Journal of Psychiatric Research 111 (2019) 110–120

Contents lists available at ScienceDirect

Journal of Psychiatric Research

journal homepage: www.elsevier.com/locate/jpsychires

Brain-enriched MicroRNA-184 is downregulated in older adults with major depressive disorder: A translational study

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ARTICLE INFO

Keywords:
Major depression
Late-life depression
miR-184
MicroRNAs
Biomarker
Drosophila melanogaster

ABSTRACT

Changes in microRNAs (miRNAs) expression have been described in major depressive disorder in young and middle-aged adults. However, no study has evaluated miRNA expression in older adults with major depression (or late-life depression [LLD]). Our primary aim was to evaluate the expression of miRNAs in subjects with LLD. We first evaluated the miRNA expression using next-generation sequencing (NGS) and then we validated the miRNAs found in NGS in an independent sample of LLD patients, using RT-qPCR. *Drosophila melanogaster* model was used to evaluate the impact of changes in miRNA expression on behavior. NGS analysis showed that hsa-miR-184 ($\log_2$foldchange = -4.21, $p = 1.2 \times 10^{-03}$) and hsa-miR-1-3p ($\log_2$foldchange = -3.45, $p = 1.3 \times 10^{-02}$) were significantly downregulated in LLD compared to the control group. RT-qPCR validated the downregulation of hsa-miR-184 ($p < 0.001$), but not for the hsa-miR-1-3p. The knockout flies of the ortholog of hsa-miR-184 showed significantly reduced locomotor activity at 21–24 d.p.e ($p = 0.04$) and worse memory retention at 21–24 d.p.e (24h post-stimulus, $p = 0.02$) compared to control flies. Our results demonstrated that subjects with LLD have significant downregulation of hsa-miR-184. Moreover, the knockout of hsa-miR-184 in flies lead to depressive-like behaviors, being more pronounce in older flies.

1. Introduction

Late-life depression (LLD) is one of the most common mental disorders in the elderly, with prevalence rates ranging from 1% to 5% (for major depressive episodes) (Byers et al., 2010). Its occurrence increases the risk of adverse health outcomes, including higher rates of medical morbidity (Blazer, 2003), mortality (Diniz et al., 2014), and increased risk of Alzheimer's disease and Vascular dementia (Diniz et al., 2013). Beyond disease, LLD has been linked to decreased health span as measured by fewer years lived without frailty, significant functional impairments, elevated direct and indirect costs (Butters et al., 2004; Ferrari et al., 2013; St John et al., 2013; Zivin et al., 2013).

The biological mechanisms of LLD are not fully understood, but they are probably heterogeneous and involve the interaction of abnormalities in multiple biological pathways (Alexopoulos and Morimoto, 2011; Diniz et al., 2011; Naismith et al., 2012; Pomara et al., 2012). The biological heterogeneity of LLD may give raise, or reflect the heterogeneity clinical presentation and outcomes among individuals,

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https://doi.org/10.1016/j.jpsychires.2019.01.019
Received 13 July 2018; Received in revised form 21 November 2018; Accepted 18 January 2019

including the presence of neurocognitive impairment. The Presence of neurocognitive impairment is a very important feature of LLD and is usually related to poorer outcomes, including higher risk of poor response to antidepressant treatment and development of dementia (Kaneriya et al., 2016; Mourao et al., 2016). Recent comprehensive, multi-modal biomarkers studies showed that subjects with LLD had significant abnormalities in biological pathways related to inflammatory response control, tissue remodeling, endothelial and vascular function, proteostasis, neurotrophic support, and enhanced molecular senescence abnormalities (Diniz et al., 2015, 2016, 2017).

MicroRNAs (miRNAs) are small (18–23 nucleotides) non-coding RNAs that are involved in the posttranscriptional regulation of gene expression by base-pairing with complementary mRNA sequences (Bartel, 2009). They can modulate the expression of multiple different genes and, thus, can have to influence multiple biological pathways. MiRNAs are produced inside the cells and can be secreted and transported by proteins, lipids, or be packed into exosomes or microvesicles, being able to modulate gene expression in different cells and tissues (Gallo et al., 2012; García-López et al., 2013; Salido-Guadarrama et al., 2014; Weber et al., 2010). They are less susceptible to RNA degradation and can be detected in different body fluids such as plasma, serum and cerebrospinal fluid (Turchinovich et al., 2012). Therefore, they can be viewed as noninvasive biomarkers that can detect and monitor the dysregulation of multiple biological pathways in different disorders.

Recent studies have identified several miRNAs enriched in human brain (Azevedo et al., 2016; Maffioletti et al., 2014). They regulate genes that are involved in the regulation of neural processes, such as neurogenesis, neuroplasticity, as well as more general processes like neuroinflammatory response, metabolism, and cell-cycle regulation (Mendes-Silva et al., 2016; Olde Loohuis et al., 2012). Accordingly, abnormalities in the miRNA expression patterns can be major players in the pathophysiology of psychiatric disorders (Geaghan and Cairns, 2015).

Previous studies have identified changes in miRNAs expression (e.g., miR-124a, miR-16, miR-26b, miR-34 family, and the miR-let-7 family) in young and middle-aged adults with major depressive disorder (Azevedo et al., 2016; Bocchio-Chiavetto et al., 2013; Lopez et al., 2014; Wan et al., 2015). More recently, they were identified as biomarkers of treatment response in major depression (Fiori et al., 2017; Roy et al., 2017). However, these studies did not screen for miRNAs using unbiased, whole-genome sequencing methods, and included relatively small sample sizes of patients and controls. Moreover, they mostly included younger adult population, and no study has evaluated miRNA expression changes in LLD.

In this study, we aimed to investigate changes in miRNA expression in subjects with LLD. We used next-generation sequencing (NGS) for the unbiased discovery of miRNA differentially expressed in these subjects. We validated the NGS findings using quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis in an independent sample of LLD and healthy controls (HC). Bioinformatics analyses were carried out to predict miRNA target genes and to evaluate the biological pathways associated with miRNA expression changes. Our main hypothesis was that microRNA previously associated with major depression in younger adults (e.g., miR-124a, miR-16, miR-26b, miR-34 family, and the miR-let-7 family) will also be associated with major depression in the elderly. Finally, we used the *Drosophila melanogaster* as a translational model to validate the role of miRNA expression in the brain in behavioral changes.

2. Material and methods

2.1. Sample recruitment and assessment

We included a convenience sample of 116 older adults (63 with LLD and 53 HC) in this study. All patients with LLD were recruited and evaluated at the Psychogeriatric Outpatient Clinic at the Federal University of Minas Gerais, Belo Horizonte, Brazil after referral for the assessment of depressive symptoms. Typical aging older adults were included as a control group. They were recruited as part of an ongoing cohort study of healthy cognitive aging at the Federal University of Minas Gerais. All participants were above 60 years old at the time of study recruitment.

All participants underwent a comprehensive psychiatric, clinical, and neurological assessments. The psychiatric assessment included the administration of the Mini Neuropsychiatric Interview (MINI) (Sheehan et al., 1998). The diagnosis of LLD was based on the DSM-5 (Diagnostic and Statistical Manual of Mental Disorders – Fifth Edition) criteria for major depressive episode (single and recurrent). The severity of depressive symptoms was rated with the Hamilton Depression Rating Scale - 21 items (HDRS-21) (HAMILTON, 1960). Inclusion criteria for controls were no history of major depressive disorder or other major psychiatric disorder, and no evidence of cognitive impairment based on the DRS scores. All participants (LLD and controls) were not under current antidepressant treatment at the time of psychiatric assessment and blood collection. Additional exclusion criteria for this study were the presence of unstable medical illness, history of auto-immune disease, chronic use of anti-inflammatory drugs medication, history of substance abuse disorder in the past year.

We administered the Mattis Dementia Rating Scale (DRS) for neurocognitive assessment and to exclude potential dementia cases in this population (Marson et al., 1997). The DRS provides scores for different cognitive domains (executive function, attention, visuoconstructional ability, language, and episodic memory) and also a total score that reflects global cognitive performance.

These subjects were randomly split into two sets: discovery set (24 with LLD and 19 HC); validation set (39 LLD and 34 HC). Given the small number of males among controls and LLD subjects, we tried to balance gender distribution in the discovery and validation set samples to avoid the bias in results due to sex.

The discovery set sample was used in the NGS analysis for the discovery of miRNAs differentially expressed between LLD and control groups. The findings on the discovery set sample were independently validated in the validation set sample using RT-qPCR.

The sociodemographic and clinical and characteristics of the sample are shown in Table 1. The study and its procedures were approved by the ethics committee of the Federal University of Minas Gerais, Belo Horizonte, MG, Brazil.

2.2. RNA extraction from plasma samples

Whole blood was collected by venipuncture in EDTA coated vacutainers and plasma was separated by centrifugation immediately after collection and stored at −80 °C until further use. Total RNA containing microRNA was extracted from 200 µl plasma using mirVana PARIS kit (Ambion, Austin, TX/USA) (Yoon et al., 2017). Extracted RNA was eluted in 50 µl of preheated nuclease-free water and measured on Qubit (Thermofisher, Waltham, MA/USA), then immediately stored at −80 °C.

2.3. NGS sequencing

Sequencing large number of individuals, which is often needed for population genetics studies, is economically challenging despite falling costs of NGS. *Pool-seq* is an alternative cost- and time-effective option to analyze several individuals (Anand et al., 2016; Liu et al., 2014). Plasma samples of 43 individuals (19 HC and 24 LLD) was used the discovery set in NGS. Control subjects were divided into 3 pools (6–7 individuals per pool) and LLD subjects were divided into 6 pools (3–5 individuals per pool). Each pooled sample was processed, and libraries were prepared using Total Ion RNA-Seq v2 kit (Life Technologies, Carlsbad, CA/USA) and were carried on Ion PI (Life Technologies) chip prepared and loaded in an automated Ion Chef (Life Technologies)

Table 1
Demographics and clinical characteristics of the sample (Discovery and Validation sets).

Discovery set				
Sample size	HC	LLD	Statistics	p-value
	19	24		
Sex (male/female)	1/18	4/20	$X^2 = -1.15$	0.25
Age (years)	69.6 ± 6.1	72.2 ± 7.9	$t = -1.19$	0.135
Years of education	9.5 ± 5.2	4.6 ± 2.4	$t = 4.03$	< 0.001
HDRS-21	2.68 ± 5.3	19.0 ± 7.3	$t = -8.20$	0.009
MMSE	28.6 ± 1.6	24.8 ± 3.9	$t = 3.72$	0.001
Dementia Rating Scale				
Attention score	36.2 ± 0.7	34.6 ± 3.4	$t = 1.88$	0.021
Initiation/Perseveration score	36.1 ± 1.7	33.9 ± 3.1	$t = 2.40$	0.05
Construction score	6.0 ± 0.0	5.18 (± 1.33)	$t = 2.57$	< 0.0001
Conceptualization score	34.7 ± 3.6	33.0 ± 7.9	$t = 0.80$	0.05
Memory score	23.2 ± 1.8	20.0 ± 4.7	$t = 2.51$	0.035
Total score	136.3 ± 5.1	126.7 ± 19.4	$t = 1.96$	0.014
Validation set				
Sample size	34	39		
Sex (male/female)	5/29	7/32	$X^2 = -1.15$	0.25
Age (years)	70.9 ± 8.1	73.1 ± 8.1	$t = -1.05$	0.670
Years of education	9.8 ± 4.8	4.5 ± 2.5	$t = 5.39$	< 0.0001
HDRS-21	2.6 ± 5.5	19.6 ± 6.8	$t = -11.25$	0.015
MMSE	28.0 ± 2.3	23.8 ± 2.8	$t = 5.71$	0.268
Dementia Rating Scale				
Attention score	35.7 ± 1.1	34.9 ± 1.4	$t = 2.38$	0.258
Initiation/Perseveration score	35.4 ± 2.5	31.9 ± 3.9	$t = 3.88$	0.01
Construction score	5.7 ± 0.7	5.3 ± 1.3	$t = 1.33$	0.006
Conceptualization score	34.0 ± 4.8	27.4 ± 6.1	$t = 4.41$	0.121
Memory score (mean ± s.d.)	23.2 ± 1.7	19.1 ± 4.0	$t = 4.94$	< 0.0001
Total score (mean ± s.d.)	134.1 ± 7.7)	118.8 ± 12.3	$t = 5.49$	0.029

Abbreviations: HC, healthy control; LLD, Late-Life Depression; HDRS-21, 21-item Hamilton Depression Rating Scale; MMSE, Mini-Mental State Examination. Continuous data is shown as mean ± standard deviation.

system. Sequencing was performed on the Ion Proton sequencer (Life Technologies) using the Ion PI ™ Hi-Q ™ Sequencing 200 Kit, and all procedures were according to the manufacturer's instructions.

FASTQ sequencing data was obtained using Ion Torrent Suite. Sequenced single-end reads were trimmed and filtered using PRINSEQ (Schmieder and Edwards, 2011). Low-quality sequences were trimmed from the ends until a base pair of *Phred* quality score ≥20 (at least 99% accurate) was not found.

2.4. Small RNA-Seq analysis

miRDeep2 was used to align mapped reads to the human reference genome sequence hg19 via the mapper.pl script (Friedländer et al., 2012). Mature and hairpin miRNA sequences were obtained from miRBase release 21 (http://www.mirbase.org/). A collapsed reads of the mapped reads were processed by miRDeep2.pl script, and miRNA read counts were generated. The miRNA read counts (at least 1 read) were used as input into the Bioconductor DESeq v. 2.0 (DESeq2) package (Anders and Huber, 2010) for further differential expression analysis. Differential expression of LLD and HC miRNAs was based on a negative binomial distribution using adjusted $p < 0.05$ and log2 values of fold difference.

2.5. miRNA extraction and RT-qPCR

For validation analysis, miRNA was extracted from 200 µl of plasma samples (34 HC and 39 LLD) using mirVana PARIS kit (Ambion, Austin, TX/USA). Before extraction, a 200 fmol of synthetic *Caenorhabditis elegans* miRNA (cel-miR-39) was spiked into each plasma sample, according to the manufacturer's recommendations and previous studies (Farina et al., 2014; Yoon et al., 2017). Extracted miRNA was eluted in 50 µl of preheated nuclease-free water and measured on NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Waltham, MA/USA), then immediately stored at −80 °C.

TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA/USA) was used for determining miRNA levels in plasma samples for seven candidate miRNAs (hsa-miR-100-5p, hsa-miR-184, hsa-miR-1-3p, hsa-miR-140-3p, hsa-miR-5572, hsa-miR-125b-5p, hsa-let-7a-5p) based in NGS results. U6 expression and a spike-in (cel-miR-39) were used as an endogenous and exogenous reference controls, respectively. These assays target only a mature form of the specific miRNA, which ensures a biologically relevant result. Reverse transcription (RT) was performed using TaqMan miRNA RT kits according to the instructions from manufacturer's recommendations. Briefly, the cDNA was synthesized from total miRNA (10 ng) using miRNA-specific primers in a 15-µl reaction volume. The RT reaction was performed using the following thermal cycling program: 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C, and then held at 4 °C. The RT product was diluted to 100ng/µl, and 4 µl of the product was used in a total reaction volume of 20 µl for relative quantification by real-time PCR using an ABI 7500HT system (Applied Biosystems). The thermal cycling program used for the quantification was as follows: 50 °C for 2 min, 95 °C for 10 min and followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The cycle threshold (Ct) number is defined as the cycle number at which the fluorescence crossed the fixed threshold. The Ct number was calculated using the second derivative method in the ABI software.

2.6. Data analysis of RT-qPCR

TaqMan gene expression assay raw Cq values of miRNAs were imported into the qbasePLUS (Biogazelle, Ghent, Belgium) software (Hellemans et al., 2007). The same cDNA samples were used for all of

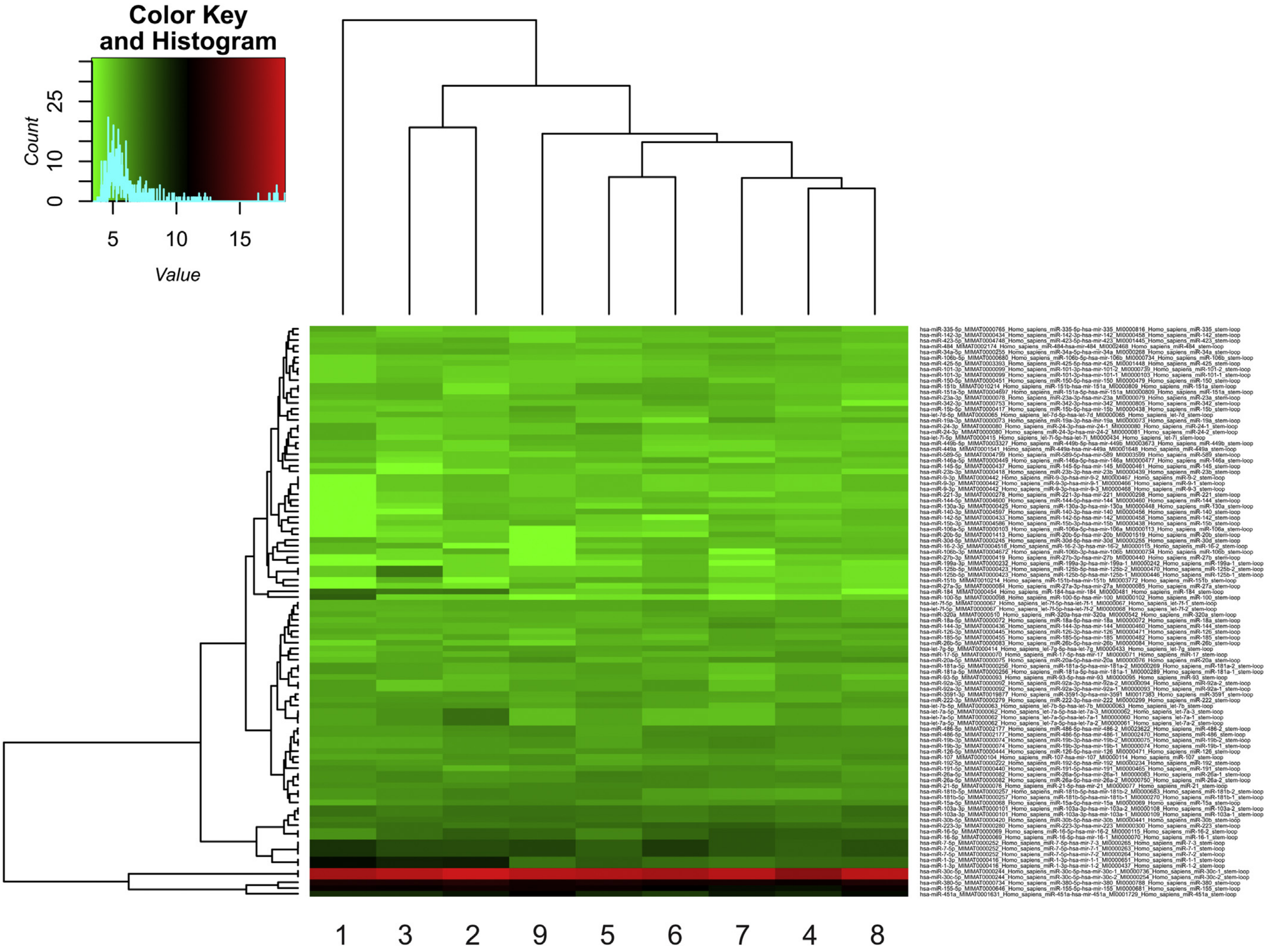

Fig. 1. NGS analysis of HC (1, 2 and 3) and LDD (4–9) pools samples: heat map of miRNAs expressed into groups.

these analyses. The qbasePLUS algorithm enabled normalization to more than one reference gene and also employed inter-run calibrator signals to minimize inter-run variability (Hellemans et al., 2007) distinguishing it from the ΔΔCt method (Livak and Schmittgen, 2001). All data were expressed as calibrated normalized relative quantities (CNRQs). GraphPad 5 (GraphPad Software, San Diego, CA/USA) was used for statistical analysis. Differences between variables were examined for statistical significance using the Student's t-test and p-values < 0.05 were considered statistically significant. The receiver operating characteristic (ROC) curve and the area under curve (AUC) were used as an accuracy index for evaluating the diagnostic performance of the selected miRNA panel.

We carried out a Student t-test to evaluate differences in socio-demographic, HDRS-21and DRS scores, and miRNAs levels between LLD and control subjects. We also carried out Pearson correlation analysis to investigate the association between miRNAs, cognitive performance, and depressive symptoms.

2.7. *Pathway enrichment analysis*

The gene set enrichment analysis was performed by using ClueGO and CluePEDIA Cytoscape plugin (Bindea et al., 2009, 2013; Shannon et al., 2003), and the Mirwalk 2.0 databases (Dweep et al., 2011) from the selected microRNA that was differently expressed in HC and LLD in NGS and RT-qPCR results. The functional enrichment of specific Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms (Kanehisa and Goto, 2000) used the right-sided hypergeometric test, followed by the Bejamini-Hochberg correction to control false-discovery rate (FDR) at a p-value of < 0.01. The GO assignments were made using the KEGG dataset released 73.0 September 2015 (Kanehisa and Goto, 2000) and the Molecular Signatures Database v5.1 (http://software.broadinstitute.org/gsea/index.jsp). A density score evaluated the significant categories (Bader and Hogue, 2003). Subsequently, we investigated the substructure of the main protein interaction network extracted from intersecting networks and focused on highly connected nodes, known as clusters, using the molecular complex detection algorithm (Bader and Hogue, 2003). A cluster is a complete n-node subgraph representing highly interconnected regions, which were obtained by using the parameter set: K-core = 4, node score cut-off = 0.3 and max depth up to 100.

2.8. *Drosophila melanogaster*

To further examine the role of miRNA expression in brain function, we evaluated the behavioral phenotypes of *Drosophila melanogaster* flies with knockout or hyper-expressing the ortholog of miRNAs that were differentially expressed in LLD. *D. melanogaster* models of human diseases provide several unique features such as powerful genetics, short life cycle, highly conserved disease pathways, and low maintenance costs. Many basic biological, physiological, and neurological properties are conserved between mammals and *D. melanogaster*, and nearly 75% of human disease-causing genes have a functional homolog in the fly

(Reiter et al., 2001). We used the elav-Gal4 and GAL4–UAS system to control the miRNA expression in the flies. Methodological details of the genetic manipulation and behavioral assays are shown in the Supplementary Material 1.

3. Results

3.1. miRNA discovery with NGS

We first examined the miRNA expression from plasma from 43 participants (24 LLD and 19 HC) using NGS. The NGS analysis generated a total of 33,264,170 raw reads which were used to construct the sRNA libraries of the HC and LLD samples. The valid reads of the LLD and HC libraries were obtained after removal of the corrupted adapter sequences, reads with length < 13 and > 26 nt and junk reads.

Using miRdeep2, which identifies known microRNAs from small RNA-seq, we identified a total of 105 circulating miRNAs in LLD and controls (Fig. 1 and Supplementary Table 1). Most of the miRNAs were expressed in both HC and LLD. Subjects with LLD showed a significant downregulation of the expression of hsa-miR-184 ($\log_2$foldchange $= -4.21$, $p = 1.2 \times 10^{-3}$) and hsa-miR-1-3p ($\log_2$foldchange $= -3.45$, $p = 1.3 \times 10^{-2}$), after adjustment for multiple analyses (false-discovery rate q-value < 0.05).

3.2. Validation of miRNA expression with RT-qPCR

We selected the two miRNAs that showed statistically significant differences in expression levels between LLD and controls (hsa-miR-184 and hsa-miR-1-3p) in NGS analysis to validate. However, the small sample size of the discovery set sample and the pooling strategy for NGS analysis can lead to a small variance in the miRNA count and, as a consequence, findings of non-statistically significant differences even for miRNA with large effect size differences (i.e., statistical error type II). To avoid the risk of false-negative results from the NGS analysis, we also included more 5 miRNAs whose *$\log_2$foldchange* was greater than 1.5 and read count of more than 10 in both groups (hsa-miR-100-5p, hsa-miR-125b-5p, hsa-miR-5572, hsa-miR-140-3p, hsa-let-7a-5p). The miRNAs specific forward primers used in RT-qPCR are shown in Supplementary Table 2.

The expression levels of the 7 miRNAs selected for validation were examined in plasma by RT-qPCR. All miRNAs were detected in our samples (Ct value < 35). Of note, cel-miR-39 and endogenous control U6 expression were stable, with no significant differences between LLD and controls.

The relative quantification of circulating miRNAs hsa-miR-184, hsa-miR-5572, hsa-miR-100-5p and hsa-miR-140-3p in plasma are shown in Fig. 2. MiRNAs hsa-miR-1-3p, hsa-miR-125b-5p, and hsa-let-7a-5p were detected in less than 30% of total samples and were not included in the statistical analysis.

The hsa-miR-184 was significantly downregulated in LLD compared to controls ($p < 0.001$) (Fig. 2). There were no statistically significant differences in the expression of hsa-miR-5572, hsa-miR-100-5p and hsa-miR-140-3p between LLD and controls. ROC curve analysis in plasma samples showed that hsa-miR-184 can discriminate HC from LLD subjects (AUC = 0.84, $p < 0.0001$) (Fig. 3A). The ROC curve analysis of the other three miRNAs was not statistically significant (Fig. 3B, C, and 3D).

Correlation analysis using the whole sample showed that hsa-miR-184 expression was significantly correlated with the severity of depressive symptoms ($r = -0.425$, $p = 0.001$), worse scores in the DRS Initiation/Perseveration item ($r = 0.292$, $p = 0.03$), and years of education ($r = 0.260$, $p = 0.04$). There were no significant correlations between hsa-miR-184 and other demographic and clinical variables.

3.3. Bioinformatics analyses

Mirwalk 2.0 dataset found 464 genes target genes of miR-184 (3'and 5′UTRs). Protein-protein interaction network build using the genes targets showed 919 nodes and edges and 64 clusters. The biological pathways and processes were mainly related to the regulation of the immune-inflammatory processes (MAP kinase, Calmodulin-kinases, NF-kappa B signaling pathway), second messenger signaling (TRP channels activation, regulation of activation of G protein in gated potassium channels calcium signaling pathway, GABA receptor activation), metabolic control (insulin signaling pathway), neurotrophic support (neurotrophic signaling pathway, VEGF signaling pathway), proteostasis and senescence-related pathways (mTOR signaling pathway) (Fig. 4 and Supplementary Tables 3 and 4).

Validation of behavioral phenotype in genetically modified *Drosophila melanogaster* knockout-expressing and over-expressing the ortholog of hsa-miR-184.

We used the negative geotaxis RING assay to evaluate the locomotor activity of the of flies. We include 3 groups of flies: ΔmiR-184 (knockout of the ortholog of hsa-miR-184), elav-Gal4 > UAS-miR-184 (over-expression of the ortholog of hsa-miR-184), and elav-Gal4 flies (parental control). We assessed locomotor activity in three different ranges of days post-eclosion (d.p.e), 0–4 d.p.e, 11–15 d.p.e and 21–25 d.p.e.

Fig. 5 shows the results for the flies' locomotor activity. We observed an age-dependent effect of miR-184 knockout expression in the flies' locomotor activity. Younger knockout flies (0–4 d.p.e.) did not show significant differences in locomotor activity, while middle-aged flies (11–15 d.p.e.) had higher locomotor activity, and older flies (21–25 d.p.e.) had significantly worse locomotor activity when compared to the control flies (Fig. 5A). Flies overexpressing the miR-184 showed a significantly higher locomotor activity in all ages when compared to control flies (Fig. 5B). As expected, flies with miR-184 knockout expression had significantly worse locomotor activity compared to flies with miR-184 overexpression (Fig. 5C).

We used the aversive odor stimulus training to investigate the effects miR-184 expression on memory retention. The flies were evaluated 6 h (short-term memory) and 24 h (long-term memory) after the aversive odor stimulus.

Flies knockout of miR-184 showed worse short-term and long-term memory compared to control flies in an age-dependent fashion (Fig. 6A). The flies overexpressing miR-184 showed better short and long-term memories in the 11–15 d.p.e. group when compared to control flies (Fig. 6B). Finally, flies knockout of miR-184 showed significantly worse short and long-term memories compared to flies overexpressing miR-184 at all d.p.e. ranges. (Fig. 6C).

Together these data from genetically modified *Drosophila melanogaster* model suggest that the knockout of the ortholog of hsa-miR-184 had a depressive-like behavioral phenotype, i.e., reduced locomotor activity and worse memory performance. Conversely, the over-expression of the miR-184 significantly reversed these phenotypes.

4. Discussion

Our study provides the first NGS analysis, with independent validation, of whole-genome circulating microRNA expression in LLD. We showed that miR-184 was significantly downregulated in LLD, and significantly correlated with the severity of depressive symptoms and executive dysfunction in this sample. Our results are in line with a previous study showing the downregulation of miR-184 in the anterior cingulate cortex (post-mortem brain tissue) of a small sample of middle-aged adults with major depression (Azevedo et al., 2016). We further biologically validated the role of miR-184 to induce behavioral changes in flies. We found that miR-184 knockout flies had less locomotor activity and worse memory capacity that were more intense at older flies. The over-expression of miR-184 reversed such phenotype changes. Our results suggest that the down-regulation of miR-184 is a potential

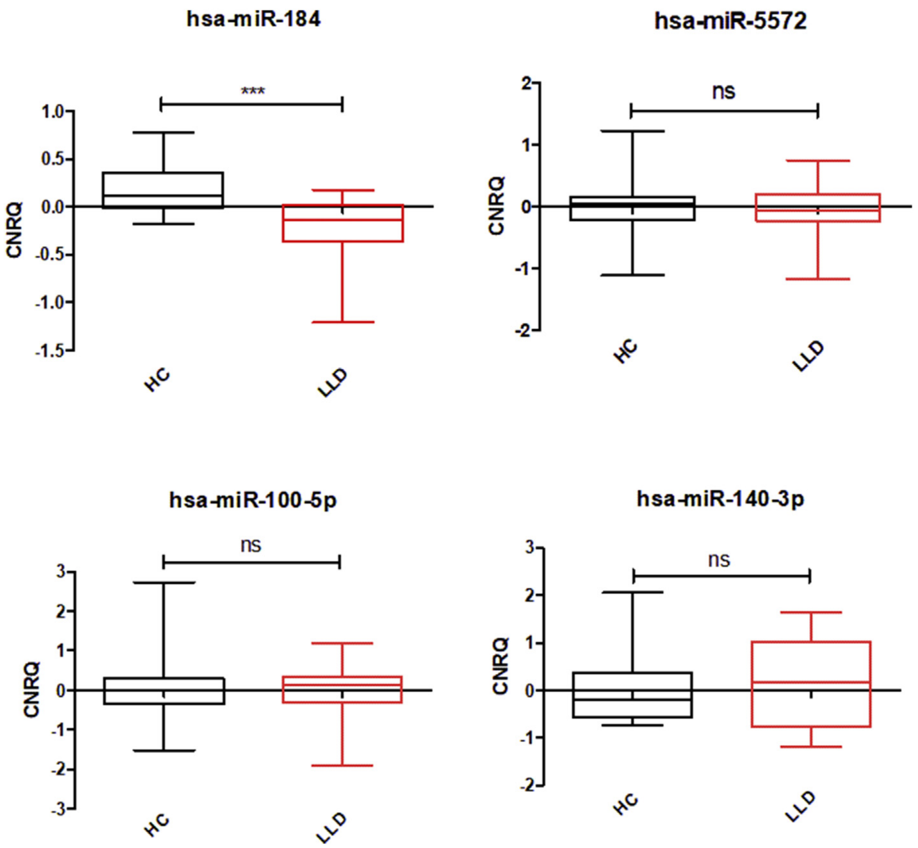

Fig. 2. Relative quantification of circulating miRNAs when comparing HC and LLD subjects determined by the Mann–Whitney *U* test.

mechanism for the development of depressive symptoms and cognitive impairment in older adults.

The miR-184 is most abundantly expressed in the brain[52,53,] and our study is the first to identify changes in expression in LLD. Enrichment pathway analysis showed that this miRNA is involved in the control of immune-inflammatory cascades, maintenance of neuronal structural integrity and plasticity, metabolic control, proteostasis and senescence regulation. Abnormalities in these cascades have been previously

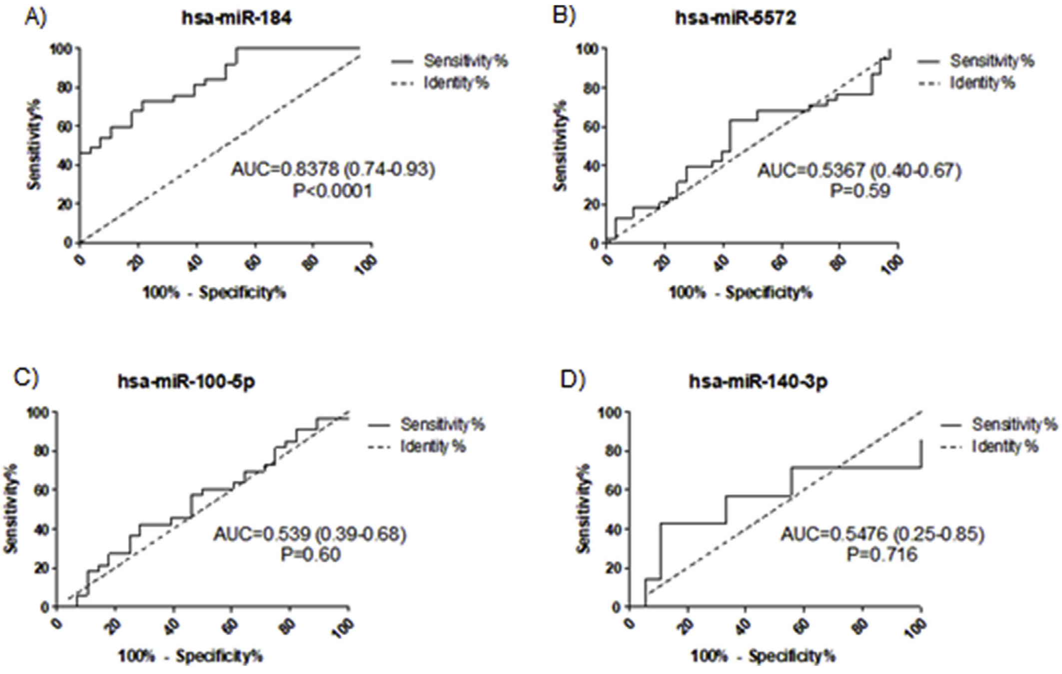

Fig. 3. ROC curve analysis to discriminate late-life depression (LLD) cases from normal controls enrolled in the validation set of 4 miRNAs. **A)** hsa-miR-184 ROC curve separates 39 LLD cases from 34 controls (AUC = 0,8373 and p-value < 0,0001), **B, C and D)** hsa-miR-5572, hsa-miR-100-5p and hsa-miR-140-3p ROC curves do not separate 39 LLD cases from 34 controls.

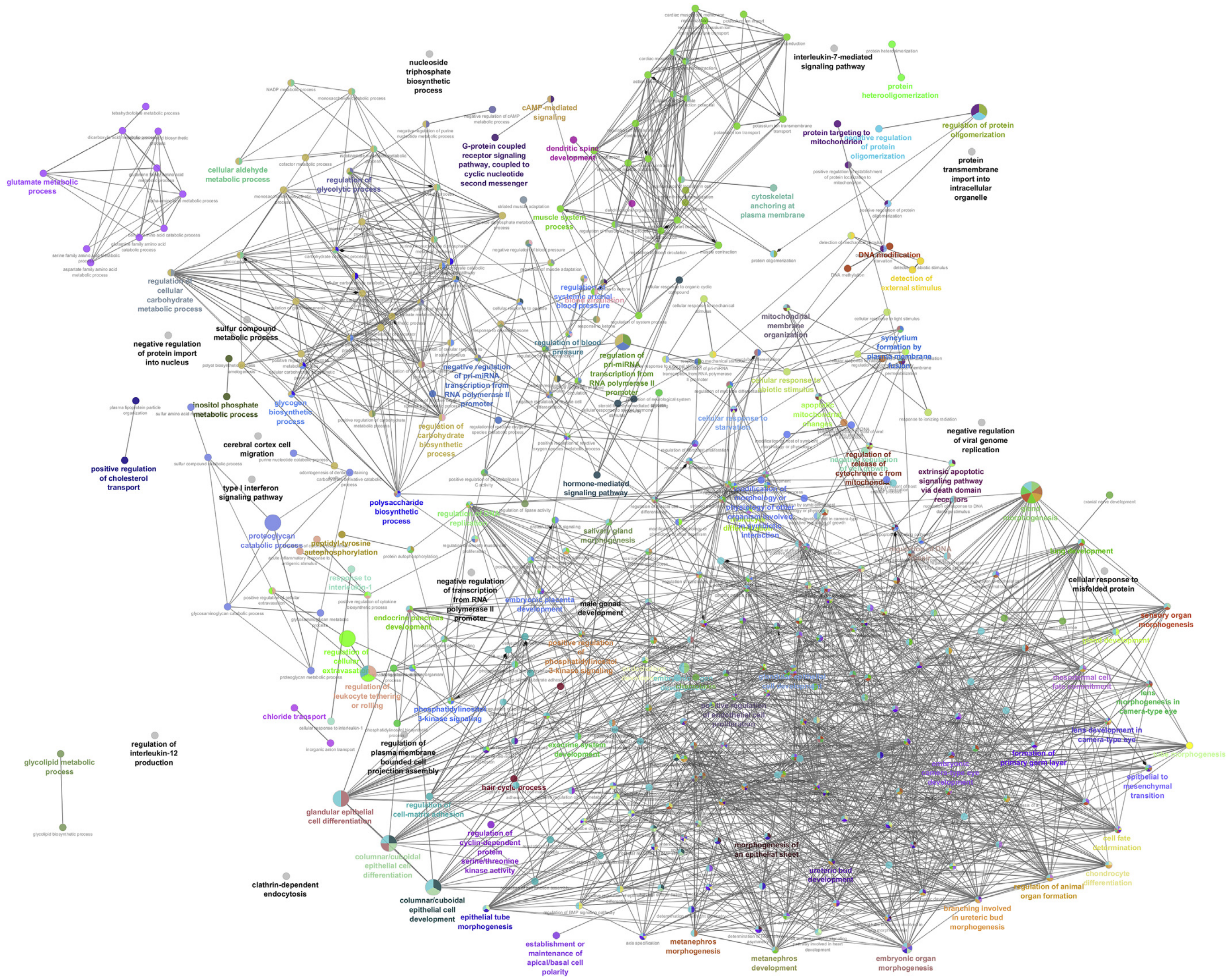

Fig. 4. Pathway network for the predicted target genes of differentially expressed miRNA-184 (p-value < 0.05). Each node represents one protein.

described in subjects with LLD using proteomics approach. For instance, our group and others have shown that individuals with LLD have greater activation of pro-inflammatory cascades, proteostasis dysregulation, reduced neurotrophic support and enhanced senescence molecular changes (Diniz et al., 2016, 2017). Moreover, changes in these biological cascades are more intense in those subjects with LLD and comorbid cognitive impairment (Diniz et al., 2015). Interestingly, in our study, the miR-184 was significantly associated with the severity of depressive symptoms and executive dysfunction, a common feature of LLD. Thus, the downregulation of miR-184 may be a common mechanism underlying the severity of depressive symptoms and cognitive impairment in older adults. Moreover, it can be a potential therapeutic target for both conditions.

Based on the findings from subjects with LLD, we used genetically modified model *Drosophila melanogaster* to further evaluate the impact of miR-184 expression on brain function and phenotype. *Drosophila melanogaster* is a powerful translational model to evaluate the effect of gene modification on biological functions, physiological and behavioral changes related. The fly shares around 75% of gene homology with humans, its maintenance is low cost and has a short lifespan. We used a well-established genetic manipulation tool (the *GAL4-UAS system*) to provide spatial control of miR-184 expression in central nervous system (Reiter et al., 2001). *Drosophila* with knockout miR-184 showed a significant reduction in the locomotor activity and worse learning and memory. These changes were more intense in older flies (21–25 d.p.e.) compared to younger counterparts. On the other hand, the overexpression of miR-184 significantly improved locomotor activity, learning and memory capacity of the flies, even at later d.p.e. Therefore, we provide robust evidence, from a translational perspective, that the downregulation of miR-184 is a biological mechanism that contributes to the development of depression, especially at older ages. Moreover, we also demonstrate that increasing the miR-184 expression can reverse the abnormal behaviors of flies.

Major depression is a biologically heterogeneous condition throughout the lifespan. Previous studies including younger subjects with major depression have shown that miR-124, miR-1202, let-7 family and other miRNAs were significantly downregulated or upregulated during depressive episodes (Azevedo et al., 2016; Bocchio-Chiavetto et al., 2013; Fiori et al., 2017; Lopez et al., 2014). In contrast, our study did not identify such miRNAs as differentially expressed in LLD. One possible explanation is that the pathophysiological mechanisms of major depression are distinct across different ages. Therefore, we can hypothesize that the biological processes of aging interact and moderate the pathophysiological changes of major depression (age*-disease interaction)[55]. Likewise, our results suggest that the biological mechanisms of major depression are age-dependent what can help to explain differences observed in long-term prognosis and treatment response in young, middle-aged and older adults. These evidence of

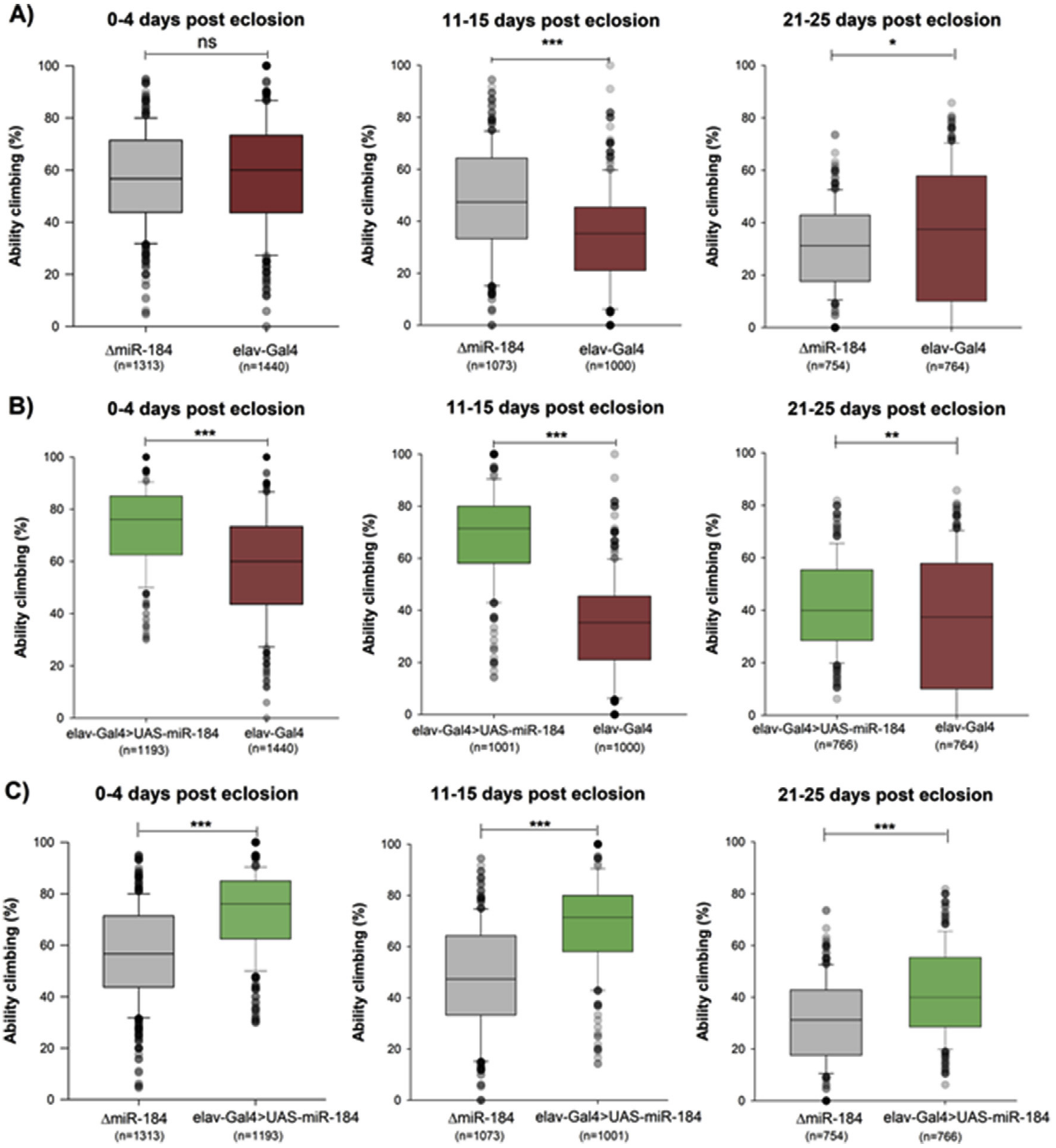

Fig. 5. Climbing ability in fruit flies. Results of the climbing ability (%) of fruit flies with different age ranges (measured in days post eclosion. **A)** Comparison of climbing scores between ΔmiR-184 and control flies elav-Gal4. There was no significant difference found in 0–4 d.p.e, but ΔmiR-184 showed better climbing scores in flies with 11–15 d.p.e ($p < 0.0001$) and worst climbing scores in flies with 21–25 d.p.e ($p = 0.04$), **B)** Comparison of climbing scores between elav-Gal4 > UAS-mir-184 and control flies elav-Gal4. Elav-Gal4 > UAS-mir-184 showed better climbing scores at all age ranges (0–4 and 11–15, $p < 0,0001$; and 21–25 d.p.e, $p = 0,0061$).**C)** Knockout flies (ΔmiR-184) showed worst climbing scores when compared to elav-Gal4 > UAS-mir-184 at all age ranges (0–4, 11–15 and 21–25 d.p.e, $p < 0,0001$).

age*disease interaction have significant implications not only for the understanding of the pathophysiological mechanisms of major depression but to the development of therapeutic interventions tailored to different ages. Nonetheless, the cross-sectional design of our study does not allow to address these hypotheses properly. Longitudinal studies with a lifespan approach are, thereof, necessary to confirm or refute these hypotheses.

Our results should be viewed in light of some limitations. We evaluated the miRNA expression in plasma, and there is a long-lasting debate in the literature whether biomarkers measured in the periphery can reliably reflect changes in the central nervous system (CNS). An important characteristic of the aging brain is the blood-brain barrier (BBB) deterioration and its increased permeability to systemic factors (Cai et al., 2017; Montagne et al., 2015) that is potentiated in older adults with a major depressive episode (Najjar et al., 2013). The BBB deterioration allows an influx and efflux of humoral factors, including microRNAs, from the CNS to the peripheral circulation (e.g., blood), and vice-versa. Also, the miR-184 is mostly expressed in the brain and not in peripheral tissues indicating that the miR-184 identified in the brain is mostly, if not all, from brain tissue. Our study included only older adults with major depression recruited at a specialty center. Our results, thus, may not be generalized to other age groups or the general elderly population. Finally, the cross-sectional design does not allow to make causal inferences about the role of miR-184 downregulation and major depression in the elderly. However, our findings that knockout flies for miR-184 also have depressive-like behaviors that are reversed with overexpression of this miRNA offers strong evidence that the downregulation of miR-184 is a contributory mechanism for the

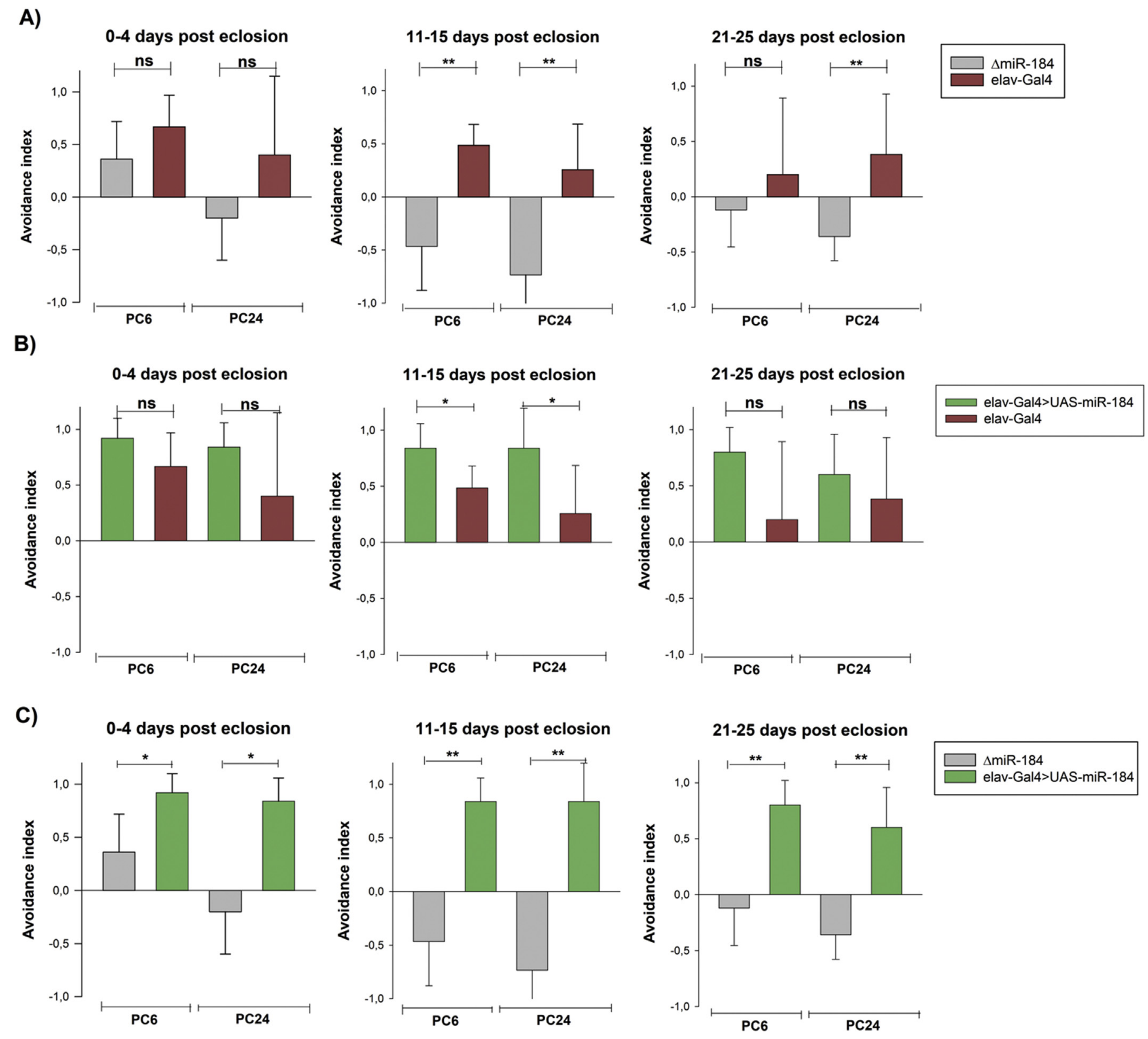

Fig. 6. Avoidance test in fruit flies. Avoidance index 6 (PC$_6$) and 24 (PC$_{24}$) hours post conditioning at aversive odor stimulus calculated in three different ranges of days post eclosion (0–4, 11–15 and 21–25). **A)** Δmir-184 showed worst PC$_6$ when compared to control elav-Gal4 (11–15 d.p.e, *p-value* = 0.0029) and worst PC$_{24}$ when compared to elav-Gal4 (11–21 with *p-value* = 0.0048 and 21–25 d.p.e with *p-value* = 0.0211). **B)** elav-Gal4 > UAS-miR-184 showed best PC$_6$ (*p-value* = 0.0284) and PC$_{24}$ (*p-value* = 0.0484) when compared to control elav-Gal4 in flies with 11–15 d.p.e and no significant differences were found in PC$_6$ and PC$_{24}$ between two groups in flies with 0–4 and 21–25 d.p.e **C)** Comparison between Δmir-184 and elav-Gal4 > UAS-miR-184 showed worse avoidance index in knockout flies at 6 and 24 h post conditioning to aversive odor stimulus in 0–4 d.p.e (PC$_6$ with *p-value* = 0.0236 and PC$_{24}$ with *p-value* = 0.0117), 11–15 d.p.e (PC$_6$ with *p-value* = 0.0066 and PC$_{24}$ with *p-value* = 0.0074) and 21–25 d.p.e (PC$_6$ with *p-value* = 0.0078 and PC$_{24}$ with *p-value* = 0.0074).

development of major depressive disorder in the elderly. Our sample included a numerically higher amount of female than male subjects in both discovery and validation set. Despite these differences in the sex frequency were not statistically significant, we cannot exclude that our results might have been biased by the skewed distribution of sex in our study sample. Finally, the absence of neuroimaging evaluation does not allow to evaluate the impact of changes in miR-184 expression on brain structure and function.

In conclusion, we found significant downregulation of miR-184 expression in LLD. The downregulation of miR-184 was significantly associated with the severity of depressive symptoms and cognitive dysfunction in these individuals. These findings were mirrored in ortholog of miR-184 knockout *Drosophila melanogaster* flies that showed behaviors of slower psychomotor activity and worse memory. Our findings suggest the role of downregulation of miR-184 as a mechanism that contributes to the development of depression and cognitive impairment in older adults.

Funding sources

This work was funded with grant from the National Council for Research (CNPq 466623/2014-3). Dr. Diniz also receives research support from the NIH (1R03MH115212-01 and 1R01MH114970-01).

Disclosures

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpsychires.2019.01.019.

microRNA	Expression level		
	HC	LLD	p-value
hsa-miR-184	0.16 ± 0.24	−0.19 ± 0.29	< 0.0001
hsa-miR-5572	0.0001 ± 0.38	−0.06 ± 0.40	0.5999
hsa-miR-100-5p	0.0268 ± 0.87	0.0063 ± 0.59	0.6074
hsa-miR-140-3p	0.0068 ± 0.74	0.2030 ± 1.04	0.7392

Abbreviations: HC, healthy control; LLD, late-life depression; ns, non-significant, ***, p-value < 0.0001. Expression levels measure as the calibrated normalized relative quantities (CNRQ). Data shown as mean ± standard deviation.

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