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Original Article

Profiling of Argonaute-2-loaded microRNAs in a mouse model of frontotemporal dementia with parkinsonism-17

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Abstract: Tauopathies are a group of neurodegenerative diseases characterized by the pathological aggregation of the microtubule-associated protein tau. These include more than 20 diseases, with Alzheimer's disease being the most frequent. While pathological and neurotoxic effects of tau are well documented, the mechanisms by which tau can promote neurodegeneration are less clear. Increasing evidence suggests a functional role for microRNAs in the pathogenesis of tauopathies, with altered expression and function of microRNAs in experimental models and patient brain. To determine whether a pathological expression of tau leads to altered microRNA expression, we investigated a mouse model (VLW), which overexpresses tau carrying three mutations identified in patients suffering from frontotemporal dementia with parkinsonism-17. Argonaute-2-bound microRNAs were co-immunoprecipitated using hippocampal tissue to identify active microRNAs within the model and quantified using a genome-wide high-throughput qPCR-based microRNA platform. While similar numbers of microRNAs are present between wild-type and VLW mice, a prominent increase in Argonaute-2-bound levels of microRNAs could be observed in VLW mice. This included microRNA-134, microRNA-99a and microRNA-101. Subsequent experiments revealed this increase in Argonaute-2 loading of microRNAs to correlate with increased microRNA expression. Our *in vivo* study suggests that a pathological tau overexpression may lead to an increase in active microRNAs, possibly contributing to dysregulation of gene expression and tau-induced pathology.

Keywords: Tauopathy, frontotemporal dementia with parkinsonism-17, MicroRNA, Argonaute-2

Introduction

Tauopathies are a family of neurodegenerative disorders featuring a prominent tau neurofibrillary pathology [1]. Alzheimer's disease (AD) is the most common tauopathy; there are, however, many others including familial frontotemporal lobar degeneration, Pick's disease, progressive supranuclear palsy, and corticobasal degeneration [1]. Huntington's disease has also recently been described as a tauopathy [2]. Frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) is a unique example of a tauopathy induced purely by mutation(s) to the tau gene, resulting in tau aggregation with a complete absence of the amyloid-β deposition and other co-morbidities which regularly accompany tauopathies and associated disorders [3].

Tau is a microtubule-associated phosphoprotein, which is mainly found in mature neurons, where it is enriched in the axonal compartment. Tau is encoded by a single gene (*MAPT*) on chromosome 17, giving rise to six major isoforms containing either three (3R) or four (4R) microtubule-binding domain repeats [4]. The main function of tau includes the stabilization

of microtubules [4]. Tau is, however, involved in a myriad of different functions including axonal transport [5] and the induction of long-term depression (LTD) [6]. Tau is subject to various post-transcriptional modifications, including phosphorylation, glycation, ubiquitination and truncation among others, with tau phosphorylation the best characterized [4]. In tauopathies, tau becomes abnormally hyperphosphorylated. This hyperphosphorylated form causes tau to detach from the microtubules, to accumulate in the cytoplasm forming insoluble filaments and tau aggregates [7]. Despite hyperphosphorylated tau having a highly destructive effect on neurons [5], the exact neurotoxic mechanisms caused by tau remain unclear, however, numerous putative mechanisms have been proposed. Among many others, these include the loss-of-function as a stabilizer of microtubule structures in the axon, leading to depolymerization, the formation of insoluble aggregates which damage the cytoplasm and axonal compartment or the formation of tau oligomers inducing synaptic dysfunction [5, 8, 9]. Delineating the actions of tau within these diseases is of great interest, not only for understanding tauopathies, but also a plethora of other neurological disorders within which tau undergoes abnormal regulations such as the neurological disease, epilepsy, in which tau contributes to an increased state of hyperexcitability [10, 11].

MicroRNAs (miRNA) are short non-coding RNAs which function to regulate protein expression at a post-transcriptional level [12]. Dysregulation of miRNA contributes to the pathogenesis of numerous brain diseases including tauopathies [13]. MiRNAs are well established as critical molecules within the cell. In the central nervous system (CNS), miRNAs influence numerous pathways, including neuronal function, dendrite outgrowth and development [14, 15]. MiRNAs function within an RNA-induced silencing complex (RISC) to inhibit translation or degrade targeted mRNA transcripts [12]. After the initial processing by the type III RNA polymerase, Dicer, which cleaves the pre-miRNA into its mature state, miRNAs are bound by the endonuclease Argonaute-2 (Ago-2), a key protein within the RISC complex. The loaded miRNA sequence then guides RISC to target complimentary mRNA transcripts to degrade or inhibit translation of the mRNA. This mechanism of complimentary sequence-targeting enables a single miRNA to target and regulate multiple transcripts and potentially entire pathways [16].

The integral role miRNAs play in tau regulation has been demonstrated in numerous studies. Genetic ablation of DICER in mice led to a reduction of miRNAs, which correlated directly with an increase in tau phosphorylation and neurodegeneration and which was primarily attributed to the loss of miR-15a [17]. Upregulation of miR-146a in AD mouse models resulted in increased phosphorylation of tau by the targeting of rho-associated, coiled-coil-containing protein kinase 1 (ROCK1) [18]. MiR-219 was shown to target *tau* transcripts and is reduced in AD and tangle-predominant dementias patient brains, with reduction of miR-219 resulting in exacerbated tau toxicity in Drosophila models and mammalian cell cultures [19]. Actual investigations into the miRNA expression of tauopathy patients, with the exception of AD [20, 21], have been limited to progressive supranuclear palsy, a rare disease characterised by the presence of neurofibrillary tangles (NFTs) composed of tau sharing many characteristics with FTDP-17 [3, 22, 23]. However, despite the apparent interest on the impact of miRNA on tau with investigations in Drosophila, AD mouse models and even tauopathy patients, there has not been a study of active Ago-2-bound miRNAs within tau pathology.

In the present study we used a high-throughput PCR-based platform to screen Ago-2-bound miRNA in wild-type (WT) and mice overexpressing mutated tau carrying three FTDP-17 mutations (VLW) [24]. Results suggest an overall increase in miRNA-loading to the RISC which may promote excessive miRNA-based silencing of target transcripts. Our results therefore propose a tau-driven activation of miRNAs and increases in miRNA expression, further suggesting targeting of tau as a therapeutic strategy to treat brain diseases with an underlying tauopathy.

Material and methods

Animals

All animal procedures were performed in accordance with the principals of the European Communities Council Directive (86/609/EEC)

and National Institute of Health's *Guide for the Care and Use of Laboratory Animals*. All studies involving animals were approved by the Research Ethics Committee of the Centro de Biología Molecular Severo Ochoa Institutional Animal Care and Utilization Committee (Comité de Ética de Experimentación Animal del CBM, CEEA-CBM), Madrid, Spain (PROEX293/15). Animal housing and maintenance protocols followed the guidelines of the Council of the European Convention ETS123 and were performed in accordance with the principals of the European Union adopted Directive (2010/63/EU). VLW transgenic mice overexpress a human 4-repeat tau isoform carrying three FTDP-17 mutations (G272V, P301L and R406W) under the *thy1* promoter [24]. The tau mutations G272V and P301L are both located within the microtubule binding repeats, thereby disrupting binding of tau to the microtubules leading to a reduction in microtubule assembly, with each affecting a specific tau isoform (3R and 4R) [25]. The R406W mutation is located at the C-terminus end of the molecule. This mutation in tau has been shown to lead to an increase in tau phosphorylation which is a prominent feature in tauopathies [25]. The combination of these mutations in tau induces both the loss of function of tau in 3R and 4R isoforms as well as tau hyperphosphorylation typical of tauopathies, aiming in this way for a synergistic pathological effect in transgenic VLW mice. Subsequent studies showed that these mice present an increase in hyperexcitability states and experience spontaneous epileptic seizures [10], an increase in acetylcholinesterase levels and activity [26] and early-onset deterioration of γ-aminobutyric acid (GABA)ergic septohippocampal innervation [27]. All transgenic mice are bred under a C57Bl/6 background and only heterozygous VLW mice were used. Hippocampi were extracted from four months old euthanized VLW and age matched C57Bl/6 WT control mice, immediately put on dry ice and stored for further processing.

Ago-2 (mi) RNA-immunoprecipitation (RIP)

Ago-2 immunoprecipitation was carried out as previously reported using RNase free solutions [28]. Briefly, hippocampi obtained from VLW and control WT mice were homogenized manually using an immunoprecipitation buffer containing 300 mM NaCl, 5 mM Mg_2Cl, 50 mM Tris HCl (pH = 7.5), 0.1% NP-40, proteases inhibitors Arotinin and Leupeptin (Merck, Darmstadt, Germany) and phosphatases inhibitors (PMSF (Roche, Basel, Switzerland) and vanadate (Merck, Darmstadt, Germany)). Nucleus, cell debris and membranes were removed by centrifugation at 16,000× g for 15 min at 4°C. Supernatant was removed and considered as the total cell lysate. Then, 5 µg of Ago-2 antibody (Cat number: C34C6, Cell Signaling Technology, Danvers, MA, USA) was added to 400 µg of the protein lysate and incubated overnight at 4°C in a carousel. The following day, 20 µl of a 50% protein-A/G-agarose bead solution (Santa Cruz Biotechnology, Heidelberg, Germany) was added to the samples, mixed and incubated for 1 h at 4°C. Finally, beads were centrifuged and pellet washed twice with immunoprecipitation buffer followed by Trizol extraction as previously described [28, 29].

MicroRNA profiling by OpenArray

MiRNA profiling was performed using the OpenArray platform from Thermo Fisher (Waltham, Massachusetts, United States), as described previously [28, 30]. Briefly, the OpenArray reverse transcription reaction was performed according to the manufacturer's protocol using 3 µl of total RNA obtained from Ago-2 immunoprecipitation. Before loading onto the OpenArray, cDNA was pre-amplified following the manufacturer's recommendation. Then, the PreAmp product was diluted with 0.1X TE (1/40) and 22.5 µl of diluted PreAmp product was added to the same volume of 2X TaqMan OpenArray Real time PCR Master Mix (Cat No. 4462164, AB). Finally, the mix of Pre-Amp product and Master-Mix was loaded onto a 384-well OpenArray plate. OpenArray panels were automatically loaded by the OpenArray AccuFill System (Thermo Fisher, Waltham, Massachusetts, United States). Each panel enables the quantification of miRNA expression of three samples and up to four panels can be cycled simultaneously. Samples were randomized between panels and run simultaneously on a QuantStudio 12K Flex Real-Time PCR system. 754 mouse miRNAs were amplified from each sample together with 16 replicates of four internal controls (ath-miR159a (negative control), RNU48, RNU44 and U6 rRNA). The number of miRNAs identified in each sample was: 165 for WT1, 151 for WT2, 140 for VLW1, 210 for VLW2 and 258 for VLW3. Only miRNAs which were expressed in all samples were used for further

analysis, totaling 87 miRNAs. Data was normalized using a global normalization method where each Ct value was normalized to the geometric median of all Ct values in one sample. MiRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method, using the average of the ΔCt values of WT1 and WT2 as a reference. Due to the low numbers, no statistical test has been carried out on the OpenArray and results are based on fold-changes between conditions. The heat map and Volcano plot were generated using ExpressionSuite Software Version 1.0.3 (Thermo Fisher, Waltham, Massachusetts, United States).

Quantitative polymerase chain reaction (qPCR)

MiRNA qPCR was carried out as previously described [29]. Briefly, 1 μg of total RNA was reverse transcribed using stem-loop Multiplex primer pools (Thermo Fisher, Waltham, Massachusetts, United States). Retro transcriptase (RT)-specific primers for the mouse miRNAs miR-134 (ID: 001186), miR-101 (ID: 002253), miR-99a (ID: 000435), miR-15b (ID: 000390) and miR-212 (ID: 002551) were used (Thermo Fisher, Waltham, Massachusetts, United States) and qPCRs were carried out on a QuantStudio 12k Flex Real Time System (Thermo Fisher, Waltham, Massachusetts, United States) using Taqman miRNA assays (Thermo Fisher, Waltham, Massachusetts, United States). RNU6 was used for normalization. The relative fold change in expression was calculated using the $2^{-\Delta\Delta CT}$ method [31].

Western blotting

Western blotting was performed as described previously [32]. Following quantification of protein concentration, 30 μg of protein samples were boiled in gel-loading buffer and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membranes and probed with the following primary antibodies: Argonaute-2 (Cell Signaling Technology, Danvers, MA, USA) and β-Actin Merck (Darmstadt, Germany). Next, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Isis Ltd, Bray, Ireland) and protein bands visualized using chemiluminescence (Pierce Biotechnology, Rockford, IL, USA). Gel bands were captured using a Fujifilm LAS-3000 (Fujifilm, Tokyo, Japan) and analyzed using Alpha-EaseFC4.0 software.

Statistical analysis

Graphs were generated using GraphPad Prism™ software. For individual PCRs, all statistical analysis was performed on SPSS. Measures of significance between groups were tested by Mann-Whitney (non-parametric t-test) because the data does not follow a normal distribution. Minimum measures of significance were set at $P < 0.05$.

Results

Increased Argonaute-2 microRNA loading in FTDP-17 tau overexpressing mice

To study the effects of a pathological tau overexpression on miRNA function, we used a mouse model, which overexpresses human tau, carrying three mutations identified previously in patients suffering from FTDP-17 (G272V, P301L and R406W (VLW)) [24, 25]. In this model, FTDP-17 tau expression is driven by the neuronal promoter *thy-1* and transgene expression is largely restricted to neurons of the dentate gyrus (DG), the cornus ammonis (CA) 1 and CA3 subfield of the hippocampus and the neocortex. Transgenic tau expression can also be found in the striatum and spinal cord [24, 32], similar to what has been reported for patients suffering from FTDP-17 [33]. Notably, the VLW mouse model lacks tau aggregates but shows increased levels of tau hyperphosphorylation. Tau filaments formed in the mouse display a pre-tangle appearance [24]. Overexpression of tau in the model is modest (~2.5 fold) compared to other tauopathy models such as P301S (~5.5 fold) [34], with the phenotype more likely to be derived from abnormal tau interactions than the formation of insoluble aggregates [24].

To elucidate the influence of pathological mutated FTDP-17 tau expression on miRNA activity, Ago-2-bound miRNAs were co-immunoprecipitated from the hippocampi of FTDP-17 tau overexpressing mice (VLW) and matched WT controls. Ago-2-bound miRNA content was analyzed by a custom OpenArray system [28], a high density qPCR assay plate enabling analysis of hundreds of custom reprinted selected miRNAs with a high reproducibility [35]. Genome-wide miRNA profiling was performed

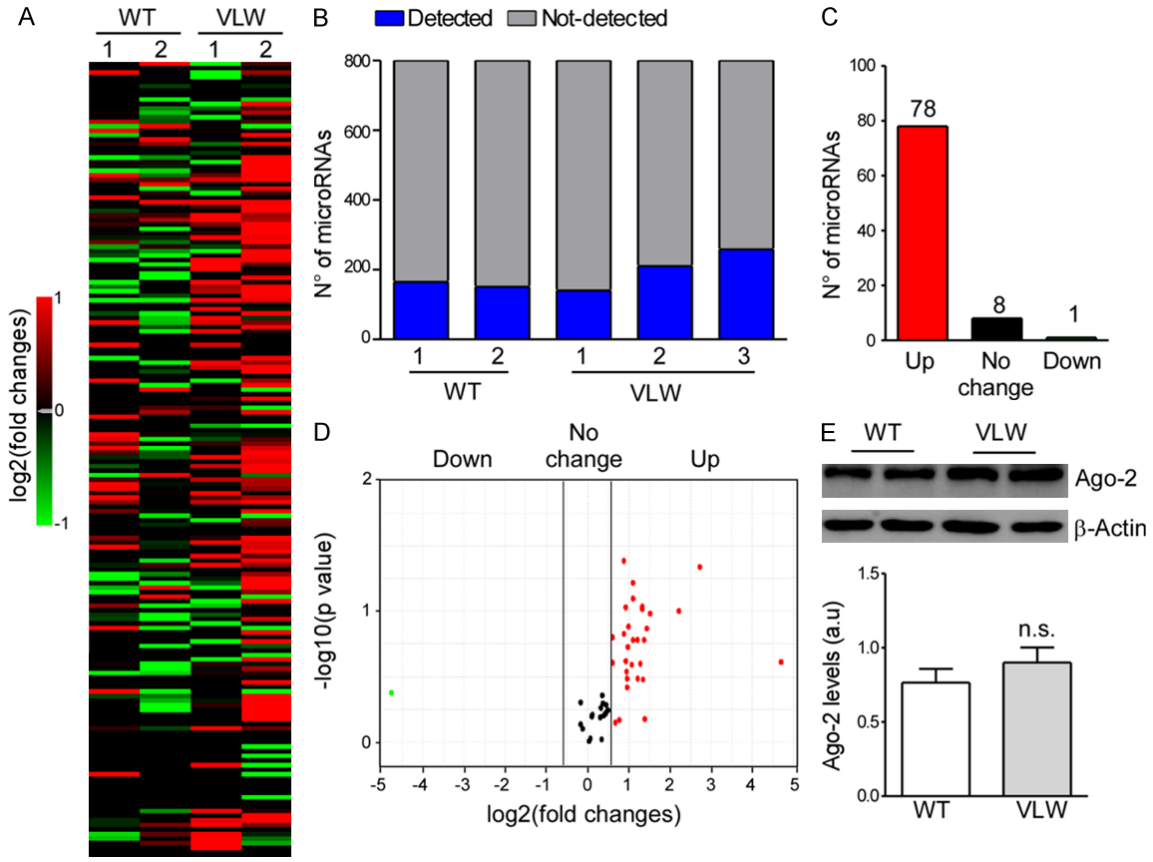

Figure 1. *Argonaute-2-bound miRNA profiling in FTDP-17 tau overexpressing mice.* A. Heatmap of differentially expressed miRNA in the hippocampus of WT and VLW mice profiled on OpenArray, each row representing an individual miRNA and each column representing an individual sample (n = 2 (Control (WT)) and 2 (VLW)). Expression ratio indicated by colour from green (low) to red (high) with intensity of expression indicated by scale bar. B. Graph showing similar numbers of miRNAs identified by OpenArray to be bound to Ago-2 between WT and VLW mice. C. Graph showing increased levels of miRNAs in VLW mice bound to Ago-2 when compared to WT control mice (87 miRNAs are consistently expressed across all conditions and samples, among these 79 are differentially expressed from control with 78 upregulated (1.5-fold expression or greater) and 1 downregulated (0.75-fold expression or less). D. Volcano blot showing the distribution of miRNAs according to *p* value (Y axis) and fold changes (X axis). E. Representative Western blot (n = 1 per lane) and graph showing no significant difference in Argonaute-2 (Ago-2) expression in the hippocampus between WT and VLW mice (n = 5 per group; P = 0.354).

using the OpenArray platform to identify functional Ago-2-loaded miRNAs (**Figure 1A**). OpenArray was carried out on 2 WT and 3 age-matched VLW mouse brains. Due to the low number of samples, no statistical testing was performed on the OpenArray data. Instead, miRNAs were selected for further experiments based on fold-change.

While a similar number of unique miRNA transcripts seem to be uploaded onto Ago-2 in both WT and VLW mice (WT: 158±7.0; VLW: 202.1± 34.26) (**Figure 1B**), the concentration of these uploaded miRNAs were increased in VLW mice, with 78 miRNAs being upregulated and only a single miRNA down-regulated (**Figure 1B-D** and **Table 1**). Among altered miRNAs, 15 were identifed within VLW mice with higher levels of Ago-2 loading (>26 Ct) and significant changes from control (>5 RQ) (**Table 1**). This includes Let-7c, miR-125a, miR-223, miR-193b, miR-409-3p, miR-125b-1-3p, miR-134, miR-125b, miR-380-5p, miR-128a, miR-99a, miR-101, miR-544, miR-140 and miR-532-3p. No significant change in Ago-2 expression could be observed in the hippocampus between VLW and WT mice (**Figure 1E**) suggesting that increased Ago-2 levels are not responsible for the observed increase of Ago-2 miRNA loading.

Our results suggest, therefore, that a pathological overexpression of mutant FTDP-17 tau

Table 1. Differentially regulated miRNAs bound to Ago-2 in VLW mice

Relative abundance	Ago-2-bound miRNAs altered in VLW mice (*fold change*)
UP in VLW (>5 fold change)	*Let-7c (25.4), miR-125a-5p (19.7), miR-223 (12.4), miR-193b (11.6), miR-409-3p (10.0), miR-125b-1-3p (9.9), miR-134 (7.9), miR-125b (7.9), miR-380-5p (7.3), miR-128a (7.1) miR-99a (7.0), miR-101 (6.5), miR-544 (6.4), miR-140 (5.5), miR-532-3p (5.2)*
Up in VLW (>1.5 fold change)	*miR-100 (4.4), miR-26b (4.3), miR-26a (4.1), miR-192 (4.1), miR-672 (4.1), miR-135b (4.0), miR-384-5p (3.8), miR-434-3p (3.8), miR-30c (3.8), miR-320 (3.7), miR-323-3p (3.6), miR-421 (3.5), miR-872* (3.5), miR-19b (3.5), miR-99b (3.5), miR-376a (3.5), miR-30e (3.3), miR-331 (3.3), miR-138 (3.2), miR-184 (3.2), miR-434-5p (3.1), miR-539 (3.1), miR-106a (3.0), miR-1274 (3.0), miR-137 (3.0), miR-376b* (3.0), miR-136 (3.0), miR-376c (3.0), miR-222 (3.0), miR-218 (3.0), miR-126 (2.9), miR-487b (2.9), miR-484 (2.9), miR-186 (2.9), miR-410 (2.8), miR-744 (2.8), miR-16 (2.8), miR-411 (2.8), miR-1897-5p (2.7), miR-15b (2.7), miR-146b (2.6), miR-324-3p (2.4), miR-1937 (2.4), miR-139-3p (2.4), miR-9* (2.3), miR-433 (2.3), miR-150 (2.3), miR-146a (2.1), miR-2134 (2.1), miR-7a* (2.0), miR-191 (2.0), miR-720 (2.0), miR-7* (1.9), miR-877 (1.9), miR-664 (1.9), miR-2146 (1.9), miR-204* (1.8), miR-491 (1.8), miR-204 (1.6), miR-140-3p (1.6), miR-375 (1.6), miR-574-3p (1.6), miR-93* (1.6)*
Unchanged (1.5-0.75)	*miR-212 (1.5), miR-592 (1.4), miR-1839-3p (1.4), miR-125b-2-3p (1.3), miR-448 (1.1), miR-1937c (1.0), miR-1839-5p (1.0), miR-339-3p (1.0)*
Down in VLW (< 0.75)	miR-29b* (0.6)

Differentially regulated miRNAs bound to Ago-2 in VLW mice. MiRNAs bound to Ago-2 are listed based on decreasing levels of relative abundance. Fold change observed in OpenArray are listed within brackets for each individual miRNA. (*) denotes a distinct miRNA produced from the opposite-3'/-5' arm of the same precursor as the original miRNA.

leads to an increase in Ago-2 loading of miRNAs, possibly increasing miRNA activity.

Increased hippocampal expression of Argonaute-2-bound microRNAs in FTDP-17 tau overexpressing mice

Our results indicate that a suprisingly large number of miRNAs seemed to be upregulated within the Ago-2 complex in the hippocampus of FTDP-17 VLW mice. Ago-2 binding has been previously shown to correlate with miRNA expression [36] and tau has been shown to alter the expression and activity of a variety of different transcritpion factors [4, 37]. To determine whether tau pathology leads to an increase in miRNA expression and whether this correlates with the observed increase in Ago-2 binding, we selected five miRNAs to be analyzed in an extra set of mice. These miRNAs were selected according to their consistent expression on the OpenArray across all conditions or previous links to the pathogenesis of tauopathies. We quantified, by indiviual RT-qPCR, three of the most strongly upregulated and consistently expressed miRNAs on all samples analyzed by the OpenArray (miR-134 (7.9 fold change), miR-99a (7.0 fold change) and miR-101 (6.5 fold change)) (**Table 1**), which have previously been associated with tauopathies and neurological disorders [29, 38, 39]. Despite miR-15b only showing a modest increase of 2.7, we decided to evaluate whether miR-15b was also regulated in our mouse model, because data has demonstrated that miR-15b represses the expression of the β-sectretase BACE1 [40]. Finally, we analyzed miR-212, a miRNA that was stably expressed across FTDP-17 VLW mice and WT control at comparable levels. MiR-134, miR-99a and miR-101 showed significantly higher expression in the hippocampus of FTDP-17 tau overexpressing mice compared to WT control mice, mirroring the OpenArrray Ago-2 results (**Figure 2A-C**). Although miR-15b lacked a significantly elevated change from control, it showed a strong trend towards elevated expression in VLW mice (**Figure 2D**). Again, this mirrors the OpenArray and demonstrates that even minor differences detected by the OpenArray within the Ago-2 complex mimicked changes in miRNA expression at a cellular level. As predicted from our OpenArray results, miR-212 showed no change between conditions (**Figure 2E**).

Our results, therefore, show that increased Ago-2 loading of miRNAs correlates with an increase in total expression of miRNAs and further confirms that increased miRNA expression seems to be the general response to pathological FTDP-17 tau overexpression.

Discussion

The main findings of the present study suggest that a pathological overexpression of FTDP-17 tau in the hippocampus leads to an increase in hippocampal miRNA expression and Ago-2 miRNA loading, possibly leading to more active miRNA and a reduction in miRNA-targeted genes which may contribute to tau-induced pathology.

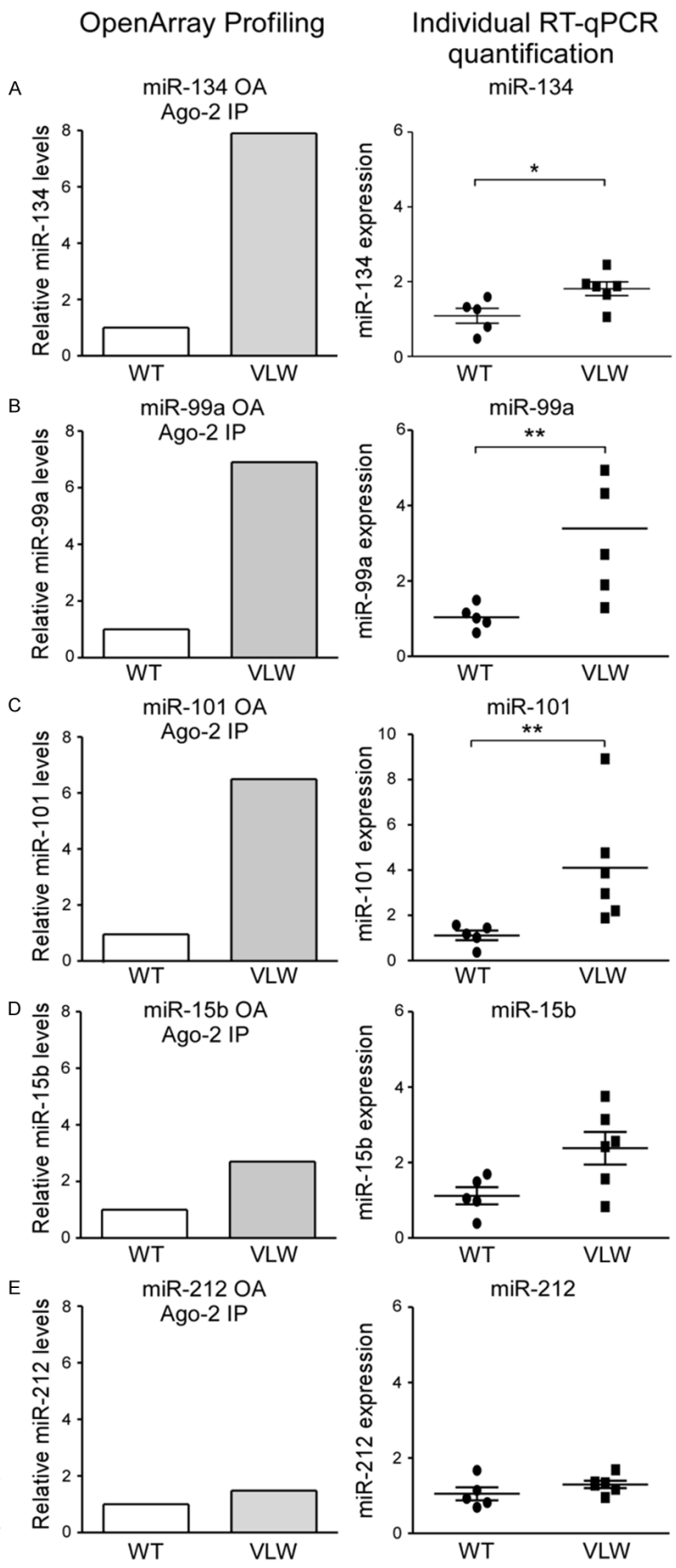

Figure 2. *Quantification of miRNAs by individual RT-qPCR*. Graphs showing relative expression of OpenArray analysis (n = 2 (WT) and 3 (VLW)) and RT-qPCR (n = 5 (WT) and 6 (VLW)) in hippocampal tissue on selected miRNAs: miR-134 (A), miR-99a (B), miR-101 (C), miR-15b (D) and miR-212 (E). OA = OpenArray, IP = immunoprecipitation, *P < 0.05, **P < 0.01.

Whereas previous studies analyzing the miRNA profile of tauopathies focused on the expressional response of miRNAs [20-23], our study represents a novel investigation into miRNA regulation during tauopathy, specifically profiling the active miRNA pool bound to Ago-2. Various studies have been undertaken to identify the miRNA profile during tauopathies, with a particular emphasis on AD using both animal models and patient brain [20, 21]. A previous study by Cogswell et al., using a RT-qPCR 96 well plate platform, identified several dysregulated miRNA within the hippocampus of AD patients which were similarly identified in the VLW model including miR-125b, miR-100 and miR-26a [41]. A subsequent study carried out by Lau et al., using deep sequencing of hippocampal and cortical brain tissue from late onset AD showed upregulated miR-223 as another shared miRNA between patients and the mouse model [20]. It is possible that the alterations to these miRNAs within AD patients are driven by the same tau-based disruptions as within the VLW model and further study into the mechanism driving alterations to these miRNAs could yield novel insights and therapeutic targets for the action of tau within AD. With regards to

APP/PS1 model, numerous miRNA shared a common trend of upregulation including Let-7c, miR-146a, miR-125a, miR-139, miR-433 and miR-320. This would indicate a similar response to the neuronal damage present in both models, but striking contrasts were also observed with miR-125b, miR-101 and miR-128a showing downregulation within the APP/PS1 mouse model with all three miRNAs strongly upregulated within the VLW model. These inverse patterns may indicate an alteration directly connected to distinct tau and amyloid-based pathology respectively. Notably, APP/PS1 mice were profiled with total miRNA rather than the Ago-2 bound miRNA as in our study, and comparison between these analyses must therefore take this into account. The expression patterns of total miRNA has, however, been shown to directly correlate with the abundance of miRNA bound to Ago-2, thus the differential abundance of an Ago-2 bound miRNA is likely to directly correlate with total expression of the miRNA [36]. Probably representing the closest comparable study as regards to the miRNA transcriptome in the presence of pathological tau, a recent study analyzed miRNA expression in patients suffering from progressive supranuclear palsy which shares a similar pathology to FTDP-17 patients [22]. In this study, patients were analyzed using TaqMan Array MicroRNA cards recognizing 372 different miRNAs. The authors showed only four differentially expressed miRNAs between control and patients (miR-147, miR-518e, miR-504 and miR-525-3p). None of these miRNAs showed altered expression in the VLW model, probably due to the difference in the model used (e.g. mice vs patients or mutated tau vs no tau mutations). Interestingly, a previous study investigating the role of miRNAs in progressive supranuclear palsy and their role on tau splicing and the impact on the 4R:3R-tau ratio showed that miR-9 and miR-137, both significantly increased in the VLW model, can regulate the 4R:3R-ratio in neurons [23].

One of the main findings of our study is the strong increase in both expression and Ago-2 loading of miRNAs in the VLW model. What causes the increase in miRNA expression and Ago-2 loading, we do not know. It is tempting to speculate that the increase in Ago-2 loading is a consequence of an increase in miRNA levels. Previous studies have shown miRNA expression to correlate with Ago-2 loading [36]. Whether the expression of mutant tau leads to an increase in the transcription of miRNAs is unclear. Pathological tau has been shown to increase the activity/expression of numerous transcription factors [4, 37], which may lead to the increased expression of miRNAs within cells. We also do not know what cell types contribute to the increase in miRNA levels. This is possibly neurons, as mutated tau overexpression is driven by the neuronal *thy-1* promoter. The contribution of glial cells, however, cannot be excluded and should be addressed in future experiments. Nevertheless, the increase in neuron-enriched miRNAs elevated in Ago-2-bound miRNA, including the cluster miR-99a and Let-7c [42], the members of the miR-125 family (miR-125a and miR-125b) [43], miR-134 [44], miR-128 [45] and miR-101 [46] strongly suggest this to be neuronal.

With neurodegeneration a common feature among all tauopathies [1], miRNAs capable of inducing or contributing to neurotoxicity were of particular interest. The miRNA cluster miR-99a along with Let-7c, contained within chromosome 21, shows a noticeable upregulation in the VLW mouse model. MiR-99a directly downregulates the transforming growth factor-β (TGF-β), which has key roles in WNT signaling and synaptic signaling [47]. Loss of TGF-β has been shown to increase neuronal cell death and microgliosis [48, 49]. Higher expression of Let-7c holds a unique neurodegenerative capability through activation of Toll-like receptors by functioning as an agonist [50]. Taken together, miRNAs within this cluster display destructive effects within neurological environments and are found at increased levels within several diseases displaying cognitive impairments [39]. Therefore, their elevated activity within the VLW model may represent a key neurotoxic mechanism induced by aberrant tau expression.

Regulation of tau phosphorylation is a central characteristic of tauopathies [1]. MiR-125b has been shown to promote tau phosphorylation by targeting and downregulating tau phosphatases dual specificity phosphatase 6 (DUSP6) and protein phosphatase 1, catalytic subunit, alpha isoform (PPP1CA) in mice [43]. MiR-125b has also been identified at elevated levels in AD patient brain [51].

Seizures and epileptiform activity have long been associated with the pathology of tauopa-

thies [52]. In line with this, tau overexpressing VLW mice present increased hyperexcitability states [10]. However, how the expression of mutant tau induces this susceptibility to seizures is unclear [10]. MiR-101 has been described as a critical regulator of excitation and inhibition within neural networks for developing a stable network. Transient blockading of miR-101 in adult mice results in an impairment of hippocampal dependent cognition and blockade of miR-101 during early development results in hyperexcitable networks in the mouse [46]. The targets of miR-101 include the Na-K-Cl cotransporter (NKCC1), ankyrin-2 (ANk2) and kinesin like protein (Kifla), key proteins controlling synaptic outgrowth [46]. The increase of miR-101 in the VLW mice and its integral role in maintaining balanced signaling environment may be a contributing factor in the development of the hyperexcitable phenotype in VLW mice [10] with higher levels of miR-101 potentially resulting in imbalances of inhibitory and excitatory neurons/inter-neurons. Another miRNA possibly leading to an increased hyperexcitability state in the VLW model and found at elevated levels bound to Ago-2 was miR-134. MiR-134 has been shown to be increased following neuronal activity [44] and inhibition of miR-134 reduced the epileptic phenotype in mouse models of epilepsy [29, 53]. Finally, miR-128, also strongly up-regulated in VLW mice, has also been shown to be implicated in hyperexcitability states in the brain by suppressing the expression of various ion channels and signaling components of the extracellular signal-regulated serine/threonine kinase ERK2 with loss of miR-128 leading to fatal epilepsy [45]. Thus, increased miR-128 levels in the hippocampus may represent an attempt to oppose hyperexcitability states in VLW mice. This noticeable influence of tau pathology within the model on key miRNAs may have implications on the incidence of epileptic activity in tauopathies such as AD, where an increased hyperexcitability state is postulated as one of the key events driving pathology [52].

MiR-29b*, the sole downregulated miRNA identified in the profile, has intriguing implications within AD as a similar downregulation of miR-29b* has previously been identified in AD patient brains [54]. Work using primary cell cultures have provided evidence that miR-29b* targets and down regulates the β-secretase BACE1, the loss of miR-29b* regulation of BACE1 levels contributes to an increased production of amyloid-β [54]. Thus, a downregulation of miR-29* in the presence of pathological tau may indicate a novel mechanism by which hyperphosphorylation of tau contributes to β-amyloid build up in AD.

A major limitation of our study is the low sample number used for Ago-2 miRNA profiling by OpenArray. This precluded meaningful statistical testing or correction for multiple comparisons. However, basing the selection of miRNAs on fold-change nevertheless resulted in reproducible validation in subsequent miRNA-specific assays on individual independent samples. A further caveat of our study is that we performed validation of specific miRNAs using total rather than Ago-2-bound miRNA. However, previous data has demonstrated that Ago-2 miRNA loading correlates closely with the total expression of miRNAs [36]. Our findings support this, showing the OpenArray results closely correlated with levels of miRNA expression. Finally, there are several transgenic tau models, each showing different stages of pathology. Whereas the VLW model may be considered to be a mild model of tauopathy [24], other models such as the P301S tauopathy mouse model [34] show a much stronger phenotype and should be analyzed in future studies.

In conclusion, our results indicate that an overexpression of pathological mutant tau leads to an increase in Ago-2 loaded miRNA, possibly leading to a reduction in protein translation and impacting on tau-induced pathology.

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Disclosure of conflict of interest

None.

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