

## ORIGINAL ARTICLE

## Opposing actions of environmental enrichment and Alzheimer's disease on the expression of hippocampal microRNAs in mouse models

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Alzheimer's disease (AD) is the most common form of dementia in the elderly. Although there are no drugs that modify the disease process, exposure to an enriched environment (EE) can slow the disease progression. Here, we characterize the effects of AD and EE on the post-transcriptional regulators, microRNAs (miRNAs), which may contribute to the detrimental and beneficial effects of AD and EE, respectively, on synaptic plasticity-related proteins and AD pathology. We found for the first time miRNAs that were inversely regulated in AD and EE, and may affect synaptic proteins and modulators, molecular factors associated with AD pathology, and survival and neuroprotective factors. MiRNAs that were upregulated only in 3xTgAD mice model of AD compared with their control mice were localized to synapses, predicted to downregulate essential synaptic proteins and are highly associated with regulating apoptosis, AD-associated processes and axon guidance. Studying the progressive change in miRNAs modulation during aging of 3xTgAD mice, we identified miRNAs that were regulated in earlier stages of AD, suggesting them as potential AD biomarkers. Last, we characterized AD- and EE-related effects in the mouse hippocampus on tomosyn protein levels, an inhibitor of the synaptic transmission machinery. While EE reduced tomosyn levels, tomosyn levels were increased in old 3xTgAD mice, suggesting a role for tomosyn in the impairment of synaptic transmission in AD. Interestingly, we found that miR-325 regulates the expression levels of tomosyn as demonstrated by a luciferase reporter assay, and that miR-325 was downregulated in AD and upregulated following EE. These findings improve our understanding of the molecular and cellular processes in AD pathology, following EE, and the interplay between the two processes, and open new avenues for the studies of understanding and controlling AD.

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## INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia in the elderly. Its pathology is associated with extracellular A $\beta$  plaques, intracellular tau tangles and cell death in the brain.<sup>1–3</sup> Progression of this disease is characterized by cognitive impairment and deterioration of brain performance.<sup>1,3</sup> These devastating processes are associated with loss of neuronal synapses<sup>1</sup> and alterations in synaptic plasticity, and are most pronounced in the hippocampus, entorhinal cortex and default mode network, brain areas that are among the first to be affected in AD.<sup>3–5</sup>

AD progression is influenced by both genetic and environmental factors,<sup>6–8</sup> and gene–environment interactions may influence and trigger pathogenic pathways that determine the severity and progression of the disease.<sup>6–8</sup> Interestingly, education and socioeconomic background in humans may affect AD pathogenesis,<sup>9,10</sup> with several epidemiological studies showing that subjects with a lower education level are at higher risk of developing AD.<sup>11</sup> Moreover, although no cure is currently available for AD, exposure to an enriched environment (EE) has been shown to have a protective effect in mouse models by slowing disease progression and reducing AD-like cognitive impairment,<sup>12–17</sup> although how exactly EE may be beneficial to AD pathology is yet not fully understood.

Mice exposed to EE experience aerobic exercise, an enlarged exploration area, and increased sensory and visual stimulation compared with mice in the usual laboratory cage environment. Since Rosenzweig's initial studies,<sup>18</sup> behavioral manipulations such as EE or running wheels have been shown to improve cognitive function and sensory–motor performance.<sup>19–21</sup> Moreover, different EE protocols have significantly improved or prevented damage to brain performance in neurodegenerative disease animal models, including AD models.<sup>13,14,17,22–27</sup> In humans, individuals who continue to be involved in intellectually stimulating activities maintain higher and prolonged intellectual abilities,<sup>28,29</sup> while those with good physical fitness showed improved memory in old age.<sup>30</sup>

Although substantial changes such as in the expression levels of synaptic proteins following EE in mice and in AD patients were reported,<sup>31–38</sup> the regulation of these changes in the molecular level, and the interplay between these changes and microRNAs (miRNAs) regulation have received less consideration. We were therefore interested in studying the impact of EE on miRNAs expression in the context of AD pathology in the 3xTgAD mouse model of AD and their effects on the synaptic transmission process and AD pathology.

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MiRNAs are small non-coding RNAs averaging 22 nucleotides in length, which have a role in a central post-transcriptional regulatory mechanism for gene expression. These RNAs bind the 3' untranslated region (3'UTR) of mRNA transcripts and facilitate its degradation or inhibit its translation.<sup>39–43</sup> MiRNAs are highly concentrated in synaptic compartments,<sup>44–46</sup> and regulate synaptic function and plasticity.<sup>47,48</sup> MiRNAs also inhibit expression of several neurodegeneration-related genes,<sup>49</sup> including those involved in AD. The expression levels of miRNAs in EE<sup>50</sup> and their general roles in AD pathology<sup>51–55</sup> were only recently studied. However, the roles of miRNAs in AD and EE, as well as the protective effect of EE on AD pathology by miRNAs are not fully defined,<sup>56–58</sup> and are therefore the main motivators of this study.

To learn about miRNAs regulation in AD, we used the well-established transgenic model of AD, the 3xTgAD mouse strain, which is characterized by developing both amyloid deposits and neurofibrillary tangle-like pathology in AD-relevant brain regions.<sup>59</sup> In this model, intracellular A $\beta$  immunoreactivity is apparent between 3 and 4 months of age in the neocortex, and by 6 months of age in the CA1 subfield of the hippocampus. Extracellular A $\beta$  deposits first became apparent in 6- to 8-month-old mice within the frontal cortex and by 12 months in other cortical regions and in the hippocampus, suggesting that there is an age-related, regional dependence to A $\beta$  deposition in these mice. Hyperphosphorylated tau immunoreactivity is first evident in CA1 neurons and layer IV cortical neurons at about 10 months and progressively increases thereafter. A $\beta$  pathology develops earlier than the tau pathology, consistent with the amyloid cascade hypothesis.<sup>60</sup>

In this study, we measured miRNA levels in the hippocampus of mice that were either exposed to EE or mice model of AD, and correlated these with the expression of the synaptic protein tomosyn (also known as syntaxin-binding protein 5). Tomosyn, a cytosolic protein that is highly enriched in the hippocampus,<sup>61</sup> is an inhibitor of the synaptic vesicle priming step and the synaptic transmission machinery via interference with the formation of soluble NSF attachment protein receptor (SNARE) complexes.<sup>62–69</sup> By doing so, we show the inverse effect of EE or AD on tomosyn expression levels and discover for the first time key miRNAs that were inversely regulated following EE and in AD. Some of these miRNAs could be synapse related, where they regulate the properties of the synaptic transmission machinery. Other miRNAs possibly regulate survival factors, leading to apoptosis, whereas other miRNAs affect the immune system response and the expression of AD-related proteins such as BACE1 ( $\beta$ -site APP-cleaving enzyme 1) and amyloid precursor protein (APP).

These miRNAs may therefore be responsible for the beneficial effects of EE in AD and are important objectives for further studies to better understand the pathogenesis and ultimately cure AD.

## MATERIALS AND METHODS

### Experimental animals

**Environmental enrichment.** Three-week-old wild-type (WT) male C57BL/6J mice were obtained from Harlan Laboratories (Frederick, MD, USA) (for immunofluorescence:  $n = 6$  EE group,  $n = 12$  control group, for miRNA/protein measurements:  $n = 10$  EE group,  $n = 10$  control group).

**AD model.** The 3xTgAD line was originally generated by co-microinjection of human APP (K670M/N671L) and tau (P301L) transgenes under the control of the Thy 1.2 promoter into mutant PS-1 (M146V) knock-in mice.<sup>59</sup> 3xTgAD mice were backcrossed to C57BL/6J mice for eight generations; male 3xTgAD mice and age-matched male C57BL/6J mice were used as sources of brain tissue for protein and miRNA analyses. The age groups included: young (4-month-old,  $n = 15$ ), old (12-month-old,  $n = 15$ ) and very old (16-month-old,  $n = 15$ ), approximately equivalent in humans to 15-year-old, 50-year-old and 70-year-old humans, respectively. All animals were housed in a controlled environment and were provided with food and tap water *ad libitum*. Room lights were on between 0500 and 1900 h.

All experiments were performed in accordance with the Tel-Aviv University Animal Care Committee.

### EE paradigm

The EE paradigm we used in this study was self-defined by our laboratory, taking into consideration that the goal of EE is to provide animals with opportunities to express their full range of species-typical behavioral patterns, and a certain degree of control over their environment. Our paradigm was designed to avoid aggressive competition between animals, and to avoid super-enrichment and stress. Included in our enrichment cages were running wheels that enable exercise that stimulates neurogenesis.<sup>70</sup> Specifically, 3-week-old mice were exposed to EE or to regular environment for 8 weeks. The EE cages were equipped with two running wheels for spontaneous exercise, bedding and nesting material, a house and toys that were changed every 10 days. The control group of mice was housed in a smaller, regular cage without running wheels, or any stimulating object, with six mice per cage, as in the EE group.

### Immunofluorescence staining

Free-floating coronal sections were processed for immunofluorescence staining as described previously.<sup>61</sup> Briefly, sections were incubated over two nights at 4 °C with primary antibodies against tomosyn (1:100 home-made affinity-purified rabbit anti-tomosyn polyclonal, as was described in Hatsuzawa *et al.*<sup>71</sup>) or synaptophysin (1:200 mouse anti-synaptophysin monoclonal, S-5768 clone SVP-38; Sigma, St Louis, MO, USA). They were then incubated with affinity-purified goat secondary antibodies (Alexa fluor; Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature (RT) at a 1:1000 dilution. To minimize variability, sections from all animals were stained and treated simultaneously. The intensities of immunofluorescence staining were determined by the in-house software written in Matlab. Images (magnification  $\times 10$ ) were thresholded (same for all images of each antibody) until background staining was minimized, and the intensity was then averaged over the region of interest that was manually selected.

### Immunohistochemical staining

Free-floating coronal sections were incubated overnight at 4 °C with biotinylated mAb AT8 (1:50; Biotest, Boca Raton, FL, USA), which targets phosphorylated S202 and T205 tau residues. The sections were then incubated with HRP-labeled Avidin (Vector Labs, Burlingame, Canada) for 30 min, after which they were stained with diaminobenzidine (DAB) (SK-4100; Vector Labs) for 10 min. To minimize variability, sections from all animals were stained and treated simultaneously.

### RNA extraction

Total RNA was extracted from whole hippocampi of mice using the TRIZOL reagent (Invitrogen). The final RNA concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, DE, USA).

### MiRNA profiling

For profiling, samples from all mice from the same experimental group were pooled together. First-strand complementary DNA (cDNA) was synthesized from total RNA using Megaplex reverse transcriptase reaction with the High Capacity cDNA kit (Applied Biosystems, Foster City, CA, USA). This reaction contains a specific stem-loop primer for each mature target miRNA. Each stem-loop primer is designed to hybridize to only the fully mature miRNA, and not to precursor forms of its target. The TaqMan Low-Density Arrays (TLDA) are quantitative real-time PCR (RT-PCR) assays based on Applied Biosystems technology. The mixture for each sample, containing cDNA, RNase-free water and TaqMan Universal PCR Master Mix (No AmpErase UNG; Applied Biosystems) was then transferred into a loading port on Rodent TLDA cards A and B, according to the manufacturer's instructions. The card was centrifuged twice, sealed and PCR amplification was carried out using an ABI Prism 7900HT Sequence Detection System under the following thermal cycler conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of (30 s at 95 °C and 1 min at 60 °C). Results were analyzed with the SDS software (Applied Biosystems) and the RQ (relative quantity) Manager Software, for automated data analysis. MiRNA relative levels were calculated based on the comparative threshold cycle (Ct) method. In short, the Ct for each miRNA and endogenous control U6 snRNA in each sample were used to create  $\Delta$ Ct values (CtmiRNA – CtU6 snRNA). Thereafter,  $\Delta\Delta$ Ct values were calculated by subtracting the  $\Delta$ Ct of

the control group from the Ct value of the tested group. The RQs were calculated using the equation:  $RQ = 2^{-\Delta\Delta Ct}$ . We note that as we pooled the data from all animals within a group, we report only miRNAs with meaningful fold change ( $>2$  or  $<0.5$ ) without *P*-values. For miRNA-targeted pathway analysis, we used DIANA-miRPath v2.0,<sup>72</sup> a web-based computational tool based on the enrichment analysis of multiple miRNA target genes. The application analyses which of the KEGG pathways may be modulated by a set of miRNA targets.

#### qRT-PCR

For qRT-PCR analysis of mature miR-1 and miR-148, 2.5  $\mu\text{l}$  of 4 ng  $\mu\text{l}^{-1}$  total RNA was used for synthesis of first-strand cDNA using a MultiScribe reverse transcriptase reaction with the High Capacity cDNA kit (Applied Biosystems) and TaqMan MiRNA Assay RT primer (Applied Biosystems) for each miRNA.

Mixtures containing cDNA, RNase-free water and TaqMan MiRNA Assay Real-Time probe (Applied Biosystems) for each miRNA were loaded on a 96-well plate. PCR amplification was carried using a ViiA 7 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) under the following thermal cycling conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of (15 s at 95 °C and 1 min at 60 °C). The expression of tested mature miRNAs was normalized to the expression of U6 snRNA, and the relative quantification method,  $2^{-\Delta\Delta Ct}$ , was used to calculate the expression relative to the mean of the U6 snRNA.

#### 3'UTR constructs for the luciferase assay

Fragment of 2094 bp of Stxbp5l (tomosyn2) that includes the relevant miRNA binding sites was cloned into psiCHECK-2 plasmid (Promega, Madison, WI, USA) downstream to the Renilla Luciferase Reporter gene, into *XhoI*-*NotI* restriction sites. Firefly Luciferase Reporter that is part of psiCHECK-2 plasmid serves as an internal control (under a different promoter). The 3'UTR fragment was PCR amplified from mice tail gDNA and *XhoI*-*NotI* restriction sites were added (restriction sites are in italics). Primers that were used for this purpose: stxbp5l-Fwd: CCACTCGAG GAGGGTCATTGTGTGACATC; stxbp5l-Rev: GGTGCGGCCGCTACAGTTAACA CTTTGCAAATG.

As a control for specific binding of selected miRNA to its binding sites in relevant 3'UTR, deletion mutation for miRNA seed sites was performed. The mutagenesis was carried out by PCR of the plasmid using the enzyme Phusion DNA Polymerase (Thermo Scientific) with GC buffer. PCR conditions are (1) 98 °C for 2 min, (2) 98 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s per kb) X18 and (3) 72 °C for 10 min.

Since Stxbp5l 3'UTR includes two binding sites for miR-325, the deletion of both binding sites was performed in the 3'UTR. As in Stxbp5l, the miR-325 binding sites are located at the beginning and at the end of the 3'UTR frame, two different primers were used. The primers used for mutagenesis and their reverse complement primers are stxbp5l-miR325-location1-Fwd: CTTTGAATTGATTCAATCTGTCTACTC; stxbp5l-miR325-location2-Fwd: GTAGTCTGTAGAGATAAATTGTATATGTTTG. Deleted seed sites in Stxbp5l: TCAATAA. After digesting the methylated source plasmid with DPN1 (New England BioLabs, Hitchin, UK), mutated plasmid was sequenced to verify that the desired mutagenesis was achieved and no additional unexpected mutations were created.

#### MiRNA constructs for the luciferase assay

Pre-miRNA of miR-325 was cloned into the *BamI*-*EcoRI* restriction sites of the miRNA expression vector miRVec (provided by Dr N Shomron, Tel Aviv University). For this purpose, the DNA fragment of ~100 bp upstream and downstream of the pre-miRNA was PCR amplified from mice tail gDNA and *BamHI*-*EcoRI* restriction sites were added (restriction sites are in italics). Primers that were used for this purpose: miR-325-Fwd: CACGAATCTACTA TAGCCACTACTAGC; miR-325-Rev: GTGGGATCCGGGTTTTATCAGCTTATTC.

#### Dual luciferase assay

HEK293T cells were seeded in 24-well plates in DMEM supplemented with 10% FBS and 1% Pen/Strep. The cells were transfected 24 h after seeding with a total of 500 ng DNA: 485 ng miRVec containing the desired pre-miRNA or an empty vector, 5 ng psiCHECK-2 containing the desired 3'UTR with or without site-directed mutations and 10 ng GFP vector. The transfection was done using the JetPEI Transfection Reagent (Polyplus Transfection, Illkirch, France), according to the manufacturer's protocol.

Firefly and Renilla Luciferase activities were measured 48 h after transfection using the Dual-Luciferase Reporter Assay System kit (Promega) and a Veritas microplate luminometer (Promega), according to Promega's protocol. The assay was conducted after verification of  $>50\%$  transfection efficiency, as was measured by GFP fluorescence.

#### Statistical analysis

All tests in this study were analyzed using an unpaired two-tailed Student's *t*-test. Results are expressed as mean  $\pm$  s.e.m.

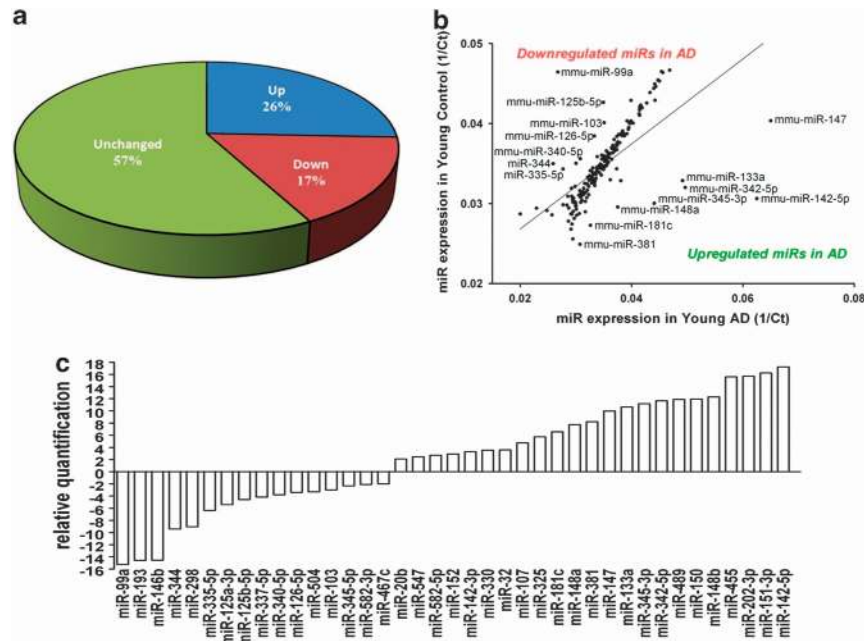
## RESULTS

*Alterations in miRNAs levels in the hippocampus of 3xTgAD mice.* We were first interested in defining the degree of miRNAs modulation and their potential roles in AD pathology. Given that each miRNA can regulate several genes,<sup>42,73,74</sup> and that miRNAs are typically transcribed in clusters,<sup>42</sup> we set to test the involvement of miRNAs in post-transcriptional regulation of synaptic transmission and AD pathology. Since AD is known to be involved with progressive deterioration in cognitive and physiological aspects, we extracted miRNAs from whole hippocampi derived from young (4-month-old, Figures 1a–c) and very old (16-month-old, Figures 2a–c) 3xTgAD mice and their age-matched WT counterparts, and characterized changes in miRNA levels by the TLDA. Significantly, broad and strong expression levels of tau tangles were measured in 3xTgAD mice compared with control mice (Supplementary Figure 1), validating these mice as modeling AD. MiRNAs that were upregulated (fold change  $\geq 2$ ; Figure 3a) or downregulated (fold change  $\leq 0.5$ ; Figure 3b) were found by comparing expression levels of the different miRNAs between the four experimental groups. We divided these miRNAs into four categories representing different processes (Figure 3): (1) *AD in very old mice*; by subtracting the list of miRNAs of very old WT mice from those of very old 3xTgAD mice (VOAD – VOC), (2) *AD in young mice*; by subtracting the list of miRNAs of young WT mice from those of young 3xTgAD mice (YAD – YC), (3) *Aging in WT mice*; by subtracting the list of miRNAs of young WT mice from those of very old WT mice (VOC – YC) and (4) *Aging in AD*; by subtracting the list of miRNAs of young 3xTgAD mice from those of very old 3xTgAD mice (VOAD – YAD).

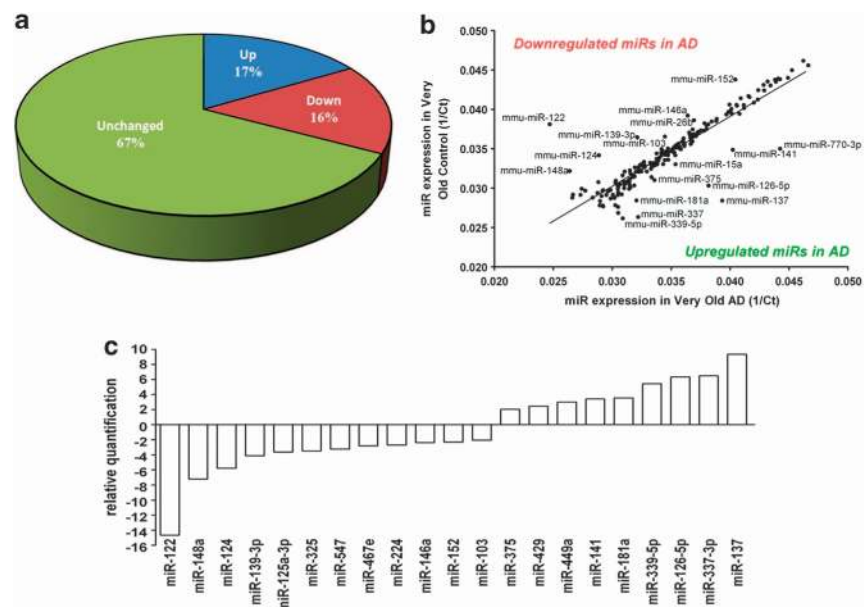
By analyzing the intersections between the above processes we were able to specifically discuss the roles of miRNAs in more defined subgroups of these processes: for example, by intersecting the lists of modulated miRNAs from *AD in very old mice* group and from *AD in young mice* group, one can learn about miRNAs that are AD-related. Moreover, by excluding modulated miRNAs from the *Aging in WT mice* group from *Aging in AD* group, miRNAs that are modulated only in the aging process of mice model of AD are revealed (and that are not associated with normal aging). This way we were able to characterize the following subgroups of miRNAs, presented in Figure 3: (1) *AD specific in very old mice*, (2) *AD specific*, (3) *AD early markers*, (4) *AD in young and Aging in WT*, (5) *AD and Aging specific*, (6) *AD in very old mice and Aging in AD*, (7) *Aging-specific in WT mice*, (8) *Aging specific* and (9) *Aging specific in AD*.

We used prediction algorithm to elucidate which mRNAs might be targeted by the miRNAs we observed (TargetScan Mouse v6.2; Baek et al.<sup>75</sup> and Selbach et al.<sup>76</sup>). Several miRNAs that their expression levels were upregulated (miR-15a and miR-34a) or downregulated (miR-298, miR-101a and miR-294) in the hippocampus of 3xTgAD mice as compared with their control mice were of special interest due to their high relevancy to AD (Table 1). These miRNAs were either previously demonstrated or predicted to regulate the expression of Bcl2, Bace1, Bace2, Mapt, App and Cox transcripts (Table 1), which are known to have a role in AD. Other miRNAs of interest that were upregulated in the hippocampus of 3xTgAD mice (miR-1 and miR-148a) may affect the synaptic transmission process, as they were either previously





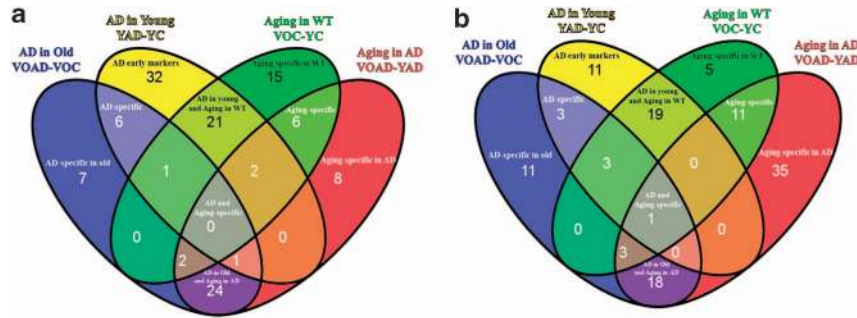
**Figure 1.** Hippocampal microRNAs (miRNAs) global expression signature in young 3xTgAD mice model of Alzheimer's disease (AD). General distribution of miRNAs modulation derived from young (4-month-old) mice model of AD compared with their age-matched wild-type (WT) C57BL/6J mice. **(a)** Distribution chart of miRNA changes showing miRNAs that were upregulated (Up), downregulated (Down) or unchanged (Unchanged). **(b)** Scatter-plot representation of miRNA expression changes. While most of the miRNAs were not modulated following exposure to enriched environment (EE) (presented around and on the regression line), some of the miRNAs were upregulated (below the regression line), and some were downregulated (above the regression line), compared with control mice. Values are presented as 1/threshold cycle (1/Ct). Properly detected miRNAs with Ct < 40 and fold change > 2 or < 0.5 are presented. **(c)** Relative quantification (RQ) of miRNAs that were upregulated with RQ > 2 or downregulated with RQ < -2.



**Figure 2.** Hippocampal microRNAs (miRNAs) global expression signature in very old 3xTgAD mice model of Alzheimer's disease (AD). General distribution of miRNAs modulation derived from very old (16-month-old) mice model of AD compared with their age-matched wild-type (WT) C57BL/6J mice. **(a)** Distribution chart of miRNA changes showing miRNAs that were upregulated (Up), downregulated (Down) or unchanged (Unchanged). **(b)** Scatter-plot representation of miRNA expression changes. While most of the miRNAs were not modulated following exposure to enriched environment (EE) (presented around and on the regression line), some of the miRNAs were upregulated (below the regression line), and some were downregulated (above the regression line), compared with control mice. Values are presented as 1/threshold cycle (1/Ct). Properly detected miRNAs with Ct < 40 and fold change > 2 or < 0.5 are presented. **(c)** Relative quantification (RQ) of miRNAs that were upregulated with RQ > 2 or downregulated with RQ < -2.

demonstrated or predicted to regulate the expression of known synaptic genes, such as Snap25, Stx6, Vamp1, Vamp2, Calm1, Hspa1, Gja1, CaMKII, Syt, NMDA-R and Vamp mRNAs (Table 1).

Analyzing for common cellular pathways (using DIANA-miRPath v2.0; Vlachos et al.<sup>72</sup>) that involve the upregulated miRNAs in very old AD mice (miR-1, miR-15a, miR-429 and miR-873), we found



**Figure 3.** Common and differential changes in microRNAs (miRNAs) in the hippocampi of young and very old 3xTgAD mice model of Alzheimer's disease (AD) and their control age-matched mice. Venn diagrams presenting the number of miRNAs that their expression levels were (a) upregulated or (b) downregulated in each group of comparison: Blue—AD in very old mice, Yellow—AD in young mice, Green—aging in wild-type (WT) mice and Red—aging in AD mice model. OAD, Old AD; OC, Old Control; YAD, Young AD; YC, Young Control. The intersections between groups result with definition of subgroups. Several subgroup titles are indicated where relevant.

**Table 1.** Modulated miRNAs in the hippocampus of young and very old 3xTgAD mice model of Alzheimer's disease

miR	Group	Expression regulation	Fold change	Predicted genes targeted	Physiological function of miR
miR-1	AD-specific in very old	Up	2.23	Snap25, Stx6, Vamp1, Vamp2, Calm1 (luciferase), Hspa1 (luciferase), Gja1 (luciferase)	Enriched in the synapse Target genes associated with the 'SNARE interactions in vesicular transport' pathway
miR-15a	VOAD, YAD, Aging AD	Up	3, 2.2, 2.2	Bcl2 (luciferase)	Decrease the survival factors associated with Bcl2, leading to apoptosis
miR-34a	AD-early markers	Up	2.17	Bcl2, Cplx2, Vamp2, Syt1	Was upregulated also in the double-transgenic mouse model of AD Targeting genes of synaptic proteins Decrease the survival factors associated with Bcl2, leading to apoptosis Might function as a marker for AD in early stages
miR-148a	YAD, Aging WT	Up	213, 9.3	CaMKII (luciferase) Syt, NMDA-R, Vamp and more	Enriched in the synapse Highly involved with 'Calcium signaling' pathway May impair LTP, synaptic plasticity and neurotransmission by targeting CaMKII
miR-298	YAD, Aging WT	Down	Ct > 40	Bace1 (luciferase) Bace2 (luciferase) Mapt (TAU)	Increased Aβ formation in AD (Boissonneault et al. <sup>104</sup> ) Increased levels of tau in AD
miR-101a	VOAD, YAD, Aging WT	Down	Ct > 40 0.0002	APP (luciferase), COX-2 (luciferase)	Downregulated also in human AD cortex Increased APP expression Increased Aβ accumulation Enhanced inflammatory response: COX-2 was shown to be upregulated in human AD and it is associated with neuronal loss
miR-294	VOAD, YAD, Aging WT	Down	Ct > 40	APP	Increased APP expression

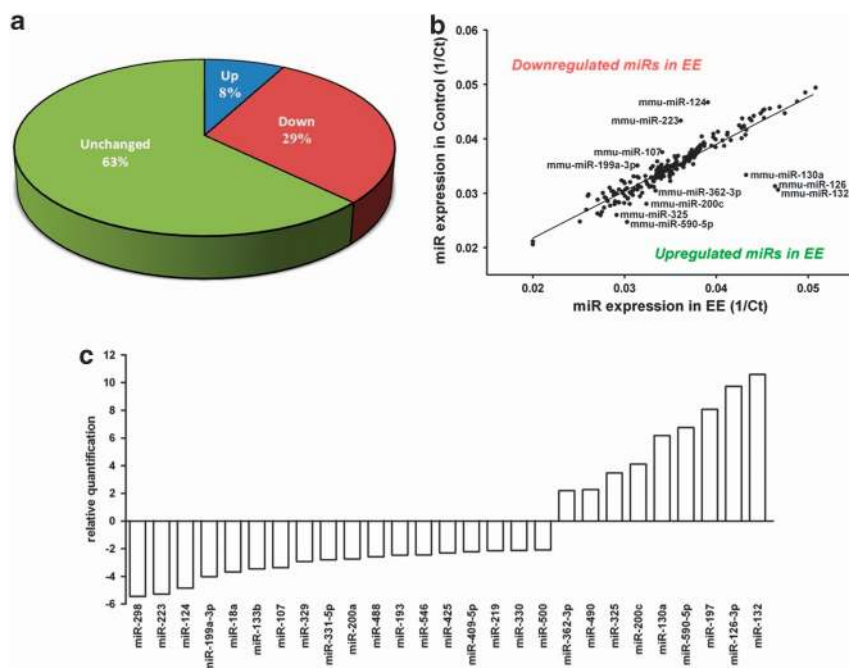
Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; COX-2, cyclooxygenase-2; Ct, threshold cycle; LTP, long-term potentiation; miRNAs, microRNAs; SNARE, soluble NSF attachment protein receptor; VOAD, Very Old AD mice; WT, wild type; YAD, Young AD mice.

List of modulated miRNAs derived from young (4-month-old) and very old (16-month-old) 3xTgAD mice compared with their age-matched WT C57B control mice. Table describes miR number, expression regulation, predicted and verified target genes and the physiological function. Luciferase = mRNA was shown to be a target of the relevant miRNA by the luciferase reporter assay. Ct > 40 means the threshold cycle too high for the calculation to be quantitative, however it could be used in a qualitative manner.

that statistically significant pathways such as SNARE interactions in vesicular transport, axon guidance, long-term depression and transforming growth factor-β signaling are predicted to be substantially downregulated in 3xTgAD mice (Supplementary Table 1). These findings may explain the impaired synaptic transmission and physiology related to AD pathogenesis.

By studying the miRNAs that were upregulated specifically in

the 'AD early markers' subgroup (see Figure 3) we were able to define the pathways that were possibly downregulated in early stages of AD-like pathology. These statistically significant pathways were involved with SNARE interactions in vesicular transport, calcium signaling, long-term potentiation (LTP), long-term depression (LTD), axon guidance, regulation of actin cytoskeleton and transforming growth factor-β signaling (Supplementary



**Figure 4.** microRNAs (miRNAs) global expression signature in the hippocampus of wild-type (WT) C57BL/6J mice following environmental enrichment. General distribution of miRNAs modulation derived from WT C57BL/6J mice that were environmentally enriched, as compared with WT C57BL/6J control mice that were exposed to regular environment. **(a)** Distribution chart of miRNA changes showing miRNAs that were upregulated (Up), downregulated (Down) or unchanged (Unchanged). **(b)** Scatter-plot representation of miRNA expression changes. While most of the miRNAs were not modulated following exposure to enriched environment (EE) (presented around and on the regression line), some of the miRNAs were upregulated (below the regression line), and some were downregulated (above the regression line), compared with control mice. Values are presented as 1/threshold cycle (1/Ct). Properly detected miRNAs with Ct < 40 and fold change > 2 or < 0.5 are presented. **(c)** Relative quantification (RQ) of miRNAs that were upregulated with RQ > 2 or downregulated with RQ < -2.

Table 2). These findings have important implications to the ability to detect AD in its early stages and define the pathways that are highly affected in early stages, and may therefore be exploited for future detection methodology and treatment. Similar pathways were also downregulated while analyzing miRNAs from the 'AD-specific' subgroup (Supplementary Table 3).

Next, we were interested in analyzing the changes in miRNA levels with advancing age and AD-like pathologies in 3xTgAD mice. To do so, we measured the expression levels of miRNAs in young and very old 3xTgAD mice, compared them with their control age-matched controls to calculate their fold change, and searched for substantial changes in fold change along the disease pathology. Specific miRNAs (Supplementary Table 4) demonstrated substantial changes in their modulation with advancing age in 3xTgAD mice (miR-126-5p, miR-148a, miR-152, miR-188-5p, miR-197, miR-325, miR-337-3p and miR-547). These specific miRNAs are predicted to have roles in synaptic transmission and plasticity pathways, suggesting the relevance of these pathways to the pathology of AD.

To verify that these changes in miRNA levels are AD pathology-specific and not due to natural aging, we measured the expression levels of miRNAs in young and very old WT C57BL/6J mice and searched for substantial changes in fold change (Supplementary Figure 2). Analyzing for common cellular pathways that involve the upregulated miRNAs in very old WT C57BL/6J mice as compared with young WT C57BL/6J mice (miR-146b, miR-152, miR-107, miR-141, miR-148a, miR-496, miR-142-5p, miR-330, miR-329 and miR-184), we found that statistically significant pathways, such as MAPK signaling pathway, focal adhesion, axon guidance, regulation of actin cytoskeleton, LTP and LTD, are predicted to be substantially downregulated in aged WT C57BL/6J mice (Supplementary Table 5). Analyzing for common cellular pathways that involve the downregulated miRNAs in very old WT C57BL/6J

mice as compared with young WT C57BL/6J mice (miR-135b, miR-101a, miR-126-5p, miR-103, miR-467b, miR-200b, miR-130b, miR-448, miR-500, and miR-429), we found that statistically significant pathways such as renal cell carcinoma, colorectal cancer, chronic myeloid leukemia and glioma are predicted to be substantially upregulated in aged WT C57BL/6J mice (Supplementary Table 6).

*EE modulates hippocampal miRNA levels in WT C57BL/6J mice.* Upon characterizing miRNAs modulation in AD, we characterized EE-related miRNAs modulation with the main aim to study whether EE- and AD-related miRNA expression changes are inversely correlated. To address this, we extracted miRNAs from the hippocampi of WT C57BL/6J male mice that were exposed to EE as well as control mice. Of the total pool of miRNAs detected by the TLDA in mice that were exposed to EE, several were dramatically regulated compared with control group as was indicated by high fold change (Figure 4).

Interestingly, certain miRNAs downregulated in response to EE as compared with their control mice (miR-147, miR-128, miR-148a, miR-218, miR-1, miR-495, miR-467a and miR-191; Supplementary Table 7) are predicted to affect mRNA targets that are enriched in synapses, and are involved with calcium signaling in presynaptic terminals (Sy13, CaMKII), synaptic plasticity (Bdnf, CaMKII, NMDA-R) neurotransmission regulation (Sy13, CaMKII, NMDA-R, Vamp, rims), AD pathology (Bdnf) and tau degradation (Bdnf, BAG2). Some of these miRNAs, indicated as changed by the TLDA assay, were validated using qRT-PCR analysis (Supplementary Figure 3). Upregulated cellular pathways that were statistically significant and include miRNAs that were considerably downregulated were involved with axon guidance, regulation of actin cytoskeleton and LTP (Supplementary Table 8). Overall, these downregulated

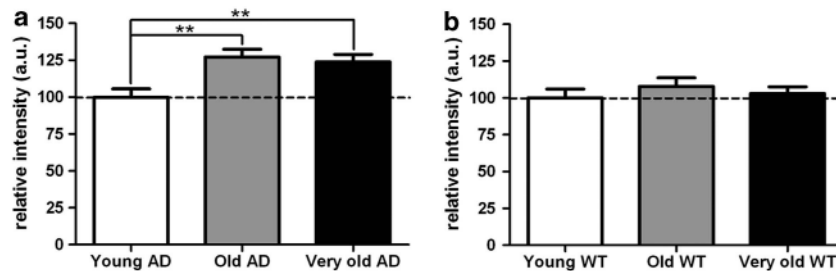
**Table 2.** Inversely regulated miRNAs in the hippocampi of 3xTgAD mice model of Alzheimer's disease and WT C57BL/6J mice following EE

miR	Expression in AD (FC)	Expression in EE (FC)	Predicted genes targeted	Physiological function
miR-325	Down VOAD (0.09)	Up (11)	Stxbp5l (tomosyn2)	Tomosyn2 expression levels regulation
miR-1	Up VOAD (2.23)	Down (0.73)	Snap25, Stx6, Vamp1, Vamp2, Bsn, Calm1 (luciferase), Hspa1 (luciferase), Gja1 (luciferase)	Enriched in the synapse Target genes associated with the 'SNARE interactions in vesicular transport' pathway
miR-148a	Up YAD (FC > 100)	Down (Ct > 40)	CaMKII(luciferase), Syt, NMDA-R, Vamp and more	Enriched in the synapse Highly involved with 'Calcium signaling' pathway May impair LTP, synaptic plasticity and neurotransmission by targeting CaMKII
miR-666-5p	Up VOAD (16.4)	Down (0.24)	Synb ( $\beta$ -synuclein)	$\beta$ -Synuclein is a presynaptic inhibitor of $\alpha$ -synuclein aggregation that impairs synaptic function (Kramer and Schulz-Schaeffer <sup>91</sup> ) $\beta$ -synuclein levels were shown to be decreased in AD
miR-147	Up YAD (FC > 100)	Down (Ct > 40)	Syt13, Bdnf	Synaptic transmission Neurons survival signaling Synapses and neurons growth and differentiation Long-term memory BDNF is highly active in the hippocampus
miR-369-3p	Down VOAD (0.5)	Up (1.4)	TNF $\alpha$	TNF $\alpha$ is well characterized as part of the AD pathology $\beta$ -amyloid induction of production TNF $\alpha$ levels correlated with clinical deterioration
miR-129-3p	Up YAD (1.48)	Down (Ct > 40)	CTSB	Cathepsin B degrades $\beta$ -amyloid precursor protein, decreasing AD pathology (Mueller-Steiner, 2006) <sup>117</sup>
miR-26b	Up YAD (1.37)	Down (0.64)	TACE	TACE (tumor necrosis factor- $\alpha$ converting enzyme) cleaves APP to prevent the formation of $\alpha/\beta$ -amyloid and create non-amyloidogenic products
miR-126-5p	Up VOAD (82)	Down (Ct > 40)	Gria2, Gabra5, Gabra6	Synaptic transmission Synaptic plasticity
miR-330	Up YAD (11.57)	Down (0.23)	Cplx2	Synaptic transmission
miR-338-3p	Up VOAD (Ct > 40)	Down (0.35)	Snap29, Snapin	Synaptic transmission
miR-27a	Up YAD (1.26)	Down (0.57)	Unc13c	Synaptic transmission
miR-27b	Up YAD (1.31) VOAD (1.15)	Down (0.79)	Unc13c	Synaptic transmission
miR-128a	Up YAD (1.59) Aging WT (1.55)	Down (Ct > 40)	APBA2, Unc13c	APBA2 is a neuronal adapter protein that interacts with APP so that it stabilizes the protein and inhibits the production of proteolytic APP fragments including the A $\beta$ peptide Synaptic transmission
miR-429	Up YAD (2.25) VOAD (5.52)	Down (0.35)	Snap25, Synj	Synaptic transmission
miR-301b	Up YAD (1.65)	Down (0.79)	Snap25	Synaptic transmission
miR-107	Up YAD (26.8) Aging WT (55.5)	Down (0.097)	Vamp1	Synaptic transmission
miR-219	Up YAD (3.1) Aging WT (1.97)	Down (0.22)	Rims1	Synaptic transmission
miR-133b	Up YAD (2.54)	Down (0.09)	Syt2	Synaptic transmission
miR-770-3p	Up YAD (47.2)	Down (0.027)	APPBP2, Syntaxin12	APPBP2 involves with transport and processing of the amyloid precursor protein Synaptic transmission
miR-489	Up YAD (Ct > 40)	Down (Ct > 40)	Vapa (Vamp-Associated Protein A)	Synaptic transmission
miR-223	Up YAD (2.45)	Down (0.027)	Vamp2	Synaptic transmission

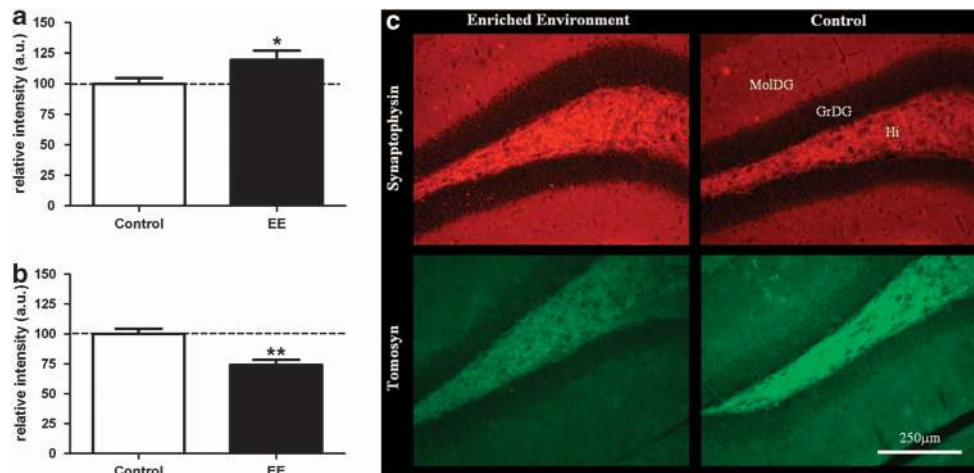
Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; APBA2, amyloid  $\beta$  A4 precursor protein-binding family A member 2; BDNF, brain-derived neurotrophic factor; Ct, threshold cycle; EE, enriched environment; FC, fold change; LTP, long-term potentiation; miRNAs, microRNAs; SNARE, soluble NSF attachment protein receptor; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; VOAD, Very Old AD mice; WT, wild type; YAD, Young AD mice.

List of inversely modulated miRNAs derived from young (4-month-old) and very old (16-month-old) 3xTgAD mice compared with their age-matched C57BL/6J WT, and C57BL/6J WT mice that were exposed to EE and their control mice. Table describes miR number, expression regulation, predicted and verified target genes and the physiological function. Luciferase = mRNA was shown to be a target of the relevant miRNA by the luciferase reporter assay. Ct > 40 means the threshold cycle too high for the calculation to be quantitative, however, it could be used in a qualitative manner.





**Figure 5.** Tomosyn protein expression levels increase in the mouse hippocampus as Alzheimer's disease (AD) pathology progresses. Analysis of immunofluorescence staining of tomosyn protein in coronal brain slices derived from hippocampi of 3xTgAD mice and their wild-type (WT) C57BL/6J age-matched control mice. **(a)** In the hippocampus of 3xTgAD mice, the expression levels of tomosyn protein were significantly increased along AD pathology compared with their levels in young 3xTgAD mice. **(b)** No significant difference in the expression levels of tomosyn protein in the hippocampus of young WT C57BL/6J mice compared with old and very old WT C57BL/6J mice. All values presented are normalized so that young AD (for **a**) or young WT (for **b**) values are 100 a.u.  $^{*}P < 0.005$ .



**Figure 6.** Effects of enriched environment (EE) on levels of synaptic proteins in the mouse hippocampus. Immunofluorescence staining of coronal slices of hippocampi from wild-type (WT) C57BL/6J mice exposed to regular (Control) or EE. In WT C57BL/6J mice subjected to EE **(a)** synaptophysin protein levels are significantly higher in the hippocampus, while **(b)** tomosyn protein levels are significantly lower. **(c)** Representative immunofluorescence images of synaptophysin protein labeling (upper row) and tomosyn protein labeling (lower row) in the hilus of WT C57BL/6J mice that were exposed to EE (left column) and their control (right column). MolDG, molecular layer of the dentate gyrus; GrDG, granular layer of the dentate gyrus; Hi, hilus.  $^{*}P < 0.05$ ,  $^{**}P < 0.005$ .

miRNAs may be involved in neuromodulation, which warrants further investigation.

#### *Inverse regulation of miRNA levels in AD and in response to EE.*

Of special interest are miRNAs that showed inverse modulation in mice following EE and in the mice model of AD (Table 2), as these may be the key players in the rescue effects associated with EE on AD pathology. For example, miR-325 was downregulated in AD and upregulated following EE (Table 2), and was predicted to regulate the mRNA of tomosyn, an inhibitor of synaptic transmission.<sup>62–64,69,77</sup> To verify targeting of tomosyn2 (Stxbp5l) by miR-325, we performed the dual luciferase reporter assay, and found that miR-325 significantly reduced luciferase activity by 45% (Supplementary Figure 4), indicating a direct regulation of tomosyn2 by miR-325.

Additional miRNAs that were inversely regulated were shown or are predicted to regulate genes encoding for synaptic proteins such as SNAP25, synaptobrevins, bassoon, calmodulin, synaptotagmins, munc13, complexin and syntaxin. The fact that these proteins are key players in the different steps of the vesicle life cycle and of synaptic plasticity demonstrates the high relevancy of miRNAs in regulating the synaptic transmission process in AD and following exposure to EE.

Moreover, some of these inversely regulated miRNAs regulate survival factors, neurotrophic factors and AD-related proteins such as  $\beta$ -synuclein and those that are responsible for tau degradation (Table 2). Out of all the inversely regulated miRNAs, several showed a remarkable inverse modulation while comparing their expression in mice following EE on one hand and on the other hand their expression in young (Supplementary Table 9) or very old 3xTgAD mice (Supplementary Table 10).

*Tomosyn protein levels are elevated in the hippocampi of 3xTgAD mice.* Our findings show that miR-325 regulates tomosyn expression levels and was downregulated in AD and upregulated following EE. To test whether the inverse regulation of miR-325 is correlated with inverse expression of tomosyn protein expression levels, we examined tomosyn protein levels in the hippocampus of mice model of AD and in WT C57BL/6J mice following EE. To study on the role of tomosyn in the progressive deterioration in cognitive and physiological aspects of AD, we used three different ages of the 3xTgAD mice and their age-matched WT control mice ('Young AD' at 4-month-old, 'Old AD' at 12-month-old and 'Very old' at 16-month-old).

Immunofluorescence staining of coronal slices demonstrated that tomosyn protein expression levels significantly increased in the



hippocampus of old and very old 3xTgAD mice compared with young 3xTgAD mice (Figure 5a). Importantly, in WT mice, no significant changes were measured in tomosyn protein levels during aging (Figure 5b).

*EE decreases hippocampal tomosyn protein levels in WT C57BL/6J mice.* EE was previously shown to improve synaptic transmission,<sup>78</sup> as well as learning and memory ability,<sup>19</sup> and mitigated the cognitive deterioration and pathology in AD mouse models.<sup>27</sup> To verify that the EE paradigm we designed indeed affects synaptic properties, we first characterized the differences in the expression levels of synaptophysin in the hippocampi of WT mice undergoing EE for 8 weeks and their control group. The synaptic vesicle protein synaptophysin is the most commonly used marker for synapses and has been found to be related to efficacy of synaptic transmission,<sup>79</sup> placing it as a good marker to demonstrate the effects of EE on synaptic properties.<sup>6,80</sup> Indeed, synaptophysin protein expression levels were significantly higher in the hippocampus of mice that were exposed to EE compared with their control mice (Figures 6a and c, upper row). Examining the effect of EE on the expression levels of tomosyn protein, we found that in contrast to synaptophysin levels, tomosyn protein expression levels were significantly decreased in the hippocampus of mice that were exposed to EE compared with their control mice (Figures 6b and c, lower row). These results demonstrate the opposite correlation between changes in synaptic proteins and in miRNAs in the mouse hippocampus of mice model of AD and following EE.

## DISCUSSION

In this study, we studied the miRNA regulators that may contribute to the beneficial and detrimental effects of EE and AD, respectively, on synaptic plasticity-related proteins and AD pathology, and explored the modulation of the synaptic protein tomosyn in mouse models of EE and AD.

### Inverse modulation of miRNAs

By screening for miRNAs that were inversely regulated in EE and AD, we were able to define for the first time the possible contribution of miRNAs to the rescue effect of EE on AD pathology. These inversely regulated miRNAs may affect not only synaptic proteins, but also molecular factors that are associated with AD pathology. By doing so, EE can enhance the expression of proteins like cathepsin B, tumor necrosis factor- $\alpha$  converting enzyme (TACE),  $\beta$ -synuclein and key players in the synaptic transmission machinery, contributing to the positive effects on animal physiology following EE. Moreover, EE also influences survival factors and neuroprotective factors that are essential for neuronal viability, and by thus can inhibit the neuronal-loss process attributed to AD pathology.

Of special interest to synaptic transmission, one of the miRNAs that were inversely regulated, miR-325, was demonstrated in our study to regulate tomosyn expression, which decreases synaptic transmission and release probability of vesicles,<sup>62–64,69,77</sup> and in mammals is encoded by two genes, *Tomosyn1* and *Tomosyn2*.<sup>65</sup> We found that miR-325 was downregulated in very old 3xTgAD mice and upregulated in EE-treated mice as compared with their control mice. We found, by using the luciferase reporter assay, that one of the demonstrated mRNA targets of miR-325 is *Stxbp5l*, which translates to the tomosyn2 protein. Utilizing an immunohistochemical approach, we demonstrated that tomosyn protein expression levels are correspondingly upregulated in AD as expected by a decrease in miR-325 expression levels in AD, and downregulated following EE as expected by an increase in miR-325 levels following EE. This direct link between the inverse regulation of miRNA and the corresponding regulation of the targeted protein expression demonstrates the complex

possible rescue effects of EE on the synaptic transmission machinery in AD.

MiR-1, which was upregulated in very old 3xTgAD mice as compared with their age-matched controls and downregulated in EE-treated mice as compared with their control mice, was shown to be highly concentrated in the synaptic fraction of the brain.<sup>44</sup> Target prediction indicates that some of the target genes for miR-1 are associated with synaptic proteins in the 'SNARE interactions in vesicular transport' pathway, such as *snap25*, *syntaxin6*, *vamp1* and *vamp2*, which are essential for the synaptic transmission machinery. Additionally, miR-1 was shown to directly regulate Calmodulin (Calm1), Heat-shock protein 1 (*Hspa1b*, *Hspd1*) and Gap junction protein (*Gja1*; by the luciferase reporter assay).<sup>81</sup>

Similarly to miR-1, miR-148a, which was upregulated in young 3xTgAD mice as compared with their age-matched controls and downregulated in EE-treated mice as compared with their control mice is also enriched in the synapse,<sup>82</sup> and was shown in the reporter assay to directly regulate CaMKII (Calcium/calmodulin-dependent protein kinase II  $\alpha$ ).<sup>83</sup> CaM-kinase II (CAMK2) is a prominent kinase that is involved in LTP, neurotransmitter release and synaptic plasticity. Therefore, it is plausible that upregulation of miR-148a as part of AD pathology results in a reduced level of CAMK2 that can explain some of the physiological effects attributed to the disease such as reduced LTP.<sup>83,84</sup> Additionally, miR-148a was demonstrated to be upregulated in human patients with schizophrenia,<sup>85</sup> a mental disorder that affects mainly cognition and involves synaptic dysfunction as in AD. Interestingly, one of the main reasons for the synaptic dysfunction in schizophrenia is the glutamatergic system, including malfunctioning NMDA receptors.<sup>86</sup> Indeed, our target analysis suggested that miR-148a may target a variety of synaptic proteins such as NMDA receptor, synaptotagmin and VAMP, suggesting a key role for miR-148a in regulating properties of the glutamatergic system in AD and schizophrenia. Finally, miR-148a has been confirmed as a negative regulator of AD-related neuroinflammation processes that can inhibit the production of inflammatory cytokines and negatively regulate the activation of immune cells, by thus preventing the overactivation of immune response.<sup>83</sup> Overall, these data may shed new light on the molecular mechanism responsible for the impaired synaptic transmission and neuroprotection associated with AD pathology, and the potential contribution of EE to inhibit these impairments in AD.

Other inversely regulated miRNAs that we found were more relevant to AD pathology. For example, miR-128, which in our study was downregulated following EE and upregulated in young mice model of AD as compared with their control mice, similarly to another study done on AD in human hippocampal samples.<sup>51</sup> Interestingly, miR-128 was recently shown in the reporter assay to regulate the expression of the co-chaperone BAG2 that is involved with tau degradation.<sup>87</sup> In addition, miR-128 is predicted to regulate APBA2 (Amyloid  $\beta$  A4 precursor protein-binding family A member 2), which is a neuronal adapter protein that interacts with APP so that it stabilizes the protein and inhibits the production of proteolytic APP fragments including the A $\beta$  peptide.

Another inversely regulated miRNA associated with AD pathology is miR-666-5p, which was downregulated following EE and upregulated in very old mice model of AD as compared with their control mice. This miRNA may be specifically involved with the neurodegenerative process of AD, as it is predicted to target *Synb*,  $\beta$ -synuclein. Beta-synuclein is a presynaptic protein that is found primarily in brain tissue, suggested to act as an inhibitor of  $\alpha$ -synuclein aggregation.<sup>88</sup> By doing so, it may take a role in the protection of the central nervous system from the neurotoxic effects of  $\alpha$ -synuclein.<sup>88</sup>  $\alpha$ -Synuclein was previously shown to aggregate at presynaptic terminals (for review, see refs.<sup>89,90</sup>), resulting in a severe pathological impact on synaptic function and almost complete loss of dendritic spines at the post-synaptic area.<sup>91</sup> Our results of upregulation of miR-666-5p in the hippocampus of mice model of AD fit well with the decreased levels of  $\beta$ -synuclein that were

demonstrated in humans in a different study in AD.<sup>92</sup> An optional role for anti-aggregatory  $\beta$ -synuclein-derived peptides in mice demonstrated a significant reduction in Lewy bodies' formation and prevention of functional deficits in AD.<sup>93</sup> Therefore, our data of downregulation of miR-666-5p following EE may explain the molecular mechanism of EE in mitigating the AD phenotype by controlling and upregulating  $\beta$ -synuclein expression, thus regulating the accumulation of  $\alpha$ -synuclein.

The inversely regulated miR-147, which was upregulated in young mice model of AD and downregulated in mice following EE as compared with their control mice, is predicted to regulate the brain-derived neurotrophic factor (BDNF). The *bdnf* mRNA has one conserved 7mer binding site for miR-147. BDNF has a role in the hippocampus,<sup>94</sup> and is associated with synaptic transmission,<sup>95</sup> activity-dependent synaptic plasticity such as LTP,<sup>95,96</sup> neuronal survival signaling (for review<sup>97</sup>), synapses and neurons growth and differentiation (for review<sup>98</sup>), as well as learning and memory.<sup>99,100</sup> The BDNF role in AD pathology prevention was also demonstrated in *in vivo* studies that demonstrated BDNF relevancy in this important neuroprotective process.<sup>101</sup> Hence, our data of downregulation of miR-147 following EE may explain the contribution of EE to AD pathology by increasing BDNF levels that are low in AD mice.<sup>102</sup>

The inverse regulation in our study was demonstrated not only in the miRNA level, but also in the protein level. We found that EE increases the level of the presynaptic protein synaptophysin that is positively involved in synaptic transmission, and decreases the level of tomosyn, a protein that inhibits synaptic transmission. The fact that synaptophysin is associated with the efficacy of synaptic transmission,<sup>79</sup> and that tomosyn is a negative regulator of the neurotransmission process,<sup>62–69,103</sup> together with our findings, add to our understanding of how exposure to EE may improve neurotransmission. In contrast, in old and very old 3xTgAD mice, tomosyn levels in the hippocampus were significantly higher compared with young 3xTgAD mice. Tomosyn's accumulation in the hippocampus of aged mice model of AD but not in aged WT control mice may therefore contribute to the synaptic failure in AD specifically. However, further experiments are needed to establish the direct involvement of tomosyn in synaptic dysfunction in AD.

#### AD-related modulation of miRNAs

We also found several AD-related miRNAs that were substantially upregulated only in 3xTgAD mice as compared with their control mice. Generally, and as will be discussed further, these miRNAs were previously shown to be highly concentrated in synapses, are predicted to downregulate essential synaptic proteins, and are highly associated with regulating processes involved with apoptosis, axon guidance and AD-associated processes. Moreover, while working in coordination, these miRNAs may affect dramatically essential cellular pathways such as calcium signaling, axon guidance and SNARE interactions, and potentially by thus result with the cognitive deterioration attributed to AD.

Out of the miRNAs that were modulated in mice model of AD as compared with their control mice, some are of special interest since they were shown or are predicted to regulate AD-related proteins. For example, miR-298, which we found to be downregulated in young mice model of AD as compared with their age-matched controls, was previously shown to exert multiple effects on: (1) BACE1 regulation and A $\beta$  formation,<sup>104</sup> (2) LTP regulation,<sup>105</sup> and (3) is predicted to target Mapt, which is the gene responsible for tau protein formation. Our data of AD-induced miR-298 downregulation support that this modulation can consequently lead to increased A $\beta$  and tau tangles formation.

MiR-101a, which in our study was downregulated in both very old and young mice model of AD as compared with their age-matched controls, was shown to be downregulated also in other studies done in humans cortex.<sup>106,107</sup> Importantly, miR-101a was

shown to downregulate the expression of APP in hippocampal neurons<sup>108</sup> and in human cell culture.<sup>109</sup> Additionally, miR-101 was shown to regulate the inflammation-associated gene Cyclooxygenase-2 (COX-2).<sup>108,110,111</sup> COX-2 was shown to be upregulated in the AD brain, and is also associated with neuronal loss. Therefore, it is possible that the downregulated levels of miR-101 we found may result in higher expression levels of COX-2 gene and an enhanced inflammatory response in AD mice. Interestingly, two miRNAs that we found to be upregulated in 3xTgAD mice were shown to regulate the antiapoptotic protein Bcl2, a key player in the genetic program of eukaryotic cells favoring survival rather than apoptosis;<sup>112</sup> miR-15a<sup>113,114</sup> and miR-34a<sup>112</sup> that was also shown to be upregulated in the double-transgenic mouse model of AD.<sup>115</sup>

More relevant to synaptic transmission, miR-34a is predicted to regulate genes of key synaptic proteins, such as complexin2, VAMP2 and synaptotagmin1. Since in our study miR-34a was found to be part of the 'AD early markers' subgroup, this may indicate that these are the first processes that promote the pathophysiology of AD in later stages. The demonstrated regulation of miR-15a and miR-34a on the survival factor Bcl2 together with the upregulation of these miRNAs we measured in the hippocampi of mice model of AD suggest an important functional explanation to the neuronal loss known in AD pathology.

Last, the progressive change in expression of certain miRNAs during aging of 3xTgAD mice supports the conceptual option of miRNAs as responsible for the AD pathology deterioration, an exciting option that highlights the importance of the field of miRNAs to AD detection and prevention.

Overall, this study improves our understanding of the molecular and cellular processes in AD pathology, following EE, and the interplay between the two processes. The high interest of recent years in the involvement of miRNAs in AD is encouraging and promising to advance AD diagnostic, treatment and prevention. Since we found several miRNAs that were specifically regulated in early stages of AD, these can be further used as AD biomarkers, a research field that has great potential.<sup>116</sup> This study opens new possibilities to further investigate the pathological consequences of AD and EE, and highlights specific miRNAs that can be manipulated in the hippocampi of mice model of AD to achieve phenotypic rescue. Ultimately, this will lead to an essential improvement in our pharmacological capabilities to manage AD pathology.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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