they found that there was an apparent clustering between samples derived from gray matter relative to those derived from white matter, and also cases with more AD pathology tended to cluster together relative to control cases. Moreover, miRNA expression may be correlated to neuropathological hallmarks in AD: amyloid plaques (with or without degenerating neuritis) and neurofibrillary tangles. Because of the major pathological changes affected gray matter, it is not surprising that a number of miRNAs altered in AD was higher in gray matter than white matter, but a handful of miRNAs was altered specifically in white matter. These data indicate an emerging need: while the expression of individual miRNA may be impactful alone, it is necessary to assess the aggregate impact of multiple miRNAs and the connection between AD pathological features and miRNA alteration (Wang et al., 2011). In fact, miRNA expression profiles highlight almost two altered pathways in AD, which involve inflammatory alteration response and $A\beta_{1-42}$ production and physiology. However, bioinformatic tools and miRNA expression profile offer a data set that have to be validate in cellular system in order to understand which are the mRNA targets and, eventually the physiopathological implications. Variation in tissue sampling may contribute to heterogeneous results. Banked tissue is essential to the study of neurological diseases but the use of postmortem tissue introduces a number of possible confounds. A variety of antemortem factors may influence the quality of harvested tissue including fever, hypoxia-ischemia and acidosis, while critical postmortem variables include postmortem interval (PMI), brain or cerebrospinal fluid pH, ambient temperature in the postmortem period, harvesting procedures, storage temperature and accidental or systematic thawing and freezing. Interindividual differences in postmortem tissue studies are typically large and often prevent the attainment of statistical significance. Much of interindividual variability may be due to highly variable agonal condition; RNA yield and quality may be affected by the extraction method employed. Many report that global measures of total RNA quality are relatively stable over a wide range of PMIs while some others report a definite loss with increasing time (Birdsill et al., 2010). However, in all extraction techniques employed a negative correlation between PMI and RNA quality-yield is point out. Furthermore, miRNA half-life appears significantly correlated with content of AU and UA dinucleotides in the RNA sequence. MiRNA-9, for example, which has 26.1% of AU in the sequence, has a half-life shorter (c.a 1 h) than miRNA-125b, 9.5% AU rich and 3.5h half-life. These data suggest the importance to compare normal and pathological tissue with similar PMI. In fact, analysis of miRNAs in autopsied brain tissue with post-mortem intervals many-fold greater than the half-lives of miRNAs may lead to inaccurate conclusions concerning their absolute abundance and hence the contribution of miRNAs to gene regulation in the brain during development, aging and in disease processes (Sethi et al., 2009). Studies performed in brain tissues with short PMI indicate that miR-9, miR-146 and miR-125 were significantly

upregulated in AD-affected temporal lobe. Importantly comparative expression analysis showed no change of these microRNAs in tissues derived from patients affected with amyotrophic lateral sclerosis, Parkinson's disease, schizophrenia. These data suggest that these miRNAs may contribute to the pathogenesis characteristic of AD. Microarray system is suitable to perform miRNA expression profiling but it did not show specific cellular distribution. Instead in situ hybridation (ISH) should be a method to directly understand the specific roles of miRNAs in the human brain at the cellular and sub-cellular levels. ISH is more labor-intensive and "low-throughput" compared to other RNA expression profiling techniques, but allows far greater resolution of given RNA's expression (Nelson et al., 2009). For example, ISH shows important cerebral cortical lamina-specific patterns of miRNA expression that would be lost on most tissue level expression studies, and these lamina-specific patterns may be relevant to human brain disease.

3. Exploring microRNA function in Alzheimer's disease

MiRNA target prediction programs, cell-based functional assays and studies in mouse models have been used to identify some of the molecular and biological functions of microRNAs dysregulated in AD brain tissues.

3.1 miRNAs dysregulated in AD modulate BACE and other targets

The amyloid precursor protein (APP), its proteolytic product amyloid beta ($A\beta$), generated by beta secretase (BACE) and gamma secretase, are all associated with both familial and sporadic forms of Alzheimer disease (AD) (Fig.4). BACE1 gene was shown post-transcriptionally regulated by miR-107 (Wang et al 2008) and miR-29a/b-1 (Hebert et al 2008). Interestingly miR-107 expression levels decreased during AD pathology progression (Wang et al 2008). On the other hand miR-29 was downregulated in anterior temporal cortex from sporadic AD patients in which BACE 1 protein was abnormally upregulated while BACE 1 mRNA levels were unchanged (Hebert et al 2008). Using BACE1 3'UTR luciferase reporter carrying wild type or mutated miRNA responsive sites BACE1 post-transcriptional regulation by those microRNA was demonstrated. Moreover, upon either overexpression or downregulation of miR-29a/b-1 in human cell culture both BACE1 protein levels and APP cleavage product $A\beta$ were, respectively, reduced and increased (Hebert et al 2008) (Fig.4).

Although these studies clearly indicate a relationship of both miR-29 and miR-107 with a gene associated to AD pathology identification of all mRNA targets affected by each microRNA deregulated in AD is the real challenge of future investigations. Indeed other studies indicate that miR-29 may play a more complex role in neurons during AD neurodegeneration. Neuron navigator 3 (NAV3), a regulator of axon guidance, was validated as miR-29 target and by immunohistochemistry NAV3 expression was found enhanced in degenerating pyramidal neurons in the cerebral cortex of AD (Shyoia et al., 2010). Moreover studies in sympathetic neurons have shown that miR-29b function as an inhibitor of neuronal apoptosis (Kole et al., 2011) by inhibition of multiple redundant BH3 only proteins (Bim, Bmf, Hrk, Puma and N-Bak) that are key initiators of apoptosis. Therefore loss of miR-29 expression may lead to neuron vulnerability and neurodegeneration. Additional roles for miR-107 in AD pathogenesis are demonstrated by studies in a transgenic mouse model of AD which over-expresses human APP carrying familial AD mutations (Yao et al., 2010). In particular reduced levels of miR-103 or miR-107 are associated with elevated cofilin protein levels and formation of rod-like structures in this

AD mouse model. Rod-like structures composed of actin and the actin-binding protein cofilin are also found in Alzheimer's disease (AD) patients (Whiteman et al., 2009).

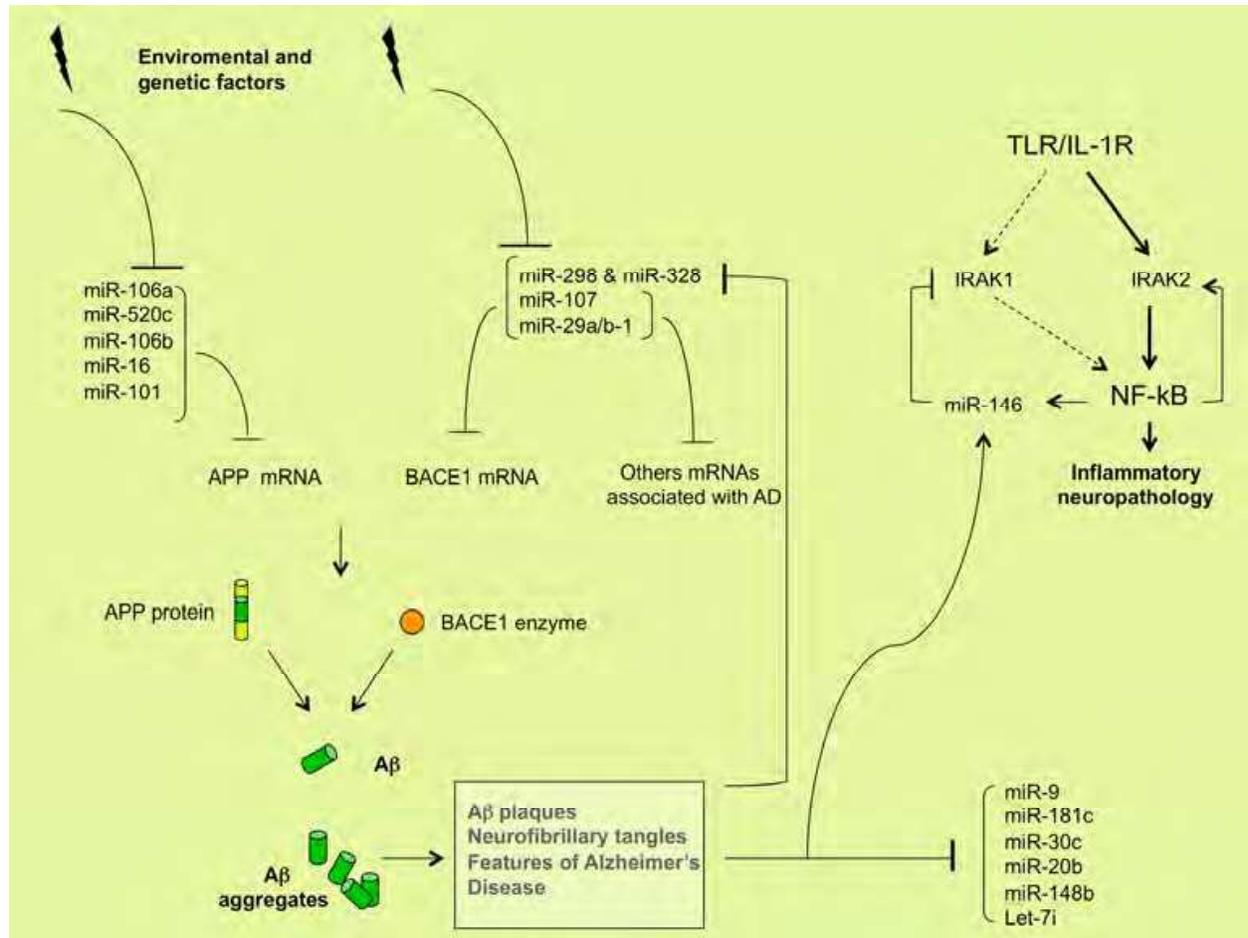


Fig. 4. MiRNAs deregulation in Alzheimer's disease pathology.

3.2 Several miRNAs regulate APP

Among mutations causing familial AD, duplication of the APP gene has also been detected (Rovelet-Lecrux et al., 2006). In addition, individuals with trisomy 21 (the APP gene is located on chromosome 21) are at increased risk of developing AD late in life (Podlisny et al., 1987). Several studies have reported that APP expression is post-transcriptionally regulated by microRNAs. A list of microRNAs: miR-106a and miR-520c, miR-20a family members (including miR-20a, miR-106b and miR-17-5p), miR-16 and miR-101, was demonstrated to downregulate APP expression (Patel et al., 2008; Hebert et al., 2009; Liu et al., 2010; Vilardo et al., 2010). The expression levels of these miRNAs were reduced in AD tissues (Fig. 4). When overexpressed in human cell lines, both miR-106a and miR-520c reduced endogenous APP levels by 50% compared to cells transfected with a non-targeting miRNA (Patel et al., 2008). However, only miR-106a is expressed in neuronal context. Overexpression of miR-20a (and miR-17-5p/106b) post-transcriptionally reduced endogenous APP expression in both non-neuronal and neuronal cells. Moreover, reduction of miR-20a, miR-17-5p and miR-106b levels was correlated with an increase in APP protein levels in developing mouse brain and in primary cultured neurons. Although two putative

miR-20 family responsive elements (REs) are predicted within the APP 3'UTR only one RE functionally interacted with miR-20 family miRNAs. When miRNA expression levels in human AD brains were analyzed, miR-106b was significantly reduced with respect to control samples. However, since APP levels varied among the different samples, a tight correlation between miR-106b and APP levels in AD tissues was not obtained (Hebert et al., 2009). In an age-associated AD animal model, senescence-accelerated mouse prone 8 (SAMP8), which has age-related learning and memory deficits post-transcriptional regulation of APP by miR-16 was suggested. Overexpression of miR-16, both in vitro and in vivo, led to reduced APP protein expression. Furthermore, miR-16 and APP displayed complementary expression patterns in SAMP8 mice and BALB/c mice embryos (Liu et al., 2010). More recently, the regulation of APP expression by the RISC/miRNA pathway was examined in primary rat hippocampal neurons. First, silencing of Ago2 in hippocampal neurons increased APP protein levels, suggesting that APP translation may be regulated by an miRNA pathway. Among miRNAs potentially targeting the APP 3'UTR, miR-101 was selected for further study because two putative miR-101 target sites, which are conserved among the human, rat and murine APP genes, are present in the 3'UTR. MiR-101 is a brain-enriched miRNA and its expression is inversely correlated with APP in developing rat hippocampal neurons and tissues. Using site directed mutagenesis, a functional interaction between miR-101 and one of the two REs was demonstrated. The inhibition of endogenous miR-101 increased APP levels, whereas lentiviral-mediated miR-101 overexpression significantly reduced APP and A β load in hippocampal neurons (Vilardo et al., 2010). These data support the hypothesis that miR-101 is a repressor of APP expression. Moreover, as described for other miRNAs, miR-101 is downregulated in the human AD cerebral cortex (Hebert et al 2008; Nunez-Iglesias et al., 2010) (Fig.4). Interestingly besides APP expression regulation, miRNAs were also involved in the regulation of neuronal APP mRNA alternative splicing which also affects β -amyloid peptide production. APP exons 7 and 8 inclusion was observed in postmitotic neurons of conditional Dicer knock-out mice (Hebert et al., 2010), while over-expression of miR-124, an abundant neuronal-specific miRNA, reversed these effects in cultured neurons. Similar results were obtained by depletion of endogenous polypyrimidine tract binding protein 1 (PTBP1) in cells, a recognized miR-124 target gene. Furthermore, PTBP1 levels correlate with the presence of APP exons 7 and 8, while PTBP2 levels correlate with the skipping of these exons during neuronal differentiation. Expression studies in AD brain showed that miR-124 expression was down-regulated (Smith et al., 2011).

3.3 Changes of miRNA in AD are implicated in inflammation, aging and oxidative stress

Inflammatory signaling plays determinant roles in brain homeostasis and neuroprotection however, altered or excessive signaling in these injury defense systems contributes to the irreversible degeneration of brain cells, in neurodegenerative disorders such as Alzheimer disease (AD). The inflammatory processes are involved during progression of AD pathogenesis, and A β 42 peptides, cytokine IL-1 β upregulation and oxidative stress are inflammatory mediators (Lukiw, 2004). A mouse and human brain abundant miRNA-146 was upregulated in AD brain and associated with the down-regulation of complement factor H, an important repressor of inflammatory signaling in the complement cascade, in

AD brain (Lukiw et al 2008). Furthermore in the hippocampus and neocortex of Alzheimer disease (AD) brain as well as in stressed human astroglial (HAG) cells in primary culture, increased expression of an NF- κ B-regulated miRNA-146 down-regulates expression of the interleukin-1 receptor-associated kinase-1 (IRAK-1), an essential component of Toll-like/IL-1 receptor signaling (Cui et al 2010). Family of interleukin-1 receptor-associated kinases (IRAKs) in the human genome, including IRAK-1, IRAK-2, IRAK-4, and IRAK-M, are key mediators in the immune pathways utilized by TLR/IL-1R (TIR) signaling. By means of their integral kinase IRAKs initiate diverse downstream signaling processes that can eventually lead to the induction of pro-inflammatory transcription factors such as NF- κ B. In control and AD samples a significant up-regulation of miRNA-146a coupled to down-regulation of IRAK-1 and a parallel up-regulation of IRAK-2 was noted. Finally independent regulation of IRAK-1 and IRAK-2 in IL-1 β +A β 42 peptide-stressed HAG cells and inducible, NF- κ B-sensitive, miRNA-146a-mediated downregulation of IRAK-1 coupled to an NF- κ B-induced up-regulation of IRAK-2 expression was demonstrated (Cui et al., 2010). This regulatory network provides an important basis for a self-perpetuating inflammatory signaling loop (Fig.4). For sporadic Alzheimer's disease, which accounts for the majority of Alzheimer's disease cases, the most important risk factor is aging. Several miRNAs that are modulated in various aging model system, might contribute to AD. It was proposed that an alteration of miRNAs expression control in mid-life may be the putative force inducing molecular frailty in individual cell signaling, and in time leading to tissue-wide dysfunction (Wang, 2007). For example, miR-34a is increased in AD, peripheral blood mononuclear cell, aging mouse liver and *C. elegans* (Cogswell et al., 2008; Schipper et al., 2007; Maes et al., 2008; Ibanez-Ventoso et al., 2006). MiRNA lin-4 has been elegantly shown to influence lifespan and healthspan via its lin-14 mRNA target and the insulin signaling pathway, and several *C. elegans* age-regulated miRNAs have sequence similarity with both fly and human miRNAs (Ibanez-Ventoso & Driscoll, 2009). The question is whether there are evolutionary-conserved homologs of lin-4 and lin-14 that control human longevity and if miRNAs are implicated in many age-associated pathologies. Other miRNAs are upregulated during aging as let-7f, miR-30d, miR-432, miR-517 and downregulated as let-7i and miR-451 (Maes et al., 2009). Are they involved in gene regulation of aging related mechanism in AD? There is a great deal of evidence that suggests that oxidative stress plays a crucial role in the initiation and progression of Alzheimer's disease. In AD peripheral blood mononuclear cells, an impairment in DNA repair and antioxidant gene responses was observed and correlated to the up-regulation of miR-181b, miR-200a, miR-517* and miR-520, that may repress DNA repair and the response to oxidative stress (Shipper et al., 2007).

3.4 Studies in mouse models exploring microRNAs function in Alzheimer's disease

Mouse models have been recently used to investigate microRNA function in AD pathology. Specific deletion of Dicer in the adult forebrain leading to loss of microRNAs, was accompanied by a mixed neurodegenerative phenotype (Hebert et al., 2010). Although neuronal loss was observed in the hippocampus, cellular shrinkage was predominant in the cortex. Interestingly, neuronal degeneration coincides with the hyperphosphorylation of endogenous tau at several epitopes previously associated with neurofibrillary pathology.

Changes of miRNA expression might trigger molecular events inducing AD pathology or generate a feed-forward mechanism during AD progression. Two recent studies performed on mouse models of AD suggest that progression of AD pathology may produce alteration of microRNAs expression (Fig. 4). In APP^{swe}/Psen1 transgenic mice, an AD mouse model which recapitulates some features of the disease, it was observed that BACE1 mRNA decreased and protein levels increased in the hippocampus at 19 months of age (Boissonneault et al., 2009). Two microRNAs, miR-298 and miR-328, were found to regulate BACE 1 protein expression in mouse cultured neuronal cells. In transgenic mice, the expression of miR-298 and miR-328 decreased in the granular neurons of the hippocampus during aging. However, while the miR-328 sequence is perfectly conserved between mouse and human, that of miR-298 is only 72% identical. More recently the hypothesis that A β itself causes neuronal miRNA deregulation which could contribute to the pathology associated with AD was explored (Schonrock et al 2010). A list of miRNAs, miR-9, miR-181c, miR-30c, miR-20b, miR-148b and Let-7i, was also downregulated in primary hippocampal cell cultures treated with A β . The miRNAs changes overlap with those occurring in the hippocampus of APP 23 mice (expressing human APP751 cDNA containing the Swedish double mutation (K651M and N652L) at 7 months of age. At this age, mice reach the critical period of A β plaque formation where insoluble A β 42 peptides increase five-fold compared to younger animals and small plaques can be seen in hippocampus and neocortex. In addition, the mice display major cognitive deficits affecting visuo-spatial learning abilities. These deregulated miRNAs overlap also with those found in sporadic human AD brain and potentially affect important biological pathways essential for proper brain function relevant to AD. The overlap between human AD brain and in vitro/in vivo AD models indicates that among the complex AD pathology, downregulation of miR-9, miR-181c, miR-30c, miR-20b, miR-148b and Let-7i could be attributed at least in part to the presence of A β (Fig.4).

4. Conclusion

Until now altered expression of miRNAs in Alzheimer's Disease brain tissues was demonstrated, however different studies identified various microRNAs. Increasing the numbers of miRNA expression profiles using large cohort of sporadic AD patients may allow us to better understand whether the variability among different profiles may be due to disease specific interindividual differences. It is difficult to determine if the changes in miRNA expression detected in the brains or CSF of patients are primary or secondary events, or both. Nevertheless early or late in the evolution of the disease, they could contribute to the pathogenesis of the observed lesions and neuronal loss. Unique patterns of miRNA expression profile in the CSF of AD could be useful as molecular biomarkers for disease diagnosis and eventually prediction of therapeutic responses. Target prediction analysis and cell-based assay linked miRNAs dysregulated in AD to APP and BACE1 genes which are associated with the pathology. Now the effort is to identify all targets of individual microRNA and to evaluate the impact that more than one microRNA may play on a specific target. In this regard computational prediction programs may be complemented by experiments in which the changes in transcripts and protein levels due to miRNA induction or knockdown are analysed using microarray platforms and SILAC (stable-isotope labelling with aminoacid in cell culture) and mass spectrometry (Selbach et al 2008). Also the co-immunoprecipitation of mRNA targets with Ago2 combined with

microarray analysis of RNA (RIP-Chip assay) represents an alternative approach (Tan et al 2009). As demonstrated for the upregulation of miR-146 by the transcription factor NFkB the characterization of promoters as well as transcriptional activators and repressors regulating microRNAs expression will be instrumental to delineate the relationship of microRNAs with networks involved in AD pathology. The use of transgenic animal models, manipulated in putative brain-expressed microRNAs associated to high-throughput methodologies, integrating transcriptomic, mirnomic and proteomics, will help to evaluate the potential association between RNA-mediated gene regulation and the pathogenesis of Alzheimer Disease and might indicate unexpected gene networks underlying Alzheimer's Disease.

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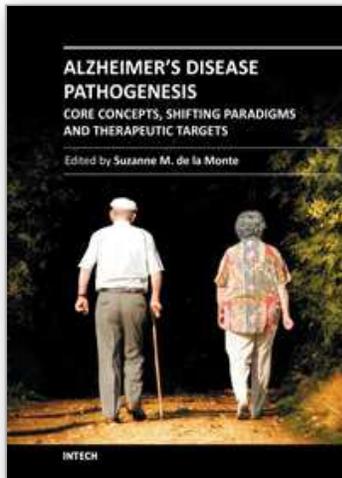
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Alzheimer's Disease Pathogenesis-Core Concepts, Shifting Paradigms and Therapeutic Targets

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Alzheimer's Disease Pathogenesis: Core Concepts, Shifting Paradigms, and Therapeutic Targets, delivers the concepts embodied within its title. This exciting book presents the full array of theories about the causes of Alzheimer's, including fresh concepts that have gained ground among both professionals and the lay public. Acknowledged experts provide highly informative yet critical reviews of the factors that most likely contribute to Alzheimer's, including genetics, metabolic deficiencies, oxidative stress, and possibly environmental exposures. Evidence that Alzheimer's resembles a brain form of diabetes is discussed from different perspectives, ranging from disease mechanisms to therapeutics. This book is further energized by discussions of how neurotransmitter deficits, neuro-inflammation, and oxidative stress impair neuronal plasticity and contribute to Alzheimer's neurodegeneration. The diversity of topics presented in just the right depth will interest clinicians and researchers alike. This book inspires confidence that effective treatments could be developed based upon the expanding list of potential therapeutic targets.

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