Special Collection on Concussion - Original Paper

American Society for Neurochemistry

ASN Neuro
Volume 13: 1–12

DOI: 10.1177/17590914211009857
journals.sagepub.com/home/asn
SAGE

High-Throughput Sequencing of BACHD Mice Reveals Upregulation of Neuroprotective miRNAs at the Pre-Symptomatic Stage of Huntington's Disease

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Abstract

Huntington's disease (HD) is a genetic disorder marked by transcriptional alterations that result in neuronal impairment and death. MicroRNAs (miRNAs) are non-coding RNAs involved in post-transcriptional regulation and fine-tuning of gene expression. Several studies identified altered miRNA expression in HD and other neurodegenerative diseases, however their roles in early stages of HD remain elusive. Here, we deep-sequenced miRNAs from the striatum of the HD mouse model, BACHD, at the age of 2 and 8 months, representing the pre-symptomatic and symptomatic stages of the disease. Our results show that 44 and 26 miRNAs were differentially expressed in 2- and 8-month-old BACHD mice, respectively, as compared to wild-type controls. Over-representation analysis suggested that miRNAs up-regulated in 2-month-old mice control the expression of genes crucial for PI3K-Akt and mTOR cell signaling pathways. Conversely, miRNAs regulating genes involved in neuronal disorders were down-regulated in 2-month-old BACHD mice. Interestingly, primary striatal neurons treated with anti-miRs targeting two up-regulated miRNAs, miR-449c-5p and miR-146b-5p, showed higher levels of cell death. Therefore, our results suggest that the miRNAs altered in 2-month-old BACHD mice regulate genes involved in the promotion of cell survival. Notably, over-representation suggested that targets of differentially expressed miRNAs at the age of 8 months were not significantly enriched for the same pathways. Together, our data shed light on the role of miRNAs in the initial stages of HD, suggesting a neuroprotective role as an attempt to maintain or reestablish cellular homeostasis.

Keywords

BACHD, Huntington's disease, microRNA, miR-146b-5p, miR-449c-5p, pre-symptomatic stage

Received January 16, 2021; Revised March 4, 2021; Accepted for publication March 18, 2021

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by a poly-glutamine expansion in the amino-terminal region of the huntingtin (htt) protein (MacDonald et al., 1993). Symptoms initiate between 35 and 50 years of age and include involuntary body movement, loss of cognitive function, psychiatric disorders and inevitable death (Li and Li, 2004; Vonsattel, 2008). Neuropathological analyses show selective and progressive neuronal cell loss in the striatum and cortex and, as disease progresses, it also affects the hippocampus and hypothalamus (Vonsattel et al., 1985; Vonsattel, 2008). Although the mechanisms underlying HD-related neurodegeneration have not yet been fully

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elucidated, it is becoming evident that gene transcription dysregulation is a key event associated with HD progression (Sugars and Rubinsztein, 2003).

MicroRNAs (miRNA) are short non-coding RNAs that negatively regulate the expression of genes by binding to the 3'-untranslated region in a sequence specific manner, promoting degradation or translational repression of target mRNAs (Diederichs and Haber, 2007). miRNAs have an important role in a wide range of biological processes, such as the development and functioning of the brain, where hundreds of different miRNAs are expressed to regulate thousands of transcripts (Petri et al., 2014). Several studies indicate that differentially expressed miRNAs are likely to play a role in the progression of neurodegenerative diseases (Maciotta et al., 2013). For instance, alterations in the expression of miRNAs were observed in both mouse models and patients suffering from HD (Packer et al., 2008; Lee et al., 2011; Hoss et al., 2014; Reed et al., 2018). Moreover, genome-wide-analysis indicate that the expression of miRNAs change progressively in an age and CAG length-dependent manner in HD mouse models (Langfelder et al., 2018). Although HD age of onset is directly related to the number of glutamines present in the htt protein, it has been shown that differentially expressed miRNAs present in the pre-frontal cortex of post-mortem HD patient influence disease onset (Hoss et al., 2014). Thus, miRNAs are likely to have a crucial role in HD progression.

It has been shown that miRNAs regulate neuroprotective and neurotoxic processes associated with neurodegenerative diseases (Junn and Mouradian, 2012; Abe and Bonini, 2013). miRNAs implicated in neuronal survival/cell death pathways were shown to be differentially expressed in mouse models and patients suffering from HD, regulating transcripts very relevant to HD progression, including brain derived neurotrophic factor (BDNF) (Johnson et al., 2008; Packer et al., 2008; Müller, 2014; Reed et al., 2018). For instance, it has been shown that overexpression of miR-22, which is down-regulated in HD, triggers anti-apoptotic actions and targets mRNAs implicated in HD etiology, including REST corepressor 1 (Jovicic et al., 2013). However, changes in expression of miRNAs may also bring benefits, slowing HD progression. A miRNA profiling study on human HD prefrontal cortex identified five miRNAs, including miR-196a-5p, that are present in the *Hox* gene cluster and are upregulated in HD, as compared to control (Hoss et al., 2014). Interestingly, overexpression of miR-196a-5p triggers the downregulation of genes involved in apoptosis, increasing cell viability, and ameliorating HD-related phenotype in animal models (Cheng et al., 2013; Kunkanjanawan et al., 2016). Therefore, miR-196a-5p increased expression in HD appears to elicit a compensatory mechanism that ameliorates HD pathology. Although these studies highlight the importance of miRNAs in HD-related neuronal toxicity, only a few miRNAs differentially expressed in HD have been shown to be directly implicated in cell death processes. It is also important to assess whether changes in miRNA expression are more pronounced during the pre-symptomatic phase or when neurodegeneration is already advanced. Therefore, the main goal of this study was to employ genome-wide-analysis to identify miRNAs differentially expressed in a mouse model of HD, the BACHD mice, that could be important for neuroprotection/neurotoxicity during the pre-symptomatic and symptomatic stages of HD.

Materials and Methods

Material

Neurobasal medium, N2 and B27 supplements, GlutaMAX, penicillin and streptomycin, Live/Dead viability assay, TRIzol™, kit Platinum® SYBR® Green qPCR SuperMix-UDG, were purchased from Thermo Fisher Scientific. TruSeq Small RNA Library Prep Kit was purchased from Illumina. All other biochemical reagents were purchased from Sigma-Aldrich.

Mouse Strains

FVB/NJ (wild type, RRID:IMSR_JAX:001800) (WT) and FVB/N-Tg(HTT*97Q)IXwy/J (BACHD) transgenic mice (Gray et al., 2008) were purchased from Jackson Laboratory (RRID:IMSR_JAX:008197, http://jaxmice.jax.org/strain/008197.html) (Bar Harbor, ME, USA). C57BL/6 mice (25–30 g) were purchased from the animal facility of the XXX. Mice were housed in an animal care facility at 23°C on a 12 h light/12h dark cycle with food and water provided *ad libitum*. Animal care was in accordance with the XXX – Ethics Committee on Animal Experimentation, CEUA 237/2015. A total of 16 wildtype (WT) and 16 BACHD male mice and 6 female C57BL/6 mice were used in this study.

RNA Isolation

After reaching the age of either 2 or 8 months, WT and BACHD mice were euthanized and the cerebral cortex and striatum were individually collected through microdissection in 0.9% NaCl solution nuclease free (n = 4 per group). RNA was isolated using TRIzol™ reagent as per the manufacturer's instructions (Thermo Fisher Scientific). RNA was resuspended in 15 µL of nuclease-free water and its concentration and integrity were analyzed by spectrophotometer (NanoDrop™, Thermo

Scientific) and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), respectively.

Small RNA Library Preparation and Bioinformatic Analyses

For each time point (2 and 8 months-old) and genotype (WT and BACHD), total RNA with high integrity (RIN > 8 measured by Bioanalyzer) was used as input for small RNA library preparation utilizing the TruSeq Small RNA Library Prep Kit (Illumina) following the manufacturer protocol. Three libraries were built from independent mice as biological replicates per condition. The 12 libraries generated were sequenced in 2 independent lanes according to the age of mice. Sequencing was performed using the Illumina HiSeq 2500 system by the GenomEast platform at Strasbourg/France, a member of the 'France Génomique' consortium (ANR-10-INBS-0009). After sequencing, raw reads were submitted to adaptor trimming using cutadapt v1.12 (Martin, 2011), discarding sequences smaller than 18 nt. The remaining sequences were mapped to the *Mus musculus* reference genome (GRCm38) using Bowtie v1.1.2 (Langmead et al., 2009) allowing no mismatches and discarding reads mapping on multiple regions. DESeq2 v1.30.0 (Love et al., 2014) was used for quantification and normalization of mapped output files. Size profiles of small RNAs matching the *M. musculus* genome and 5′ nt frequency were calculated using in-house Perl v5.16.3, BioPerl library v1.7.1 and R v3.6.0 scripts. Plots were made in R using the ggplot2 v2.2.0 or nVennR v0.2.2 packages (Pérez-Silva et al., 2018). miRNA expression values reported in this work are normalized count values represented as heatmap or by transformation on percentage (WT expression values as 100%). Sample-level quality control was conducted across all samples. All differential expression analyses were carried out with linear models using miRNA expression as the outcome variable. Libraries were deposited in the NCBI Sequence Read Archive (SRA) under BioProject number PRJNA686706.

The miRNA enrichment analysis was performed using the miEAA v2.0 tool, available at https://ccb-compute2.cs.uni-saarland.de/mieaa2 (Kern et al., 2020). The over-representation analysis (ORA) was performed using differentially expressed miRNAs for each time point as the test set, while the total list of detected miRNAs for each time point was used as reference set. The analysis was conducted against the following datasets indexed on the webtool: Gene Ontology, KEGG, miRbase, miRWalk, MNDR, RNALocate, SM2miR, and miRPathDB. The *p*-value was adjusted using FDR (Benjamini-Hochberg) method for each category independently. Benjamini-Hochberg FDR adjustment is also reported as 'Q-value' in results (Supplemental Table 1). ORA results are available in Supplemental Table 1.

Quantitative RT-qPCR

Two strategies for miRNA quantification using RT-qPCR were used: (1) After RNA isolation, 3 µg of total RNA were used for cDNA synthesis using the kit NCode™ (miRNA First-Strand cDNA Synthesis) as per manufacturer's instructions (Thermo Fisher Scientific). miRNA expression levels were quantified by RT-qPCR using Platinum® SYBR® Green qPCR SuperMix-UDG. This strategy was used to quantify the expression of the following miRNAs: mmu-miR-449a-5p (NR_029961.1); mmu-miR-146b-5p (NR_030468.1); mmu-miR-7a-5p (LM379197.1); mmu-miR-486-5p (LM379823.1) and snRNA-Rnu6 (NR_003027.2) as an endogenous normalizer. The following primers were designed according to manufacturer's instructions: mmu-miR-449a-5p (forward: 5′- GTGGCAGTGTAT TGTTAGCTGGT-3′); mmu-miR-146b-5p (forward: 5′-GCTGAGAACTGAATTCCATAGGCT-3′); mmu-miR-7a-5p (forward: 5′- GTGGAAGACTAGTGATTT TGTTGT-3′); mmu-miR-486-5p (forward: 5′- TCCTG TACTGAGCTGCCCCGAG-3′); snRNA-Rnu6 (forward: 5′- CACGCAAATTCGTGAAGCGTTCC-3′). Reverse primer is provided in the kit. Verification of undesired secondary structures or primer dimer were performed using "OligoAnalyser 3.1" tool (Integrated DNA Technologies©), available at https://www.idtdna.com/calc/analyzer. All primers used in this work were validated by serial dilution and showed efficiencies comprising between 90 and 110%. (2) After RNA isolation, 50 ng of total RNA was used for cDNA synthesis using kit TaqMan® Small RNA Assay as per manufacturer's instructions (Thermo Fisher Scientific). miRNA expression levels were quantified by RT-qPCR using Taqman® Universal PCR Master Mix. This strategy was performed to quantify the following miRNAs: mmu-miR-100-5p (LM379177.1); mmu-miR-409-5p (LM380366.1) and mmu-miR-93-5p (LM379109) as a normalizer. For both strategies (1) and (2), the expression of miRNAs used as endogenous normalization controls (snRNA-Rnu6 and mmu-miR-93-5p) showed no differential expression between conditions. Changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method. Both strategies reproduced the miRNA yields observed in the small RNA-sequencing quantifications. RT-qPCRs reactions were performed using the QuantStudio™ 7 Flex real-time PCR system platform (Applied Biosystems).

Neuronal Primary Culture Preparation

Neuronal cultures were prepared from the striatal region of E15 mouse embryo brains. After dissection, striatal

tissue was submitted to trypsin digestion followed by cell dissociation using a fire-polished Pasteur pipette. Cells were plated on poly-L-ornithine-coated dishes in Neurobasal medium supplemented with N2 and B27 supplements, 2 mM GlutaMAX, 50 µg/mL penicillin, and 50 µg/mL streptomycin. Cells were incubated at 37°C and 5% CO_2 in a humidified incubator and cultured for 12 days *in vitro* with medium replenishment every other 4 days.

miRNA Blockade in Primary Cell Cultures

miRNAs were blocked using seed-targeting 8-mer locked nucleic acid (LNA) oligonucleotides, which were designed and synthesized with a phosphorothioate backbone as described by Obad et al. (2011). Sequences are shown in Table 1. At the day in vitro (DIV) 6, neuronal cell cultures were incubated with either vehicle (saline) or LNAs at a final concentration of 2 µM. The same amount of LNAs was added to neuronal cell cultures every other day, ending on the DIV 12. As a positive control for neurotoxicity, neurons were incubated for 20 h with 50 µM glutamate.

Cell Death Assay

Cell death was determined by Live/Dead viability assay, as described previously (Doria et al., 2013). Briefly, neurons were stained with 2 µM calcein acetoxymethyl ester (AM) and 2 µM ethidium homodimer-1 for 15 min and the fractions of live (calcein AM positive) and dead (ethidium homodimer-1 positive) cells were determined by microscopy. Neurons were visualized by fluorescence microscopy FLoid® Cell Imaging Station (Thermo Scientific, Waltham, MA, USA) and scored by a blinded observer. A minimum of 300 cells were analyzed per well in triplicates using the ImageJ™ software. Dead cells were expressed as a percentage of the total number of cells.

Data Analysis and Statistics

Statistics concerning bioinformatic analyzes were described in a previous session. Data are represented as means ± SEM for the number of independent experiments as indicated. GraphPad Prism™ v8.4.3 software was used to evaluate statistical significance in RT-qPCR and cell viability assays, using unpaired *t*-test or one-way (Anova). Fisher exact test for a contingency table was conducted using the webtool Graphpad Quickcalcs (available at https://www.graphpad.com/quickcalcs/).

Results

Changes in miRNA Expression Are More Pronounced During the Pre-Symptomatic Phase of HD

In order to assess the expression profile of miRNAs in HD, we deep-sequenced small RNAs in the striatum of 2- and 8-month-old BACHD and wild-type (WT) mice, representing the asymptomatic and symptomatic stages of the disease, respectively. In 2-month-old BACHD mice, we observed a total of 44 differentially expressed miRNAs, which corresponds to 2.95% of all detected miRNAs (Figure 1A and Supplemental Figure 1A). 20 miRNAs were up-regulated, while 24 miRNAs were downregulated when compared to controls (Figure 1B). At the age of 8 months, we identified a total of 28 differentially expressed miRNAs, corresponding to 1,87% of all detected miRNAs at this timepoint (Figure 1A and B and Supplemental Figure 1B). 14 miRNAs were significantly up-regulated and 14 miRNAs were down-regulated in BACHD mice compared to WT (Figure 1B). Considering both 2 and 8 months of age, 5 miRNAs were commonly up-regulated (miR-449a-5p, miR-449c-5p, miR-146b-5p, miR-7a-5p, and miR-486-5p) and 1 miRNA (miR-133a-3p) was down-regulated (Figure 1C and D). Moreover, our results showed that more miRNAs were differentially expressed at 2 than at 8 months of age ($p = 0.0353$, Fisher's exact test, one-tailed). Thus, we decided to focus on 2 months of age.

In order to validate our observations, we quantified the expression levels of seven differentially expressed miRNAs in 2-month-old mice using RT-qPCR. miRNA expression levels measured by RT-qPCR exhibited a similar pattern as that of the small RNA-sequencing data (Figure 2A and B). At 2 months of age, 5 miRNAs (miR-409-5p, miR-133a-3p, miR-146b-5p, miR-449a-5p, miR-486-5p) were confirmed to be significantly down or up-regulated in BACHD mice, comparing to WT controls, while 2 other miRNAs (miR-100-5p and miR-7a-5p) showed a similar trend of down- or up-regulation as that of sequencing data, although these differences did not reach statistical significance in

Table 1. Locked Nucleic Acid (LNA-AntimiR) Oligonucleotides.

miRNA (miRBase ID)	miRNA (5′–3′) seed underlined	LNA Sequence (5′–3′)
mmu-miR-449c-5p (MIMAT0001542)	AGGCAGUGUAUUGUUAGCUGGU	CACTGCCT*
mmu-miR-146b-5p (MIMAT0003475)	UGAGAACUGAAUUCCAUAGGCU	AGTTCTCA*
cel-miR-67-5p (MIMAT0020316)	CGCUCAUUCUGCCGGUUGUUAUG	AATGAGCG*

* All nucleotides carry a phosphothiate modification.

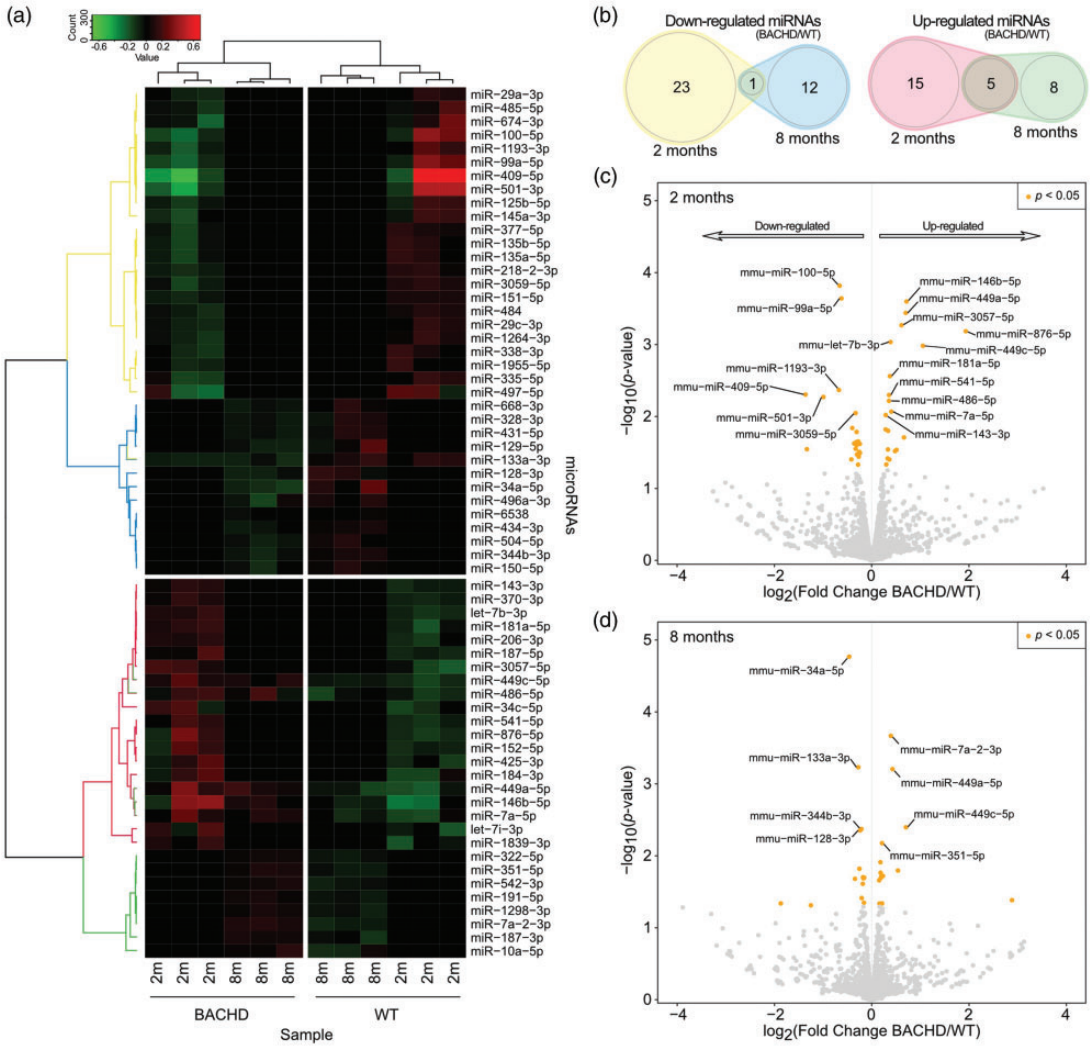

Figure 1. Alterations in miRNA Levels Are Prominent in Early Stages of Huntington's Disease. A: Heatmap representing the miRNAs differentially expressed in the striatum of BACHD, as compared to that of WT mice, at 2- and 8-months of age, grouped by hierarchical clustering. Up- and down-regulation are represented in red and green, respectively. The intensity of the color corresponds to the ratio of expression between BACHD vs WT groups. B: Number of miRNAs differentially expressed in BACHD, as compared to WT mice, grouped by age and fold-change significance. C and D: Volcano Plot showing the miRNA fold change comparing BACHD to WT according to the significance in (C) 2- and (D) 8-month-old mice. Read counts were submitted to a negative-binomial test using generalized linear model (GLM) regression and Wald test for significance testing. P-values were adjusted using the Benjamini–Hochberg method. n = 3 independent samples.

the case of RT-qPCR results (Figure 2B). Thus, the RT-qPCR results corroborated the small RNA-sequencing data, even though minor statistical differences were observed possibly due to differences in technique sensitivity.

Previous studies have shown a prominent cell loss coupled with alterations in miRNA expression in the cerebral cortex of patients affected by HD (Hedreen et al., 1991; Hoss et al., 2014, 2015). To test whether the miRNAs differentially expressed in the striatum could also be altered in the cortex, we assessed miRNA levels by RT-qPCR in the cerebral cortex of BACHD animals and compared the results to that of WT. We observed no significant alterations in the expression levels of miR-133a-3p, miR-146b-5p, miR-449a-5p, miR-486-5p, miR-7a-5p in the cortex, when comparing BACHD and WT mice (Supplemental Figure 2). These results suggest that the miRNAs alterations observed in the striatum are tissue specific.

Differentially Expressed miRNAs Potentially Modulate Neuroprotective Pathways at an Early Stage of HD in BACHD Mice

To have a broad view of pathways and groups of genes that could be affected by miRNAs at 2- and 8-months of

age, we analyzed the enrichment of putative targets using Over-Representation Analysis (ORA) implemented at the miEAA webtool (Kern et al., 2020). We used as input only the significantly up- or down-regulated miRNAs for each time point. Interestingly, our analysis showed that up-regulated miRNAs in 2-month-old BACHD mice potentially target genes involved in cell metabolism, growth, survival, and response to stress, such as members of the mTOR, PI3K and AMPK pathways (Figure 3). However, those same pathways were not significantly represented among the targets of differentially expressed miRNAs at the age of 8 months (Figure 3). miRNAs targeting pathways involved in neuronal damage such as seizure, Rett syndrome, HD, and Alzheimer's disease were down-regulated at the age of 2 months. Taking these results together, we hypothesize that, at initial stages of the disease, miRNAs could be involved in a neuroprotective modulation, possibly as an attempt to maintain or reestablish homeostasis. However, at later stages, when HD symptoms are already evident, the neuroprotective modulation seems to be lost.

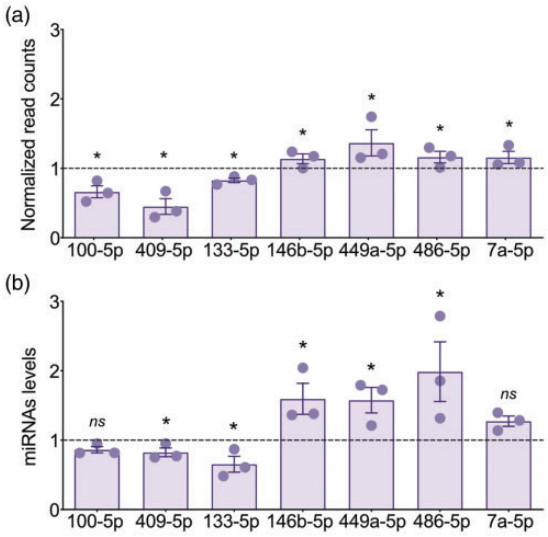

Figure 2. Fold-Change of Selected miRNAs in Early Stages of Huntington's Disease in BACHD Mice. A and B: Graphs show the relative miRNA expression levels of miR-100-5p, miR-409-5p, miR-133a-3p, miR-146b-5p, miR-449a-5p, miR-486-5p, and miR-7a-5p in the striatum of 2-month-old BACHD mice. A: Normalized read counts from high-throughput sequencing data. B: Relative miRNA expression levels accessed by RT-qPCR. RT-qPCR assays were normalized either by miR-93-5p in the case of miR-100-5p and miR-409-5p, or RNU6 levels in the case of miR-146b-5p, miR-449a-5p, miR-486-5p and miR-7a-5p. WT is represented as 100% (dashed line). Bar represent mean ± SEM. n = 3. * indicates statistical significance ($p < 0.05$), ns nonsignificant.

Blockade of miR-146b-5p and miR-449c-5p Promotes Neurotoxicity of Primary Cultured Striatal Neurons

As the differentially expressed miRNAs were predicted to be important for the enrichment of neuroprotective pathways such as PI3K-Akt and mTOR cell signaling pathways (Supplemental Table 1), we decided to test whether two upregulated miRNAs, miR-449c-5p and miR-146b-5p, could be important for neuronal cell death/survival. miR-449c-5p and miR-146b-5p were chosen because these miRNAs were previously shown to target genes involved in neuronal processes such as neuronal differentiation, cell maturation and inflammation (Wu et al., 2014; Fededa et al., 2016; Muñoz-San Martín et al., 2019; Yang and Zhao, 2020). Moreover, miR-449c-5p and miR-146b-5p were shown to be involved in the

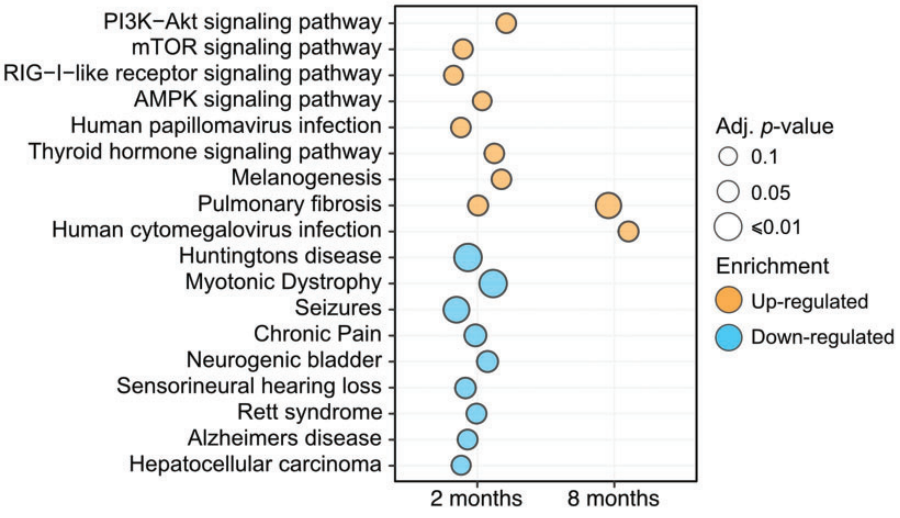

Figure 3. Differentially Expressed miRNAs Potentially Target Neuroprotective Pathways in 2-Month-Old BACHD Mice. Enrichment analysis of miRNA targets was accessed by Over-Representation Analysis, using as input differentially expressed miRNAs against datasets available in the literature (see Methods for details).

enrichment of 2 or more pathways involved in cell survival and/or neuroprotection (Song et al., 2014; Wu et al., 2014; Yang and Zhao, 2020). As a control, we also analyzed miR-7a-5p, as this miRNA is not present in the enrichment of the aforementioned pathways. Of note, these selected miRNAs were also differentially expressed at 8 months of age, although not to the same extent as in 2-month-old mice. Thus, to investigate the role of miR-449c-5p and miR-146b-5p in neuronal cell survival, we blocked miRNAs activity in primary neuronal cell cultures obtained from the striatum of mouse embryos. The primary cell cultures employed in this study were composed almost exclusively by neurons (99,5%), mainly medium-sized spiny neurons, as previously characterized by our group (Doria et al., 2013; Olmo et al., 2017). Striatal neurons were incubated in the presence or absence of anti-miRNA LNA oligonucleotides (Table 1) for 6 days to block miRNA activity. Neurons treated with anti-miR-146b-5p LNA and anti-miR-449c-5p LNA exhibited higher levels of neuronal cell death, as compared to that of vehicle-treated neurons (Figure 4A, C, D, and F). Neurons treated with anti-miR-7a showed no significant increase in neuronal cell death above basal levels (Figure 4A, B, and F). As a neurotoxic insult, neurons were treated with glutamate for 20 h, which triggered higher levels of neuronal cell death, as compared to vehicle-treated neurons (Figure 4A, E, and F). Importantly, miR-146b-5p and miR-449c-5p blockade appear to be as toxic to neurons as glutamate, as we found no significant difference in neuronal cell death levels when comparing neurons treated with anti-miR-146b-5p and anti-miR-449c-5p to that of glutamate treated neurons (Figure 4C to F). Therefore, our results suggest that miR-146b-5p and miR-449c-5p are involved in a neuro-survival mechanism.

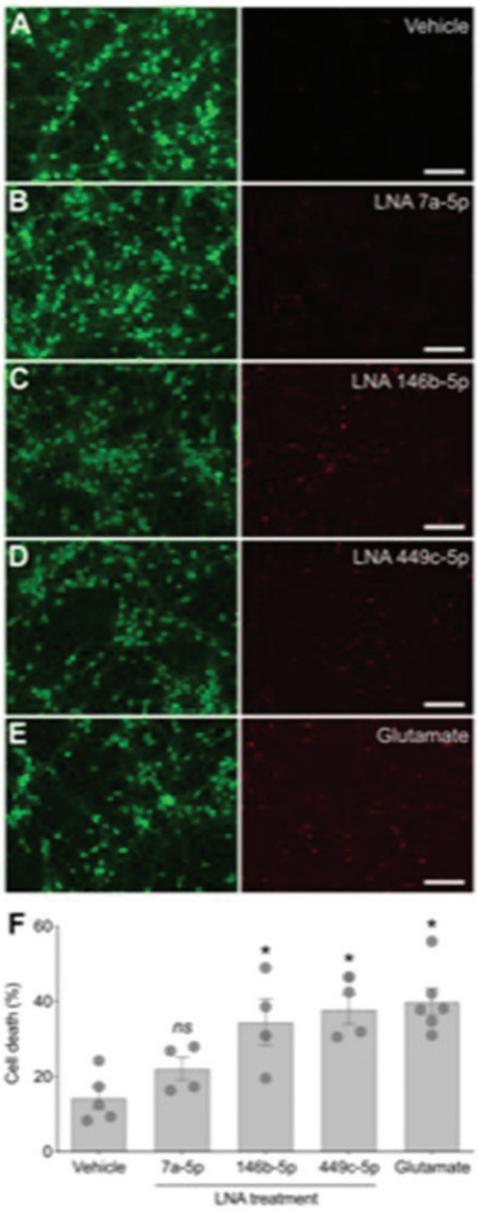

Figure 4. miR-146b-5p and miR-449c-5p Blockade Promote Neurotoxicity in Striatal Neurons. A–E: Representative images from primary cultured striatal neurons labeled with calcein AM (green, live cells) and ethidium homodimer-1 (red, dead cells) under the following conditions: (A) vehicle, (B) miR-7a LNA blocker (2 μM), (C) miR-146b-5p LNA blocker (2 μM), (D) miR-449c-5p LNA blocker (2 μM), and (E) glutamate (50 μM). Scale bar corresponds to 50 μm. F: Graph shows the percentage of neuronal cell death in primary cultured striatal neurons that were either untreated or treated with the indicated LNA blocker or glutamate. Bars represent mean ± SEM. n = 4–5. * indicates statistical significance ($p < 0.05$); ns, not significant.

Discussion

Huntington's disease is characterized by a progressive clinical course and the onset of symptoms most often takes place in adults (Wexler et al., 2004). Although many studies investigate the pathogenesis of HD in later stages, few studies discuss the pathological mechanisms in the asymptomatic phase of the disease. As it has been previously described in the case of HD and other neurodegenerative diseases, miRNAs play a regulatory role, fine-tuning RNA levels in the cell (Dong and Cong, 2019). Here, we focused on investigating the miRNA repertoire in the striatum of BACHD mice at the ages of 2 and 8 months to unveil the early and late transcriptional alterations that take place in this tissue. Interestingly, our analyses revealed that 2-month-old BACHD mice display a larger number of differentially expressed miRNAs in comparison to 8-month-old mice, which suggests molecular adaptations throughout the course of the disease.

Despite the fact that it is now clear that miRNAs are very important to regulate gene expression and for brain

homeostasis, one of the main issues regarding the involvement of miRNAs in diseases is the contradicting findings obtained across different studies. HD is not an exception and, indeed, the miRNAs identified in this study differ from previously published data obtained from animal models and HD patients. Changes in the abundance of miRNAs in HD have been reported in several studies using human samples (Martí et al., 2010; Hoss et al., 2014; Reed et al., 2018). For instance, Hoss et al. (2014) identified five up-regulated miRNAs (miR-10b-5p, miR-196a-5p, miR-196b-5p, miR-615 -3p and miR-1247-5p) in the prefrontal cortex (Brodmann Area 9) from twelve HD and nine control patients. Interestingly, enrichment analysis revealed cell pathways that are involved in neuronal differentiation, neurite growth, cell death and survival (Hoss et al., 2014). However, due to technical restrictions, the analyses were limited to the cerebral cortex or regions other than the caudate-putamen, which is the main region affected by the disease.

Although HD is caused by the mutation of a single gene, which makes it easier to develop reliable animal models, disease onset and the course of HD progression can vary considerably among animal models (Pouladi et al., 2013). Thus, the discrepancies regarding differentially expressed miRNA in HD could be due to the different mouse models analyzed. For instance, we have used a transgenic mouse model of HD, the BACHD mice, and in the study by Langfelder et al, knock-in mouse models expressing htt containing different lengths of poly-glutamine repeats were employed. In the former study, a smaller number of miRNAs differentially expressed were found in 2-month-old mice, as compared to 6- and 10-month-old mice (Langfelder et al., 2018). However, Langfelder et al (2018) revealed that altered miRNAs might regulate neuronal survival pathways, which is consistent with our observations. Thus, although it is possible that different models of HD may show particular transcriptional responses linked to the transgene and also to mouse background, the resulting effect can lead to a similar conclusion. More studies using a broad range of HD mouse models are necessary to elucidate this hypothesis. In this direction, Lee et al. (2011) have examined the miRNA profile in striatum samples from YAC128 mice, another transgenic HD mouse model (Lee et al., 2011). Although authors did not evaluate the biological pathways putatively targeted by miRNAs, they identified a set of downregulated miRNAs that were also found to be down-regulated in our study, including miR-29c-3p, miR-218-5p, miR-344-3p, and miR-674-3p, which were previously linked to the regulation of stress responses and inflammatory pathways (Bae et al., 2005; Park et al., 2009; Gao et al., 2010). Of note, we observed the enrichment of stress and inflammatory pathways at early stages of the disease, while the latter study shows the modulation of miRNAs potentially targeting stress and inflammation genes in 12-month-old mice (Lee et al., 2011). Again, differences in mouse background and/or the speed of disease onset in YAC128 and BACHD mice could explain these observations. A more detailed investigation analyzing data from different models of HD could shed light on groups or individual miRNAs that potentially regulate neuroprotective pathways in asymptomatic and symptomatic stages of HD.

Our results show that the changes in miRNA expression were small (fold-changes from 1.93 to -1.36 in 2-month-old and from 2.88 to -1.87 in 8-month-old mice), when comparing BACHD and WT mice. Therefore, it is possible that the alteration of one single of these miRNAs would not have a big impact on brain equilibrium. However, in neurodegenerative diseases, it has been proposed that the interplay among different groups of miRNAs, even when exhibiting small changes in expression, might be more relevant than a pronounced change in the expression of one or two miRNAs (Leichter et al., 2017; Rajgor, 2018). In fact, only when we performed analysis including all the differentially expressed miRNAs, we were able to observe significant enrichment of pathways and that is the main reason why putatively affected pathways were so different when comparing 2- and 8-month-old mice, even though some of the miRNAs affected at 2 months of age were also altered at the age of 8 months. Moreover, it is very interesting that our results show enrichment for gene sets comprising cell signaling pathways important for neuronal survival at 2 months of age and that disease-related pathways were likely altered at a later disease stage. For instance, PI3K/AKT pathway is especially relevant in HD, as activation of this pathway leads to neuroprotection (Datta et al., 1999; Kandel and Hay, 1999), as well as to the phosphorylation of mutant htt protein, which functions to reduce htt aggregate formation and neuronal cell death (Humbert et al., 2002; Warby et al., 2009). Moreover, the mTOR pathway is important to regulate autophagy, which was shown to reduce mutant htt aggregates and ameliorate HD-related pathology (Ravikumar et al., 2003, 2004). Of note, PI3K/AKT and mTOR cell signaling pathways were enriched in 2-month-old mice, but not at 8 months of age. Thus, changes in the expression of one single miRNA might not mean much, especially because the changes we observed were small. However, the interplay among different miRNAs and the cumulative effect of these small changes in expression may result in the activation of neuroprotective pathways in an attempt to maintain brain homeostasis at early disease stages, an effect that is lost with disease progression.

It is worth mentioning that we observed changes in the expression of miRNAs that belong to the same cluster, including miR-449a and -449c, which have been

categorized as a family because they share the same seed sequence and, therefore, they possibly target the same mRNAs, affecting similar biological pathways. Interestingly, miR-449a-5p displays abundant expression in the brain (Wu et al., 2014). Considering that the expression levels of both miR-449a and -449c were up-regulated in early and late stages of the disease, we investigated their role in striatal neurons. Blockade of miR-449c-5p led to neuronal cell death, suggesting that this miRNA is neuroprotective. Studies using cancer cell lines revealed that the miR-449 family modulates apoptosis and cell cycle by regulating genes and pathways including cyclin D1, BCL2, and p53 pathway components (Bou Kheir et al., 2011; Hu et al., 2014; Yong-Ming et al., 2017). Microarray analysis identified a down-regulation of miR-449a-5p and miR-34a-5p in prefrontal cortex samples of a senescence-accelerated mouse model (SAMP8) and further analysis correlated this down-regulation with an increase of sodium channel subunit beta-2 (SCN2B) expression levels. In addition, miR-449a-5p upregulation or SCN2B downregulation increased neurite extension of cultured neurons, suggesting that miR-449a might play an important role in neuronal growth and associated progression of aging by targeting SCN2B (Tan et al., 2020). Other studies revealed that miR-34/449 are involved in specific developmental alteration on brain substrates. Genetic ablation of the miR-34/449 cluster in mice culminates in the reduction of brain size and in the developmental disruption of forebrain structures, including the putamen and the olfactory tubercle (Song et al., 2014; Wu et al., 2014). Taking it all together, there is strong evidence supporting the role of miR-449 cluster in the modulation of neuronal development and survival. Thus, the up-regulation of miR-449a and miR-449c observed in 2-month-old mice suggests an early response to maintain or restore brain homeostasis against the deleterious stimulus triggered by mutant htt. However, more experiments are necessary to evaluate the *in vivo* contribution of miR-449 cluster in neuroprotection, especially regarding PI3K and mTOR pathways.

Several studies demonstrated immune response alterations in HD gene carriers (Träger et al., 2014; Rocha et al., 2016; Valekova et al., 2016). Interestingly, miR-146b-5p, which is up-regulated in our 2-month-old dataset, was shown to modulate the activity of the RIG-I-like receptor signaling pathway. For instance, miR-146a up-regulation in an obesity cell model reduces inflammatory activity by targeting TRAF6 and also reducing IL-8 levels, important components of the RIG-I pathway (Roos et al., 2016). Furthermore, studies in thyroid cancer revealed that the constitutive overexpression of miR-146b-5p in thyroid cell lines leads to a significant decrease in its target PTEN, culminating in PI3K/AKT pathway hyperactivation (Ramírez-Moya et al., 2018). The same authors also observed that miR-146b overexpression led to the reduction of FOXO1 and p27 levels in the nucleus and increased cell survival. Of note, our analyses indicate that miR-146b-5p increased levels contribute to the regulation of PI3K-Akt signaling pathway (look Supplemental Table 1). Corroborating our findings, Ghose et al. (2011) showed, in both cell and mouse models of HD, that mutant htt increases the expression of p53 and p65 subunits of NF-κB (RelA/NF-κB), which in turn decreases the levels of miR-146a-5p (Ghose et al., 2011). Thus, miR-146a-5p increased levels might be an important factor, ameliorating the neurotoxic effects caused by mutant htt.

In conclusion, the results shown in this study clearly indicate that the changes in miRNA expression are more pronounced at early stages of the disease progression and that these alterations might be neuroprotective, as blockade of the up-regulated miRNAs, miR-146-5p and miR-449c-5p, triggered neuronal cell death. However, as the neuroprotective pathways are no longer enriched at 8 months of age, we propose that these early changes attempting to delay disease progression are lost as mouse age.

Summary

- Changes in miRNA levels are more pronounced during the pre-symptomatic phase of Huntington's disease in BACHD mouse model.
- miRNAs altered in 2-month-old BACHD mice potentially target neuroprotective pathways.
- Blockade of miR-146-5p and miR-449c-5p promote neuronal cell death.

Acknowledgments

We thank Siad Cedric Amadou and Suellen Rosseto Mendonça for contributing with experiments, support with animal breeding, and sample management.

Author Contributions

I. G. O., R. P. O., and F. M. R. wrote the manuscript. I. G. O., R. P. O., A. N. A. G., and F. M. R. designed and prepared the figures. I. G. O., R. P. O., A. N. A. G., and R. G. W. P. performed experiments and analyses. F. M. R. and J. T. M. conceived the project and obtained funding. All authors reviewed the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article:

This work was supported by Pro-Reitoria de Pesquisa (PRPq)/ Universidade Federal de Minas Gerais (UFMG), CNPq (Grant Number 429049/2018-8) and FAPEMIG (Grant Number PPM-00212-18) Grants to F. M. R., as well as CNPq Grant (Number 457168/2013-7) to J. T. M. and F. M. R.

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Supplemental material

Supplemental material for this article is available online.

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