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## MicroRNA expression patterns in human anterior cingulate and motor cortex: a study of dementia with Lewy bodies cases and controls

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### Abstract

**Overview**—MicroRNAs (miRNAs) have been implicated in neurodegenerative diseases including Parkinson’s disease and Alzheimer’s disease (AD). Here, we evaluated the expression of miRNAs in anterior cingulate (AC; Brodmann area [BA] 24) and primary motor (MO; BA 4) cortical tissue from aged human brains in the University of Kentucky AD Center autopsy cohort, with a focus on dementia with Lewy bodies (DLB).

**Methods**—RNA was isolated from gray matter of brain samples with pathology-defined DLB, AD, AD+DLB, and low-pathology controls, with n=52 cases initially included (n=23 with DLB), all with low (<4hrs) postmortem intervals. RNA was profiled using Exiqon miRNA microarrays. Quantitative PCR for post-hoc replication was performed on separate cases (n=6 controls) and included RNA isolated from gray matter of MO, AC, primary somatosensory (BA 3), and dorsolateral prefrontal (BA 9) cortical regions.

**Results**—The miRNA expression patterns differed substantially according to anatomic location: of the relatively highly-expressed miRNAs, 150/481 (31%) showed expression that was different between AC versus MO (at p<0.05 following correction for multiple comparisons), most (79%) with higher expression in MO. A subset of these results were confirmed in qPCR validation focusing on miR-7, miR-153, miR-133b, miR-137, and miR-34a. No significant variation in miRNA expression was detected in association with either neuropathology or sex after correction for multiple comparisons.

**Conclusion**—A subset of miRNAs (some previously associated with  $\alpha$ -synucleinopathy and/or directly targeting  $\alpha$ -synuclein mRNA) were differentially expressed in AC and MO, which may help explain why these brain regions show differences in vulnerability to Lewy body pathology.

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## Keywords

Synuclein; noncoding; epigenetic; neuropathology; neuroanatomy; gender; miR-15/107; miR-107; miR-15; miR-16; miR-133; brain; sensory; frontal

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## Introduction

Neurodegenerative diseases (NDs) are devastating brain disorders with an enormous impact on public health. Among the prevalent NDs are Alzheimer's disease (AD) and dementia with Lewy bodies (DLB), and "gold-standard" diagnoses can only be made with certainty at autopsy (Beach et al., 2012; Nelson et al., 2009b; Nelson et al., 2010a). According to consensus-based recommendations, DLB pathology includes aberrant cerebral cortical deposits of insoluble  $\alpha$ -synuclein/Lewy bodies (McKeith et al., 2004; McKeith et al., 2017). The prevalence of Lewy body pathology is 10%–30% in most series of dementia patients (Heidebrink, 2002; Neltner et al., 2016; Rahkonen et al., 2003; Sonnen et al., 2009; Zaccai et al., 2005). It is relatively challenging to study "pure" DLB cases since most persons with cortical Lewy body pathology also have comorbid AD pathology (Irwin et al., 2017; Nelson et al., 2009b; Nelson et al., 2010a; Nelson et al., 2010b). Notably, focused duplication of the gene (*SNCA*) that encodes  $\alpha$ -synuclein is enough to produce  $\alpha$ -synuclein/Lewy body pathology (Gwinn et al., 2011; Nishioka et al., 2006; Singleton et al., 2003), so, other gene regulatory factor(s) that cause increased  $\alpha$ -synuclein expression could potentiate the pathology.

An enigmatic characteristic of NDs is that they tend to affect specific brain areas initially, and then progress to additional brain regions in a predictable sequence. Symptoms reflect the spatiotemporal progression. In DLB, Lewy bodies are observed in the anterior cingulate gyrus (Brodmann Area [BA] 24; AC) early in the disease (Thal et al., 2004). By contrast, the primary motor cortex (BA 4; MO) and primary somatosensory (BA 3) neocortical regions are relatively resistant to pathology (Thal et al., 2004).

Brain region-specific gene expression may contribute to the initiation and/or propagation of pathology in some parts of the brain, while causing other anatomically-defined brain areas to be less vulnerable. Testing this hypothesis requires factoring in gene regulatory phenomena in addition to mRNA levels, because mRNA is an imperfect proxy for protein levels, and transcription is only one among many different nodes of gene expression regulation in the human brain (Nelson and Keller, 2007). Thus, new information about factors that affect gene regulation post-transcriptionally may be helpful to achieve better understanding of ND pathogenesis.

MicroRNAs (miRNAs) are short (~22 nucleotides in length) noncoding RNAs that have been implicated in NDs (Liu et al., 2008; Nelson et al., 2008a). Encoded by small genes that can be located practically anywhere in the genome (Londin et al., 2015), miRNAs regulate gene expression in multiple ways – predominantly by interacting with mRNA "targets" post-transcriptionally – and each miRNA has the potential to regulate dozens or even hundreds of targeted mRNAs (Kiriakidou et al., 2004; Liu et al., 2008; Wang et al., 2010). Relative to protein-coding genes, the human brain miRNA repertoire is smaller, with under 1000

moderately- or highly-expressed brain transcripts (Hebert and Nelson, 2012). However, individual miRNAs can be expressed at up to an order of magnitude higher transcript copies than highly expressed mRNAs (Hebert and Nelson, 2012). Furthermore, individual miRNAs have previously been implicated in AD and Lewy body pathology (Hebert and De Strooper, 2009; Nelson et al., 2008a; Pietrzak et al., 2016; Ubhi et al., 2014; Weinberg et al., 2015).

Fundamental characteristics of any miRNA include the cells it is expressed in and the factors that are associated with expression variance. For this reason, miRNA expression profiling is an important tool for understanding miRNA biology in health and in disease states.

Although there have been prior expression profiling studies of miRNAs in human tissues, many brain areas have not been assessed thoroughly to date, and there are many extant unanswered questions. For example, the miRNA profiles in AC and MO regions have not been compared with each other and the relevance of miRNAs to DLB is unknown.

To address these issues, we evaluated the expression of miRNAs in well-characterized human brains. We focused specifically on AC and MO tissue from aged human brains that had been snap-frozen in liquid nitrogen at autopsy from the University of Kentucky Alzheimer's Disease Center (UK-ADC) cohort. We were interested in testing whether the miRNA expression patterns varied according to anatomic region, sex, and/or neuropathology.

## Results

Human brain samples were obtained at autopsies after short post-mortem interval (PMI), followed by dissection of gray matter for RNA isolation and miRNA expression profiling. The rationale for the areas of brain chosen for miRNA analyses is depicted in Fig. 1. Some aspects of RNA quality are depicted in Fig. 2. Patient characteristics, including clinical and pathological parameters, and PMI, are shown in summary form in Table 1. Provided in supplemental material are the following additional data: case-by-case clinical, pathological, PMI, and RNA Integrity Number (RIN) data (Supplemental Table 1). Note that a total of 7 microarrays failed QC due to RIN values <4.0. We also note that, as we have reported previously, "pure DLB" is relatively rare and predominantly seen in males (Nelson et al., 2009b; Nelson et al., 2010a; Nelson et al., 2010b), whereas most cases with DLB pathology have concomitant AD pathology (Irwin et al., 2017). Thus, a group of cases with AD pathology but no Lewy bodies was used as a control cohort. The total number of tested subjects with DLB pathology was 23, of which 16 had substantial comorbid AD pathology.

All the miRNA expression data for each case are provided in Supplemental Tables 2 and 3. All of the values of miRNA expression depicted in Tables 2–5 were the result of a statistical approach that enabled the use of microarray data from the two rounds of evaluations, as described in the Methods section. A total of 481 relatively high-expressing miRNAs met the inclusion criterion ( $\log_2$  expression >7 in at least one data set) and were the basis of further analyses.

### MiRNAs differentially expressed by brain area

Even after correcting for multiple comparisons, 150 of the relatively highly-expressed miRNAs were detected at different levels in the MO and AC samples across all conditions and microarray iterations. Of these, 119 (79%) were higher expressed in MO (versus AC), whereas 31 (21%) were higher expressed in AC. Table 2 shows the 10 miRNAs most highly expressed in MO and the 10 miRNAs most highly expressed in AC in terms of the statistical comparison. All of the miRNAs found to be differentially expressed at a  $p < 0.05$  level after correction for multiple comparisons are listed in Supplemental Table 4.

### MiRNAs with variation that is associated with sex and DLB pathology

After correcting for multiple comparisons to decrease likelihood of false discovery, there was no miRNA that showed significant differences associated with either sex or pathology-confirmed DLB status. Shown in Table 3 are the miRNAs showing nominal association with sex or DLB status ( $p < 0.05$ , but not corrected for multiple comparisons).

### MiR-15/107 family

Members of the miR-15/107 family of miRNAs, which share regulatory targets (Finnerty et al., 2010), have been linked to both AD pathology and to regulation of  $\alpha$ -synuclein and/or synucleinopathy (Alderman and Yang, 2016; Gui et al., 2015; Li et al., 2014b; Liu et al., 2014; Liu et al., 2016; Moncini et al., 2016; Nelson and Wang, 2010; Parsi et al., 2015; Wang et al., 2008b; Yao et al., 2010; Zhang and Cheng, 2014). Some of the miR-15/107 miRNAs also are enriched in brain (Wang et al., 2014). Shown on Table 4 are microarray values of members of the miR-15/107 family: miR-15a, miR-15b, miR-16, miR-103, miR-107, miR-424, miR-497, miR-503, and miR-646. None of these miRNAs showed an association with sex and DLB pathology that was statistically significant. However, three of the four highest-expressing miR-15/107 family genes in the current sample – hsa-miR-15a-5p, hsa-miR-15b-5p, and hsa-miR-16-5p – were expressed at higher levels in MO than AC. MiR-107 showed a trend for lower expression in association with AD pathology (not shown), as expected (Nelson and Wang, 2010; Wang et al., 2008b).

### Other miRNAs previously implicated in $\alpha$ -synucleinopathy

Although we found that a subset of miRNAs were differentially expressed in the AC and the MO (see Table 2), these altered miRNAs were different than those previously implicated in  $\alpha$ -synucleinopathy: miR-7, miR-153, miR-1643, miR-34b, miR-34c, miR-214, miR-26b, miR-320, miR-20, miR-128, miR-133, miR-433, miR-205, miR-224, miR-301b, and miR-373 (Choi et al., 2014; de Mena et al., 2010; Doxakis, 2010; Fragkouli and Doxakis, 2014; Janeczek and Lewohl, 2013; Junn et al., 2009; Kabaria et al., 2015; Kim et al., 2013; Kong et al., 2015; Li et al., 2014a; Ma et al., 2013; Majidinia et al., 2016; Mouradian, 2012; Niu et al., 2016; Patil et al., 2015; Recasens et al., 2016; Tagliafierro et al., 2017; Wang et al., 2008a; Wang et al., 2015; Xie and Chen, 2016; Zhou et al., 2016). Results for these miRNAs are shown in Table 5. None of these miRNAs showed association with sex or pathology that was statistically significant. However, a subset of these – hsa-miR-133a/b-3p, hsa-miR-34c-5p, hsa-miR-20a-5p, hsa-miR-7-5p, hsa-miR-34b-5p, hsa-miR-153-3p, hsa-miR-320a, hsa-miR-7-2-3p – were expressed differentially in the AC and MO regions.

## Replication (qPCR) experiment for miRNA expression variance associated with anatomical areas

Quantitative PCR for post-hoc replication was performed on six low-pathology control cases (Fig. 3). As these experiments were performed for the purpose of replication, none of these cases were among those used in the microarray experiments. In addition to evaluating RNA from MO and AC in these cases, we also analyzed RNA from primary somatosensory and dorsolateral frontal cortical regions. Average age of death of the six individuals was 80.5 years, average PMI was 3.5 hrs (See summary data on the six patients in Supplemental Table 5). Tested with TaqMan single-tube miRNA RT-qPCR were the following miRNAs: miR-133b, miR-137, miR-153, miR-34a, and miR-7. As was found with the microarrays, miR-133b and miR-34a were expressed higher in the MO than AC, while miR-7 and miR-137 were expressed at lower level in MO than AC. Intriguingly, and extending the results from the microarrays to study new brain areas, primary sensory cortex (BA 3) also had relatively high miR-133b and miR-34a and low miR-7 and miR-137. By contrast, frontal cortex (BA9) had relatively low miR-133b and miR-34a and high miR-7 and miR-137. Some of the specific predicted miRNA recognition sites on  $\alpha$ -synuclein mRNA (with miRNAs that are expressed at relatively low levels in AC) are shown in Fig. 4. A schematic depiction of the overall hypotheses tested and our interpretation of the data are presented in Fig. 5.

## Discussion

The purpose of the current study was to test whether variation in miRNA levels detected in human brain tissue was associated strongly with any of the following: DLB pathology; sex; or neuroanatomical region (AC vs MO). The hypotheses that were being tested, and our interpretation of the results, are depicted in Fig. 5. Of these factors, we only found evidence of the anatomical region being associated strongly with variation in miRNA expression: many miRNAs were differentially expressed in AC and MO. For example, miR-133b and miR-34a were expressed at relatively high levels in primary motor and somatosensory cortices, whereas miR-7 and miR-137 were expressed at higher levels in AC and frontal cortex. These data are compatible with the hypothesis that brain areas with lower vulnerability to Lewy body pathology express higher miR-133b and miR-34a, but lower levels of miR-137 and miR-7.

This study had technical and theoretical limitations. Whereas the miRNA microarray results were referred to as a proxy for “miRNA expression”, there are other factors, including miRNA stability/degradation (Sethi and Lukiw, 2009) and miRNA expression platform-related idiosyncrasies, that influenced our results. Some of the experimental conditions that affected RNA stability were at least partly mitigated by the short PMI (<4hrs), and the samples having been snap-frozen in liquid nitrogen at autopsy. However, the presence of purely technical variation introduced by the profiling platform is underscored by the different results from the two iterations of the miRNA microarray, and we have seen even greater variation between different platforms previously (Nelson et al., 2008b; Wang et al., 2008c). Microarrays, like PCR-based and “deep sequencing” methods, each have biases (Hebert and Nelson, 2012). For these reasons, we posit that there is no true gold-standard

miRNA expression profiling method and the different methods must be used to complement each other, as in the current study using qPCR to validate and replicate the microarray results. Although our study represents one of the larger miRNA microarray studies to date focused on human brain, we did not have a sample size appropriate for evaluating subtle, or context-specific, associations between tested potential dependent variables and miRNA expression. This problem is exacerbated because we are testing multiple parameters and also many different miRNAs, so that substantial statistical corrections were required to account for the multiple tests.

MiRNAs have been previously linked to neurodegeneration (Delay and Hebert, 2011; Hebert and De Strooper, 2007; Nelson et al., 2008a; Rademakers et al., 2008) and implicated in  $\alpha$ -synucleinopathic conditions (Asikainen et al., 2010; Kim et al., 2007; Lim et al., 2010; Minones-Moyano et al., 2011; Schonrock et al., 2010). This is one of the first studies published to date that has focused on DLB and miRNA expression (see (Hebert et al., 2013)). To date there is no miRNA that is specifically linked to DLB as far as we know. Correspondingly, we did not discover a strong association between DLB pathology and any particular miRNA's expression. However, the finding of region-specific miRNA pattern may be clinically relevant to DLB and other diseases.

Lewy body pathology apparently spreads through the brain in a manner that is predictable; the AC is affected in relatively early stages of DLB, whereas the MO is generally not affected until late stages (Dickson et al., 2009; Toledo et al., 2016). This spatiotemporal sequence is inferred by cross-sectional (autopsy) studies and awaits confirmation until suitable biomarkers can be performed in longitudinal studies of living persons. The reason(s) for this common pattern of disease progression are unknown.

MiRNAs have been identified as “master regulators” of gene expression (Garofalo and Croce, 2011; Leung and Sharp, 2006) and so, theoretically, the different expressions of miRNAs in AC and MO could possibly be a factor underlying the differential vulnerability of these brain regions to Lewy body pathology. If this were true, then, the impact of a given miRNA, and a given brain area, could differ dramatically according to the circumstances—for example, whereas the MO is affected late in DLB, the same cortical region may be affected relatively early in persons showing clinical and pathological features associated with the ALS/FTLD disease spectrum (Brettschneider et al., 2013).

Prior studies of gene expression in human brains can be correlated with our results. Particular miRNAs have been shown to be potential regulators of  $\alpha$ -synuclein expression in the brain (Choi et al., 2014; de Mena et al., 2010; Doxakis, 2010; Fragkouli and Doxakis, 2014; Janeczek and Lewohl, 2013; Junn et al., 2009; Kabaria et al., 2015; Kim et al., 2013; Kong et al., 2015; Li et al., 2014a; Ma et al., 2013; Majidinia et al., 2016; Mouradian, 2012; Niu et al., 2016; Patil et al., 2015; Recasens et al., 2016; Tagliafierro et al., 2017; Wang et al., 2008a; Wang et al., 2015; Xie and Chen, 2016; Zhou et al., 2016). Among these, the following miRNAs were shown to be differentially expressed in MO and AC in the current study: hsa-miR-133a/b-3p, hsa-miR-34c-5p, hsa-miR-20a-5p, hsa-miR-7-5p, hsa-miR-34b-5p, hsa-miR-153-3p, hsa-miR-320a, and hsa-miR-7-2-3p. Using another approach, Pietrzak et al assessed RNA expression in the AC from eight DLB subjects and 10 controls,

and then applied bioinformatics to identify potential miRNA binding sites enriched in mRNAs (Pietrzak et al., 2016). This method yielded a list of candidate miRNAs that may participate in DLB pathogenesis. Among the 10 miRNA candidates identified (Pietrzak et al., 2016) – miR-25, miR-124, miR-506, miR-363, miR-82, miR-26a, miR-367, miR-1297, miR-140, miR-936 – we only found evidence that one (miR-25) was differentially expressed in AC and MO areas.

We reported previously that males are approximately three times more likely than females to die with relatively ‘pure’ neocortical Lewy body pathology (Nelson et al., 2010b). We also showed that clinicians’ knowledge of this sex-linked dimorphism in Lewy body pathology would improve clinical diagnostic accuracy (Nelson et al., 2010b). Smaller prior studies showed the same trend – males have higher DLB/cortical Lewy body risk (Barker et al., 2002; Fujishiro et al., 2008; Hishikawa et al., 2003; Kraybill et al., 2005; Weiner et al., 1996) and also higher Parkinson’s disease risk (Shulman and Bhat, 2006; Shulman, 2007). MiRNA expression is one possible explanation for sex differences; sexually dimorphic and sex hormone-regulated miRNA expression have been described (Bannister et al., 2009; Bizuayehu et al., 2012; Cutting et al., 2012; Jalava et al., 2012; Murata et al., 2010; Zhou et al., 2011) but these phenomena were previously not studied in human brain. In the present study, we could not find compelling evidence for sexually dimorphic miRNA expression in either AC or MO regions.

In conclusion, the current study was designed to detect large effect size phenomena related to variation in miRNA expression in human AC and MO. We analyzed relatively high-quality RNA derived from well characterized human brains. Using both miRNA microarrays and post-hoc studies with qPCR, we identified miRNAs that are differentially expressed – across sex and disease states – between AC and MO, and may represent part of the reason for the differential vulnerability of those two brain regions to brain pathology. It is hoped that the data we are providing with this manuscript will enable other researchers to both generate and test hypotheses related to the roles of brain miRNAs in health and disease.

## Methods and materials

### RNA isolation from a human cerebral cortex

Samples were derived from short-PMI autopsies, representing different NDs as defined according to pathology. All methods were in compliance with a University of Kentucky IRB protocol. Premortem clinical evaluations and pathological assessments were as described previously (Nelson et al., 2007; Schmitt et al., 2000; Wang et al., 2008c). Tissue used for pathologic evaluation was dissected from MO and AC (immediately adjacent to the tissues sampled for RNA studies), immersion-fixed in formalin and evaluated for AD- and DLB-type pathology using conventional methods (Nelson et al., 2009a). None of these cases has ever been evaluated previously using a microarray or any other high-throughput profiling method. The inclusion criteria that were applied for this convenience sample of DLB brains: PMI <4hrs; no evidence of frontotemporal dementia; no cancer in the brain parenchyma; and no large infarctions in the brain, or microinfarcts found within 3cm of the brain tissue samples. For broader inclusion/exclusion criteria of this autopsy cohort, see (Schmitt et al., 2012). Criteria of operationalizing AD was Braak NFT stage>III with A $\beta$  plaques more than

“Sparse”, whereas controls had Braak NFT stages <IV and had intact cognition in their last clinical examination.

RNA was isolated as previously described in detail (Wang et al., 2008b; Wang et al., 2008c). Briefly, RNA was extracted from the gray matter of snap-frozen cerebral cortical brain tissue. All biochemical analyses were performed blind with respect to patient information. Tissue (0.5–2 gms) that had been snap-frozen in liquid nitrogen and then transferred to a –80° C freezer was thawed in ice-cold isotonic lysis buffer with RNasin (Promega, Madison, WI; 250 U/ml) and Complete protease inhibitor pills (Roche, Basel Switzerland). Prior to RNA extraction (as the tissue thawed), meninges and large blood vessels were removed and gray matter was carefully dissected away from white matter. Trizol LS (Invitrogen, Carlsbad, CA) was used according to manufacturer’s instructions, except an additional overnight –20°C precipitation step was included during isopropanol precipitation. RNA quality was confirmed using A260/A280 readings and also the Agilent Bioanalyzer for RIN.

### Microarray methods

The quality of the total RNA after rethawing was again verified by Exiqon as part of the quality control process, using an Agilent 2100 Bioanalyzer profile. Two different batches of microarrays were analyzed, the first using the 6th generation (Product #208310) Exiqon miRNA microarray, miRBase version 17, and second round performed with 7th generation (Product #208500), miRBase version 20. Briefly, 1 µg total RNA from sample and reference was labeled with Hy3™ and Hy5™ fluorescent label, respectively, using the miRCURY™ LNA Array power labeling kit (Exiqon, Denmark) following the procedure described by the manufacturer. The Hy3™-labeled samples and a Hy5™-labeled reference RNA samples (referred to hereafter as Cy3 and Cy5) were mixed pair-wise and hybridized to the miRCURY™ LNA array version 11.0 (Exiqon, Denmark), which contains capture probes targeting all miRNAs for human, mouse or rat registered in the miRBASE version 14.0 at the Sanger Institute. The hybridization was performed according to the miRCURY™ LNA array manual using a Tecan HS4800 hybridization station. After hybridization the microarray slides were scanned and stored in an ozone free environment (ozone level below 2.0 ppb) in order to prevent potential bleaching of the fluorescent dyes. The miRCURY™ LNA array microarray slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc., USA) and the image analysis was carried out using the ImaGene 8.0 software (BioDiscovery, Inc., USA). The quantified signals were background corrected (Normexp with offset value 10 as described previously (Ritchie et al., 2007)) and normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm.

### Analytic and statistical methods

We analyzed miRNAs from RNA with RIN greater than 4.0 and mean expression ( $\log^2$ ) value greater than 7.0 in at least one of the two data sets in order to avoid analyzing miRNAs that were not expressed or lacking in data quality in both experiments. To analyze the data, a mixed model was fit to each miRNA with a random effect for each individual in the study and fixed effects to adjust for age and Exiqon array iteration in miRNA expression. This



model accounts for variability in miRNA expression across individuals, as well as for differences in expression due to age and Exiqon array iteration. In addition, each model included fixed effects for DLB (for cases where DLB severity is greater than 0) vs. non-DLB (cases where DLB severity is 0), sex, and brain region (AC vs. MO), plus all two-way interactions among these variables, and an interaction among DLB, sex, and brain. Each interaction allows each combination of sex, brain region, and DLB status to have a different association with miRNA expression, rather than forcing, for instance, differences in brain region to be the same in males and in females. Thus, in summary, the mixed models allow researchers to detect effects of DLB, brain region, and sex on miRNA expression, while accounting for variability across individuals and differences across age and array iteration. After the mixed models were fit, tests were performed to look for evidence of effects of interest. Specifically, tests were performed comparing the average miRNA expression across brain regions. Multiple testing corrections were made for fixed effect tests using a Benjamini-Hochberg correction. Significance was noted when the adjusted p-values for the corresponding test were less than 0.05.

### **RT-qPCR validation in separate cases of a subset of miRNAs**

Quantitative PCR for post-hoc replication was performed on separate cases (n=6) and included RNA isolated from MO, AC, primary somatosensory, and frontal cortical regions as described above, all from the same cases. Different cases were used in comparison to those above—only controls lacking substantial DLB or AD pathology were evaluated for these replication experiments. RNA was extracted as above and evaluated with TaqMan® single-tube miRNA RT-qPCR (Thermo Fisher Scientific) according to the manufacturer's instructions. An equal quantity (350 ng) of RNA was used in the TaqMan® single-tube assays. TaqMan® assays were designed to follow MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al., 2009).

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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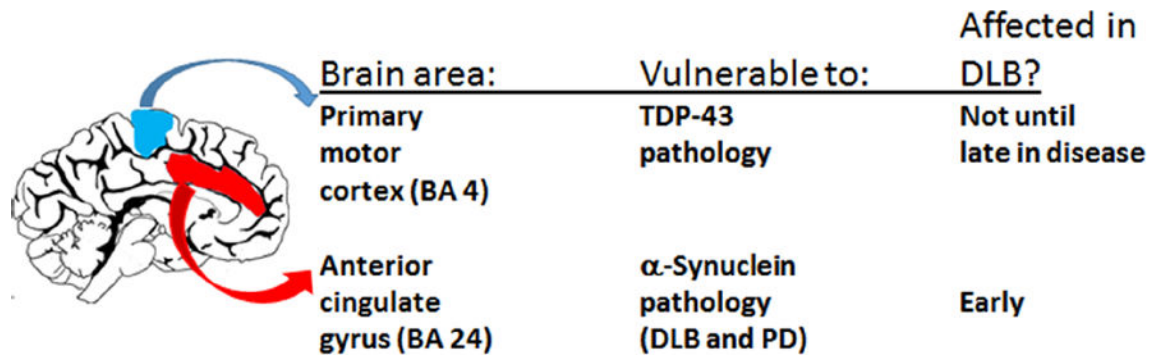
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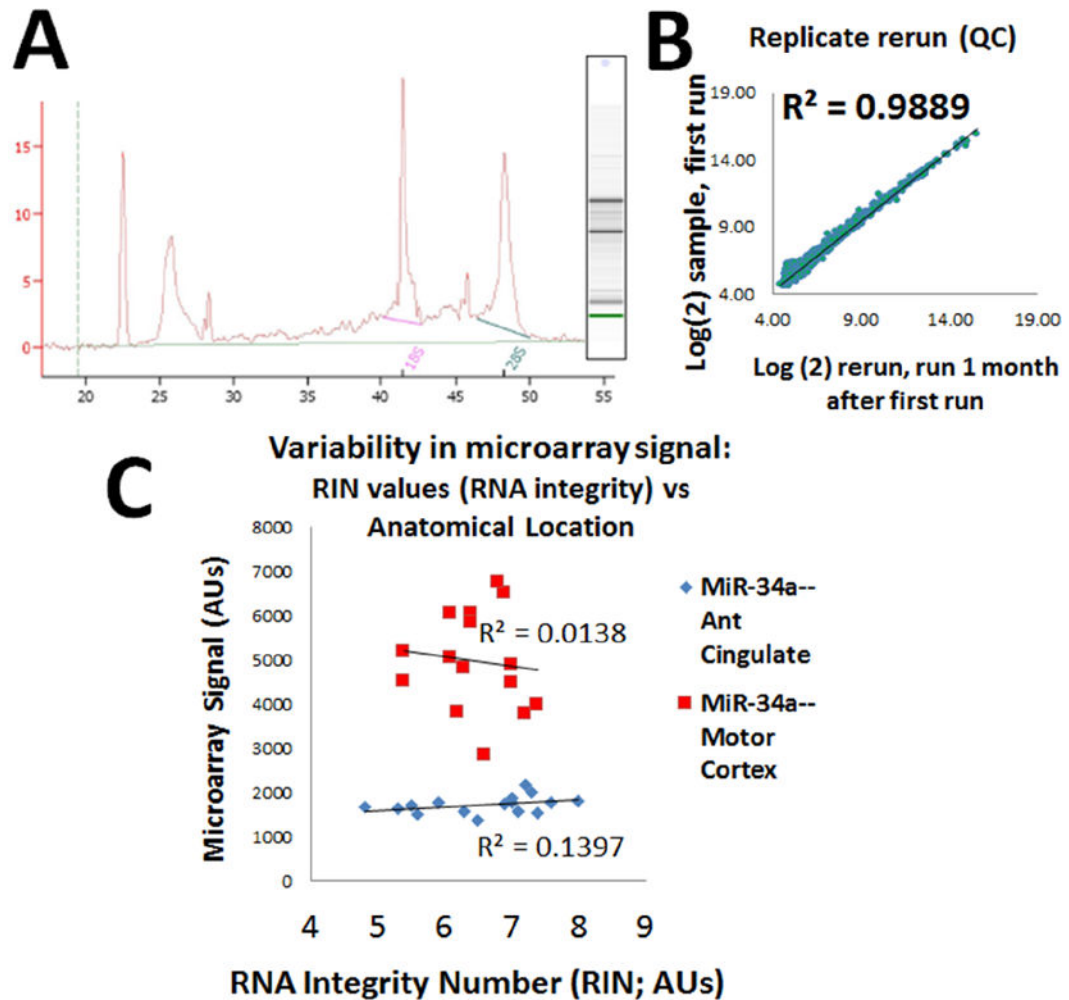
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**Figure 1. Rationale for selecting brain tissue for RNA extraction and miRNA profiling in the current study**

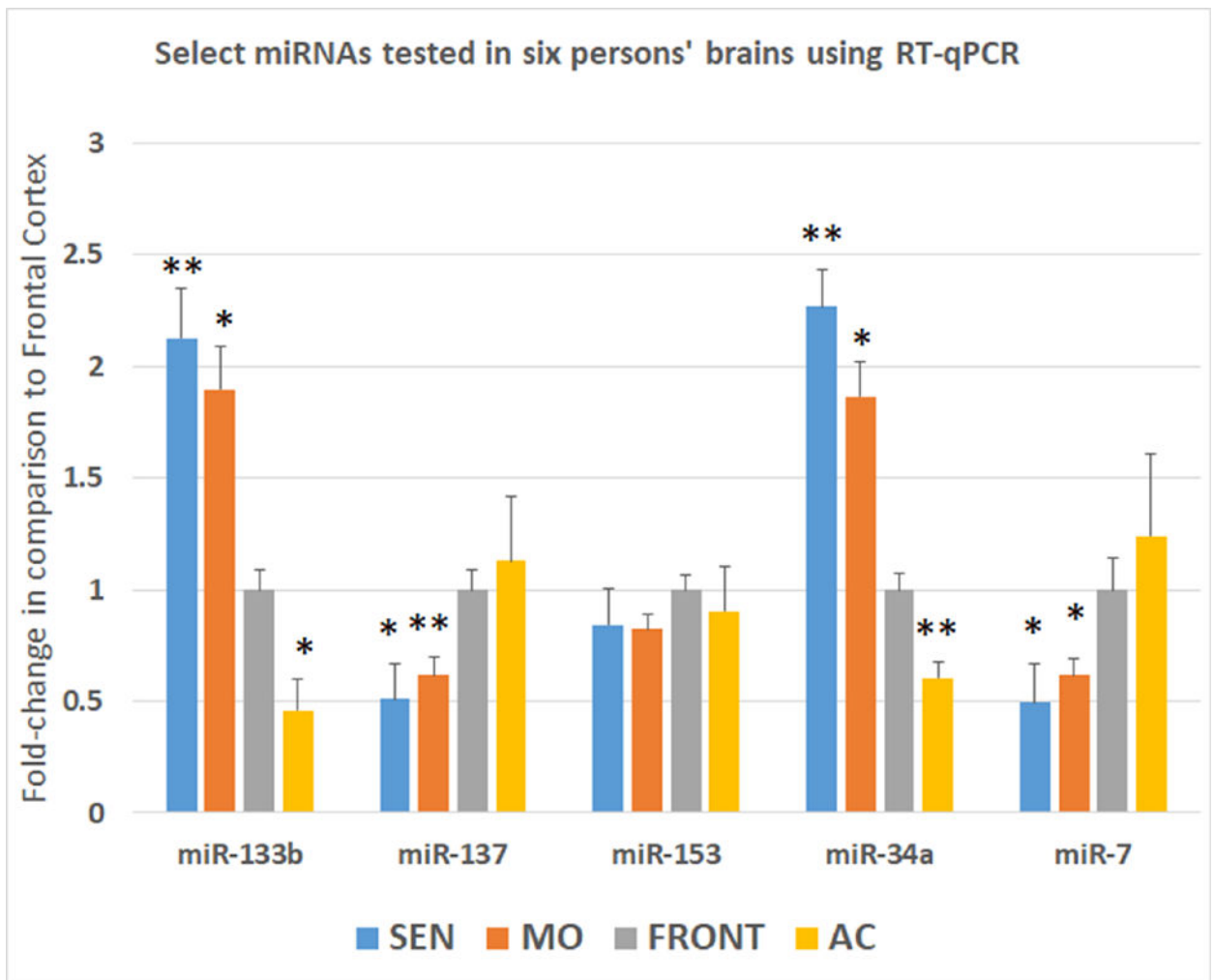
Particular brain areas show different vulnerabilities to specific brain diseases. Whereas the anterior cingulate gyrus (AC; Brodmann area 24) is affected early in DLB, the primary motor cortex (MO; Brodmann area 4) is generally not affected until late in the disease, if at all.



**Figure 2. Focus on technical factors using high-quality RNA probed with state-of-the-art miRNA microarrays**

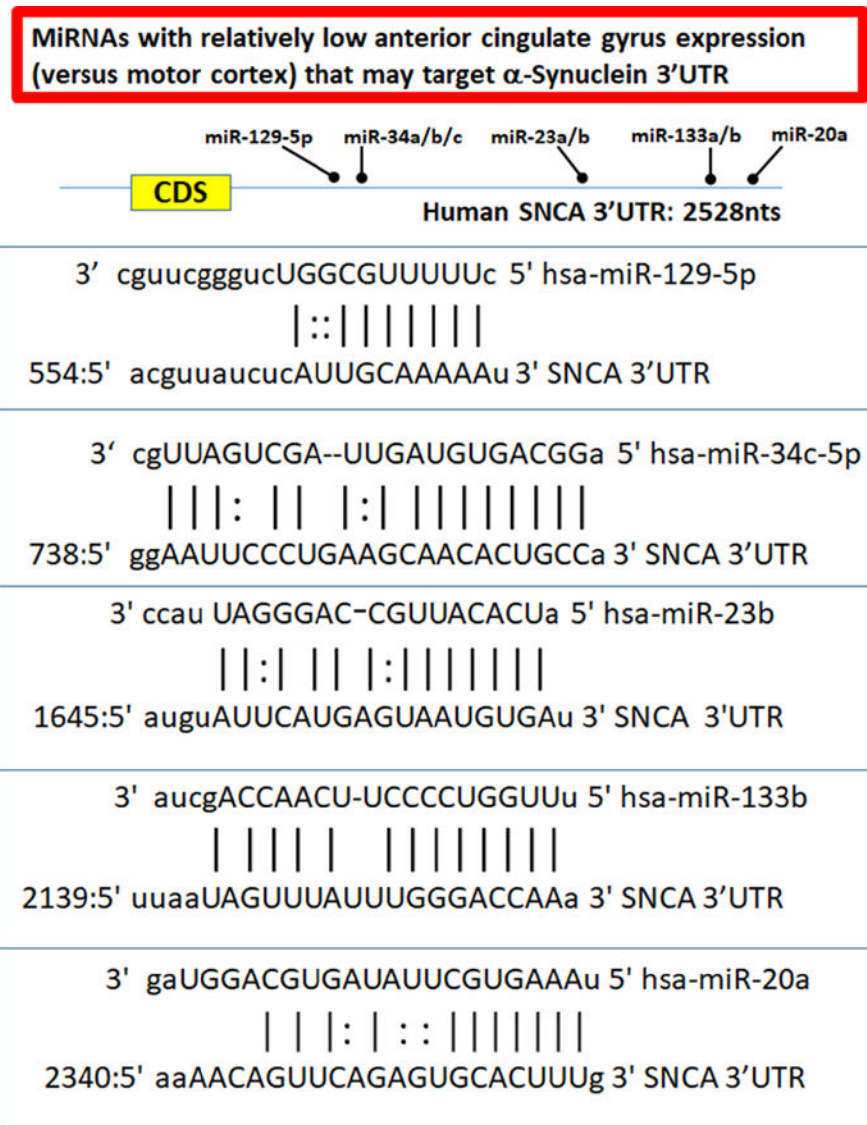
Quality control (QC) included Agilent BioAnalyzer (electropherogram, A), and the RNA Integrity Number (RIN) were mostly between 6-8, with inclusion criteria of RIN >4.0. Gray matter dissection was performed as described previously (Nelson et al., 2008b; Nelson and Wang, 2010; Wang et al., 2008c; Wang et al., 2011). For added QC, we performed extra technical replicates of six different samples a month after the primary microarrays were run and these showed within-case correlations (B is a representative correlation), demonstrating outstanding replicability. C. Variability in microarray signals correlated poorly with RIN number indicating that RNA integrity ( $\geq 4$ ) and RNA degradation had weak impact on miRNA expression results in our preliminary samples. Shown here are results for miR-34a which are representative.



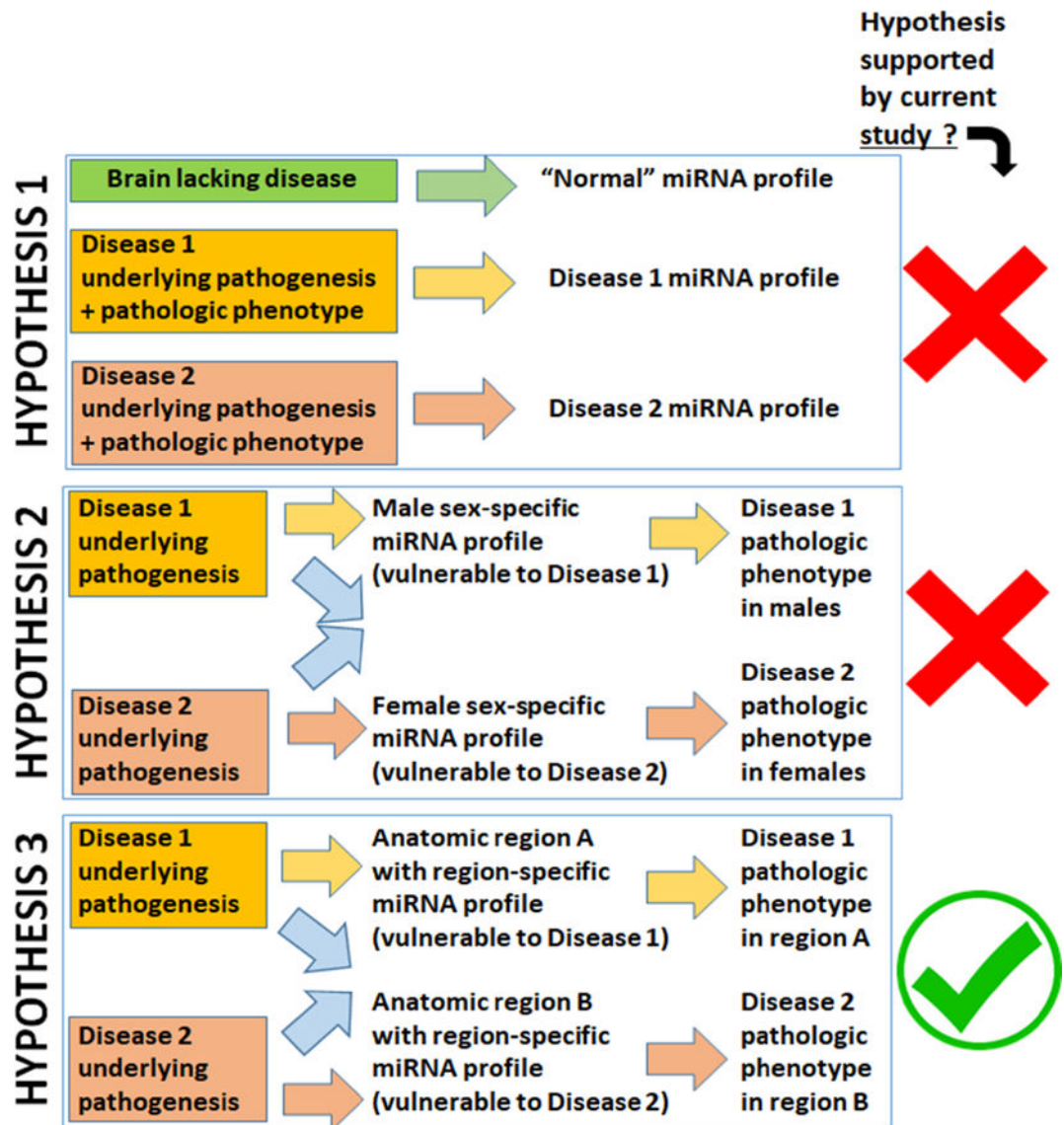


**Figure 3. RT-qPCR results on a replication cohort (n=6) separate from those studied with miRNA microarrays, in order to compare the expression of miRNAs in primary sensorimotor cortex (SEN—blue bars); primary motor cortex (MO—orange); dorsolateral frontal cortex (FRON—gray); and anterior cingulate gyrus (AC—yellow)**

A subset of miRNAs were chosen that had differential expression in MO and AC according to the microarray studies, and had also been implicated in Lewy body disease and/or  $\alpha$ -synuclein regulation. Note that miR-133b and miR-34a were expressed at higher levels in primary somatosensory cortex and motor cortex, whereas miR-137 and miR-7 were expressed at lower levels in these brain areas. However, miR-153 did not show differential expression in these samples. \*-p<0.05; \*\*-p<0.01 using Student's t-test. Error bars = StDev.

**Figure 4.**

One hypothesis for why neurons in the anterior cingulate gyrus are vulnerable to  $\alpha$ -synuclein/Lewy body pathology is that relatively low expression of miRNAs that target the 3'-untranslated region (3'UTR) of  $\alpha$ -synuclein in the anterior cingulate gyrus could be partly responsible. The results of the current study were correlated with a miRNA web-based miRNA/mRNA target prediction algorithm (Betel et al., 2010; John et al., 2004) to highlight the potential target sites on the 3'UTR of the *SNCA* transcript that may be relevant to this hypothesis. These putative miRNA recognition elements all are "conserved miRNAs with good mirSVR scores" according to the website (<http://www.microrna.org/microrna/faq.do>).



**Figure 5.**

Schematic depiction of three separate hypotheses related to brain miRNA expression that could help explain differences in vulnerability to neurodegenerative diseases that are at least partially sex-specific and region-specific. **HYPOTHESIS 1:** the combination of underlying pathogenic mechanisms and the pathologic phenotype alter the neurochemical milieu and result in a condition-specific miRNA profile. The current study did not find support for this hypothesis with the caveat that the sample sizes were relatively small and thus statistically underpowered to correlate subtle variations in miRNA expression patterns with various pathologic and demographic parameters. **HYPOTHESIS 2:** there are sex-specific miRNA expression patterns that help to explain the differential vulnerability of males and females to subtypes of neurodegenerative diseases. The current study did not find evidence of robust sex-specific miRNA expression patterns in the sampled brain regions. **HYPOTHESIS 3:** there are neuroanatomic area-specific patterns of miRNA expression that may help cause those brain regions to have region-specific vulnerability to pathologic phenotypes. Since

there were robust differences detected in miRNA patterns across brain regions, including among miRNAs that are hypothesized to target disease-relevant transcripts, we interpret the data from the current study to be compatible with this hypothesis.

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**Table 1**  
**Summary information on cases used in the current study**

For detailed information on each case, see Supplemental Table 1

Pathology-defined case categories	n	n, F	Avg age at death	PMI (Hrs Avg)
Ctrl	16	8	82.4	2.8
DLB	7	2	82.7	2.6
AD	13	8	89.8	2.5
AD+DLB	16	10	79.0	2.7

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**Table 2**

**A subset of miRNAs that show differential expression in anterior cingulate gyrus and motor cortex (top 10 up-regulated in motor cortex [blue] and top 10 up-regulated in anterior cingulate [yellow] by p-value; note that 130 other differentially expressed miRNAs are presented in Supplemental Table 4)**

Values shown are estimates of the average miRNA expression value for the corresponding combination of sex, brain region, and pathology adjusted for age and year.

RNA	Adj Pval For Brain*	Anterior Cingulate				Motor Cortex			
		Males		Females		Males		Females	
		DLB	Non-DLB	DLB	Non-DLB	DLB	Non-DLB	DLB	Non-DLB
hsa-miR-31-3p	5.21E-07	6.6021	6.7391	6.5896	6.684	6.9523	6.9757	6.9071	7.0142
hsa-miR-31-5p	3.18E-06	7.7926	7.8246	7.82	7.7891	8.0879	8.1552	8.0402	8.1721
hsa-miRPlus-G1246-3p	5.90E-06	6.971	7.111	7.0029	7.0199	7.3388	7.2277	7.2391	7.2529
hsa-miR-34a-5p	6.84E-06	7.5044	7.2532	7.3762	7.1129	8.0542	8.0157	8.0519	8.1428
hsa-miR-133b	8.17E-06	6.0775	5.9338	6.1173	5.9231	6.6235	6.3554	6.4687	6.5011
hsa-miR-338-3p	8.65E-06	9.5422	9.7136	9.3534	9.577	9.9171	9.9867	9.9809	10.1153
hsa-miR-584-5p	4.56E-05	6.9292	6.9521	7.0193	6.7815	7.2134	7.1281	7.2317	7.1843
hsa-miR-365a/b-3p	4.66E-05	7.862	8.013	7.8536	7.9969	8.2927	8.1894	8.1764	8.2443
hsa-miR-758-3p	6.63E-05	6.2306	6.1672	6.1978	6.2106	6.049	5.9753	5.8999	6.0197
hsa-miR-185-5p	6.63E-05	8.1934	8.3073	8.187	8.2434	8.5712	8.4618	8.4916	8.5461
hsa-miR-33b-5p	6.63E-05	6.7699	6.778	6.7368	6.749	7.1108	7.0492	7.0398	7.0707
hsa-miR-135a-5p	7.47E-05	8.3028	8.6913	8.4991	8.7826	8.1154	8.0386	7.8877	8.0347
hsa-miR-551b-3p	7.90E-05	7.0652	7.3022	7.2082	7.3993	7.0217	6.7284	6.7882	6.7126
hsa-miR-218-5p	0.000299	7.6044	7.7582	7.658	7.773	7.4514	7.289	7.3475	7.2747
hsa-miR-1185-5p	0.001029	6.6961	6.6594	6.6661	6.7199	6.5659	6.449	6.4068	6.5388
hsa-miR-154-3p	0.001091	6.1423	6.205	6.1887	6.2029	6.0806	5.9777	5.8626	6.0123
hsa-miR-543	0.001818	6.1106	6.1487	6.0874	6.1801	6.0588	5.8923	5.9416	5.9761
hsa-miR-132-5p	0.002194	6.8244	6.9952	6.8267	6.9137	6.7527	6.7007	6.5669	6.7445
hsa-miR-137	0.002194	6.3179	6.8285	6.5601	6.8326	6.1137	5.7082	5.9383	5.8436
hsa-miR-7-5p	0.002496	10.3032	10.5859	10.3652	10.6905	10.1936	10.1601	10.0723	10.0952

\* -p-value is adjusted for multiple comparisons

**Table 3**  
**No miRNAs showed differential expression in sex by DLB after multiple comparison adjustment**

The two miRNAs presented here have unadjusted p-values less than 0.05 and may show a trend. estimates of the average miRNA expression value for the corresponding combination of sex, brain region, and pathology adjusted for age and year.

RNA	Unadj Pval For Sex*DLB	Anterior Cingulate				Motor Cortex			
		Males		Females		Males		Females	
		DLB	Non-DLB	DLB	Non-DLB	DLB	Non-DLB	DLB	Non-DLB
hsa-miR-516a-3p/hsa-miR-516b-3p	0.022494	6.4476	6.2454	6.1295	6.2893	6.6242	6.312	6.4782	6.4181
hsa-miR-4328	0.046346	6.8247	6.5422	6.7308	6.7731	6.8403	6.6068	6.6816	6.8325

**Table 4**  
**MiRNA expression for miRNAs in the miR-15/107 family; those up-regulated in motor cortex are shaded blue**

Estimates of the average miRNA expression value for the corresponding combination of sex, brain region, and pathology adjusted for age and year.

RNA	Anterior Cingulate				Motor Cortex				Adj p-value for brain
	Males		Females		Males		Females		
	DLB	Non-DLB	DLB	Non-DLB	DLB	Non-DLB	DLB	Non-DLB	
hsa-miR-16-5p	10.7802	10.9397	10.7707	10.9432	11.0162	11.1346	10.8987	11.1181	0.0348
hsa-miR-103a-3p	10.3849	10.4951	10.4098	10.5276	10.4741	10.5314	10.3938	10.5017	NS
hsa-miR-15a-5p	9.7437	9.9063	9.7371	9.8953	9.9933	10.0579	9.8941	10.0603	0.0133
hsa-miR-15b-5p	9.0169	9.2194	8.9979	9.2099	9.3371	9.3348	9.2122	9.3747	0.0017
hsa-miR-107	9.0015	9.2017	9.0256	9.255	9.0791	9.146	9.0793	9.1631	NS
hsa-miR-424-5p	7.1216	7.164	7.0938	7.1006	7.1603	6.9496	7.0832	7.0907	NS
hsa-miR-497-5p	6.2485	6.1855	6.2816	6.2125	6.3988	6.1022	6.2365	6.2629	NS
hsa-miR-503-3p	5.5115	5.4314	5.4414	5.3967	5.5273	5.4061	5.3884	5.4512	NS
hsa-miR-503-5p	5.3822	5.3436	5.4269	5.3724	5.3249	5.3651	5.3579	5.3747	NS
hsa-miR-646	4.8733	4.7618	4.8528	4.7567	4.7535	4.764	4.68	4.8211	NS

\*-p-value is adjusted for multiple comparisons



Table 5

**miRNA expression for miRNAs previously implicated in  $\alpha$ -synucleinopathy; those up-regulated in motor cortex are shaded blue and those up-regulated in anterior cingulate gyrus are shaded yellow**

Values shown are estimates of the average miRNA expression value for the corresponding combination of sex, brain region, and pathology adjusted for age and year.

RNA	Anterior Cingulate				Motor Cortex				Adj p-value for brain*
	Males		Females		Males		Females		
	DLB	Non-DLB	DLB	Non-DLB	DLB	Non-DLB	DLB	Non-DLB	
hsa-miR-133b	6.0775	5.9338	6.1173	5.9231	6.6235	6.3554	6.4687	6.5011	8.17E-06
hsa-miR-133a-3p	5.848	5.7429	5.8764	5.802	6.2502	6.0379	6.1812	6.14	9.91E-05
hsa-miR-34c-5p	6.0231	6.0994	6.0893	5.9919	6.2591	6.2074	6.2372	6.2648	0.0001
hsa-miR-20a-5p	9.6119	9.9028	9.6552	9.8962	9.9988	10.088	9.904	10.0526	0.0016
hsa-miR-7-5p	10.3032	10.5859	10.3652	10.6905	10.1936	10.1601	10.0723	10.0952	0.0025
hsa-miR-34b-5p	5.6875	5.5041	5.4729	5.4263	5.805	5.7586	5.6199	5.7868	0.0048
hsa-miR-153-3p	7.3275	7.3024	7.3644	7.4377	7.2315	7.0217	6.9554	7.1223	0.0216
hsa-miR-320a	7.8979	8.0834	7.9415	8.0285	8.2249	8.0812	8.1417	8.1644	0.0267
hsa-miR-7-2-3p	6.171	6.0477	6.1892	5.9223	6.3006	6.1158	6.3905	6.2319	0.0301
hsa-miR-373-3p	5.5635	5.4798	5.5206	5.5004	5.5702	5.5429	5.6026	5.6076	NS
hsa-miR-320b	7.8607	8.0713	7.9686	8.0195	8.1417	8.0489	8.0914	8.1176	NS
hsa-miR-205-5p	6.3984	6.3697	6.3739	6.459	6.4445	6.408	6.4608	6.4703	NS
hsa-miR-128-3p	8.3193	8.4338	8.4448	8.5202	8.4255	8.2395	8.2868	8.2694	NS
hsa-miR-301b	5.5309	5.5481	5.5393	5.5584	5.5139	5.5696	5.6117	5.6708	NS
hsa-miR-20b-3p	6.3735	6.4784	6.4437	6.4443	6.45	6.4575	6.4907	6.4973	NS
hsa-miR-20b-5p	7.808	8.1349	7.875	8.1615	7.922	8.1938	7.9443	8.1676	NS
hsa-miR-224-3p	6.3124	6.399	6.4666	6.2922	6.4324	6.3306	6.4809	6.3485	NS
hsa-miR-26b-5p	9.3938	9.6238	9.4095	9.6125	9.4779	9.6279	9.4428	9.6327	NS
hsa-miR-373-5p	5.7349	5.6502	5.6515	5.672	5.72	5.6069	5.7453	5.6778	NS
hsa-miR-214-3p	6.4538	6.8021	6.9107	6.2816	6.7823	6.4591	6.6973	6.4082	NS

\* -p-value is adjusted for multiple comparisons