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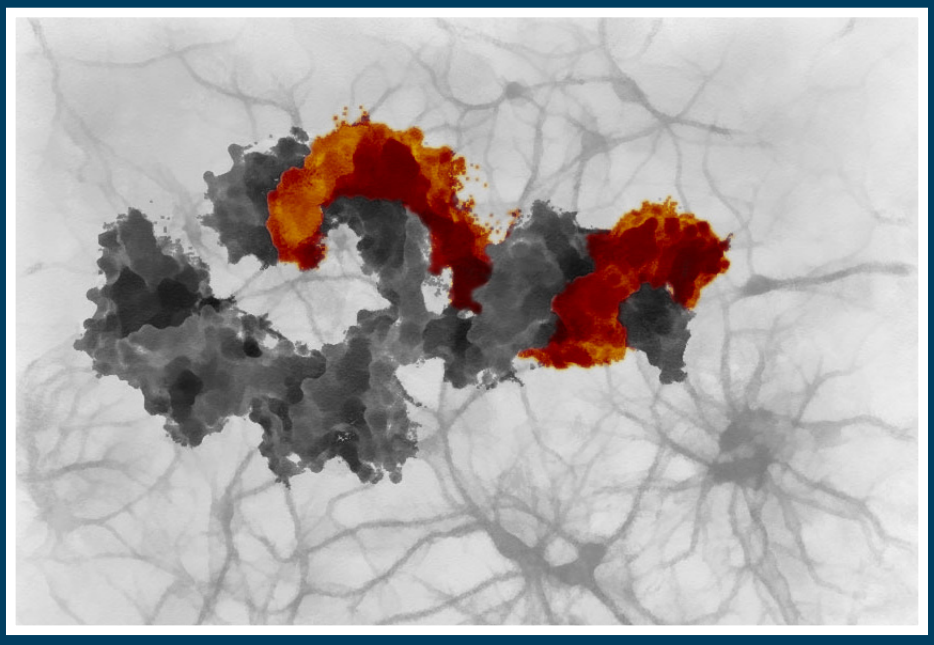

NOVEL ROLES OF NON-CODING BRAIN RNAs IN HEALTH AND DISEASE

Topic Editor
Hermona Soreq

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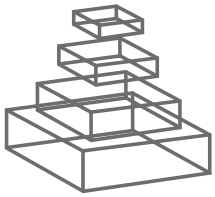
MOLECULAR NEUROSCIENCE



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NOVEL ROLES OF NON-CODING BRAIN RNAs IN HEALTH AND DISEASE

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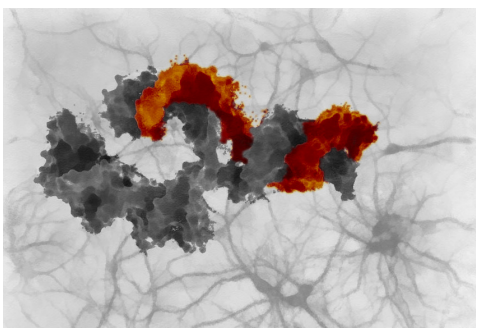

Neuronal miRNA (red) wraps around its transcript target (black) on a background of primary neurons (grey).

Non-coding RNAs (ncRNAs), and in particular microRNAs are rapidly becoming the focus of research interest in numerous basic and translational fields, including brain research; and their importance for many aspects in brain functioning merits special discussion. The wide-scope, multi-targeted and highly efficient manner of ncRNA regulatory activities draws attention to this topic by many, but the available research and analysis tools and experimental protocols are still at their infancy, and calls for special discussion given their importance for many aspects in brain functioning. This eBook is correspondingly focused on the search for, identification and exploration of those non-coding RNAs whose activities modulate the multi-leveled functions of the eukaryotic brain. The different articles strive to cover novel approaches for identifying and establishing ncRNA-target relationships, provide state of the art reports of the affected neurotransmission pathways, describe inherited and acquired changes in ncRNA functioning and cover the use of ncRNA mimics and blockade tools for interference with their functions in health and disease of the brain. Non-coding RNAs are here to stay, and this exciting eBook provides a glimpse into their impact on our brain's functioning at the physiology, cell biology, behavior and immune levels.

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EDITORIAL
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Novel roles of non-coding brain RNAs in health and disease

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Keywords: microRNAs, long non-coding RNAs, cholinergic signaling, schizophrenia, epilepsy, ischemic stroke, Alzheimer's disease, Parkinson's disease

Non-coding RNAs (ncRNAs), and in particular microRNAs (miRNAs) are rapidly becoming the focus of research interest in numerous basic and translational fields, and their importance for many aspects in brain functioning reveals novel roles and merits special discussion. The wide-scope, multi-targeted, and highly efficient manner of ncRNA regulatory activities draws attention to this topic by many, but the available research tools and experimental protocols are still insufficient, and their importance for many aspects in brain functioning keeps changing. Much of the research effort in this field has initially been devoted to cancer research, but the regulatory role of ncRNAs is considered global. Consequently, molecular neuroscientists picked it up as well, although the brain presents special challenges for ncRNA and miRNA research. To reflect the rapid recent development of ncRNA and miRNA research in the nervous system, this Research Topic eBook is focused on the search for and exploration of those ncRNAs and miRNAs whose activities modulate the multi-leveled functions of the eukaryotic brain in health and disease. It strives to cover the state of the art expertise and describe novel roles for known and recently identified ncRNAs and miRNAs and cover experimental approaches for identifying and establishing ncRNA-target relationships, reports of the affected pathways, inherited and acquired changes in ncRNA functioning and the use of ncRNA mimics and blockade tools for interference with their functions in health and disease.

This eBook covers several key topics of interest in the molecular neuroscience field that try to bridge the gap between ncRNAs, miRNAs, and the wider research community. As researchers, we are interested in advancing this field for the improvement of both basic and translational studies aimed at progressing toward better human health and wellbeing. Therefore, this volume is opened by a review contributed by the Gerhard Schratt group that presents a comprehensive characterization of the nuclear miRNA repertoire of post-mitotic neurons (Khudayberdiev et al., 2013). This is followed by a thorough discussion of the flexibility and stability of miRNAs in brain development and function that was written by the Christophe Beclin group (Follert et al., 2014) and by insights on the functional interactions between miRNAs and copy number variations in the aging brain contributed by the Ronald Bontrop group (Persengiev et al., 2013). Yet other authors focused their articles on particular neuronal roles of specific miRNAs. Thus, Alexander Murashov and Di Wu described the role of miRNA-431 in regulating axon regeneration in mature sensory neurons by targeting the Wnt antagonist Kremen1 (Wu and Murashov, 2013), while Bettina Nadorp presented a new view of the different genes involved in specific neurotransmission pathways as co-regulated by miRNAs (Nadorp and Soreq, 2014). To this end, she initiated a bioinformatics effort combined with *in vivo* experimental work to discover and validate the role of predicted overlapping microRNA regulators of acetylcholine packaging and degradation in neuroinflammation-related disorders.

Engineered animal models represent an important tool for exploring ncRNA and miRNA functions in the brain, and several of the articles in this eBook reflect this aspect. Some of the covered research efforts took global experimental approaches in diverse engineered animal models; thus, the Sebastian Kadener group reported Genome-wide assessment of post-transcriptional control in the fly brain, highlighting the rapid changes in this dynamic field of research (Mezan et al., 2013). Yet others referred to the diagnostic potential, like the Andre Fisher group that covered the rapidly evolving field of miRNA biomarkers for Central Nervous System disease (Rao et al., 2013). Hartl and Grunwald-Kadow and co-authors outlined new roles for "old" miRNAs in nervous system functions and diseases (Hartl and Grunwald Kadow, 2013). Another, even newer topic in this field is that of long ncRNAs in neurodevelopmental disorders, a subject which is likely to develop exponentially in the coming years and was the focus of an article by the Armaz Aschrafi group (van de Vondervoort et al., 2013).

The rapidly gained reputation of miRNAs lead to escalating numbers of joint basic-clinical studies, and many of those put a major emphasis on the nervous system diseases as related to changes in miRNAs. The most prevalent neurodegenerative disease, Alzheimer's disease was the focus of two separate articles: Sebastian Hebert and colleagues discussed the future prospects of circulating miRNAs to become a useful diagnostic tool and create novel biomarkers for early identification of Alzheimer's disease (Dorval et al., 2013), whereas the Euginia Wang group presented an in-depth study of the prospects of one specific miRNA to become such a biomarker (Bhatnagar et al., 2014): miRNA-34c, which was previously shown to associate with aging and whose levels are shown in our eBook to increase in the Alzheimer's circulating plasma. The next two articles shift the interest to nervous system diseases affecting younger patients, like chronic pain and epilepsy. Here, Michaela Kress and co-authors address the topics of pain regulation by miRNAs in nociceptive circuits as predictors

of future clinical applications (Kress et al., 2013), and David Henshall covers the issue of miRNAs involvement in status epilepticus (Henshall, 2013). A key issue in miRNA research involves the emerging need to combine experimental work with state of the art biostatistics and bioinformatics analyses. Combined bioinformatics/genetics and miRNA studies appear in the Markus Nothen review of the highly focused role of miRNAs as the cause of schizophrenia in those rare patients who are 22q11.2 deletion carriers, and this study was expanded to discuss the possible implications for idiopathic disease at large (Forstner et al., 2013). MiRNAs in sensorineural diseases of the ear were the focus of a mini-review by the Karen Avraham group, and may be perceived as a first sign of new discoveries on miRNA contributions in sensory impairments (Ushakov et al., 2013). Ischemic stroke is another nervous system disease with an expanding impact in these days of continuously prolonged life expectancy in Western societies. In our eBook, Julie Anne Saugstad and co-workers discuss modified miRNAs following focal cerebral ischemia in male and female mouse brains (Lusardi et al., 2014).

Apart from the miRNAs themselves, our eBook also refers to the protein complexes involved in miRNA functioning, also in the context of neurodegenerative disease. The RISC complex and its causal involvement in Parkinson's disease is the focus of an article by the Matthew Wood group (Heman-Ackah et al., 2013). Last, but not least are expanded repeat diseases that were covered by two independent studies: RNA pathogenesis via Toll-like receptor-activated inflammation in expanded repeat neurodegenerative diseases by the Catherine Suter group (Richards et al., 2013) and Small ncRNAs as source of complexity added to the RNA pathogenic mechanisms in trinucleotide repeat expansion diseases (Marti and Estivill, 2013). ncRNAs are here to stay, and their impact on our brain's functioning at the physiology, cell biology, behavior, and immune levels is worth an in-depth journey.

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A comprehensive characterization of the nuclear microRNA repertoire of post-mitotic neurons

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MicroRNAs (miRNAs) are small non-coding RNAs with important functions in the development and plasticity of post-mitotic neurons. In addition to the well-described cytoplasmic function of miRNAs in post-transcriptional gene regulation, recent studies suggested that miRNAs could also be involved in transcriptional and post-transcriptional regulatory processes in the nuclei of proliferating cells. However, whether miRNAs localize to and function within the nucleus of post-mitotic neurons is unknown. Using a combination of microarray hybridization and small RNA deep sequencing, we identified a specific subset of miRNAs which are enriched in the nuclei of neurons. Nuclear enrichment of specific candidate miRNAs (miR-25 and miR-92a) could be independently validated by Northern blot, quantitative real-time PCR (qRT-PCR) and fluorescence *in situ* hybridization (FISH). By cross-comparison to published reports, we found that nuclear accumulation of miRNAs might be linked to a down-regulation of miRNA expression during *in vitro* development of cortical neurons. Importantly, by generating a comprehensive isomiR profile of the nuclear and cytoplasmic compartments, we found a significant overrepresentation of guanine nucleotides (nt) at the 3′-terminus of nuclear-enriched isomiRs, suggesting the presence of neuron-specific mechanisms involved in miRNA nuclear localization. In conclusion, our results provide a starting point for future studies addressing the nuclear function of specific miRNAs and the detailed mechanisms underlying subcellular localization of miRNAs in neurons and possibly other polarized cell types.

Keywords: miRNA, isomiR, neuronal development, plasticity, deep sequencing, microarray

INTRODUCTION

MicroRNAs (miRNAs) are an important class of small regulatory non-coding RNAs with a size of 18–25 nucleotides (nt). The canonical miRNA biogenesis pathway starts with the generation of the primary miRNA (pri-miRNA) transcript by RNA polymerase II mediated transcription. The pri-miRNA transcript is cleaved by the microprocessor complex, containing among other proteins Drosha and Di George Syndrome critical region gene 8 (DGCR8) proteins, which results in ∼70 nt hairpin-like precursor miRNAs (pre-miRNA). Pre-miRNAs are subsequently exported to the cytoplasm by the nuclear export receptor Exportin-5 (Zeng and Cullen, 2004), where they are further cleaved by Dicer to produce an intermediate RNA duplex. One strand of this duplex (known as guide miRNA) binds to an Argonaute family protein (AGO) 1–4, the core component of the miRNA-associated RNA-induced silencing complex (miRISC). MiRISC mainly functions in the cytoplasmic compartment by translational inhibition and/or degradation of target mRNAs. MiRNAs are implicated in many steps of neuronal development and the function of mature neurons, including synaptic plasticity, learning and memory (Fiore et al., 2011). Interestingly, several recent studies suggest that miRNAs, in addition to their well-defined role in the cytoplasm, may also be involved in the regulation of gene expression in the nucleus of mammalian cells.

First, it was shown that miRNAs are present in the nuclear compartment. Some of them are even enriched in the nuclei or nucleoli of cancer cell lines (Hwang et al., 2007; Liao et al., 2010; Park et al., 2010; Li et al., 2013), myoblasts (Politz et al., 2009) and neural stem cells (Jeffries et al., 2011). Second, the key components of the miRNA pathway, such as Ago (Tan et al., 2009), Dicer (Sinkkonen et al., 2010) and multiple glycine/tryptophan repeat containing protein - GW182 (Till et al., 2007; Nishi et al., 2013), are detected in the nucleus. Third, Ago proteins associate with splicing factors (Ameyar-Zazoua et al., 2012) and regulate siRNA-mediated alternative splicing (Allo et al., 2009). Fourth, some miRNAs were shown to post-transcriptionally regulate gene expression in the nucleus (Hansen et al., 2011; Tang et al., 2012). Finally, several miRNAs (and siRNAs) were identified to control gene expression by binding to the promoter of target genes, thereby triggering epigenetic changes, such as DNA methylation (Morris et al., 2004) and histone modification (Kim et al., 2008; Place et al., 2008; Benhamed et al., 2012).

Epigenetic modifications and alternative mRNA splicing, apart from being important in neuronal differentiation, are also implicated in activity-dependent gene expression in mature neurons (Norris and Calarco, 2012; Zovkic et al., 2013), an essential mechanism for synaptic plasticity, learning and memory. Furthermore, genes undergoing alternative mRNA splicing are overrepresented

in the brain (Yeo et al., 2004), suggesting that specific molecular mechanisms that lead to transcript diversity must be present in the brain. However, whether miRNAs can regulate gene expression by any of the aforementioned mechanisms in the neuronal nucleus is not known. A prerequisite for the study of miRNA function in the nucleus of post-mitotic neurons is the *a priori* knowledge of the nuclear miRNA repository. However, to date nuclear miRNAs have only been identified from proliferating cells, and it can be expected that terminally differentiated cells like neurons have a completely different miRNA expression profile.

In the present study, using microarray and deep sequencing technologies, we identified miRNAs which are enriched in the nuclei of rat primary cortical neurons. Our results suggest that employing a combination of microarray and deep sequencing technologies to determine nuclear-enriched miRNAs can yield more accurate results than using each method separately. Accordingly, we could validate differential expression of specific nuclear-enriched miRNAs by Northern blot, quantitative real-time PCR (qRT-PCR) and fluorescence *in situ* hybridization (FISH). By cross-comparison to published reports we observed that expression levels of nuclear-enriched miRNAs in general decline during development of neurons, suggesting that these miRNAs could play a role in early developmental stages of neurons. Importantly, by generating a comprehensive isomiR profile of the nuclear and cytoplasmic compartments, we found that the most 3′-terminal nucleotide of miRNA species is a robust predictor of nuclear enrichment. In conclusion, our results provide a roadmap for future studies addressing the detailed mechanisms underlying subcellular localization of miRNAs in neurons and possibly other polarized cell types.

MATERIALS AND METHODS

PRIMARY NEURONAL CULTURE

Primary cortical and hippocampal neuron cultures were prepared from embryonic Day 18 (E18) Sprague-Dawley rats (Charles River Laboratories) as previously described (Schratt et al, 2006). Cortical and hippocampal cultures were maintained in Neurobasal (NB) medium containing 2% B27 supplement, penicillin-streptomycin (100 U/ml penicillin, 100 µg/ml streptomycin), and GlutaMax (1 mM). All reagents were purchased from Life Technologies. Glia-depleted cultures were obtained by supplementing FUDR solution (10 µM) starting from day *in vitro* 0 (DIV0). FUDR solution was prepared by mixing equimolar amount of fluorodeoxyuridine (Sigma) and uridine (Sigma). Glia-enriched cultures were maintained in the standard medium, except B27 supplement was exchanged to 10% FBS (Life Technologies). When indicated, cells were treated for 2 h with 40 ng/mL of BDNF (PeproTech) or 55 mM of KCl solution.

NUCLEAR FRACTIONATION PROTOCOL

For nuclear fractionation, 40 million cells from cortical cultures at DIV7 were used. Cells were washed once with 10 mL of ice-cold 1 × Phosphate buffered saline (PBS; Life Technologies) and were scraped into ice-cold 1 × PBS using cell lifters (Corning). Then cells were pelleted by centrifugation at 100 g speed for 5 min at 4°C. Subsequently, cell pellet was resuspended in 600 µl of ice-cold hypotonic homogenization buffer [HHB; 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM Na-EGTA, 10 mM Tris-HCl pH = 7.4, 1 mM DTT, 2 u/µl RNasin Plus RNase inhibitor (Promega)] and was incubated on ice for 30 min. After supplying cell suspension with 600 µl of 0.2% Igepal CA630 containing HHB, it was homogenized with 40 stokes in a Dounce potter. From the obtained cell lysate, nuclear and cytoplasmic fractions were separated by centrifugation at 720 g speed for 5 min at 4°C. The nuclear fraction (pellet) was washed three times with 1.5 mL of isotonic homogenization buffer (IHB; HHB, supplemented with 250 mM sucrose). The total RNA from nuclear (pellet) and cytoplasmic (supernatant) fractions was extracted using peqGOLD TriFast reagent (Peqlab) per manufacturer's instructions. On average, 15–20% of the total RNA derived from the fractionation originated from the nucleus. For determination of nuclear and cytoplasmic protein markers, the nuclear pellet obtained after washes with IHB was resuspended in RIPA buffer [10 mM NaCl, 1% Triton X-100, 0.5% Sodiumdeoxycholate, 1 mM EGTA, 0.05% SDS, 50 mM Tris-HCl pH = 8.0, fresh 5x protease inhibitor cocktail (Roche)].

WESTERN BLOTTING

Western blotting was performed as previously described (Siegel et al., 2009). The following primary antibodies were used: anti-HDAC2-rabbit monoclonal (Abcam) and anti-beta Actin-mouse monoclonal (Sigma).

RNA EXTRACTION, SIZE SELECTION OF SMALL RNAs AND MICROARRAY PROCEDURE

Twelve microgram of total RNA from nuclear and cytoplasmic fractions was supplemented with spike-in oligoribonucleotides (18 nt, 5-Phos-AGCGUGUAGGGAUCCAAA-3; 24 nt, 5-Phos-GGCCAACGUUCUCAACAAUAGUGA-3; 30 nt, 5-Phos-GGCAUUAACGCGGCCGCUCUACAAUAGUGA-3; 50 femtomoles of each; http://bartellab.wi.mit.edu/protocols.html) and mixed with the same volume of Gel loading buffer II (Life Technologies). RNA was separated using denaturing urea 15% PAGE gel (SequaGel System, National Diagnostics), which was run in 1 × TBE (89 mM Tris/89 mM Borate/2 mM EDTA) buffer at 30 Watts. Gel was stained with 2 × SYBR GOLD dye (Life Technologies; in 1 × TBE) for 10 min and gel pieces corresponding to small RNAs of 15–35 nt size were cut out. Small RNAs were eluted by incubation of gel pieces in 300 mM NaCl solution overnight at 4°C with constant rotation. Precipitation of RNA was carried out by addition of 2.5–3 volume of 100% EtOH to a supernatant and incubation at −20°C for at least 2 h. Pellet was resuspended in 20 µl of DEPC-treated H_2O. For miRNA profiling analysis, 14 µl of small RNA, obtained from each sample, were sent to microRNA Microarray Service provided by LC Sciences (Texas, USA). In brief, three biological replicates of nuclear fractionated samples (three nuclear and three cytoplasmic samples) were labeled with Cy3 (nuclear) and Cy5 (cytoplasmic), and then were hybridized on a single microarray chip (dual-sample hybridization). The signal values were derived by background subtraction and global normalization. A transcript to be listed as detectable should have met at least two conditions: signal intensity higher than 3 × (background standard deviation) and spot CV < 0.5. CV was calculated by (standard deviation)/(signal

intensity). When repeating probes were present on an array, a transcript was listed as detectable only if the signals from at least 50% of the repeating probes were above detection level. The data obtained from LC Sciences was further normalized to a signal intensity value of 24 nt spike-in oligoribonucleotides. The probes on the array were based on miRBase version 16 that contained 679 rat miRNAs. For expression analysis, only miRNAs that possessed average signal intensity values of at least 35 (higher than $\log_2$[average signal intensity] = 5) after background subtraction (where signal intensity values of miRNAs that were same as the background signal were considered as zero), in either of the cellular fractions, were considered. Nuclear enrichment score (NEnS) was calculated by taking logarithm base 2 of the ratio of (average nuclear signal intensity value)/(average cytoplasmic signal intensity value). Statistical analysis was performed on signal intensity values with Student's *t*-test (two-tail, paired). The calculation of Pearson's coefficient between different microarray datasets was performed in Excel (Analysis ToolPak add-in) and was based on $\log_2$ transformed signal intensity values of miRNAs.

DEEP SEQUENCING

Small RNA libraries were constructed and sequenced by EMBL genomic core facility (Heidelberg, Germany). In brief, four small RNA libraries (2 nuclear and 2 cytoplasmic) representing two biological replicates were prepared using small RNA sample prep assay (Illumina) as per manufacturer's instructions. Each of the small RNA libraries was sequenced for 36 cycles in a single lane of one Illumina HiSeq flow cell. Raw sequencing reads were trimmed from 3′ adapter (TCGTATGCCGTCTTCTGCTTG) and filtered according to quality using default parameters of Fastx-Toolkit for fastq data on a Galaxy, a web-based genome analysis tool [(Goecks et al., 2010); https://main.g2.bx.psu.edu/]. Sequencing reads that contained only adapter sequence or those that initially (before trimming) did not contain adapter sequence, as well as reads shorter than 15 nt were discarded. Furthermore, only reads that have at least two identical sequence counts in each of the libraries were considered for analysis ("clean reads"). Clean reads were mapped to the rat mature miRNAs (miRBase v19) using default parameters (one mismatch, 3 nt in the 3′ or 5′-trimming variants, 3 nt in the 3′-addition variants) of Miraligner software (Pantano et al., 2010). The rest of the unmapped reads were first mapped to rat premiRNAs (miRBase v19) and then to other classes of non-coding RNAs [snoRNAs, snRNAs, rRNAs, tRNAs, mitochondrial tRNAs, mitochondrial rRNAs, miscRNAs; sequences were retrieved from Ensembl genome database (rn4) using BioMart portal, http://central.biomart.org/], piRNAs (http://www.ncrna.org/frnadb/, http://www.noncode.org), mRNAs (mRNA_coding sequence, 3′UTR, -1000_transcription_start_site+5UTR; sequences were retrieved from Ensembl genome database (rn4) using BioMart portal, http://central.biomart.org/] and finally to rat genome (ftp://ftp.ccb.jhu.edu/pub/data/bowtie_indexes/; USCS rn4) in a sequential order using bowtie-0.12.8 software (Langmead, 2010) allowing up to 2 mismatches. All read counts that were mapped to the sequences from aforementioned RNA/DNA databases were used to normalize between nuclear and cytoplasmic small RNA libraries. After normalization, miRNAs represented by at least 100 reads in one of the cellular compartments were considered for further analysis. Nuclear enrichment score (NEnS) was calculated by taking logarithm base 2 of the ratio of (average nuclear read count)/(average cytoplasmic read count). The rank based comparison of microarray and deep sequencing was performed by Rank Sum function of RankProdIt [http://strep-microarray.sbs.surrey.ac.uk/RankProducts/; (Laing and Smith, 2010)].

QUANTITATIVE REAL-TIME PCR

The total RNA extraction from neuronal cultures was performed using peqGOLD TriFast reagent per manufacturer's instructions. RNA samples were treated with TURBO DNase (Ambion). For detection of small nuclear RNAs (U1, U4, U6) and mRNAs (GAPDH), 200 ng of total RNA sample was reverse transcribed with iScript cDNA synthesis kit (Bio-Rad) and quantitative real-time PCR (qRT-PCR) was performed on the StepOnePlus Real-Time PCR System (Applied Biosystems), using iTaq SYBR Green Supermix with ROX (Bio-Rad). For detection of mature miRNAs, 50 ng of total RNA sample was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit and qRT-PCR was performed on the StepOnePlus Real-Time PCR System (Applied Biosystems), using TaqMan MicroRNA Assay (Applied Biosystems). Each sample was measured in dub—or triplicates. qRT-PCR data from nuclear fractionated samples were analyzed by $2^{-\mathrm{dCt}}$ $[2^{-(\mathrm{NUC\ Ct}-\mathrm{CYT\ Ct})}]$ method (ΔCt method). Data obtained from whole-cell RNA (developmental, neuron, glia-specific expression) were analyzed by ΔΔCt method, where Ct values were first normalized to an internal control (e.g., U6) and then to the reference sample, which was arbitrarily set to 1. For statistical analysis (Student's and Welch's *t*-tests) the data, which was normalized only to U6 was used. Primers used for the qRT-PCR are provided as supplementary data (Table S8).

NORTHERN BLOT

From ten to twenty microgram of total RNA were separated using denaturing urea 15% PAGE gel (Mini-PROTEAN system; Bio-Rad) in 1x TBE and blotted onto a GeneScreen Plus nylon membrane (PerkinElmer) in pre-cooled 0.5x TBE. Radioactively labeled Decade marker (Ambion) was used as molecular marker. RNAs were crosslinked to the membrane by UV irradiation (1200 mJ), followed by baking of the membrane for 30 min at 80°C. The membrane was pre-incubated in hybridization buffer (5 × SSC, 20 mM Na_2HPO_4 (pH = 7.2), 7% SDS, 2 × Denhardt's solution, 40 μg/mL salmon sperm DNA) for at least 2 h at 50°C at constant rotation, followed by incubation overnight at 50°C in hybridization buffer containing the denatured [32P] labeled DNA probe. The membrane was washed twice for 10 min and twice for 30 min at 50°C with non-stringent wash solution (3 × SSC, 25 mM NaH_2PO_4 (pH = 7.5), 5% SDS, 10 × Denhardt's solution) and once for 5 min at 50°C with stringent wash solution (1 × SSC, 1% SDS). Signals were detected by autoradiography using the Cyclone Plus Phosphor Imager (PerkinElmer). The membrane was stripped (0.1% SDS, 5 mM Na-EDTA, preheated to 95°C) for 1 h and re-used several times to detect additional miRNAs and U6 snRNA. DNA probes are provided as supplementary data (Table S8).

FLUORESCENCE *In-situ* HYBRIDIZATION (FISH)

FISH was performed on dissociated hippocampal neurons at DIV5. Cells were fixed with 4% PFA/4% sucrose/DEPC-PBS for 15 min at room temperature and washed three times with DEPC-PBS. After permeabilization using 0.2% Tween/DEPC-PBS for 2 min, cells were washed twice with DEPC-PBS and treated for 5 min with 0.1 M TEA (Triethanolamine-acetic acid in DEPC-H_2O, pH 8.0) and for 10 min with freshly prepared 0.25% Acetic Anhydride in 0.1 M TEA. Cells were washed three times with DEPC-PBS and pre-incubated in hybridization buffer at 55°C for 1 h. Subsequently, hybridization was carried out overnight at 55°C, using hybridization buffer supplemented with denatured (5 min 85°C, 5 min on ice) DIG (or FITC)-labeled LNA probes (Exiqon; 5 pmol per well in the 24-well format) directed against relevant miRNA. Cells were washed twice in 2x SSC and twice in 0.2x SSC, 30 min each. After two washes with PBS, cells were permeabilized with 0.2% Tween/PBS for 2 min and washed again twice with PBS. Depending on the condition, for signal amplification and co-immunostaining, cells were incubated with first set of antibody dilutions [anti-MAP2–mouse (Sigma) + anti-DIG–FITC (Roche) for U6, miR-25 and miR-92a; anti-MAP2–mouse + anti-FITC–Alexa488–rabbit (Life Technologies) for miR-9] in blocking solution [0.5% Blocking Reagent in PBS (Roche)] for 1.5 h at room temperature. After four washes with PBS, second set of antibodies (anti-Mouse–Alexa546 (Life Technologies) + anti-FITC–Alexa488–rabbit; anti-Mouse–Alexa546, respectively) was applied for 30 min at room temperature. Then cells were washed four more times with PBS and incubated in the last antibody [anti-Rabbit-Alexa488 (Life Technologies)] solution for 30 min. Cells were washed three times with PBS (second wash with Hoechst dye—1:20,000) and mounted on microscope slides using Aqua- Poly/Mount (Polysciences). FISH experiments were analyzed using the 63x objective of the LSM 5 Pascal laser scanning confocal microscope (Zeiss), with identical settings for specific probes. For z-stacks, three consecutive optical sections were taken at a 0.4 μm interval with a resolution of 1024 × 1024 pixels. Maximum projections of the z-stack images were used for subsequent analysis of the signal intensities in nucleus and cytoplasm with the ImageJ software. LNA probes are provided as supplementary data (Table S8).

IMMUNOCYTOCHEMISTRY

Immunostaining of endogenous MAP2 anti-MAP2–mouse (Sigma) and GFAP [anti-GFAP-rabbit (DakoCytomation)] in dissociated hippocampal neurons (DIV18) was performed as described (Siegel et al., 2009).

DEVELOPMENTAL EXPRESSION SCORE

DES was calculated by log2 transforming the ratio of miRNA expression values obtained from prefrontal cortex of post-natal Day 3 (P3) and embryonic Day 10 (E10) rats in the published report by Yao and colleagues (Yao et al., 2012).

IsomiR ANALYSIS

IsomiRs with at least 10 reads in one of the cellular fractions were considered for analysis presented in **Figures 7B,C**. The relative nuclear enrichment score (rNEnS) was calculated as a ratio of nuclear vs. cytoplasmic percentage proportion of a certain miRNA variants (isomiRs) and therefore should be distinguished from NES (which is an absolute value). For example, miRNA isoforms of miR-1 are isomiR-1.1 (constitutes 20% of miR-1 with 20 read counts in the nucleus; 30% with 60 reads in the cytoplasm), isomiR-1.2 (30% and 30 reads, nucleus; 50% and 100 reads, cytoplasm) and isomiR-1.3 (50% and 50 reads, nucleus; 20% and 40 reads, cytoplasm). The rNEnSs for these isomiRs are 20/30 = 0.66, 30/50 = 0.6 and 50/20 = 2.5, respectively, although NEnS for the same isomiRs constitute 20/60 = 0.33, 30/100 = 0.3, and 50/40 = 1.25, respectively. The usage of rNEnS allows to determine the impact of 3′-terminal nucleotide modification of isomiRs on preferential nuclear localization, since it calculates overall proportion of isomiR read counts in the specific cellular compartment independent of whether it is underrepresented in the other cellular compartment. The frequency of nt at 3′ last 5 nt was calculated using WebLogo [(Crooks et al., 2004); http://weblogo.berkeley.edu/].

STATISTICAL ANALYSIS

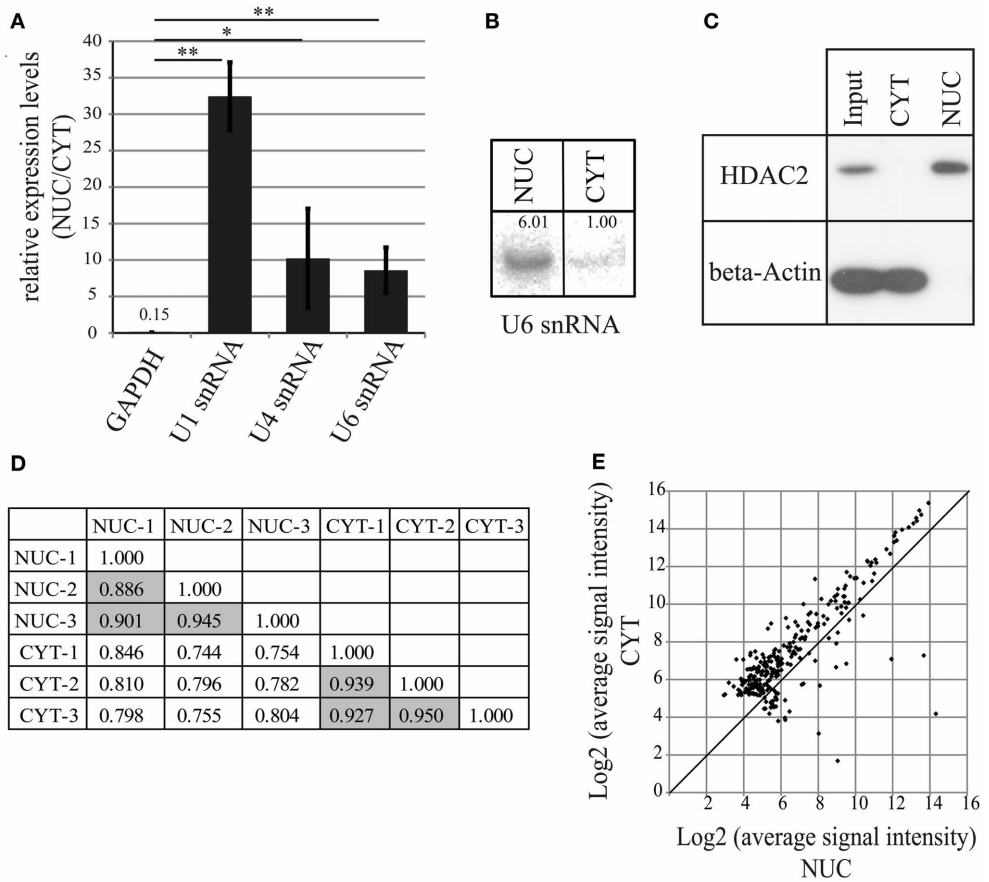
Experiments are reported as mean ± standard deviation (*SD*) and based on three (if not otherwise stated) independent replications. Statistical significance was calculated using Student's (for samples with equal variance) and Welch's *t*-tests (for samples with unequal variance), and for multiple comparisons Bonferroni correction was applied (Benjamini et al., 2001).

RESULTS

MICROARRAY PROFILING OF NUCLEAR AND CYTOPLASMIC miRNAs

To characterize miRNAs preferentially localizing to neuronal nuclei, we decided to undertake a biochemical fractionation approach that separates the nuclear and cytoplasmic compartments of rat primary cortical neurons cultured for 7 days *in vitro* (DIV). After isolation of total RNA from both compartments, the efficacy of nuclear fractionation was determined by the quantification of expression levels of small nuclear RNAs (snRNA U1, U4, U6; all strictly localized in the nucleus) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA by qRT-PCR (**Figures 1A, S1A**). As expected, snRNAs were highly enriched in the nuclear compartment, whereas GAPDH mRNA was strongly depleted. Similar to the results obtained from qRT-PCR, we observed a 6-fold enrichment in the nuclear compartment for U6 snRNA with Northern blot assay (**Figure 1B**). Furthermore, the results from Western blotting showed exclusive expression of the protein markers HDAC2 and beta-Actin in the nuclear and cytoplasmic fraction, respectively (**Figure 1C**). Together, these results demonstrate that the used fractionation protocol can effectively separate nuclear and cytoplasmic compartments.

As a common practice, the raw data obtained from high throughput methods such as microarray are first normalized before the differential expression between two samples is calculated. Since we wanted to calculate the absolute enrichment of miRNAs in the nuclear compartment compared to the cytoplasmic compartment, we supplemented total RNA samples with spike-in oligoribonucleotides (18 nt, 24 nt, 30 nt) for normalization. Furthermore, in order to detect hybridization signals



	NUC-1	NUC-2	NUC-3	CYT-1	CYT-2	CYT-3
NUC-1	1.000					
NUC-2	0.886	1.000				
NUC-3	0.901	0.945	1.000			
CYT-1	0.846	0.744	0.754	1.000		
CYT-2	0.810	0.796	0.782	0.939	1.000	
CYT-3	0.798	0.755	0.804	0.927	0.950	1.000

FIGURE 1 | MiRNA profiling by microarray after nucleo-cytoplasmic fractionation of neurons. (A) qRT-PCR analysis of marker genes to validate the fractionation protocol. The fold enrichment (y-axis) of marker genes in the nucleus was calculated by the 2^{-dCt} $[2^{-(NUC\ Ct-CYT\ Ct)}]$ method. Bar plots show mean ± standard deviation (*SD*; $n = 3$). Statistical significance was determined using Student's *t*-test with Bonferroni correction (*$p < 0.05$; **$p < 0.01$). **(B)** Northern blot analysis of the nuclear marker U6 snRNA in nuclear and cytoplasmic fractions. Intensity of the signal was quantified using ImageJ. **(C)** Detection of nuclear (HDAC2, histone deacetylase 2) and cytoplasmic (beta-Actin) marker proteins in the subcellular fractions using Western blotting assay. Whole cell lysate was used as an input sample. **(D)** Comparison of different biological replicates from microarray experiments. Pearson's correlation coefficients between indicated samples are shown. Data on gray background represents correlation coefficients for biological replicates from the same cellular fraction. **(E)** Distribution of miRNA expression in the nucleus and the cytoplasm. Scatterplot of $\log_2$ transformed signal intensity values for miRNAs from nuclear (x-axis) and cytoplasmic (y-axis) fractions (267). Dots above the diagonal indicate cytoplasmic enrichment, below, nuclear enrichment of the respective miRNAs.

originating primarily from mature miRNAs, we size-selected total small RNAs (from 15 to 35 nt) from equal amounts (12 μg) of nuclear and cytoplasmic total RNA by 15% denaturing urea polyacrylamide gel electrophoresis (PAGE).

To determine expression levels of nuclear and cytoplasmic mature miRNAs, size-selected small RNA samples (3 nuclear and 3 cytoplasmic samples) were analyzed by miRNA microarrays (LCSciences), containing probes for 679 rat mature miRNAs (miRBase version 16). In total, we were able to detect 267 mature miRNAs which were common to both nucleus and cytoplasm (Table S1). To check the reproducibility of microarray profiling, we compared data obtained from three different biological replicates of fractionations. All three biological replicates performed with cytoplasmic fractions exhibited similar expression patterns (Pearson's correlation coefficient, $r = 0.93$–95; **Figure 1D**). Likewise, all nuclear fractions showed comparable expression, albeit with a slightly lower correlation coefficient ($r = 0.89$–0.94; **Figure 1D**). Together, these data suggest that fractionations were reproducible and the microarray profiling procedure and normalization was appropriate. Interestingly, samples from nuclear and cytoplasmic compartments had a lower correlation coefficient ($r = 0.74$–0.85) between datasets (**Figure 1D**), implying that the miRNA expression profiles of nuclear and cytoplasmic compartments are distinct. The average expression of the majority of miRNAs was lower in the nucleus compared to the cytoplasm (**Figure 1E**), indicating that most of the miRNAs, as expected, are preferentially located in the cytoplasm.

To identify a set of nuclear-enriched miRNAs, we first calculated a nuclear enrichment score [NEnS; $\log_2$(NUC/CYT)] for all miRNAs, by $\log_2$ transforming the average ratio of nuclear/cytoplasmic signal intensity values (Table S1). The NEnS for individual miRNAs ranged from 10.14 to −3.50 with a

median of −1.12, suggesting that on average miRNA expression in the cytoplasm is ∼2-fold higher than in the nucleus. From a total of 267 miRNAs, 91 miRNAs (34.1%) displayed a statistically significant differential distribution between nuclear and cytoplasmic compartments (student's *t*-test, $p < 0.05$; Table S2). Among them, 87 (32.6%) miRNAs were preferentially found in the cytoplasm, and only 4 (1.5%; miR-133b*, miR-365*, miR-328a*, miR-92a) in the nucleus. Three of these miRNAs (miR-133b*, miR-365*, and miR-328*) were not previously reported to be expressed in neuronal cells. Therefore, to validate our results and to obtain a more comprehensive coverage of nuclear miRNAs, we decided to perform in addition deep sequencing of small RNAs from our fractionation experiment.

DEEP SEQUENCING OF SMALL RNAs FROM NUCLEAR AND CYTOPLASMIC FRACTIONS

In comparison to microarrays, deep sequencing-based profiling of small RNAs is more sensitive and allows to study the expression of miRNAs at nucleotide resolution. Furthermore, it allows to discriminate mature and precursor forms of miRNAs. Importantly, it also provides information about variable isoforms of miRNAs, so called isomiRs, and the nature of the associated nucleotide modifications. We used the Illumina-platform for deep-sequencing of small RNA libraries obtained from different compartments of rat primary cortical neurons (DIV7). To ascertain reproducibility of the results, we used two biological replicates for each cellular fraction. Moreover, to obtain a deep coverage of all possible isomiRs and to eliminate the effect of multiplexing artifacts which can result from different barcodes used in small RNA libraries, each of the small RNA libraries (2 nuclear and 2 cytoplasmic) were sequenced in individual lanes of one Illumina HiSeq flow cell.

In total, we obtained ∼62 and 66 million sequence read counts for nuclear and cytoplasmic fractions, respectively. After filtering reads according to the length (>15 nt), contamination (adapter sequences), quality and abundance (at least 2 identical reads per unique sequence), ∼19 and 41 million "clean" reads, respectively, remained for further analysis. These reads were mapped to the publicly available rat RNA and genomic databases (rn4; **Table 1**; see Methods). To compare the abundance of read counts between two cellular fractions, a normalization according to the total number of mapped clean reads (nuclear—17,883,861; cytoplasmic—37,917,208) was performed. Interestingly, the number of normalized read counts from the nucleus matching to mature miRNA was 3–4-fold lower (depending on biological replicate) than in the cytoplasm (**Figure 2A** and Table S3). As expected, the nucleolar/nuclear small RNAs (snoRNA and snRNA) were highly enriched (63–89-fold and 16–17-fold, respectively) in the nuclear fraction; in contrast, cytoplasmic tRNAs were depleted (3-fold) in this fraction, again showing the purity of the cellular fractions used for sequencing. Furthermore, the expression of mature miRNAs in two biological replicates for each cellular fraction showed very high Pearson's correlation coefficient (nuclear, $r = 0.99$; cytoplasmic, $r = 0.98$), demonstrating a high reproducibility of the experiments (**Figures 2B,C**).

Table 1 | The summary of small RNA deep sequencing.

	Total NUC read counts	Total CYT read counts	Total read counts
Total	62,931,202	65,995,793	128,926,995
disc*_<15 nt	7,163,839	7,572,786	14,736,625
disc_adapter only	870,091	1,389,233	2,259,324
disc_non-clipped	29,780,435	10,399,717	40,180,152
disc_qual_low	1,156,960	2,065,820	3,222,780
disc_one read per condition	4,968,122	3,930,256	8,898,378
mature miRNA (miRBase v19)	3,171,512	23,941,026	27,112,538
precursor miRNA (miRBase v19)	72,675	576,251	648,926
snoRNA	5,880,089	166,704	6,046,793
snRNA	2,352,776	300,101	2,652,877
rRNA	181,268	436,816	618,084
tRNA	247,157	1,688,530	1,935,687
Mt_tRNA	66,111	463,741	529,852
Mt_rRNA	87,352	147,452	234,804
miscRNA	218,657	466,009	684,666
piRNA	387,290	672,414	1,059,704
mRNA_coding sequence	104,619	330,211	434,830
mRNA_3UTR	96,117	92,012	188,129
mRNA_1000 TSS + 5UTR	138,915	289,055	427,970
rat genome (rn4)	4,879,323	8,346,886	13,226,209
mappable_all	17,883,861	37,917,208	55,801,069
not mapped	913,165	2,663,540	3,576,705

*Numbers represent raw (non-normalized) read counts. *disc_ discarded.*

In total, we identified 335 miRNAs represented by at least 100 reads in one of the cellular compartments (Table S4). The size distribution of reads mapping to mature miRNAs peaks at 23 nt (**Figure 2D**), but not at 22 nt as was previously observed (Lee et al., 2010), probably owing to the high expression of miR-9 (49 and 44% of total reads in nuclear and cytoplasmic fractions, respectively). The overall distribution of read length in the nucleus was similar to the cytoplasm, but as mentioned above, with less total reads. The NEnS for individual miRNA ranged from 1.88 to −5.58 and the median was −2.08, when all detected miRNAs are considered (Table S4). Only two miRNAs, miR-143 and miR-126* possessed a positive NEnS, indicating that these miRNAs are enriched in the nucleus according to deep sequencing. Since only two biological replicates were generated, the statistical parametric analysis was not applicable.

COMPARISON OF MICROARRAY AND DEEP SEQUENCING

Two hundred and twenty miRNAs were commonly detected by both microarray and deep sequencing methods, whereas 47 and 115 were specific for microarray or deep sequencing, respectively (**Figure 3A**). The comparison of miRNA expression [$\log_2$(signal

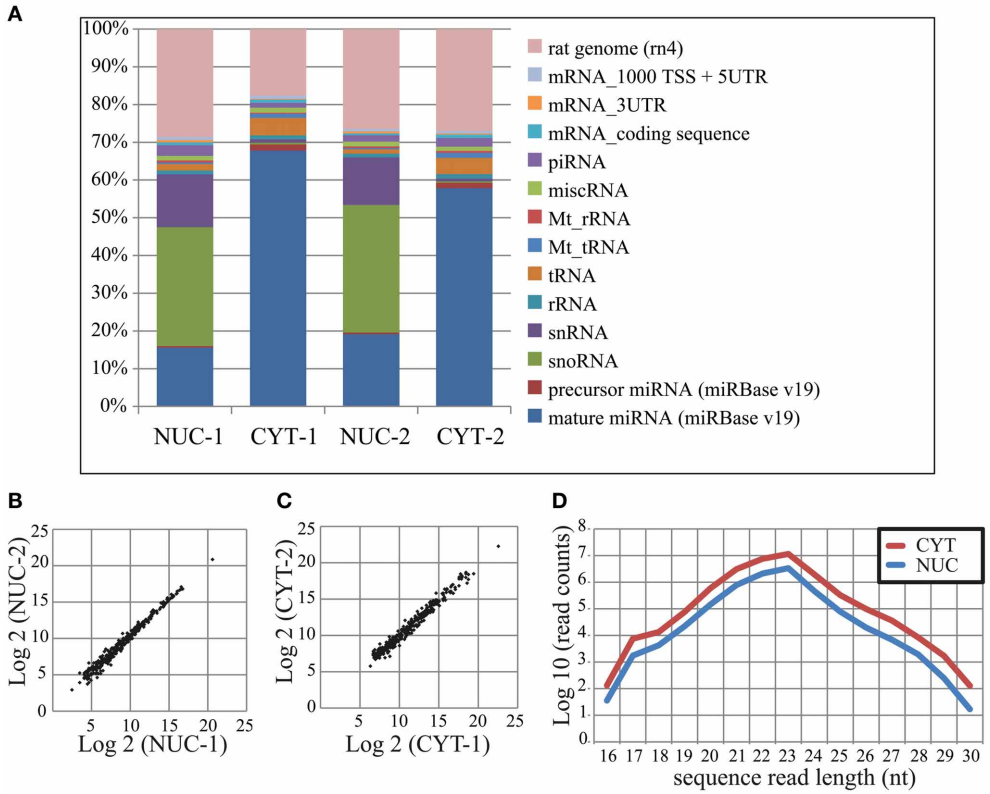


FIGURE 2 | Small RNA deep sequencing after nucleo-cytoplasmic fractionation of neurons. (A) Proportional distribution of mapped sequencing read counts in the small RNA libraries. The total read counts were set to 100% for each small RNA library. **(B,C)** Scatterplots of $\log_2$ transformed read counts mapping to mature miRNAs from the nuclear **(B)** and cytoplasmic **(C)** fractions (two biological replicates each). **(D)** The sequence length distribution (x-axis) of reads mapping to mature miRNAs. The read counts (y-axis) were $\log_{10}$ transformed.

intensity or read count)] data obtained with these two methods showed a Pearson's correlation coefficient of 0.63 and 0.71 for nuclear and cytoplasmic miRNAs, respectively (**Figures 3B,C**), suggesting that overall there is a correlation in the expression patterns between the datasets obtained from different methods. However, the correlation coefficient is much lower compared to biological replicates (**Figures 1D**, **2B,C**). The major effect contributing to the difference between the data is probably that deep sequencing is more sensitive than microarray as illustrated in **Figures 3B,C**. The points corresponding to the low expressed miRNAs (data points $\log_2 = \sim 5$ on y-axis) according to microarray are shifted toward the right side of the x-axis, indicating that deep sequencing, in contrast to microarray, can effectively detect and discriminate between low expressed miRNAs. This is even more apparent in the nuclear fraction (**Figure 3B**). Since the NEnS of miRNAs is calculated from the $\log_2$ transformed ratio of nuclear and cytoplasmic expression levels (signal intensity or read counts), a cross-platform difference in detection efficacy of miRNA expression might result in rather different NEnS for the same miRNA depending on the method. Indeed, NEnS scores for miRNAs obtained from microarray and deep-sequencing experiments showed no correlation (Pearson's correlation coefficient, $r < 0.1$, data not shown), and therefore statistical parametric analysis was not applicable. Hence we sought to employ alternative statistical methods to compare datasets from microarray and deep sequencing.

Rank-based non-parametric statistics employs the ranks instead of actual expression levels to identify differentially expressed genes (Hong et al., 2006). Therefore, this type of analysis is less sensitive to "noise" between the data obtained using different high-throughput platforms, such as microarray (Hong and Breitling, 2008) and deep sequencing (Llorens et al., 2013), and allows determining the genes, in our case miRNAs, which are consistently high-ranked in data obtained using different methods. We used the Rank Sum method to identify miRNAs, which possess consistently high (higher than other miRNAs) NEnS ranking in both microarray and deep sequencing, and therefore potentially might be enriched in the nucleus (Hong et al., 2006; Laing and Smith, 2010). For this analysis miRNAs that were detected by both platforms (220) were considered. As illustrated in the rank-based heatmap, miRNAs are color-coded from red to white in descending rank order for each biological replicate separately (**Figure 4**, columns 1–5 and Table S5) and together (**Figure 4**, column 6 and Table S5). Despite some differences in the ranking, the overall ranking of miRNAs is highly similar not only between different biological replicates, but also between different technological platforms. After applying Benjamini-Hochberg false discovery rate (FDR)

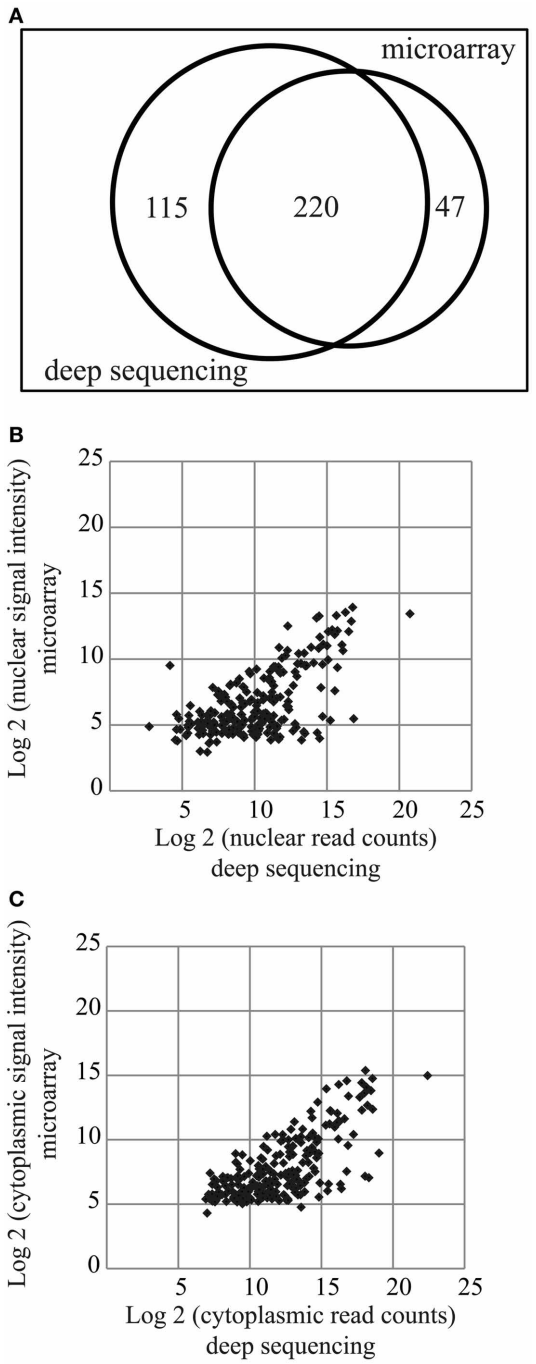


FIGURE 3 | Comparison of miRNA expression profiles obtained from miRNA microarrays and small RNA deep sequencing. (A) Venn diagram illustrating miRNAs detected by the two different methods. 220 miRNAs were detected by both methods. **(B,C)** Scatterplot of $\log_2$ transformed signal intensity values (microarray, y-axis) and read counts (deep sequencing, x-axis) for miRNAs detected in the nuclear **(B)** or cytoplasmic **(C)** fractions.

of 0.05 for multiple testing (Benjamini et al., 2001), we identified 8 miRNAs, which were significantly higher ranked among the biological replicate experiments of microarray and deep sequencing (**Table 2**), suggesting that these miRNA might be

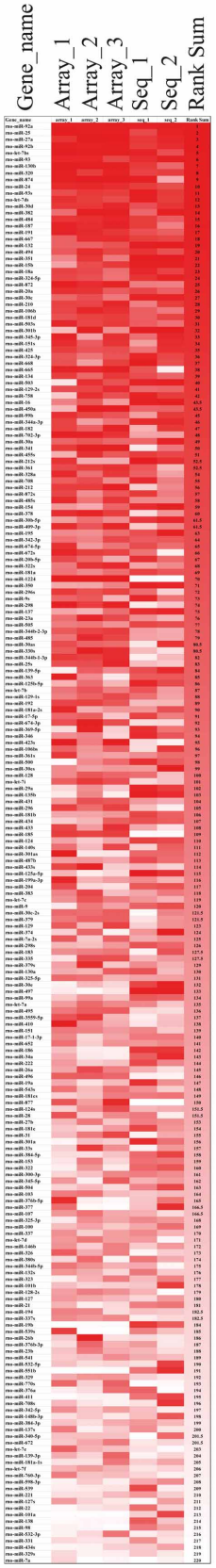


FIGURE 4 | Continued

FIGURE 4 | Alignment of miRNAs according to their miRNA nuclear enrichment scores (NEnS) obtained with microarrays and deep sequencing. MiRNAs were ranked from high (red) to low (white) NEnS for each experiment separately (array_1, _2, _3, seq_1, _2), and then the average ranking was calculated and arranged in descending order based on the Rank Sum method (Laing and Smith, 2010).

Table 2 | Top 10 high-ranked and top 10 low-ranked miRNAs according to Rank Sum method.

miRNA_name	Rank sum rank
rno-miR-92a	1
rno-miR-25	2
rno-miR-27a	3
rno-miR-92b	4
rno-let-7b*	5
rno-miR-93	6
rno-miR-130b	7
rno-miR-320	8
rno-miR-874	9
rno-miR-24	10
rno-miR-127*	211
rno-miR-22	212
rno-miR-101a	213
rno-miR-138	214
rno-miR-98	215
rno-miR-532-3p	216
rno-miR-331	217
rno-miR-434*	218
rno-miR-329*	219
rno-miR-7a	220

preferentially localized to the neuronal nuclei compared to the vast majority of miRNAs. Importantly, the synaptic miR-7a and miR-138 (Siegel et al., 2009) were among the 10 most low ranked miRNAs (i.e., cytoplasmic; **Table 2**), suggesting that the rank-based analysis method is able to faithfully detect differences in the intracellular distribution of miRNAs.

VALIDATION OF NUCLEAR-ENRICHED miRNA CANDIDATES IDENTIFIED BY PROFILING APPROACHES

To validate results obtained using microarray and deep sequencing with a Rank Sum analysis, we decided to perform Northern blot, which allows size-separation and visualization of miRNAs with different sizes, including mature miRNA. As shown in **Figure 5A** (and **Figures S1B,C**), the mature form of four highly ranked miRNAs (miR-92a, miR-25, miR-27a, and miR-92b) was higher or equally expressed in the nuclear fraction compared to the cytoplasm. In contrast, a low–ranked miRNA, miR-138 (rank = 214), showed the opposite expression pattern. Interestingly, if only one method, for instance deep sequencing, is taken into account to calculate nuclear-enrichment, then the ranks for miR-92a, miR-25, miR-27a, and miR-92b are 7, 31, 28 and 34, respectively (Table S5). According to the same method miR-132 is ranked 3, implying that this miRNA should be more nuclear enriched than the other four. However, miR-132 possessed slightly less signal in the nucleus compared to the cytoplasm by Northern, which is more in line with the ranking (rank = 19) when both methods (Rank Sum) are taken into account (Table S5). A similar rank correction is observed for miR-19b (deep seq rank = 49; Rank Sum rank = 184), for which Northern showed a similar depletion of signal in the nucleus compared to the cytoplasm as miR-138 (**Figure 5A**). Likewise, the miRNAs highly ranked using only microarray data are either not detected by deep sequencing (miR-133b*, miR-365*, and miR-328*) or their ranking (Rank Sum) is decreased considerably (miR-1224; **Figure 5A** and Table S5). This is in line with the Northern blot data which suggests that the nuclear signal for these miRNAs is possibly originating from by-products of pre-mRNA splicing or non-coding RNA transcription, but not from the mature miRNA (**Figure 5A**). Taken together, these results confirm that some of the highly ranked miRNAs (miR-92a, miR-25, miR-27a, and miR-92b) are indeed enriched in the nucleus and also indicates the robustness of the rank-based statistical analysis to identify nuclear-enriched or -depleted miRNAs.

In addition to Northern blot assay, we further validated nuclear enrichment of the two top candidate miRNAs, miR-25 and miR-92a using TaqMan qRT-PCR. In agreement with results from Northern blot, miR-25 and miR-92a showed a significant nuclear enrichment compared to GAPDH, with a NUC/CYT fold change of 1.81 and 1.41, respectively (**Figure 5B**). As expected, the nuclear marker gene U6 was enriched (11.48) in the nucleus, whereas the cytoplasmic marker gene GAPDH was depleted (0.36), once more demonstrating that the cellular fractionation protocol was efficient in separating nuclei and cytoplasm.

For all experiments so far, we used total RNA from nuclear and cytoplasmic compartments. This RNA was obtained from a biochemical fractionation method that relies on differential centrifugation. With this method, it is difficult to achieve complete separation of compartments, and therefore the obtained results might not entirely reflect the natural distribution of miRNAs in intact neurons. Moreover, biochemical preparations likely contain a mixture of RNA from different cell types, e.g., neurons and glia. Thus, we performed in addition fluorescent *in situ* hybridization (FISH) with LNA probes to precisely determine localization of nuclear-enriched miRNAs (miR-25 and miR-92a) in intact primary rat hippocampal neurons (DIV5) at the single cell level (**Figure 5C**). After application of a FISH probe against miR-25 and miR-92a a stronger fluorescent signal in the neuronal nucleus compared to the cytoplasm was observed, indicating that these miRNAs are preferentially localized in the nucleus of intact neurons. Conversely, the cells hybridized with a probe against miR-9 (Rank Sum rank = 120) displayed a stronger fluorescent signal in the cytoplasm compared to the nucleus. Accordingly, quantification of FISH signal from many cells revealed that the ratio between nuclear and cytoplasmic signals for miR-25 and miR-92a was significantly higher ($p = 0.002$ and 0.0006, respectively, Student's t-test) than miR-9 (**Figure 5D**). Taken together, our results from Northern, qRT-PCR and FISH strongly suggest that miR-25 and miR-92a are enriched in the nucleus of post-mitotic primary rat neurons.

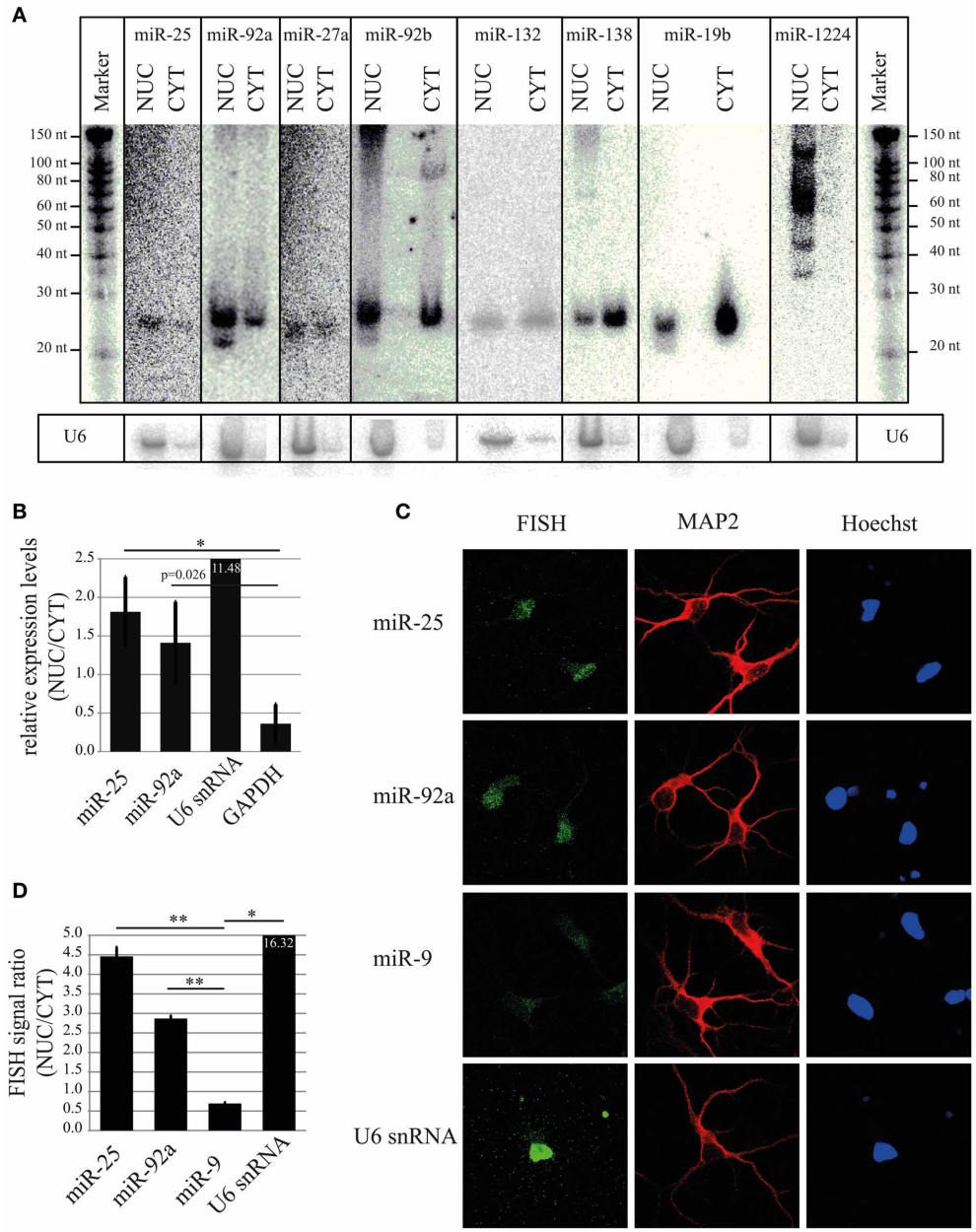


FIGURE 5 | Validation of nuclear expression for selected miRNA candidates. (A) Northern blot analysis of nuclear-enriched (miR-25, miR-92a, miR-27a, miR-92b) and -depleted miRNAs (miR-138, miR-19b). As a control for the fractionation efficacy, U6 snRNA was probed. **(B)** qRT-PCR analysis of nuclear-enriched miRNAs (miR-25 and miR-92a). The fold enrichment (y-axis) of miRNAs and marker genes (U6 snRNA and GAPDH) in the nucleus was calculated by the 2^{-dCt} $[2^{-(NUC\ Ct-CYT\ Ct)}]$ method. Bar plots show mean ± *SD* ($n = 3$). Statistical significance was determined using Student's *t*-test with Bonferroni correction ($^{*}p < 0.05$). **(C)** Subcellular localization of the indicated miRNAs at DIV5 hippocampal neurons as assessed by fluorescent *in situ* hybridization assay (FISH) using Digoxigenin (DIG) labeled miRCURY LNA probes (green). FISH for U6 was used as a positive control for nuclear localization. MAP2 protein was used to identify neurons (red). Hoechst counterstain was used to label nuclei (blue). **(D)** Quantification of nuclear localization from FISH experiment presented in **(C)**. Signal intensities within the nucleus and cytoplasm were determined with ImageJ. The ratios of nuclear/cytoplasmic signal intensities are shown as an indicator for nuclear enrichment. Bar plots show mean ± *SD* ($n = 2$; 10 cells per condition of a single experiment). Statistical significance was determined using Student's *t*-test with Bonferroni correction ($^{*}p < 0.05$; $^{**}p < 0.01$).

DEVELOPMENTAL EXPRESSION LEVELS OF miRNAs AND THEIR NUCLEAR ENRICHMENT

In order to obtain a first indication at which developmental stage miR-25 and miR-92a might function in neurons, we performed a developmental time-course experiment, quantifying the relative expression levels of mature miRNAs at 4, 11, 18, 25 DIV in cortical neurons using qRT-PCR (**Figure 6A**). The expression levels of both miR-25 and miR-92a were significantly declining

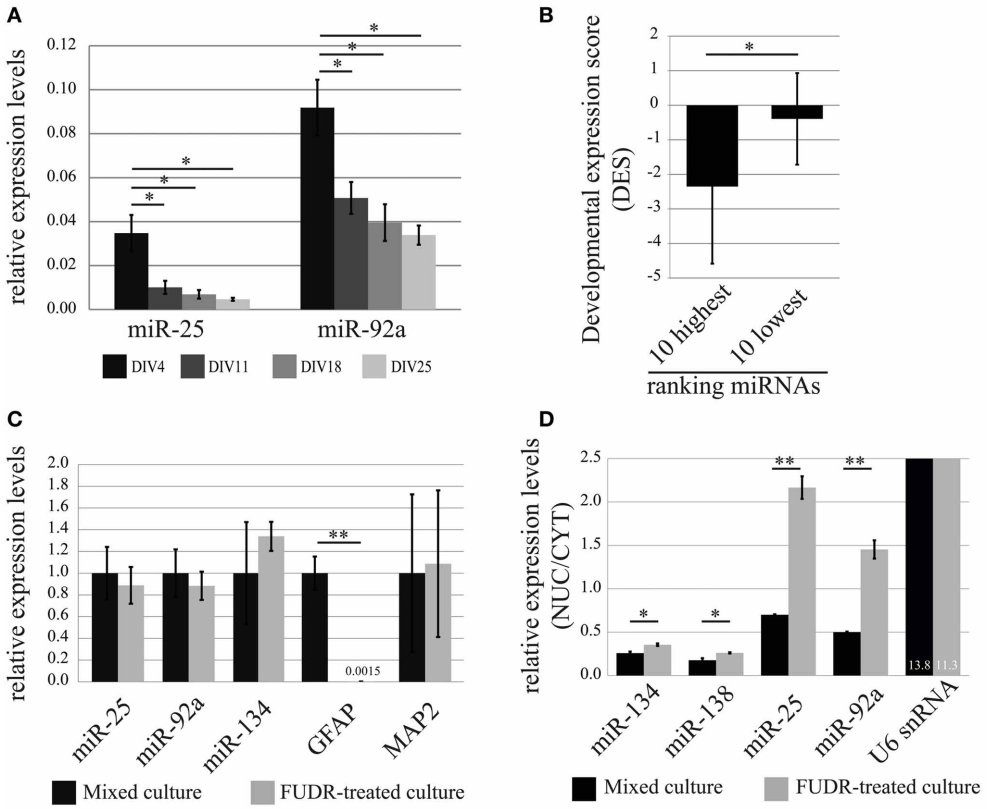


FIGURE 6 | Developmental stage and cell-type-specific expression of the nuclear-enriched miRNAs, miR-25 and miR-92a. (A) Relative expression (normalized to U6 snRNA) levels of miR-25 and miR-92a during *in vitro* development of primary cortical neurons was determined by qRT-PCR analysis. Bar plots show mean ± *SD* ($n = 2$). Statistical significance was determined using Student's *t*-test with Bonferroni correction (*, $p < 0.05$). **(B)** Developmental expression score (DES; $\log_2$(P3/E10) from (Yao et al., 2012); y-axis) comparison of 10 highest and lowest ranked miRNAs. Error bars represent standard deviation from the mean DES within each group. Statistical significance was determined using Student's *t*-test ($p = 0.028$). **(C)** Expression of miR-25 and miR-92a in mixed cultures and neuronal-enriched cultures (FUDR-treated). The relative expression levels of indicated RNAs were obtained by the ddCt method. RNA levels in mixed cultures were arbitrarily set to 1. Bar plots show mean ± *SD* ($n = 3$). *SD* for mixed culture condition was determined after normalization to an internal control RNA (U6 snRNA). Statistical significance was determined based on U6 snRNA normalized values using Student's *t*-test with Bonferroni correction (**$p < 0.01$). **(D)** Nuclear-enrichment of miRNA expression in mixed and neuron-enriched (FUDR-treated) cultures. The expression level of miRNAs was determined using qRT-PCR analysis with TaqMan microRNA assay. Bar plots show mean ± *SD* ($n = 2$). Statistical significance was determined using Student's *t*-test with Bonferroni correction (*$p < 0.05$; **$p < 0.01$).

with the progress of neuronal development, whereby the decrease in expression of miR-25 was more pronounced compared to miR-92a. At the end of the developmental time-course (DIV25), expression levels of miR-25 and miR-92a were reduced by 80% and 60%, respectively, compared to DIV4. Taken together, our results indicate that expression of miR-25 and miR-92a is down-regulated during post-mitotic neuronal development. However, since measurements were started at DIV4, we cannot rule out that the peak of expression for these miRNAs is actually even earlier in development.

Based on our observations, we tested the hypothesis that developmental down-regulation might be a common feature of nuclear-enriched miRNAs. We therefore calculated a developmental expression score (DES; see Methods) for each miRNA present in our ranking list based on a recent study which reported genome-wide miRNA expression profiles during development of the rat cortex *in vivo* (Yao et al., 2012). A negative DES would hereby indicate that the expression level of the respective miRNA is down-regulated during rat cortex development. Indeed, we observed a trend toward an increase of the average DES from high (nuclear-enriched) to low (cytoplasmic-enriched) ranking miRNAs, suggesting that down-regulation during rat cerebral cortex development is a common feature of nuclear-enriched miRNAs (**Figure S1D** and Table S6). Accordingly, the DES of two extreme groups consisting of the 10 highest and lowest ranked miRNAs (hence, the most reliable in terms of nucleo-cytoplasmic localization), differ significantly ($p = 0.028$; Student's *t*-test) with an average DES of −2.35 and −0.39, respectively (**Figure 6B**).

Taken together, these findings suggest that nuclear-enriched miRNAs in general might be expressed at early stages of neuronal development and that their decline in expression levels during development correlates with nuclear enrichment.

MiR-25 AND miR-92a ARE SPECIFICALLY ENRICHED IN NEURONAL NUCLEI, BUT NOT IN GLIA

Results from recent publications suggest that miR-25 and miR-92a might be preferentially expressed in glia compared to neurons (Jovicic et al., 2013). In order to investigate the contribution of glial cells to the expression of miR-25 and miR-92a in our primary cortical neuronal cultures, we further decided to test expression levels of these miRNAs in glia-depleted and glia-enriched neuronal cultures. Primary cortical neurons prepared with our standard protocol contain a substantial amount of proliferating glial cells (10–20% of total cells at DIV7, data not shown). We therefore considered the possibility that glia-derived miRNAs could significantly contribute to the results concerning nuclear-enrichment of miRNAs in neurons. To obtain glia-depleted culture, we cultured cells in the presence of a potent inhibitor of cell proliferation, 2′-Deoxy-5-fluorouridine (FUDR), and relative expression levels of miRNAs were assessed by qRT-PCR. Depletion of glial cells in our cultures was verified by the quantification of the astrocytic marker gene glial fibrillary acidic protein (GFAP), which was almost completely absent in FUDR-treated cultures (**Figures 6C, S2A**). The expression of neuronal marker genes, miR-134 and MAP2 were not significantly affected by FUDR-treatment, suggesting that the overall contribution of RNA from glial cells to the total RNA in our mixed cultures is small. Importantly, the nuclear-enriched miRNAs, miR-25 and miR-92a, in contrast to GFAP, were only slightly reduced in FUDR-treated cultures, showing that the expression of these miRNAs in our mixed cultures is predominantly derived from neurons, with a small contribution from glia.

Experiments carried out on glia-depleted cultures indicate that the overall contribution of glial cells to the expression of nuclear-enriched miRNAs in our mixed cultures is small. However, they do not rule out that the expression of these miRNAs in an individual glial cell is in fact higher compared to that in an individual neuron. We therefore established a culture protocol that strongly favors the growth of glial cells (approximately 50–60% are glial cells; data not shown). In glia-enriched cultures, we could detect higher expression of GFAP, and lower expression of miR-134 and MAP2 compared to mixed culture, suggesting that these culture conditions indeed favored the growth of glial cells (**Figure S2B**). Interestingly, miR-25 and miR-92a displayed a 2.2 and 1.5-fold, respectively, higher expression in glia-enriched cultures compared to mixed culture, suggesting that expression of these miRNAs is in fact higher in individual glial cells compared to neurons.

Finally, we wanted to compare nuclear enrichment in glial cells and neurons. For this we fractionated mixed and FUDR-treated cultures in nuclei and cytoplasm, and then measured RNA expression by TaqMan qRT-PCR (**Figure 6D**). Interestingly, the nuclear enrichment of both miR-25 and miR-92a was on average 3-fold higher in FUDR-treated cultures compared to mixed cultures. In contrast there was no significant change in the nuclear enrichment of miR-134, miR-138 and U6 snRNA, demonstrating the specificity of the assay. These results suggest that miR-25 and miR-92a are specifically enriched in the nucleus of neurons, but not glial cells, where they might instead preferentially localize to the cytoplasm.

In summary, although miR-25 and miR-92a are clearly expressed in glial cells, the major contributors to their expression in mixed cultures are neurons. Furthermore, nuclear-enrichment of these miRNAs is a specific feature of neurons. These results are consistent with FISH and suggest a specific function of miR-25 and miR-92a in the nucleus of post-mitotic neurons.

INSPECTION OF NUCLEAR miRNAs FOR COMMON SEQUENCE CHARACTERISTICS

Since localization of RNAs to distinct cellular compartments is known to be dependent on specific cis-acting sequences (Jambhekar and Derisi, 2007), we decided to search for common cis-acting elements that might target miRNAs to the neuronal nucleus. In this regard, it was shown that a 3′ hexanucleotide motif (AGUGUU) is sufficient to direct miR-29b into the nucleus of HeLa cells (Hwang et al., 2007; Jeffries et al., 2010). Furthermore, it was reported that in human neural progenitor cells, 7 out of 21 miRNAs with preferential nuclear localization possess an ASUS (S = G or C; this motif is also included in the aforementioned miR-29b) motif within the last 3′ 9 nt (Jeffries et al., 2011). However, the ASUS motif was neither enriched nor depleted in the last 3′ 10 nt of two extreme groups consisting of the top 20 high-ranked and top 20 low-ranked miRNAs (Table S5), suggesting that in contrast to the results from non-neuronal systems (Jeffries et al., 2011) the ASUS motif does not function as a nuclear localization signal in neurons.

Furthermore, it was reported that miRNAs which have the same seed sequence and a similar composition of the nine 3′-terminal nt, are likely to be enriched in the same cellular compartment [nuclear or cytoplasmic; (Jeffries et al., 2011)]. In agreement, we found that three members of a miRNA family [miR-92a (rank = 1), miR-25 (rank = 2) and miR-92b (rank = 4)] which in addition to the seed share a common 3′-terminus are high ranked, whereas another member of the same family with a different 3′-terminus [miR-363 (rank = 85); **Figure S3**] is low ranked. However, some other miRNA pairs with similar nucleotide composition, such as miR-27a (rank = 3)/miR-27b (rank = 153) or miR-130b (rank = 7)/miR-130a (rank = 130) were not ranked together (**Figure S3**), suggesting that having the same seed together with a similar 3′-terminus alone is not sufficient to confer nuclear enrichment in neurons. Therefore, in addition to the similarity of seed and 3′-terminus, other sequence elements might also be important for nuclear localization of miRNAs.

A closer inspection of the nuclear rank list revealed that highly ranked miRNAs have a tendency to contain a guanine (G) at the 3′-terminus, whereas low ranking miRNAs often end with a uridine (U; **Figure S4**). However, a statistical analysis of the two extreme groups (top 10 high-ranked and 10 low-ranked miRNAs) did not show a significant difference (data not shown). We further investigated if the high-ranked miRNAs share any other sequence motifs. However, none of the online available multiple alignment and motif finding tools (ClustalW, MEME, LocARNA, Gibbs motif sampler) found any over-represented motifs among nuclear-enriched miRNAs (data not shown).

Taken together, bioinformatic inspection of miRNAs for putative cis-acting sequence elements revealed that in contrast to

previously published results, the ASUS motif at the 3′ region of miRNAs is evenly distributed through-out our ranking list, suggesting that in neurons this motif is unlikely to participate in nuclear localization. Moreover, a similar nucleotide composition alone is not a faithful predictor of nuclear enrichment, implying that nuclear localization of miRNAs probably involves multiple, sequence- and structure dependent mechanisms.

IsomiRs WITH A 3′-TERMINAL GUANINE PREFERENTIALLY LOCALIZE TO A NUCLEUS

Analysis of mature miRNA localization did not reveal the presence of a common sequence element responsible for nuclear accumulation. However, a slight trend for the presence of a 3′-terminal guanine was observed (**Figure S4**). We therefore took advantage of the high sequence coverage of our deep sequencing datasets, which allows the analysis of individual isomiRs, even those expressed at low levels. IsomiRs are variants of canonical miRNAs containing 5′ and 3′-end variations, which either result from a variability in the cleavage of Drosha and Dicer [templated nucleotide addition (TA) or trimming] or from non-templated nucleotide addition (NTA; **Figure 7A**) catalyzed by nucleotidyltransferases.

As previously reported by several groups (Lee et al., 2010; Zhou et al., 2012), we also found that the abundance of isomiR types was miRNA specific. For instance, the sequence reads for the canonical form (miRBase v19) and for 3′-terminal single nucleotide templated addition (TA_1) forms of miR-138 were equally abundant and together comprised more than 50% (cytoplasm) −70% (nucleus) of the total reads for this miRNA (**Figure S5**). In contrast, for miR-25 and miR-92a the canonical and TA_1 forms, respectively, were overrepresented by 70% in both cellular compartments (**Figure S5**). In order to determine the overall abundance of specific isomiRs in the nuclear and the cytoplasmic fractions we calculated the percentage of the isomiRs, considering the entire nuclear or cytoplasmic sample. MiR-9 was excluded from the analysis, since the read counts for this miRNA comprise 49 and 44% of the total reads in nuclear and cytoplasmic fractions, respectively, and therefore might change the overall isomiR profile considerably. Our analysis showed that canonical miRNAs added to 42.93 and 43.76% of the total nuclear and cytoplasmic sequence reads, respectively (**Figure 7B**). As previously reported (Wyman et al., 2011; Zhou et al., 2012), the most abundant form of isomiRs were non-templated additions of single adenine (7.16%-nucleus; 8.40%-cytoplasm) and uracil (6.37%-nucleus; 6.58%-cytoplasm) nt, both of which were slightly overrepresented in the cytoplasm. Interestingly, an overall relatively rare non-templated addition of a single guanine (NTA_G) was 4-fold higher in the nucleus (1.30%) compared to the cytoplasm (0.31%). Furthermore, isomiRs with templated addition of a single guanine (TA_G) were also more prominent in the nuclear (2.59%) than in the cytoplasmic (1.56%) fraction (**Figure 7C**). This calculation is based on the abundance of the sequence reads,

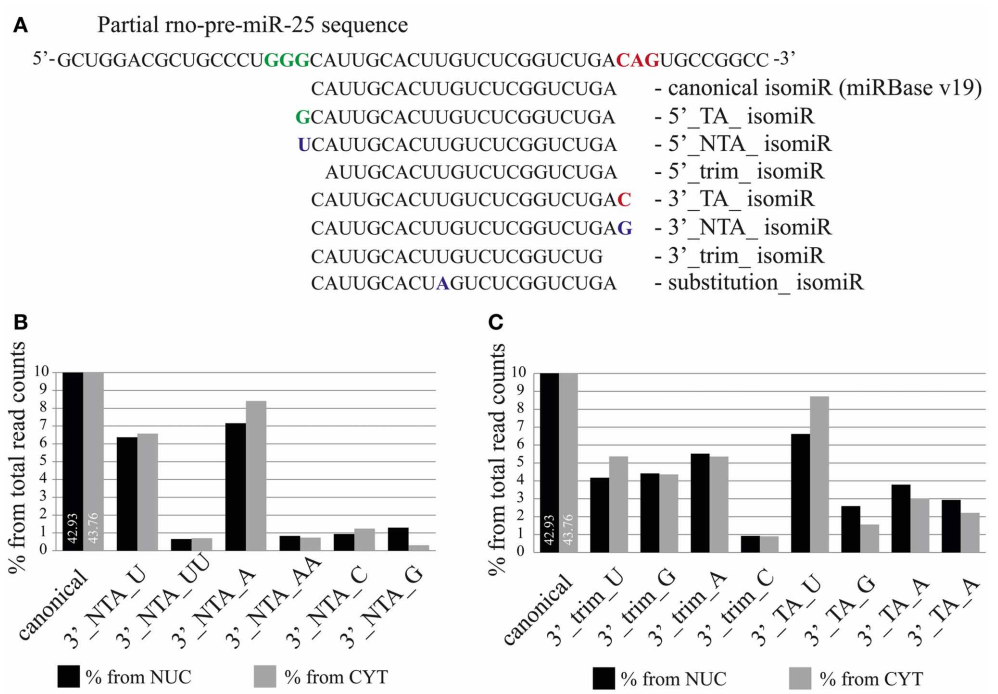


FIGURE 7 | The distribution of isomiRs between nuclear and cytoplasmic fractions of neurons. (A) Definition of different isomiR species. For illustration, miR-25 isomiRs aligned to the pre-mir-25 sequence are shown (TA, templated addition; NTA, non-templated addition; trim, trimmed). **(B,C)** Proportion of specific isomiRs from the total sequence reads which mapped to miRNAs in the nuclear (black) and the cytoplasmic (gray) fractions. **(B)** Proportion of isomiRs with specific 3′ non-templated additions. **(C)** Proportion of isomiRs with 3′ trimmed and templated additions. The respective added or trimmed nucleotides are indicated for specific isomiRs.

and distributions might be skewed by a few isomiRs of very abundant miRNAs. We note that the top 15 highly expressed miRNAs together account for 77.5% (cytoplasm) of all sequencing reads. In order to avoid the influence of the read counts, we first calculated a relative nuclear enrichment score (rNEnS; % of the nuclear fraction/% of the cytoplasmic fraction for a respective miRNA; see Methods) of isomiRs and then quantified the type (A, U, G, C) and occurrence of nt at the 3′-terminus of each unique sequence. Although we will be not able to differentiate the source of the last nucleotide variation (trimming, templated or non-templated additions) with this analysis, we can obtain an estimate how the 3′-terminal nucleotide influences nuclear localization. Strikingly, guanine at the 3′-terminus of isomiRs with a high rNEnS was strongly overrepresented compared other nt (**Figures 8A,B**). However, as the rNEnS declined guanine at the 3′-terminus became less frequent, whereas other nt (A, U, and C) were now more prominent. A closer inspection of specific miRNA isomiRs (**Figure S6**) confirmed our observation that isomiRs with a high rNEnS tend to possess a guanine nucleotide at their 3′-terminus. This data implies that 3′ guanine could promote nuclear accumulation of isomiRs.

In order to determine whether 3′-terminal G (canonical, trimmed, NTA, or TA), independent of the remaining sequence, has an impact on nuclear localization, we calculated rNEnS for different isomiRs (**Figures 8C–F**). Strikingly, the average rNEnS for non-templated (NTA_G; 4.08) and templated guanine added (TA_G; 2.99) isomiRs were significantly higher ($p =$ 4.2E-13 and 6.8E-07, respectively; welch t-test) than the average rNEnS of all canonical isomiRs (1.17), irrespective of the 3′-terminal nucleotide (**Figure 8C**). In contrast, the average rNEnS for NTA_C (0.95; $p =$ 4.9E-06) and NTA_A (0.99; $p =$ 2.6E-06) was lower than for all canonical isomiRs. These results suggest either the possibility of targeted guanylation in the nucleus or enhanced localization to the nucleus of isomiRs already possessing a 3′ guanine due to NTA_G or TA_G. We next calculated the impact of the 3′-terminal nucleotide in canonical isomiRs. Surprisingly, the average rNEnS for canonical isomiR_Gs (1.63) was also significantly higher than canonical isomiR_C (1.1; $p =$ 1.8E-09), _A (1.0; $p =$ 4.3E-12) and _U (1.1; $p =$ 1.6E-10) (**Figure 8D**). Furthermore, one nucleotide trimming of canonical isomiR_C, _A and _U and thereby exposing a guanine, but not other nt at the 3′-terminus, increased significantly the rNEnS for these isomiRs (Trim_N>G; N = A, C, or U; **Figures 8F, S7**). Conversely, one nucleotide trimming of canonical isomiR_G and thereby exposing nt other than guanine at the 3′-terminus significantly decreased the rNEnS (**Figure 8E**). Together, these results strongly argue that the guanine nucleotide at the 3′-terminus *per se* might lead to a preferential nuclear localization of isomiRs.

Taken together, we found that isomiRs possessing a 3′-terminal guanine nucleotide show preferential localization to the nucleus. The "origin" of this 3′-terminal guanine (NTA, TA, trimmed, or canonical) further influences the extent of nuclear localization.

DISCUSSION

It is increasingly recognized that miRNAs, in addition to their well described role as post-transcriptional regulators of mRNA translation/stability in the cytoplasm, are also involved in transcriptional (Kim et al., 2008; Place et al., 2008; Benhamed et al., 2012) and post-transcriptional (Hansen et al., 2011; Tang et al., 2012) regulatory processes in the nuclei of proliferating cells. However, a function of miRNAs in the nucleus of post-mitotic cells has not been described. As a first step in the determination of a putative nuclear role of miRNAs, we assessed the complete miRNA nuclear-enrichment profile and sequence-specific requirements that might aid (or be responsible for) the nuclear localization of miRNAs (and their isomiRs) in rat post-mitotic primary neurons.

In this study, we applied the two most common high-throughput profiling technologies, microarray and deep sequencing, to identify the nucleo-cytoplasmic distribution of miRNAs. In line with previous reports (Liao et al., 2010; Jeffries et al., 2011) we also detected the expression of almost all cytoplasmic miRNA counterparts in the nucleus. However, unlike these previous publications, our results from both profiling methods suggested that the majority of miRNAs are enriched in the cytoplasm and only a small subset in the nucleus. The discrepancy between these earlier findings and our current observations might be due to different cell types [cancer cell line (Liao et al., 2010), neural progenitor cells (Jeffries et al., 2011), post-mitotic neurons (this study)] used in these studies. It is also possible that the data normalization (Jeffries et al., 2011) and the power (Liao et al., 2010) of statistical analysis might have contributed. The normalization method performed by Jeffries et al. (2011) assumes that only a minority of genes are differentially expressed between conditions (i.e., normalized to the mean/median expression value of all miRNAs detected within the single replicate experiment). Without *a priori* knowledge of nucleo-cytoplasmic distribution of miRNAs, this type of data normalization might not be appropriate to measure the absolute differences in the expression levels of miRNAs (although this does not affect the nuclear-enrichment ranking between miRNAs) in nuclear and cytoplasmic compartments, since it equalizes otherwise initially different expression profiles in these compartments. To overcome this limitation and to measure absolute miRNA expression levels we therefore used exogenous controls, spike-in oligoribonucleotides (microarray) and total RNA/genomic mapped reads (deep sequencing) for cross-compartmental normalization of miRNA expression. Liao and colleagues used only one biological replicate for deep sequencing, thereby lacking any statistical power. In contrast, we used five biological replicates (3 for microarray and 2 for deep sequencing) and identified nuclear-enriched miRNAs in neurons based on the non-parametric Rank Sum method. Interestingly, the application of both microarray and deep sequencing gives more reliable results than each method separately with regard to the identity of nuclear-enriched miRNAs. Based on further validation results (Northern blot) of nuclear- and cytoplasmic-enriched miRNAs we presume that at most 5% of the 220 miRNAs analyzed by both profiling methods are truly nuclear-enriched miRNAs, although additional experiments are required to validate the expression of more high ranked miRNA candidates.

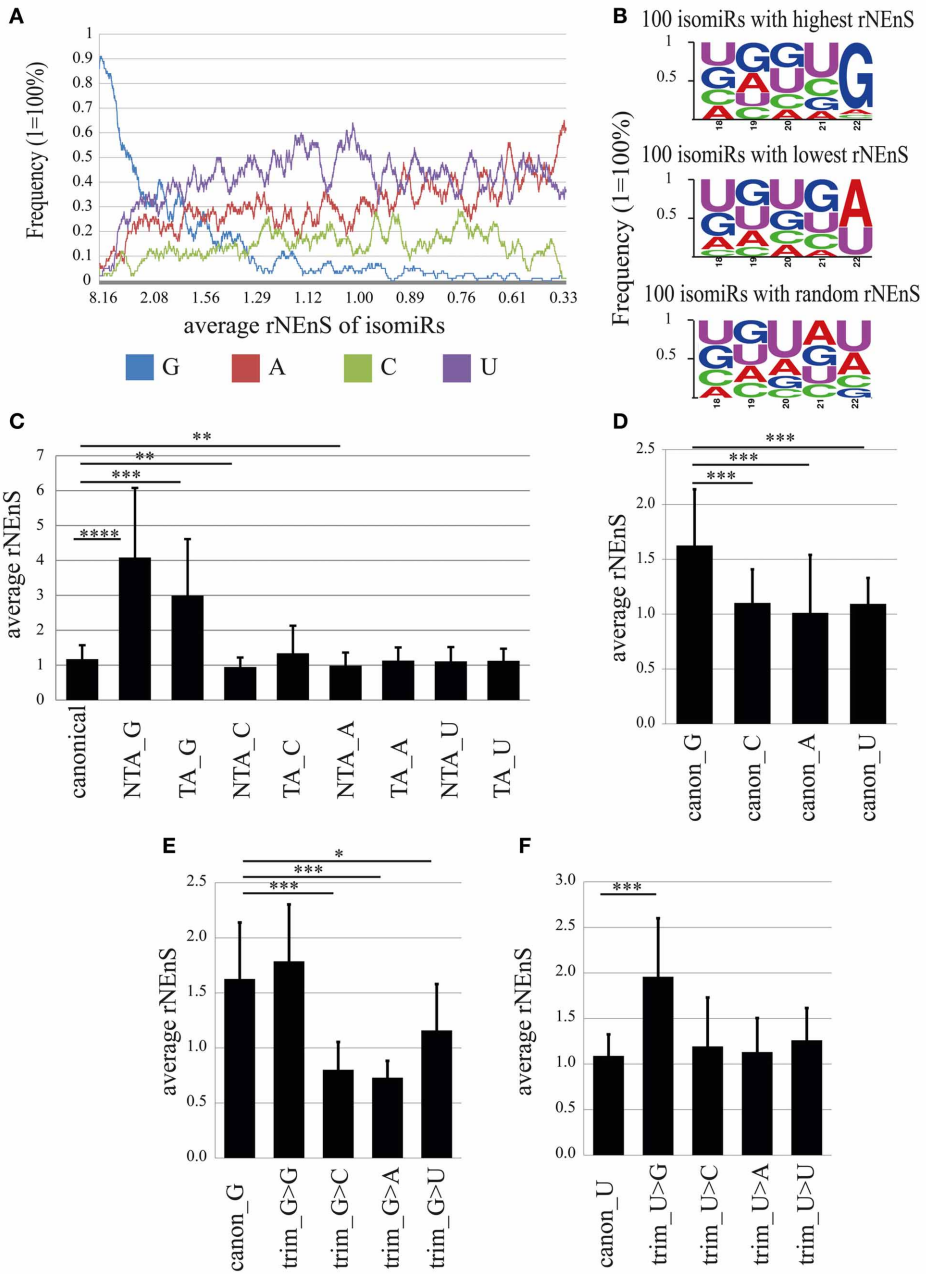


FIGURE 8 | The impact of the 3′-terminal nucleotide on nuclear localization. (A) Frequency of different nucleotides at the 3′-terminus of isomiRs depending on the relative nuclear enrichment score (rNEnS). Nucleotide frequency (y-axis) was calculated using moving window technique, where window length was set as 100 and the average frequency values were calculated by moving the window with one step at a time from isomiRs (in total 4661) possessing high to low rNEnS. In the x-axis, the average rNEnS of isomiRs using moving window technique with the same parameters as above is depicted. **(B)** Frequency of different nucleotides in the 3′-terminal 5 nts of 100 isomiRs with highest (upper panel), lowest (middle panel) and random rNEnS (lower panel). **(C)** Impact of 3′ non-templated (NTA) and templated additions (TA) on the relative nuclear localization. Bar plots show mean ± *SD* [*n* =from 31 (TA_G) to 306 (canonical)]. Statistical significance was determined using Welch's *t*-test (unequal variance) with Bonferroni correction (*$p < 0.05$; **$p < 0.001$; ***$p < 0.00001$; ****$p <$ 1.0-E10). **(D)** rNEnS for canonical isomiRs containing different 3′-terminal nucleotides. Bar plots show mean ± *SD* (canon_G, $n = 57$; canon_C, $n = 62$; canon_A, $n = 75$; canon_U, $n = 112$). Statistical significance was determined using Welch's *t*-test (unequal variance) with Bonferroni correction (*$p < 0.05$; **$p < 0.001$; ***$p <$ 1.0-E7). **(E,F)** Impact of trimming on the nuclear localization of isomiRs. **(E)** rNEnS for canonical isomiR_G that underwent 3′ trimming, thereby exposing the indicated nucleotide. Bar plots show mean ± *SD* (canon_G, $n = 57$; trim_G>G, $n = 12$; trim_G>C, $n = 14$; trim_G>A, $n = 17$; trim_G>U, $n = 9$). Statistical significance was determined using Welch's *t*-test (unequal variance) with Bonferroni correction (*$p < 0.05$; **$p < 0.001$; ***$p <$ 1.0-E5). **(F)** rNEnS for canonical isomiR_U that underwent 3′ trimming, thereby exposing the indicated nucleotide. Bar plots show mean ± *SD* (canon_U, $n = 112$; trim_U>G, $n = 32$; trim_U>C, $n = 29$; trim_U>A, $n = 10$; trim_U>U, $n = 20$). Statistical significance was determined using Welch's *t*-test (unequal variance) with Bonferroni correction (*$p < 0.05$; **$p < 0.001$; ***$p <$ 1.0–E5).

In addition to the overall distribution of miRNAs between the nuclear and cytoplasmic compartments, there is also little overlap regarding the identity of nuclear-enriched miRNAs among our and earlier reports, which might be accounted for by cell-type and differentiation stage (proliferating vs. non-proliferating) specific differences in miRNA expression. For example, members of the miR-25 family (miR-25 and miR-92a) are found to be preferentially localized in the cytoplasm of human neural stem cells (Jeffries et al., 2011), whereas we found that these miRNAs are enriched in the nuclei of post-mitotic neurons. Furthermore, the miR-25 family members are overexpressed in different cancer types (Kim et al., 2009; Li et al., 2009), and are implicated in the inhibition of pro-apoptotic and anti-proliferative genes such as tumor protein 53 (Kumar et al., 2011) and BCL-2 family protein BIM; (Tsuchida et al., 2011; Zhang et al., 2012), a regulation which presumably occurs in the cytoplasm. Therefore, it is likely that in the early stages of neural development (e.g., in neural progenitors), miR-25 family members localize to the cytoplasm and are involved in the post-transcriptional regulation of proteins involved in the control of cell cycle and proliferation. Indeed, overexpression of miR-25 increased the proliferation of mouse neural stem/progenitor cells [NSPC; Brett et al., 2011] and also induced re-entry into mitosis of post-mitotic neurons from zebrafish spinal cord by directly inhibiting the expression of p57 cell-cycle inhibitor (CDKN1C) (Rodriguez-Aznar et al., 2013). Likewise, miR-25 family members might suppress the expression of neuronal phenotype promoting genes in the cytoplasm of glial cells, since the gene ontology (GO) terms, such as neuron development and differentiation, are enriched in the predicted mRNA targets for these miRNAs. In contrast, we found that miR-25 and miR-92a preferentially localize to the nucleus of post-mitotic neurons, where they might be involved in the regulation of gene expression in the nuclei of post-mitotic neurons. Recently, miR-25 was reported to inhibit the expression of the sarco(endo)plasmic reticulum Ca2 ATPase (SERCA2) by binding to the 3′-UTR of SERCA2 mRNA in the cytoplasm of post-mitotic neurons (Earls et al., 2012). It is therefore likely that miR-25, and possibly other nuclear-enriched miRNAs, are also involved in post-transcriptional gene regulation in mature neurons. A future challenge will be to specifically manipulate the nuclear and cytoplasmic pools of miRNAs to elucidate compartment-specific functions.

Since we observed a positive correlation between developmentally down-regulated and nuclear-enriched miRNAs, it is tempting to speculate that developmental stage-specific changes in biogenesis and/or degradation of these miRNAs might contribute to an enrichment of specific miRNAs in the nucleus. In addition to miRNA degradation in the cytoplasm, it is conceivable that targeting (or confinement) of miRNAs to the nucleus may be a mechanism to "remove" miRNAs from the cytoplasm to avoid regulation of cytoplasmic mRNA targets. Since the subcellular compartment of miRNA degradation remains unknown (Ruegger and Grosshans, 2012), it is possible that nuclear localization could be used to target miRNAs for degradation. Accordingly, some of the exoribonucleases such as ribosomal RNA-processing protein 41 (RRP41), exoribonuclease 1 (ERI-1) and 5′ to 3′ exoribonuclease XRN2, which are involved in miRNA degradation in metazoans (Ruegger and Grosshans, 2012), were shown to shuttle between the nucleus and cytoplasm (Ansel et al., 2008; Schmid and Jensen, 2008; Nagarajan et al., 2013) and participate in nuclear functions, e.g., ribosomal RNA biogenesis. A possible nuclear degradation of miRNAs is further supported by the observation that transfected siRNAs and endogenous miRNAs are enriched in the nucleolus (Ohrt et al., 2006; Politz et al., 2009). In this respect, studying the stability and localization of mature miRNAs upon their specific delivery into the nucleus or cytoplasm might help to identify the cellular compartment(s) important for degradation of mature miRNAs. Interestingly, it was shown that the turn-over of miRNAs in neurons can be regulated in an activity-dependent manner (Krol et al., 2010). It would be therefore important to determine the role of the nucleus in the rapid turnover of miRNAs in response to activity.

Irrespective of miRNA turnover, specific miRNAs (such as miR-25 and miR-92a) might perform distinct functions depending on the cell type and/or the developmental (metabolic) stage of a cell. For example, in neural stem cells and glial cells, some miRNAs repress neuron-promoting (and anti-proliferation) genes, e.g., by targeting the respective mRNAs and preventing their "leaky" expression in the cytoplasm. In post-mitotic neurons, the same miRNAs might be imported to the nucleus, where they could be involved in transcriptional or post-transcriptional regulation of gene expression as has been shown for some miRNAs and siRNAs in cancer cell lines (Kim et al., 2008; Place et al., 2008; Allo et al., 2009; Tang et al., 2012). In the future, the analysis of miRNA nucleo-cytoplasmic expression during differentiation of neural stem cells to fully differentiated neurons will be required to determine the exact time-point when the cytoplasmic function of specific neuronal miRNAs is switched to a function in the nucleus.

One of the mechanisms that miRNAs could employ to regulate transcriptional gene expression in the nucleus is by introducing epigenetic modification marks to DNA (methylation) and histone (acetylation and methylation) proteins. To clarify whether nuclear-enriched miRNAs are directly involved in epigenetic control of gene expression, specific manipulation of nuclear miRNAs followed by transcriptional and/or epigenetic profiling will be needed.

We also investigated whether cis-acting elements in mature miRNAs might direct them into the nucleus. Surprisingly, we identified that isomiRs, and to a smaller extent canonical miRNAs, containing 3′-terminal guanine nt are preferentially localized within the nucleus. In addition, we found that the source of the 3′-terminal G strongly influences nuclear fate. For example, isomiRs with NTA_G are the most nuclear enriched, followed by isomiR_Gs obtained from one 3′ nucleotide trimming, and then canonical isomiR_G for which the 3′-terminal is generated by Dicer/Drosha. IsomiR_Gs, independent of the source of the 3′-terminal guanine, could favor nuclear localization in at least two ways. First, the 3′-terminal guanine could confer higher stability in the nucleus. Second, binding of specific proteins to isomiR_Gs could mediate active transport from the cytoplasm to the nucleus. The active import of isomiR_Gs (as well as other isomiRs) to the nucleus might be performed by Argonaute proteins, which were shown to shuttle between the nuclear and

cytoplasmic compartments (Weinmann et al., 2009; Nishi et al., 2013). Since Argonaute proteins (AGO 1–3) show different global small RNA binding pattern (Dueck et al., 2012), one could speculate that one of the AGO isoforms might specifically associate with isomiR_Gs and import them to the nucleus. In addition, RNA-binding proteins other than AGO might also be involved in nucleo-cytoplasmic shuttling of isomiRs. Non-templated addition of guanine to the 3′-terminus appears to further enhance nuclear accumulation. NTA_G could either happen in the nucleus after import, or in the cytoplasm followed by nuclear import. The identity of the guanylyltransferase(s) responsible for the production of NTA_Gs is unknown. Known metazoan RNA guanylyltransferases which are part of the mRNA cap-synthesis complex are unlikely to be involved in isomiR_G production, since these guanylyltransferases transfer a guanine monophosphate nucleoside to the nascent 5′ diphosphate mRNA end (Ghosh and Lima, 2010), but not the 3′ end. Apart from guanylation at the 3′ of miRNAs, the only example where terminal guanylyltransferase activity was observed is specific guanylation of European yellow lupine (*Lupinus luteus*) 5 s rRNA at the 3′ end in Hela cell extract (Wyszko et al., 1996). However, the responsible enzyme as well as physiological significance of this modification is not known. Interestingly, isomiRs with non-templated guanine addition are more abundant in mouse hippocampus (Zhou et al., 2012) and cerebellum (Wyman et al., 2011) compared to other tissues, suggesting that 3′ non-templated addition of guanine could be a brain-specific phenomenon. In this regard, determination of the identity and subcellular localization of guanylyltransferase responsible for NTA_G in neurons will be highly informative.

Taken together, our results indicate that mammalian neurons have a distinct subset of nuclear-enriched miRNAs, and that their localization to the nucleus might be linked to the developmental stage-specific down-regulation of miRNA expression. Furthermore, we uncovered that the type of nucleotide at the 3′-terminus of miRNA/isomiR can significantly influence subcellular localization of miRNAs in neurons. In the future, it will be important to characterize the physiological role of nuclear-enriched miRNAs in neurons, as well as the molecular mechanisms underlying nucleo-cytoplasmic localization, with a focus on the role of 3′-terminal guanylation. This will not only increase our understanding of neuronal development, but also provide important new insights into general aspects of miRNA metabolism.

AUTHOR CONTRIBUTIONS

Sharof A. Khudayberdiev, Federico Zampa, and Marek Rajman performed experiments; Sharof A. Khudayberdiev performed data analysis (if not otherwise stated); Sharof A. Khudayberdiev and Gerhard Schratt wrote the manuscript; Gerhard Schratt supervised the project.

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SUPPLEMENTARY METERIAL

The Supplementary Meterial for this article can be found online at: http://www.frontiersin.org/journal10.3389/fnmol.2013.00043/abstract

Figure S1 | (A) Cycle threshold (Ct) values for markers measured with qRT-PCR in **Figure 1A**. **(B)** Denaturing 15% PAGE gel showing equal loading of RNAs before membrane transfer for Northern blotting. The gel was stained with 2x SYBR Gold dye (Life Technologies) for 5 min and was imaged using E-BOX VX2 gel documentation system (PeqLab). **(C)** Northern blot analysis of miR-25 and miR-92a using cytoplasmic and nuclear RNA from neurons treated with KCl and BDNF. **(D)** MicroRNA ranking (Rank Sum) and distribution of an average developmental expression score (DES). DES was calculated by $\log_2$ transforming the ratio of miRNA read counts from prefrontal cortex of post-natal Day 3 (P3) and embryonic Day 10 (E10) rats in the published report of Yao et al. (2012). DES of 179 (out of 220) miRNAs that were detected both by us and Yao et al. (2012) were employed for analysis. The average DES (y-axis) was calculated using moving window technique, where window length was set as 10 and the average values were calculated by moving the window with one step at a time from high to low ranking miRNAs. In the x-axis, the ranking number of miRNAs in descending order is depicted.

Figure S2 | (A) Immunostaining of primary hippocampal cultures for neuronal (MAP2, red) and astrocytic (GFAP, green) marker proteins after treatment with FUDR. Cultures were treated with 10 μM FUDR at DIV3 and fixed at DIV18. **(B)** Expression of indicated RNAs in mixed cultures and glia-enriched (10 % FBS-treated). The relative expression levels of indicated RNAs were obtained by the ddCt method. RNA levels in mixed cultures were arbitrarily set to 1. Bar plots show mean ± *SD* ($n = 2$). SD for mixed culture condition was determined after normalization to an internal control RNA (U6 snRNA). Statistical significance was determined based on U6 snRNA normalized values using Student's t-test with Bonferroni correction ($^*p < 0.05$; $^{**}p < 0.01$).

Figure S3 | Sequence similarity of miRNAs. Members of miRNA families possessing the same seed sequence are depicted in separate rectangular boxes. Red colored letters indicate conserved nucleotides between depicted family members. Blue colored letters indicate partial conservation (miR-25/92a/92b).

Figure S4 | Frequency of guanine (G, blue) and uracil (U, violet) nucleotides at the 3′-terminus of mature miRNAs (miRBase v18) depending on the NEnS. Frequency of nucleotide was calculated using moving window technique, where window length was set as 20 and the average occurrence values were calculated by moving the window with one step at a time from high to low ranking miRNAs. In the x-axis, the ranking number of miRNAs in descending order is depicted.

Figure S5 | Proportion of specific isomiRs from the total sequence reads mapped to the respective miRNAs in the nuclear and the cytoplasmic fractions.

Figure S6 | Relative NEnS and 3′-terminal guanine nucleotide of specific miRNA (miR-124 and miR-25) isomiRs. On the y-axis, the $\log_2$ transformed read counts from nuclear fraction, and on the x-axis, rNEnS for each isomiR is depicted. Black colored bars indicate isomiRs that possess guanine nucleotide at the 3′-terminus.

Figure S7 | Impact of trimming on nuclear localization of isomiRs. **(A)** rNEnS for canonical isomiR_A that underwent 3′ trimming, thereby exposing the indicated nucleotide. Bar plots show mean ± *SD* (canon_A, $n = 75$; trim_A>G, $n = 25$; trim_A>C, $n = 18$; trim_A>A, $n = 8$;

trim_A>U, $n = 10$). Statistical significance was determined using Welch's *t*-test (unequal variance) with Bonferroni correction (*$p < 0.05$; **$p < 0.001$; ***$p < 1.0$–E5). **(B)** rNEnS for canonical isomiR_C that underwent 3′ trimming, thereby exposing the indicated nucleotide. Bar plots show mean ± *SD* (canon_C, $n = 62$; trim_C>G, $n = 13$; trim_C>C, $n = 17$; trim_C>A, $n = 2$; trim_C>U, $n = 9$). Statistical significance was determined using Welch's *t*-test (unequal variance) and none of the comparisons passed the Bonferroni correction (*, $p < 0.05$).

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MicroRNAs in brain development and function: a matter of flexibility and stability

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Fine-tuning of gene expression is a fundamental requirement for development and function of cells and organs. This requirement is particularly obvious in the nervous system where originally common stem cell populations generate thousands of different neuronal and glial cell types in a temporally and quantitatively perfectly orchestrated manner. Moreover, after their generation, young neurons have to connect with pre-determined target neurons through the establishment of functional synapses, either in their immediate environment or at distance. Lastly, brain function depends not only on static circuitries, but on plastic changes at the synaptic level allowing both, learning and memory. It appears evident that these processes necessitate flexibility and stability at the same time. These two contrasting features can only be achieved by complex molecular networks, superposed levels of control and tight interactions between regulatory mechanisms. Interactions between microRNAs and their target mRNAs fulfill these requirements. Here we review recent literature dealing with the involvement of microRNAs in multiple aspects of brain development and connectivity.

Keywords: microRNA, neurogenesis, neural stem cells, fate determination, synaptogenesis, synaptic function, LTP

INTRODUCTION: MICRORNA GENESIS AND FUNCTION

MicroRNAs are small RNA molecules of around 22 nucleotides, processed from longer primary transcripts (pri-miRNAs) in successive maturation steps. MicroRNA genes contain an imperfect palindromic sequence that creates a secondary stem–loop structure within the pri-miRNA. This stem–loop structure contains the mature microRNA and its passenger strand (Denli et al., 2004; Gregory et al., 2004; Landthaler et al., 2004; Han et al., 2006) and serves as substrate for two double-strand RNases, Dicer and Drosha (Carmell and Hannon, 2004). Targeting occurs by partial complementarity between the mRNA's 3'UTR and a 6–8 nucleotides long sequence at the 5' end of the microRNA. This partial complementarity allows a single microRNA to target multiple mRNAs simultaneously and, vice versa, a single mRNA may be regulated by different microRNAs (Klein et al., 2005; Kosik, 2006). Thus, bioinformatic predictions and proteomic evidence indicate a vast amount of potential microRNA/mRNA interactions (Bartel, 2009). In addition to other regulatory mechanisms (feedback loops among transcription factors, epigenetic mechanisms, etc), microRNAs have been implicated in the control of neurogenesis and brain function. We will discuss several examples in this review.

MicroRNAs CONTROLLING NEUROGENESIS: FROM STEM CELLS TO NEURONS

Maintenance and differentiation of neural stem cells is controlled by the equilibrium between the relative amounts of key proteins that promote or inhibit entry into the neurogenic program. Multiple examples show that this equilibrium is achieved, at least in part, by microRNAs that act in complex feedback loops with their targets.

THE TLX SYSTEM: STABILIZATION BY FEEDBACK LOOPS

One example for such complex regulation is provided by control and interactions of the orphan nuclear receptor Tailless (TLX). TLX is expressed in stem cells of the developing and adult brain where it controls their maintenance and proliferation (Shi et al., 2004; Liu et al., 2008; Zhang et al., 2008). MiR-9 is a highly brain enriched microRNA that targets and regulates TLX (Zhao et al., 2009) expression and is itself negatively regulated by the nuclear receptor (Zhao et al., 2009; **Figure 1**). Moreover, two members of the let-7 microRNA-family also control TLX expression, thus acting upstream of the TLX/miR-9 feedback loop (Zhao et al., 2010, 2013). Interestingly, both miR-9 and let-7b also share CyclinD1, another key cell cycle regulator during neurogenesis, as a target (Guo et al., 2013; Zheng et al., 2013). Finally, during cortical development TLX acts in concert with the lysine specific de-methylase 1 (LSD1) that is controlled by miR-137, which, in turn, is repressed by TLX dependent recruitment of LSD1 to the microRNA locus (Sun et al., 2011; **Figure 1**).

REST INHIBITION TO OPEN THE DIFFERENTIATION LOCK

A second example for the sophisticated regulatory interactions that control neural stem cells status implicates the zinc finger protein REST (RE1-silencing transcription factor). REST and its co-repressor CoREST are part of a protein complex that binds to the so-called RE1 site of target promoters and thereby down-regulates neuronal genes in non-neural tissues (Andres et al., 1999; Ballas and Mandel, 2005; Bithell, 2011). The REST complex contains additional proteins like the phosphatases CtdspL, Ctdsp1, Ctdsp2 (Yeo et al., 2005) and, interestingly, LSD1, providing an intersection with the TLX system (Lee et al., 2005). Since the REST complex opposes neuronal

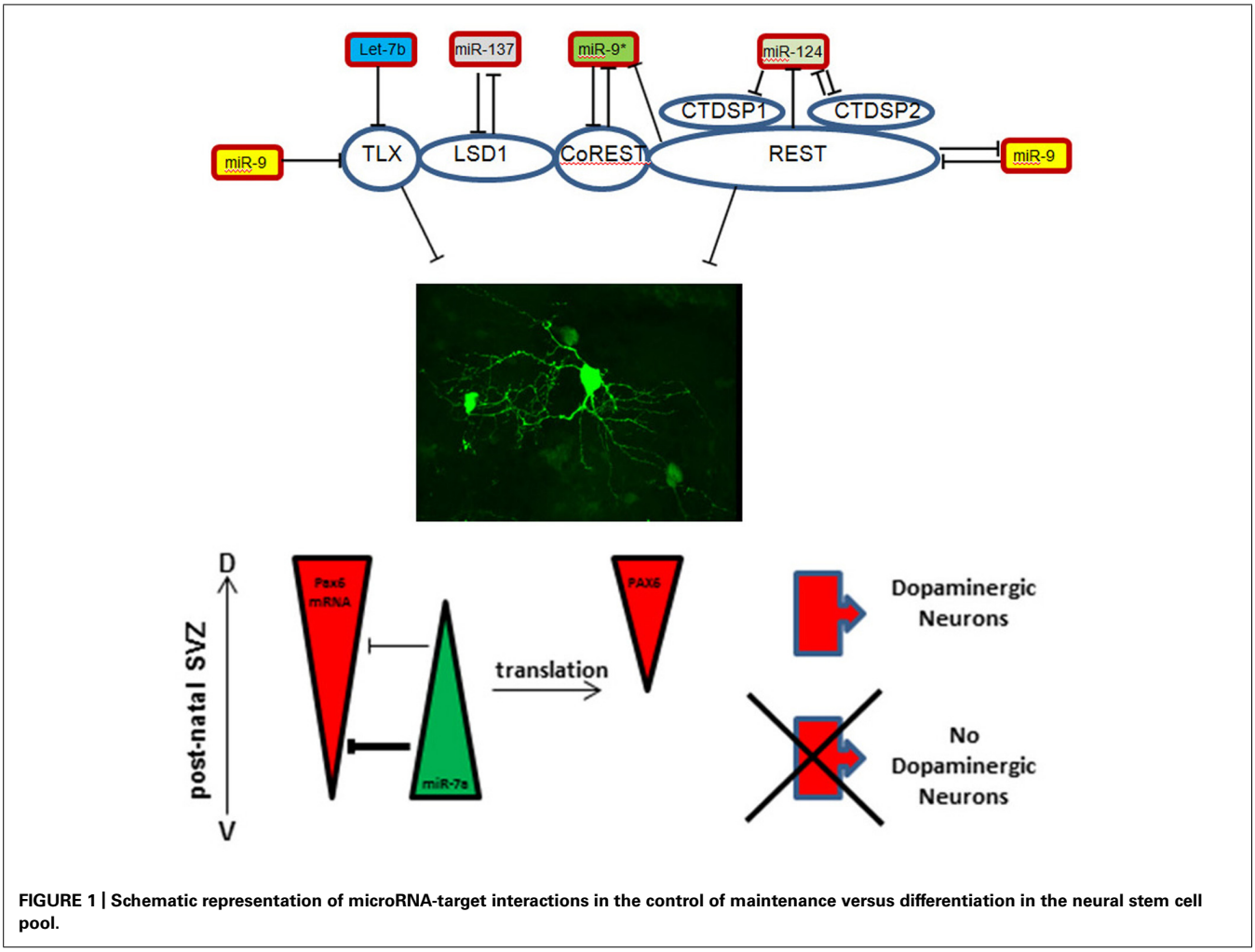

FIGURE 1 | Schematic representation of microRNA-target interactions in the control of maintenance versus differentiation in the neural stem cell pool.

differentiation, and thus maintains the immature state (Ballas and Mandel, 2005), it has to be released from its binding site to allow neurogenesis. As for TLX, miR-9 targets and down-regulates REST while its counterstrand miR-9* targets CoREST (Packer et al., 2008; **Figure 1**). Conversely, the miR-9/miR-9* genomic loci both contain RE1 sites upstream of the protein coding sequence and are regulated by the REST complex (Packer et al., 2008).

Another regulator of the REST control system is miR-124, one of the most abundant microRNAs in the brain. During development miR-124 promotes neuronal differentiation by targeting REST, again implicating a feedback loop since REST itself acts as inhibitor of miR-124 expression (Conaco et al., 2006; Visvanathan et al., 2007). In addition, a synergistic function of miR-124 and miR-9*, the passenger strand of miR-9, has been reported (Yoo et al., 2009). Both microRNAs repress the subunit BAF53a of the neural-progenitor-specific BAF (npBAF) chromatin-remodeling complex, which allows a switch to the BAF53b subunit (Yoo et al., 2009). This subunit switch is important for post-mitotic phases of neural development. Additionally, miR-124 was shown to target the RNA-binding protein Ptbp1, a repressor of neuron-specific splicing (Makeyev et al., 2007) as well as laminin γ1 and integrin β1, both repressed during neuronal differentiation (Cao et al., 2007). Finally, miR-124 was shown to be involved in postnatal neurogenesis through its inhibition of the neural stem cell (NSC) maintenance factor Sox9 (Cheng et al., 2009). Taken together this indicates that miR-124 promotes neuronal differentiation, both, during embryonic development and in postnatal stages, thereby acting on multiple molecular layers from transcription and splice factors to extracellular matrix molecules.

EPIGENETIC MECHANISMS

Surprisingly, in contrast to its above-described pro-neurogenic role in the embryo, miR-137 has been implicated in the maintenance of stem cell proliferation in the adult forebrain through cross-talk with epigenetic mechanisms involving MeCP2 and Ezh2 (Szulwach et al., 2010).

Moreover, miR-184 is another microRNA which links epigenetic processes to neurogenesis (Liu et al., 2010). The authors reported that the loss of methyl binding protein MBD1 increased the expression of miR-184 and identified Numblike (Numbl), a Notch1 antagonist important for survival of SVZ derived neuroblasts (Kuo et al., 2006), as a direct target.

DETERMINATION OF NEURONAL FATE

A key feature of brain development is that common neural stem cells are able to generate a large diversity of cell types. The role of microRNAs on lineage and subtype specification in the brain just starts being explored. During postnatal neurogenesis miR-7a has been reported as an important contributor to fate specification of OB dopaminergic inter-neurons. The regulation by miR-7a impacts on gene dosage and the precise expression pattern of the transcription factor Pax6 which is a critical dopaminergic fate determinant in the SVZ (Hack et al., 2005; de Chevigny et al., 2012b). This is part of the control system determining neurotransmitter phenotype of OB inter-neurons (de Chevigny et al., 2012a). Interestingly, during cortex development miR-7a was found to promote oligodendrocyte generation by targeting Pax6 and NeuroD4 (Zhao et al., 2012). Thus, mir-7a is able to control different types of fate decision by controlling the same targets in different transcriptional contexts (**Figure 1**).

Mir-133 has been implicated in midbrain dopaminergic differentiation *in vitro* through regulation of Pitx3. Moreover, Parkinson's patients have been shown to be deficient for this microRNA, suggesting a feedback circuit in the fine-tuning of dopaminergic behaviors (Kim et al., 2007). However, these findings have been challenged by the recent observation that miR-133b-deficient mice show normal numbers and function of dopaminergic neurons (Heyer et al., 2012). Thus, the situation needs clarification.

Another interesting microRNA in regard to specification events is miR-34a. This microRNA is reported to promote generation of post-mitotic neurons from isolated mouse embryonic NSCs by targeting the NAD-dependent deacetylase sirtuin-1 (Sirt1; Aranha et al., 2011). In contrast, miR-34a is reported to enhance Notch1 signaling in neural progenitors, by repressing the Notch pathway repressor Numbl that ultimately antagonizes neuronal differentiation (Fineberg et al., 2012). Taken together, this might indicate that miR-34a acts strongly context dependent based on the transcriptional and cellular environment.

In conclusion, investigation of regulatory interactions between microRNAs and their targets in the control of neurogenesis revealed complex regulatory circuits based on feedback regulations, synergistic actions of several microRNAs and intersections between signaling systems.

MicroRNAs AT THE SYNAPSE

Synapses are the main structures that allow communication between neurons. Synapses of a given neuron may coexist in different states, differing in strength, thus the capacity of the synapse to respond to presynaptic release of neurotransmitter. The property of a synapse to modify its strength is called synaptic plasticity which comes in two flavors. Long term potentiation (LTP) is induced by high frequency stimulation of presynaptic neurons (Bliss and Lomo, 1973) and results in an increase in the density of AMPA receptors at the post-synaptic membrane, leading to enhanced Na^+ flux (Johnston et al., 2003; Malenka and Bear, 2004). This, in turn, increases the likelihood of synaptic signal transmission. LTP is specific to a given synapse and spreading to the neighboring synapses is efficiently inhibited. In contrast, during long term depression (LTD), low-frequency stimulation decreases the strength of a synapse (Massey and Bashir, 2007). Overlying these processes, homeostatic mechanisms exist at the pre- and post-synaptic compartments that dampen these opposing phenomena (LTP and LTD) to avoid hyper or hypo-excitability of synapses in response to permanent high or low-frequency stimulation (Malenka and Bear, 2004; Lee et al., 2010). This situation, where synapses exhibit variable strength in the brain, draws a landscape of favored neuronal circuits where transmission will occur with higher probability than others.

Establishment of defined neuronal circuits in particular states is considered to be the basis of both, memory and learning. For long-term memory, information has to be stably stored over prolonged periods, implying a high degree of stability of a given circuit state. In contrast, learning in response to stimuli from the outside world has to be associated with rapid changes at the synaptic level leading to rapid changes in circuit status. It is evident that these seemingly opposing cellular processes occur also at the molecular level. Thus, regulatory fine-tuning mechanisms must exist, that allow synaptic stability and flexibility at the same time.

MicroRNAs REGULATING FORMATION AND STABILITY OF THE SYNAPSE

Molecularly, LTP (the situation is not clear for LTD) is characterized by a change in the biochemical composition of the activated synapse, with specific recruitment of key synaptic proteins. These mechanisms are mainly under the control of CamKII signaling. It has recently been shown that the synaptic accumulation of several important LTP-inducing proteins is a consequence of local synaptic translation (Hornberg and Holt, 2013; Swanger et al., 2013) establishing a link between the protein content of a given synapse and its strength.

As for regulation at the stem cell level, over the past years a variety of mRNA/microRNA interactions have been described, that fulfill the requirement of providing flexibility and stability at the same time. Indeed, a subset of microRNAs was found strongly enriched in synapse preparations of forebrain tissue (Lugli et al., 2008). Moreover, MOV10, a helicase that is part of the RISC complex (Chendrimada et al., 2007), is accumulated at synapses and actively degraded upon activity. Absence of MOV10 displaced a subset of major synaptic mRNA into the polysomal fraction, demonstrating microRNA-mediated control of translation at the synapse (Banerjee et al., 2009).

In parallel to these more global approaches, several specific microRNAs were shown to be involved in synaptic plasticity, whereby they act at different levels. In some cases they participate in silencing synapses by inhibiting expression of structural proteins while in other cases they favor synaptic potentiation. Moreover, some microRNAs have been involved in synaptic homeostasis, by limiting the over-expression of synaptic proteins upon activation. Interestingly, several microRNAs that control synaptic protein expression have been implicated in drug addiction.

Several microRNAs prevent expression of synaptic proteins in the presence of the corresponding mRNAs. Upon stimulation relieve of this translational block allows the rapid activation of the synapse. The first microRNA shown to be involved in synapse formation was miR-134 (Schratt et al., 2006). Its precursor is transported specifically to dendrites via binding to the DEAH-box helicase DHX36 (Bicker et al., 2013). Once arrived

in the dendrites, pre-miR-134 is processed into mature miR-134, which inhibits spine formation in cultured hippocampal neurons (Schratt et al., 2006) and dendritogenesis in cortical neurons (Christensen et al., 2010) via the kinase Limk1 and the translational repressor Pumillo2 (Schratt et al., 2006; Fiore et al., 2009). Upon neuronal activation, the inhibitory effect of miR-134 is relieved and spine formation occurs (Schratt et al., 2006). In line with its role in opposing spine formation, miR-134 was recently shown able to impair synaptic plasticity through the inhibition of SIRT1 gene in a gain-of-function setting (Gao et al., 2010; **Figure 2**).

However, two recent papers interrogate the assumption that miR-134 is a general opponent to active excitatory synapses formation. First, it was shown that inhibition of miR-134 reduces spine density in hippocampal pyramidal neurons *in vivo* (Jimenez-Mateos et al., 2012) thereby protecting from epileptic seizure (Jimenez-Mateos et al., 2012). This suggests a pro-synaptogenic role of the microRNA in excitatory neurons. Second, whereas all these previous miR-134 related observations were made in excitatory neurons, a recent paper showed that activity of miR134 in cortex is restricted to inhibitory GABAergic inter-neurons where it down-regulates DHHC9, the palmitoyltransferase of the regulatory GTPase HRAS (Chai et al., 2013). To reconcile these contrasting results the authors propose that miR-134 exerts its function on excitatory neurons indirectly, through the associated inter-neurons (Chai et al., 2013).

MicroRNAs REGULATING SYNAPTIC PLASTICITY

Palmitoylation is a post-translational modification that is commonly used to mediate activity-dependent changes in synapses (Kang et al., 2008). MiR-138 is present at the post-synapse where it regulates dendritic spine morphology through translational inhibition of the de-palmitoylating enzyme Lypla1 (Siegel et al., 2009). Moreover, miR-125 was found to regulate synaptic plasticity in cortical neurons through translational inhibition of the post-synaptic protein PSD-95. Interestingly, binding of miR-125 to PSD-95 is mediated by the phosphorylated form of FMRP, the gene responsible for the fragile-X syndrome (Muddashetty et al., 2011). In response to stimulation of metabotropic mGluR receptors FMRP is dephosphorylated and miR-125 is released from PSD-95 3'UTR mRNA, which can then be translated (**Figure 2**).

Kv1.1 is a voltage-gated potassium transporter that controls action potential frequency (Brew et al., 2003). Exact dosage of this transporter is important as even a mono-allelic mutation induces episodic ataxia in human patients (Zerr et al., 1998) and appropriate levels of Kv1.1 protein at synapse are assured by

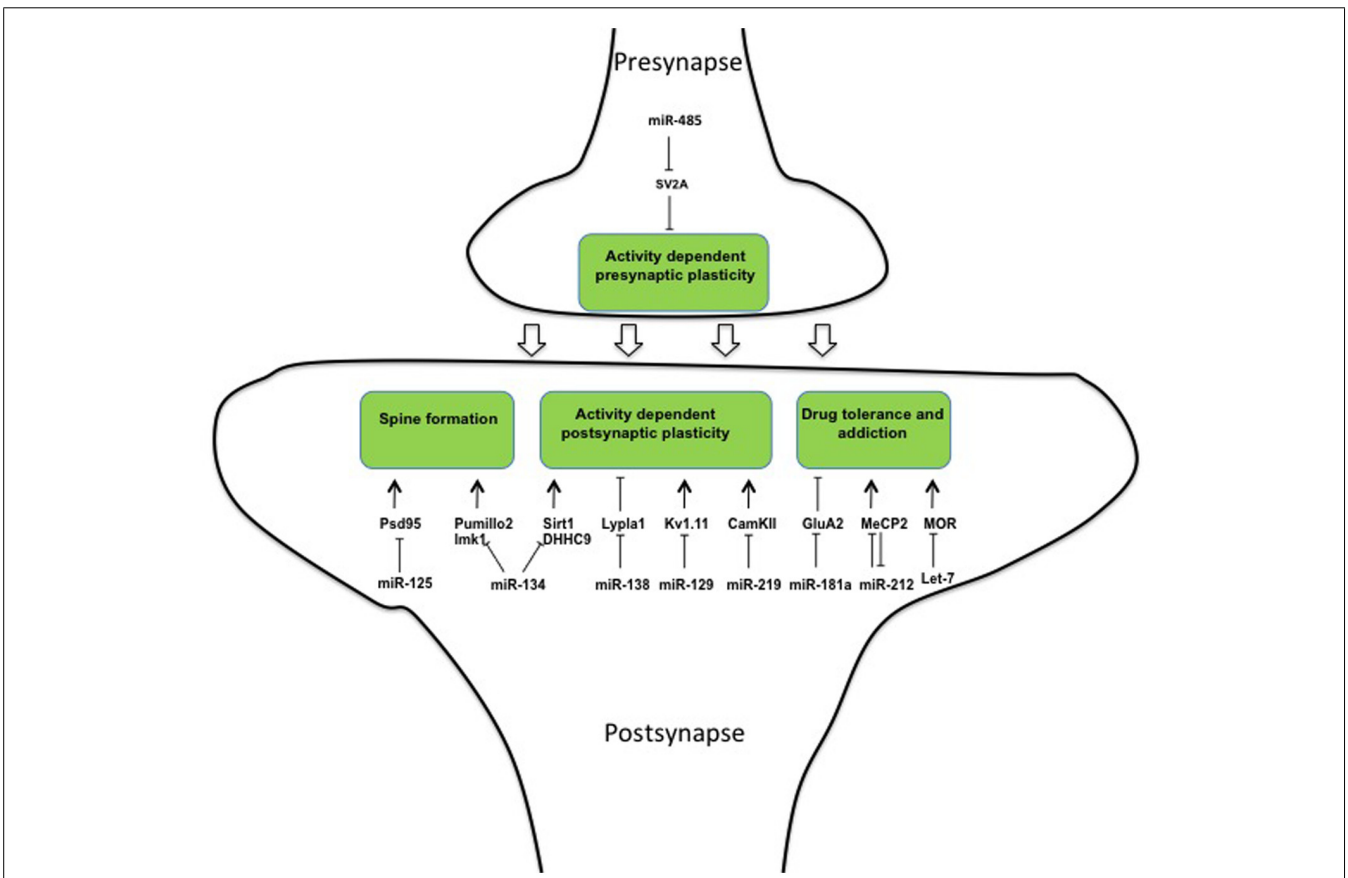

FIGURE 2 | MicroRNAs regulate different aspects of synapse formation, stabilization, and plasticity.

positive and negative regulation of its translation. In this system the neuron-specific microRNA miR-129 binds and inhibits Kv1.1 mRNA translation (Sosanya et al., 2013). However, miR-129 competes for Kv1.1 mRNA-binding with the RNA-binding protein HuD, which acts as a positive regulator of Kv1.1 protein expression. The master regulator of this system, which orchestrates between positive and negative regulation, is the mTOR kinase. Activity of mTOR results in increased amounts of intra-cellular HuD that displaces miR-129 from Kv1.1 mRNA, thus allowing translation to occur (Raab-Graham et al., 2006; **Figure 2**).

MicroRNA miR-219 expression in the prefrontal cortex parallels expression of the NMDA-receptor. Moreover, CamKII, a major mediator of LTP and NMDA signaling, was shown to be a direct target of miR-219 (Kocerha et al., 2009). Finally, miR-219 down-regulation alleviates behavioral modifications associated with alterations in NMDA-receptor signaling, in accordance with a functional role of miR-219 in synaptic plasticity (Kocerha et al., 2009). Thus, a multitude of regulatory interactions between microRNAs and target genes have been implicated in the negative control of synapse formation and transmission.

However, there is also evidence that microRNAs promote synaptic plasticity upon activation. Transgenic mice over-expressing miR-132 in forebrain neurons exhibit increased spine density (Hansen et al., 2010) while miR-132 inhibition reduces spine formation (Magill et al., 2010). These results, together with the observation that miR-132 accumulates in response to activity (Nudelman et al., 2010), suggest a positive role for this microRNA for synapse formation and plasticity. However, the situation might be more complicated, since miR-132 has also been shown to inhibit the CpG-binding protein MeCP2 (Klein et al., 2007), an inducer of spine formation.

The inhibitory activity of microRNAs may also be used to dampen structural changes at synapses upon activation and thus be involved in homeostatic plasticity. After stimulation of cultured hippocampal neurons, miR-485 expression was increased at pre-synapses (Cohen et al., 2011). Here, the microRNA was shown to regulate the pre-synaptic protein SV2A (**Figure 2**) and by this to reduce the probability of neurotransmitter release as shown by a lower miniature excitatory synaptic current (mEPSC) frequency. This inhibition in pre-synaptic function partially prevented clustering of post-synaptic proteins such as PSD95 and AMPA receptor subunits (Cohen et al., 2011).

FROM SYNAPTIC FUNCTION TO DRUG ADDICTION

Several reports demonstrate the involvement of the microRNA pathway in homeostatic plasticity occurring in response to drug intake. Indeed, psychotropic drugs act generally through stimulation of specific synaptic receptors. Repeated stimulation of these receptors reinforces the strength of the involved neuronal circuitries. This leads to compulsive consumption of the drug if the potentiation at the synapse is not dampened. Several microRNAs were shown to be involved in the response to chronic drug exposure and to drug addiction. MicroRNA miR-181a is specifically accumulated at post-synapses of nucleus accumbens. Moreover, its concentration increases during cocaine abuse (Saba et al., 2012). At the post-synapse, one of the miR-181a targets is the AMPA subunit GluA2 (Saba et al., 2012; **Figure 2**). It is known that drug of abuse favors the exchange from GluA2 containing AMPARs to GluA2 lacking AMPARs and this molecular modification at the synapse is required for drug-craving after prolonged cocaine withdrawal (Conrad et al., 2008). It appears possible that this mechanism is responsible for the role of miR-181a in the alterations in "cocaine place preference" (CCP) that have been shown in rodents (Chandrasekar and Dreyer, 2011) and also in the altered neuro-adaptation associated with cocaine abuse (Saba et al., 2012).

Neuro-adaptation leads to profound structural alterations that can, depending on the individual, lead to variations in sensitivity to a drug over time (Bowers et al., 2010; Dacher and Nugent, 2011). This variation explains why some subjects will become addicts and others will not. miR-212 was shown to play a central role in neuro-adaptation and to oppose loss of control toward drug consumption. Upon chronic cocaine exposure miR-212 and its cluster neighbor miR-132 are over-expressed in the dorsal striatum (Hollander et al., 2010). Under extended access to cocaine gain- and loss-of-function experiments showed that miR-212 interfered with the self-administered dose. These results suggest that miR-212 is involved in the dampening of plasticity induced by chronic cocaine exposure, which causes the compulsive behavior. At the molecular level, the action of miR-212 is mediated through the inhibition of a so far unidentified repressor of Raf1, which is itself an activator of CREB. This indirect activation of CREB, reduces the motivational properties of the drug by dampening the reward circuitry (Dinieri et al., 2009). Moreover, miR-212 has been shown to target MePC2, as already mentioned a DNA-binding protein involved in synaptic structural plasticity, providing a parallel pathway accounting for the anti-addictive role of the microRNA toward cocaine (Im et al., 2010). Interestingly, MeCP2 inhibits expression of miR-212 (**Figure 2**), and by this limits the action of miR-212 in the control of cocaine intake, highlighting again the importance of feedback loops in the regulatory actions of microRNAs (Im et al., 2010).

In addition to this considerable amount of information implicating microRNAs in the control of addiction to cocaine, microRNAs are involved in the behavior toward Opioids. These are potent analgesics of considerable clinical value, but have several drawbacks limiting their use, including tolerance and addiction. Opioid signaling is mediated in neurons through the mu opioid receptor (MOR) and tolerance occurs through the decrease in MOR expression at the synapse. He et al. (2010) showed that the microRNA let-7, on one hand, inhibits MOR translation and, on the other hand, accumulates upon chronic morphine treatment in mice (**Figure 2**). Moreover, knocking-down let-7 reduced -but did not entirely prevent- opioid tolerance in treated mice, demonstrating a role of the microRNA in dampening opioid signaling upon chronic stimulation (He et al., 2010).

CONCLUSION

MicroRNAs have been shown to be implicated in virtually all biological functions ranging from embryonic development, aging, infections, genetic disease to cancer (Tang et al., 2007).

However, microRNAs do in general not have simple functions as on/off switches, but serve whenever fine-tuning of gene expression in space, time and dose is necessary. In the brain the necessity for such fine-tuning is evident (Schratt, 2009). In the stem cell compartment the generation of neurons from initially

quite homogeneous stem cells population has to be orchestrated in space and time to generate the thousands of different neuronal and glial cell types in the correct place and number. For proper function, these cells have to form complex cellular circuitries that are tightly regulated at the levels of connectivity and synaptic signal intensity. Here we reviewed the functions of gene and microRNA interactions in different aspects of these processes. We find that many of the microRNAs in the brain are implicated in many aspects of the neurogenic process, thereby regulating different targets sequentially and often synergistically with other microRNAs. Another common feature of these interactions is that they control homeostasis of otherwise fragile systems, thereby often implicating complex feedback loops. Finally, the brain has to react instantaneously to outside stimuli and microRNA mediated control of gene expression allows bypassing the transcriptional control level. Given all these properties and requirements, it is predictable that in the future a multitude of further interactions, loops and functions implicating microRNAs will be described.

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Insights on the functional interactions between miRNAs and copy number variations in the aging brain

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MicroRNAs (miRNAs) are regulatory genetic elements that coordinate the expression of thousands of genes and play important roles in brain aging and neurodegeneration. DNA polymorphisms affecting miRNA biogenesis, dosage, and gene targeting may represent potentially functional variants. The consequences of single nucleotide polymorphisms affecting miRNA function were previously demonstrated by both experimental and computational methods. However, little is known about how copy number variations (CNVs) influence miRNA metabolism and regulatory networks. We discuss potential mechanisms of CNVs-mediated effects on miRNA function and regulation that might have consequences for brain aging. We argue that CNVs, which potentially can alter miRNA expression, regulation or target gene recognition, are possible functional variants and should be considered high priority candidates in genotype–phenotype mapping studies of brain-related disorders.

Keywords: miRNAs, CNV, brain aging, neurodegeneration, non-coding RNA

INTRODUCTION

The establishment of human cognitive abilities is a gradual process that takes place mostly in the period between birth and adulthood, although some developmental processes extend beyond this period (Sowell et al., 2004; Thompson et al., 2004; Zhan et al., 2013). During this time window, the brain undergoes dramatic molecular transformations, which are manifested both structurally and functionally (de Graaf-Peters and Hadders-Algra, 2006). Notably, shortly after the brain development is accomplished, the process of brain aging commences at early adulthood, which is revealed by the gradual decline of the brain ability to absorb and process the flow of information (Sowell et al., 2004; Peters et al., 2008; Salthouse, 2009; May, 2011; Zhan et al., 2013). However, more recent research has revealed that changes in brain circuits are not exclusively restricted to the early stages of brain development, and has supported the concept of continuous neuroplasticity throughout live (May, 2011; Taubert et al., 2012). Novel experience as a result environmental changes and new learning experience have been recognized as stimulating factors of brain function and underlying neuroanatomic networks. Experiments with animals have showed that mice living in active environment exhibited a reduced neuronal age-dependent degeneration and achieved a greater threshold for age-dependent deficits (Kempermann et al., 2002; Fryer et al., 2011).

The aging process is confronted by various neuroprotective mechanisms that are genetically programed and underlie the dynamics of the brain adaptive responses. The sole purpose of the multiple cellular and functional events that take place during brain aging is to maintain neural cells functionality and structural integrity. In cases where the neuroprotective mechanisms are overwhelmed by the accumulation of toxic products, the result is progressive neurodegeneration, as observed in Alzheimer's disease (AD), cerebellar ataxias, and Parkinson's disease (PD). The neuroprotective mechanisms can be augmented by dietary and behavioral modifications, but the genetic predisposition to accelerated aging is likely to be the main driving factor that triggers and maintains the advance of neurodegeneration.

miRNA MACHINERY REACTION TO THE BRAIN AGING

Non-coding RNAs and microRNAs (miRNAs) in particular, play an essential role in the regulation of a number of cell processes, including cell proliferation, development, differentiation, stress responses, blast transformation, and apoptosis. The rapid accumulation of knowledge in the field of miRNA research has revealed its role in regulating gene expression at transcriptional and post-transcriptional levels. Meanwhile, the role of miRNAs in senescence remains poorly understood. miRNAs regulate several pathways associated with the aging mechanisms, and recent genome-wide analysis of miRNA expression revealed age-related changes in their expression level (Kosik, 2006; Krichevsky et al., 2006; Cogswell et al., 2008; Hebert and De Strooper, 2009). These data have underscored the significance of miRNA in brain aging and neurodegeneration.

MicroRNA can affect pathways involved in aging, and miRNA profiling has shown significant alterations in their expression level. Importantly, recent data have shown the significance of miRNA in brain aging and neurodegeneration (Kosik, 2006; Krichevsky et al., 2006; Cogswell et al., 2008; Hebert and De Strooper, 2009). The genome-wide expression analysis of miRNAs in aging individuals revealed a general decline in miRNA levels that was linked to potential loss of control of genes that regulate the cell cycle progression and cell differentiation programing (Noren Hooten et al., 2010). Nine miRNAs (miR-103, miR-107, miR-128, miR-130a, miR-155, miR-24, miR-221, miR-496, and miR-1538) were identified to be significantly lower in the peripheral blood mononuclear

cells of old individuals as compared to the young subjects were identified in this study.

The ability of miRNAs to regulate oxidative stress and cell death is displayed in relationship to the growth harmone/insulin-like growth factor (GH/IGF) pathway and several AD-related oxidative damaging proteins (Nakasa et al., 2008; Stanczyk et al., 2008; Wang et al., 2008). Oxidative DNA damage may occur due to free reactive oxygen species (ROS) binding to nucleic acids and thus preventing transcription and causing DNA damage (Cooke et al., 2003). miR-210 and miR-373 inhibit the expression of key DNA repair proteins following hypoxic stress (Crosby et al., 2009). p53, a critical factor for maintaining the genome integrity, is activated by DNA oxidative damage, which is partially due to the miR-29-induced repression of negative regulators of p53, p85a, and CDC42 (Park et al., 2009).

Apoptosis is an extremely important signaling events influenced by miRNAs, particularly in the context of aging and age-related diseases. Several members of the miR-34 family participate in the p53 network, which induces apoptosis, cell cycle arrest, and senescence (Chang et al., 2007; He et al., 2007). It appears that activation of apoptosis – through internal or external stimuli, leads to repression of miRNAs that would otherwise silence genes involved in activating the apoptosis cascade. The reciprocal action, once an apoptotic cascade is activated, is the upregulation of miRNAs targeting proliferative or cell-survival genes (Wang, 2007). These results illustrate the complexity of miRNA interactions and their contribution to the regulation of programed cell death mechanisms.

MicroRNAs play a role in the control of brain metabolism and subsequently the dynamic of miRNA expression levels reflects the cellular responses to aging progression and deterioration of neuronal functionality. Several miRNAs are selectively expressed in brain tissues (Landgraf et al., 2007) and the inactivation of miRNA processing enzyme Dicer was found to lead to rapid degeneration of Purkinje cells (Schaefer et al., 2007). The global signature of miRNA expression in the adult brain appears to be species-specific, as shown by several comparative studies carried out on different species (Lee et al., 2000; Fraser et al., 2005; Berezikov et al., 2006). Selected miRNAs have been shown to be involved in AD, spinocerebellar ataxias, PD, and other neurodegenerative pathologies (Lukiw, 2007, 2012; Cogswell et al., 2008; Nelson et al., 2008; Persengiev et al., 2012b; Dimmeler and Nicotera, 2013). Genome-wide screens of miRNAs and ncRNAs in the aging brain found that miRNA expression is differentially regulated in the cortex and cerebellum of humans and non-human primates. This observation is likely to reflect the temporal functional status of neuronal activity in the cortex and cerebellum. Despite the observation for the lack of unifying specific miRNA pattern associated with the brain aging, the ontological analysis of targeted genes revealed that they represent a relatively conserved group (Persengiev et al., 2011). Importantly, miR-144 was identified to be the sole miRNA that was consistently upregulated in the aging chimp and human cerebellum and cortex (Persengiev et al., 2011, 2012a). The mechanism underlying the selective increase of miR-144 transcripts is unknown at this point, but indicates that miR-144 might play a coordinating role in the post-transcriptional suppression of specific genes in the aging brain. The mechanisms that govern miRNA expression during brain development and aging are highly structured and largely unknown. Complex gene expression patterns are regulated at several levels, including regulation by *cis*-acting *trans*-regulatory factors or regulation on the basis of epigenetic modifications such as gene methylation and histone modifications that depend on the genomic landscape. Thus, the adaptive responses of the brain cells during the aging process, which is reflected by brain phenotypic changes and the associated pathologies, will depend on either the physical presence or accessibility of multiple regulatory elements.

COPY NUMBER VARIATIONS ASSOCIATED WITH miRNA GENES AND BRAIN ANOMALIES

Copy number variations (CNVs) in non-coding regions can have profound effects on human phenotype (Klopocki and Mundlos, 2011). CNVs most common outcome is altering the copy number of an entire gene that is predisposed to a dosage effect. In a different scenario, CNVs can result in position effects and cause long-distance effects as far as 1 Mb from the translocation breakpoints. CNVs have been associated with several neuropsychiatric disorders, such as autism, schizophrenia, and bipolar disorder (Cook and Scherer, 2008; Lee and Scherer, 2010). Furthermore, CNVs have been associated with PD and early onset AD, which support the possibility of the existence of CNVs-driven mechanism(s) in PD and AD pathogenesis (Toft and Ross, 2010; McNaughton et al., 2012).

Copy number variations have an impact on the miRNA-mediated post-transcription regulatory network as well. miRNAs preferentially regulate the centers of protein interaction and metabolic networks (Liang and Li, 2007; Baek et al., 2008) and CNVs of miRNA genes may fluctuate the dosage balance of signal transduction pathways, metabolic flux, or protein complexes (Veitia, 2004; Veitia et al., 2008), leading eventually to individuals of the same population or different populations having different susceptibility to diseases. Although a comprehensive investigation to evaluate the CNV-miRNAs health risks among human populations is still lacking, recent experimental studies have confirmed the role of CNV-causing dysregulation of miRNAs in disease occurrence (Volinia et al., 2010). High-frequency copy number abnormalities occur in miRNA-containing regions throughout the genome in a range of human diseases (Zhang et al., 2006; Guo et al., 2008; Rossi et al., 2008; Wong et al., 2008), which is associated with altered expression of multiple genes and pathways (Reddy et al., 2009; Whitman et al., 2010). Genome-wide association studies have confirmed such associations for dozens of protein-coding genes and showed that CNVs capture at least 18% of the total detected genetic variation in gene expression (Stranger et al., 2007). The expression of miRNA genes is modified by CNVs and there is a correlation between somatic CNV and the miRNA levels. Thus, the CNV of functionally relevant miRNAs can modulate or predispose to certain complex genetic diseases.

Copy number variations are segments of genomic DNA that are roughly 1 kb to 1 Mb in length that show variable numbers of copies in the genome due to deletions or duplications and may cause the impairment of neuronal structures. The co-localization of all miRNA loci with known CNV regions was analyzed by using

bioinformatics tools (Marcinkowska et al., 2011). In total, 209 copy number variable miRNA genes (CNV-miRNAs) in CNV regions deposited in the Database of Genomic Variations (DGV) have been identified and validated. Eleven CNV-miRNAs in two sets of CNVs have been classified as highly polymorphic. The overall conclusions from this *in silico* study were that miRNA loci are underrepresented in highly polymorphic and well-validated CNV regions consistent with their essential biological functions. The potential importance and consequences of the miRNAs presence in detected CNV regions, however, has been recognized in several other studies, suggesting that rare CNV-miRNA variants might have significant functional impact (Morley and Montgomery, 2001; Sebat et al., 2004; McCarroll et al., 2008).

At this stage, little is known about CNV of miRNA genes that can cause reduced cognitive ability in normal individuals during aging. miRNA copy number change can cause aberrant miRNA expression and/or deregulation of their target genes in subjects with neurodegenerative disorders, intellectual disability, and congenital abnormalities. For instance, the potential role of CNVs in AD has been investigated and identified a number of genes overlapped by CNV calls (Heinzen et al., 2010; Swaminathan et al., 2012a,b). Case-control association revealed several loci containing CHRFAM7A, RELN, DOPEY2, CSMD1, HNRNPCL1, IMMP2L, SLC35F2, NRXN1, ERBB4, and HLA-DRA genes that are associated with AD. The NRX1 gene has been linked to AD, autism, and schizophrenia (Szatmari et al., 2007; Latella et al., 2009) and ERBB4 is likely to play a role in AD progression (Woo et al., 2010). Overall, there appears that gene duplications and deletions across AD cohorts might account for the differences in the individual susceptibility to the neurodegeneration progression.

Copy number variations were established to be a major contributor of the pathology of brain disorders, but almost all studies have focused on the protein-coding genes present in the CNV loci, while the impact of miRNAs present in these regions has been overlooked. In a more recent study the biological and functional significance of miRNAs present in CNV loci and their target genes has been addressed by using an array of computational tools (Vaishnavi et al., 2013). The study found that nearly 11% of the autism-associated CNV loci harbor miRNAs, most of which were not previously reported to be associated with autism. A systematic analysis of the CNV-miRNAs based on their interactions with the target genes enabled the authors to pinpoint 10 miRNAs, miR-590-3p, miR-944, miR-570, miR-34a, miR-124, miR-548f, miR-429, miR-200b, miR-195, and miR-497 as core factors. The newly identified autism-associated miRNAs were predicted to form a regulatory loop with transcription factors and their downstream target genes. In addition, miRNAs present in deleted and duplicated CNV loci may explain the difference in dosage of the crucial autism genes and can also affect core components of miRNA processing machinery through negative feedback loops. Interestingly, the most common genomic disorder in humans, the hemizygous deletion of a 1.5–3 Mb region of chromosome 22q11.2, which increases the risk of developing schizophrenia by approximately 25-fold includes *DGCR8* miRNA processing gene (Brzustowicz and Bassett, 2012). The exact mechanism by which this deletion increases risk is unknown, but the observation strongly suggests that altered miRNAs metabolism may be a factor in the pathogenesis of schizophrenia. Overall, the findings support a possible role of copy number change in miRNA expression and processing with consequences affecting cognition, brain disorders, and/or CNV-mediated developmental delay.

EFFECTS OF COPY NUMBER VARIATIONS ON miRNA FUNCTION

Heritable information is transformed into cellular and organismal functions by the orderly expression of the entire set of genes in the genome. The complex process of gene expression regulation functions at several levels can be affected by structural alteration in the genomic architecture. Variations in the human genome occur on several levels. Originally, they were described as single-nucleotide changes within or outside of the coding sequence, or as microscopically visible alterations (CNVs) that affect parts of or even entire chromosomes. The effects include regulation in *cis* by promoters, enhancers, and repressors; regulation in *trans* by, e.g., transcription factors or miRNAs; or regulation on the basis of epigenetic modification such as DNA methylation. These genomic segmental differences reflect the dynamic nature of the genome and are believed to account for a large part of human phenotypic variability, including the predisposition to disease.

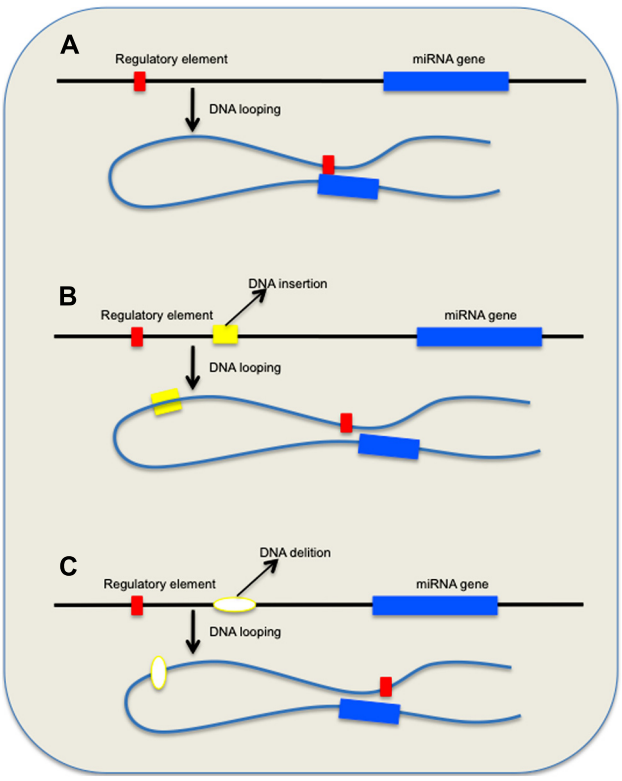

FIGURE 1 | Effect of genomic variations on long-range DNA interactions between remote regulatory elements and miRNA genes. **(A)** DNA looping allows factors associated with distant regulatory element(s) to bind miRNA consensus sequences and control gene expression. **(B,C)** Scenarios that depict the effect of DNA insertions and deletions on the repositioning of the remote regulatory element(s) and eventual loss of physiological control mechanism.

Selected genomic loci have been associated with non-coding pathogenic CNVs and their associated human disease phenotypes. CNVs were found to be distributed genome-wide that encompass non-coding sequences, thereby affecting the regulation of gene expression (Klopocki and Mundlos, 2011). More recently, a genome-wide scan identified 125 regions in which the same haplotypes are segregating in humans and chimpanzees, all with the exception of two encompassed non-coding regions (Leffler et al., 2013). In another study, a systematic search for DNA sequences missing in humans and present in chimpanzees, revealed that the identified sequences were almost exclusively from the non-coding regions of the genome (McLean et al., 2011). In addition, the study discovered that the absence of the penile bone in humans, which is present in chimpanzees, macaques, and mice, is due to the loss of a regulatory element that influences the expression of the androgen receptor gene. It is likely that these approaches will identify many more species-specific changes that relate to changes in phenotype.

Polymorphisms in miRNA genes can affect the expression of many downstream-regulated genes (Georges et al., 2007; Borel and Antonarakis, 2008). Single nucleotide mutations (SNPs) are most common form of polymorphism that affects the function of miRNAs, e.g., the structure of miRNA precursors, the efficiency of miRNA biogenesis and miRNA-target recognition. A series of *in silico* and experimental studies have revealed many SNPs located in different parts of miRNA genes (Duan et al., 2007; de Jong et al., 2013). The occurrence of SNPs in predominantly in the regions surrounding miRNA-coding elements, while sequences of mature miRNAs featured as the most conserved (Saunders et al., 2007). Functional analysis demonstrated that rare mutations naturally occurring within pre-miRNA sequences affect miRNA biogenesis and impair miRNA-mediated gene silencing (Duan et al., 2007; Sun et al., 2009). Recently, large genome-wide association study has demonstrated that SNPs located outside (>14 kb) of pre-miRNA sequences can modulate miRNA expression both as *cis*- and *trans*-regulators, as well (Borel et al., 2011). miRNA target sites are also conserved genetic elements and SNPs with potential to either disrupt or create new miRNA target sites are underrepresented in both experimentally validated and computationally predicted miRNA target sites (Chen and Rajewsky, 2006; Saunders et al., 2007; de Jong et al., 2013). Analysis of CNVs in the human and chimpanzee genomes demonstrates the potentially greater role of CNVs in evolutionary change than single base-pair sequence variation (Cheng et al., 2005). Comparisons of the human and chimpanzee genomes revealed that there are more than twice as many nucleotides involved in CNVs as there are in changes to individual nucleotides, 2.7% compared to 1.2%. Furthermore, the data revealed that while the majority of CNVs were shared between the human and chimpanzee genomes, approximately one-third of the CNVs observed in the human genome were unique and therefore acquired later in evolution. Additional studies have further revealed that CNVs are often linked to genetic diseases apparent in humans (Stankiewicz and Lupski, 2002). However, little is known about CNVs interactions with miRNAs.

Copy number variations have the propensity to alter the general organization of the chromatin in the affected chromosome regions that may have significant functional impact. Recent findings emphasized that nuclear architecture and chromatin organization play important role in the regulation of gene expression (Stankiewicz and Lupski, 2002), and that these components are essential epigenetic mechanisms for both the normal physiology as well as in the pathogenesis of a number of human maladies (Parada et al., 2004a). Portions of DNA, known as DNA loops, protrude from euchromatic portions of chromosomes, and the genes on these segments may localize to transcriptionally active chromatin centers that contain intergenic or intragenic miRNA genes (Osborne et al., 2004). Chromosome looping that enables remote segments of DNA from the same chromosome or from different chromosomes to interact and to modify the expression of distant genes presents a plausible mechanism that links the global misregulation of miRNA expression in AD and other neurodegenerative diseases to CNVs (**Figure 1**). As a consequence of

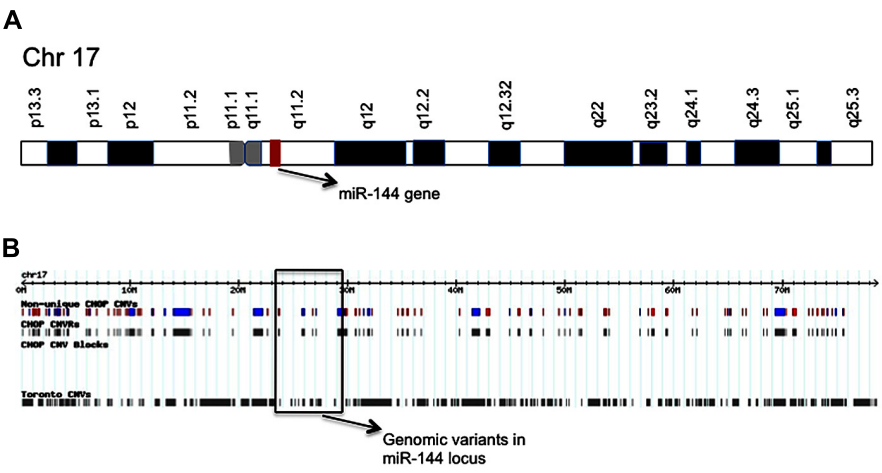

FIGURE 2 | Schematic of chromosome 17 showing the location of miR-144 gene (A); and distribution map of genetic variants identified in chromosome 17 (B). The location of miR-144 gene is shown and CNVs in its respective genomic region are marked.

CNV-induced chromatin reorganization, accessibility of miRNA binding elements within 3′ untranslated region (UTR) of target genes, miRNA promoters availability, as well as the expression of long ncRNAs that serve as sponges for miRNAs might be dramatically altered (Sanyal et al., 2012; Memczak et al., 2013). CNVs that are in close proximity of these loops may also trigger recombination and chromatin rearrangements (Parada et al., 2004b).

Interestingly, the aging-specific miR-144 is located on chromosome 17 in a region reported to be polymorphic, including several inversions and duplications, according to CNV database (**Figure 2**; **Table 1**). The significance of CNVs in the vicinity

Table 1 | Genomic variations in the vicinity of miR-144 genomic location on chromosome 17 according to the Database for Genomic Variants.

Locus	Landmark	Variation type	Cytoband	Position (Mb)	Known genes in the locus
chr17:27013684-27014304	chr17:27,013,684..27,014,304	InDel	17q11.2	27.0	
chr17:27107800-27123735	chr17:27,107,800..27,123,735	Copy number	17q11.2	27.1	
	chr17:27,120,270..27,121,891	Copy number	17q11.2	27.1	
chr17:27122880-27122983	chr17:27,122,880..27,122,983	InDel	17q11.2	27.1	
chr17:27130078-27131878	chr17:27,130,078..27,131,878	Copy number	17q11.2	27.1	
	chr17:27,130,682..27,131,776	Copy number	17q11.2	27.1	
chr17:27130696-27131659	chr17:27,130,930..27,131,420	InDel	17q11.2	27.1	
	chr17:27,130,738..27,131,656	InDel	17q11.2	27.1	
	chr17:27,130,736..27,131,659	InDel	17q11.2	27.1	
	chr17:27,130,696..27,131,638	InDel	17q11.2	27.1	
chr17:27245834-27562095	chr17:27,459,989..27,461,612	Copy number	17q11.2	27.5	UTP6
	chr17:27,412,804..27,436,507	Copy number	17q11.2	27.4	SUZ12
	chr17:27,465,972..27,469,974	Copy number	17q11.2	27.5	LRRC37B
	chr17:27,245,834..27,562,095	Copy number	17q11.2	27.2	SH3GL1P1
	chr17:27,466,732..27,471,357	Copy number	17q11.2	27.5	ARGFXP2
	chr17:27,333,922..27,335,931	Copy number	17q11.2	27.3	RHOT1
chr17:27384860-27385274	chr17:27,384,860..27,385,274	InDel	17q11.2	27.4	LRRC37B
chr17:27460863-27461165	chr17:27,460,863..27,461,165	InDel	17q11.2	27.5	
chr17:27614844-27619890	chr17:27,614,844..27,619,890	Copy number	17q11.2	27.6	RHBDL3
chr17:27621887-27622597	chr17:27,621,887..27,622,597	InDel	17q11.2	27.6	RHBDL3
chr17:27627845-27628095	chr17:27,627,845..27,628,095	InDel	17q11.2	27.6	RHBDL3
chr17:27633422-27634030	chr17:27,633,422..27,634,030	InDel	17q11.2	27.6	RHBDL3
chr17:27668824-27669757	chr17:27,668,824..27,669,757	InDel	17q11.2	27.7	RHBDL3
	chr17:27,669,594..27,669,594	InDel	17q11.2	27.7	
chr17:27788363-27788659	chr17:27,788,363..27,788,659	InDel	17q11.2	27.8	
chr17:27837365-27838765	chr17:27,837,365..27,838,765	Copy number	17q11.2	27.8	CDK5R1
chr17:27917975-27917975	chr17:27,917,975..27,917,975	InDel	17q11.2	27.9	MYO1D
chr17:28279105-28280814	chr17:28,279,105..28,280,814	Copy number	17q11.2	28.3	TMEM98
chr17:28341799-28342792	chr17:28,341,799..28,342,792	InDel	17q11.2	28.3	
chr17:28501812-28502008	chr17:28,501,828..28,502,008	InDel	17q11.2	28.5	ACCN1
	chr17:28,501,812..28,502,002	InDel	17q11.2	28.5	
chr17:28620758-28620884	chr17:28,620,758..28,620,884	InDel	17q11.2	28.6	ACCN1
chr17:28630652-28631318	chr17:28,630,652..28,631,318	InDel	17q11.2	28.6	ACCN1
chr17:28643047-28645208	chr17:28,643,047..28,645,208	Copy number	17q11.2	28.6	ACCN1
chr17:28670843-28673962	chr17:28,670,843..28,673,962	Copy number	17q11.2	28.7	ACCN1
chr17:28708062-28708198	chr17:28,708,062..28,708,198	InDel	17q11.2	28.7	ACCN1
chr17:28779244-28781640	chr17:28,779,244..28,781,640	Copy number	17q11.2	28.8	ACCN1

miR-144 gene is encoded on the minus strand and contains two exons on Chr:17q11.2; 23,396,926-27,188,636 locus.

of miR-144 gene is unclear at this point, but long-range regulatory chromatin interactions play an important role in gene regulation. Both intrachromosomal and interchromosomal long-range associations have been demonstrated, and DNA binding factors have been implicated in the maintenance of these interactions (Cremer et al., 2000; Branco and Pombo, 2006). Several distant DNA segments may interact with a single gene and influence its expression pattern. Monoallelically expressed genes, most notably imprinted genes, are frequently found to be regulated by these long-range interactions. In support of this concept, FLT3-internal tandem duplications (ITDs) on chromosome 13, an adverse prognostic marker in specific aging individuals, were found to affect negatively the expression of GATA-3 transcription factor and miR-144 (Whitman et al., 2010). Members of GATA transcription factor family are believed to play a role in the control miR-144 transcription. GATA-4 transcription factor been reported to be critical regulator of miR-144 expression and is supposed to be the responsible gene for the congenital heart defects (CHDs) in the chromosomal 8p23 deletion syndrome, a complex malformation syndrome with clinical symptoms manifested by facial anomalies, microcephaly, mental retardation, and CHDs (Guida et al., 2010; Zhang et al., 2010). These findings emphasize the importance of studying the geography and architecture of the nucleus as an important factor in the regulation of miRNA expression.

CONCLUSIONS AND OUTLOOK

The existing CNVs in the human genome cover approximately 360 Mb, or 12% of the human genome, as reported by the CNV Project database (http://www.sanger.ac.uk/research/areas/humangenetics/cnv/). CNVs encompass more nucleotide content per genome than SNPs, underscoring CNVs' significance to genetic diversity. A genome-wide map of CNVs shows that no region of the genome is exempt, and that between 6% and 19% of each individual's chromosomes exhibit CNVs (Redon et al., 2006).

The mechanisms that operate during the progress of brain aging and associated neurodegenerative diseases are complex and their malfunction is rarely due to the failure of a few cell death or neuronal differentiation genes. Because susceptibility to premature aging and cognitive decline is a result of the malfunction of numerous genes, miRNAs dysregulation that inevitably would alter the expression of multiple genes might provide the basis for neuronal cell deterioration.

Multiple factors participate in the control of miRNA expression. Here, we discuss the emerging role of CNVs in miRNA regulation and the potential impacts on brain aging and neurodegeneration. Our simple notion is that the long-range interactions between DNA segments affected by CNVs might directly modify miRNA expression pattern, and as consequence miRNA-mediated inhibition of genes that are important for maintaining neuron homeostasis. We argue that CNVs-miRNA interactions are an important part of increased brain susceptibility to external and internal stress during the aging process. A more complete understanding of CNVs effect on the global nuclear geography and chromatin organization in the vicinity of miRNA-encoding regions will allow defining the chromosome regions that represent risk factors for the brain anomalies. Therefore, the challenge now is to annotate CNVs, which potentially can alter miRNA expression and determine whether they are functional variants and should be considered high-priority candidates in genotype–phenotype mapping studies of brain-related disorders.

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MicroRNA-431 regulates axon regeneration in mature sensory neurons by targeting the Wnt antagonist *Kremen1*

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MicroRNAs (miRNAs) are small, non-coding RNAs that function as key post-transcriptional regulators in neural development, brain function, and neurological diseases. Growing evidence indicates that miRNAs are also important mediators of nerve regeneration, however, the affected signaling mechanisms are not clearly understood. In the present study, we show that nerve injury-induced miR-431 stimulates regenerative axon growth by silencing *Kremen1*, an antagonist of Wnt/beta-catenin signaling. Both the gain-of-function of miR-431 and knockdown of *Kremen1* significantly enhance axon outgrowth in murine dorsal root ganglion neuronal cultures. Using cross-linking with AGO-2 immunoprecipitation, and 3′-untranslated region (UTR) luciferase reporter assay we demonstrate miR-431 direct interaction on the 3′-UTR of *Kremen1* mRNA. Together, our results identify miR-431 as an important regulator of axonal regeneration and a promising therapeutic target.

Keywords: miRNA, axon, regeneration, Wnt, *Kremen1*, miR-431, sensory neurons

INTRODUCTION

Axon loss is the hallmark of traumatic brain and spinal cord injury (SCI) as well as many neurodegenerative diseases including Alzheimer's (Coleman and Perry, 2002). A body of research is focused on understanding the mechanisms of axon degeneration and promoting axon regeneration, however, the molecular mechanisms of neural repair remain poorly understood (Fang and Bonini, 2012). Growing evidence indicates that microRNA (miRNA) pathway controls regulatory mechanism involved in neural repair and regeneration (Strickland et al., 2011; Wu et al., 2011, 2012; Yu et al., 2011a; Zhang et al., 2011; Zhou et al., 2012). miRNAs are short, non-coding RNAs that silence gene expression by imperfect binding to 3′-untranslated region (UTR) of mRNA (Bartel, 2004; Filipowicz et al., 2008). miRNAs ability to simultaneously regulate the expression of several genes suggests that they are critical regulators of complex transcriptional networks (McNeill and Van Vactor, 2012). In the nervous system, miRNAs have been implicated in neurodevelopment (Smith et al., 2010), neurogenesis (Shi et al., 2010), and neurological disorders (Hebert and De Strooper, 2007; Kim et al., 2007). Recent observations have identified a group of miRNAs which reside within the distal axonal domain of superior cervical ganglia neuron suggesting miRNA role in the maintenance of axonal structure and function (Natera-Naranjo et al., 2010). In addition, several miRNAs have been associated with axon regeneration in peripheral nervous system (PNS) neurons (Strickland et al., 2011; Yu et al., 2011a; Zhang et al., 2011; Zhou et al., 2012) and axon development in cortical neurons (Dajas-Bailador et al., 2012).

Recent studies from our laboratory have demonstrated that ablation of Dicer, a key enzyme required for miRNA biogenesis, markedly impairs the regenerative axon growth *in vivo* and *in vitro*, indicating that the intact Dicer-dependent miRNA pathway is critical for successful peripheral nerve regeneration (Wu et al., 2012). In the current study, we examine the mechanism of miRNA action in axon regeneration. Here we show that injury-induced miR-431 stimulates regenerative axon growth by silencing *Kremen1*, a negative regulator of Wnt/beta-catenin signaling pathway. Both the gain-of-function of miR-431 and loss-of-function of *Kremen1* significantly enhance regenerative axon growth in dissociated dorsal root ganglia (DRG) neuronal cultures. Using cross-linking with AGO-2 immunoprecipitation (CLIP), and 3′-UTR luciferase assay we demonstrate miR-431 direct interaction on the 3′-UTR of *Kremen1* mRNA. Collectively, our observations provide the first evidence for a role of miRNA in regulating Wnt/beta-catenin signaling pathway in nerve regeneration and identify miR-431 as an important regulator and a potential therapeutic target.

MATERIALS AND METHODS

ANIMALS

Eight-week-old CD-1 male mice were obtained from Charles River laboratories (Wilmington, MA, USA). The animal use protocol was approved by the institutional Animal Care and Use Committee of East Carolina University, an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. Animals were housed individually under standard laboratory conditions, with a 12 h light/dark schedule and unlimited access to food and water.

CONDITIONING NERVE LESION

Before surgery, anesthesia was induced using an intraperitoneal ketamine (18 mg/ml)-xylazine (2 mg/ml) mixture (0.05 ml/10 g of body weight). The procedure followed a protocol described previously (Islamov et al., 2004). Exposure of the right sciatic nerve was performed with sterile surgical instruments through an incision on the middle thigh of the right hind limb. Approximately 5 mm of nerve was exposed from the sciatic notch to the trifurcation of the nerve. The exposed sciatic nerve was crushed in the mid-thigh for 15 s with a fine hemostat. The wounds were closed with 3M™ Vetbond™ Tissue Adhesive (3M, Saint Paul, MN, USA) and the animals were left to recover for 5 days.

DISSOCIATED DRG CULTURES

Mouse L4/5 DRG neurons were collected 5 days after a conditioning sciatic nerve crush from both the intact side and injured side. DRGs were dissociated with collagenase and 0.25% trypsin in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA). The dissociated DRGs were plated on poly-L-lysine and laminin (Invitrogen), coated plates. DRGs were grown in DMEM/F12 containing 10% horse serum, L-glutamine, and N2 supplement (Gemini Bio-product, West Sacramento, CA, USA) at 37°C for 18 h. To inhibit glial cell growth cytosin β-D-arabinofuranoside (Arac, 10 μM) and 5,6-dichlorobenzimidazole riboside (DRB, 80 μM; Sigma, Saint Louis, MO, USA) or 50 nM 5-fluoro-2′-deoxyuridine (Sigma) were added to the growth medium.

PC12 CELL CULTURES

PC12 cells were cultured in DMEM containing 10% horse serum, 5% fetal bovine serum 2 mM glutamine, and penicillin and streptomycin (100 unitl/ml). The cells were plated on collagen-coated cell culture dishes. For nerve growth factor (NGF)-induced differentiation of PC12 cells, NGF (50 ng/ml) was added to cell culture medium to initiate neurite outgrowth. Medium was refreshed every 2–3 days.

TRANSFECTION OF miRNA MIMICS AND INHIBITORS

In order to determine the biological effects of each individual miRNA on regenerative axon growth, we performed functional analyses for injury-induced miRNAs. Gain-of-function experiments were performed with Ambion® Pre-miR™ miRNA Precursor Molecules (Ambion, Austin, TX, USA), which are also called miRNA mimics. With transfection reagent, these small, chemically modified double-stranded RNA molecules can be introduced into cells and be taken up into the RNA-induced silencing complex (RISC), mimicking endogenous mature miRNAs activity. Loss-of-function analyses were performed with Ambion® Anti-miR™ miRNA inhibitors. The miRNA inhibitors are chemically modified, single-stranded nucleic acids designed to specifically bind to complementary miRNAs. The binding between endogenous miRNA and miRNA inhibitors down-regulates endogenous miRNAs activity.

All miRNA mimics and miRNA inhibitors were obtained from Ambion. Transient transfections of DRGs were performed using Lipofectamine™ LTX and Plus Reagent (Invitrogen) according to the manufacturer's protocol. To extend the time window for effective transfection of miRNA precursors and inhibitors, as well as, initiation of miRNA machinery, we incubated DRG neurons with 20 μM of SP600125 for the first 24 h according to a protocol previously described (Davare et al., 2009). SP600125 is a specific inhibitor of JNK and reversibly inhibits axonogenesis (Davare et al., 2009). We then released the block on axonogenesis from the SP600125 by washing out SP600125 and change culture media. DRG neurons were then cultured for an additional 24 h to allow axon formation.

IMMUNOFLUORESCENT STAINING AND IMAGE ANALYSIS

The cells cultured on coverslips were fixed with 4% paraformaldehyde for 5 min and washed with phosphate buffered saline with Tween (PBST). After blocking with 10% goat serum for 1 h at room temperature, the samples were incubated with the indicated primary antibodies diluted at optimized concentrations at 4°C overnight. This was followed by incubation with secondary antibodies conjugated with FITC-, TX Red-, or Alexa Fluor® (Invitrogen). Negative controls included samples processed in parallel with non-immune serum or without primary antibodies. After mounting the slides with anti-fading media (Invitrogen), images were viewed with an Olympus IX81 fluorescent microscope and captured with CellSens Dimension software (Olympus America Inc., Center Valley, PA, USA). The images we acquired were all single plane fluorescent images.

Quantification of axon length and measurement of axon branches were performed following previously described lab protocol (Murashov et al., 2005). For each coverslip, 30 images were taken, and from each, 10–15 neurons, which were completely distinguishable from neighboring cells, were chosen for further analysis. The axon length was quantified by tracing the image of neurites with the ImageJ software (NIH, Bethesda, MD, USA). The longest axon for each neuron was measured and recorded. The number of neurite branches per neuron was also determined from each neuronal population manually. Only primary branches, which are routinely defined as neurites originating from the neuronal soma and are at least longer than two times the diameter of the cell body were counted (Liu et al., 2002).

IMMUNOBLOTTING ANALYSIS

Tissue samples were homogenized in ice-cold homogenization buffer (20 mM Tris, 2 mM EGTA, 2 mM EDTA, 6 mM β-mercaptoethanol, 1mM PMSF, and 10% Triton) containing protease inhibitor cocktail (Sigma), and centrifuged at 10,000 *g* for 10 min at 4°C. The supernatants were collected in fresh tubes and stored at −20°C. Proteins concentrations were quantified using the Bio-Rad reagent (Bio-Rad, Hercules, CA, USA) and samples for western blot analysis were prepared by boiling with standard stop buffer for 5 min. Equal amounts of solubilized proteins were loaded per lane on sodium dodecyl sulfate gels and separated by electrophoresis. The separated proteins were then transferred to immobilonP membranes (Millipore Corporation, Bedford, MA, USA).

Membranes were blocked in Odyssey blocking buffer (LI-COR, NE, USA) for 1 h at room temperature on a shaker, and then probed with a primary antibody in Odyssey blocking buffer at 4°C

overnight. The membranes were washed three times with PBST, and then incubated with IRDye® conjugated secondary antibodies for 1 h at room temperature with gentle shaking. The fluorescent signals on membrane were detected with the Odyssey® Infrared Imaging System (LI-COR). Densitometry values were normalized to α-tubulin, to obtain the relative signal intensity.

LIST OF ANTIBODIES

Primary antibodies

Mouse monoclonal neuro-specific β III tubulin antibody (TUJ-1) Covance Research Products, Inc. (Denver, PA, USA). Goat polyclonal antibodies against *Kremen1* (R&D Systems, Minneapolis, MN, USA). Rabbit anti-GAP-43 polyclonal antibodies (Millipore, Billerica, MA, USA). Mouse monoclonal anti-α-tubulin antibodies Zymed (Zymed Laboratories, Carlsbad, CA, USA).

Secondary antibodies

IRDye 800CW goat anti-Mouse IgG, IRDye 680LT goat anti-Rabbit IgG, and IRDye 800CW donkey anti-goat IgG secondary antibodies (LI-COR Corporate, NE, USA). For fluorescence studies, secondary FITC-, TX Red-conjugated IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) or Alexa Fluor 594 donkey anti-goat from Invitrogen were applied.

Cross-linked immunoprecipitation (CLIP) analysis

Argonaute CLIP method to identify *in vivo* targets of miRNAs followed procedure described previously (Jaskiewicz et al., 2012). DRG neuronal cell cultures were transfected with 100 nM of miR-431 mimic or a scrambled miRNA mimic negative control. Two days post-transfection, the cells were rinsed once in PBS and then placed in UVP CL-1000 cross-linker (UVP, Upland, CA, USA) with the cover off. Cells were irradiated once for 400 mJ/cm^2 and once more for 200 mJ/cm^2 to establish protein-RNA reversible cross-linking. Cells were lysed in cell lysis buffer (100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES, PH 7.0, 0.5% NP-40, 1 mM DTT, 100 U/ml RNasin RNase inhibitor (Promega), 2 mM vanadyl-ribonucleoside complexes solution (Sigma)) supplemented with a mixture of protease inhibitors (Invitrogen). Cells were then detached with a cell scraper and lysate was transferred to a tube on ice. Cell lysates were centrifuged at 16,000 *g* for 15 min at 4°C and the supernatants (the protein lysates) were transferred to sterile tubes for further immunoprecipitation. Prior to the immunoprecipitation, protein G agarose beads (Sigma) were equilibrated by washing twice with a wash buffer (0.5% NP-40, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM Tris, pH 7.5, 5 mM DTT, with protease inhibitor) containing 1 mg/ml yeast tRNA and 1 mg/ml BSA. After pre-clearing the protein lysate with equilibrated protein G-agarose beads, 5 μl of each sample was saved as an input fraction. The protein lysate was immunoprecipitated with specific mouse monoclonal antibodies against Ago-2 (Wako, Richmond, VA, USA) or control serum and bounded by protein G agarose beads with agitation at 4°C overnight. After precipitation, the beads were washed three times with washing buffer. Afterward, the bonds between RNA and protein were disrupted by heating at 50°C for 30 min. RNA was then extracted and purified using Trizol (Invitrogen) and used for qRT-PCR.

Luciferase assays

Luciferase assays were performed using the pMIR-REPORT^{TM} miRNA expression reporter vector system (Ambion). pMIR-REPORT firefly luciferase (FL) plasmids were purified with Miniprep kit (Qiagen, Valencia, CA, USA) and digested with restriction enzymes *Spe*I and *Hind*III (New England BioLabs, Ipswich, MA, USA). Linearized vectors from the restriction digestion were retrieved by agarose gel electrophoresis and gel purification of DNA using Gel Extraction Kit (Omega Bio-Tek, Inc., Norcross, GA, USA). The 3′-UTR regions of mouse *Kremen1* gene were amplified from mouse *Kremen1* cDNA clones (Source Bioscience, Nottingham, UK). The primers were designed as: 5′-ATAACTAGTGCTCCGCTCCAAGCTCGAGTTTGC 3′ and 5′-GCGAAGCTTTCTCTTTTGTAAAAGTTAAGTACC 3′. Restriction enzyme sites for *Spe*I and *Hind*III were introduced into the PCR product to facilitate directional cloning. The 3′-UTR of *Kremen1* was inserted into downstream of FL gene in the pMIR-REPORT vector with T4 ligase (New England BioLabs), and subsequently transformed in DH5α competent cells (Invitrogen). Luciferase assays were performed using the Dual-Luciferase assay kit (Promega). PC12 cells (40,000) were cultured and co-transfected in 24-well plates with 400 ng of FL reporter construct, 100 nM miR-431 mimics or mimic negative controls, and 40 ng of pRL-TK control vector encoding renilla luciferase (RL; Promega). The transfection was performed with Lipofectamine 2000. Forty hours after transfection, the cells were harvested in passive lysis buffer and firefly and RL activities were measured in a Turner Biosystems 20/20n Luminometer (Turner Biosystems, Sunnyvale, CA, USA). The luciferase data is expressed as a ratio of FL to RL to normalize for transfection variability between samples. Luciferase experiments were repeated at least three independent times in triplicate.

miRNA and gene expression array analyses for DRG RNA

Total RNA for the microarray expression analysis was isolated from L4 to L5 DRGs, pooled from 10 mice at 4 days after sciatic nerve crush. Total RNA extraction was performed with miRVANA^{TM} miRNA isolation kit following the manufacturer's instruction (Ambion). These pooled RNA samples were sent to UNC Lineberger Comprehensive Cancer Center Genomics Core for microarray analysis. After a quality control, they were hybridized to 8 × 15 miRNA one-color arrays (Agilent, Santa Clara, CA, USA). The same RNA samples were also hybridized to 4 × 44K mouse gene expression microarrays (Agilent) at the same Genomics Core. All microarray experiments were performed in duplicate and repeated twice. Normalization and further analyses of microarray data were performed with GeneSpring software (Agilent). Differentially expressed miRNAs were determined using a combination of t tests, with FDR correction of 0.1, and further defined by p-value < 0.05 after correction for multiple hypotheses. The analysis with GeneSpring allowed for identification of a different expression pattern of miRNAs in the crushed groups compared with the control groups. Statistically significant upregulated or downregulated miRNAs were then selected for further analysis. All microarray data have been submitted to GEO (access number pending).

Real-time PCR (RT-PCR)

Total RNA was isolated from L4-L5 DRGs using *mir*Vana™ miRNA Isolation Kit (Ambion). Total RNAs from DRG neuronal cell cultures were purified with RNAqueous Micro Scale RNA Isolation Kit (Ambion). RNA was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Reverse transcription was performed with NCode™ VILO™ miRNA cDNA Synthesis Kit and SuperScript VILO cDNA Synthesis Kit (Invitrogen) for miRNA expression analysis and mRNA expression analysis, respectively. The real-time PCRs were carried out using EXPRESS SYBER® GreenER™ qPCR SuperMix Universal (Invitrogen) in triplicates for each cDNA sample on Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). Primers specific for each miRNA and mRNA were obtained from Invitrogen. As an internal control, primers for S12 (mitochondrial ribosome small subunit) were added for RNA template normalization, and the relative quantification of gene and miRNA expressions were calculated against S12 using a $2^{-\Delta\Delta CT}$ method. We routinely use S12 for qPCR studying axonal injuries. Other standard controls like beta-actin and GAPDH usually change in response to crush injury. All experiments were carried out three times independently.

List of primers

miR-21: 5′-TAGCTTATCAGACTGATGTTGA-3′
miR-431: 5′-CAGGCCGTCATGCAAA-3′
miR-744: 5′-GGGCTAGGGCTAACAGCA-3′
miR-124: 5′-GCGGTGAATGCCAAAAA-3′
miR-29a: 5′-TAGCACCATCTGAAATCGGTTA-3′
Kremen1: 5′-ACAGCCAACGGTGCAGATTAC-3′ and 5′-TGTTGTACGGATGCTGGAAAG-3′
GAP-43: 5′TGGTGTCAAGCCGGAAGATAA-3′ and 5′-GCTGGTGCATCACCCTTCT-3′
S-12: 5′-TGGCCCGGCCTTCTTTATG-3′ and 5′-CCTAAGCGGTGCATCTGGTT-3′

Statistical analysis

Data from multiple independent experiments were analyzed with GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA, USA). The results were expressed as mean ± standard error of the mean in graphic and text representations. To determine the difference between three or more groups, a one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests was utilized. For the analysis of two independent groups, Student's *t*-test was used. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

miRNAs ARE DIFFERENTIALLY EXPRESSED IN DRG UPON SCIATIC NERVE INJURY

We analyzed miRNA expressions in DRGs using microarrays at 4 days after sciatic nerve crush. DRGs were collected from both the pre-conditioned side, as well as the contralateral uninjured side. RNA from the contralateral uninjured side served as a control group. At 4 days post-injury, pre-conditioned DRG neurons show robust regenerative axon growth (Forman et al., 1980). RNA from the pre-conditioned DRG was considered the actively regenerating group. By comparing the miRNA expression pattern from pre-conditioned DRG and control DRG, miRNAs that were upregulated and down-regulated during the process of regeneration were determined. Several miRNAs demonstrated differential expression based on regenerative growth condition. Using 1.5-fold cut-off, statistical analyses revealed that 19 miRNA were differentially expressed in the pre-conditioned DRG compared to the non-conditioned contralateral DRG. Of those 19, 11 miRNAs had higher expression level in pre-conditioned group and the other eight miRNAs had lower expression level in DRG during regeneration (**Figure 1A**). miR-431, miR-714, miR-744, miR-877, miR-130b, miR-21, miR-323-3p, miR-325, miR-409-3p, miR-154*, and miR-681 were significantly increased 4 days post-sciatic nerve crush in pre-conditioned DRGs, while miR-190, miR-1, miR-33, miR-32, miR-153, miR-335-5p, miR-193, and miR-488 showed significantly decreased expression. The most upregulated miR-431 was selected for further analyses.

We validated the microarray data for miR-431 using real-time qPCR. We also included miR-744 and miR-21 as positive controls and miR-124 and miR-29a as non-regulated controls in our real-time PCR experiments. These last two miRNAs play various roles in neurodevelopment and maintenance of neuronal cell homeostasis (Cheng et al., 2009; Shioya et al., 2010); however, they did not show changes in their expression in our array data. In agreement with the microarray data, miR-431, miR-744, and miR-21 were significantly upregulated in regenerating neuronal cells. We detected 2.4-fold upregulation of miR-431, a twofold upregulation of miR-744, and a 2.5-fold upregulation of miR-21, respectively (**Figure 1B**). At the same time, RT-qPCR experiments showed that miR-29a and miR-124 did not change their expression during regeneration.

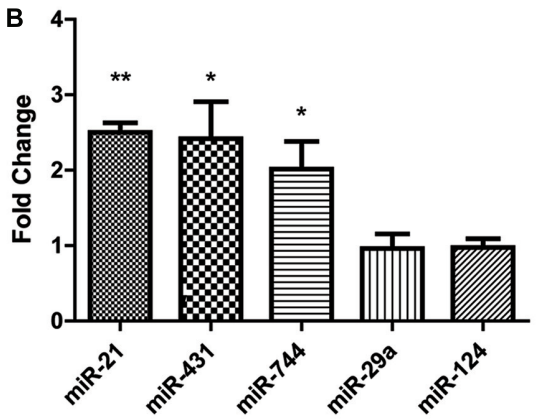
GAIN-OF-FUNCTION OF miR-431 INCREASES REGENERATIVE OUTGROWTH

To investigate the role of miR-431 in regenerative axon growth, we manipulated the level of miR-431 in dissociated DRG neurons. We observed a positive association between miR-431 expression and neurite outgrowth in dissociated DRG neuronal cell culture (**Figure 2A**). Increased mir-431 level was achieved by applying miR-431 mimic to DRG neuronal cell cultures at a final concentration of 100 nM. Overexpression of miR-431 significantly increased axon length. Additionally, blocking miR-431 activity with miR-431 inhibitor significantly inhibited neurite extension (no treatment control group: 100 ± 5%; miR-431 mimic group: 130 ± 6%; mimic negative group: 91 ± 4%; miR-431 inhibitor group: 75% ± 7%; inhibitor negative control: 90 ± 8%; **Figure 2B**). Moreover, manipulating miRNA-431 levels also affected axon branching, and led to a decrease in the number of branches per neuron due to transfection with miR-431 inhibitor (no treatment control group: 100 ± 9%; miR-431 mimic group: 110 ± 10%; mimic negative group: 82 ± 7%; miR-431 inhibitor group: 64% ± 6%; inhibitor negative control: 86 ± 10%; **Figure 2C**).

We next studied GAP-43 expression in DRG neurons with miR-431 mimic and inhibitor treatments, as a strong association

A

miRNA	Fold change	
mmu-miR-431	2.611244	up
mmu-miR-714	2.1570964	up
mmu-miR-744	2.1376243	up
mmu-miR-877	2.081734	up
mmu-miR-130b	1.7698824	up
mmu-miR-21	1.7598088	up
mmu-miR-323-3p	1.6689959	up
mmu-miR-325	1.5642644	up
mmu-miR-409-3p	1.5461996	up
mmu-miR-154*	1.517914	up
mmu-miR-681	1.5049202	up
mmu-miR-190	2.063784	down
mmu-miR-1	1.7755992	down
mmu-miR-33	1.6966436	down
mmu-miR-32	1.6579413	down
mmu-miR-153	1.6068152	down
mmu-miR-335-5p	1.595247	down
mmu-miR-193	1.5837495	down
mmu-miR-488	1.5546329	down



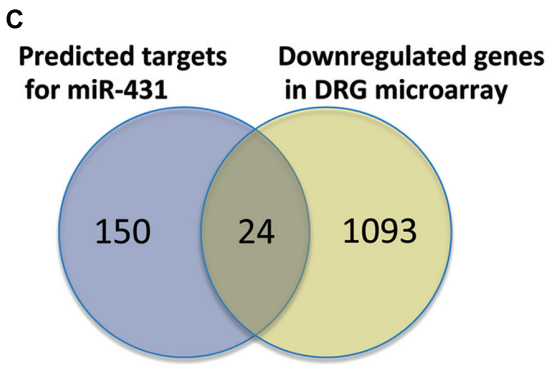


FIGURE 1 | Sciatic nerve injury induced changes in miRNA expression profile in DRG. (A) Total RNA for the microarray expression analysis was isolated from DRG 4 days after sciatic nerve crush. Agilent arrays were done in duplicates and repeated twice. Normalization and analyses were performed with GeneSpring software. miRNAs with a statistically significant upregulation or down-regulation over 1.5-fold were listed in the table. **(B)** Three miRNAs that were significantly upregulated were selected for further validation. Real-time qPCR for miRNA validated the relative changes in miRNA level. miRNA expression was normalized to reference *(Continued)*

FIGURE 1 | Continued
gene s12. The graph indicates a significant increase of miR-744, miR-431, and miR-21 in DRG after sciatic nerve crush, whereas the expression level of miR-124 and miR 29a did not change (*$p < 0.05$, **$p < 0.01$, $N = 3$). **(C)** Venn diagram of overlap in predicted miR-431 target genes and down-regulated genes in DRG after conditioning sciatic nerve lesion. The potential targets of miR-431 were chosen using three algorithms http://www.targetscan.org, http://www.microrna.org, and http://diana.cslab.ece.ntua.gr. Down-regulated genes were selected using fold change cut-offs of >2 and significance *p*-values of <0.05 expression based on microarray data for DRGs 4 days post-sciatic nerve injury. Overlap shows 24 genes having predicted binding site for miR-431 and significantly down-regulated expression level in DRG microarray. A one-way ANOVA followed by Bonferroni's multiple comparison tests was utilized. For the analysis of two independent groups, Student's *t*-test was used.

between neurite outgrowth and expression of GAP-43 has been reported in previous studies (Benowitz and Routtenberg, 1997). We observed significant increase in GAP-43 immunostaining caused by transfection with miR-431 (**Figure 2D**). GAP-43 mRNA level was further studied with RT-qPCR. **Figure 2E** clearly demonstrates a significant increase in GAP-43 mRNA in the cultures treated with 100 nM of miR-431 mimics, as compared to the group treated with the scrambled miRNA mimic control. This relates to immunofluorescent data demonstrating significant increase in axon outgrowth after overexpression of miR-431.

IDENTIFICATION OF miR-431 mRNA TARGETS

We used three databases[1] to generate a list of mRNAs with potential binding site for miR-431 in their 3′-UTR. The potential candidates were further selected based on evaluation of the gene expression microarray data for DRGs 4 days post-sciatic nerve injury (SNI). We hypothesized that an increased expression of miR-431 in preconditioned DRG, would negatively associate with expression of the target mRNAs in the same RNA samples. Using GeneSpring 10 software package (Agilent) we performed joint analysis of miRNA and gene expression data. This allowed us to narrow the list of potential targets to 24 genes. These 24 genes met both criteria, of having a predicted binding site for miR-431 in their 3′-UTR and significantly down-regulated expression level in DRG microarray (**Figure 1C**).

To investigate which genes may be regulated by miR-431, we initially screened potential targets in neuronal PC12 cells overexpressing miR-431. Transient overexpression of miR-431 was achieved using transfection of PC12 cells with miR-431 mimic. The expression of potential targets was studied with real-time RT-qPCR. The experiments revealed that only six genes (*Braf, Eif2s2, Kremen1, Msi2, Tnrc6b, Zkscan1*) were significantly down-regulated by miR-431 in PC12 cells (**Table 1**). We then applied the same approach to test these six genes with overexpression of miR-431 in primary DRG neurons. In the RT-qPCR experiments, overexpression of miR-431 led to significant suppression of the expression of only three genes including *Braf*, *Kremen1*, and *Zkscan1* (**Table 1**). Based on the literature data indicating that *Kremen1* is an antagonist of Wnt signaling pathway (Nakamura and Matsumoto, 2008), which

[1]http://www.targetscan.org, http://www.microrna.org, http://diana.cslab.ece.ntua.gr

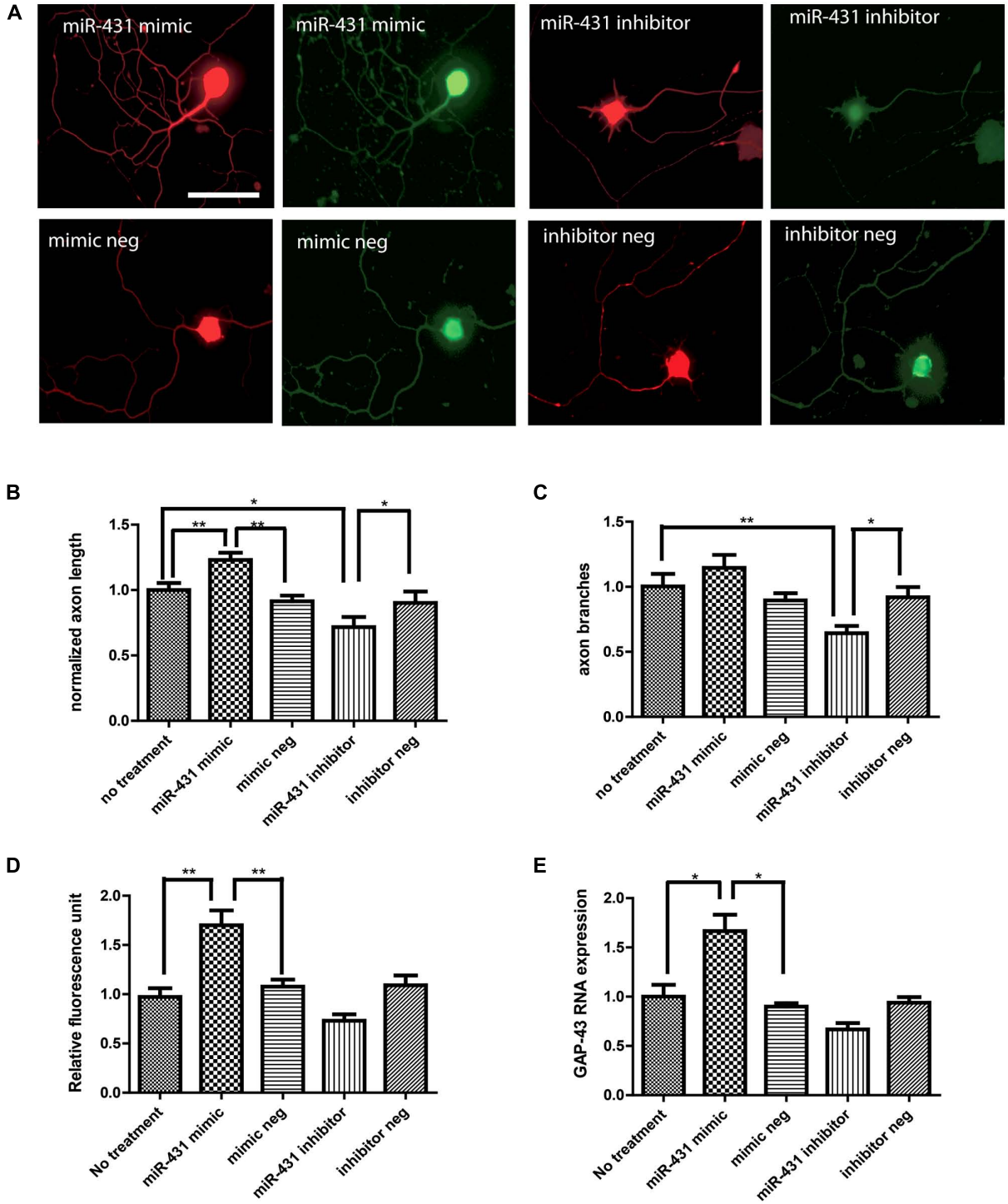


FIGURE 2 | miR-431 increases axon outgrowth in DRG neurons. Effects of miR-431 mimic and inhibitor on axon outgrowth. (A) Left panel shows the effect of the transfection of DRG neurons with miR-431 mimic. Right panel depicts the effect of transection with miR-431 inhibitor. Negative controls for miR-431 mimic and inhibitor are indicated on the lower images. Cells were stained with primary antibodies against neuronal β-tubulin and signals were visualized with TX-Red conjugated secondary antibody (scale bar: 50 μm). The expression of GAP-43, a marker for axon regeneration, was detected using an anti-GAP-43 antibody and visualized with FITC-conjugated secondary antibodies. The effect of miR-431 on axon length **(B)** and on axon branching **(C)** was quantified. Overexpression of miR-431 significantly increased axon extension, whereas suppression of miR-431 significantly blocked axon branching. The fluorescence signal intensity against GAP-43 was quantified in **(D)**. The significant increase in GAP-43 immunofluorescence reflects increase in regenerative axon growth. **(E)** Significant increase in GAP-43 expression on mRNA level quantified by RT-qPCR (*$p < 0.05$, **$p < 0.01$, $N = 50$). A one-way ANOVA followed by Bonferroni's multiple comparison tests was utilized.

Table 1 | Effect of miR-431 overexpression on levels of potential target genes in PC12 cells and primary DRG culture.

Gene	PC12 cells		DRG culture	
	Relative value	**SEM**	**Relative value**	**SEM**
Braf	**0.323**	**0.1245**	**0.5133**	**0.07055**
Cwf1912	5.833	0.8762		
Dlst	1.85	0.1531		
Eif2s2	**0.4367**	**0.06766**	0.8333	0.2028
Fgf12	0.68	0.1531		
Hip1	1.07	0.2608		
Kremen1	**0.3833**	**0.05364**	**0.6733**	**0.0393**
Luc712	0.69	0.1054		
Msi2	**0.4133**	**0.05364**	1.04	0.07024
Ncam1	1.103	0.2284		
Nudcd3	0.91	0.1002		
Slc30a10	1.163	0.02906		
Son	4.033	0.5044		
Tcf712	2.043	0.4937		
Tnrc6b	**0.58**	**0.0755**	0.9467	0.245
Vezt	1.253	0.1141		
Wnk3	0.9433	0.1601		
Zeb2	0.9733	0.1742		
Zkscan1	**0.2967**	**0.06009**	**0.45**	**0.06429**

Transient overexpression of miR-431 was achieved using transfection with its mimic. Relative mRNA levels of the potential target genes were evaluated by real-time RT-qPCR. Bold numbers indicate significant decrease in gene expression. Only three genes Kremen1, Braf, and Zkscan1 were significantly down-regulated in primary neuronal culture.

is critical for axonal remodeling (Purro et al., 2008), we focused our subsequent experiments on characterization of *Kremen1*–miR-431 interaction.

To investigate a direct interaction between target mRNAs and miR-431 in RISC, CLIP of the Ago-2 protein, the central component of the RISC was carried out. Applying miR-431 mimic to DRG neurons increased the expression level of miR-431 ∼7.75-fold in DRG neuronal cell cultures (**Figure 3A**). Electrophoresis of CLIP samples confirmed the miR-431 induced association of *Kremen1* mRNA with RISC, suggesting *Kremen1* as the target gene for miR-431 (**Figure 3B**). **Figure 3B** shows the RT-PCR of *Kremen1* mRNA presented in the total RNA (input) and IP fractions from DRG cultures treated with miRNA mimic and the mimic negative control. In the total RNA samples from DRG cultures, overexpression of miR-431 reduced the amount of stable *Kremen1* mRNA when compared to the miRNA mimic negative control group. In the Ago-2 immunoprecipitated RNA samples, overexpression of miR-431 clearly increased the level of Ago-2 associated *Kremen1* mRNA. In the IP negative control group (non-immune serum), no detectable *Kremen1* mRNA was observed, confirming the specificity of the precipitation (**Figure 3B**).

LUCIFERASES REPORTER ASSAY CONFIRMS miR-431 TARGET *Kremen1* 3′UTR

Kremen1 has one binding site for miR-431 at its 3′-UTR, at the position 2530–2536 bp. It corresponds perfectly to nucleotides 2–7 of the mature miRNA in mouse, rat, and human. In addition, the seed target site is close the poly-A tail, which increases its accessibility. To confirm miR-431 direct interaction on *Kremen1* 3′ UTR, we established a *Kremen1* 3′UTR-FLs construct with the 3′-UTR of *Kremen1* inserted downstream of the FL gene. This construct allowed us to quantitatively evaluate the regulatory effect of miR-431 on the 3′-UTR of *Kremen1*. PC12 cells were transiently transfected with miR-431 mimics or mimic negative controls, *Kremen1* 3′UTR-FL construct, and RL plasmid DNA as internal control. As shown in **Figure 3F**, co-transfection of miR-431 mimic and *Kremen1* 3′UTR-FL construct resulted in significant decrease in FL activity. Luciferase activity reduced to 48% compared with the vector control, whereas co-transfection of mimic negative controls and *Kremen1* 3′UTR-FL construct did not affect the expression of FL gene (**Figure 3F**). Together, these data suggest that miR-431 actively modulates *Kremen1* protein and RNA expression within DRG neurons through association with *Kremen1* 3′UTR.

miR-431 MODULATES *Kremen1* EXPRESSION AT mRNA AND PROTEIN LEVELS IN PRIMARY NEURONAL CULTURES

To show that miR-431 regulates endogenous *Kremen1* in DRG neurons, we transfected cells with either miR-431 mimics, miR-431 inhibitors, mimic negative control, or inhibitor negative control. Since miRNA-mediated gene regulation can destabilize target mRNA and reduce the level of the target mRNA, we used RT-qPCR to determine the effect of miR-431 on *Kremen1*. We observed that transient transfection with miR-431 mimic, decreased the mRNA level of *Kremen1* to 30%. Application of miR-431 inhibitors significantly elevated the mRNA level of *Kremen1* (**Figure 3C**). These results demonstrated that miR-431 level is inversely correlated to *Kremen1* expression at mRNA level in DRG neurons.

We then performed proteomic analysis of *Kremen1* in DRG neurons. Whereas endogenous miR-431 was inhibited by transfection with miR-431 inhibitor, the expression level of the *Kremen1* protein was significantly higher than in control groups. Quantification of three independent experiments revealed that miR-431 reduced *Kremen1* protein levels by 50% when compared with the mimic negative control group. On the other hand, inhibition of endogenous miR-431 resulted in a significant increase of *Kremen1* expression by 45% (**Figures 3D,E**).

Kremen1 EXPRESSION IN DRG *IN VIVO*

After establishing a physical interaction between miR-431 and *Kremen1*, we next investigated the expression patterns of *Kremen1* during axon regeneration. From gene expression array data, *Kremen1* expression in DRG decreased at 4 days after SNI, suggesting its expression was down-regulated as the peripheral nerve regenerated. To further reveal physiological role miR-431 *Kremen1* interaction, we analyzed expression of *Kremen1* at RNA and protein levels from control and regenerating DRGs. RT-qPCR revealed that *Kremen1* RNA expression decreased four-fold at 4 days after sciatic nerve crush, when axons exhibit

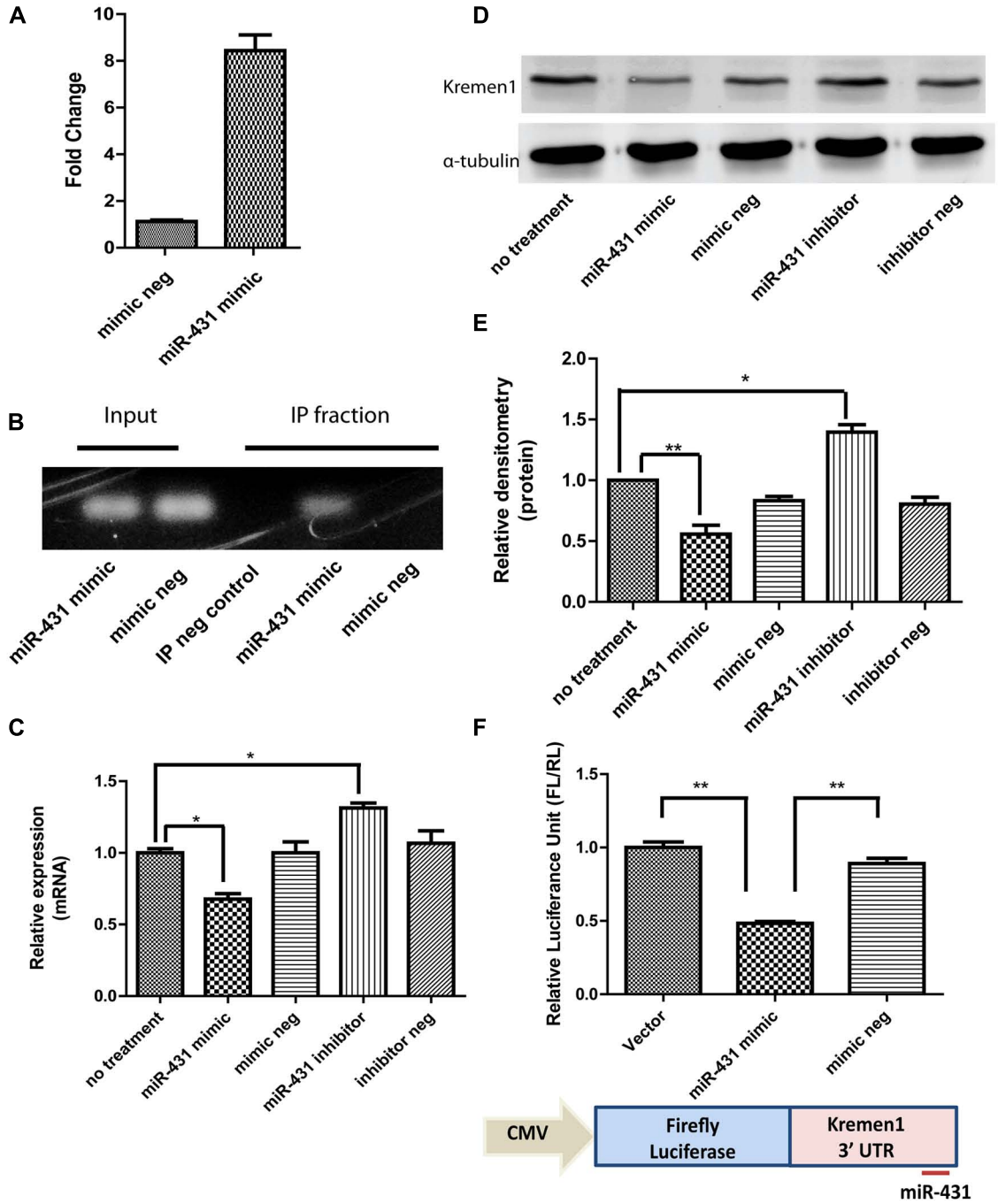


FIGURE 3 | miR-431 regulates *Kremen1* expression. (A) RT-qPCR confirmed the increase of miR-431 level in DRG neuron after the transfection of miR-431 mimic. **(B)** Although overexpression of miR-431 decreased *Kremen1* mRNA in total cell lysates (input), it enhanced the binding between *Kremen1* mRNA and Ago-2 complex. In the Ago immunoprecipitated fractions, there was an increased amount of *Kremen1* mRNA. The lack of signal in the non-specific serum IP sample (IP neg. control) confirmed the specificity of the IP. **(C)** miR-431 negatively regulated *Kremen1* expression at mRNA level. Treatment of miR-431 mimics in DRG neuronal cultures significantly inhibited *Kremen1* expression as compared with that of control groups. On the contrary, suppression of miR-431 activity significantly enhanced the expression of *Kremen1* mRNA. **(D)** Western blot analysis of *Kremen1* expression exhibited similar negative correlation of miR-431 and *Kremen1* expression. Cells transfected with miR-431 mimics had decreased protein level of *Kremen1*, whiles cells transfected with miR-431 inhibitors had an increased expression of *Kremen1*. α-tubulin was used as the loading control and was used to normalize densitometry values. **(E)** The quantification of densitometric levels of *Kremen1*. **(F)** PC12 cells were transfected with *Kremen1* 3′UTR-firefly Luciferase constructs for luciferase assays. Co-transfection with miR-431 mimics significantly reduced the luciferase activity ($*p < 0.05$, $**p < 0.01$), whereas co-transfection with mimic negative controls did not affect the expression of firefly luciferase gene. A one-way ANOVA followed by Bonferroni's multiple comparison tests was utilized.

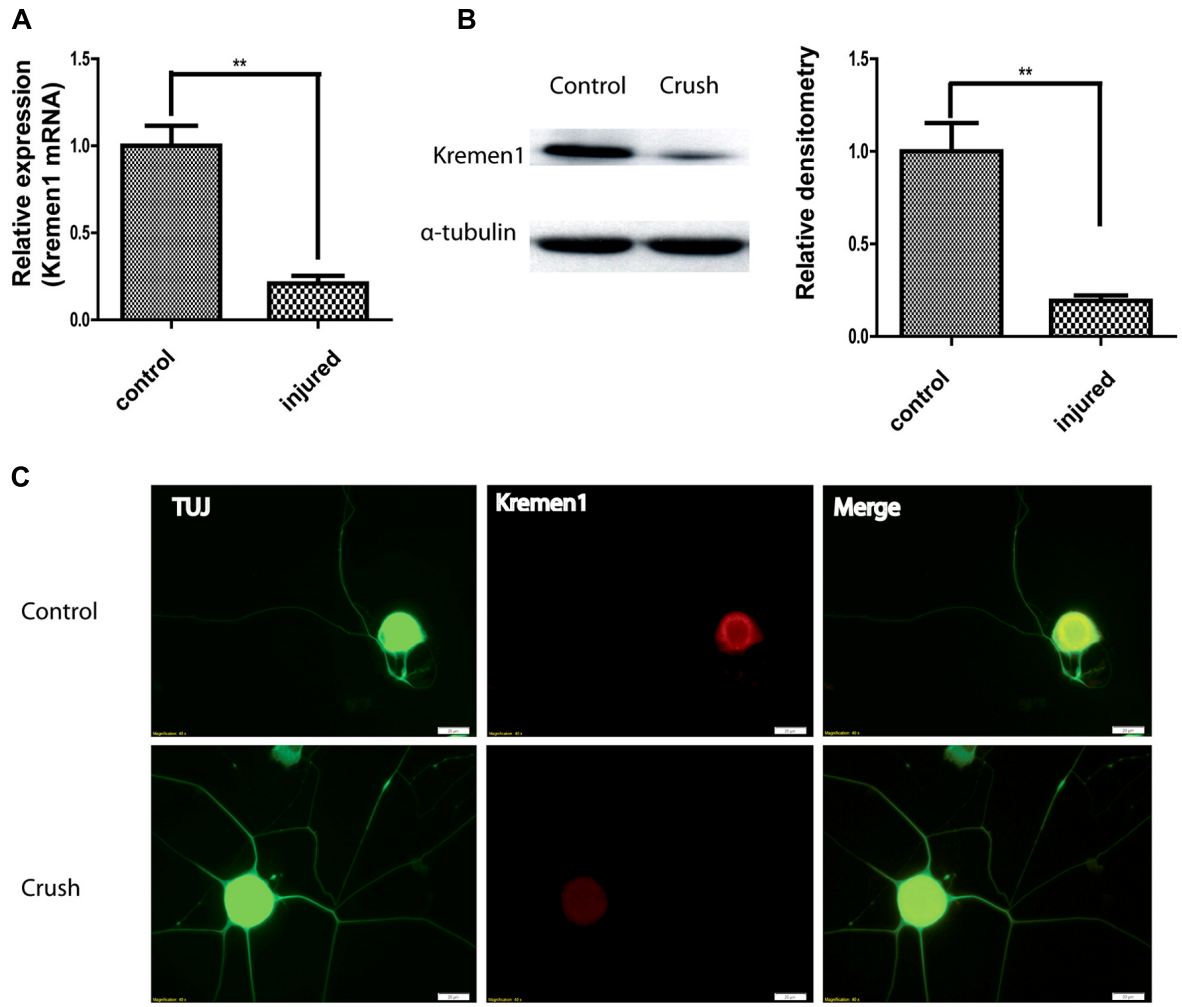


FIGURE 4 | Nerve crush injury reduces *Kremen1* expression. (A) Total RNA was isolated from control or crush-injured mouse DRG, and relative expression of *Kremen1* was determined using RT-qPCR. GAPDH and S12 were used to normalize for RNA loading. **(B)** Western blot analysis of total DRG lysates at 4 days post-crush injury. α-Tubulin was shown as a loading control. As shown in the quantified densitometry data, there was a significant decrease of *Kremen1* expression during nerve regeneration. **(C)** Immunofluorescent staining in dissociated DRG neurons demonstrated the expression of *Kremen1* within neurons. *Kremen1* as a transmembrane receptor was shown to be located in cell bodies, but not axons. TUJ staining was used to visualize neuronal cells. Preconditioning of sciatic nerve clearly promotes regenerative axon growth in DRG neurons, and this phenomenon is accompanied by a decrease in *Kremen1* expression. Scale bar: 20 μm. (** $p < 0.01$) For the analysis of two independent groups, Student's *t*-test was used.

robust regenerative growth (**Figure 4A**). Similarly, we found that *Kremen1* protein was reduced in DRGs at 4 days post-injury. The Western blot data showed a significant 80% decrease in *Kremen1* expression after SNI when compared to control (**Figure 4B**).

The expression of *Kremen1* in DRG neuron was further examined using indirect immunofluorescence (IIF). IIF with antibodies against *Kremen1* revealed the localization of *Kremen1* in dissociated DRG neurons. In both pre-conditioned and control groups, the immunoreactivity of *Kremen1* was detected mainly in neuronal cell bodies, however, there was less *Kremen1* immunostaining in the group with sciatic nerve crush (**Figure 4C**). These data further support a functional relationship between miR-431 and *Kremen1* in regenerating DRG neurons and suggest a role of *Kremen1* in peripheral nerve regeneration.

FUNCTIONAL ANALYSIS OF *Kremen1* ROLE IN AXON REGENERATION

Given the effects of miR-431 on *Kremen1* expression and the role of miR-431 in neurite outgrowth, we investigated the effect of *Kremen1* knockdown on regenerative axon growth. Two groups of DRG neurons were transfected with either siRNA specifically targeting *Kremen1* mRNA, or scrambled siRNA (negative control). The differences in the regenerative growth between *Kremen1* siRNA group and control scrambled siRNA group were quantified based on axon elongation and branching. The experiments revealed that knockdown of *Kremen1* significantly increased axon length in dissociated DRG cultures (**Figure 5**). The axon length in the *Kremen1* knockdown group increased ∼30% in comparison to the scrambled siRNA control group. This effect on axon outgrowth is similar to the effect of miR-431 overexpression on axon outgrowth reported earlier (**Figure 2B**). Taken together, these results

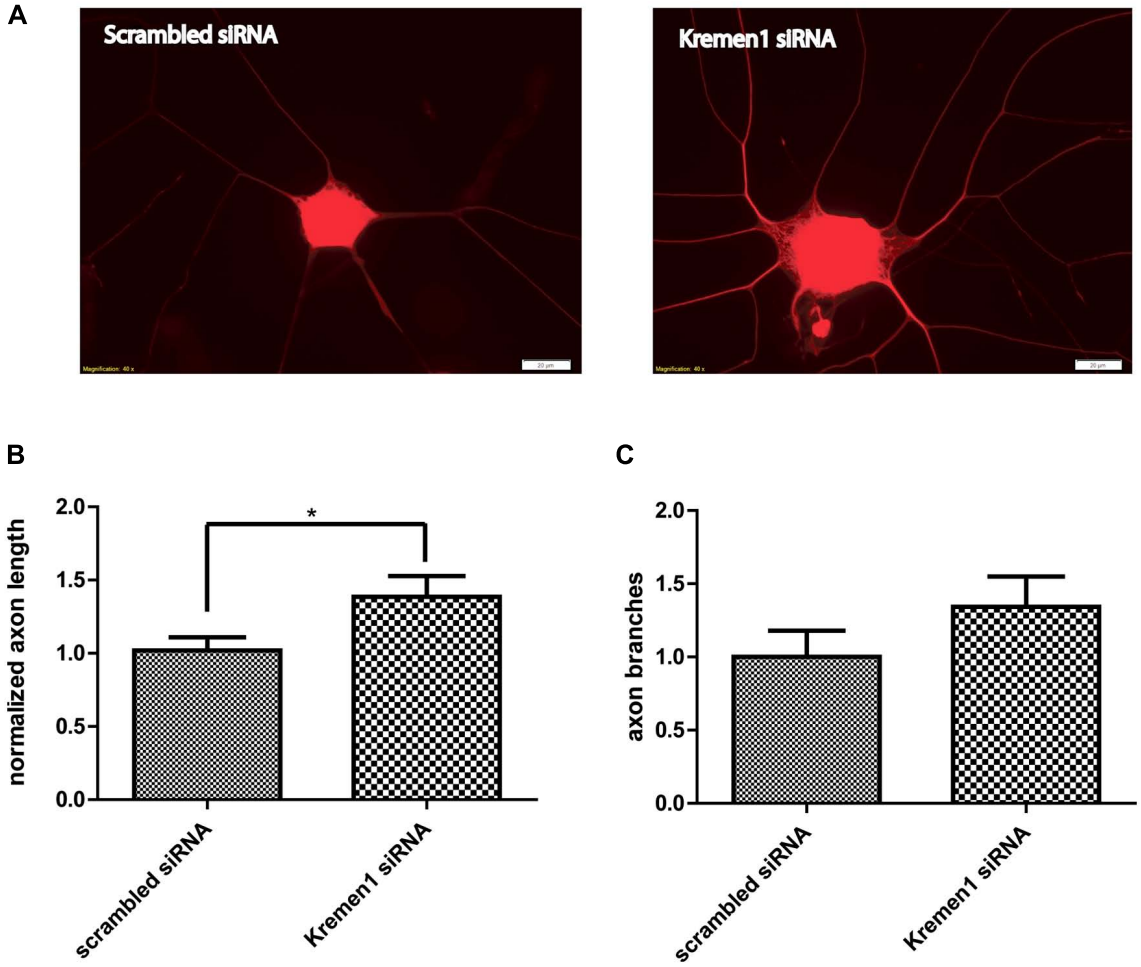


FIGURE 5 | Knockdown of *Kremen1* increases neurite outgrowth. **(A)** Neurite outgrowth in *Kremen1* siRNA and scrambled siRNA treated DRG neurons was detected by TUJ immunostaining. Representative images show that *Kremen1* siRNA significantly decreased *Kremen1* expression level, which was accompanied by an increase of axon outgrowth. Scale bar: 20 μm. As the quantification performed in miR-431 functional analysis, we measured the length of the longest axon for each neuron **(B)** and counted the number of branches for each neuron **(C)**. Inhibition of *Kremen1* significantly increased the length of axon, however, its effect on neurite branching was not significant. $*-p < 0.05$. For the analysis of two independent groups, Student's *t*-test was used. Scale bar: 20 μm.

indicate that miR-431 mediates increase of axon growth through *Kremen1* repression.

DISCUSSION

ALTERED miRNA EXPRESSION FOLLOWING NERVE INJURY

Our microarray experiments identified a group of injury-regulated miRNAs in DRG neurons after conditioning sciatic nerve lesion. Alterations in miRNAs have been recently shown in several studies profiling miRNA expression after nerve injuries in the central nervous system. Microarray based analysis of miRNA in the rat cerebral cortex after traumatic brain injury revealed that a set of miRNAs were differentially expressed at 6, 24, 48, and 72 h after injury. At all-time points post-injury, miR-21 was consistently highly expressed in the cerebral cortex (Lei et al., 2009). Changes in miRNA expression have also been studied by microarray analysis in hippocampus after traumatic brain injury. At three and 24 h after controlled cortical impact injury, 35 miRNA exhibited increased expression levels and 50 miRNA exhibited decreased expression level (Redell et al., 2009). Following a contusive SCI in adult rats (Liu et al., 2009), 60 miRNAs showed significant changes in their expression level in the injured spinal cord at 4 h, 1, and 7 days. Among those 60 miRNAs, 30 were upregulated, 16 were down-regulated, and 14 showed early upregulation at 4 h followed by down-regulation at 1 and 7 days post-SCI (Liu et al., 2009). Recently, observations on miRNA expression have been extended to the PNS. miRNA expression has been profiled following SNI in proximal stumps of injured sciatic nerve and DRG by microarray and deep sequencing in several studies (Strickland et al., 2011; Yu et al., 2011b; Zhou et al., 2012). Following sciatic nerve transection, 20 miRNA transcripts displayed a significant change in expression levels at 7-day post-axotomy in rat DRG (Strickland et al., 2011). Both miR-21 and miR-431 showed significant upregulation in DRG after SNI, comparably to our current data. Taken together, Strickland's and our

study, demonstrate that miR-21 and miR-431 are implicated in peripheral nerve regeneration across species. Strickland's study further revealed that miR-21 promoted the regenerative growth of the injured neuron by targeting the Sprouty2 protein (SPRY2; Strickland et al., 2011).

In our studies, we focused on miR-431, which was the most upregulated miRNA in DRG microarray after nerve injury in our experiments. miR-431 was initially identified as central nervous system specific miRNA as it was cloned from brain tissue of mouse embryos (Wheeler et al., 2006). Whole mount *in situ* hybridization revealed miR-431 localization to the developing spinal cord and brain with particularly strong expression in the pons. The pons is particularly rich in synapses because ninety percent of the descending axons passing through the midbrain synapse on neurons in the pons (Wheeler et al., 2006). However, to date, limited information is available about miR-431 physiological function. Recent observation has linked expression of miR-431 to regulation of cell viability (Tanaka et al., 2012). miR-431 was upregulated by the addition of human fibroblast interferon (HuIFN-β) in a non-cancer HuIFN-β sensitive cell line RSa, with concomitant suppression of IGF1R signaling and reduction of cell viability (Tanaka et al., 2012). However, at this time, the function of miR-431 in the nervous system remains uncertain.

THE FUNCTION OF miR-431 IN REGENERATIVE AXON GROWTH

To determine the role of miR-431 in axon regeneration, miR-431 gain- and loss-of-function were investigated in DRG neuronal cultures. Application of miR-431 mimics markedly increased the intracellular miR-431 level and promoted regenerative axon outgrowth. miR-431 gain-of-function correlated with longer axons, more branches, and higher GAP-43 expression, a marker of regeneration. In contrast, transfection of miR-431 inhibitors impaired the regenerative axon growth, as significantly shorter axons and fewer branches were observed in DRG cultures. Analyses of 24 putative targets of miR-431, showed that only six were suppressed in PC12 cells and even less genes were suppressed in DRG primary neurons. This could be related to the specificity of miR-431 to these genes, and to the fact that down-regulation of less specific targets is more easily detected in PC12 cells. The difference may be also related to the fact that the cells were from different species; PC12 were from rat and DRG culture was from mouse.

We have further identified *Kremen1* as the target that mediates the effects of miR-431 on neuronal cells. miR-431 expression inversely relates to *Kremen1*. The direct interaction between miR-431 and *Kremen1* mRNA was confirmed by CLIP, and 3′-UTR luciferase reporter assay. *Kremen1* expression was down-regulated by miR-431 at the mRNA and protein levels. This may mean that miR-431 cleaves the mRNA of this gene rather than repressing its translation. To the best of our knowledge this is the first observation of direct mRNA target cleavage by miR-431. At the same time, our data do not exclude possibility that there is another miRNA or transcription factor that may regulate *Kremen1* too.

Kremen1 was originally discovered as a transmembrane protein containing the kringle domain. Later reports confirmed that both *Kremen1* and its relative Kremen2 were high-affinity receptors for Dickkopf1 (Dkk1), the inhibitor of Wnt/β-catenin signaling (Mao et al., 2002). The canonical Wnt/β-catenin signaling is mediated by two receptor families, Frizzle protein and lipoprotein-receptor-related protein 5 and 6 (LRP5/6). *Kremen1* functionally cooperates with Dkk1 to form a ternary complex composed of *Kremen1*, Dkk1, and LRP5/6, and induces rapid endocytosis and removal of the Wnt receptor LRP5/6 from the cell membrane, which inhibits the transduction of Wnt/β-catenin signaling. Wnt/β-catenin signaling plays a vital role in diverse developmental and physiological processes, including cell-fate determination, tissue patterning, and stem cell regulation (Diep et al., 2004). Wnt/β-catenin signaling pathway also contributes to adult neurogenesis. Blocking Wnt signaling abolishes neurogenesis in adult hippocampal progenitor cells *in vitro* and suppresses neurogenesis *in vivo* (Lie et al., 2005). With ectopic expression of Dkk1, canonical Wnt/β-catenin signaling is markedly reduced in both the hippocampus and cortex (Solberg et al., 2008).

Studies have also established a role for Wnt signaling in regulating synaptic plasticity and axonal growth (Hall et al., 2000; Wang et al., 2006; Budnik and Salinas, 2011). Wnt signaling regulates axon terminal remodeling (Budnik and Salinas, 2011), formation of growth cones and lamellipodia (Hall et al., 2000), microtubules organization (Purro et al., 2008), and synaptic assembly (Ahmad-Annuar et al., 2006). Loss- and gain-of-function studies in animal models demonstrated that loss of Wnt7a results in a strong deficit in the accumulation of synaptic markers at the cell synapses (Ahmad-Annuar et al., 2006). In contrast, in cultured mouse cerebellar granule cells, Wnt7a increased neurite elongation and branching as well as the expression of synaptic markers (Lucas and Salinas, 1997). Likewise, targeted disruption of Wnt receptor genes in mice produced severe defects in axon growth and guidance, resulting in a loss of thalamocortical, nigrostriatal tracts, and the anterior commissure (Wang et al., 2002, 2006). Moreover, SCI induced a time-dependent increase in Wnt expression, phosphorylation of Wnt receptors, and activity of β-catenin protein. Thus, the activation of the Wnt pathway after SCI suggests the involvement of Wnt pathway in nerve regeneration (Fernandez-Martos et al., 2011).

These abundant evidences from studies in animal models, cell and organ culture firmly established an important role of Wnt signaling in neurite outgrowth and axonal guidance. The function of Wnt signaling could potentially link our observation on increased miR-431 and decreased *Kremen1* expression to the enhanced axonal outgrowth. In our study, *Kremen1* loss-of-function produced an increase in axon outgrowth mimicking the effect of miR-431 gain-of-function but did not increase branching. The axon elongation is a critical factor for axon regeneration. The excessive branching can be detrimental to axon regeneration, especially in the PNS. Evidence suggests that axonal elongation and branching are differentially regulated in hippocampal neurons (Pujol et al., 2005).

Taken together, our studies identified miR-431 as an endogenous, injury-regulated inhibitor of *Kremen1*, which promotes regenerative axon growth in adult sensory neurons. Further studies are necessary to fully define the role of miR-431 in axonal regeneration. These findings may not only contribute to our understanding of fundamental biological process, but also could have important implication for improving the therapeutic strategies for nerve injury.

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Predicted overlapping microRNA regulators of acetylcholine packaging and degradation in neuroinflammation-related disorders

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MicroRNAs (miRNAs) can notably control many targets each and regulate entire cellular pathways, but whether miRNAs can regulate complete neurotransmission processes is largely unknown. Here, we report that miRNAs with complementary sequence motifs to the key genes involved in acetylcholine (ACh) synthesis and/or packaging show massive overlap with those regulating ACh degradation. To address this topic, we first searched for miRNAs that could target the 3′-untranslated regions of the choline acetyltransferase (ChAT) gene that controls ACh synthesis; the vesicular ACh transporter (VAChT), encoded from an intron in the ChAT gene and the ACh hydrolyzing genes acetyl- and/or butyrylcholinesterase (AChE, BChE). Intriguingly, we found that many of the miRNAs targeting these genes are primate-specific, and that changes in their levels associate with inflammation, anxiety, brain damage, cardiac, neurodegenerative, or pain-related syndromes. To validate the *in vivo* relevance of this dual interaction, we selected the evolutionarily conserved miR-186, which targets both the stress-inducible soluble "readthrough" variant AChE-R and the major peripheral cholinesterase BChE. We exposed mice to predator scent stress and searched for potential associations between consequent changes in their miR-186, AChE-R, and BChE levels. Both intestinal miR-186 as well as BChE and AChE-R activities were conspicuously elevated 1 week post-exposure, highlighting the previously unknown involvement of miR-186 and BChE in psychological stress responses. Overlapping miRNA regulation emerges from our findings as a recently evolved surveillance mechanism over cholinergic neurotransmission in health and disease; and the corresponding miRNA details and disease relevance may serve as a useful resource for studying the molecular mechanisms underlying this surveillance.

Keywords: acetlycholinesterase, butyrylcholinesterase, choline acetyltransferase, vesicular acetylcholine transporter, microRNA-186, cholinergic signaling, primate-specific microRNAs

INTRODUCTION

MicroRNAs (miRNAs) are small, 20–25 nucleotides long, non-coding RNA molecules, each of which can predictably target many protein-coding messenger RNA (mRNA) transcripts to silence them post-transcriptionally. Mammalian miRNAs bind target mRNAs via a short "seed" sequence, such that many miRNAs can target the same mRNAs, and different mRNAs may be targeted by a single miRNA gene (Bartel, 2009). Interestingly, miRNAs often target different mRNAs all involved in a particular biological function (Chen et al., 2004). This has been extensively studied in various cancers (Kefas et al., 2008; Papagiannakopoulos et al., 2008; Levy et al., 2010; Lupini et al., 2013), but the control by miRNAs of specific neurotransmission processes remained largely unexplored. In principle, one would predict that the synthesis, packaging in neuronal vesicles and destruction or re-uptake of a certain neurotransmitter should be co-regulated; this, in turn implies that some miRNAs may co-suppress two or more of the mRNA transcripts involved in regulating the levels of certain neurotransmitters, and that modified expression of such miRNAs might be involved in diseases associated with impaired regulation of this neurotransmission pathway. Based on this working hypothesis, we studied miRNA-mediated regulation of mRNA transcripts involved in the synthesis, vesicle packaging, and destruction of acetylcholine (ACh).

MiRNA-binding sequence motifs are primarily located at the 3′-untranslated region (3′-UTR) of the mRNA transcript (Bartel, 2009). Therefore, we interrogated the 3′-UTR domains in the choline acetyltransferase (ChAT) gene, which is responsible for ACh synthesis and the vesicular acetylcholine transporter (VAChT), encoded from the first intron in the ChAT gene (Erickson et al., 1994; Eiden, 1998). The VAChT transcript has its own 3′-UTR, which might suggest that it can be regulated by distinct miRNAs. Given that released ACh is degraded by the ACh hydrolyzing enzymes acetyl- and butyrylcholinesterase (AChE, BChE) (Meshorer and Soreq, 2006), and since increased AChE synthesis may be linked with decreased ChAT production (Kaufer et al., 1998), we further searched for potential overlaps in the predicted miRNAs between the human ChAT/VAChT 3′-UTRs and

the alternative 3′-UTR choices of the major variants of AChE mRNA, the "synaptic" AChE-S and the "read-through" variant AChE-R which shares its 3′-UTR with the "erythrocytic" AChE-E isoform and is known to be induced under stress (Meshorer and Soreq, 2006). Of note, the AChE-S variant possesses a considerably shorter 3′-UTR compared to AChE-R (Hanin and Soreq, 2011) with further likelihood of differential miRNA regulation for the AChE-R and AChE-S targets. The BChE gene, completing the series of regions to be analyzed, has a different 3′-UTR with lower G, C-content (Soreq and Seidman, 2001), demonstrating sequence differences. All of these regions are functionally involved in cholinergic signaling, and we therefore designated the corresponding miRNAs "CholinomiRs." A schematic overview of the transcripts involved in this pathway is given in **Figure 1**.

There were only a few overlaps between the predicted CholinomiRs regulating the synthesis and destruction of ACh. In contrast, we identified numerous overlaps between those CholinomiRs controlling ACh packaging and its synthesis; suggesting that miRNAs play an important role in selectively co-regulating cholinergic signaling by adapting the rates and efficacy of ACh packaging and destruction. Changes in these CholinomiRs, of which many are primate-specific, were further reported by others in inflammation and anxiety, brain damage, pain, cardiac, and neurodegenerative diseases, all of which are known for cholinergic signaling impairments.

To test the relevance of our predictions for *in vivo* conditions, we subjected mice to the long-lasting predator scent stress (Zimmerman et al., 2012) and tested, 1 week later, for changes in one miRNA, miR-186 and its predicted targets AChE-R and BChE. Given our previous findings of miRNA regulation of cholinergic-mediated production of intestinal miR-132 (Shaked et al., 2009), we quantified miR-186 levels in intestinal sections and measured cholinesterase activities. We found that predator scent stress induces intestinal increases in both the cholinesterases-targeting miR-186 and in the activities of the targeted cholinesterases. Our findings support the hypothesis

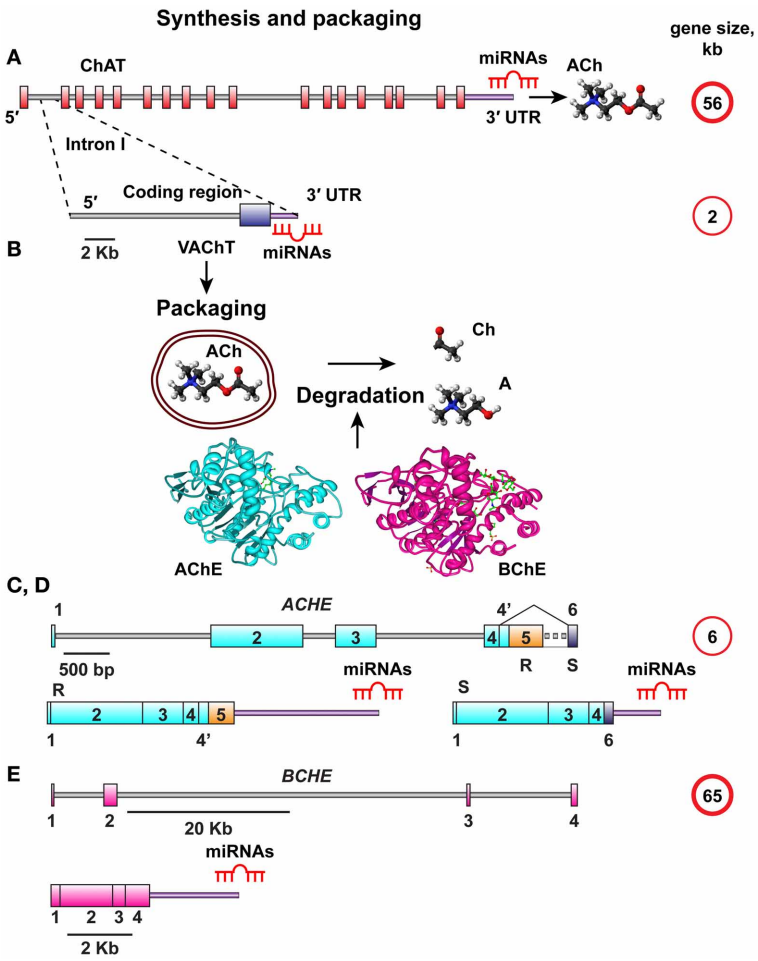

FIGURE 1 | Schematic overview of ACh synthesis, packaging, and destruction. (A) The choline acetyl transferase (ChAT) gene responsible for ACh synthesis is 56 kb in size and all its splice variants have the same 3′UTR. **(B)** The vesicular acetylcholine transporter (VAChT) controlling ACh packaging into vesicles is encoded from the first intron of the ChAT gene. The VAChT gene is only 2 kb in length, yet it has its own 3′UTR. Once released, ACh should be degraded into Acetate and Choline by one of the splice variants of AChE or BChE, depending on its site of release. **(C,D)** The AChE gene is 6 kb in size, and its mRNA transcript is spliced to yield the major AChE-S and AChE-R splice variants with distinct 3′-UTR domains. **(E)** The BChE gene is 65 kb in size and yields only one known splice variant.

that overlapping CholinomiR regulation serves as a recently evolved surveillance mechanism that can balance cholinergic signaling in brain and peripheral systems. The detailed lists of these miRNAs and their potential involvements with different diseases may be a valuable resource for researchers interested in both basic and translational aspects of key neuroinflammation and pain-related disorders.

MATERIALS AND METHODS

BIOINFORMATICS APPROACHES

3′-UTR sequences of the human ChAT, VAChT, AChE-S, AChE-R, and BChE transcripts were acquired from the NCBI nucleotide database (Entrez Nucleotide, 2010 http://www.ncbi.nlm.nih.gov/nuccore/). These sequences are 380, 58, 219, 963, and 477 nucleotides long, respectively. MiRNA-mRNA interactions were addressed by using four different algorithms, miRBase (Last update in June 2013, http://www.mirbase.org/), TargetScan (Last update in June 2012, http://www.targetscan.org/vert_50/), microcosm (version 5) (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) and miRanda (Last update in August 2010, http://www.microrna.org/microrna/home.do) which were last updated in June 2013, June 2012 and August 2010, respectively. All predictions ensured a threshold P-value < 0.05, and analysis specifications allowed both evolutionarily conserved and non-conserved miRNAs, which further enabled us to differentiate between primate-specific and evolutionarily conserved miRNAs.

To gain more information on the identified miRNAs and assess their prospects to interact with their targets, we determined the G, C content of all identified miRNAs using G, C content calculator algorithms (http://www.endmemo.com/bio/gc.php). We further focused on the overlapping miRNAs targeting more than one 3′-UTR of the 5 studied transcripts and distinguished primate-specific from evolutionarily conserved miRNAs that appeared in our list using the HomoloGen conservation score[1] that reports genomic conservation values between tested genes from different species. For data-mining regarding the relation to specific diseases we utilized PubMed and Google Scholar.

EXPERIMENTAL *in vivo* TESTS

To experimentally test the putative association between changes in the identified miRNAs and their protein targets under stressful conditions, we exposed C57/B6J mice to predator scent and additionally injected them for four consecutive days with 50 μg kg^{-1} saline, essentially as in (Zimmerman et al., 2012). 7 days later, we removed intestinal sections from these mice and matched male control mice, as in (Shaked et al., 2009). The mice were housed four per cage, at 21 ± 1°C, in a 12-h light/dark cycle. The RNA extraction procedure followed that of (Maharshak et al., 2013) for human intestinal biopsies. Extracted RNA from the intestinal sections was used to quantify miR-186 and RNU6 levels by qRT-PCR (PerfeCTa® microRNA Assay). RNU6 snRNA levels were used to normalize the levels of miR-186. The following primers were employed: RNU6 (Quanta, Cat. # HS-RNU6), miR-186-5p (Quanta, Cat. # HSMIR-0186-5p), PerfeCTa® Universal PCR Primer (Quanta, Cat. # 95109-500). Protein extraction from the intestinal sections was performed by using Solution D (0.01 M TRIS, 1 M NaCl, 1 mM EGTA, 1% T-X100). Cholinesterase activities in the tissue homogenates were determined by quantifying acetylthiocholine hydrolysis rates (Ellman et al., 1961) in the presence or absence of iso-OMPA to selectively block BChE activities, all as under (Arbel et al., 2014). Animal procedures followed the ethics instructions at The Hebrew University of Jerusalem (Ethics number of research: NS-10205-4).

STATISTICAL ANALYSIS

Collected data was summarized and displayed as mean ± standard deviation (Erickson et al., 1994) using SPSS software (version 19.0, SPSS INC, Chicago, IL, USA). Normally distributed variables were compared using Student's t test. The level of significance used for all of the above analyses was 2-tailed $p < 0.05$.

RESULTS

3′-UTR sequences of the human ChAT, VAChT, AChE-S, AChE-R, and BChE transcripts were acquired from the NCBI nucleotide database. These sequences span 380, 58, 219, 963, and 477 nucleotides, respectively and differ in their G, C content. We identified 42, 67, 55, 125, and 205 complementary miRNAs predicted to bind to the interrogated 3′-UTRs, respectively (**Table 1**, for details see Supplementary Table 1). Thus, the density of miRNA binding sites in these transcripts did not simply reflect their length, and the AChE-R and BChE mRNA transcripts encoding for soluble, secreted proteins emerged as particularly susceptible for miRNA targeting. Interestingly, we found no overlap between the VAChT and ChAT targeting miRNAs, suggesting differential regulation of the ACh synthesis and packaging processes in spite of these transcripts being subject to joint transcriptional control. In contradistinction, 26% of the ChAT targeting miRNAs are also predicted to target cholinesterases, and ACh packaging and degradation seemed to share yet more miRNAs: of 67 VAChT-targeting miRNAs, 55% predictably recognize binding sites in cholinesterases as well, and 5 of them co-target both the alternatively spliced "synaptic" AChE-S and the stress and inflammation-inducible "read-through" AChE-R transcript, enabling more profound suppression of ACh hydrolysis (for details see Supplementary Table 2). Notable examples involve the conserved neurodevelopment-associated hsa-miR-125b (Martino et al., 2009) and the primate-specific hsa-miR-608 and -765 (primate specificity determined by HomoloGen conservation score[1]).

Our working hypothesis predicted that miRNAs targeting more than one of the five 3′-UTR domains would be more likely than others to be causally involved in regulating this entire pathway. Also, many believe that recent evolutionary processes reshaped the miRNA landscape in primates, contributing to human higher brain functions (Khaitovich et al., 2006). To find out if this re-shaping process affected the regulation of the cholinergic system, we searched for primate specificity within the group of 76 identified miRNAs that target more than one cholinergic transcript (Supplementary Table 3). About half (49%) of the VAChT and cholinesterases co-targeting miRNAs were found to

[1]http://www.ncbi.nlm.nih.gov/

Table 1 | Overlapping miRs are largely primate-specific.

Targeted transcripts	Length of 3′UTR [nt]	G, C-content of 3′UTR	No of predicted miRNAs	G, C-content of targeting miRNAs [%]	% of primate specific miRNAs
ChAT	58	59	42	57	
VAChT	380	62	67	58	
AChE-S	219	66	55	59	
AChE-R	963	62	125	55	
BChE	477	28	205	45	
VAChT + AChE-S			10	58	40
VAChT + AChE-R			24	61	54
VAChT + BChE			3	41	33
VAChT + ChAT			0	0	0
AChE-S + AChE-R			23	61	37
AChE-S + BChE			1	59	0
AChE-S + ChAT			1	82	0
AChE-R + BChE			16	46	44
AChE-R + ChAT			4	62	25
BChE + ChAT			6	52	66
AChE-S + AChE-R + VAChT			6		50
AChE-S + AChE-R + ChAT			1		100
AChE-S + BChE + VAChT			1		0

Shown are the 3′-UTR lengths, G, C-content and the numbers of miRNAs predicted to target each of the ChAT, VAChT, AChE-S, AChE-R, and BChE transcripts alone and those predictably targeting more than one of these transcripts and the fractions of these miRNAs that are primate-specific.

be primate-specific (Supplementary Table 3), suggesting recently evolved miRNA-mediated mechanisms for co-regulation of ACh packaging and degradation (**Table 1**).

Of the cholinesterase targeting miRNAs, we found 16 that predictably target both BChE and AChE-R, whereas 23 miRNAs show "seed" complementarity to both AChE-R and AChE-S. However, BChE, and the synaptic AChE-S variant share only one single miRNA (**Table 1**), hsa-miR-491-5p (further details on shared miRNAs can be found in Supplementary Table 2). BChE has a much lower G, C content than the other four transcripts (Soreq et al., 1990); therefore, we wanted to find out whether this is reflected in its putatively targeting miRNAs. Not surprisingly, we found the average G, C content of BChE-targeting miRNAs to be 45%, approximately 12% lower than the average G, C content of the miRNAs targeting the other four transcripts (**Table 1**, Supplementary Table 4).

Next, we searched for the relevance of miRNAs targeting more than one transcript involved in ACh metabolism to known disease phenotypes (Supplementary Table 5). Compatible with the cancer-associated bias in the miRNA field, we found numerous studies on cancer-related miRNAs that target more than one transcript involved in ACh metabolism (Supplementary Table 5), and yet more are likely to accumulate in the near future. However, after excluding all of these cancer-related publications, we revealed 28 out of the identified 76 miRNAs that associate with other diseases. These could be classified into five groups: inflammation and anxiety, brain damage, cardiac or neurodegenerative diseases and pain-related syndromes (Supplementary Table 6). **Table 2** lists these 28 miRNAs including the transcripts they are targeting as well as their reported disease associations. Interestingly, 67% of these miRNAs play key roles in inflammation-associated diseases and 61% of them target more than one disease group.

Figure 2 presents the miRNAs co-targeting AChE-S and AChE-R, BChE, and AChE-R or VAChT and AChE-R in a pie chart classifying the shared targets and relevant diseases and highlighting the observed interactions between these miRNAs and the disease phenotypes, as well as the primate specificity of part of these miRNAs. Disease group association is shown as surrounding lines of differential thicknesses, reflecting the number of miRNAs in each group. A notable example is the evolutionarily conserved neural-expressed miR-125b, which targets both VAChT and AChE-R (**Table 2**) and associates with all five groups: inflammation and anxiety (Manca et al., 2011; Xu et al., 2011; Danielsson et al., 2012; Matsukawa et al., 2013), brain damage (Rink and Khanna, 2011), neurodegenerative diseases (Lukiw and Alexandrov, 2012) and cardiac diseases (Voellenkle et al., 2010) and diverse pain syndromes (Imai et al., 2011; Kynast et al., 2013; Monastyrskaya et al., 2013; Sakai et al., 2013). Known association of each of these diseases with impaired cholinergic signaling (Shenhar-Tsarfaty et al., 2013a,b) supports the notion of physiological significance for surveillance by the overlapping miRNAs. A primate-specific example involves miR-765, which co-targets AChE-R and VAChT. Redell (Redell et al., 2010) found that miR-765 is upregulated in the plasma of patients after traumatic brain injury, compatible with changes in serum cholinesterases in post-stroke patients (Ben Assayag et al., 2010). Additionally, miR-765 targets the neurotrophin-3 receptor 3′UTR, and Muiños-Gimeno

Table 2 | Disease association of predicted CholinomiRs.

Target	miRNA	Inflammation and anxiety	Brain damage	Cardiac diseases	Neurodegenerative diseases	Pain
R, V	hsa-miR-125a-5p	Murata et al., 2013	Rink and Khanna, 2011			
	hsa-miR-125b	Manca et al., 2011; Rink and Khanna, 2011; Xu et al., 2011; Danielsson et al., 2012; Lukiw and Alexandrov, 2012; Matsukawa et al., 2013	Rink and Khanna, 2011	Voellenkle et al., 2010	Lukiw and Alexandrov, 2012	
	hsa-miR-298		Liu et al., 2010			
	hsa-miR-663	Xu et al., 2011				
	hsa-miR-675	Lu et al., 2012				
	hsa-miR-7	Shaoqing et al., 2011; Etoh et al., 2013; Oshikawa et al., 2013		Archacki et al., 2003	Junn et al., 2009; Wong et al., 2013a,b	Sakai et al., 2013
	hsa-miR-765	Muinos-Gimeno et al., 2009	Redell et al., 2010			
R, B	hsa-miR-129-5p				Wang et al., 2011	
	hsa-miR-186	Lerman et al., 2011		Bostjancic et al., 2009		
	hsa-miR-199a-5p	Paraskevi et al., 2012		Abdellatif, 2011		Monastyrskaya et al., 2013
	hsa-miR-200b	Chen et al., 2012	Lee et al., 2010			Imai et al., 2011
	hsa-miR-200c	Paraskevi et al., 2012	Lee et al., 2010			
	hsa-miR-429		Lee et al., 2010			Imai et al., 2011
	hsa-miR-590-3p				Villa et al., 2011	
S, R	hsa-miR-7f-2*	Xie et al., 2011	Tan et al., 2009		Schipper et al., 2007	
	hsa-miR-7g	Takagi et al., 2010				
	hsa-miR-24	Iliopoulos et al., 2009	Zhu and Fan, 2012	Wang et al., 2012c; Zhang et al., 2013		
	hsa-miR-124-3p		Doeppner et al., 2013; Dutta et al., 2013; Matsumoto et al., 2013; Sun et al., 2013			Kynast et al., 2013
	hsa-miR-132	Shaked et al., 2009; Murata et al., 2010; Hanieh and Alzahrani, 2013; Maharshak et al., 2013; Shaltiel et al., 2013	Lusardi et al., 2010; Chen-Plotkin et al., 2012; Valiyaveettil et al., 2013	Katare et al., 2011; Jin et al., 2012; Eskildsen et al., 2013	Lau et al., 2013; Lungu et al., 2013; Wong et al., 2013a	
	hsa-miR-194			Matsumoto et al., 2013		
	hsa-miR-198			Hoekstra et al., 2010		

(Continued)

Table 2 | Continued

Target	miRNA	Inflammation and anxiety	Brain damage	Cardiac diseases	Neurodegenerative diseases	Pain
	hsa-miR-423	Lerman et al., 2011		Keller et al., 2011; Goren et al., 2012; Dickinson et al., 2013; Oliveira-Carvalho et al., 2013	Bhattacharyya and Bandyopadhyay, 2013	
	hsa-miR-4329			Gupta et al., 2013		
R, C	hsa-miR-149	Diaz-Prado et al., 2012		Van Rooij et al., 2008		
S, V	hsa-miR-149*	Santini et al., 2013				
B, C	hsa-miR-193b*	Wang et al., 2012a,b				
S, B, V	hsa-miR-491-5p	Iborra et al., 2013	Yuan et al., 2013		Wang et al., 2011	
S, R, V	hsa-miR-920			Meder et al., 2011		

Shown are miRNAs sorted by their putative targets and the relevant reports on their disease group association. Primate specific miRNAs are marked in orange. The abbreviations C, V, S, R, and B stand for ChAT, VAChT, AChE-S, AChE-R, and BChE, respectively.

(Muinos-Gimeno et al., 2009) discovered a single nucleotide change located in the miR-765 binding site of this receptor's mRNA 3′UTR to be associated with panic disorder. Neurotrophin receptors regulate cholinergic signaling (Naumann et al., 2002), which associates with stress reactions (Kaufer et al., 1998; Sklan et al., 2004) as well as with RNA metabolism changes in the Alzheimer's brain (Berson et al., 2012; Lau et al., 2013); predicting that disrupted association of miRNAs with these receptors would impair cholinergic signaling and could increase the risk of anxiety-related diseases.

To experimentally test for *in vivo* association of stress-induced changes in the identified miRNAs and their putative target genes, we selected the evolutionarily conserved miR-186 which predictably targets the two soluble cholinesterases, BChE and AChE-R. Of those, AChE-R increases under psychological stress were well documented (Kaufer et al., 1998; Cohen et al., 2002; Meshorer et al., 2005; Shaltiel et al., 2013), including the serum (Sklan et al., 2004), but BChE's involvement was never approached. In our current study, we quantified miR-186 levels in intestinal biopsies prepared from male C57BJ mice 7 days following 10 min exposure to cat litter (predator scent stress) and injection for four consecutive days with 50 μg kg^{-1} saline (Zimmerman et al., 2012) compared to matched controls ($n = 5$ mice per group). In the intestinal biopsies, miR-186 expression normalized to the house-keeping short RNA RNU6 showed a 1.6-fold increase ($p < 0.016$) in pre-stressed mice. In parallel, these mice showed a 1.8-fold elevation in total cholinesterase activities ($p < 0.003$, Student's t test) as well as a less pronounced 1.6-fold increase in AChE levels ($p < 0.054$). **Figure 3** presents these findings, demonstrating directly associated changes in the intestinal levels of the miR-186 and its two cholinergic regulating targets.

DISCUSSION

Combined use of four different bioinformatics algorithms identified a large number of miRNAs putatively targeting the 3′UTRs of ChAT, VAChT, AChE-S, AChE-R, and BChE. MiRNAs can notably regulate whole biological pathways; for example, miR-181 controls mouse hematopoiesis (Chen et al., 2004), miR-608 targets two inflammation-related transcripts, CDC42 and IL6 (Jeyapalan et al., 2011; Kang et al., 2011) and miR-221 controls multiple cancer pathways (Lupini et al., 2013). To challenge the possibility that certain miRNAs likewise regulate ACh metabolism and belong to the family of CholinomiRs, we searched for miRNAs targeting more than one of the five transcripts involved in the process of ACh synthesis, packaging and degradation. Intriguingly, packaging more than synthesis of ACh emerged as being co-regulated with its degradation, suggesting a dynamically controlled surveillance of these two processes. Furthermore, these findings highlight the option of differential post-transcriptional regulation of VAChT and ChAT, which share the same promoter but have distinct 3′-UTR domains.

The numbers of miRNAs we identified are likely to be underestimated due to the exclusion of all the cancer-related miRNAs, which may be linked to cholinergic signaling as well. However, it is noteworthy that many of the miRNAs associated with ACh metabolism are primate-specific. This implies that there are no mouse models to study their function and influence, decreasing the likelihood of experimental animal studies of these miRNAs. Nevertheless, we found many human disease-association studies that demonstrate putative links of these miRNAs to inflammation and possibly reflecting the inflammatory blockade by ACh (Tracey, 2010). In comparison, only a few of the identified miRNAs appear to be largely modified in neurodegenerative diseases such as Alzheimer's disease, perhaps because cholinergic neurons decline early in the Alzheimer's brains (Mcgeer et al., 1984), so that it may be too late to find miRNAs playing a role in ACh metabolism in late stage brain samples from Alzheimer's patients. An exception is the AChE-targeted miR-132, which shows a drastic decline in the Alzheimer's brain (Lau et al., 2013).

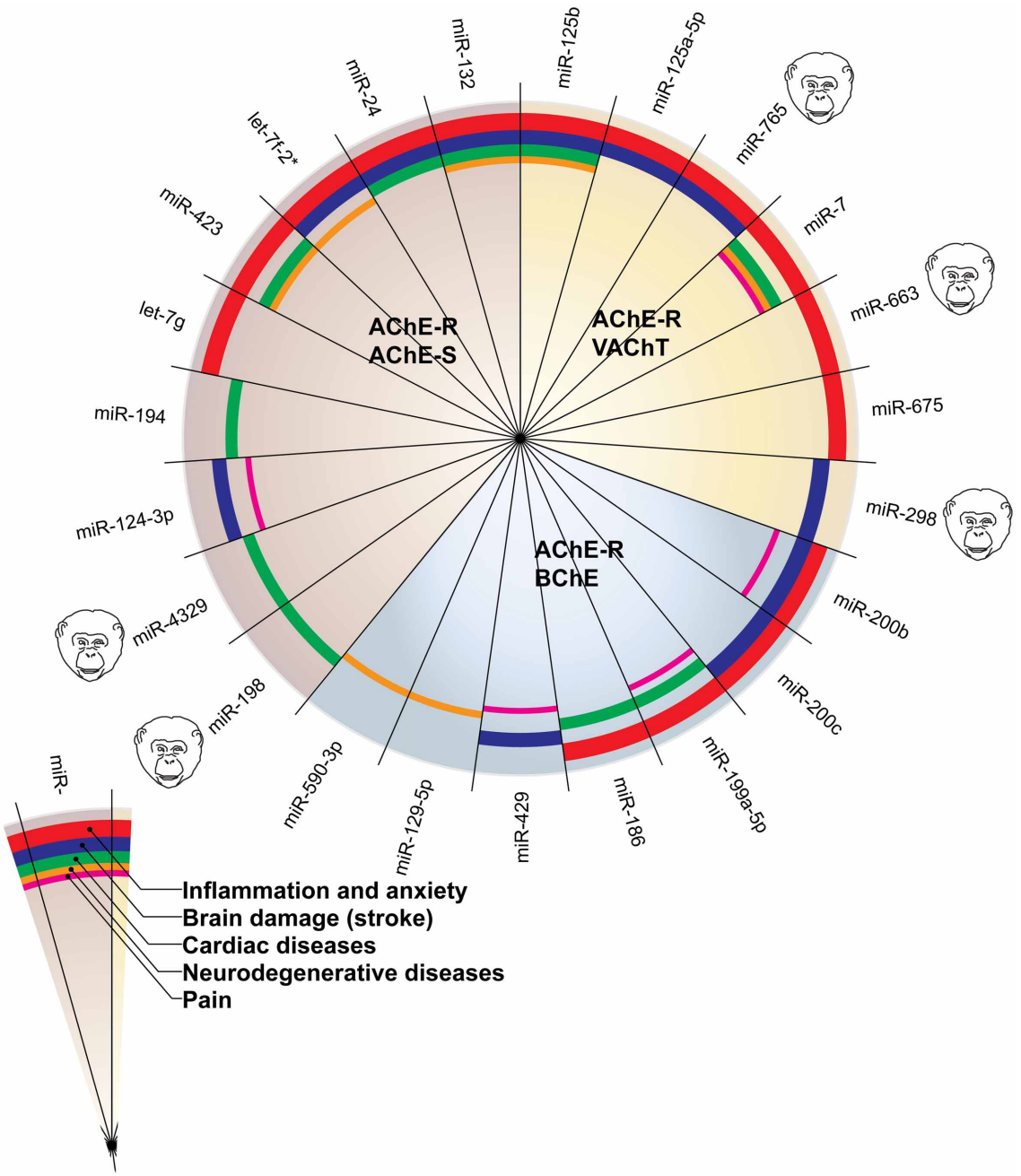

FIGURE 2 | MiRNAs targeting AChE-S and AChE-R, BChE and AChE-R or VAChT and AChE-R show associations to different disease groups related to cholinergic signaling. The pie chart classifies the shared targets, as well as the relevant diseases and highlights the observed interactions between them. Disease group association is shown as surrounding lines of differential thicknesses, reflecting the number of miRNAs in each group. The red, blue, green, orange and purple colored lines symbolize disease associations with the groups of "inflammation and anxiety," "brain damage (e.g., stroke)," "cardiac diseases," "neurodegenerative diseases," and "pain," respectively. Note that miR-132 and miR-125b associate with all disease groups. The monkey heads next to specific miRNA numbers symbolize primate specificity.

The apparent relevance of CholinomiRs to diverse pain syndromes is particularly intriguing (Kress et al., 2013). Thus miR-199a-5p is expressed in the bladder's smooth muscle and urothelium and may play a role in bladder pain syndrome (Monastyrskaya et al., 2013) by suppressing LIN7C, ARHGAP12, PALS1, RND1 and PVRL1. In addition, miR-199a-5p is predicted to target both BChE and AChE-R, suggesting that its increase would up-regulate cholinergic stimulation in the bladder, which could also contribute to pain reactions. Likewise, miR-200b and miR-429 predictably target BChE and AChE-R, and changes

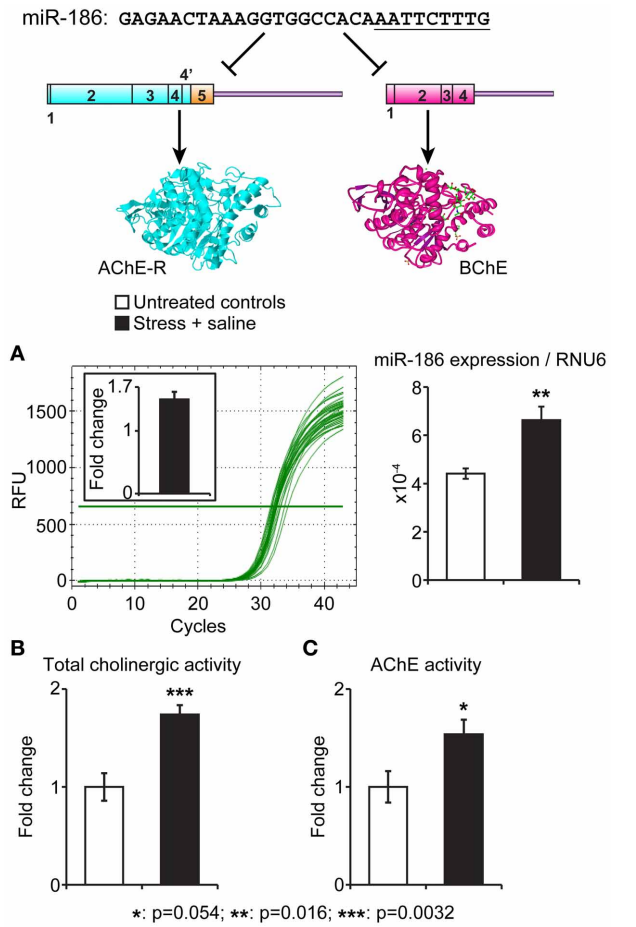

FIGURE 3 | Intestinal miR-186 increases under predator scent stress are accompanied by elevated BChE and AChE activities. Top: The nucleotide sequence of the BChE and AChE-R-targeting miR-186 and schemes of its AChE-R and BChE mRNA targets. The "seed" sequence is underlined. **(A)** qRT-PCR quantification normalized to RNU6 levels demonstrates reproducible RFU values and 1.6-fold excess of miR-186 in intestinal sections from stressed mice, 7 days post-predator scent exposure ($p = 0.016$). **(B,C)** Highly significant intestinal elevation of total acetylthiocholine hydrolytic activity ($p = 0.0032$) accompanied by less pronounced AChE activity measured in the presence of 10 μM iso-OMPA ($p = 0.05$). $N = 5$ mice per group, in all tests.

in their levels were reported under neuropathic pain following sciatic nerve ligation within the limbic forebrain's nucleus accumbens (Imai et al., 2011). This has been attributed to miR-200b/429 targeting of DNA methyltransferase 3a (DNMT3a), which indeed accumulated in post-synaptic neurons in the nucleus accumbens under a neuropathic pain-like state. Such an increase may lead to long-term silencing of several genes at the transcriptional level, and enhanced cholinergic stimulation might contribute to this effect. An inverse effect has intriguingly been observed for miR-7a, which predictably targets VAChT and AChE-R and may hence reduce the packaging efficiency of ACh and **limit** cholinergic signals: miR-7a alleviates the maintenance of neuropathic pain through regulating neuronal excitability by targeting the β2 subunit of the voltage-dependent sodium channel (Sakai et al., 2013). In all of these cases, the cholinergic targets may thus modulate the observed pain-related effects.

Our experimental test of miR-186 relevance for stress-related conditions revealed a direct dual association between elevated miR-186 and parallel increases in BChE levels in intestinal tissues from predator scent-injection-stressed mice. These effects were long-lasting and were only significant for BChE, highlighting for the first time, the changes in this protein as associated with psychological stress. That both miR-186 and its BChE target show intestinal increases under stress may indicate that these miRNA changes reflect a feedback response limiting excessive ACh stimulation; supporting this notion, serum BChE increases in post-stroke patients were associated with better prospects of recovery (Shenhar-Tsarfaty et al., 2013a). Further studies will be required to explore the molecular mechanisms underlying this involvement.

Several limitations need to be taken into account regarding this study. First, the search algorithms for miRNA candidates appear to differ substantially, each yielding different results. In our current study, we studied those miRNAs found in each of these algorithms, to improve the prospects of identifying as many relevant miRNAs as possible. Second, as our study spanned all of the miRNAs that predictably target the 3′-UTRs in all of the transcripts of interest, further studies will be required to functionally validate these miRNAs not only as single targeting but also as dually targeting more than one of these ACh metabolism-related transcripts. Third, we utilized a data-mining approach as before (Hanin and Soreq, 2011), and relied on explorative studies which link the identified miRNAs to disease association, but it remains unclear if such associations reflect the disease outcome or inversely, an effort of the system to protect itself from the disease.

Taken together, our current study highlights the importance of interrogating the extent and dynamics of miRNA regulation at a pathway level as a novel approach for studying the molecular mechanisms underlying specific processes in health and disease. Moreover, the large fraction of primate-specific miRNAs that were identified in our study calls for paying special attention to such cases. Given that miRNAs are considered "druggable" molecules, for example by antisense oligonucleotide manipulations (Shaked et al., 2009; Shaltiel et al., 2013), it is imperative to search for high throughput datasets from human tissue studies of relevant diseases and perhaps engineer new mouse models with knocked-in primate-specific miRNAs, such as miR-608. This can become a novel approach for identifying targets for therapeutic intervention with diseases where primate-specific miRNAs are subjected to major changes. In either case, our current approach represents the peak of an iceberg; however, it provides an initial proof of principle for the concept of joint regulation over different transcripts involved in specific neurotransmission pathways. This study should further be regarded as a first step in a long pathway, since we only focused on five transcripts out of many involved in only one pathway, the cholinergic signaling pathway; but there are many more transcripts, such as neurotrophin or nicotinic and muscarinic ACh receptors playing a role in cholinergic signaling. Our findings thus indicate overlapping miRNA regulation as a new surveillance mechanism that can balance cholinergic neurotransmission and may be of value for both

basic and translational aspects of neuroinflammation-related disorders.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnmol.2014.00009/abstract

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Genome-wide assessment of post-transcriptional control in the fly brain

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Post-transcriptional control of gene expression has central importance during development and adulthood and in physiology in general. However, little is known about the extent of post-transcriptional control of gene expression in the brain. Most post-transcriptional regulatory effectors (e.g., miRNAs) destabilize target mRNAs by shortening their polyA tails. Hence, the fraction of a given mRNA that it is fully polyadenylated should correlate with its stability and serves as a good measure of post-transcriptional control. Here, we compared RNA-seq datasets from fly brains that were generated either from total (rRNA-depleted) or polyA-selected RNA. By doing this comparison we were able to compute a coefficient that measures the extent of post-transcriptional control for each brain-expressed mRNA. In agreement with current knowledge, we found that mRNAs encoding ribosomal proteins, metabolic enzymes, and housekeeping genes are among the transcripts with least post-transcriptional control, whereas mRNAs that are known to be highly unstable, like circadian mRNAs and mRNAs expressing synaptic proteins and proteins with neuronal functions, are under strong post-transcriptional control. Surprisingly, the latter group included many specific groups of genes relevant to brain function and behavior. In order to determine the importance of miRNAs in this regulation, we profiled miRNAs from fly brains using oligonucleotide microarrays. Surprisingly, we did not find a strong correlation between the expression levels of miRNAs in the brain and the stability of their target mRNAs; however, genes identified as highly regulated post-transcriptionally were strongly enriched for miRNA targets. This demonstrates a central role of miRNAs for modulating the levels and turnover of brain-specific mRNAs in the fly.

Keywords: post-transcriptional regulation, RNA-sequencing, polyA tail, *Drosophila melanogaster*, brain, miRNA

INTRODUCTION

Steady-state levels of mRNAs are a consequence of a balance between transcription and degradation rates. Work done in this area in the last few decades has demonstrated that mRNA molecules are subjected to post-transcriptional regulation of different kinds. These modes of regulation include among others deadenylation, stabilization or degradation by RNA-binding proteins, nonsense-mediated decay reduction (NMD), and miRNA-mediated regulation (Bevilacqua et al., 2003; Alonso, 2005; Halbeisen et al., 2008; Wen and Brogna, 2008; Brogna and Wen, 2009; Meisner and Filipowicz, 2010; Braun et al., 2012). Post-transcriptional regulation usually impacts mRNA stability by influencing or determining the degradation rate. In these cases, cellular control over steady-state levels is achieved mainly by tight post-transcriptional regulation mechanisms rather than by regulating the transcription rate *per se*.

Although several studies have comprehensively assessed post-transcriptional control and RNA turnover rates, these assessments have been restricted either to unicellular organism e.g.,(Andersson et al., 2006; Shock et al., 2007; Miller et al., 2011; Morey and Van Dolah, 2013; Rustad et al., 2013) or cells in culture (Filipowicz et al., 2008; Sharova et al., 2009; Rabani et al., 2011). In other cases, turnover rates have been extrapolated by comparing the levels of nascent and total RNA levels. Although powerful, this type of methodology requires large amount of material and/or laborious procedures (Core et al., 2008; Menet et al., 2012; Rodriguez et al., 2013).

Post-transcriptional regulation of mRNA stability and decay is dictated mainly by trans-acting factors like miRNAs, siRNAs, and RNA binding proteins. These factors act on *cis* elements usually located in the 3′ untranslated region (UTR) of the target mRNA [e.g., AU rich elements, miRNA binding sites (Chen and Shyu, 1994; Kai and Pasquinelli, 2010)]. Their mode of action involves the direct or indirect recruitment of the mRNA degradation machineries like deadenylases, decapping enzymes, and the exosome complex, (for review see Houseley and Tollervey, 2009). A major/convergent point of control on mRNA stability is the length of the polyA tail. Indeed, most pathways that control mRNA turnover affect directly or indirectly the length of the polyA tails (Fabian et al., 2010; Huntzinger and Izaurralde, 2011).

MiRNAs are small (20–23 nucleotide) non-coding RNAs that serve as post-transcriptional regulators of gene expression (Bartel, 2009). MiRNAs are produced in two sequential cleavage steps by the microprocessor complex and the RNAse III enzyme *dicer*

(Denli et al., 2004). Their mechanism of action involves the formation of imperfect hybrids with 3′ UTRs of target mRNAs, which results in translational repression, recruitment of the deadenylase GW182, and mRNA degradation (Fabian et al., 2010; Huntzinger and Izaurralde, 2011). miRNAs associate with the target mRNA as part of a large silencing complex called RISC, which in *Drosophila* includes the protein AGO-1 (Bartel, 2009).

Control of mRNA stability has a central importance in the brain: local translational control and mRNA degradation and stabilization in response to changes in neuronal function and activity are critical for proper brain function. Indeed many RNA-regulators (miRNAs and RNA-binding proteins) are important actors in behavioral processes (Kadener et al., 2009; Liu et al., 2012; Luo and Sehgal, 2012; Lim and Allada, 2013; Zhang et al., 2013) and neuronal function in general. Moreover, miss-regulation of RNA stability can lead to neuronal-related pathologies (Aw and Cohen, 2012; Liu et al., 2012). Despite the importance of post-transcriptional control in the brain, no studies to date have globally assessed mRNA stability and the extent of post-transcriptional control in this tissue.

In this study, we performed a genome-wide assessment of post-transcriptional control in the fly brain. We did so by comparing the levels of polyA-selected and rRNA-depleted RNA samples. As rRNA-depleted RNAs include both nascent and unstable RNAs, for a given transcript the relative amounts between the rRNA-depleted and polyA selected samples is a surrogate of the amount of post-transcriptional control and should be inversely related to the stability of this mRNA. We validated our results by showing that, first, housekeeping genes (like those encoding ribosomal proteins and key metabolic enzymes) are the most stable mRNAs identified using our approach and, secondly, that the mRNAs under the control of the circadian clock, and hence expected to have high turnover rates are actually enriched among the less stable transcripts according to our prediction. Interestingly we found that mRNAs ranked as highly stable or unstable are enriched for genes with very specific Gene Ontology (GO) categories. In particular, mRNAs encoding proteins related to neuronal function and physiology are strongly enriched among the less stable mRNAs. Moreover, we found that the mRNAs predicted to be highly regulated post-transcriptionally by our criteria, are highly enriched for miRNA binding sites. In order to determine whether specific miRNAs mediate most of this regulation, we profiled miRNA expression in the *Drosophila* brain using oligonucleotide miRNA microarrays. Surprisingly, we did not find a correlation between the level of expression of miRNAs in the *Drosophila* brain and the extent of post-transcriptional control of the predicted targets. This demonstrates that although miRNAs have a central function in regulating brain mRNAs, the regulation likely involves many layers and complex mechanisms.

RESULTS

USE OF THE polyA PLUS TO TOTAL RNA RATIO TO ASSESS GLOBAL mRNA STABILITY

In a recent study, Hughes et al., generated RNA-seq data from rRNA-depleted RNA (also called total RNA, TR) and polyA+ RNA (PA) isolated from fly brains (Hughes et al., 2012). Contrary to polyA+ RNA, rRNA-depleted RNA includes all forms of RNA, among them nascent RNAs (pre-mRNA) and RNA with short (or no) polyA tails. Hence, transcripts with strong post-transcriptional control would be more enriched in this preparation than in the polyA+ RNA fraction. Therefore, we reasoned that for a given mRNA, the ratio between the abundance in the TR and the PA libraries should be proportional to the amount of post-transcriptional control. A low PA/TR signal indicates strong post-transcriptional control: mRNAs with short polyA tails tend to be found more abundantly in the total RNA fraction, as these transcripts bind weakly to the oligo dT beads used to isolate polyA+ mRNAs (Meijer et al., 2007; Meijer and de Moor, 2011; Kojima et al., 2012).

We limited our analysis to the transcripts produced by RNA polymerase II as RNAs transcribed by other polymerases lack a polyA tail and hence will only be present in the TR fraction. The data was processed as indicated in **Figure 1A**. As shown in **Figure 1B**, the data have a quasi-normal distribution after log transformation ($n = 32898$). As expected, transcripts that are not polyadenylated, such as some of the histones transcripts, are toward the left end of the curve as these have low PA/TR ratios (**Figure 1B**, red and blue dots).

Rather than being a direct reflection of mRNA stability, low PA/TR ratios may indicate nuclear retention or specific control of polyA tail length not related to mRNA turnover. In order to test the validity of our approach, we looked at the PA/TR ratio of specific groups of mRNAs that are known to have long or short half-lives (**Figures 1B**, **2A**). We first analyzed mRNAs encoding housekeeping protein. We observed that mRNAs encoding proteins with the GO terms ribosomal and TCA cycle enzymes were significantly enriched in the group of mRNAs with high PA/TR ratios (high stability; $p = 3.13e^{-147}$and $p = 1.92e^{-12}$, respectively, **Figure 2A**). On the other hand, we found circadian-regulated mRNAs among the subset of genes with low PA/TR ratios ($p = 1.62e^{-47}$); circadian-regulated mRNAs are by definition short-lived as they display mRNA oscillations and do not accumulate through the day. Therefore, we conclude that our approach can be used to identify differentially stable mRNAs.

GENE GROUPS IN THE EXTREME OF THE PA/TR DISTRIBUTION BELONG TO SPECIFIC GENE ONTOLOGY CATEGORIES

Evaluation of PA/TR values of genes associated with other GO terms gave interesting results. Genes involved in immune response were enriched among the group of genes with low PA/TR ratio ($p = 7.6e^{-9}$); genes in the oxidative stress response group had higher PA/TR ratio ($p = 0.00037$) (**Figure 2A**). Interestingly, genes associated with neuronal-related GO terms such as axon and neuronal cell body were significantly enriched among the mRNAs with low PA/TR ratios ($p = 3.94e^{-43}$ and $p = 1.012e^{-18}$, respectively), suggesting that mRNAs encoded by genes in this group are under high post-transcriptional regulation (**Figure 2A**).

To determine which types of mRNAs are in the most stable or unstable groups of genes, we determined the types of transcripts that are particularly enriched in the extremes of the PA/TR distribution. These transcripts should be extremely stable (high PA/TR ratio) or unstable (low PA/TR ratio). We selected the transcripts in the top 5% or bottom 5% of the PA/TR ranking and tested

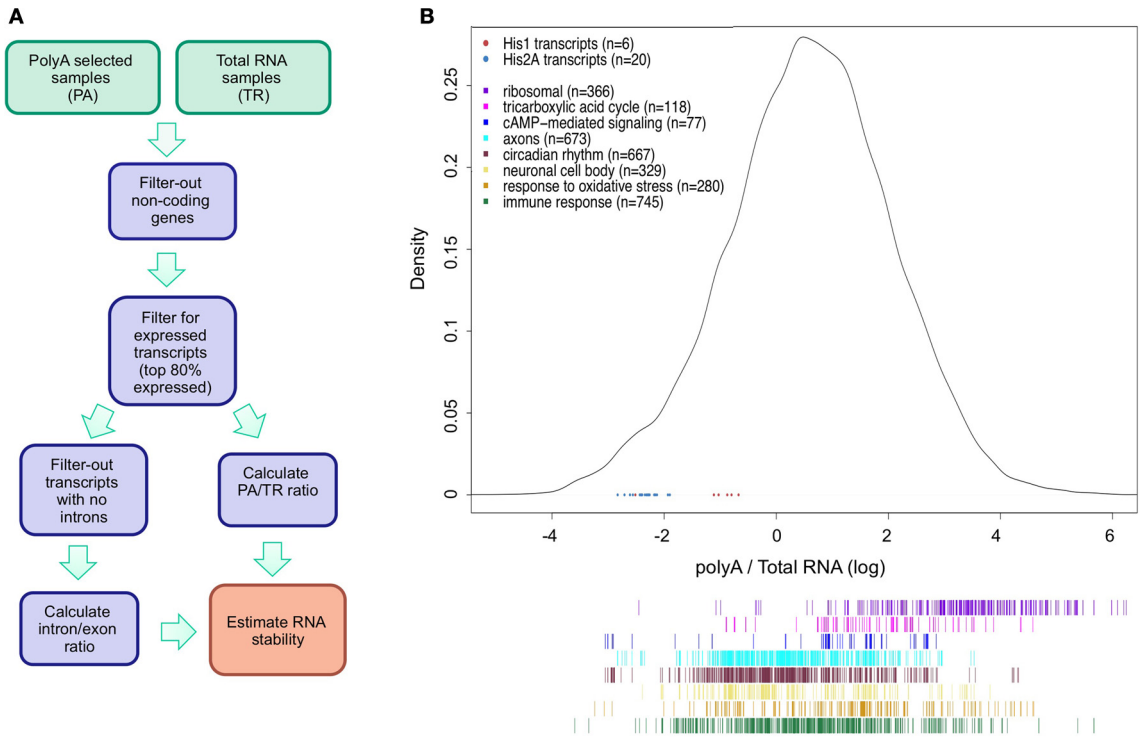

FIGURE 1 | Analysis of mRNA stability using polyA and total RNA-seq data. (A) Schematic illustration of the strategy used to estimate RNA stability. **(B)** Density plot of the distribution of polyA/total-RNA (PA/TR) values for all transcripts ($n = 32898$). Data was log-transformed to achieve normal distribution. Blue and red dots at the lower part of the plot represent the PA/TR values of His1 (red) and His2A (blue) transcripts. Colored lines under the density plot represents transcripts associated to different GO terms groups. Color Key for Histone and GO terms transcripts is presented at the top left panel.

whether these transcripts are enriched for specific GO terms (**Figures 2B,C**). As expected, transcripts with high PA/TR ratios were enriched for genes with GO terms related to housekeeping functions like ribosomal, enzymes and cytoskeleton organization (**Figure 2B**). Interestingly, we found that genes encoding proteins involved in cell cycle, luminal proteins, and nuclear mRNA splicing were also enriched in this fraction, suggesting that their mRNAs are long lived (**Figure 2B**).

In addition, we found that many more GO terms were enriched in mRNAs with low PA/TR ratios (**Figure 2C**). Notably, genes involved in brain-related processes were highly enriched in the less stable, short-lived mRNA group. These include genes involved in neurological system processes, cognition, sensory perception, behavior, and synapse organization. In addition, genes involved in transcriptional control (such as DNA binding proteins) belonged to the group of short-lived messages. The strong quantitative and qualitative differences between the genes enriched in both extremes of the PA/TR ratio, reinforces the notion that post-transcriptional control is central in brain physiology and function.

As stated above, the PA/TR ratio may reflect factors other than mRNA stability. We therefore used an independent measurement to further analyze the genes in the top 5% and bottom 5% of the PA/TR distribution. Intronic data has been used in the past as surrogate of transcription. As the total RNA-seq data includes signal from introns and exons, this dataset can also be used to independently test mRNA stability by calculating the relative amounts of introns and exons for a given mRNA. Hence, we calculated the ratio of intronic vs. exonic signal (I/E) for those genes at the extremes of the PA/TR distribution. We expected that mRNAs with high turnover rates and for which we computed low PA/TR ratios will have high I/E ratios and that those genes in the upper end of the PA/TR distribution would display an opposite trend. In order to avoid misinterpretations of the results due to different scaling factors, we based our comparison on the ranking of the different ratios. We observed that the mRNAs ranked as very stable (top 5%) using the PA/TR ratio were among the transcripts with lowest I/E ratios (less nascent compared to mature mRNA, hence more stable (**Figure 3A**). In addition, those mRNAs ranked as very unstable in the PA/TR ratio measurement had highest I/E ratio, further validating our approach (**Figure 3B**).

PA/TR RATIO CORRELATES WITH TRANSCRIPT ABUNDANCE ONLY FOR LOWLY EXPRESSED mRNAs

In order to further validate the ability of the PA/TR ratio to evaluate mRNA stability, we decided to examine whether the PA/TR has any bias for low or high expressed mRNA. For assessing this possibility, we used a linear regression model that takes into account the relationship between transcript expression levels (RPKM values of the poly A selected RNA) and its predicted stability (PA/TR ratio). Indeed, this model show a

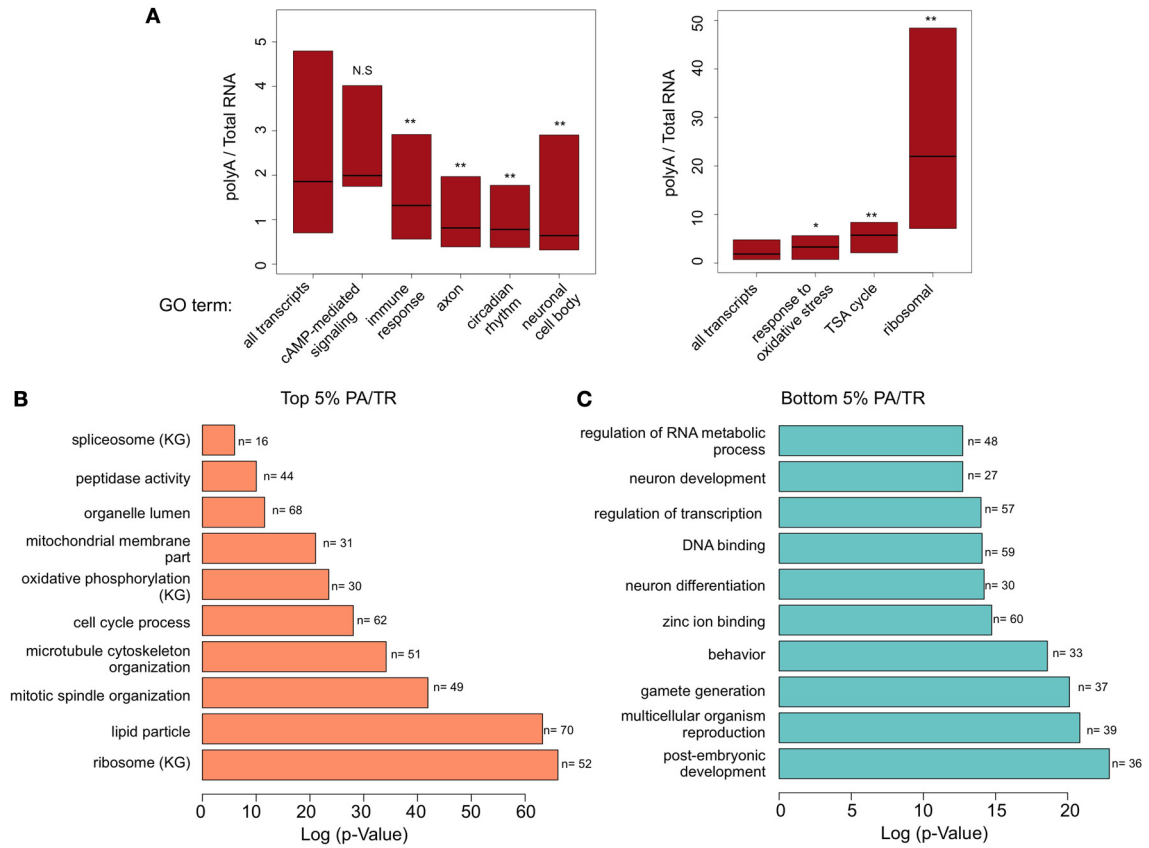

FIGURE 2 | Gene ontology (GO) enrichment analyses (A) Box plot representation (quartiles and median) of transcripts associated with different GO terms. Number of transcripts at each group is presented at **Figure 1B** at the top left panel. Mann-Whitney *U*-test was performed to determine statistical significance of the differences. $^{*}p < 0.005$; $^{**}p < 0.0001$. NS, non-significant. **(B)** Results of DAVID functional annotation analysis to examine GO enrichment in genes with the top 5% and **(C)** bottom 5 % PA/TR values. The data presented is log transformed *p*-Value (FDR corrected) of GO terms or KEGG pathways (KG) found to be enriched in the tested group of genes.

positive correlation between the mRNA abundance and stability ($n = 32898$, $r = 0.29$, $p < 0.00001$). However, only ∼9% of the change in PA/TR ratio can be explained by the expression levels (R-squared = 0.0879) demonstrating that the PA/TR ratios are not a mere reflection of mRNA abundance. Moreover, when filtering out the very low expressed mRNAs (those expressed less than 1 RPKM), the explained fraction is reduced to only 3.5% ($n = 31482$, R-squared = 0.0346). Interestingly, for the lowly expressed transcripts, the explained fraction is more than 30% ($n = 1416$, R-squared = 0.3181) **Figure 4A** shows a scatter plot of the correlation (the red line represents RPKM value of 1).

In order to look in more detail into the relationship between the PA/TR ratio and mRNA abundance, we selected groups of transcripts based on their expression levels (e.g., 1–1.5, 10–11, 20–21 until 80–81 RPKM) and compare their PA/TR ratio distribution (**Figure 4B**). ANOVA test demonstrated that there is no significant difference in the distribution of PA/TR values across the range of 20-80 RPKM ($p = 0.09$), showing that in this range, transcripts with four times difference in expression levels can have the same PA/TR ratio. Indeed, only the two groups with lower expression (RPKM 1–1.5 and 10–11) showed significantly different distribution, as they are clearly enriched for transcripts with low PA/TR ratio ($p < 0.0001$ for both). These results demonstrate that PA/TR ratio does not correlate with transcripts abundance globally. However, transcripts with very low mRNA abundance have in average lower PA/TR ratio, but we favor the interpretation that this is a result rather than a bias of the PA/TR ratio (see discussion).

GLOBAL ASSESSMENT OF miRNA ABUNDANCE IN THE *Drosophila* BRAIN

Our meta-analysis revealed that several types of mRNAs are highly regulated at the post-transcriptional level. MiRNAs or RNA binding proteins could mediate this regulation. Since there is no publicly available genome-wide expression data available for miRNAs in the *Drosophila* brain, we generated our own dataset. We purified RNA from dissected brains and determined the abundance of individual miRNAs using oligonucleotide microarrays. In order to minimize effects due to the time of collection, we isolated RNA from brains of flies collected at six different times of the days. **Figure 5A** shows heat-map representation of top 50 miRNA expressed in the *Drosophila* brain. miRNA expression levels were averaged across the six time points for further analysis.

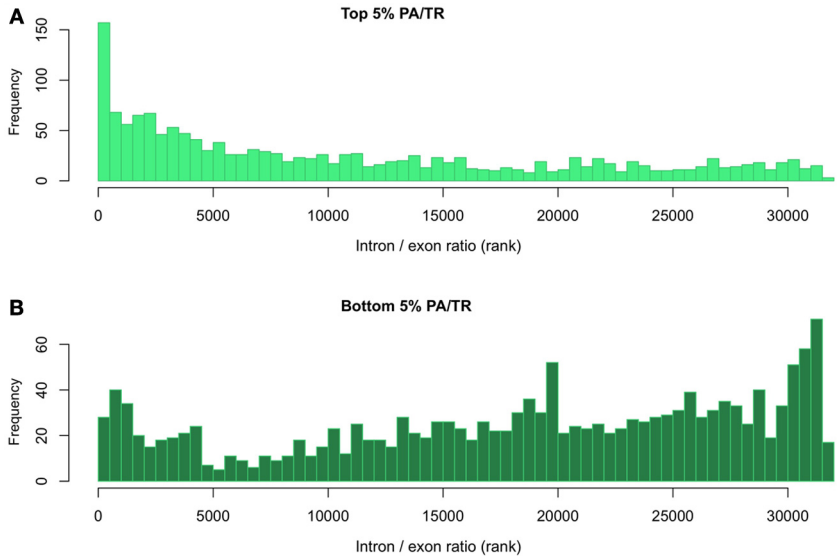

FIGURE 3 | Evaluation of mRNA stability using the relationship between the intronic and exonic signals. (A) Distribution of intron/exon ratio of transcripts with top 5% and **(B)** bottom 5% PA/TR values. All transcripts were ranked according to their intron/exon RPKM ratio. The rank values of the top or bottom 5% PA/TR were extracted and plotted.

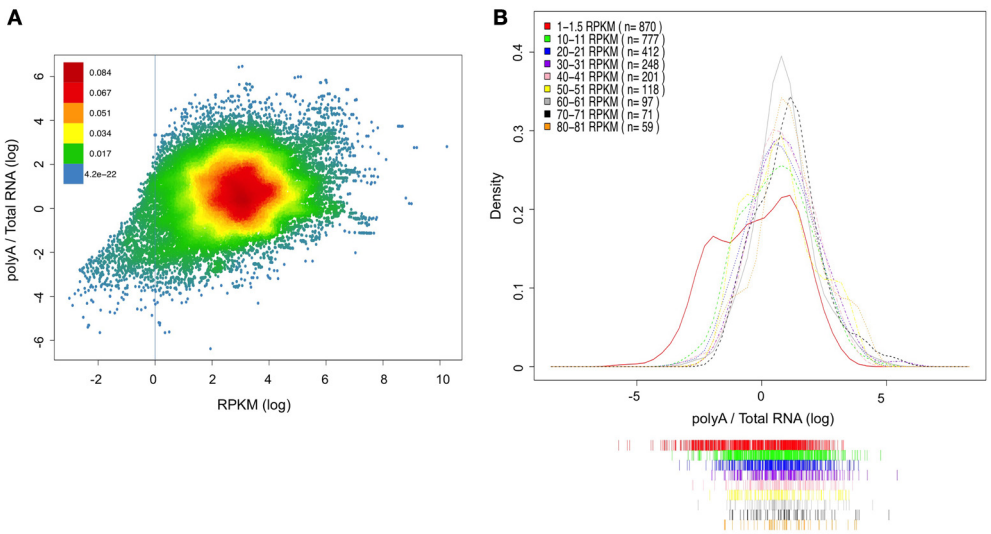

FIGURE 4 | Association between gene expression and PA/TR ratio. (A) Correlation between RPKM value of each transcript and its PA/TR ratio. The data is presented as log transformed values and the density of the dots at the plot is represented by the different colors (see color key). Red line represents level of 1 RPKM (log value of 0). **(B)** PA/TR distributions of groups of transcripts with various RPKM values. The RPKM range in each group is indicated at the top left panel. The data is presented as log transformed values.

TRANSCRIPTS WITH LOW PA/TR RATIOS ARE ENRICHED FOR miRNA BINDING SITES, BUT THEIR STABILITY IS NOT CORRELATED TO THE ABUNDANCE OF THE PREDICTED REGULATORY miRNA

In order to test whether miRNA-mediated regulation has a major impact on processes in the brain, we tested whether the less stable mRNAs were enriched for predicted miRNA targets. We used dataset of predicted targets of conserved miRNA families (using TargetScanFly) and estimated the PA/TR ratio of these transcripts. We found that those mRNAs which have been predicted to be regulated by miRNA ($n = 15206$) are enriched among the less stable transcripts. Mann-whitney U-test and bootstrapping approach (10,000 bootstrap samples) showed that the difference is statistically significant ($p < 0.0001$). This demonstrates a key role for miRNAs in regulating mRNA stability in the fly brain (compare the distribution of the PA/TR ratios for all mRNAs and those that have been predicted or not to be miRNA-regulated in **Figure 5B**).

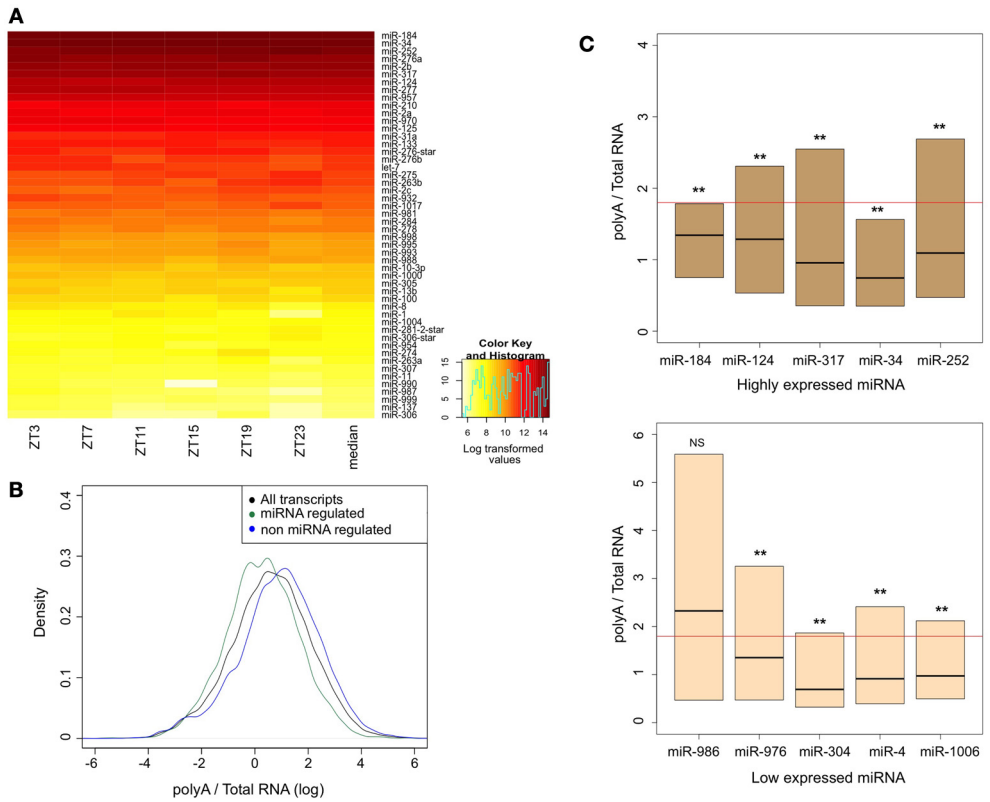

FIGURE 5 | Assessment of miRNA expression in the brain and the stability of their targets. (A) Heat-map representation of top 50 miRNA expressed in the *Drosophila* brain. Fly brains were collected across six time points of the circadian day (ZT, zeitgeber time). RNA was extracted and loaded to Affymetrix array chips. miRNA expression levels were averaged across the six time points for further analysis. **(B)** Density plot comparing the distribution of polyA/total-RNA values for all transcripts (black line; $n = 32898$) with those of miRNA-regulated genes (green line; $n = 15206$) and non miRNA-regulated genes (blue line; $n = 16,456$). Data was log-transformed to achieve normal distribution. Mann-Whitney *U*-test and bootstrapping approach (10,000 bootstrap samples) showed significant difference between the groups ($p < 0.0001$). **(C)** Box plot representation (quartiles and median) of PA/TR values of different miRNA target genes. For each list of miRNA targets, Mann-Whitney *U*-test was used to determine statistical significance of the differences. Numbers of transcripts at each group are summarized at Supplementary Table 2. Horizontal line represents the median values for all transcripts. $^{**}p < 0.0001$. NS, no significant.

Last, we tested whether there is a correlation between miRNA expression levels in the brain and the stability (calculated from the PA/TR ratio) of their target mRNAs. We divided miRNAs into groups based on their expression levels. For each miRNA we calculated the PA/TR ratio of its predicted targets and tested for significant differences between its targets values and the entire transcript population. For almost all the miRNAs, their predicted targets had significantly lower PA/TR values than the entire transcript population: Out of 94 miRNA families only seven were not found in the group with lower PA/TR targets (Supplementary Table 2). Surprisingly, we did not find any correlation between the expression levels of the miRNAs and the PA/TR ratio. Predicted targets of both highly expressed and lowly expressed miRNA had low PA/TR ratio (**Figure 5C**), and applying Spearman's correlation test did not show significant correlation between miRNA expression and PA/TR values ($p = 0.109$). These results demonstrate that although miRNA regulation is a key regulatory mechanism in the brain, there is a complex, non-linear correlation, between transcripts containing miRNA target sequences and miRNA expression levels.

DISCUSSION

In this work we utilized previously published RNA-seq data and newly generated brain-specific miRNA expression data to globally estimate mRNA turnover rates in the *Drosophila* brain and to evaluate the mechanism behind this regulation. In order to estimate globally mRNA turnover rates, we compared the levels of each transcript in polyA+ purified and rRNA-depleted RNA samples. More specifically, we generated a PA/TR ratio that should directly correlate with the extent of post-transcriptional control and inversely with mRNA stability. We validated our approach by showing that mRNAs known to be highly stable like those encoding proteins related to the ribosome and cytoskeleton function, have a high PA/TR ratio. At the opposite end of the stability spectrum, mRNAs known to have high turnover rates like those encoding synaptic, circadian, and other proteins display PA/TR ratios indicative of short half-lives. Interestingly we found that mRNAs encoding proteins involved in key neuronal functions are among the most highly regulated mRNAs at the post-transcriptional level. MiRNAs seem to play a key part in mRNA stability in the brain, as transcripts with very low PA/TR

ratio are strongly enriched for miRNA binding sites. However, miRNA regulation is likely to be complex and redundant, as we did not find correlation between miRNA levels and the PA/TR ratio of their predicted mRNA targets in the brain.

Although we have validated our strategy, we acknowledge that it provides an indirect measure of mRNA stability. This is because the PA/TR ratio may reflect nuclear retention, inefficient splicing, and other modes of regulation like cytoplasmic polyadenylation instead of mRNA turnover. However, we believe that it can be certainly assured that genes with low PA/TR ratio are under strong post-transcriptional control. Indeed, modes of post-transcriptional regulation that does not lead to mRNA decay (e.g., cytoplasmic polyadenylation) could constitute an important point of control for certain mRNAs like those that are translated in synapses. It is well known that synaptic-translated mRNAs are associated with the miRNA machinery and specific RNA binding proteins until their translation. The PA/TR ratio thus measures more generally the extent of post-transcriptional control of mRNA levels rather than being a measure of mRNA turnover.

We found that for most transcripts, there is no correlation between their expression and their stability measured by the PA/TR ratio (**Figures 4A,B**). However, we found that very lowly express genes are among the less stable mRNAs. We don't believe that this is the result of bias in the analysis or calculation of the PA/TR coefficient but rather a biological meaningful result. In other words, we believe that our results indicate that lowly expressed genes are the result of not so low expression coupled to high mRNA turnover. This could be a way to diminish gene expression noise, as it is known that lowly transcribed genes are subjected to high expression noise. Indeed middle transcription followed by strong post-transcriptional control has been proposed to be an efficient way to generate low mRNA levels without much noise (Hornstein and Shomron, 2006). Given the key function of the genes with low expression in the brain, this seems a fair tradeoff. It should be pointed out that the data we utilized for this study is extremely deep (∼20 million reads per sample for polyA selected RNA, and ∼40 million paired-end reads per sample for non-polyA), so even the very low genes are well represented (in terms of total amounts of reads) in the PA samples, therefore, we don't think that our PA/TR ratio has diminished performance in this extreme of the expression distribution.

Although our results suggest a key role for miRNAs in post-transcriptional control, we were surprised to find that there is no correlation between the levels of brain miRNAs and the extent of post-transcriptional control of their predicted targets. This could be due to several factors. First, it is known that mRNAs are usually targeted by several miRNA species, with certain miRNAs expressed in some cell types but not others (Bartel, 2009). Second, miRNA abundance is not always reflective of the functional activity. Indeed, sequencing of AGO-1 associated (RISC-bound) miRNAs is a more accurate measurement of the abundance of functional miRNAs as only a fraction of miRNAs present in a cell are incorporated into a RISC and are thus functional at a given time (Krol et al., 2010). Third, our correlations are based on miRNA-target predictions. Although algorithms like Target-Scan usually display low false positive rates, many meaningful interactions might be missed (Yue et al., 2009). Hence the lack of correlation could be due to failure in the miRNA-target prediction algorithm, although we feel that this is unlikely as we observed the lack of correlation using only the evolutionary conserved miRNAs. Last, as the brain is highly heterogeneous in neuronal cell types, it is possible that miRNAs expressed at very low levels globally have key functions in specific neuronal groups. A last consideration is that our approach does not consider expression levels. Two genes with equal PA/TR ratios but very different expression levels may respond very differently to a given miRNA. Based on this consideration, we believe that identification and analysis of a dataset of AGO-1-associated mRNAs and miRNAs would shed additional light on post-transcriptional regulation in the brain (Varghese et al., 2010; Aw and Cohen, 2012; Weng and Cohen, 2012).

In sum, our comparison of levels of total or polyA-selected RNA allowed us to evaluate the extent of post-transcriptional control for all brain-expressed mRNAs. The lack of a strong correlation between the expression levels of miRNAs in the brain and the stability of their target mRNAs indicates that much remains to be learned about the modulation of brain-specific mRNAs in the fly. Our work provides a valid approach for analysis of mRNA stability and indicates a central role for miRNAs in regulating mRNA levels in the brain.

MATERIALS AND METHODS

RNA-SEQ DATA ANALYSIS

We used RNA-seq data published by Hughes et al., available at GEO (accession number: GSE29972) (Hughes et al., 2012). In this paper, the authors generated RNA libraries with polyA selected (PA) or ribosomal-depleted RNA (TR). Our analysis was based on the processed data published by the authors, which includes RPKM values calculated as described. Two replicates of CS samples from ZT0 and ZT12, both polyA selected and ribo-depleted, were used for the analysis. Non-coding genes and lowly expressed transcripts (bottom 20% RPKM values) were filtered out. For each transcript, we divided polyA RPKM values with non-polyA RPKM values of the corresponding sample to determinate PA/TR ratio. The average of PA/TR values of both replicates and time points was used for further analyses. To determine Intron/Exon (I/E) ratio we divided the average exonic and intronic RPKM values and the data was ranked prior to comparison to PA/TR ratio. PA/TR data was log-transform to achieve normal distribution for data visualization and prior to applying linear regression model. All data used in this study is included in Supplementary Table 1 (see also **Figure 1**). All analyses described in this paper were performed using R version 3.0.1.

GENE ONTOLOGY ENRICHMENT ANALYSIS

Gene Ontology database (http://www.geneontology.org/) was used to obtain lists of genes associate with different GO terms. For each list of genes, we extracted PA/TR values of the transcripts, calculated median and used the non-parametric Mann-Whitney *U*-test to determine statistical significance. DAVID functional annotation tool (http://david.abcc.ncifcrf.gov/home.jsp) (Huang et al., 2009) was used to examine GO enrichment in the groups

of genes with top 5% or bottom 5% PA/TR values. The list of expressed transcripts (top 80% RPKM values) was used as the background in the analysis. The data presented is log transformed *p*-Values after FDR correction.

Drosophila's BRAIN SAMPLE PREPARATION

For profiling the expression of miRNA, 3–5 days old *Drosophila.M* Canton-S flies were entrained in 12:12 LD cycles. Fly brains were collected across six time points of the circadian day. At each time point twenty five brains were dissected, and completely cleaned from trachea and fat tissue, in ice cold PBSX1. Brains collected into an eppendorf were immediately immersed in Lysis/Binding buffer (Ambion, AM1560) and kept on ice for the rest of the dissection. By the end of each dissection round brains were homogenized using a rotor blade and frozen in liquid nitrogen.

RNA EXTRACTION

Extraction of small RNA containing-total RNA was performed using the mirVana miRNA isolation kit (Ambion, AM1560). Organic extraction using Acid-Phenol:Chloroform was done according to the manufacture's protocol. Following elution samples were treated with TURBO DNase (Ambion, AM2238) according to the manufacture's protocol. Finally, RNA was recovered by isopropanol precipitation supplemented with glycerol.

AFFYMETRIX GENECHIP miRNA 2.0 ARRAY

Pre-miRNA and mature miRNA expression levels were studied using Affymetrix GeneChip miRNA 2.0 Array. 600ng from each of the miRNA containing-total RNA were loaded to six array chips. Affymetrix Expression ConsoleTM Software was used to normalize and calculate summary values from Affymetrix CEL files. Data were background-corrected by the RMA method. Heatmap was generated using the heatmap.2 function of the gplots package in R. miRNA expression levels were averaged across the six time points for correlation analysis.

miRNA TARGET GENES ANALYSIS

List of conserved miRNA families and their targets was obtained from TargetScanFly (http://www.targetscan.org/fly/). PA/TR values of miRNA target genes were extracted and the median for each miRNA targets group was calculated. Mann-Whitney *U*-test was used to estimate statistical significant comparing to all transcript population and the *p*-Values were FDR corrected. For estimating the significance of the differences between all miRNA targets and non-miRNA targets PA/TR values bootstrapping approach was also applied (10,000 bootstrap samples). Spearman correlation test was used to examine relationship between miRNA expression levels and PA/TR value of their targets.

AUTHOR CONTRIBUTIONS

Shaul Mezan: Performed the experimental work and helped with the writing of the manuscript. Reut Ashwal-Fluss: Lead the analysis of the data and helped with the writing of the manuscript. Rom Shenhav and Manuel Garber: Helped with the analysis of the data. Sebastian Kadener: Designed the experimental and guided the analytical part. Wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnmol.2013.00049/abstract

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MicroRNAs as biomarkers for CNS disease

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For many neurological diseases, the efficacy and outcome of treatment depend on early detection. Diagnosis is currently based on the detection of symptoms and neuroimaging abnormalities, which appear at relatively late stages in the pathogenesis. However, the underlying molecular responses to genetic and environmental insults begin much earlier and non-coding RNA networks are critically involved in these cellular regulatory mechanisms. Profiling RNA expression patterns could thus facilitate presymptomatic disease detection. Obtaining indirect readouts of pathological processes is particularly important for brain disorders because of the lack of direct access to tissue for molecular analyses. Living neurons and other CNS cells secrete microRNA and other small non-coding RNA into the extracellular space packaged in exosomes, microvesicles, or lipoprotein complexes. This discovery, together with the rapidly evolving massive sequencing technologies that allow detection of virtually all RNA species from small amounts of biological material, has allowed significant progress in the use of extracellular RNA as a biomarker for CNS malignancies, neurological, and psychiatric diseases. There is also recent evidence that the interactions between external stimuli and brain pathological processes may be reflected in peripheral tissues, facilitating their use as potential diagnostic markers. In this review, we explore the possibilities and challenges of using microRNA and other small RNAs as a signature for neurodegenerative and other neuropsychatric conditions.

Keywords: biomarker, microRNA, next-generation sequencing, CSF, plasma, exosome

INTRODUCTION

Central nervous system disorders encompass a broad spectrum of neurodegenerative, oncological, inflammatory, and developmental conditions. Several mechanisms exist that evolved in order to isolate and protect the CNS from insult; interestingly, these effectively also act as barriers to diagnosis. Surrogate markers of disease are thus critical to facilitate disease detection, stratification of patients into subpopulations, prediction of prognosis, evaluation of response to treatment, and eventually allow better understanding of etiopathology.

To be of maximum diagnostic benefit, biomarkers would predict disease early, before the onset of clinical symptoms. Finding and testing such biomarkers would be best achieved by a longitudinal study in a large patient population at risk of developing the disease, a resource-intensive process that requires a long commitment and careful planning. However, the more common cross-sectional association studies are equally valuable in biomarker discovery. Brain imaging techniques and their modifications, as well as genotype studies to identify susceptibility alleles—the latter frequently employed in predicting tumor prognosis—are being used successfully to understand complex neurological conditions. In parallel, as techniques evolve rapidly and new hypotheses emerge, we see novel methods being applied to biomarker discovery. Thus, with the recent rapid acceleration in the field of non-coding RNA research, the potential predictive and diagnostic uses of these molecules have also attracted significant attention. Among non-coding RNA, microRNAs have been most intensely studied and their biology has repeatedly been proven critical for diverse cellular functions. More importantly, recent evidence indicates that miRNAs can be detected in peripheral tissues and can be used to "capture" changes in the cell of origin, including neurons. This has generated substantial interest in the use of small non-coding RNAs, in particular miRNAs, as biomarkers for CNS pathology. One advantage of molecular markers such as small RNAs over imaging technology is that samples can be frozen down for retrospective analysis, which enables larger studies. This manuscript aims to provide an overview of recent advances in the field of miRNA-based biomarker discovery for CNS disease.

SOURCES OF RNA BIOMARKERS

As RNA is continually transcribed, translated, and turned over in response to physiological and pathological stimuli, the RNA profile of a cell, interpreted appropriately, could serve as a reflection of its current functional state. Current technologies enable transcriptome analysis on an unprecedented scale. In the human CNS we often need to rely on extracranial or peripheral sources of RNA to obtain a live readout of the disease state. The choice of potential sources for representative RNA is wide and includes body fluids such as blood, plasma, or cerebrospinal fluid as well as non-neuronal tissue or cells such as lymphocytes (**Figure 1**). The question that arises when using non-neuronal tissue or body

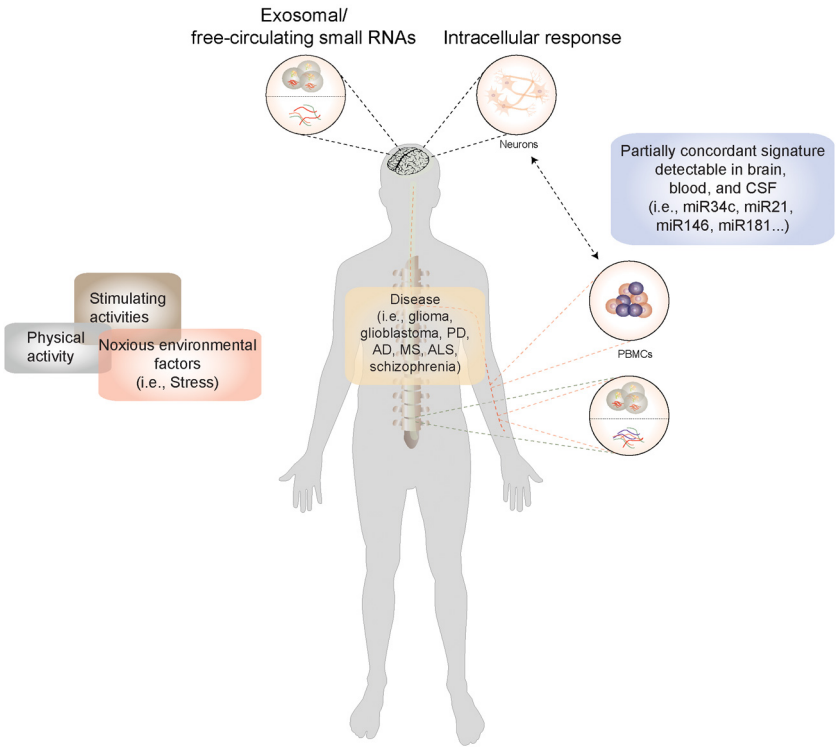

FIGURE 1 | A model for miRNA-based biomarker development: Disease-causing factors impact the brain both directly and indirectly (via immune and other cells), eliciting changes in gene and microRNA expression patterns. Many of these stimuli concurrently exert their influence on non-neuronal cells, where they also elicit a response. In CNS diseases, in the absence of direct access to diseased tissue, microRNA expression patterns from peripheral cells such as blood cells could be used a proxy for genome-environment interaction in the CNS. Moreover, microRNAs circulate stably in cerebrospinal fluid and plasma in extracellular vesicles and in lipoprotein complexes, and can be isolated from these body fluids and profiled. Information derived from peripheral sources could thus be used to construct a picture of neuronal function both in the healthy and the diseased state.

fluids as a source is: To what degree do they resemble biological processes in the brain, arguably the most unique of organs with a distinct composition and cellular milieu? Nevertheless, a biomarker is formally defined as a proxy that allows remote and early detection of a biological process (i.e., disease) regardless of its mechanistic role in the condition being diagnosed. In the ideal situation, it would also reflect the biology of the original tissue, thus providing insight into disease mechanism, and even serve as a potential therapeutic target. Two major sources of peripheral RNA exist, namely extracellular RNA and RNA within peripheral mononuclear blood cells (PBMCs). While the former is still beginning to be explored, for the latter evidence has accumulated to indicate that a certain correlation exists between the molecular events occurring in the brain and those that can be detected in blood cells (**Figure 1**).

RNA FROM BLOOD CELLS

The use of genetic material from blood cells to screen for biomarkers of neurological conditions has been used as early as 1975 (Issidorides et al., 1975). Peripheral blood mononuclear cells (PBMCs), one of the major cellular components of blood, are particularly interesting in the context of biomarkers due to their ability not only to respond to internal and external stimuli, but also to "store" the information at the epigenetic level (Tang et al., 2001; Gavin and Sharma, 2009, 2010). Studies in monozygotic twins have demonstrated that over time PBMCs accumulate differences at the DNA methylation and histone acetylation level (Fraga et al., 2005). Furthermore, PBMCs have been successfully used to characterize the disease biosignature in neuropsychiatric conditions such as schizophrenia and bipolar disorder (Tang et al., 2001; Segman et al., 2005; Tsuang et al., 2005; Bowden et al., 2006; Iga et al., 2006; Anderson et al., 2008). Several lines of evidence suggest that both brain and blood cells can respond to environmental stimuli and reflect this response at the epigenetic level in their genome and that this response is indeed to some extent concordant between both tissue types (Desjardins et al., 2008; van Heerden et al., 2009; Li et al., 2011; Ursini et al., 2011; Yuferov et al., 2011; Davies et al., 2012; Provencal et al., 2012). Firstly, gene expression profiles in PBMCs have revealed common patterns of transcriptional activity in blood and neurons (Sullivan et al., 2006). Thus, for example, DNA methyltransferases DNMT1 and DNMT3a have been found to be upregulated in both post-mortem brain tissue and PBMCs from schizophrenia patients (Zhubi et al., 2009) and whole chromosome mRNA expression profiles were found to be partially consistent between blood and brain in Huntington's disease patients (Anderson et al., 2008). In

mice, a model of early life stress (i.e., maternal separation) was shown to induce a concordant transcriptional response in PBMCs and several brain regions (Desjardins et al., 2008).

Further, transcriptomic information obtained in peripheral blood has been successfully applied to predict healthy/disease status or to differentiate between disease stages (Tang et al., 2001; Tsuang et al., 2005; Du et al., 2006; Desjardins et al., 2008). This is possible due to the fact that PBMCs and neurons are actually exposed to very similar biochemical environments and can thus mount a concordant cellular response to incoming stimuli. Interestingly, in most of these cases, genes found to be differentially expressed in blood were also directly associated with neuropsychiatric disease and to be altered in postmortem brain (Tang et al., 2001; Tsuang et al., 2005; Du et al., 2006; Desjardins et al., 2008).

Secondly, the levels of certain epigenetic markers, such as DNA methylation patterns or miRNA expression, have been shown to directly correlate between PBMCs and neuronal tissue. A recent study by Davies and colleagues demonstrated a globally correlated inter-individual pattern of DNA methylation between cortical brain areas and PBMCs in healthy human postmortem tissue (Davies et al., 2012). In Rhesus monkeys, a model of early life stress based on surrogate mother rearing induced significant changes in DNA methylation in the prefrontal cortex, as well as in PBMCs (Provencal et al., 2012). Although the response in brain was more drastic, a positive and significant correlation in epigenetic changes was found between both tissue types (Provencal et al., 2012). At the individual gene level, the prodynorphin promoter has also been recently shown to display a consistent methylation pattern between blood cells and caudate/cingulate cortex in human post-mortem tissue (Ursini et al., 2011) and changes in methylation observed in human blood samples within the COMT gene (Catechol-O-methyltransferase, a critical enzyme for dopamine processing in the brain) were replicated and significantly correlated between blood and prefrontal cortex in the orthologous genomic location in rats (Li et al., 2011). Additionally, there is evidence to suggest that the level of other epigenetic markers, such as miRNA levels, also show parallel patterns of expression in blood and brain. Thus, levels of miR34a were recently shown to increase during aging in blood PBMCs, as well as in plasma and brain, and to correlate with a concomitant decrease in SIRT1 expression, one of the main targets of this miRNA (van Heerden et al., 2009).

Taken together, there is a solid base to suggest that PBMCs and perhaps other blood cells have the potential to provide a transcriptional and epigenetic biosignature that can be useful for both biomarker development and drug discovery and that these can be used as a proxy to study epigenetic mechanisms of neuropathology and its progression.

EXTRACELLULAR RNA

After the discovery that cells export RNA packaged in 40–90 nm sized vesicles called exosomes, and that this RNA could be taken up and translated by recipient cells (Valadi et al., 2007), extracellular vesicles rapidly attracted attention as a potential medium for intercellular communication. Similar findings in exosomes from primary glioblastoma cells, indicating that malignant vesicles may play a role in modulating tumor microenvironment (Skog et al., 2008), brought researchers to the idea of using the information carried by these vesicles to study organs/tumors remotely. Cell-derived RNA can also be found in a host of other membrane enclosed vesicular bodies variously called nanovesicles (Kogure et al., 2011), shedding vesicles, microvesicles (Ratajczak et al., 2006), or microparticles (Patz et al., 2013).

Exosomal and other extracellular vesicles are known to play a role in neuronal function, but the nature and degree of their involvement is still being studied. Exosomal release is modulated by glutamatergic synaptic activity, indicating that this may be a part of normal synapse physiology, and that the contents of these vesicles could be relevant for interneuronal communication (Lachenal et al., 2011). Exosomes also play a role in signaling between the pre- and post-synapse. Exosomal transfer of synaptotagmin 4 from the pre- to the post-synaptic compartment enables the presynapse to influence postsynaptic retrograde signaling (Korkut et al., 2013). These and several other lines of evidence led to the hypothesis that intercellular communication via exosomal content is a key underexplored physiological mechanism in the nervous system (Smalheiser, 2007). Thus, the RNA content of brain-cell-derived vesicles is a promising source of biomarkers for CNS disease. Extracellular RNA can also be found outside vesicles (Wang et al., 2010), in complex with lipoproteins such as HDL (Vickers et al., 2011) or with Argonaute2 (Arroyo et al., 2011; Turchinovich et al., 2011). This population comprises primarily miRNA, which appears to circulate stably in this form (Mitchell et al., 2008).

Recently, evidence that extracellular RNA can be extracted from various body fluids including saliva (Palanisamy et al., 2010), plasma (Hunter et al., 2008), urine (Alvarez et al., 2012), and CSF (Patz et al., 2013) has accumulated (**Figure 1**). Next generation sequencing (NGS)-generated profiles of the RNA contents of extracellular vesicles are beginning to be published (Burgos et al., 2013; Ogawa et al., 2013). However, the cellular source of this RNA is not always clear. RNA isolated from body fluids is likely to originate from a heterogenous mixture of cell types. The majority of RNA that circulates in the plasma is presumably of hematologic or endothelial cell origin, and the degree to which other tissues contribute is difficult to estimate. Studying the degree of variation of circulating miRNA molecules from the canonical sequence (the so-called isomiR profile) could allow an estimation of relative contributions of its tissue of origin (Williams et al., 2013). Although CSF is a relatively closed system, the cellular subpopulation of origin of CSF vesicles is also heterogenous, comprising vesicles derived from oligodendrocytes (Scolding et al., 1989), microglia, and macrophages (Verderio et al., 2012) as well as neurons (Saman et al., 2012).

Rapid progress is currently being made in the relatively new field of extracellular RNA isolation and profiling. Body fluids such as blood or CSF are thus likely to be a rich future source of small RNA biomarkers for CNS disease (**Figure 1**).

CURRENT microRNA DETECTION AND ANALYSIS TECHNOLOGIES

CNS biomarker studies have employed RNA from several different sources, and the decision about choice of source RNA involves

several factors. Using whole blood, serum, or plasma is clearly a minimally invasive approach and for those trying to develop or test a biomarker, these samples are probably easiest to access from registries or biological material repositories. Moreover, for ultimate clinical use, an accurate blood-based biomarker would be highly valuable. On the other hand, the presence of the blood-CSF barrier makes it likely that molecular entities isolated directly from CSF are more accurate reflections of brain physiological and pathological processes. Thus, RNA from CSF could be a more sensitive marker of changes that are diluted when trying to detect them in peripheral tissue. Using non-coding RNA as a molecular marker for disease involves several steps: The RNA must be isolated from the source and purified, enriched, or amplified before it is quantified, analyzed, and connected back to biological function. At each step of the process a formidable array of alternatives exists, and technologies in this field continue to evolve rapidly.

EXTRACELLULAR RNA ISOLATION METHODS

RNA can be extracted from extracellular vesicles with relative ease, using one of several methods. The most commonly used isolation methods employ commercial kits based on a combination of a lysis step and column precipitation. Guanidinium thiocyanate-phenol-chloroform extraction is also effective, either by itself or in combination with a column. Most methods result in high quality and pure RNA, equally compatible with most downstream applications. However, each method results in a different RNA yield, in terms of quantity as well as RNA size profile (Eldh et al., 2012). One possible reason for that is that all the current vesicular isolation methods yield a heterogenous mixture of vesicles that vary in intracellular source (cell membrane vs. endosomal), RNA content, and lipid membrane composition. The difference in membrane composition likely translates to a difference in susceptibility to lysis, as different buffers are likely to target vesicle subpopulations with varying degrees of efficacy. Moreover, some of the commercially available methods are specifically designed to enrich small RNA species, while others are non-selective. The outcome is that the RNA population used for biomarker studies depends heavily on the RNA extraction method employed.

These differences in isolated RNA species are even wider when RNA is isolated directly from serum, plasma, CSF, or other biological fluids. The miRNA content is likely to include protein and lipid-complex associated free RNA in addition to vesicular RNA. A comparison of RNA extraction methods used directly on plasma and CSF showed large differences in yield (Burgos et al., 2013). The degree of variation in RNA size profile and content is not clear.

RNA can also be isolated from whole blood using commercially available tubes designed for the purpose. A comparison of 2 commercial kits using proprietary lysis reagents for direct RNA isolation from peripheral blood found that the overlap between the results obtained (in terms of gene expression changes) could be as low as 46% (Menke et al., 2012); this effect is particularly pronounced when the fold change in gene expression is small (Asare et al., 2008).

miRNA DETECTION/QUANTIFICATION

One step in miRNA detection is the sensitivity and accuracy of the technologies employed in their detection. In the case of small RNAs, there is a number of methods, from classical Northern Blotting to microarrays (Cissell and Deo, 2009; de Planell-Saguer and Rodicio, 2011). But if there is one technology that has allowed the leap in this field, it has been NGS. Although there has been great development in the techniques for small RNA detection and quantification, it was really the implementation of small RNA sequencing (small RNASeq) that made the difference in our knowledge of these molecules. In fact, the number of novel miRNAs has started growing exponentially since the implementation of small RNASeq sequencing (http://www.dddmag.com/articles/2012/12/starting-small). Techniques previously used to probe the cellular small RNAome are diverse and each of them has unique advantages and disadvantages to it, mainly associated with (1) whether detection is done in solid state or in solution and (2) whether or not previous knowledge of the target molecules is required [reviewed in Cissell and Deo (2009), de Planell-Saguer and Rodicio (2011)]. Briefly, solid-based technologies are more amenable to high-throughput strategies but are generally more time-consuming and have a difficult application *in vivo*, whereas solution-based techniques give much faster output and can be used *in vivo* but miss the global picture (Cissell and Deo, 2009; de Planell-Saguer and Rodicio, 2011). But arguably the currently hottest technique used for small RNA detection is small RNA sequencing. In this approach, total RNA is extracted and a size selection step ensures enrichment for small RNAs (18–22 nt in size). After adapter ligation, these are then subjected to sequencing, resulting in millions of reads that represent the abundance of each small RNA/miRNA molecule in the sample [although the degree of correlation between the actual abundance and read count is not free of debate (Linsen et al., 2009)]. This approach expands the dynamic range of signal for small RNA detection massively and provides unbiased interrogation of all known and unknown small RNA species without prior knowledge of the target, thereby virtually overcoming the limitations of all the other available technologies. If anything, one of the major limitations for the end-user of small RNASeq is the analysis (see following section).

As sequencing technologies continue to evolve rapidly while becoming more and more accessible to researchers, this method has taken over by far as the golden standard for small RNA expression analysis and novel discovery. It has been successfully used to model brain development (Yao et al., 2012), to characterize different mammalian tissues (Landgraf et al., 2007), and to study and develop biomarkers for different kinds of cancer (Moore et al., 2013), to name a few examples. Furthermore, one of the earliest studies to apply genome-wide small RNA profiling in neurons lead to the discovery of miR34c as a potential biomarker and a therapeutic target for Alzheimers's disease (Zovoilis et al., 2011). Additionally, because sequencing does not depend on previous target knowledge, there are more and more studies uncovering novel miRNAs and other small RNA species in the brain (Jacquier, 2009; Lee et al., 2009; Ling et al., 2011; Inukai et al., 2012). Naturally, sequencing-based approaches do entail some limitations. In addition to the still relatively

complex analysis, the major disadvantages relate mainly to scalability and input material requirements. One of the steps in sample preparation is PCR amplification. It is a well-known source of biases and, if overdone, can cause excessive duplication levels, which leads to information loss during the analysis. Although the amount of input material is generally not problematic in most model system approaches, when dealing with human tissue, and, in particular, in the field of biomarker development, where sample access is limited (i.e., in the case of blood or cerebrospinal fluid), the ability to scale down starting material requirements is critical. The field of small RNASeq is still under heavy development and there is reason to believe that downscaling can indeed be achieved with high fidelity, at least pertaining to miRNA detection (authors' unpublished data). As sequencing technologies continue to develop, we will be able to detect small RNAs from very low amounts of starting biological material.

DATA ANALYSIS AND PATTERN DISCOVERY

RNA-Seq data analysis entails serial steps including quality control, alignment to reference genome, read quantification (read counting), and statistical comparison of conditions of interests (Pepke et al., 2009). A comprehensive review of the method is out of the scope of this article, but it is worth mentioning that in the case of small RNAs, there are some additional considerations to be made. Because of the short length of target molecules, sequencers will read into the adapter primers used during the library preparation. These sequences have to be trimmed before alignment, since they would otherwise interfere with this step. The alignment step itself is also distinctive from the approach generally taken for RNA-Seq. Although alignment to the genome is possible, most current strategies take a hierarchical approach in which reads are serially aligned to different databases of small RNA species. After alignment, read counting and differential expression analysis can be carried out using standard procedures as those used in RNASeq (Pepke et al., 2009). Although the analytical procedure for small RNASeq is still under development, a number of publicly available tools exist that deal with the most standard approaches [the pros and cons of some of which are reviewed in Zhou et al. (2011)].

As small RNA studies evolve from investigation of single candidates to global transcriptional profiling, novel methods of analysis need to be adopted to interpret the large amounts of data generated. When targeted approaches are used, investigators typically use *p*-values or *p*-values corrected for multiple testing. With larger datasets, where differential expression analysis is the norm, filtering, and normalization is often of critical importance. These data also lend themselves very well to machine learning approaches, which have already been used in miRNA biomarker studies for multiple sclerosis and glioblastoma (Roth et al., 2011; Noerholm et al., 2012).

In biomarker research, the most commonly used unsupervised learning approaches are clustering and principle component analysis (PCA), typically used to detect a feature pattern without prior knowledge about sample grouping. In situations where the RNA profiles of the groups under comparison exhibit a high level of dissimilarity, they cluster into distinct groups by an unsupervised clustering algorithm. Alternatively, a "modified unsupervised clustering" where clustering is performed after feature selection may also be used (Noerholm et al., 2012). In most studies, the differences in RNA expression profiles are often subtle, requiring selection of candidates followed by application of supervised machine learning algorithms. Optimally applied, supervised machine learning algorithms such as support vector machines (the most popular so far in RNA biomarker studies), random forests, or artificial neural networks are trained to make classifications based on selected features and then tested on an independent data set to estimate prediction accuracy. However, flawed application of these specialized analysis techniques can lead to reporting of falsely high accuracy rates, hindering reproducibility.

For biomarkers to be used in the clinical setting, they should be applicable (with a certain margin of error) to a single individual. Therefore, predictions of sensitivity, specificity, and accuracy are often more useful than estimates of significant differences between patient and control groups.

LANDMARK CNS BIOMARKER WORK

Blood cells, plasma, and CSF have all been used as starting material to develop miRNA biomarkers for CNS malignancies as well as neurodegenerative and other neurological diseases. One of the first studies to compare miRNA profiles from blood mononuclear cells between patient and control populations showed mir-34a and mir 181b to be upregulated in mononuclear cells from the blood of patients with Alzheimer's disease. In addition, gender and APOE4 status were also found to influence the PBMC miRNA profiles within the group of AD patients (Schipper et al., 2007). This approach has since been used to identify potential biomarkers for other CNS diseases such as multiple sclerosis, schizophrenia (Lai et al., 2011; Gardiner et al., 2012), Parkinson's disease (Martins et al., 2011; Soreq et al., 2013), and amyotrophic lateral sclerosis (De Felice et al., 2012). For multiple sclerosis in particular, a large number of studies exist that profile miRNA in peripheral blood immune cells (Keller et al., 2009; Cox et al., 2010; De Santis et al., 2010; Lindberg et al., 2010; Martinelli-Boneschi et al., 2012).

Plasma and serum have also been investigated as a source of miRNA biomarkers for multiple sclerosis (Siegel et al., 2012). Cerebrospinal fluid miRNA has been studied in Alzheimer's disease (Cogswell et al., 2008), multiple sclerosis (Haghikia et al., 2012), and to a larger extent in glioblastoma (Baraniskin et al., 2012; Teplyuk et al., 2012). A single study of miRNA in pooled CSF microparticles from patients with neurotrauma showed that the contents of CSF could also be useful in diagnosing brain injury (Patz et al., 2013) (**Table 1**). Among the CNS malignancies, a variety of starting biological materials has been used; the majority of studies investigate samples from patients with glioblastoma, probably because drawing CSF pre and post-operatively is routine procedure in glioblastoma diagnosis. (Roth et al., 2011; Baraniskin et al., 2012; Ilhan-Mutlu et al., 2012; Teplyuk et al., 2012; Wang et al., 2012), and a single study of patients with astrocytoma (Yang et al., 2013) (**Table 1**).

Over the last year there has been a sharp increase in published studies about circulating microRNA as biomarkers for various

Table 1 | Summary of microRNA biomarker studies for central nervous system diseases.

Disease	Patient population	Biological Material	RNA isolation and detection		Statistical Analysis		Results	Reference
			Isolation	Detection	Quantification and statistics	Prediction and accuracy estimation		
Glioma	10 patients with Glioma versus 10 controls with other neurological diorders, primary diffuse large B-cell lymphoma of the CNS (PCNSL), brain metastases	CSF	miRVAna RNA kit(Ambion)	qPCR	Mann-Whitney U tests and Kruskal-Wallis tests with Dunn's multiple comparison, Kruskal-Wallis tests with Dunn's multiple comparison	ROC analysis and decision trees	MiR-15b and miR-21 were differentially expressed in CSF samples from patients with gliomas	Baraniskin et al., 2012
Glioblastoma	10 Patients with glioblastoma versus 50 patients with other brain malignancies versus 10 healthy controls	Plasma	miRcute miRNA isolation kit	qPCR	Mann–Whitney test	ROC curves	miR-21, miR-128 and miR-342-3p were significantly altered in gliomas and in glioblastoma multiforme	Wang et al., 2012
Astrocytoma	122 Patients with astrocytoma grades II-IV and 123 healthy controls	Serum	Trizol	Solexa sequencing, followed by qPCR validation in an independent cohort	Student's *t*-test and ANOVA	ROC curves for each miroRNA and for the group of microRNAs	Seven miRNAs including has-miR-15b*, -23a, -133a, -150*, -197, -497 and -548b-5p significantly decreased in the serum of patient with grade II-IV astrocytoma	Yang et al., 2013
Glioblastoma	20 patients Glioblastoma versus 20 healthy controls	cellular fraction of whole blood	miRNeasy Mini Kit (Qiagen)	Microarray	Unpaired two-tailed parametric t-test. Pvalues obtained for each individual miRNA were adjusted for,multiple testing by Benjamini–Hochberg	Support vector machines algorithm	52 miRNAs differentially regulated	Roth et al., 2011
Glioblastoma	10 patients with glioblastoma and 10 healthy volunteers	Plasma	Exiqon microRNA isolation protocol	Taqman qPCR	Mann-Whitney U test and Paired t test	None	MicroRNA-21 is raised in the plasma of patients with glioblastoma and decreases significantly after surgical tumor removal	Ilhan-Mutlu et al., 2012
Glioblastoma and brain metastases	19 Patients with glioblastoma versus 74 patients with brain metastases and 15 controls with non-neoplastic brain conditions	CSF	miRVAna RNA kit(Ambion)	Taqman qPCR	Wilcoxon signed rank test	Support vector machines algorithm	MiR-10b Is Present and MiR-21 Is Elevated in CSF of Glioblastoma and Brain Metastasis Patients,MiR-200 Family in the CSF Is Indicative of Brain Metastasis	Teplyuk et al., 2012
Parkinson's disease	19 Parkinson's disease patients and healthy controls	PBMCs	miRNeasy Mini Kit (Qiagen)	miRCURYTM LNA microarrays, validated by qPCR	Differential expression analysis, Combined with al.pha synuclein CHiP-Seq for pathway analysis,miR-30b, miR-30c and miR-26a emerged as key modulators	None	18 miRNAs differentially expressed,	Martins et al., 2011
Parkinson's disease	7 patients Parkinson's disease before and after deep brain stimulation versus 6 healthy controls	leukocytes	Leukolock RNA isolation system(Ambion)	SOLiD RNA sequencing	Differential expression analysis, Followed by combinatorial analysis with splice-junction and exon arrays to generate a miRNA-spliced target disease netowrk		16 microRNAs differentialy expressed in patients versus controls, 11 microRNAs changed after DBS, 5 of these overlapped(reversal of miRNA pattern to healthy after DBS)	Soreq et al., 2013
Alzheimer's disease	6 AD patients (Braaks stage 5) and 9 non-demented controls (Braaks stage 1)	CSF	Proprietary galss-fiber based methods(Asurage n)	Taqman qPCR array	Between Groups Analysis, t test	None	Sixty miRNAs differentially expressed between early AD and advanced AD, including all members of the miR-30 family	Cogswell et al., 2008
Alzheimer's disease	20 AD patients versus 22 controls	CSF	miRCURY kit for biofluids	Taqman qPCR	Differential expression, two-tailed t test	ROC curves	Has-miR-146a decreased in the CSF of AD patients	Müller et al., 2014

(Continued)

Table 1 | Continued

Alzheimer's disease	6 AD patients versus 6 controls	CSF	TRIzol reagent (Invitrogen) and/or mirVana RNA kit (Ambion)	MicroRNA array confirmed by LED-Northern dot blot	Analysis of Variance (ANOVA)	None	Hsa-miR-9, -125b, -146a, -155, -34a and -28 higher in AD than in controls	Alexandrov et al., 2012
Alzheimer's disease	94 AD Patients,21 healthy controls and 72 patients with other neurological diseases. Controls and non-AD patients were from an independent center.	Whole blood	Carlsbad CA) and/or an Ambion mirVana RNA	RNA sequencing partially validated by qPCR	Wilcoxon-Mann-Whitney test followed by correction for multiple testing by Benjamini-Hochberg adjustment	Radial basis function support vector machines,ROC curves	12-miRNA signature (hsa-let-7f-5p,let-7d-3p, -miR-1285-5p,-107, -103a-3p,- 26b-5p, -26a-5p, -532-5p, -151a-3p, -161, -112, -5010-3p)	Leidinger et al., 2013
Alzheimer's disease	2 independent cohorts (11 AD, 9 MCI and 20 healthy controls) and (20 AD and 17 healthy controls)	Plasma	isolation kit	Nanostring nCounter miRNA expression assay, validated by qPCR	Fold change and differential expression analysis	Linear discriminant analysis, individual and group microRNA ROC curves in independent cohort	7-miRNA signature (hsa-let-7d-5p, let-7g-5p,miR-15b-5p, -142-3p, -191-5p, -301a-3p and -545-3p)	Kumar et al,. 2013
Alzeimer's disease and mild cognitive impairment	7 Patients with 'probable AD', 7 patients with mild cognitive impairment and 7 cognitively normal controls	Serum	miRNeasy Mini Kit (Qiagen) and RNeasy MinElute Cleanup Kit (Qiagen)	miScript SYBR Green PCR	Differential expression, 2 tailed t tests and Mann-Whitney test	None	Hsa-miR-137, -181c, -9, -29a and -29b were downregulated in both AD and mild cognitive impairment when compared to controls	Geekiyanage et al. 2012
Alzheimer's disease	16 AD patients versus q6 controls	PBMCs	Trizol with RNeasy Mini columns	Microarray with qPCR validation	Significance Analysis of Microarrays	None	Hsa-miR-34a and -181b higher in AD	Schipper et al.., 2007
Alzeimer's disease and mild cognitive impairment	20 AD patients, 20 patient with mild cognitive impairment and 20 cognitively normal controls	Plasma	MirVana Paris kit (Ambion) and/or prprietary glass-fiber-based method (Asuragen)	Taqman qPCR	Mann-Whitney U-tests of MicroRNA-pair ratios compared in the 3 groups	ROC curves for miRNA pairs	Two sets of miRNA pairs,(hsa-miR-128/-491-5p, -132/-491-5p and -874/-491-5p) and (hsa-miR-134/-370, -323-3p/-370 and -382/-370) differentiate MCI and AD from controls but not from each other	Sheimerman et al., 2012
Multiple Sclerosis	20 patients with relapsing-remitting MS (RRMS) versus healthy controls	cellular fraction of whole blood	miRNeasy Mini Kit (Qiagen)	Microarray	t tests with Benjamini-Hochberg correction for multiple testing	SVM	165 miRNAs differentially regulated, hsa-miR-145 emerged as the best single differentiating microRNA	Keller et al., 2009
Multiple Sclerosis	53 patients with MS versus 39 patient with other neurological diseases	CSF	miRNeasy Mini Kit (Qiagen)	qPCR array,BioCat, confirmed by qPCR	Mann-Whitney U tests	ROC curves	Hsa-miR-922, -181c , and -633 differentially regulated in MS , -181c and -633 could differentiatee relapsing-remitting from secondary progressive MS	Haghikia et al., 2012
Multiple Sclerosis	4 MS patients and 4 healthy controls	plasma	MirVana Paris kit (Ambion)	Microarray	T test	None	six plasma miRNAs (has-miR-614, -572, -648, -1826, -422a and -22) that were significantly up-regulated and one plasma miRNA (miR-1979) that was significantly downregulated in MS individuals.	Siegel et al.., 2012
Multiple Sclerosis	19 MS patients and 14 controls	PBMCs	Trizol reagent	Illumina® Beadarray with qPCR validation	Discovery sample and verified in replication sample, Wilcoxon rank sum test and one way Anova test, including Holm–Sidak for multiple comparisons	None	104 miRNAs deregulated, of which let-7g and miR-150 confirmed by qPCR Combined with mRNA expression anaysis	Martinelli-Boneschi et al., 2012
Multiple Sclerosis	8 relapsing–remitting MS patients and 10 healthy, age- and gender-matched volunteers, 15 additional patients and 10 healthy volunteers were included into the validation cohort.	CD41 lymphocytes	miRNeasy Mini Kit (Qiagen)	TaqMan Array, validated by taqmn qPCR in a separate cohort	Differential expression analysis	None	Ten, four and six differentially expressed miRNA in CD4+, CD8+ and B-lymphocytes, respectively, of MS compared with HV, miR-17-5p upregulated in MS patients confirmed in validation set	Lindberg et al., 2010

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Table 1 | Continued

Multiple Sclerosis	12 Relapsing-remitting MS patients versus 14 healthy controls, validated in a separate cohort	regulatory T cells	TRIzol® Plus RNA purification Kit	Agilent Human miRNA microarray	Differential expression analysis followed by the application of the Benjamini and Hoechberg correction	None	23 human miRNAs differentially expressed between CD4+CD25high bona fide Treg cells from MS patients vs. healthy donors	De Santis et al., 2010
Multiple Sclerosis	59 MS patients and 37 controls	Whole blood	Paxgene colelction followed by trizol isolation	Illumina sentrix array matrix, microarray	Significance Analysis of Microarray	None	26 were down-regulated and 1 up-regulated in MS whole blood, miR-17 and miR-20a most significantly different	Cox et al.., 2010
Multiple Sclerosis	Patients with relapsing-remitting multiple sclerosis and controls	PBMCs, plasma and serum	Nucelospin miRNA kit (Macherey-Nagel)	miRCURY microarray validated by qPCR	Microarray analzyed by test for differential expresion, qPCR analyzed by Mann-Whitney-U test	ROC curves	has-mIR-145 was 3-foldupregulated in MS patients	Søndergaard et al., 2013
Multiple Sclerosis	15 MS patients and 12 Controls	PBMCs and serum	MirVana Paris kit (Ambion)	Taqman qPCR	Student's t test	None	Levels of hsa-miR-223 and -23a were significantly altered in PBMCs and serum of MS patients	Ridolfi et al.2013
Amyotrophic lateral sclerosis	8 sALS patients and 10 healthy controls with an independent validation cohort(14+14)	leukocytes	Trizol reagent	microarray	Differential expression analysis of microarray	None	8 miRNAs that were significantly up- or downregulated in sALS patients	De Felice et al.., 2012
Schizophrenia	Learning set of 30 cases and 30 controls,validated in an independent testing set of 60 cases and 30 controls	PBMCs	Trizol reagent	microarray with qPCR validation	Wilcoxon rank-sum test , followed by stepwise logistic regression analysis	Logistic regression analysis, SVM, ROC curve	seven-miRNA signature (hsa-miR-34a,-449a, -564, -432, -548d, -572 and -652) was derived	Lai et al., 2011
Schizophrenia	112 schizophrenia patients and 76 controls with no psychiatric illnesses	PBMCs	Trizol reagent	microarray platform (Illumina), validated by qPCR	Significance analysis of microarray	None	Set of deregulated microRNAs originating from a single imprinted locus at the maternally expressed DLK1-DIO3 region on chromosome 14q32	Gardiner et al., 2012
Manic episodes. bipolar disorder	21 patients and 21 controls	Plasma	Acid phenol:chloroform extraction	Taqman qPCR	ANOVA test with Tukey post-test).	None	Hsa-miR-134 is decreased in patients with a manic episode and levels go back to normal in patients treated for 4 weeks.	Rong et al., 2011
Depression	40 Patients with depression and 40 healthy controls	Serum	extraction	SYBR green qPCR	Wilcoxon rank sum test	None	Hsa-miR-132 and -182 are raised in patients with depression	Li et al., 2013

neurological diseases. Many of these used unbiased, genome-wide profiling approaches to compare patients with controls and derive. For Alzheimer's disease alone there are now a total of 5 published studies from various blood fractions and 3 from CSF. While these individual studies report high accuracy rates, and some of them include large numbers of patients, it is curious that their results do not match or even overlap with each other. The blood studies all used different fractions of blood and comparisons are perhaps unrealistic, but the CSF studies also showed differing results. For example, hsa-miR-146a is reported in one of the 3 studies to be upregulated in AD (Alexandrov et al., 2012), in a second study to be downregulated (Müller et al., 2014), while the third shows no effect on it at all, reporting a downregulation of hsa-miR-146b instead (Cogswell et al., 2008) (**Table 1**). Perhaps in the future, a larger number of studies and their metaanalysis would shed more light on which non-coding RNAs are truly useful biomarkers of disease.

FROM BIOMARKERS TO FUNCTION

Although several classes of non-coding RNA have been discovered (Taft et al., 2010), miRNAs are the most extensively characterized. Computational tools that predict miRNA targets are quite frequently used to ascribe function to putative miRNA biomarkers. Since miRNAs and the genes they target are expressed in a tissue- and pathology-specific manner, predicted targets usually require experimental confirmation. Tools that combine prediction algorithms with large scale wet lab experimental methods such as polysome profiling, immunoprecipitation of members of the RISC complex or degradome sequencing are likely to provide more specific results (Thomson et al., 2011). Since the publication of a miRNA mRNA map based on argonaute HITS-CLIP data from the brain (Chi et al., 2009), more specific predictions are also available.

As our understanding of non-coding RNA biology develops, we see that miRNAs are evolutionarily conserved across species but have overlapping targets and are often functionally redundant. While landmark advances have been made toward understanding the role of single miRNAs in the CNS (Kim et al., 2007; Rajasethupathy et al., 2009; Edbauer et al., 2010; Zovoilis et al., 2011), we see a gradual shift from studying the single-miRNA-target interaction toward viewing these critical regulators as part of a network, tuning or buffering key gene regulation node (Zhang and Su, 2009).

Clearly, miRNAs exert their influence on biological pathways in concert with transcription factors and other modulators of gene expression. A few of the more recent biomarker studies attempt to view the larger picture by concurrently profiling miRNA expression, gene expression, and protein-DNA interaction. In particular, researchers studying biomarkers for Parkinson's disease have pioneered these analyses by combining miRNA expression with tissue-specific gene isoform expression (Soreq et al., 2013) or data from ChIP-sequencing data with miRNA target prediction (Martins et al., 2011) to build a picture of the regulatory network in health vs. disease.

Biomarkers are ultimately validated when they can be connected with molecular mechanisms across different levels of biological complexity. A systems biology approach could achieve this by integrating data, where it is available, across different levels such as genes, molecules, phenotypes, cell, and tissues. Various computational tools are available to integrate these data types and more are being developed (Villoslada and Baranzini, 2012). Simple, readily available and widely used methods to link a set of differentially expressed genes with biological processes or pathways include gene ontology term search and *gene set enrichment analysis*. The availability of large and complex data sets and computing power has spurred rapid advances in network biology.

Moreover, RNA data can be analyzed in combination with patient information, disease history, genomic data like APOE4 allele, disease-specific clinical tests like MEP (motor-evoked potential for MS or mini-mental state examination for dementia), and data from proteomics and other high throughput approaches. Proteomics-based biomarkers for neurodegenerative and other neurological diseases have been studied and new avenues for biomarker discovery such as metabolomics continue to emerge; an LC/MS based approach (Trushina et al., 2013) to study the metabolic profiles of CSF and plasma from AD patients found around 150 metabolites each in CSF and plasma that were significantly different in patients with Alzheimer's disease or patients with mild cognitive impairment (MCI) than healthy individuals, allowing them to identify putative pathways that may be altered (Trushina et al., 2013). These kinds of data could lend themselves to a combinatorial analysis provided that patient information and other variables are fully documented and available.

CURRENT LIMITATIONS AND FUTURE MILESTONES OF miRNA-BASED BIOMARKER DISCOVERY

The use of non-coding RNA and miRNA in particular has gained significant attention since the discovery that these RNA species can be detected extra- and intracellularly in peripheral tissue. The growing use of powerful detection methods such as massive sequencing has given a significant boost to the search for minimally invasive disease indicator. In addition, the discovery of the existence of free or exosomal circulating RNA in blood and CSF has also fostered research in this direction. Although this is still a relatively young field, it is rapidly evolving and promises great advances in the field of biomarker discovery, especially for nervous system pathology. The CNS is the least accessible of all tissues and would therefore greatly benefit from advances in this field. Current limitations to this approach include those inherently associated with biomarker discovery (i.e., working with material from different sources, extraction methods, patient history, etc.), as well as those specifically associated with sequencing-based detection methods and extraction strategies.

As is often the case when working with human tissue, samples from different sources show wide variability in profile as a result of handling, sample preparation and preservation. These are especially pronounced when a highly sensitive technique like sequencing is used. In addition, because the source of tissue are primarily human patients that may be on medication, proper consideration of these (potentially confounding) cofactors is essential, as medication pursues restoration of the biological balance and this may include alterations in the molecule of interest. When RNA profiles are altered after drug treatment, it can be a challenge to dissect the direct effects of treatment on RNA expression from

those connected with disease remission (Rong et al., 2011). An analysis of highly cited (more than 400 citations) biomarker publications (including protein, genetic, and other blood biomarkers) showed that individual studies usually report high association between the marker and disease outcome; however when the same biomarker is subsequently compared with larger studies or meta-analyses, the effect size is often significantly smaller than initially believed (Ioannidis and Panagiotou, 2011).

Another issue inherently associated with large human studies and generally with studies handling big datasets is information availability and reproducibility. As is known from the field of microarrays, data is often incomplete or incompletely annotated and the analyses hard to reproduce (Ioannidis et al., 2009) and this is still an issue in the field of small RNA-based biomarker development (Ioannidis et al., 2009).

In addition to these limitations, there is also those specifically associated with the extraction and quantification methods used for peripheral miRNA detection. As already mentioned in section Current microRNA Detection and Analysis Technologies, a variety of extraction techniques exist, each with specific biases that can greatly influence the relative weight of a certain molecular species in the sample. In addition, because the technology is rapidly evolving, there is still no clear-cut consensus as to what is the best approach to analyze large-scale small RNA profiles. These issues will settle with time, as techniques become more robust and analysis methods stabilize, but until then, they are to be carefully considered in the experimental design.

Finally, as already mentioned, there is the issue of how faithful the peripheral profile is to the original biological situation in the CNS. Although this is not most critical for biomarker discovery *per se* (as mentioned above, a biomarker can be simply defined as a "handle" that allows detection of a remote biological process and does not necessarily need to correlate with it), often studies strive to uncover molecules that can serve as a biomarker *and* be used as therapeutic targets. Evidence from PBMCs indicates that there is indeed a considerable coherence between the central neuronal response and the peripheral response in blood and that there is a cross-talk between these two tissues. It remains to be experimentally established whether this correlation can also serve to better understand neuronal physiology in the healthy and the disease situation. In this respect, the development of novel, unbiased technologies to detect even the smallest amounts of miRNAs peripherally in combination with studies in model systems has proven critical.

All in all, despite current limitations, miRNA-based biomarkers constitute an exciting field in biomedical research. For neuroscience, where the search for remotely accessible markers to understand the brain is essential for human studies, the field has elicited considerable interest and as the costs of NGS continue to decrease, it is likely to become a routine approach to generate individual patient profiles and allow targeted therapeutic intervention.

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New roles for "old" microRNAs in nervous system function and disease

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Since their discovery, microRNAs became prominent candidates providing missing links on how to explain the developmental and phenotypical variation within one species or among different species. In addition, microRNAs were implicated in diseases such as neurodegeneration and cancer. More recently, the regulation of animal behavior was shown to be influenced by microRNAs. In spite of their numerous functions, only a few microRNAs were discovered by using classic genetic approaches. Due to the very mild or redundant phenotypes of most microRNAs or their genomic location within introns of other genes many regulatory microRNAs were missed. In this review, we focus on three microRNAs first identified in a forward genetic screen in invertebrates for their essential function in animal development, namely *bantam*, *let-7*, and *miR-279*. All three are essential for survival, are not located in introns of other genes, and are highly conserved among species. We highlight their important functions in the nervous system and discuss their emerging roles, especially during nervous system disease and behavior.

Keywords: *let-7, bantam, miR-279,* nervous system, development, behavior, regeneration, degeneration

INTRODUCTION

The discovery of microRNAs was a big step towards the understanding of post-transcriptional regulation of gene expression. Several hundreds of microRNAs capable of interacting with a plethora of target mRNAs were discovered in model organisms. While sequence based prediction tools of microRNA-target interaction evolved quickly, phenotypical analysis of microRNA function lagged behind, in part due to the lack of clear phenotypes in mutants of single microRNAs. In addition, phenotypes were expressed only under certain environmental and experimental conditions suggesting that microRNAs act predominately by fine-tuning gene expression. As a consequence, microRNAs were rarely discovered in genetic screens. However, three microRNAs were found in genetic forward screens in invertebrates. All three are essential for survival, are not located in introns of other genes, and are highly conserved among species.

INITIAL DISCOVERY OF *let-7, bantam,* AND *miR-279*

Let-7 was the first microRNA described in *C. elegans*. It is conserved across different species and found in all common model organisms. In *C. elegans let-7* participates in the so-called heterochronic pathway, which regulates the transition between different developmental stages in the worm by timing the division and differentiation of stem cells. *Let-7* is upregulated in the last larval stage (L4), and by downregulating *lin-41* mRNA, allows the animal to fully mature. Weak mutant alleles of *let-7* lead to a reiteration of larval patterns of cell division, and the animal fails to differentiate. Strong alleles of *let-7* mutants cause a severe phenotype of a bursting vulva (Reinhart et al., 2000). The second example, *bantam*, has no mammalian homolog and has been intensively studied in *Drosophila*. Mutations of this microRNA affect the proliferation of the wing disk and lead to failure in the G1-S transition of wing disk cells (Brennecke et al., 2003; Herranz et al., 2008). The third microRNA, *miR-279*, is highly conserved in insects. The first phenotype described was found in the olfactory system of *Drosophila*. In this system, *miR-279* regulates the differentiation of a subclass of olfactory receptor neurons by downregulating two transcription factors, Nerfin-1 and Escargot (Cayirlioglu et al., 2008; Hartl et al., 2011).

Here, we review recent advances in the understanding of the function of *let-7*, *bantam*, and *miR-279* in neural development, regeneration and degeneration, and behavior.

Let-7 REGULATES CELLULAR DIFFERENTIATION OF THE NMJ AND OTHER BRAIN STRUCTURES

In silico analysis predicted a strong interaction of *let-7* with the transcription factor Abrupt. Recent studies verified this microRNA-target relationship in the neuromuscular junction (NMJ) and the mushroom body (MB) in *Drosophila*. In both tissues, the microRNA controls the developmental transition to an adult shape. When missing, the NMJ retains a juvenile shape and is not able to fully differentiate (Sokol et al., 2008). Although no anatomical phenotype was detected, *let-7* mutant flies show defects in locomotion, flight, and also fertility (Sokol et al., 2008). The authors could show that the phenotype is accompanied by increased levels of the broad-complex, tramtrack, and bric-a-brac (BTB) transcription factor Abrupt in *let-7* mutants (Caygill and Johnston, 2008) corroborating a previous finding that Abrupt ensures the remodeling of the larval NMJ to achieve its adult shape and function (Hu et al., 1995).

Two other recent studies in *Drosophila* show that the *let-7* complex (*let-7-C*) is a key regulator of the development of the MB (Kucherenko et al., 2012; Wu et al., 2012). *Let-7-C* gives rise to three different microRNAs, namely (*let-7, miR-100,* and *miR-125*), which can act individually but also synergistically on mRNA regulation. The *Drosophila* MB is a complex brain structure essential for olfactory learning and memory as well as context-dependent innate behavior (Heisenberg, 2003; Fiala, 2008; Bracker et al., 2013). It is comprised of Kenyon cells (KCs) that can be further classified into four different subtypes. During development, they are derived from multi-potent progenitor cells and are born in a fixed order (γ → α′/β′ → pioneer α/β → α/β; Zhu et al., 2003, 2006). Two factors guiding the precise timing of MB neuron subtypes are the transcription factors, "Chronologically inappropriate morphogenesis" (Chinmo) and Abrupt (Zhu et al., 2006; Kucherenko et al., 2012). Chinmo affects the differentiation of MB subtypes in a concentration dependent manner. While high levels of Chinmo in post-mitotic neurons specify γ and α′/β′, low levels of Chinmo drive the differentiation of late-born MB neurons (pioneer α/β, α/β; Zhu et al., 2006). In order to generate different levels of Chinmo throughout MB development, *let-7* and *miR-125* co-transcribed from the *let-7-C locus,* contribute to the progressive downregulation of *chinmo in vivo*. *Let-7* and *miR-125* regulate *chinmo* expression directly via binding sites in the 3′UTR of the transcription factor. The third microRNA of the *let-7-C, mir-100*, seems not to be involved in the post-transcriptional regulation (Wu et al., 2012). All *let-7-C* microRNAs are strongly upregulated in the transition from the late pupal to early adult stage. *In vivo*, precocious expression of *let-7* and *miR-125* in larval stage 1 leads to a sharp decrease of Chinmo levels already in larval stage 3. As a consequence, the adult MB shows strong morphological defects and mis-differentiation of its cell types. The second study revealed that *let-7-C* also influences the timing of α′/β′ to α/β transition via the Chinmo related BTB transcription factor Abrupt (Kucherenko et al., 2012). The differentiation of the late born α/β neurons depends on the expression of *let-7C*. By contrast, Abrupt is essential to establish the identity of α′/β′ neurons. Thus, *let-7-C* mediated downregulation of Abrupt regulates the transition between different subsets of MB neurons. Notably, the expression of *let-7-C* appears to be dependent on Ecdysone signaling, a key regulator for morphological transitions during insect development (Robbins et al., 1968). While both studies describe effects on MB morphology, the effect of the *let-7-C* mutation on MB morphology differs in the two studies. Wu et al. only find delays in the transition towards different MB subtypes. By contrast, Kucherenko et al. detect a significant reduction of α/β lobe volume. Reasons for these phenotypical differences are not known, but may have to do with the role of microRNAs as buffers rather than instructors of gene expression. Therefore different experimental conditions such as nutrition and temperature might influence the severity of the phenotype. Nevertheless, the results of the two publications show that *let-7-C* is used to sharpen the expression of two potent transcription factors in order to produce different neuronal subtypes. While Chinmo has a broader effect on MB development and seems to affect the generation of all MB subtypes, Abrupt only affects the transition of the late born neurons.

MECHANISMS OF *let-7* REGULATION

In order to precisely time the expression of potent transcription factors during development, microRNA expression needs to be tightly regulated in expression. To ensure precise timing of activity for instance during neuronal differentiation, *let-7* interacts with one of its classical targets in an autoregulatory cycle. In embryonic stem (ES) and embryonic carcinoma (EC) cells, the pluripotency factor Lin-28 binds *pre-let-7* and inhibits the last step during *let-7* processing and thereby prevents the formation of a mature microRNA (Rybak et al., 2008). In neural stem cells, *lin-28* is repressed by *let-7* and *miR-125*. This leads to a neural stem cell commitment towards differentiated neurons (Rybak et al., 2008).

The transcription factor SRY (sex-determining region)-box 2 (SOX2) directly binds the Lin-28 promotor and regulates its expression (Cimadamore et al., 2013). Expression and activation of Lin-28 inhibits *let-7* and leads to the maturation of the NPCs, which are derived from human ES cells (Cimadamore et al., 2013). In this context, loss of SOX2 as well as overexpression of *let-7* (specifically of *let-7i*) led to the inhibition of neuronal differentiation (Cimadamore et al., 2013).

Bantam DETERMINES CELLULAR GROWTH IN THE NERVOUS SYSTEM

During development, two processes must be coordinated: first, cells differentiate to obtain their cellular fate and function, and second, the cell number is multiplied forming the basis of growth and proliferation. Recent studies implicate the microRNA *bantam* in glia proliferation in the *Drosophila* brain and optic lobe. *Drosophila* larvae undergo an extreme growth in the third instar stage. Epidermal growth is often linked to the well-studied Hippo pathway. In a recent study, Reddy and Irvine (2011) show an involvement of the pathway in non-epithelial glia cells for the first time. In the Hippo pathway, Merlin acts as the upstream regulator of the core kinase cascade in the Hippo pathway. Merlin depletion or expression of an activated form of Yorkie leads to glia overgrowth in the optic lobe and the brain of *Drosophila*. Similar to the wing disk, Merlin activates the expression of Yorkie and in turn, Yorkie was found to activate the expression of *bantam* (Nolo et al., 2006; Thompson and Cohen, 2006). As a consequence of *bantam*, expression levels of Myc are increased, probably as an indirect effect due to suppression of the ubiquitin ligase Mei-P26 (Herranz et al., 2010). Interestingly, neurons are insensitive to increased levels of *bantam* and hence, glia cells are affected exclusively.

Another study describes a role for *bantam* during the development of another neural structure, the optic lobe. *Bantam* is highly expressed in mitotically active cells in the developing optic lobe. Depletion of *bantam* levels in third instar *Drosophila* larvae leads to smaller optic lobes, whereas overexpression of *bantam* results in an increased volume of optic lobes. The effect of *bantam* is not due to a mis-differentiation of glia cells since even in the full mutant larvae glia cells are present. In this context *bantam* seems to influence the number of glia cells. The authors conclude that *bantam* maintains the pool of stem cells during development and thus influences the proliferation of the cells. In the same study, the T-box transcription factor Omb was identified as downstream target of *bantam* that can also partially rescue the gain-of-function phenotype of the microRNA. The molecular mechanism of how

bantam controls the cell cycle remains to be investigated (Li and Padgett, 2012).

miR-279 AS A MOLECULAR SWITCH OF CO_2 NEURON LOCATION

miR-279 is highly conserved in all insect species, and was first identified in a forward genetic screen for axon guidance and synapse formation factors using the olfactory system of *Drosophila* as a model system (Cayirlioglu et al., 2008). The olfactory system in *Drosophila* consists of two appendages, the antenna and the maxillary palp, which house different sets of olfactory sensory neurons (OSNs). During development each subset of OSNs sends their axons to a defined area, a glomerulus, in the antennal lobe in the brain to generate a spatial representation of odors. In the olfactory system, *miR-279* regulates development and axon targeting of a specific class of sensory neurons. Flies mutant for *miR-279* develop extra CO_2 sensory neurons on the second olfactory appendage of insects, the maxillary palp, in addition to CO_2 neurons found on control antenna (Jones et al., 2007; Kwon et al., 2007). These ectopic CO_2 neurons innervate a glomerulus in the antennal lobe associated with the detection of food cues and not with the detection of CO_2 (Cayirlioglu et al., 2008) The innervation pattern of the ectopic CO_2 neurons highly resembles the location and central brain innervation of mosquito CO_2 neurons (Ghaninia et al., 2007; Lu et al., 2007). Therefore, *miR-279* was proposed as a molecular switch in the divergence of mosquitoes and flies for the trait of CO_2 detection (Cayirlioglu et al., 2008; Jones, 2008). Since flies are highly repelled by CO_2 and mosquitoes strongly attracted to it the study provides a starting point to explore how molecular changes in regulation shape neural circuits and thereby the behavioral output (Benton, 2008; Jones, 2008). Similar to *let-7, miR-279* appears to regulate neuronal commitment and differentiation of progenitor cells (Cayirlioglu et al., 2008; Hartl et al., 2011; **Table 1**). On the mechanistic level, *miR-279* expression is regulated by the pan-neuronal transcription factor Prospero (Hartl et al., 2011), an important player in stem cell progression and sensory neuron development (Doe et al., 1991; Choksi et al., 2006). Two transcription factors both of which are also direct targets of Prospero, the snail transcription factor Escargot and the zinc-finger transcription factor Nerfin-1 were identified as essential and sufficient targets of *miR-279*: gain-of-function of both targets together induced the formation and mistargeting of ectopic CO_2 neurons efficiently (Hartl et al., 2011). Thus, the pan-neural factor Prospero refines its own activity by inducing a microRNA to regulate the expression of its own downstream target genes. Given Prospero's role during stem cell development in both flies and mice a use of the same or similar network is conceivable also in the control of tumor formation in the brain or the lymphatic system (Petrova et al., 2002; Galeeva et al., 2007).

NEURONAL REGENERATION

While aging, the nervous system progressively loses the ability to rapidly regenerate new cells. The decline in regeneration during aging is a conserved phenomenon. In *C. elegans,* the anterior ventral microtubule (AVM) axon is used to study the effect of single molecules on the regeneration of neurons. The regeneration decline in *C. elegans* occurs usually in the larval stage 3 (L3) prior to the transition to young adults. Worms mutant for microRNA biosynthesis factors Dicer-1 or Argonaute Alg-1, however, continue to regenerate the axons of AVM which in turn extend much longer as compared to wildtype controls (Zou et al., 2013). In this context, the microRNA involved and responsible for increased axonal length and regeneration is *let-7* (**Figure 1**). Mutants of *let-7* exhibited the same phenotype in AVM neurons as *alg-1* mutants. The study showed that in order to stop AVM axons from extending, Lin-41 is strongly repressed by *let-7* in late adult stages. Mutants of *lin-41* show a decreased regeneration of the axons. Interestingly, Lin-41 co-immunoprecipitates with Alg-1, which constitutes a key factor for *let-7* biogenesis. The experiments suggest that in

Table 1 | Conserved functions of *let-7, bantam, and miR-279* in the nervous system.

	microRNA	Model organism	Function	Citation
Development	*let-7*	*Drosophila*	Maturation of neuromuscular junction (NMJ)	Caygill and Johnston (2008), Sokol et al. (2008)
			Mushroom body (MB) differentiation	Kucherenko et al. (2012), Wu et al. (2012)
		Human neural precursor cells embryonic stem cells	Pluripotency	Rybak et al. (2008), Cimadamore et al. (2013)
	bantam	*Drosophila*	Glia cell growth in the brain and optic lobe	Reddy and Irvine (2011)
			Differentiation and number of glia cells in the optic lobe	Li and Padgett (2012)
	miR-279	*Drosophila*	CO_2 neuron development	Cayirlioglu et al. (2008), Hartl et al. (2011)
Regeneration	*let-7*	*C. elegans*	AVM neuron axon regeneration	Zou et al. (2013)
		Zebrafish	De-differentiation of Mueller glia cells	Ramachandran et al. (2010)
	bantam	*Drosophila*	Dendritic aborisation (da) neuron regeneration	Song et al. (2012)
Degeneration	*let-7*	Mouse	Loss of cortical neurons through extracellular *let-7*	Lehmann et al. (2012)
Behavior	*miR-279*	*Drosophila*	Regulation of circadian rhythm	Luo and Sehgal (2012)

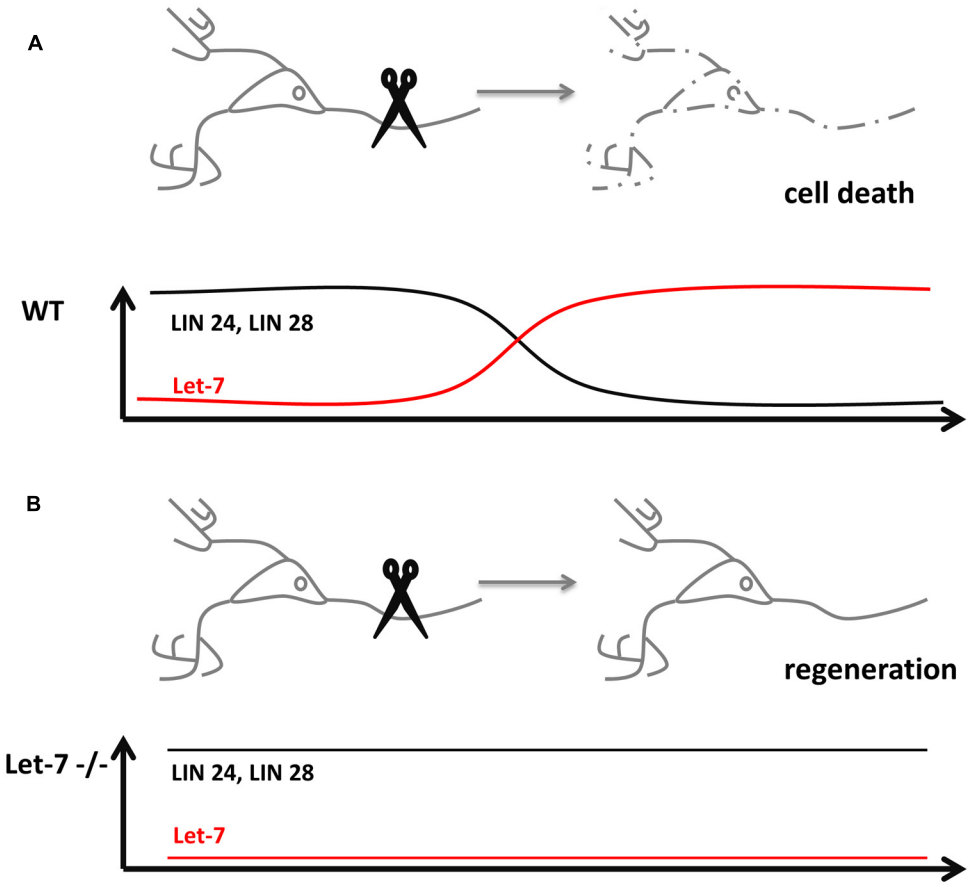

FIGURE 1 | microRNAs influence neuronal regeneration. (A) Severing of an axon usually causes cell death in wildtype neurons (dashed lines), which lost its potential for regeneration through the downregulation of the pluripotency factors Lin 24 and 28 by microRNA *let-7*. **(B)** *let-7*$^{-/-}$ neurons are still able to regenerate destroyed axons, since the pluripotency factors Lin 24 and 28 stay expressed even in post-mitotic cells.

early stages of development a Lin-41/Alg-1 complex is formed and represses the synthesis of *let-7* permitting axonal regeneration and extension. In late larval up to the young adult stages, this suppression is removed and *let-7* is processed to the mature microRNA, which effectively downregulates *lin-41*. In turn, the AVM axons are no longer able to regenerate and stop to grow. Therefore, the molecular mechanism underlying the regeneration decline of AVM axons exemplifies, how a regulatory circuit is reused in post-mitotic cells.

Another example of how a pluripotency factor is used to trigger regeneration in post-mitotic cells was studied in the fish retina. The retina of fish has the remarkable potential to fully regenerate after injury. In order to recover, Müller glia cells de-differentiate and form new progenitors. The regeneration potential is based on the high expression of the pluripotency factor Lin-28 in de-differentiated Müller glia cells, a feature shared with ES cells (Rybak et al., 2008; **Table 1**). Since Lin-28 is also a known target of *let-7*, the fish retina exemplifies the autoregulatory mechanism of the microRNA and its target in a regeneration inducing process (Ramachandran et al., 2010).

In another system, the dendritic arborisation (da) neurons found in the body wall of *Drosophila* larvae, microRNA *bantam* was found to be involved in the process of balancing the growth of dendrites and the underlying epithelium as the target area of the neurons. During a process called scaling, the microRNA functions as a signal to synchronize the neuronal growth with the epithelium. When *bantam* is missing, the dendrites overshoot and fail to cover the appropriate space. Interestingly, during this process, *bantam* is not expressed in the neurons but in the epithelial target cell and acts as a signal to downregulate Akt signaling in the neurons. How the microRNA signal is transferred is not yet found and opens up a new field of study dealing with microRNA signal transduction via direct microRNA transport between neighboring cells (Parrish et al., 2009). In a new study, the axons and dendrites of one class of da neurons are established as a model for neuronal regeneration (Song et al., 2012). Similar to the mammalian system, peripheral sensory neurons retain the potential to regenerate after injury, whereas the processes of central neurons fail to regenerate after injury. Studies how to overcome this lack of regeneration were mostly performed in the mammalian system. However, this study shows in detail that the da neurons in the *Drosophila* body wall and the ventral nerve cord can serve as a model to identify the molecular players involved in regeneration.

Not only in the axons, also da neuron dendrites in *bantam* mutants regenerate. The effect could also be mimicked through depletion of PTEN or gain-of-function of Akt in the neurons. Because Akt signaling is involved in scaling as well as in regeneration of the da neurons, regulatory cycles important during are likely reused during regeneration.

Let-7 IS INVOLVED IN THE DEGENERATION OF NEURONS

An unexpected role of *let-7* was revealed in a study on signaling mechanisms leading to neuronal degeneration (Lehmann et al., 2012). Upon a neuronal damage, e.g., during the course of Alzheimer's disease, the immune system multiplies the degradation process through a so far unidentified mechanism. In a recent study, the RNA sensing receptor Toll-like 7 (TLR 7) in cortical neurons of mice was shown to bind extracellular enriched *let-7* released by degenerating neurons (**Figure 2**). Subsequently, the TLR 7 expressing cell undergoes apoptosis. Mice injected with *let-7b* into the spinal canal lose 18% of neurons after 3 days in the cortical area where TLR 7 is endogenously expressed. A comparable loss of neurons occurred also in the striatal area of the mouse brain. After 2 weeks, the effect further increased up to a loss of 30% of neurons. In mice lacking the TLR 7 receptor, no neuronal loss was detectable. Injection of *let-7b* was in turn also sufficient to activate downstream TLR 7 signaling which was shown by the increased phosphorylation state of IRAK4 (Takeuchi and Akira, 2010).

Moreover, an increase of extracellular *let-7b* was also measured in the corticospinal fluid (CSF) of Alzheimer patients (Lehmann et al., 2012). This observation implies a conserved function of the microRNA as signaling molecule in neurodegenerative diseases. Whether depletion of *let-7b* could decrease the extent of neurodegeneration in Alzheimer disease patients appears to be an interesting hypothesis to be tested in future studies.

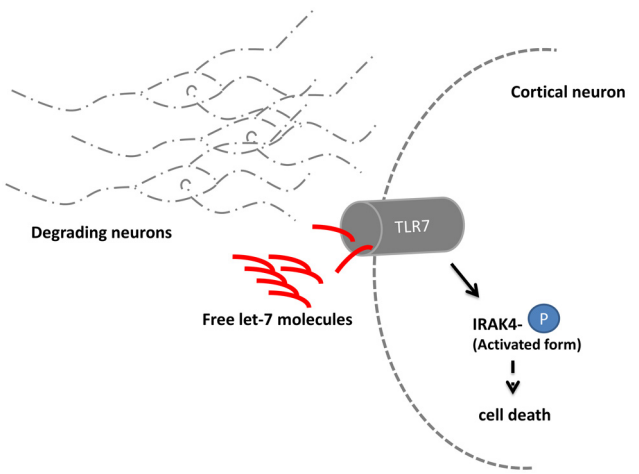

FIGURE 2 | *let-7* regulates neuronal degeneration. Neurons degrading due to e.g., the consequences of Alzheimer's disease release the microRNA *let-7*. Extracellular elevated *let-7* molecules are sensed by the Toll-like receptor 7 (TLR 7) which is expressed in cortical neurons. Upon binding the downstream molecule IRAK 4 becomes phosphorylated and induces through yet unidentified signaling molecules cell death.

microRNAs CONTROL BEHAVIOR

Recent work implicated microRNA function also in the behavior of adult animals. Using an overexpression screen, Luo and Sehgal (2012) demonstrated that *miR-279* controls the circadian rhythm of flies (**Figure 3**). Enhanced levels of *miR-279* were found to disrupt the rest-activity cycles of *Drosophila*. Cycles in circadian rhythm are linked to the oscillations of Period in so-called pacemaker neurons (Ozkaya and Rosato, 2012). These oscillations are normal in *miR-279* mutants suggesting that the microRNA is an effector of Period transmitting signals from pacemaker neurons. Interestingly, *miR-279* targets the secreted morphogen unpaired (Upd) using a JAK/STAT-dependent interaction that was recently discovered in the oocyte (Yoon et al., 2011). The *Drosophila* ovaries contain migratory border cells and non-migratory follicle cells. A gradient of the secreted morphogen unpaired (Upd) is used to establish the cell fate of the two subtypes (Yoon et al., 2011). In order to activate JAK/STAT signaling, the cytokine Upd is secreted and binds STAT (signal transducer and activator of transcription). High levels of Upd specify migratory border cells (Silver and Montell, 2001; Ghiglione et al., 2002), whereas low or transient levels specify the non-migratory follicle cells (Starz-Gaiano et al., 2008). In the oocyte, *miR-279* was found to favor the cell fate of follicle cells through repression of STAT. In the adult fly, reducing the levels of Upd in *miR-279* mutants rescues the circadian rhythm phenotype. Central pacemaker neurons target neurons positive for Upd and were proposed to be candidates for signal receivers of the central clock. This study shows for the first time, which signaling pathway transmits the PER protein oscillations of the circadian clock to downstream neurons. In addition, it represents one of the rare examples for a function of microRNAs in behavior without affecting the development of the underlying circuits.

DISCUSSION

Mutants of only a few microRNAs were found to be essential or to exhibit substantial phenotypes. Of the characterized microRNAs, most appear to fine-tune expression of target mRNAs. The microRNAs with a quantifiable and persistent phenotype are good candidates to study microRNA function and regulation in detail. Three microRNAs, *let-7*, *bantam,* and *miR-279* were discovered in forward genetic screens, because their mutants showed substantial developmental phenotypes. Notably, these microRNAs are encoded by their own genetic loci, in contrast to microRNAs that are found in intronic regions of other genes. In this review, we focused on the roles of these well studied microRNAs in the nervous system. Besides multiple developmental effects of these microRNAs, more recent studies find them to be involved in regeneration, degeneration, and also behavior.

During development the discussed microRNAs target very often pluripotency factors to ensure the proper transition between differentiation and proliferation of different cell types. In mutants, cells fail to differentiate or properly grow. Interestingly, the same microRNA target relationships also affect regeneration events in post-mitotic neurons or glia cells. As exemplified in the fish retina and fly da neurons blocking the expression of *let-7* or *bantam* enables the cell to increase the levels of pluripotency factors to allow cell growth and thereby regeneration of cells and tissues.

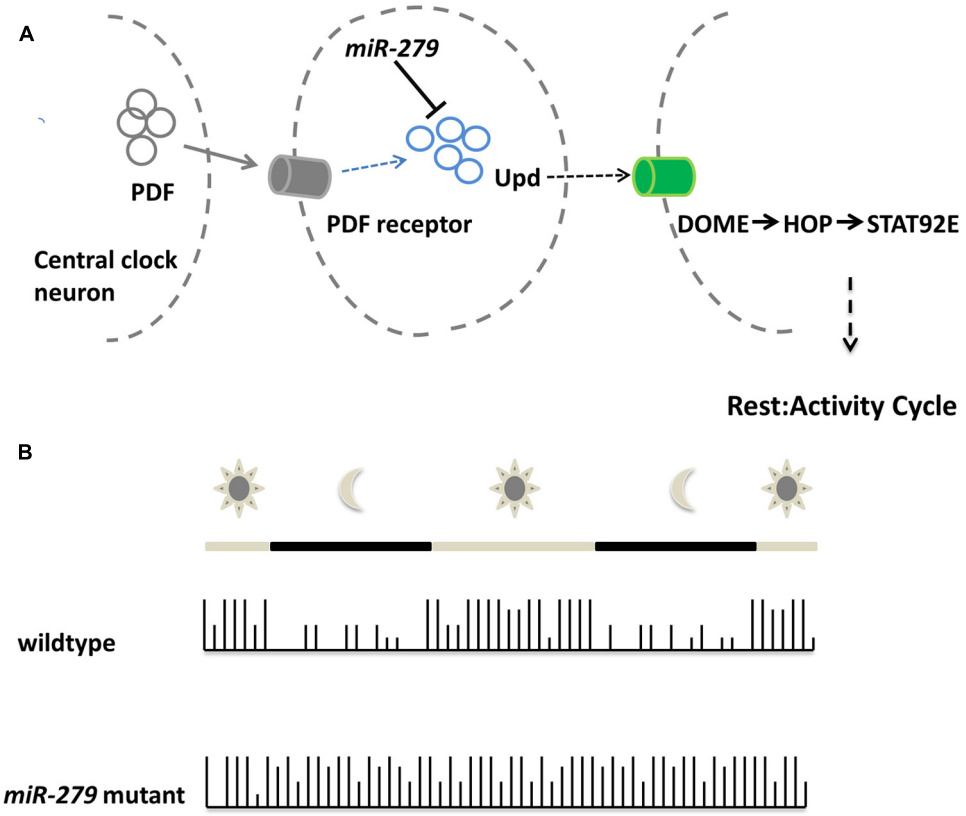

FIGURE 3 | *miR-279* regulates the circadian rhythm in *Drosophila*. **(A)** Schematic showing the molecular mechanisms of central clock neurons to downstream neurons in order to regulate the rest/activity cycle. The central clock neurons release PDF which is sensed via the pdf receptor in target cells. Upon PDF receptor activation, the expression of the cytokine Unpaired is activated, but also repressed via the microRNA *miR-279*. The released cytokine is bound by the receptor DOME in central clock downstream neurons which eventually assure the transition between rest and activity in the circadian rhythm of the fly. **(B)** During the day a wildtype fly shows alternating patterns of activity and rest. During daytime the fly shows high activity which is indicated by the high bars in the activity plot. During the night the rest cycle starts, indicated by low bars. The microRNA-279 mutant flies do not show rest/activity cycles anymore and show a overall activity throughout day and night.

In contrast, *let-7* uses a new and different mechanism in the context of neuronal degeneration. Here, an increase in *let-7* levels in degenerating neurons induces cell death of cortical neurons. In this case, *let-7* acts as an extracellular signaling molecule. Two very interesting questions arise from this finding: (i) how are microRNAs transported? and (ii) do other microRNAs work as signaling molecules? The type of microRNAs and the mechanisms of microRNA-mediated extracellular signaling might also be of interest in light of a potential use of microRNAs as therapeutic agents to prevent neuronal cell loss in neurodegenerative diseases such as Alzheimer's (Chen et al., 2012). We also discussed a study of a novel role of *miR-279* during the circadian rhythm regulation in *Drosophila*. This study provided an interesting example of the function of small regulatory RNAs in the adult animal behavior. The authors demonstrated that *miR-279* helps transmitting the JAK/STAT pathway signal to neurons downstream of pace maker neurons without affecting their development. This study is remarkable, because it showed for the first time that oscillations of Period levels are transmitted to downstream neurons. Regarding the role of microRNAs, the work encourages a search for additional microRNAs involved in the regulation of behavior.

However, the cases of the three discussed microRNAs also imply that the approach of identifying microRNAs through a forward screen approach has weaknesses, because of the highly redundant function of the majority of these molecules. Through the development and further refinement of RNAseq methods, it is now possible to identify the set of microRNAs expressed in a specific tissue or even in a single cell. Alternatively to forward genetic screens, RNAseq mediated analysis of expression of all microRNAs in a given sample could be used to specifically manipulate a combination of microRNAs. These manipulations are being facilitated by the existence of new methods such as site-directed excision with Zinc finger nucleases (Zfn), TALENs (transcription activator-like effector nuclease), or CRISPR-Cas (clustered regulatory interspaced short palindromic repeat; Gaj et al., 2013). Using a modified reverse genetic approach the function of a specific microRNA or groups of microRNAs could be revealed.

Thanks to the work of many groups, we have gained a substantial understanding of microRNA function during development of the nervous system and beyond. The recent findings that

microRNAs regulate aspects of adult animal biology such as neuronal degeneration and regeneration as well as behavior suggest that still much is to be discovered to complete our picture of microRNA-mediated gene regulation and signaling. New genetic and genomic approaches and constantly improved methods to monitor gene expression will pave the way to analyze the function not only of single essential microRNAs but also of groups of microRNAs during nervous system development and in the adult animal.

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Long non-coding RNAs in neurodevelopmental disorders

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Recent studies have emphasized an important role for long non-coding RNAs (lncRNA) in epigenetic regulation, development, and disease. Despite growing interest in lncRNAs, the mechanisms by which lncRNAs control cellular processes are still elusive. Improved understanding of these mechanisms is critical, because the majority of the mammalian genome is transcribed, in most cases resulting in non-coding RNA products. Recent studies have suggested the involvement of lncRNA in neurobehavioral and neurodevelopmental disorders, highlighting the functional importance of this subclass of brain-enriched RNAs. Impaired expression of lnRNAs has been implicated in several forms of intellectual disability disorders. However, the role of this family of RNAs in cognitive function is largely unknown. Here we provide an overview of recently identified mechanisms of neuronal development involving lncRNAs, and the consequences of lncRNA deregulation for neurodevelopmental disorders.

Keywords: long non-coding RNA, nervous system development, fragile X syndrome, genomic imprinting, autism spectrum disorders, intellectual disability, schizophrenia

INTRODUCTION

Therapeutic strategies for the amelioration of neurobehavioral dysfunction in neurodevelopmental disorders such as intellectual disabilities (ID), or autism spectrum disorders (ASD) are often insufficient for a large patient population. These disorders have complex behavioral and cognitive phenotypes that are thought to develop through disturbances in neural circuitry and synaptic function. Moreover, genetic epidemiology and population genetic studies suggested that a spectrum of allelic risk underlies complex traits like ID (Geschwind, 2008). However, the existence of risk alleles rarely confers diagnostic specificity (Hitzemann et al., 2013). One possible explanation for this may involve dysregulation of the rate of gene transcription/translation by small or long non-coding (nc)RNAs, leading to abnormal expression of ID-risk genes of phenotypic relevance (Olde Loohuis et al., 2012). Several studies have now indicated altered levels of brain-specific small and long ncRNA in ID and other neurodevelopmental disorders (Willemsen et al., 2011). lncRNAs constitute a large fraction of the total ncRNA pool, each exceeding 200 nucleotides in length. It was initially assumed that lncRNAs merely act as primary or precursor transcripts for the production of short ncRNAs (sncRNAs) such as microRNAs (miRNAs) or small nucleolar RNAs (snoRNAs; Aschrafi et al., 2008). Conversely to snoRNAs genes, however, the evolutionary conservation of lncRNAs often extends beyond the overlapping sncRNA segments (Wang et al., 2004). They have been shown to either act solely, or together with proteins, exerting a wide range of cellular roles, e.g., their regulation of transcription and RNA processing (Wang et al., 2008). The purpose of this review is to emphasize the role of lncRNAs in regulating neuronal molecular pathways, and to highlight their putative role in dysregulation of these mechanisms in neurodevelopmental disorders.

MECHANISMS OF ACTION OF lncRNAs

lncRNA TRANSCRIPTION MODULATES THE EXPRESSION OF OTHER GENES

Transcription of lncRNAs from alternative transcription start sites in the vicinity of other genes may interfere with the transcription efficiency of that gene (Martens et al., 2005; Martianov et al., 2007). These transcriptional interference mechanisms have been shown to regulate key developmental pathways, such as those involving *Hox*-genes expression (Wang et al., 2011). A complete overview of potential regulatory mechanisms of lncRNAs is provided in (Guttman and Rinn, 2012) or (Ponting et al., 2009). A schematic overview of lncRNAs cellular function is depicted in **Figure 1**.

lncRNAs MAY REGULATE RNA-PROCESSING AND PROTEIN ACTIVITY

Initial research suggested that the functions of lncRNAs relate to their interactions with the RNA-binding proteins (RBPs), a protein family highly abundant in the brain (Smart et al., 2007). Due to the long sequence and structural characteristics of lncRNAs, along with various RBPs and RNA-binding domains, numerous combinations of lncRNA/RNA-binding proteins can be formed. This allows the recruitment of various protein-complexes and a multitude of "downstream" functions. Previous studies suggested that lncRNAs, in concert with RBPs and different protein-complexes, have the capacity to induce chromatin remodeling and histone

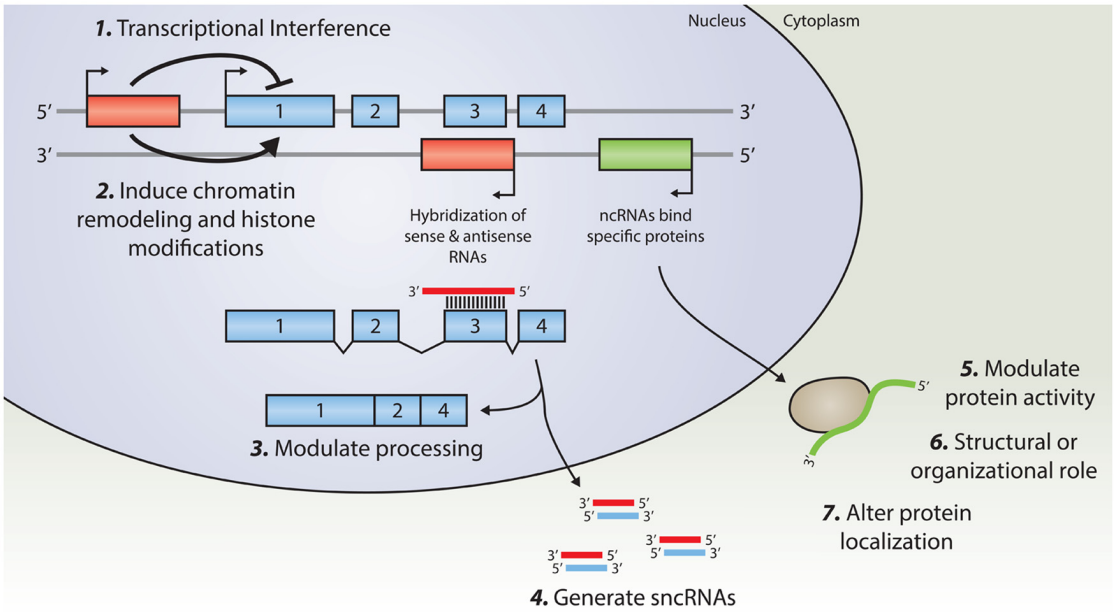


FIGURE 1 | An overview of known regulatory mechanisms for lncRNA. Transcription from an upstream promoter can negatively or positively affect the expression of a downstream gene via (1) transcriptional interference mediated by inhibition of RNA Polymerase 2 recruitment, or by (2) inducing chromatin remodeling and histone modification. Alternatively, an antisense transcript is able to hybridize to the overlapping sense transcript and modulate further processing (3), or provide a substrate for Dicer, or other nucleases, in order to generate various small non-coding RNAs (4). By binding to specific protein partners, a long noncoding transcript may modulate the activity of that particular protein (5), serve as a structural component that allows the formation of a larger RNA–protein complex (6), or alter the cellular localization of the protein (7).

modification, as well as modulating alternative splicing and protein-activity. For example, *Alu RNA* and *B2 RNA* may directly affect RNA polymerase II activity. Both are transcribed from Short Interspersed Nuclear Elements (SINEs; Yakovchuk et al., 2009). *Alu RNA* and *B2 RNA* block binding of RNA polymerase II to the promoter and change the conformation of the transcription initiation complex significantly.

CHROMATIN REMODELING AND HISTONE MODIFICATION CAN BE INDUCED BY lncRNAs

LncRNAs are capable of mediating the activity of proteins involved in chromatin remodeling and histone modification, including those at the Polycomb Repressive Complex 2 (PRC2) complex (Khalil et al., 2009; Tsai et al., 2010) and the CBP/p300 complex (Wang et al., 2008). A genome-wide study revealed that approximately one third of conserved intergenic lncRNAs associates with either the PRC2 complex or the CoREST/REST or SCMX proteins, all known chromatin-modifying proteins (Khalil et al., 2009). A prominent epigenetic mechanism exerted by lncRNAs is the X-chromosome inactivation. The extent of this control is unique among the chromosomes and is disrupted in X-linked IDs. X-chromosome inactivation is mediated via the lncRNA *Xist* that binds to one of the X-chromosomes (Zhao et al., 2008). *RepA* was found to be both part of the *Xist* lncRNA, as well as expressed by itself (Zhao et al., 2008). The *RepA* lncRNA is able to bind the histone methyltransferase Enhancer of Zester Homolog 2 (Ezh2), which is a subunit of the PRC2. The recruitment of the PRC2 complex by *Xist*, via the *RepA* sequence, allows trimethylation on lysine-27 of H3 histones (H3K27), effectively repressing gene expression, and inactivating the X-chromosome (Zhao et al., 2008). Very Recently, *Xist* was found to function in a two-step mechanism, though targeting of gene-rich islands before gene-poor domains (Simon et al., 2013).

FUNCTIONAL ROLES OF lncRNAs IN NERVOUS SYSTEM DEVELOPMENT AND FUNCTION

Multiple lines of evidence suggest that dysregulated processes as seen in neurodevelopmental disorders are based on mechanisms that are under tight regulation by lncRNAs (see below). A number of ncRNAs were found to be specifically expressed within the hippocampus (Mercer et al., 2008b), a region involved in processing and consolidation of memories. Several lncRNAs originate from genomic regions associated with protein-coding genes involved in memory formation and maintenance, such as an lncRNA transcribed antisense to *Camkk1*, which is involved in male-specific memory formation (Mercer et al., 2008a).

During brain development, differentiation of neural stem cells and progenitors is crucial. Recently, various lncRNAs have been linked to these events, implying a key role for lncRNAs not only during development, but also in several neuropathologies (reviewed by e.g., Qureshi et al., 2010). For example, a subset of lncRNAs are specifically associated with genes from the *Dlx*-family, known to be involved in brain development in mammals and Drosophila. Two of the differentially expressed lncRNAs, *Evx1as* and *Hox5b/6as* were shown to be associated with trimethylated H3K4 histones and histone methyltransferases (Dinger et al.,

2008). In addition, embryonic ventral forebrain-2 (*Evf2*) is transcribed from the *Dlx5/Dlx6* locus, antisense to the *Dlx6* gene (Feng et al., 2006). *Dlx6* is a homeobox-containing transcription factor important in forebrain neurogenesis (Stenman et al., 2003). Furthermore, 659 evolutionary conserved murine lncRNAs have been identified of which the brain-specific lncRNAs are preferentially (2 to 3-fold increase) located adjacent to brain-expressed protein-coding genes, involved in transcriptional regulation, or in nervous system development (Ponjavic et al., 2009).

Recent studies identified 945 lncRNAs, of which 174 were differentially expressed in the mouse embryoid bodies; and that are annotated to developmentally important events relating to stem cell pluripotency (Dinger et al., 2008). One of these RNAs, *Sox2OT* (*Sox2* Overlapping Transcript) is a highly conserved lncRNA that overlaps the *Sox2* gene (Fantes et al., 2003). *Sox2* is a transcription-factor critical in maintaining self-renewal properties of neural stem cells (Mizuseki et al., 1998). Similar to *Sox2, Sox2OT* is present in neural stem cells and is downregulated during differentiation (Amaral et al., 2009).

During fate-specification from neuronal oligodendrocyte bipotent progenitors into GABAergic interneurons, 56 lncRNAs were found to be upregulated, including *Gtl2, Rian, Evf2* and *Copg2as*, but also the novel *AK044422* (Mercer et al., 2010). Interestingly, *AK044422* overlaps with miR-124a, a highly conserved and highly expressed brain-specific miRNA previously implicated in regulating neuronal specification and differentiation (Makeyev et al., 2007; Visvanathan et al., 2007). Synaptogenesis is a pivotal process during neuronal development, which is altered in various neurodevelopmental disorders (reviewed by e.g., (Zoghbi, 2003; Ecker et al., 2013)). Metastasis-associated lung adenocarcinoma transcript 1 (*Malat1*) is an lncRNA that is enriched in nuclear speckles (Hutchinson et al., 2007; Clemson et al., 2009). There, it co-localizes with splicing factors to controls the expression of genes involved in synapse function and synaptogenesis (Bernard et al., 2010).

LncRNAs ARE INVOLVED IN NEURODEVELOPMENTAL DISORDERS

Several lncRNAs are either differentially expressed in or associated with neurodevelopmental disorders, such as Prader–Willi syndrome (PWS), Angelman syndrome (AS), ID, and ASD (**Table 1**). The role of lncRNAs is possibly best understood in genomic imprinting disorders such as PWS (Wevrick and Francke, 1997; Jong et al., 1999) and AS (Runte et al., 2004), both of which feature learning difficulties but otherwise have different symptoms (further discussed below).

IMPRINTING DISORDERS

Genomic imprinting is mediated by various processes such as DNA methylation and histone modification, but also by ncRNAs (Bartolomei, 2009). PWS (MIM 176270) is characterized by infantile hypotonia, early childhood obesity, short stature, hypogenitalism/hypogonadism, ID, and other behavioral problems including temper tantrums. The genetic cause of the disorder lies in a disruption of the paternal chromosome 15q11.2q13, since the maternal chromosome is inactive through imprinting (Horsthemke and Wagstaff, 2008). To date, two genes have functionally been associated with the pathology of the disorder: *NECDIN* and small nuclear ribonucleoprotein polypeptide N *(SNRPN)*. *Necdin* deficient mice show a subset of the multiple clinical manifestations of PWS (Muscatelli et al., 2000). *SNRPN* encodes the SmN splicing factor, the SNRPN upstream reading frame (*SNURF*) and partially overlaps the *UBE3A* gene. Importantly, the downstream introns of *SNRPN* contain C/D box-containing *SNORD116* (*HBII-85*) snoRNA clusters whose expression is under control of the *SNRPN* promoter (Runte et al., 2001). Several case reports indicated that paternally inherited microdeletions of this cluster cause PWS (Sahoo et al., 2008; de Smith et al., 2009; Duker et al., 2010). Moreover, two mouse models with targeted deletions in the *MBII-85* snoRNA cluster exhibited a similar phenotype as other PWS models, which included decreased activity, hypotonia at birth, and postnatal growth retardation (Skryabin et al., 2007; Ding et al., 2008).

IPW (Imprinted gene in the PWS region) is located in the proximal chromosome 15q, merely 150 kb distal to *SNRPN* and is not expressed in patients with 15q11-q13 deletions (Wevrick et al., 1994). Additionally, *ZNF127* is located in the same region and has been reported to have a disrupted expression in PWS. This gene has a potentially non-coding antisense gene, *ZNF127AS*, which might be regulating the imprinting of *ZNF127* gene (Jong et al., 1999).

Angelman syndrome (MIM 105830) is caused by a disruption of the maternal allele of chromosome 15q11-q13, covering the same genomic location as PWS. However, the symptoms are different and include intellectual disability, movement or balance disorder, typical abnormal behaviors, and severe limitations in speech and language. The genetic underpinning of the disorder is thought to be a disruption in the *UBE3A* gene (Matsuura et al., 1997). The *UBE3A-AS* gene is transcribed antisense to the *UBE3A* gene and repression of *UBE3A* is dependent on *UBE3A-AS* (Chamberlain and Brannan, 2001; Johnstone et al., 2006). However, another study suggests that silencing of the paternal *UBE3A* can also occur when *UBE3A-AS* is not present, indicating that the regulation is more complex (Le Meur et al., 2005).

INTELLECTUAL DISABILITY

Despite the highly variable genetic etiology in ID, only a limited number of molecular and cellular pathways appear to be affected by the magnitude of different gene mutations. ID genes have been shown to cluster in pathways underlying neurogenesis, neural migration, neuronal outgrowth, and synaptic function (van Bokhoven, 2011). Numerous studies have suggested that synaptogenesis and normal synaptic function is dependent on the activity of a large number of proteins, and that disturbance of individual components within the network, or alterations of their activities causes synaptic dysfunction, phenotypically culminating in ID (Aschrafi et al., 2005). Regulation of gene transcripts by small and large ncRNAs may underlie epigenetic control of synaptic activity in ID and other neurodevelopmental disorders. Previous studies have indicated that disruption of lncRNA expression and signaling impairs synaptic plasticity, and results in severe cognitive impairment in mice, and human, which are detailed below.

Table 1 | An overview of the lncRNAs identified in neurodevelopmental disorders.

Disorder	LncRNA	Significance	Reference
PWS	*SNORD116 (HBII-85)* C/D box cluster	Microdeletions including this cluster cause PWS (phenotype)	Duker et al. (2010), Sahoo et al. (2008), de Smith et al. (2009)
	IPW	Not expressed in PWS	Wevrick et al. (1994)
	ZNF127AS	Disrupted expression in PWS	Jong et al. (1999)
AS	*UBE3A-AS*	Increased or decreased expression in AS	Runte et al. (2004)
FXS	*FRM4 (FMR1-AS1)*	Silenced in FXS patients; knockdown results in alterations in cell cycle regulation and increased apoptotic cell death	Ladd et al. (2007), Khalil et al. (2008)
	BC1	Associated with fragile X syndrome	Zalfa et al. (2003, 2005)
Rett syndrome	AK087060 AK081227	Upregulated in MECP2 KO mice; AK087060 associated with the downregulation of its host gene, GABA receptor subunit Rho 2 (Gabbr2)	Petazzi et al. (2013)
DS	*NRON*	Regulates nuclear shutting of NFAT, whose reduced activity leads to DS features	Willingham et al. (2005), Arron et al. (2006)
2p15-p16.1 microdeletion syndrome	*FLJ16341*	In critical region with three protein-coding genes: BCL11A, PAPOLG, and REL	Hancarova et al. (2013)
MCOPS3	*SOX2OT*	Modulates expression of SOX2, in which genetic defects cause micropthalmia syndrome 3.	Fantes et al. (2003), Amaral et al. (2009)
ASD	*ST7OT1*	Associated with autism in one patient	Vincent et al. (2002)
	ST7OT2		
	ST7OT3		
	PTCHD1AS1	Deletions are only found in males with ASD and not in male control individuals.	Noor et al. (2010)
	PTCHD1AS2		
	PTCHD1AS3		

The disorders are listed in the first column (PWS, Prader–Willi syndrome; AS, Angelmann syndrome; FXS, fragile X syndrome; DS, down syndrome; MCOPS3, micropthalmia syndrome 3; ASD, autism spectrum disorder).

Fragile X Syndrome

Fragile X syndrome (FXS, MIM 300624) is inherited via an X-linked dominant pattern and characterized by moderate to severe mental retardation, macro-orchidism, and distinct facial features. The disorder is caused by an unstable expansion of a CGG repeat in the *FMR1* gene leading to silencing of the gene by methylation of repeat and promoter (Sutcliffe et al., 1992), resulting in decreased FMRP protein levels in the brain (Devys et al., 1993). Accumulating evidence suggests that the etiology of the disorder is influenced by lncRNAs. The promoter of *FMR1* is bidirectional and can also give rise to the lncRNA *FMR4* or *FMR1-AS1*, a gene transcribed in the antisense orientation and overlaps the CGG repeat region. *FMR4* is similar to *FRM1* in being silenced in FXS patients and upregulated in permutation carriers (Ladd et al., 2007; Khalil et al., 2008). Following siRNA knockdown of *FMR4*, alterations in cell cycle and apoptosis were reported. Conversely, overexpression of *FMR4* resulted in increased cell proliferation. Additionally, knockdown of *FMR4* did not influence *FMR1* expression and vice versa, suggesting an independent mechanism from *FMR1* (Khalil et al., 2008). Together, these findings points toward a contribution of *FMR4* in the pathology of FXS.

Recently, Pastori et al. (2013) discovered two new transcripts in the *FMR1* gene locus: *FMR5* and *FMR6*. *FMR5* was similarly expressed in brain regions from unaffected and permutation individuals and full mutation patients, whereas *FMR6* was silenced in full mutation and permutation carriers. According to the authors, this might suggest an abnormal transcription or chromatin remodeling prior to transition to the full mutation. In addition to the finding that both *FMR5* and *FMR6* are expressed in blood leukocytes, these lncRNAs are potentially useful as biomarkers in FXS.

FMRP, the protein that is encoded by *FMR1*, acts as a translational repressor of specific mRNAs at the synapse and associates with the dendritic RNA *BC1* (Zalfa et al., 2003). *BC1* enables the interaction of FMRP with the target mRNAs; and FMRP can directly bind to *BC1* and its human analog *BC200* via its N-terminus. Of note, the 5′ stem loop of *BC1* is involved in FMRP recognition and this region is complementary to FMRP target mRNAs (Zalfa et al., 2005). Taken together, the studies suggested that *BC1* is a ncRNA that is essential for the repression of mRNAs via FMRP and loss of this repression in FXS patients could result in synaptic dysfunction. It should be noted that, In Iacoangeli et al. (2008), five independent groups

reported that results published by Zalfa et al. (2003) are not reproducible. Thus, there is no confirmation, independent of the Bagni group, of a specific physical link between FMRP and BC1 RNA.

Rett syndrome

Rett syndrome (MIM 312750) is characterized by arrested development between 6 and 18 months of age in females, regression of acquired skills, loss of speech, stereotypical movements, seizures, and ID. Mutations in the *MECP2*, which binds methylated CpGs and can both activate and repress transcription, were first described to be the cause of the disorder (Amir et al., 1999). While assessing the transcriptome of male *Mecp2* hemizygous knockout mouse brains (Petazzi et al., 2013), it was revealed that the lncRNAs *AK081227* and *AK087060* were both significantly upregulated as compared to wild-type littermates. Importantly, overexpression of *AK08127* was associated with the downregulation of its host coding protein gene, the gamma-aminobutyric acid receptor subunit Rho 2. This suggest that transcriptional dysregulation of lncRNAs may have the capacity to contribute to the etiology of Rett syndrome.

Down syndrome

Down syndrome (DS) or Trisomy 21 (MIM 190685) is characterized by ID, distinct facial characteristics and congenital heart defects. The lncRNA *NRON* may be involved in DS, since *NRON* modulates cytoplasmic-to-nuclear transport of NFAT (Willingham et al., 2005). Decreased nuclear NFAT activity leads to DS-like characteristics in animal models, suggesting a possible role for *NRON* in DS (Arron et al., 2006). Recently, an inducible *XIST* was introduced on chromosome 21 using genome editing (Jiang et al., 2013). This approach created a model to investigate genomic expression changes and cellular pathologies of trisomy 21. Notably, deficits in proliferation and neural rosette formation are rapidly reversed upon silencing one chromosome 21, representing a major step toward potential development of "chromosome therapy" (see **Figure 2** for a proposed approach).

Other syndromic neurodevelopmental disorders

In the last decade, several new rare microdeletion syndromes were identified. One of these is the 2p15-p16.1 microdeletion syndrome (Rajcan-Separovic et al., 2007), characterized by ID, autistic features, microcephaly, short stature, and various dysmorphic facial features. The genomic cause of this disorder remains to be elucidated, but the susceptibility candidate genes include *BCL11A*, *PAPOLG* and *REL* and one lncRNA gene *FLJ16341*, although the function of this lncRNA is still elusive.

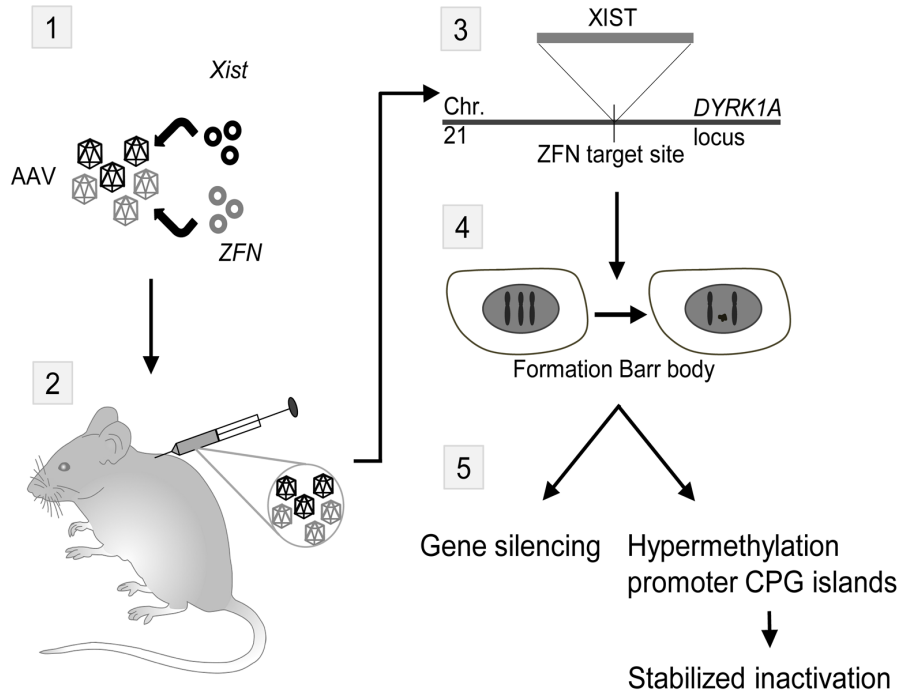
AUTISM SPECTRUM DISORDER

Autism spectrum disorders is an umbrella term for various developmental disorders, including autism, pervasive developmental disorder not otherwise specified (PDD-NOS) and the Asperger syndrome. Common symptoms of the various ASD disorders include problems of reciprocal social interactions, verbal and non-verbal communication, and rigid and stereotyped behaviors. ASD is a clinically and etiologically heterogeneous disorder with a complex genetic architecture. Not only multiple common genetic variants appear to be involved, each with small effect size, but also rare variants with strong effect size (Devlin and Scherer, 2012). The latter are mostly *de novo* mutations, as evidenced by whole-exome and genome sequencing studies in ASD patients (Talkowski et al., 2012; Vulto-van Silfhout et al., 2013), or copy number variations (CNVs; Poelmans et al., 2013). Microarray analysis shows that 5–10% of subjects with ASD have an identifiable genetic etiology in recurrent or *de novo* chromosomal rearrangements (Marshall et al., 2008). In the last decade, several studies reported aberrant expression of lncRNAs, suggesting that these might be important in the etiology of the disorder. Recently, Ziats and Rennert (2013) showed that over 200 lncRNAs were differentially expressed in a microarray of postmortem prefrontal cortex and cerebellum tissue of ASD patients. A decade earlier, Vincent et al. (2002) identified a novel autism locus, which includes the gene *RAY1/ST7*. This locus contains at least four non-coding genes (*ST7OT1-4*), both on the sense and antisense strands that potentially regulate *RAY1/ST7*. Several rare variants were detected in autism patients on either the *RAY1/ST7* or the *ST7OT1-3* genes that were not observed in a control population.

Mutations in the X-chromosome *PTCHD1* gene have been reported to involve X-linked ID and ASD (Noor et al., 2010; Filges et al., 2011). Although the exact function of the gene is still unknown, several lines of evidence suggest that it might have a causative role in a subset of ID and/or ASD patients (Filges et al., 2011). On the antisense strand of the *PTCHD1* gene, several overlapping lncRNAs (*PTCHD1AS1, PTCHD1AS2* and *PTCHD1AS3*) were detected, which may serve as regulators for *PTCHD1*, since the 5′ exons are adjacent on opposite strands.

CONCLUSION

Regulation of epigenetics processes during brain development and in activity-dependent brain functions are key to the symptomology underlying many neurodevelopmental disorders. In recent time, a wide range of cutting-edge "omics" and bioinformatics based technologies vastly accelerated our understanding of the key molecular players and mechanisms involved in regulating these epigenetic processes. In contrast to the earlier held view that lncRNAs were merely transcriptional noise, it is now apparent that lncRNAs exert important regulatory functions in the brain, both during adult and developmental stages and represent a key epigenetic mediator of these processes. The interplay between lncRNAs and chromatin remodeling factors may be key to understanding the role of epigenetics in neurodevelopmental disorders (Kramer and van Bokhoven, 2009). LncRNAs are now believed to modulate molecular events during neurogenesis, cell-fate decisions, differentiation and maturation, but are also involved in higher brain functions such as memory formation. The large number of brain-expressed lncRNAs suggests that many more such higher-order functions might also be modulated by lncRNA-mediated mechanisms, which remain to be more fully illustrated in future research efforts. Animal models of lncRNA function, e.g. knockout mice for *Malat1* (Zhang et al., 2012) and *Neat1* (Nakagawa et al., 2011), have been developed recently and might provide a better insight in lncRNA-mediated mechanisms. However, already at this stage it



Main limitations to overcome

a. Determine the optimal age for the therapeutic intervention
b. AAVs can contain vectors up to only 4.7 kb
c. Prevention of inactivation of >1 chromosome 21
d. Determine the optimal route of administration
e. Overcome inter-species differences in (neuronal) transduction efficiency

FIGURE 2 | Proposed strategy for a therapeutic application of *Xist* and zinc finger nucleases (ZFN) to treat trisomy 21. Adeno-associated viruses (AAVs) are currently the most promising CNS gene delivery vector (for review, see Gray, 2013). As shown in this scheme, the first step in the approach would be incorporation of plasmids containing *Xist* and ZFN targeted to the *DYRK1A* locus on chromosome 21 in AAVs (1). Next, injection of the viruses in rodents can be performed intracranial, intravenous or in the cerebrospinal fluid (2). Intracranial injections have been successfully performed in mammals as large as cats, but an estimated number of 20–30 required injections per hemisphere in human infants rendered this technique unfavorable over alternatives (Vite et al., 2005). AAV9 vectors have the capacity to cross the blood-brain barrier and transduce neurons and astrocytes (Foust et al., 2009), making intravascular injection of viral vectors for CNS targeted gene therapy a possibility. The third possible route of administration is injecting the viral vectors in the cerebrospinal fluid (CSF), thus transducing the central nervous system effectively even in non-human primates (Samaranch et al., 2012). After injection of the AAVs and transduction of the viruses in the cells, *Xist* will be incorporated on the ZFN target site in the *DYRK1A* locus on chromosome 21 (3). Here, it will induce the formation of a chromosome 21 Barr body (4), which will lead to gene silencing and hypermethylation of promoter CPG islands, effectively stabilizing the inactivation of the chromosome (5; Jiang et al., 2013). Eventually, this approach may lead to a CNS-wide inactivation of the third chromosome 21, thereby reducing the symptoms of the trisomy 21 disorder. However, several major limitations have to be overcome in order to translate this proposed approach to humans. First, the optimal age for the therapeutic intervention should be established. The majority of the *in vivo* gene therapy studies have been performed in juvenile or adult animals, but starting the treatment at an earlier age should be considered in order to achieve the best therapeutic effect. Moreover, the optimal route of delivery for CNS gene therapy is currently not established yet, with possibilities being intravascular injection, injection in the CSF, and to a lesser extend, intracranial injections. Third, a practical issue of using AAVs in the therapeutic approach is the limitation of AAVs to contain vectors up to only 4.7 kb in length. This is insufficient for the *Xist* containing vector used in the proof-of-principle study by Jiang et al. (2013). Last, comparing intravascular injection of vectors with a CNS target revealed that both neuronal and overall transduction efficiency in primates is considerably lower than in rodents, the latter most likely due to circulating pre-existing neutralizing AAV antibodies (Gray et al., 2011).

is clear that lncRNAs may offer a unique approach to modulate pathogenetic events in the causation of neurodevelopmental disorders.

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microRNAs in nociceptive circuits as predictors of future clinical applications

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Neuro-immune alterations in the peripheral and central nervous system play a role in the pathophysiology of chronic pain, and non-coding RNAs – and microRNAs (miRNAs) in particular – regulate both immune and neuronal processes. Specifically, miRNAs control macromolecular complexes in neurons, glia and immune cells and regulate signals used for neuro-immune communication in the pain pathway. Therefore, miRNAs may be hypothesized as critically important master switches modulating chronic pain. In particular, understanding the concerted function of miRNA in the regulation of nociception and endogenous analgesia and defining the importance of miRNAs in the circuitries and cognitive, emotional and behavioral components involved in pain is expected to shed new light on the enigmatic pathophysiology of neuropathic pain, migraine and complex regional pain syndrome. Specific miRNAs may evolve as new druggable molecular targets for pain prevention and relief. Furthermore, predisposing miRNA expression patterns and inter-individual variations and polymorphisms in miRNAs and/or their binding sites may serve as biomarkers for pain and help to predict individual risks for certain types of pain and responsiveness to analgesic drugs. miRNA-based diagnostics are expected to develop into hands-on tools that allow better patient stratification, improved mechanism-based treatment, and targeted prevention strategies for high risk individuals.

Keywords: chronic pain, biomarker, polymorphism, miRNA-based diagnostics, miRNA expression patterns, miRNA polymorphisms, antagomir, miRNA-based analgesic

INTRODUCTION

Human chronic pain disorders are bio-psycho-social diseases, which are difficult to treat due to their diversity. Chronic pain syndromes that develop after nerve damage, trauma or surgery are characterized by persistent and severe pain; they induce anxiety and depression and greatly impair patients' quality of life. One out of five Europeans suffers from chronic pain with most reporting that they endure it for more than two years (Breivik et al., 2006; Baker et al., 2010). Due to direct and follow-up costs they constitute a heavy burden for the health system (Phillips, 2006).

Of the painful neuropathies, the most frequent, painful diabetic polyneuropathy is a common complication of diabetes mellitus occurring in up to 20% of patients (Sommer, 2003; Sadosky et al., 2008). Good glycemic control can reduce the incidence of diabetic polyneuropathy but not painful diabetic polyneuropathy (PDPN) for which only symptomatic therapy of low to moderate efficacy is available to date (Vincent et al., 2011). Cellular mechanisms are emerging that include the classical changes of the diabetic milieu (Bierhaus and Nawroth, 2012; Bierhaus et al., 2012) however various studies have also identified signatures of neuroinflammation as critical components of painful diabetic polyneuropathy (Pabreja et al., 2011; Vincent et al., 2011). Pathological neuro-immune communication has also been associated with painful neuropathy that occurs in up to 50% of patients with traumatic peripheral nerve injury as a consequence of accidents, warfare or surgical procedures (Myers et al., 2006; Ciaramitaro et al., 2010; Birch et al., 2012). Also the neurogenic complex regional pain syndrome (CRPS) occurring as a complication of bone fracture, tissue injury or surgical interventions has a neuro-inflammatory component (Parkitny et al., 2013).

In the majority of cases symptoms grossly resolve, however in 30% of patients pain symptoms persist or even intensify (Marinus et al., 2011). The beneficial effect of therapy with glucocorticosteroids in the acute phase of CRPS supports pathophysiological mechanisms associated with neuro-immune dysfunction (Üceyler et al., 2007a; Fischer et al., 2010; Marinus et al., 2011). Thus, converging evidence suggests that neuro-immune alterations in the peripheral and central nervous system play a major role in the general pathophysiology of neurogenic and neuropathic pain (McMahon and Malcangio, 2009; Kuner, 2010). Non-coding RNAs (ncRNAs), including microRNAs (miRNAs) and Piwi-binding piRNAs, are intimately associated with normal cellular as well as pathological processes (Mattick, 2004; Hüttenhofer et al., 2005; Hüttenhofer and Schattner, 2006). In this review we will focus on miRNAs since they are most extensively studied so far.

Various diseases, including neuropathic pain disorders, reveal unique miRNA expression signatures that can be exploited as diagnostic and prognostic markers. Recent reports on miRNA modulation of both neuronal and immune processes further predict therapeutic potential for manipulating disease-modified miRNAs in diseases affecting both the immune system and brain function, such as neuropathic pain disorders, Alzheimer's disease, Parkinson's disease, multiple sclerosis, and anxiety-related disorders (Soreq and Wolf, 2011; O'Connor et al., 2012).

miRNAs that function within both the nervous and the immune systems possibly act as "negotiators" between these two interacting compartments (**Figure 1**). These "neurimmiRs" primarily target transcription factor genes or other regulatory genes, which enables simultaneous modulation of both immune and neuronal processes including cognition through direct or indirect alterations of neuron–glia or brain-to-body signaling (Soreq and Wolf, 2011). Thus, a given miRNA controls multiple cellular pathways, and miRNAs can act as "master switches" of the transcriptome or proteome, regulating multiple gene products and orchestrating multiple pathways including genes that encode cellular enzymes, trophic factors, receptor proteins, and ion channels many of which are individually pursued as drug targets.

Pain conditions have been suggested to deregulate the expression of miRNAs in pain pathways from primary afferent nociceptors to brain areas associated with emotional components of pain perception (Bai et al., 2007; Aldrich et al., 2009; Kusuda et al., 2011; Imai et al., 2011; Poh et al., 2011; von Schack et al., 2011). miRNAs are frequently deregulated and expressed at aberrant levels in diseased tissue, and first evidence suggests that this applies to neurogenic pain in CRPS (Orlova et al., 2011). Altered miRNA expression is frequently a consequence of genetic mutations, which may also cause loss or gain of function (Mishra and Bertino, 2009). This may account for inter-individual variation of pain sensitivity. However, the functional consequences of polymorphisms in miRNA genes and/or their binding sites, the downstream targets of miRNAs and the mechanisms by which miRNAs regulate circuitries and processes modulating nociception and endogenous analgesia are as yet unresolved.

Therapeutic miRNA regulation has been thoroughly studied and widely established in cancer research but its impact and the therapeutic prospects of miRNAs in the pain field are largely unexplored. Manipulation of miRNAs offers the possibility to control multiple targets including neuro-immune interactions, nociceptive processing and cognitive pathways. Both miRNAs and their isomiRNA versions are likely to each interact with many different targets, which may lead to downstream changes either due to the direct suppression of these targets or because of regulatory effects of those targets. Such downstream effects may be rather elaborate and are defined by some researchers "off-target" effects. However, we find that this definition may be misleading as it assumes that the physiological role of each miRNA is limited to the suppression of its direct targets. It is expected that miRNAs and miRNA derivatives will have few, if any, sequence-specific "off-target" effects. Thus, miRNA based diagnostics and therapeutics may have superior advantages by targeting multiple pain-associated genes and miRNA-based drugs may be the most appropriate therapy for the prevention or treatment of neuropathic pain.

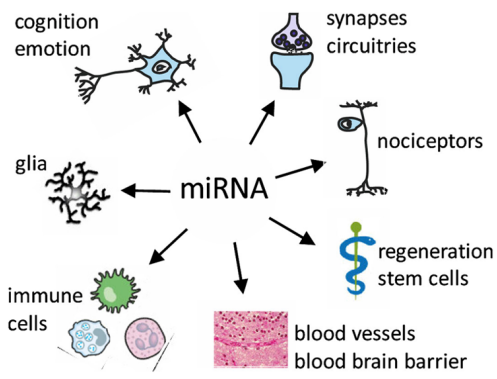

FIGURE 1 | Targets of miRNA regulation networks.

BIOMARKERS FOR NEUROPATHIC AND NEUROGENIC PAIN SYNDROMES

Painful diabetic polyneuropathy is the most frequent painful neuropathy occurring in up to 20% of diabetic patients (Sommer, 2003; Sadosky et al., 2008). CRPS is an extremely painful condition that occurs in some patients after bone or tissue injury and peripheral nerve injury (traumatic neuropathy) and results in chronic neuropathic pain in many of these patients. These well-characterized albeit aetiologically diverse (metabolic, inflammatory, traumatic) neuropathic/neurogenic pain syndromes cover a spectrum of mechanisms underlying chronic pain. Nevertheless, the medical need for these syndromes is prevalent, and each of them is prototypic for an entire group of pain disorders.

It is unclear why diabetic neuropathy or traumatic neuropathy are painful in some instances and painless in others or why some patients develop CRPS after bone fracture, and why some recover from CRPS and others do not (Marinus et al., 2011). Thus, as yet unknown factors determine whether a given disorder entails chronic neuropathic pain. A first approach to be able to predict the individual risk of pain chronification was to use sensory phenotypes as surrogate markers for possible underlying mechanisms. Quantitative sensory testing (QST) is now well established but is still insufficient to disentangle specific pathophysiological mechanisms of chronic pain (Baron et al., 2012). One of the major

hindrances in translating such findings into better therapy of neuropathic and neurogenic pain syndromes is the complexity of their pathophysiology. It is well known that alterations in many processes including ion channels, inflammatory mediators, neurotrophic factors, synaptic plasticity, and de- and regeneration, are involved, and that they even change during the course of the disease (Hehn et al., 2012). Therefore, a search for better and more specific diagnostic trait and state markers is one of the prerequisites for successful treatment in the future. Circulating miRNAs are detectable in body fluids including blood and cerebrospinal fluid and may be useful as novel biomarkers amenable to clinical diagnostic applications for various types of disease (Cogswell et al., 2008; Orlova et al., 2011; Ajit, 2012; Weiland et al., 2012; Machida et al., 2013). Therefore, it should be likewise promising to carefully assess which circulating miRNAs and novel ncRNAs are associated with neurogenic and neuropathic pain syndromes and may emerge as reliable diagnostic biomarkers for painful diabetic polyneuropathy, nerve injury pain, CRPS, headache and migraine.

NEW DRUGGABLE MOLECULAR TARGETS FOR PAIN TREATMENT

Treatment of painful diabetic polyneuropathy is far from satisfactory in many patients although this is the most intensely studied painful neuropathy in randomized controlled trials (RCTs). National and international guidelines differ in their recommendations about first and second line treatment choices. While pregabalin is favored by some (Bril et al., 2013), duloxetine or even tricyclic antidepressants are first choice in others (NICE-guideline; Attal et al., 2010; Dworkin et al., 2007). All of these drugs have adverse effects on diabetes. Furthermore, mean treatment effects comprise only two points of pain reduction on a 11-point Likert scale. In other types of neuropathy, like traumatic neuropathy or the frequent inflammatory types, there is little or no data at all from RCTs on pain treatment. Even worse, treatment of CRPS is neither standardized, nor satisfactory, nor based on multicentre RCTs. From single center studies with very limited patient numbers some evidence exists for anti-inflammatory treatment by corticosteroids or bisphosphonates in acute but not chronic stages, and for behavioral therapy for selected patients in chronic stages (de Tran et al., 2010). For the most frequently used invasive treatment modalities such as sympathetic blockers no RCT evidence of efficacy is available (Straube et al., 2010). Thus, more efficacious and specific medications are needed for both neurogenic and neuropathic pain syndromes.

Both, the novel and specific mode of action and the ability to function as master switches of entire signaling networks has triggered enthusiasm for miRNAs as promising therapeutic targets although relatively little is known about the mechanisms of cellular uptake, storage and mode of action of miRNA modulators (van Rooji and Olson, 2012). In several rodent pain models, deregulated expression of miRNAs was found in pain pathways from primary afferent nociceptors to brain areas associated with emotional components of pain perception (**Figure 2**; Bai et al., 2007; Aldrich et al., 2009; Kusuda et al., 2011; Imai et al., 2011; Poh et al., 2011; von Schack et al., 2011). First evidence supporting a future for analgesic miRNA treatment comes from

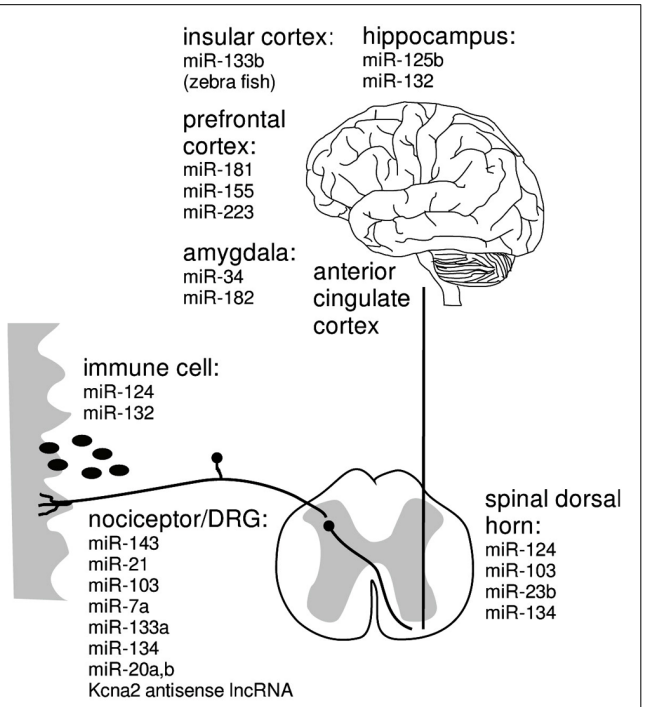

FIGURE 2 | miRNA that may be causally associated with maintained neuropathic pain in immune cells (Shaked et al., 2009; Soreq and Wolf, 2011; Ponomarev et al., 2013), nociceptors/DRG (Zhao et al., 2010, 2013; Tam Tam et al., 2011; Ni et al., 2012; Sakai and Suzuki, 2013), spinal cord (Brandenburger et al., 2012; Favereaux et al., 2011; Im et al., 2012; Ni et al., 2012; Willemen et al., 2012), insular cortex (Sanchez-Simon et al., 2010), amygdala (Meerson et al., 2010; Haramati et al., 2011; Griggs et al., 2013), prefrontal cortex (Poh et al., 2011), hippocampus (Edbauer et al., 2010).

mice intrathecally receiving miR-124, miR-103 or miR-23b which are reported to prevent and treat persistent inflammatory and neuropathic pain (Favereaux et al., 2011; Imai et al., 2011; Willemen et al., 2012). Despite the fact that these miRNA treatments reduced signatures of synaptic modification, neuroinflammation and microglial response, the full extent and the mechanisms of the analgesic effect are not understood to date (Favereaux et al., 2011; Willemen et al., 2012).

CIRCUITRIES AND PROCESSES MODULATING NOCICEPTION AND ENDOGENOUS ANALGESIA

Various studies have identified signatures of neuroinflammation as critical components of diabetic polyneuropathy (Pabreja et al., 2011; Vincent et al., 2011) in addition to the cellular mechanisms that include the classical changes of the diabetic milieu (Bierhaus et al., 2012; Bierhaus and Nawroth, 2012). Pathological neuro-immune communication has been associated with painful neuropathy following traumatic peripheral nerve injury (Myers et al., 2006; Ciaramitaro et al., 2010; Birch et al., 2012). Moreover, CRPS occurring as a complication of bone fracture or tissue injury results from neurogenic inflammatory processes (Goebel, 2011). In humans, a systemic pro-inflammatory profile distinguishes painful from painless neuropathy, and a local pro-inflammatory profile is part of the pathophysiology of small

fiber neuropathy (Üceyler et al., 2007b, 2010). Specialized peripheral neurons, the nociceptors sense inflammatory or neuropathic conditions and respond with increased excitability and sensitivity leading to persisting pain and hyperalgesia (Costigan and Woolf, 2000; Sommer and Kress, 2004; Berta et al., 2008; Üceyler et al., 2009). However, mice lacking receptors for pro-inflammatory mediators in their nociceptor neurons are frequently protected from certain signatures of pathological pain (Andratsch et al., 2009; Schweizerhof et al., 2009; Mair et al., 2011; Quarta et al., 2011). The deficiency in anti-inflammatory cytokines in patients with CRPS (Üceyler et al., 2007a) together with beneficial effect of therapy with glucocorticosteroids support pathophysiological mechanisms associated with neuro-immune dysfunction (Fischer et al., 2010).

Inflammatory processes are also activated in the spinal cord upon peripheral nerve injury and involve microglia activation and leakage at the blood nerve barrier along the entire neuraxis (McMahon and Malcangio, 2009; Beggs et al., 2010, 2012). Microglia activation occurs in diabetic neuropathy in rodents (Wodarski et al., 2009; Beggs et al., 2012; Talbot and Couture, 2012) and has been recognized to be critical for the maintenance of neuropathic pain via the release of pro-nociceptive mediators (Clark et al., 2007). Leakage of the blood nerve barrier or the blood spinal barrier is just emerging in the pathophysiology of neuropathic pain accompanied by changes in tight junction proteins (Echeverry et al., 2011). Tight junction proteins which are critically involved in maintaining the blood–brain barrier like claudin-1 are also new targets, e.g., of miR-155 (Qin et al., 2013).

Deregulated miRNAs can be a consequence or cause of local inflammatory processes such as regulation of nociceptor sensitisation by controlling phospholipase A2 activation (Sun et al., 2012). Analyses of expression profiles of dorsal root ganglia (DRG) containing nociceptor cell bodies reveal that particular miRNAs are deregulated in rodent pain models giving rise to deregulation of miRNA-targeted ion channel expression patterns and metabotropic receptor transcripts in peripheral neurons which presumably cause nociceptor dysfunction (Zhao et al., 2010; von Schack et al., 2011). miRNAs are universal regulators of differentiation, activation and polarization of microglia in normal and inflammatory conditions (Ponomarev et al., 2013). Microglia and macrophage activity is suppressed by specific miRNAs, e.g., miR-124, and it is therefore anticipated that miRNA regulation is critically involved in endogenous inhibition and resolution of inflammation by e.g., resolvins (Ponomarev et al., 2011; Recchiuti et al., 2011). Certain miRNAs are substantially suppressed in glucocorticoid-treated thymocytes by reduced expression of the key miRNA processing enzymes Dicer, Drosha, and DGCR8/Pasha (Smith et al., 2010). This observation is of great relevance since CRPS for example is regarded a prototype disorder of failed termination of inflammation (Birklein and Kingery, 2009). The spinal release of immune modulators affects both spinal synaptic processes and local inhibitory circuits, possibly by classical cytokine-prostaglandin signaling and dys-inhibition of e.g., glycinergic spinal control (Samad et al., 2001; Harvey et al., 2004). Plastic changes at synapses in the spinal dorsal horn promote neuropathic and neurogenic pain via mechanisms involving enhanced nociceptive transmission but also inhibition of spinal endogenous analgesic circuits (Hartmann et al., 2004; Harvey et al., 2004; Fossat et al., 2007; Sandkühler, 2007, 2009; Pernía-Andrade et al., 2009; Zeilhofer et al., 2009; Fossat et al., 2010; Laffray et al., 2012).

For a few miRNAs and long ncRNAs, downstream target proteins have been reported. For example, a conserved long ncRNA seems to modulate sensory neuron excitability by activation of a transcription factor MZF and downregulation of *Kcna2* potassium channel expression and this has been causally associated with neuropathic pain (Zhao et al., 2013). In addition, the functional consequences of miR-103 regulation of voltage-gated Cav1.2 calcium channels and intrinsic excitability of spinal projection neurons have been demonstrated (Favereaux et al., 2011). It is well accepted that certain hereditary forms of migraine are associated with polymorphisms of voltage-gated calcium channels Cav2.1 and Cav2.2 (Pietrobon and Striessnig, 2003). Novel evidence suggests that in particular endogenous pain control systems including GABAergic and opioidergic synaptic signals are down-regulated by miRNAs such as miR-134 or miR-181a (Ni et al., 2012; Sengupta et al., 2013). Some of them link miRNAs like let-7 or miR-339 to opioid tolerance (He et al., 2010; He and Wang, 2012; Wu et al., 2013). In analogy, miRNA neuronal dys-regulation should not only apply to neurogenic or neuropathic pain but very likely the same principles and pathways should apply to other pain syndromes like headaches and in particular hereditary and other forms of migraine.

COGNITIVE, EMOTIONAL AND BEHAVIORAL COMPONENTS OF PAIN

Neuropsychological alterations are present in 65 % of CRPS patients and in particular cognitive impairment and deficits of emotional decision-making may impact their quality of life especially in risky, emotional situations (Apkarian et al., 2004). Emotional deficits and functional alterations in corresponding brain regions are reported in chronic CRPS patients and pain-related fear is one of the strongest predictors of disability in chronic pain disorders (Geha et al., 2008; de Jong et al., 2011).

Specific areas in the brain are actively involved in pain perception and behavior in humans and rodents and structural brain changes are associated with sensory and emotional function in rodent long-term neuropathic pain. In particular, decreased volumes of primary somatosensory and frontal cortex, retrosplenial and entorhinal cortex, anterior cingulate cortex and insula are maintained for months (Seminowicz et al., 2009). Specifically, abnormalities in hippocampus volume are observed in human CRPS and the mouse spared nerve injury (SNI) model. Similar to CRPS patients, SNI mice show increased anxiety like behavior and abnormal contextual fear extinction and this is associated with reduced extracellular signal-regulated kinase (ERK) expression, decreased neurogenesis and altered synaptic plasticity (Kodama et al., 2007; Mutso et al., 2012). Mice with experimental neuropathic pain also show cognitive deficits in novel object recognition and this is associated with deregulation of glycinergic neurotransmission in the hippocampus (Kodama et al., 2011), and may relate to reported enhanced quantal neurotransmitter release in the anterior cingulate cortex of mice with neuropathic pain (Toyoda et al., 2009). Dopaminergic and glutamatergic inputs from amygdala, hippocampus and prefrontal

cortex to the nucleus accumbens participate in the putative emotional control circuits and recent human brain activity studies have examined the nucleus accumbens in the emotional aspects of pain processing (Baliki et al., 2010). These reports further link chronic pain with emotional dysfunction, and maladaptive responses of the nucleus accumbens in neuropathic pain have recently been associated with deregulated miRNAs in this region (Imai et al., 2011).

Brain-specific miRNAs are emerging as regulators of cognition, neuronal plasticity and memory by manipulating synapse structure and function, and specific miRNAs not only control cognition and emotional processes but also neuro-immune communication in the brain (Bredy et al., 2011; Soreq and Wolf, 2011). Mental retardation has been associated with miR-125b, miR-132 and other miRNAs and this arises from effects on dendritic spine morphology and synaptic physiology at hippocampal neurons. AMPA-mediated miniature mEPSC amplitude and frequency are reduced by neuronal over-expression of miR-125b and increased by miR-132 and this is due to differential regulation of glutamate NR2A and NR2B receptor mRNA levels (Edbauer et al., 2010). Other glutamate receptor subunits in the brain are regulated by dopamine through miR-181a which has recently been associated with the pain system (Saba et al., 2012). miR-132 is a highly interesting brain specific miRNA since it is up-regulated by brain derived neurotrophic factor (BDNF) and other growth factors in cortical neurons and this results in an increased expression of synaptic proteins including glutamate receptors (NR2A, NR2B and GluA1), an effect that is attenuated by glucocorticoids (Kawashima et al., 2010; Numakawa et al., 2011). Hippocampal miR-132 mediates stress-inducible cognitive deficits through acetylcholinesterase as a downstream target and specifically in the amygdala miR-34 is associated with the repression of stress-induced anxiety (Haramati et al., 2011; Shaltiel et al., 2013). More generally, happiness, anxiety and depression seem to depend on miRNA expression levels. Specific miRNAs are deregulated in patients suffering from depression and anxiety, and in pre-clinical models of psychological stress (Meerson et al., 2010). Moreover, psychoactive agents, including antidepressants and mood stabilizers, utilize miRNAs as downstream effectors (O'Connor et al., 2012). This further links neuropathic pain to emotional disorders and to the clinical benefit of antidepressants for pain treatment (Dworkin et al., 2007).

PAIN PREDISPOSING GENETIC POLYMORPHISMS

There is evidence that chronic pain, pain sensitivity and responsiveness to analgesic opioids show a sufficient heritability to make these phenotypes highly interesting sources for genetic variability which has an influence on pain (Angst et al., 2012; Hocking et al., 2012; Nielsen et al., 2012). Altered miRNA expression is frequently a consequence of genetic mutations, which may also cause loss or gain of function (Mishra and Bertino, 2009). This may account for significant inter-individual variation in the response to painful stimuli and analgesic drugs. Polymorphisms of specific molecular targets may be associated with certain pain phenotypes and this has emerged for example for a specific calcium channel subunit in a *Drosophila* screen that is conserved in mice and humans (Mogil, 2012; Neely et al., 2010). Several meta-analyses are available of the genetics of pain and associated specific loss- or gain of function polymorphisms with altered pain perception (LaCroix-Fralish et al., 2011; Mogil, 2012). A recent genome-wide association (GWA) study revealed three susceptibility loci for common migraine in the general population, however, systematic association studies are unavailable for DPN and CRPS to date (Chasman et al., 2011). In general, genetic studies have helped to understand the role and downstream mechanisms of individual proteins in pain processing, but specific single nucleotide polymorphism (SNP) related pain disorders apply to small numbers of individuals only and so far do not explain the large variability regarding susceptibility to distinct pain disorders or the responsiveness to different pain therapies in the general population (Dworkin et al., 2007; Attal et al., 2010).

The functional consequences of polymorphisms in miRNA genes and/or their binding sites, the downstream targets of miRNAs and the mechanisms by which miRNAs regulate circuitries and processes modulating nociception and endogenous analgesia are entirely unaddressed. SNPs in miRNAs or their target sites are not only bioinformatically predicted to be associated with the pathogenesis of diseases but are also experimentally validated (Wu et al., 2008; Coassin et al., 2010). It is known that SNPs are less common in miRNAs or their target sites than in other parts of the genome which points to the importance of miRNAs for cellular processes. However, on the other hand SNPs in these sites can affect the expression of a large number of genes when the production of the miRNA is influenced by that particular SNP. Moreover, SNPs in target sites of miRNAs can either modulate/disrupt existing binding sites or create new binding sites for the miRNAs that may then influence gene expression. SNPs in these regions have become a major focus of research and some of them are expected to explain pathogenetic mechanisms in disease development (Glinsky, 2008; Haas et al., 2012). For example, miRNA expression is markedly different between normal tissues and tumor tissues although otherwise miRNA expression is strictly controlled. This might be explained by somatic mutations that are introduced during carcinogenesis. The investigation of genetic variants at miRNAs or their target sites and their association with various diseases is only in its infancy. Initial studies show that these RNA chains might also be involved in neurological diseases such as Parkinson's disease (Martins et al., 2011), Alzheimer's disease (Serpente et al., 2011) or frontotemporal lobar degeneration (Villa et al., 2011). The identification of SNPs in miRNA related regions of the genome might be advantageous over classical GWA study since individual ncRNAs may control and regulate whole networks and pathways involving a multitude of functional proteins. This may open a new avenue that may potentially improve our understanding of extensive inter-individual differences in patients.

TRANSLATION OF PRE-CLINICAL AND CLINICAL RESULTS INTO SOLUTIONS FOR THE BENEFIT OF PATIENTS

As stated above, one of the major hindrances in the way of translating such findings into better therapy of neuropathic and neurogenic pain syndromes is the complexity of their pathophysiology, which even changes during the course of disease. Based on and in analogy to recent developments in the oncology field, an improved understanding of the role of miRNAs in

neuropathic pain might be highly useful for diagnostic and prognostic assessments. For example, aberrant expression or functional deregulation of miRNAs has been associated with the risk for and progression of malignancies and this knowledge is expected to advance the management of certain cancer types through the development of novel personalized miRNA-based diagnostics and therapies (Dreussi et al., 2012; Rossi and Calin, 2013). Increasing evidence indicates that certain miRNAs may be aberrantly expressed or deregulated in certain individuals after tissue injury or with diabetes. This may be associated with increased risk of pain chronification or even responsiveness to analgesic drugs (Ivanov et al., 2012). Therefore, miRNAs are expected to have potential for personalized pain medicine as biomarkers for risk assessment, drug selection and novel therapies.

Therapeutic miRNA regulation has been thoroughly studied and begins to be established in different types of cancer, and the first miRNA targeted drug has entered phase II clinical trials (Lindow and Kauppinen, 2012). In contrast, the potential therapeutic impact of miRNAs in the pain field is as yet largely unexplored. To date, therapeutic approaches have been restricted to rodent models and intrathecal administration and some inconsistencies have emerged; thus miRNA increases in a disease may be either a cause or a feedback reaction to the observed symptoms. For example, although miR-124 is up-regulated after chronic constrictive nerve injury (CCI), intrathecal administration of miR-124 can prevent and treat persistent inflammatory and neuropathic pain (Willemen et al., 2012). Likewise, miR-132 levels are increased in colon biopsies from patients with intestinal bowel disease which should predictably limit inflammation (Maharshak et al., 2013). Importantly, manipulation of miRNAs offers the possibility to control multiple targets including neuro-immune interactions, nociceptive processing and cognitive and affective pathways. Thus, miRNA based therapeutics may have superior advantages by targeting multiple pain-associated genes and miRNA-based drugs may be the most appropriate therapy for the prevention or treatment of neuropathic and neurogenic pain. At least, recent developments provide an optimistic perspective on the evolution of therapeutic ncRNAs despite the drawback of unresolved obstacles for successful delivery and unknown, however unlikely, off-target effects (Cho, 2012).

Manipulating miRNAs as a therapeutic tool presents significant theoretical and practical challenges that must be overcome before this approach becomes a reality. Specific examples involve two of the more straightforward approaches for miRNA modulation, miRNA mimics and antagomirs (**Figure 3**). miRNA mimics consist of over-expressing specific miRNAs that are reduced in the disease state. This mimic approach could be done by introducing synthetic oligonucleotides (natural or modified) or involve over-expression of such miRNAs from an introduced viral vector. Antagomirs are synthetic oligonucleotide sequences that are designed to be inversely oriented (antisense) to miRNAs that are over-expressed in the disease state and which can form Watson-Crick base pairing with the target miRNA. This can either inactivate a miRNA or result in its degradation. Similar to the miRNA mimics, these therapeutic and research tools can also consist of synthetic or modified nucleic acid sequences or be overexpressed from viral vectors.

Alternative methodologies used in experimental settings include miRNA sponges, which are exogenous DNA repeats of the target sequence and can serve to soak up excess copies of the excess miRNA (Ebert et al., 2007). The miRNA sponges may be produced under the regulation of RNA Polymerase III promoters and can generate high amounts of specific target sequences. Another novel yet promising approach involves target protection. In this application, modified antisense oligonucleotides such as LNA or morpholinos are prepared that will be complementary to a specific sequence in the target gene messenger RNA. These are added to the cells, where they bind to the target sequence, block its down-regulation by the miRNA complex and ensure sufficient expression of the target mRNA (Choi et al., 2007). Enhanced and prolonged miRNA suppression and simultaneous targeting of multiple miRNAs can be achieved by inhibitors carrying clustered hairpins based on the "Tough decoy" (TuD) design which offer the advantage of standardized suppression of families or clusters of miRNAs and can be combined with recombinant adenovirus vectors (Haraguchi et al., 2009; Xie et al., 2012; Bak et al., 2013; Hollensen et al., 2013).

An important difficulty that may be predicted for developing neuronal miRNA therapeutics is delivery, since targeting to the brain involves the significant hurdle of crossing the blood–brain barrier. Nevertheless, therapeutic efficacy of certain approaches such as the use of LNA antagomirs has been demonstrated even in primate models, and certain neuronal miRNA therapeutic approaches are now in preclinical development. These studies cover several creative approaches that have been developed to overcome the delivery problem. Thus, ∼20-mer miRNA-size oligonucleotides are indeed unlikely to cross the blood–brain barrier. However, peripheral administration of oligonucleotide controllers of inflammation-regulating miRNAs would change the levels of cytokines, and cytokines can penetrate and affect the brain. Such effects have been demonstrated for miR-132 (Shaked et al., 2009) and miR-212 (Hollander et al., 2010). Other means include direct infection of cerebral neurons with viral vectors that may be adapted for better tropism to neuronal cells (Barbash et al., 2013). Direct introduction of antisense oligonucleotides can alternatively be performed by intracerebroventricular or local stereotactic injection though these would be extremely problematic in pain syndromes. Yet more recent work described the use of rabies virus glycoprotein labeled nanoparticles to enable direct delivery of a miRNA mimic to neuronal cells (Hwang do et al., 2011).

CONCLUSION

Recently, specific miRNAs have been associated with pathological pain and the deregulation of ion channel expression in sensory neurons in rodent pain models (Zhao et al., 2010; Favereaux et al., 2011; Li et al., 2011). Pain conditions have been suggested to deregulate the expression of miRNAs in pain pathways from primary afferent nociceptors to brain areas associated with emotional components of pain perception (Bai et al., 2007; Aldrich et al., 2009; Imai et al., 2011; Kusuda et al., 2011; Poh et al., 2011; von Schack et al., 2011; Arai et al., 2013; Genda et al., 2013; Sakai and Suzuki, 2013). Unique signatures of miRNAs are associated with altered innate immune signaling and secreted miRNAs are even considered

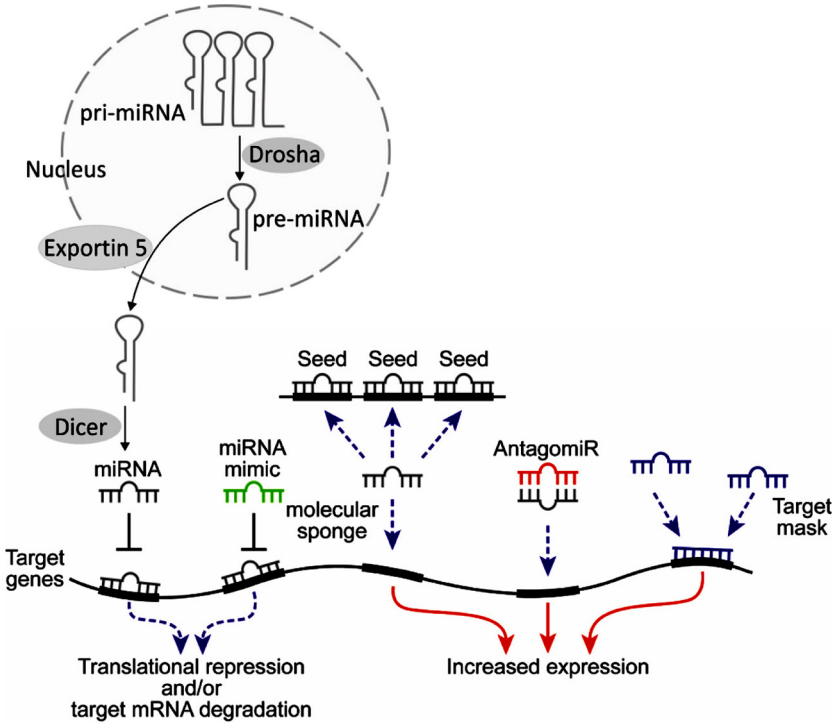

FIGURE 3 | Endogenous miRNAs are generated from primary (pri-) miRNAs via cleavage by the RNAse Drosha into pre-miRNAs in the nucleus. They are exported into the cytosol by Exportin 5 and there are cleaved into active miRNAs by the RNAse Dicer. Depending on the degree of homology, miRNAs trigger translational repression or degradation of target mRNAs (for review see He and Hannon, 2004; Bartel, 2009). Therapeutic manipulations of miRNAs may involve various methods. Host tissue miRNAs (gray) bind to complementary sequences, which are often located in the 3′-untranslated region (3′-UTR) of the target genes. This leads to translational repression, often accompanied by degradation. Mimicking this process, miRNA mimics (green) with similar sequences to those of miRNAs may be designed to target the same mRNAs. Such mimics are synthetic oligonucleotides that are chemically protected against nucleolytic degradation. Alternative routes include molecular "sponges," with several binding sites of a certain miRNA; antagomiRs (red), complementary oligonucleotides to the host miRNA which bind to it and limit its function, and target masks, which bind to part of target miRNAs and compete with their function. Thus, tools exist both for inducing gain of function (red arrows) or loss of function (dashed blue arrows).

a new form of neuroimmune communication and control immune cell activity as well as neuron function (Peng et al., 2010; Bredy et al., 2011; Chen et al., 2012; Ponomarev et al., 2013). miRNAs act at the neuro-immune interface which controls neuronal plasticity and memory but also are linked to the etiology of anxiety and mood disorders (Bredy et al., 2011; Soreq and Wolf, 2011; O'Connor et al., 2012; Shaltiel et al., 2013). Such deficits in the interaction of immune cells and neurons together with cognitive and emotional alterations in patients with neuropathic or neurogenic pain syndromes are hypothesized to converge on miRNA deregulated mechanisms along the entire neuraxis, and alterations in miRNA expression may account for the variation of susceptibility to certain types of pain or even for the responsiveness to analgesics and opioid tolerance (Parsons et al., 2008). Understanding the role of miRNAs in pain mechanisms is suggested to provide great benefit for clinical diagnostic and therapeutic applications.

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MicroRNAs in the pathophysiology and treatment of status epilepticus

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MicroRNA (miRNA) are an important class of non-coding RNA which function as post-transcriptional regulators of gene expression in cells, repressing and fine-tuning protein output. Prolonged seizures (status epilepticus, SE) can cause damage to brain regions such as the hippocampus and result in cognitive deficits and the pathogenesis of epilepsy. Emerging work in animal models has found that SE produces select changes to miRNAs within the brain. Similar changes in over 20 miRNAs have been found in the hippocampus in two or more studies, suggesting conserved miRNA responses after SE. The miRNA changes that accompany SE are predicted to impact levels of multiple proteins involved in neuronal morphology and function, gliosis, neuroinflammation, and cell death. miRNA expression also displays select changes in the blood after SE, supporting blood genomic profiling as potential molecular biomarkers of seizure-damage or epileptogenesis. Intracerebral delivery of chemically modified antisense oligonucleotides (antagomirs) has been shown to have potent, specific and long-lasting effects on brain levels of miRNAs. Targeting miR-34a, miR-132 and miR-184 has been reported to alter seizure-induced neuronal death, whereas targeting miR-134 was neuroprotective, reduced seizure severity during status epilepticus and reduced the later emergence of recurrent spontaneous seizures. These studies support roles for miRNAs in the pathophysiology of status epilepticus and miRNAs may represent novel therapeutic targets to reduce brain injury and epileptogenesis.

Keywords: argonuate, dicer, epilepsy, epileptogenesis, hippocampal sclerosis, miRNA, non-coding RNA, RNA induced silencing complex

INTRODUCTION

A prolonged, non-terminating seizure (status epilepticus, SE) is a neurological emergency that has potential to cause irreversible brain damage. Uncovering the molecular mechanisms by which seizures transition into an uninterrupted state and elucidating the downstream consequence of such seizures on the brain are important if we are to understand and improve treatment of this devastating condition. MicroRNA (miRNA) have recently been implicated in the pathophysiology of SE and their expressional responses, targets and mechanisms represent a new focus of research in this field with potential to better understand the condition, identify novel therapeutics, and develop diagnostic biomarkers.

STATUS EPILEPTICUS

Seizures are the result of abnormal, synchronous discharges of groups of neurons in the brain. Most epileptic seizures are self-terminating, often ending within a minute or less (Chen and Wasterlain, 2006). This is thought to be due to homeostatic mechanisms including inactivation of ion channels, build up of the anticonvulsant adenosine within the extracellular space, the anti-excitatory effect of tissue acidosis, and other changes (Lado and Moshe, 2008; Loscher and Kohling, 2010). However, some seizures do not self-terminate. This can result in the development of SE, which is variously defined by duration, often as 30 min of continuous seizure activity or two or more seizures without complete recovery in between. SE can follow drug withdrawal in patients with epilepsy but also occurs due to a myriad of other factors including CNS infection (Tatum Iv et al., 2001). The molecular mechanisms underlying the transition from seizure to SE are poorly understood, but may involve loss of surface receptors for the inhibitory neurotransmitter γ-amino butyric acid (GABA; Wasterlain et al., 2009).

The threshold of impending SE is defined operationally as over 5 min of continuous seizure activity (Chen and Wasterlain, 2006). Such patients require urgent care. Current treatment is with anticonvulsants such as lorazepam or midazolam (intravenous or intramuscular) or certain anti-epileptic drugs including phenytoin (Rossetti and Lowenstein, 2011; Silbergleit et al., 2012). If SE persists, additional combinations may be necessary including intravenous pentobarbital or the anesthetic propofol (Rossetti and Lowenstein, 2011). There is recent clinical evidence supporting the use of the *N*-methyl-D-aspartate (NMDA) receptor antagonist ketamine (Gaspard et al., 2013; Synowiec et al., 2013).

Status epilepticus has the capability of causing profound brain damage. The central mechanism of seizure-induced neuronal injury is glutamate-mediated excitotoxicity but there is also an important contribution from apoptosis-associated signaling pathways (Meldrum, 1991; Fujikawa, 2006; Engel and Henshall, 2009). Status epilepticus also produces synaptic reorganization, gliosis, inflammation, blood-brain barrier (BBB) damage, and lasting changes to excitability (Coulter, 1999; Vezzani et al., 2011). Despite

major progress, there remains a need to further improve our understanding of the pathophysiologic mechanisms of SE and explore novel approaches to treatment that may better interrupt SE (particularly pharmacoresistant SE) and prevent long-term deleterious consequences (e.g., provide neuroprotection and anti-epileptogenesis).

SE TRIGGERS LARGE-SCALE CHANGES IN EXPRESSION OF PROTEIN-CODING GENES

Status epilepticus results in large-scale changes to expression of genes within affected brain regions such as the hippocampus. The most recent microarray analyses in animal models that featured genome-wide coverage found changes to over 1000 genes after SE (Gorter et al., 2006; Jimenez-Mateos et al., 2008; Lauren et al., 2010). Affected biological processes include metabolism, signaling, transport, immune response, transcriptional regulation, cytoskeleton, glial function, neuronal death, and extracellular matrix organization (Lukasiuk and Pitkanen, 2007; Wang et al., 2010; Pitkanen and Lukasiuk, 2011a). There has been recent progress in identifying transcription factors driving up- and down-regulation of protein-coding genes after SE, including Activating transcription factor 5 (ATF5; Torres-Peraza et al., 2013), CCAAT/enhancer-binding protein homologous protein (CHOP; Engel et al., 2013b), Neuron restrictive silencing factor (NRSF/RE1-silencing transcription factor; McClelland et al., 2011) and Nuclear factor erythroid 2-related factor 2 (Nrf2; Mazzuferi et al., 2013). Uncovering the regulatory mechanisms controlling translation of mRNA transcripts represents a largely unexplored aspect of the molecular pathophysiology of SE.

MicroRNA

MicroRNA represents a potentially critical post-transcriptional mechanism regulating protein levels after SE. miRNA are an endogenous class of small (∼23 nt) non-coding RNA that function to regulate gene expression at a post-transcriptional level by targeting mRNAs and reducing protein production (Bartel, 2004). Biogenesis of miRNAs is a highly conserved process which begins with RNA pol II or III-dependent transcription of a primary transcript (pri-miRNA; Lee et al., 2004; Borchert et al., 2006). miRNAs can be transcribed as single units or as part of polycistronic miRNA "clusters", such as miR-17∼92 (He et al., 2005) and miR-379∼410 (Seitz et al., 2004). The pri-miRNA is processed in the nucleus to a shorter hairpin by the Drosha microprocessor complex (Lee et al., 2003; Gregory et al., 2004). The resulting pre-miRNA is exported to the cytoplasm for further processing by the RNAase III enzyme Dicer. Cleavage of pre-miRNA by Dicer produces the mature miRNA duplex. One strand is selected and incorporated into the RNA-induced silencing complex (RISC) while the other strand is typically degraded.

HOW miRNAs WORK

MiRNAs control protein output by binding to specific, complementary sequences in target mRNAs of protein-coding genes. MiRNA binding sites are most often found in the 3′untranslated region (UTR) but have also been identified at the 5′end and within the open reading frame (ORF; Bartel, 2009). In mammals, miRNAs usually do not have complete complementarity to the mRNA sequence and therefore do not trigger direct cleavage of the mRNA as occurs with the RNA interference pathway activated by short interfering RNAs (Krol et al., 2010). However, mRNA levels of targets are often reduced by miRNA targeting (Lim et al., 2005; Guo et al., 2010). Targeting involves a 7–8 nucleotide "seed" region within the 5′ end of the miRNA binding to the mRNA via Watson–Crick base-pairing, followed by a variable number of further binding sites (Bartel, 2009; **Figure 1A**). The molecular machinery driving this process is the RISC which is a multi-protein complex, comprising members of the argonaute family as well as GW182 proteins (Fabian et al., 2010). Ago2 is critical in loading the miRNA and bringing it together with the mRNA target. The effect of miRNA targeting of a mRNA can be inhibition of translation or deadenylation and subsequent degradation, or both (Fabian et al., 2010). RISCs containing miRNA and their targets may also be sequestered in processing (P) bodies, including at synapses, which is reversible, enabling later release of the mRNA for translation (Cougot et al., 2008; Saugstad, 2010).

IMPACT OF miRNAs ON PROTEIN LEVELS

There are over 1500 miRNAs in the human genome (miRBase v19). These are predicted to regulate the levels of at least one third of translated proteins, although over 60% of protein-coding genes are predicted to have miRNA regulatory sites (Friedman et al., 2009). Such extensive control is possible because a single miRNA may be capable of targeting perhaps 200 mRNAs. Not all mRNAs are targets for miRNA, however, and mRNA sequences with short 3′UTRs often lack miRNA binding sites meaning they are probably not significantly regulated in this manner. Conversely, mRNAs with tissue-specific expression or involved in developmental transitions tend to have longer 3′UTRs with more potential miRNA regulatory sites and these transcripts may be under potent miRNA control (Ebert and Sharp, 2012). MiRNAs also display cell and tissue-specific distribution (Lagos-Quintana et al., 2002; Sempere et al., 2004; Shao et al., 2010; He et al., 2012). In the brain, a large number of miRNAs display cell-specific enrichment that contributes to differentiation and distinguishes neurons from astrocytes, oligodendrocytes, and microglia (Jovicic et al., 2013).

The impact of a miRNA on protein levels of its targets is often only within the twofold range (Baek et al., 2008; Selbach et al., 2008). This may fall below the level capable of producing a phenotype, although under conditions of cell stress limited targeting may be enough to produce a larger effect. Multi-targeting of a single mRNA can produce much stronger effects, in the 10-fold range or targeting of multiple mRNAs within the same pathway (Ebert and Sharp, 2012). Nevertheless, for a given miRNA-mRNA pairing, these variables must be determined experimentally and not assumed based on bioinformatics predictions alone. A miRNA strongly predicted to target a particular mRNA may or may not be in a position to influence its translation. This is particularly important when considering the significance of miRNA changes reported in SE studies that analyzed pooled brain regions (e.g., whole hippocampus) containing multiple cell types expressing diverse transcripts and displaying varying degrees of vulnerability to damage after seizures.

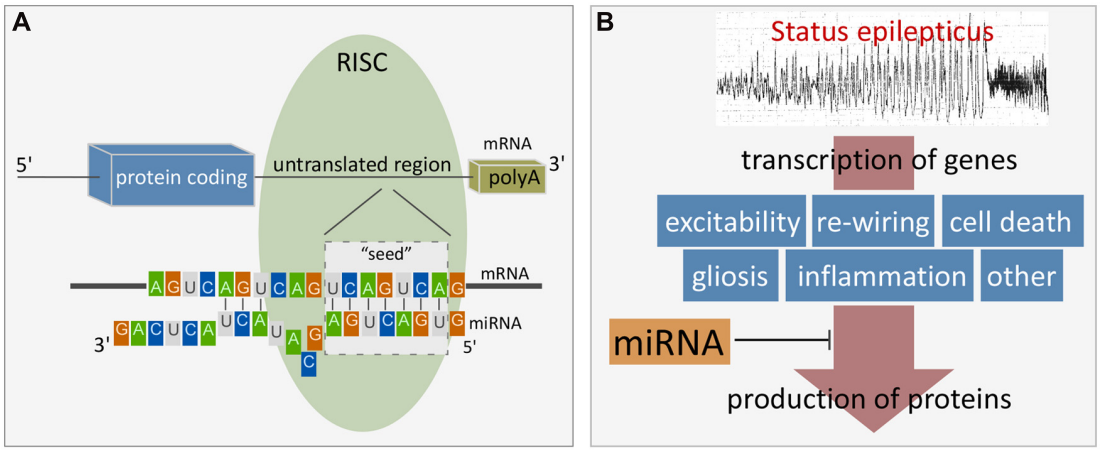

FIGURE 1 | (A) Cartoon showing the site and mechanism of miRNA targeting to mRNA. Figure highlights the seven nucleotide "seed" region critical for miRNA binding to the mRNA target. Additional binding also determines specificity and potency. Alignment is facilitated by initial miRNA loading into the RISC which contains Ago2 and GW182 proteins. Binding typically occurs within the 3′UTR. The result is either degradation of the mRNA target or inhibition of translation. **(B)** Cartoon depicting scheme whereby miRNAs influence gene expression after SE. Status epilepticus results in transcriptional up-regulation of 100s of genes. miRNAs lie downstream of this and exert influence over protein production and as a result, influence post-injury outcomes such as repair, cell death and reorganization. Such miRNAs represent potential treatment targets to interrupt pathogenesis of damage and long-term consequences (e.g., hyperexcitability).

NEURONAL ACTIVITY AND miRNA EXPRESSION

A key function of neuronal miRNAs is in regulating synaptic plasticity in response to neuronal activity (Schratt, 2009; McNeill and Van Vactor, 2012). Dendritic spines are major sites of excitatory communication in the brain and the molecular mechanisms regulating spine size are important determinants of learning, memory, and perhaps, seizure thresholds (Rochefort and Konnerth, 2012). *In situ* hybridization and other visualization techniques reveal enrichment of several miRNAs within dendrites, including miR-134 (Schratt et al., 2006) and miR-138 (Siegel et al., 2009) and neuronal depolarization or glutamate receptor activation results in changes to the expression of these miRNAs. Components of the RISC are also present within the synapto-dendritic compartment (Lugli et al., 2005) and certain dendritically localized miRNAs contain sequences within the precursor form that are identified by chaperone proteins which then shuttle them to the dendrite for later processing to the active mature form (Bicker et al., 2013). The targets of synapto-dendritic miRNAs are involved in dendritic morphogenesis and include p250GAP (by miR-132; Vo et al., 2005; Wayman et al., 2008), Lim kinase (Limk1), and Pumilio2 (by miR-134; Schratt et al., 2006; Fiore et al., 2009) and acyl protein thioesterase 1 (by miR-138; Siegel et al., 2009). This system enables prompt, localized control of neuronal morphology that is responsive to excitatory input (McNeill and Van Vactor, 2012).

ALTERED microRNA EXPRESSION FOLLOWING SE

MiRNAs may serve important roles in SE by regulating protein production during and after seizures, thereby influencing hyperexcitability, injury, and repair responses (**Figure 1B**). Several studies have profiled miRNA responses in the hippocampus in the acute wake (≤48 h) of SE (Liu et al., 2010; Hu et al., 2011; Jimenez-Mateos et al., 2011; Risbud and Porter, 2013). **Table 1** provides a summary of these studies, including the SE model and profiling platform. Combined, the four studies reveal increased expression levels of approximately 100 different miRNAs after SE while levels of about 200 miRNAs decreased. Although there are difficulties with cross-comparing data derived from different platforms and inter-study differences in the models, seizure severity and timing of sampling, a sub-set of miRNAs changed expression in at least two profiling studies. This includes six up-regulated miRNAs (miRNAs-21, -30c, -125b, -132, -199a, and -375) and nine down-regulated miRNAs (miRNAs-10b, -29a, -98, -181b,c, -374, -381, -450a, and -497; **Table 1**). In another study, miR-10b levels were found to be up-regulated after SE (Jimenez-Mateos et al., 2011). If studies that performed analyses of single miRNAs are also included then this list expands to include up-regulation of miR-34a, miR-134, and miR-155, and down-regulation of miR-9, miR-125a, miR-145, and miR-150 (Hu et al., 2012; Pichardo-Casas et al., 2012; Sano et al., 2012; Ashhab et al., 2013; Peng et al., 2013). Also, miR-146a has been consistently found to be upregulated after SE, albeit at later time points (Aronica et al., 2010; Hu et al., 2012; Omran et al., 2012). Thus, over 20 miRNAs appear to show conserved responses to SE. This is likely an under-estimate, however, since most profiling studies had limited spatio-temporal sampling and incomplete coverage of the rodent miRNAome. The roles of a selection of the profiling-identified miRNAs potentially relevant to seizures and brain function are reviewed briefly below. A potential caveat is that in most cases we do not have direct evidence that these miRNAs are functional – uploaded to the RISC – after SE.

CONSERVED miRNAs DETECTED AFTER SE IN PROFILING STUDIES

Among the four studies to have profiled miRNA responses to SE, miR-132 is the most consistently up-regulated miRNA, being

Table 1 | miRNA profiling after status epilepticus.

Reference	Platform	SE model	Time point(s) (h)	Profiled	Regulated[a]	Common
Liu et al. (2010)	Taqman	KA (rat)	24	380	31 (13 Up, 18 Down)	Up: miR-21, miR-30c, miR-125b, miR-132, miR-199a, miR-375
Hu et al. (2011)	Microarray	PILO (rat)	24	113	26 (19 Up, 7 Down)	
Jimenez-Mateos et al. (2011)	Taqman	KA (mouse)	24	380	33 (21 Up, 12 Down)	
Risbud and Porter (2013)	Microarray	PILO (rat)	4, 48	All	265 (77 Up, 188 Down)	Down: miR-10b, miR-29a, miR-98, miR-181b,c, miR-374, miR-381, miR-450a, miR-497

Table summarizes the main studies which have profiled changes to miRNAs in the first 48 h after SE, including the profiling platform, animal model of SE, time point, number of miRNAs studied and the number altered by SE. [a] *Numbers of regulated miRNAs are a guide only – studies differed in terms of what threshold was set for calling a miRNA "regulated" and the application of statistics and/or correction for multiple comparisons. Of note, in the Liu et al study, if miRNAs regulated by at least 1.5-fold are included, then 60 were regulated, with 21 up-regulated and 38 downregulated. Also, miRNAs "not detected" after SE were considered down-regulated in Jimenez-Mateos et al. (2011). Box on far right depicts the commonly regulated miRNAs from these studies (same direction in two or more studies). KA, kainic acid; PILO, pilocarpine.*

identified in 3/4 of the studies (Hu et al., 2011; Jimenez-Mateos et al., 2011; Risbud and Porter, 2013). Up-regulation of miR-132 was also reported in two other studies that looked at individual miRNA responses to SE (Nudelman et al., 2010; Peng et al., 2013) and miR-132 is over-expressed in human temporal lobe epilepsy (Peng et al., 2013). Increased levels of miR-132 are also present in Ago2-eluted samples from the hippocampus after experimental SE, implying it is functional (Jimenez-Mateos et al., 2011).

The role of miR-132 in the brain is increasingly well understood. Expression of miR-132 is associated with synaptogenesis and a number of miR-132 targets are of potential relevance to the pathophysiology of SE (see **Table 2**). Overexpression of miR-132 in hippocampal neurons in culture was shown to cause neurite (Vo et al., 2005) and dendritic (Wayman et al., 2008) sprouting, and increase excitatory currents (Edbauer et al., 2010). Over-expression of miR-132 *in vivo* (∼fivefold was achieved) resulted in an increase in spine density and was associated with a deficit in novel object recognition task (Hansen et al., 2010). In contrast, deletion of miR-132 *in vivo* is associated with decreased dendritic length and branching (Magill et al., 2010) and select defects in synaptic transmission (Pathania et al., 2012). Other predicted targets of miR-132 include MeCP2 (Lusardi et al., 2010), loss of which promotes cognitive deficits, hyperexcitability and seizures (Shahbazian et al., 2002). Targeting of acetylcholinesterase by miR-132 may increase cholinergic tone and influence excitability, hippocampal function, or inflammation (Friedman et al., 2007; Shaked et al., 2009; Shaltiel et al., 2013). The increased hippocampal miR-132 levels that accompany

Table 2 | miRNAs targeted in status epilepticus.

miRNA	Targets	Regulatory control	Biological function(s)	Effect of *in vivo* silencing
miR-34a	Bcl-2, CDK4, SIRT1, Map3k9, Syt	p53 (↑), p73 (↑)	Apoptosis, neuronal differentiation	↓ Hippocampal damage, ↓ apoptosis signaling, no change in SE severity
miR-132	MeCP2, P250GAP, AChE	CREB (↑), NRSF (↓)	Dendritic spines (shape, density), ACh breakdown, Gene silencing	↓ Hippocampal damage, no change in SE severity
miR-134	Limk1, Pum2, CREB, DCX	Mef2 (↑), YY1 (↓)	Dendritic spines (shape, complexity), synaptic plasticity, differentiation	↓ Hippocampal damage, ↓ SE severity, ↓ epileptic seizures
miR-184	Akt2, Ago2	STAT3 (↑)	Apoptosis, interleukin signaling	↑ Hippocampal damage, no change in SE severity

Table lists the four miRNAs which have been targeted in vivo in SE models. Table also includes examples of some validated miRNA targets of potential relevance to seizures/epilepsy, examples of their expressional control (transcription factors which act to either increase or decrease levels of the miRNA), examples of established biological functions and, finally, the impact of targeting the miRNA using an LNA-antagomir in vivo. AChE, acetylcholinesterase; Ago2, Argonaute 2; Bcl2; B Cell lymphoma 2; CDK4, cyclin-dependent kinase-4; CREB, cAMP-response element binding protein; DCX, doublecortin; Limk1, Lim-domain kinase 1; Map3k9, mitogen-activated protein kinase kinase kinase 9; MeCP2, methyl CpG binding protein 2; p250GAP, Rho GTPase activating protein; Pum2; Pumilio2; SIRT1, Sirtuin 1; Syt, Synaptotagmin; YY1, Yin yang 1.

SE and are present in epilepsy may, therefore, influence neuronal morphology and contribute to hyperexcitability or cognitive dysfunction.

Much less is known about the remaining conserved miRNAs from the profiling studies. Most of the functional studies have been in cancer, where their targets have been linked to controlling apoptosis, invasiveness, and cell division. While this may fit with pathways expected to be regulated after SE, it is likely that some (or perhaps most) of the brain targets of these miRNAs in the setting of SE will be different.

Based on available functional studies and known targets, including members of the Bcl-2 family and p53 pathway, increased levels of miR-21, miR-125b, and down-regulation of miR-29a and miR-497 in SE would be expected to have an anti-apoptotic effect (Chan et al., 2005; Mott et al., 2007; Sathyan et al., 2007; Le et al., 2009; Park et al., 2009; Yin et al., 2010; Yadav et al., 2011; Amir et al., 2013). In contrast, down-regulation of miR-10b and miR-98 in SE would be predicted to have a pro-apoptotic effect based on their roles as "oncomirs" (Ma et al., 2007; Ozsait et al., 2010; Foley et al., 2011; Wang et al., 2011). These miRNAs may therefore be involved in the control of apoptosis-associated signaling and regulation of seizure-induced neuronal death (Bozzi et al., 2011; Henshall and Engel, 2013).

Members of the miR-181 family have been linked to promoting cell death (Shi et al., 2008) and may control expression of Bcl-2 family proteins (Ouyang et al., 2012). Astrocytes are particularly enriched in miR-181c, and reduced miR-181b and miR-181c levels promote astrocyte-derived cytokine responses (Hutchison et al., 2013). Thus, miR-181, like miR-146a (Iyer et al., 2012), may negatively regulate inflammatory responses in astrocytes after SE.

For the remaining miRNAs down-regulated in at least two profiling studies – miR-374, miR-381, miR-450a – there is little or no relevant experimental data beyond detection in some cancer models. Whether these represent novel miRNAs with roles in the pathogenesis of SE is uncertain but could be explored in future studies.

CONTROL OF microRNA EXPRESSION AFTER SE

Although we have an increasingly expansive picture of which miRNAs change after SE and in what direction, we know little about the mechanisms controlling miRNA expression itself. No studies have directly explored miRNA regulatory control in SE, however the transcriptional control mechanism for some SE-regulated miRNAs is understood (**Table 2**). Expression of miR-34a is controlled by p53 (Chang et al., 2007; Raver-Shapira et al., 2007) as well as p73 (Agostini et al., 2011), and an inhibitor of p53 prevented miR-34a upregulation after SE (Sano et al., 2012). Expression of miR-132 is regulated by CREB, a stress-activated transcription factor that promotes neuronal survival (Lee et al., 2009). Some CREB-mediated effects may be pro-epileptogenic. Consistent with this, mice with decreased CREB levels develop fewer spontaneous seizures following pilocarpine-induced SE (Zhu et al., 2012). For miR-134, regulatory control is activity-dependent and driven by Mef2 (Fiore et al., 2009). miRNAs have also been identified under control of the transcriptional repressor NRSF. NRSF is implicated in the epigenetic silencing of multiple genes after SE and interference in NRSF function can recover expression and function of genes whose down-regulation is implicated in epileptogenesis (McClelland et al., 2011). MiR-124 is a known NRSF target that is involved in defining the neuronal phenotype. Levels of miR-124 have been reported to both increase (Peng et al., 2013) and decrease (Risbud and Porter, 2013) following SE in rats. These transcription factors and others may exert their influence on the post-SE molecular environment by modulating expression of miRNAs under their control. Their targeting represents potential approaches for modulating miRNA expression in SE.

Time-course studies have noted abrupt increases in miRNA expression, with rapid turn-on followed by restitution to baseline or lower levels after SE (McKiernan et al., 2012; Sano et al., 2012). This type of precise, dynamic response is reminiscent of miRNA responses during brain development (Krichevsky et al., 2003) and after other CNS insults (Lusardi et al., 2010), and supports tight transcriptional control of the spatiotemporal induction of miRNAs. This has implications for studies where only a single time point has been used because in the absence of a complete time course, erroneous conclusions may be drawn about the full response of a miRNA to SE.

THERAPEUTIC miRNA TARGETING IN SE

Targeting miRNAs for therapeutic benefit is gaining increasing attention in multiple fields (Brown and Naldini, 2009; Stenvang et al., 2012). If miRNAs exert significant influence over processes involved in either seizure generation or the pathophysiological consequences of SE then miRNA targeting may have therapeutic potential. Delivery of a miRNA inhibitor or replenishment of an otherwise lost miRNA (e.g., via a miRNA mimic) could alter the excitability of the brain leading to less severe seizures or mitigate the downstream consequences leading to neuroprotection.

Brain-expressed miRNAs represent challenging targets for experimental and therapeutic modulation *in vivo*. First, miRNAs display cell-specific expression and tight transcriptional regulation along with their potential for multi-targeting, the control of which is still poorly understood. This means that delivery of an inhibitor or mimic may require careful timing and the means to control where it goes in the brain. Second, small molecules (e.g., <1000 Da) do not yet exist that selectively target miRNAs, although small molecules have been identified which alter miRNA biogenesis (Shan et al., 2008; Melo et al., 2011). A leading approach is to use antisense oligonucleotides (antagomirs; Stenvang et al., 2012). Modifications such as locked nucleic acid (LNA; Wahlestedt et al., 2000) make these potent and selective miRNA inhibitors and further modifications such as placement of cholesterol (Krutzfeldt et al., 2005) or other tags [e.g., penetratin peptide (Schratt et al., 2006)] facilitate cell entry. Studies show that for a miRNA to be inhibited the antagomir must be in several fold excess (Ebert et al., 2007). The mechanism by which antagomirs reduce miRNA function appears to differ depending on the chemistry of the molecules, and includes activation of degradation mechanisms and sequestration as a heteroduplex (Stenvang et al., 2012). Shorter sequences, so-called "tiny LNAs" which share common seed regions of miRNA families may enable blockade of multiple miRNA members and further potentiate targeting efficacy (Obad et al., 2011). A potentially attractive quality of

antagomir targeting of miRNAs is prolonged suppression of the miRNA. Silencing of miRNAs by antagomirs has been reported to last several weeks in the periphery (Krutzfeldt et al., 2005; Elmen et al., 2008) and after injection into the brain (Jimenez-Mateos et al., 2012). Another challenge is that antagomirs do not cross an intact BBB (Krutzfeldt et al., 2005). To date, this has been overcome by direct intracerebroventricular microinjection of antagomirs (Jimenez-Mateos et al., 2011; Hu et al., 2012; Jimenez-Mateos et al., 2012; McKiernan et al., 2012; Sano et al., 2012). However, BBB integrity is disrupted by seizures (Marchi et al., 2012) therefore systemic injection may be sufficient to deliver antagomirs into the brain after SE. If the site of BBB disruption is limited to the area of pathologic activity then brain penetration may restrict delivery to the site of injury with minimal effects elsewhere in the brain. Alternatively, strategies can be used to temporarily breach the BBB (Campbell et al., 2008), or antagomirs can be given via intra-nasal delivery (Jimenez-Mateos et al., 2012) or encapsulated in a nanoparticle or exosome (Alvarez-Erviti et al., 2011).

Clinical trials are now underway using antagomirs for non-CNS conditions. Miravirsen targets miR-122, which is involved in hepatitis C virus replication and miravirsen was shown to be safe and effective in patients (Janssen et al., 2013). This raises the possibility of using miRNA-based therapeutics for other diseases, including CNS applications (Brown and Naldini, 2009; Stenvang et al., 2012).

miRNA TARGETING IN SE

Four miRNAs have been targeted *in vivo* in experimental models of SE using antagomirs (**Table 2**). The first to be targeted was miR-132 (Jimenez-Mateos et al., 2011). Intracerebroventricular injection of an LNA-modified antagomir targeting miR-132 reduced hippocampal levels of miR-132 when measured 24 h later in mice. Animals in which miR-132 had been silenced and then subjected to SE were found to have significantly less damage to the CA3 subfield of the hippocampus (Jimenez-Mateos et al., 2011). No effects of the antagomirs were reported on seizure severity. The mechanism of the protection is unknown and while miR-132 was confirmed in other experiments to be increased in the RISC, the mRNA targets in the RISC were not explored (Jimenez-Mateos et al., 2011). Whether the neuroprotection has any functional effects is unknown as cognitive tests have not yet been performed. However, a similar degree of neuroprotection in the same model was associated with fewer spontaneous seizures in long-term EEG monitoring studies (Jimenez-Mateos et al., 2008; Engel et al., 2010).

Silencing of two other miRNAs has been reported to alter seizure-induced neuronal death without affecting the severity of SE (**Table 2**). MiR-34a is another miRNA whose up-regulation has been reported in multiple models (Hu et al., 2012; Sano et al., 2012). Increased miR-34a levels promote apoptosis via suppressing anti-apoptotic proteins including Bcl-2 (Hermeking, 2010). However, the pro-apoptotic effect of miR-34a in neurons has been questioned (Agostini et al., 2011). More recently, miR-34a was shown to be a positive and negative regulator of neuronal differentiation, targeting synaptotagmin-1 (Agostini et al., 2011), and Numb1 (Fineberg et al., 2012). Inhibition of miR-34a using antagomir infusions into the ventricle of rodents was reported to reduce seizure-induced neuronal death in one study (Hu et al., 2012), but not in another (Sano et al., 2012). In contrast, targeting miR-184, a miRNA up-regulated by a protective episode of brief, non-harmful seizures, resulted in increased susceptibility to seizure-induced neuronal death in mice (McKiernan et al., 2012). Again, seizure severity was not affected. This supports miR-184 having protective effects against seizure-damage, although no candidate targets of this miRNA are obvious to explain this action (**Table 2**). Together with results of miR-132 and miR-34a, these studies reveal miRNAs as potential targets for modulating cell death after SE.

MiR-134 is another activity-regulated miRNA that has been found to be upregulated after SE in kainate and pilocarpine models of SE (Jimenez-Mateos et al., 2011, 2012; Peng et al., 2013). Levels of miR-134 were also confirmed to be increased in the RISC in Ago2 pull down experiments after SE and there were lower protein levels of two validated targets (Jimenez-Mateos et al., 2012). Targeting miR-134 using intracerebroventricular injections of LNA-modified antagomirs produced silencing of the miRNA lasting several weeks. When mice were injected with the antagomirs 24 h before SE, the resulting seizure severity was strongly reduced. Indeed, the seizure suppression was qualitatively similar to the effect of the anticonvulsant lorazepam in the same model (Jimenez-Mateos et al., 2012). Hippocampal damage in these antagomir pre-treated mice was also strongly reduced, although this may have been secondary to the anticonvulsant effect rather than a direct neuroprotective action.

In further experiments, the authors tested the effect of the antagomirs on the development of epilepsy. Antagomirs were injected 1 h after SE, ensuring the initial brain insult was similar between antagomir and scrambled-control SE mice. In EEG and video monitoring of the mice the antagomir-injected animals displayed ∼90% fewer spontaneous seizures during the next month (Jimenez-Mateos et al., 2012). Seizure frequency remained reduced 2 months later indicating, presumably, a permanent protective effect. Chronic pathologic changes to the hippocampus including progressive neuronal loss, gliosis, and synaptic reorganization were also reduced (Jimenez-Mateos et al., 2012). The mechanism by which silencing miR-134 produces these strong anti-seizure effects is unknown, although *in vitro* experiments suggested they may be Limk1-dependent (Jimenez-Mateos et al., 2012). These findings suggest antagomirs targeting this miRNA could have neuroprotective and disease-modifying effects which might be a new therapeutic strategy for SE.

miRNAs AS BIOMARKERS OF SEIZURE-DAMAGE AND EPILEPTOGENESIS

MiRNAs have been recognized as having potential as non-invasive biomarkers (Scholer et al., 2010; de Planell-Saguer and Rodicio, 2011). Unique expression profiles of miRNAs have been reported in blood and other biofluids in animal models and patients and these may be useful as diagnostics, helping to discriminate between diseases with a similar clinical presentation, provide better stratification of patients, predicting disease course, and responses to therapy. Biofluid miRNA profiles could also have applications in toxicology and as markers of tissue damage. Part of their attraction lies with the conserved and widespread function of miRNAs

in cell physiology and disease but there are also physico-chemical properties of miRNAs that make them suitable biomarkers. Unlike most other forms of RNA, miRNAs are remarkably stable in biofluids, remaining detectable in serum for weeks and they are also resistant to freeze-thaw and pH changes (Chen et al., 2008; McDonald et al., 2011; Blondal et al., 2013). The stability is attributable, at least in part, to binding to Ago2 (Arroyo et al., 2011; Turchinovich et al., 2011) and presence in membrane-enclosed circulating microvesicles such as exosomes (Hunter et al., 2008; Gallo et al., 2012).

There is an emerging consensus that biomarkers would be useful diagnostics in epilepsy (Pitkanen and Lukasiuk, 2011b; Simonato et al., 2012; Engel et al., 2013a). Molecular biomarkers of SE could be used to gauge insult severity, prognosis, and inform the choice of anticonvulsants or administration of anti-epileptogenic treatments, were they to become available. Evidence is emerging that miRNA signatures in biofluids can distinguish between different forms of neurological disease or acute brain injuries (Rong et al., 2011; Balakathiresan et al., 2012; Baraniskin et al., 2012; Haghikia et al., 2012). To date only a single study has looked at miRNA changes in the blood following SE (Liu et al., 2010). This revealed that kainate-induced seizures in rats produce unique miRNA expression profiles in blood that are different from those produced by other acute neurological injuries, including stroke, and hemorrhage (Liu et al., 2010). The study reported up-regulation of 15 miRNAs and decreased levels of 43 miRNAs in blood, although none passed correction for multiple comparisons (Liu et al., 2010). Nevertheless, this supports biofluid miRNA changes as a source of molecular biomarkers of SE. Notably, a number of the commonly regulated miRNAs identified in the hippocampus in profiling studies are found in serum and plasma, including miR-29a, miR-125b, and miR-375 (Blondal et al., 2013). Just as significantly, several miRNAs increased by SE in the hippocampus are not normally present in these biofluids, including miR-132 and miR-134 (Blondal et al., 2013), supporting their detection post-SE as a potential biomarker of seizures or injury.

REMAINING CHALLENGES

What are some of the future challenges? There is a need to identify the targets of seizure-regulated miRNAs. This could be achieved using techniques such as HITS-CLIP, whereby RISC-loaded RNAs are cross-linked to proteins followed by Ago2-immunoprecipitation and sequencing (Chi et al., 2009). Knowledge of the *in vivo* targets of miRNAs in SE models would also lead to better understanding of the mechanisms by which antagomirs produce their effects. The specificity of antagomirs in the brain has yet to be established, although some studies have looked at potential off-target effects (Jimenez-Mateos et al., 2011, 2012). Future studies should explore ways to deliver antagomirs via systemic routes while also including assessment of cognitive effects of miRNA silencing. This is particularly relevant for miR-132 and miR-134 because these directly regulate dendritic spines and small changes to levels of miRNA regulating dendritic spines have been found to produce behavioral phenotypes (Hansen et al., 2010).

There are several directions that could be taken to explore the potential of miRNAs as biomarkers in SE. For example, comparing profiles in different biofluids or between different models, and identifying miRNAs with predictive value for epileptogenesis. More clinical data are needed. For example, are miRNAs profiles in the brain or biofluids altered following SE in patients?

Several of the other commonly regulated miRNAs have yet to be targeted in animal models but likely represent focuses of the future. Combinations of miRNA targeting or delivery could offer ways to more completely block deleterious consequences of SE such as epileptogenesis. Experiments could also explore whether antagomirs can have effects on already established epilepsy. Can a disease-modifying effect be produced once epilepsy is established?

Clearly, miRNA functions are directly relevant to seizure thresholds, but clinical applications of miRNA-based therapeutics for SE would most likely be as disease-modifying post-treatments rather than acute anticonvulsants. This is because antagomirs take time to produce miRNA knockdown and measurable effects on the de-repressed targets. This means they are not realistic prospects for stopping SE, although perhaps there would be an application in super-refractory SE (Shorvon, 2011). Nevertheless, faster or more efficient targeting tools may emerge or indeed we may simply identify better miRNA targets or find ways to target the proteins under their control.

SUMMARY

MiRNAs represent a major additional layer of gene expression control in SE, regulating protein levels within cells in the seizure-damaged brain. As functional studies begin to explore the importance of individual miRNAs in SE we are seeing influences on neuronal death, excitability, gliosis, and neuroinflammation. Many or even most processes dysregulated after SE may be controlled to some degree by miRNA expression. The arrival of miRNA-based inhibitors in clinical trials in other diseases herald translation to the clinic that may eventually also be possible for SE. Translation will be facilitated by focusing on the most critical miRNAs, identifying the molecular targets of miRNAs altered by SE and exploring antagomir delivery routes. Last, miRNAs represent an interesting class of biomarker that may have applications for tracking the severity of injury after SE and whether or not a patient is at risk of long-term consequences such as development or exacerbation of epilepsy.

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MicroRNAs as the cause of schizophrenia in 22q11.2 deletion carriers, and possible implications for idiopathic disease: a mini-review

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The 22q11.2 deletion is the strongest known genetic risk factor for schizophrenia. Research has implicated microRNA-mediated dysregulation in 22q11.2 deletion syndrome (22q11.2DS) schizophrenia-risk. Primary candidate genes are *DGCR8* (DiGeorge syndrome critical region gene 8), which encodes a component of the microprocessor complex essential for microRNA biogenesis, and *MIR185*, which encodes microRNA 185. Mouse models of 22q11.2DS have demonstrated alterations in brain microRNA biogenesis, and that *DGCR8* haploinsufficiency may contribute to these alterations, e.g., via down-regulation of a specific microRNA subset. *miR-185* was the top-scoring down-regulated microRNA in both the prefrontal cortex and the hippocampus, brain areas which are the key foci of schizophrenia research. This reduction in *miR-185* expression contributed to dendritic and spine development deficits in hippocampal neurons. In addition, *miR-185* has two validated targets (RhoA, Cdc42), both of which have been associated with altered expression levels in schizophrenia. These combined data support the involvement of *miR-185* and its down-stream pathways in schizophrenia. This review summarizes evidence implicating microRNA-mediated dysregulation in schizophrenia in both 22q11.2DS-related and idiopathic cases.

Keywords: 22q11.2 deletion syndrome, schizophrenia, microRNA, *MIR185, DGCR8,* copy number variants, genetic risk factor

INTRODUCTION

The 22q11.2 deletion syndrome (22q11.2DS), also known as the velocardiofacial/DiGeorge syndrome, is a phenotypically heterogenous disease which is caused by a hemizygous microdeletion on the long arm of chromosome 22 in the region q11.2. The overall prevalence is 1 in 2,000–4,000 live births (Murphy et al., 1999; Botto et al., 2003; Robin and Shprintzen, 2005). The disorder is associated with a high risk for psychiatric disorder.

In particular, 22q11.2DS patients have an estimated 20–25% risk for schizophrenia or related psychotic disorders such as schizoaffective disorder (Murphy et al., 1999; Chow et al., 2006; Bassett and Chow, 2008; Philip and Bassett, 2011). The deletion is therefore the strongest known genetic risk factor for schizophrenia (odds ratio = 20.3; Levinson et al., 2011), and accounts for approximately 1–2% of all schizophrenia cases (Karayiorgou et al., 1995, 2010; Bassett and Chow, 2008; International Schizophrenia Consortium, 2008; Stefansson et al., 2008). Individuals with 22q11.2DS have variable cognitive and behavioral deficits (Karayiorgou et al., 2010) including relative impairments in social judgment, motor skills, verbal learning, and executive functioning (Chow et al., 2006; Philip and Bassett, 2011). In addition, adults with a 22q11.2 microdeletion have a two- to threefold increase in the risk of generalized anxiety disorder compared to the general population (Philip and Bassett, 2011). The major clinical features of 22q11.2DS-related schizophrenia are largely indistinguishable from those of the idiopathic disease (Murphy et al., 1999; Chow et al., 2006; Bassett and Chow, 2008). Identification of schizophrenia-risk gene/s in the 22q11.2DS deletion region may therefore generate insights into the pathophysiology of schizophrenia in general (Earls et al., 2012).

The size of the 22q11.2 deletion varies. The majority of 22q11.2 deletions (around 90%) are 3 Mb in size and span approximately 60 known genes, while the remaining 10% are 1.5 Mb in size and encompass around 35 genes (Edelmann et al., 1999; Shaikh et al., 2000). Both the larger and the smaller 22q11.2 microdeletions usually result from non-allelic homologous recombination, which is mediated by flanking low-copy repeats (Edelmann et al., 1999). Although the 22q11.2DS phenotype is highly variable, its severity is not correlated with the size of the deletion. This suggests that the minimal 1.5 Mb deletion region is crucial in terms of etiology (Carlson et al., 1997; Karayiorgou et al., 2010).

Initial research to identify schizophrenia-risk genes in the 22q11.2 deletion region proved unsuccessful. The identification of heterozygous loss-of-function mutations in non-deleted schizophrenia patients would be the most obvious human genetic evidence for the involvement of a specific gene in disease susceptibility. Hopes were raised by the identification of heterozygous point mutations in the T-box 1 gene (*TBX1*), which encodes a T-box transcription factor, that resulted in the characteristic

abnormal facies and cardiac defects of 22q11.2DS in patients without a 22q11.2 deletion (Yagi et al., 2003; Zweier et al., 2007). However, no such mutation has yet been identified in schizophrenia patients.

In contrast, recent research in the *Df(16)A*$^{+/-}$ mouse model has generated breakthroughs in our understanding of the underlying biological mechanisms of 22q11.2DS schizophrenia-risk. This engineered mouse strain carries a heterozygous chromosomal deletion which spans a segment syntenic to the human 22q11.2 locus. *Df(16)A*$^{+/-}$ mice show deficits in the synaptic connectivity of hippocampal neurons, including a lower density of dendritic spines and glutamatergic synapses (Mukai et al., 2008). In addition, *Df(16)A*$^{+/-}$ mice display hyperactive behavior and deficits in spatial working memory-dependent learning (Stark et al., 2008). Further characterization of this animal model has suggested that the 22q11.2 microdeletion results in alterations in the biogenesis of brain microRNAs (Stark et al., 2008; Xu et al., 2010). Primary candidate genes in the region are the DiGeorge syndrome critical region gene 8 (*DGCR8*), which encodes a component of the microprocessor complex essential for microRNA biogenesis (Tomari and Zamore, 2005), and the *MIR185* gene (Karayiorgou et al., 2010), which encodes microRNA 185. Both genes are located within the minimal 1.5 Mb deletion region at 22q11.2 (Karayiorgou et al., 2010).

The microRNAs are a class of 21–25-nucleotide small non-coding RNAs. They control the expression of their target genes by binding to target sites in messenger RNAs (mRNAs), typically in their 3′ untranslated regions (He and Hannon, 2004; Meola et al., 2009). In most cases, microRNAs negatively regulate target gene expression through a combination of repression of mRNA translation and promotion of mRNA decay. Each microRNA usually controls up to several hundred target mRNAs, while one mRNA target can be synergistically regulated by multiple microRNAs (Sathyan et al., 2007; Didiano and Hobert, 2008; Drew et al., 2011). This allows microRNAs to integrate different intracellular signals and to regulate various signaling pathways (Johnston and Hobert, 2003; Choi et al., 2007). Accumulating evidence suggests that microRNAs contribute to the basic mechanisms underlying brain development and plasticity (**Table 1**; Fineberg et al., 2009; Schratt, 2009; Im and Kenny, 2012). Neural microRNAs play an important role at various stages of synaptic development, including dendritic arborization (Vo et al., 2005; Yu et al., 2008), synapse formation, and synapse maturation (Caygill and Johnston, 2008; Siegel et al., 2009). Arguably the two most extensively studied examples in the context of synapse development are *miR-132* and *miR-134*. CREB-induced *miR-132* promotes dendritogenesis and spine growth by down-regulating p250GAP (Wayman et al., 2008; Magill et al., 2010). *miR-134* on the other hand is required for activity-dependent dendritic arborization and the restriction of spine growth by targeting Pumilio-2 and Lim-domain containing protein kinase (Limk1), respectively (Schratt et al., 2006; Fiore et al., 2009). Furthermore, investigation of a mouse model displaying conditional knock-out of the microRNA biogenesis enzyme *Dicer* (Schratt, 2009) revealed disrupted morphogenesis of the hippocampus and cortex (Davis et al., 2008), suggesting that undisturbed microRNA processing might be necessary for normal brain development (Xu et al., 2010). These data suggest the possible involvement of microRNA-dependent dysregulation in the pathogenesis of various psychiatric disorders (Forero et al., 2010; Xu et al., 2010), including schizophrenia (Beveridge et al., 2008).

The present review summarizes the various lines of evidence implicating microRNAs as the causal factor for schizophrenia in 22q11.2DS carriers and emerging evidence from expression studies and genome-wide association studies (GWAS) that these mechanisms may also be involved in the development of idiopathic schizophrenia.

THE ROLE OF *DGCR8*

Investigation of *Dgcr8*$^{+/-}$ mice confirmed that heterozygous *Dgcr8* deficiency was responsible for the reduced biogenesis of microRNAs observed in *Df(16)A*$^{+/-}$ mice (Stark et al., 2008; Schofield et al., 2011). *Dgcr8*$^{+/-}$ mice displayed 22q11.2DS-associated cognitive and behavioral deficits, and altered short-term plasticity in the prefrontal cortex (PFC; Stark et al., 2008). This indicates that *DGCR8* heterozygosity, and the resulting alterations in microRNA expression, are sufficient to produce some of the neural deficits observed in 22q11.2DS (Schofield et al., 2011). On the neuronal cell level, *Dgcr8* deficiency resulted in structural changes in dendritic spines and reduced dendritic complexity in the hippocampus (Stark et al., 2008). Schofield et al. (2011) identified alterations in the electrical properties of layer V pyramidal neurons in the medial PFC of *Dgcr8*$^{+/-}$ mice, as well as a decrease in the complexity of the basal dendrites and reduced excitatory synaptic transmission. These functional results suggest that precise microRNA expression is critical for the development of PFC circuitry (Schofield et al., 2011), circuitry which has been reported to be altered in schizophrenia patients (Ursu et al., 2011).

Dgcr8$^{+/-}$ mice also displayed a decrease in the number of cortical neurons, structural deficits in dendritic spines in the PFC, and alterations in synaptic potentiation and short-term plasticity (Fenelon et al., 2011). These alterations might influence functional connectivity (Schreiner et al., 2013), and could be implicated in the observed cognitive and behavioral deficits. In particular, they may explain observed alterations in prepulse inhibition (Stark et al., 2008), which have also been reported in schizophrenia patients (Powell et al., 2009).

Ouchi et al. (2013) showed that heterozygous *Dgcr8* deficiency in mice led to reduced progenitor cell proliferation and neurogenesis in the adult hippocampus. This is of particular interest since alterations in the anatomy, histology, and function of the hippocampus have been consistently reported in schizophrenia patients (Tamminga et al., 2010). Several schizophrenia-associated genes were down-regulated in the hippocampus of *Dgcr8*$^{+/-}$ mice (Ouchi et al., 2013), including the insulin-like growth factor 2 (IGF2), which was recently found to play a crucial role in hippocampal functions such as memory consolidation and fear extinction (Agis-Balboa et al., 2011; Chen et al., 2011a). Interestingly, restoration of IGF2 expression in the hippocampus rescued the observed spatial working memory deficits in *Dgcr8*$^{+/-}$ mice, suggesting that IGF2 contributes – at least in part – to the learning and spatial working memory deficits that are associated with 22q11.2DS-related schizophrenia (Ouchi et al., 2013).

Table 1 | List of individual microRNAs involved in neural development and synapse development/plasticity and their mRNA targets in mice and men.

MicroRNA	Function	Target/s	Reference
microRNAs involved in neural development			
let-7	Promotes neuronal differentiation	HMGA, LIN28, TLX	Nishino et al. (2008), Rybak et al. (2008), Zhao et al. (2010)
	Neural tube closure	MLIN41	Maller Schulman et al. (2008)
miR-7a	Inhibits differentiation of forebrain dopaminergic neurons	PAX6	de Chevigny et al. (2012)
miR-9	Promotes neuronal differentiation	FOXG1, TLX, STAT3, REST, FGF8, FGFR1, FOXP2	Krichevsky et al. (2006), Leucht et al. (2008), Packer et al. (2008), Shibata et al. (2008, 2011), Zhao et al. (2009), Yoo et al. (2011), Clovis et al. (2012)
	Promotes proliferation of early human embryonic stem cell-derived neural progenitor cells	STMN1	Delaloy et al. (2010)
miR-9*	Promotes neuronal differentiation	BAF53a	Yoo et al. (2009, 2011)
	?	coREST	Packer et al. (2008)
miR-17	Inhibits neural differentiation	?	Beveridge et al. (2009)
miR-17/92	Promotes axonal outgrowth in embryonic cortical neurons	PTEN	Zhang et al. (2013)
	Controls spinal neural progenitor patterning	Olig2	Chen et al. (2011b)
miR-34a	Antagonizes neuronal differentiation	Numbl	Fineberg et al. (2012)
	Promotes neuroblastoma and medulloblastoma differentiation	?	Agostini et al. (2011), de Antonellis et al. (2011)
miR-92b	Limits the production of intermediate cortical progenitors	?	Nowakowski et al. (2013)
miR-124	Promotes neuronal differentiation	SCP1, PTBP1, SOX9, DLX2, JAG1, BAF53a, RhoG, Lhx2	Makeyev et al. (2007), Visvanathan et al. (2007), Cheng et al. (2009), Yoo et al. (2009, 2011), Sanuki et al. (2011), Akerblom et al. (2012), Franke et al. (2012)
miR-125	Promotes neuronal differentiation	GLI1, SMO, LIN28, SMAD4	Ferretti et al. (2008), Rybak et al. (2008), Boissart et al. (2012)
miR-128	Inhibits NSC proliferation	BMI1	Godlewski et al. (2008)
miR-132	Promotes synaptic integration and survival of newborn dentate gyrus and olfactory bulb neurons	Nurr1, FoxP2	Luikart et al. (2011), Clovis et al. (2012), Pathania et al. (2012)
	Promotes differentiation of dopamine neurons	Nurr1	Yang et al. (2012)
miR-133b	Modulates maturation of dopaminergic neurons	PITX3	Kim et al. (2007)
miR-137	Promotes neural differentiation of embryonic stem cells	Klf4, Tbx3	Jiang et al. (2013)
miR-200	Promotes olfactory progenitor differentiation	SOX2, ETF3	Choi et al. (2008), Peng et al. (2012)
miR-324-5p	Promotes neuronal differentiation	GLI1, SMO	Ferretti et al. (2008)
miR-326	Promotes neuronal differentiation	GLI1, SMO	Ferretti et al. (2008)
miR-541	Promotes neurite outgrowth of PC12 cells	Synapsin-1	Zhang et al. (2011)
microRNAs involved in synapse development/plasticity			
miR-29a/b	Inhibits spine maturation	Arpc3	Lippi et al. (2011)
miR-34c	Negative constraint of memory consolidation	SIRT1	Zovoilis et al. (2011)

(Continued)

Table 1 | Continued

MicroRNA	Function	Target/s	Reference
miR-124	Regulates neuronal process complexity	RhoG, Cdc42	Yu et al. (2008), Franke et al. (2012)
miR-125a	Reduces number of branched spines	PSD-95	Muddashetty et al. (2011)
miR-125b	Negatively regulates spine morphology	NR2A	Edbauer et al. (2010)
miR-129	Reduces intrinsic neuronal excitability	Kv1.1	Sosanya et al. (2013)
miR-132	Promotes dendritogenesis Promotes spine growth Facilitates memory acquisition Positively regulates LTP Essential for experience-dependent plasticity in visual cortex Negatively regulates circadian clock resetting	P250RhoGap, MeCP2, RFX4	Vo et al. (2005), Cheng et al. (2007), Wayman et al. (2008), Edbauer et al. (2010), Impey et al. (2010), Mellios et al. (2011), Tognini et al. (2011), Scott et al. (2012), Hansen et al. (2013), Remenyi et al. (2013), Wang et al. (2013)
miR-134	Necessary for activity-dependent dendritogenesis	Pum2	Fiore et al. (2009)
	Restricts spine growth	Limk1	Schratt et al. (2006)
	Interferes with memory formation and LTP	Creb1	Gao et al. (2010)
miR-137	Inhibits dendritic morphogenesis	Mib1	Smrt et al. (2010)
miR-138	Negatively regulates dendritic spine size	Apt-1	Siegel et al. (2009)
	Represses axon regeneration	SIRT1	Liu et al. (2013)
miR-146a	Inhibits AMPAR endocytosis	MAP1B	Chen and Shen (2013)
miR-181a	Reduces AMPAR expression and spine formation	GluA2	Saba et al. (2012)
miR-188	Controls dendritic plasticity	Nrp-2	Lee et al. (2012)
miR-219	Regulates circadian clock length	SCOP	Cheng et al. (2007)
miR-375	Reduces dendrite density	HuD	Abdelmohsen et al. (2010)
miR-483-5p	Rescues dendritic spine defects in MeCP2-overexpressing neurons	MeCP2	Han et al. (2013)
miR-485	Regulates presynaptic homeostatic plasticity	Synapsin-1	Cohen et al. (2011)

NSC, neural stem cell; LTP, long-term potentiation; ? – unknown.

The question now arises as to which specific microRNAs are regulated by *DGCR8*. The investigation of *Dgcr8*$^{+/-}$ mice identified 59 down-regulated microRNAs in the PFC and 30 down-regulated microRNAs in the hippocampus (Stark et al., 2008). These down-regulated microRNAs include *miR-185*, which is also located in the minimal 1.5 Mb deletion region at 22q11.2.

THE ROLE OF *MIR185*

Studies of 22q11.2DS mouse models have identified *miR-185* as the top-scoring down-regulated microRNA in schizophrenia-associated brain areas (Stark et al., 2008; Benetti et al., 2009). A recent study by Xu et al. (2013) confirmed the drastic reduction in *miR-185* expression levels in the hippocampus and PFC of *Df(16)A*$^{+/-}$ mice, and showed that this reduction contributed to deficits in dendritic complexity and spine development in hippocampal neurons. In addition, *Dgcr8* deficiency resulted in an approximately 20% reduction in *miR-185* expression in the hippocampus (Xu et al., 2013). This suggests that the pronounced reduction of *miR-185* expression in *Df(16)A*$^{+/-}$ mice – a reduction which is much more pronounced than would be expected by the 50% decrease in gene dosage – may be due to the combined effect of the hemizygosity of the *MIR185* gene and the impaired maturation of the *pri-miR-185* transcript secondary to reduced *Dgcr8* levels (**Figure 1**; Xu et al., 2013). The large reduction in *miR-185* expression renders *miR-185* unique among the genes that are affected by the 22q11.2 microdeletion (Xu et al., 2013).

A recent human study confirmed a down-regulation of *MIR185* expression to 0.4× normal levels in the peripheral blood of patients with 22q11.2DS (de la Morena et al., 2013). This finding suggests that pronounced *miR-185* down-regulation also occurs in patients with 22q11.2DS.

Previous research has shown that *MIR185* is present or enriched in synapses (Lugli et al., 2008; Earls et al., 2012). This may indicate that *MIR185* is of relevance to neural function, since a number of microRNAs have been shown to play a critical role in synaptic plasticity (Schratt, 2009).

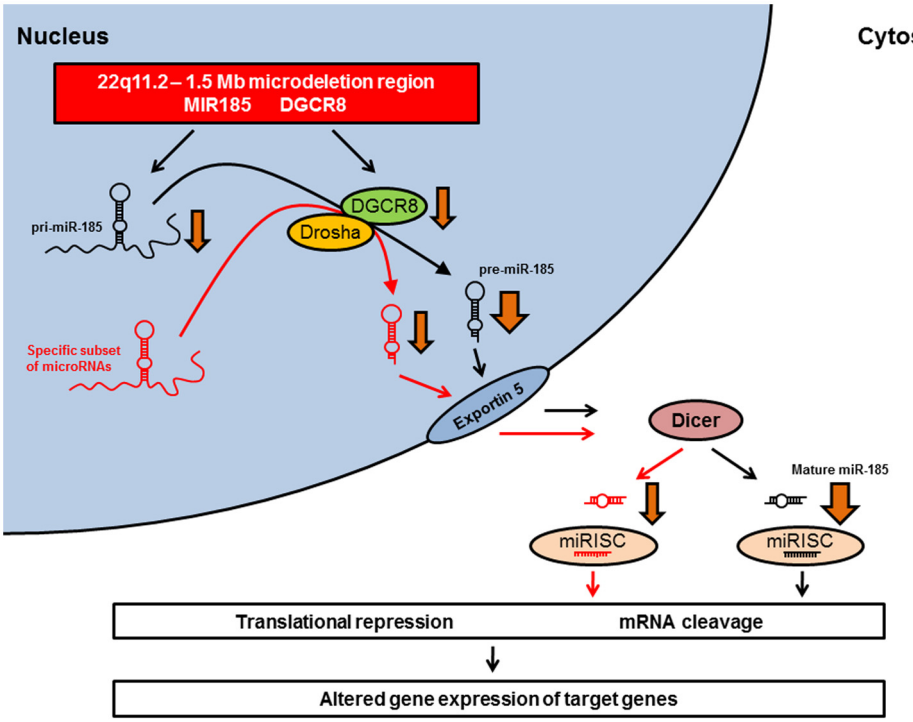

FIGURE 1 | Dysregulation of microRNA biogenesis in 22q11.2DS animal models. The *MIR185* and the *DGCR8* genes are located within the minimal 1.5 Mb microdeletion region on chromosome 22q11.2 and the equivalent region of mouse chromosome 16. The microdeletion leads to a hemizygosity of *MIR185* and *DGCR8*. The heterozygous *Dgcr8* deficiency is responsible for the reduced biogenesis of a specific subset of microRNAs (red) observed in *Df(16)A*$^{+/-}$ mice (Stark et al., 2008). These down-regulated microRNAs include *miR-185* (black). The pronounced reduction of *miR-185* expression (indicated by a broader arrow) may be due to a combined effect of the hemizygosity of the *MIR185* gene and the impaired maturation of the *pri-miR-185* transcript secondary to reduced *Dgcr8* levels (Xu et al., 2013). The resulting alterations in mature microRNA expression levels may lead to altered gene expression of target genes, which might produce some of the neural, cognitive, and behavioral deficits observed in 22q11.2DS. miRISC, microRNA-induced silencing complex.

Earls et al. (2012) identified *MIR185* as a regulator of sarco(endo)plasmic reticulum Ca(2+) ATPase (SERCA2) which maintains Ca^{2+} levels in the endoplasmatic reticulum. The depletion of *MIR185* contributes to SERCA2 upregulation and has been proposed as a mechanism leading to abnormal hippocampal synaptic plasticity in 22q11.2DS mouse models. The microRNA regulation of SERCA2 translation may also be implicated in the elevation of SERCA2 protein observed in the post-mortem brains of idiopathic schizophrenia patients (Earls et al., 2012). These results suggest that microRNA-mediated SERCA2 upregulation at central synapses might be a mechanistic link between 22q11.2DS and idiopathic schizophrenia (Earls et al., 2012).

Further support for the involvement of *MIR185* in schizophrenia is provided by findings that two of its validated targets (RhoA, Cdc42; Liu et al., 2011) are associated with altered expression levels in schizophrenia (Hill et al., 2006; Ide and Lewis, 2010). Cdc42 (cell division cycle 42) is a member of the RhoGTPase family (Hill et al., 2006) and promotes dendritic spine formation (Irie and Yamaguchi, 2002; Tada and Sheng, 2006; Wegner et al., 2008) by regulating the polymerization of the actin cytoskeleton into filopodia (Nobes and Hall, 1995). Cdc42 is activated by Collybistin/ARHGEF9 (Reid et al., 1999; Reddy-Alla et al., 2010), which has recently been identified as a candidate blood biomarker in psychosis (Kurian et al., 2011). RhoA (Ras homologous member A) also belongs to the RhoGTPase family and regulates the destabilization of the actin cytoskeleton (Hill et al., 2006). The activation of RhoA leads to a reduction in the number of dendritic branches and the density of dendritic spines (Nakayama et al., 2000; Hill et al., 2006).

EXPRESSION OF microRNAs IN IDIOPATHIC SCHIZOPHRENIA

Post-mortem studies of human brain tissue have revealed alterations in microRNA expression in patients with schizophrenia. This research is reviewed elsewhere (Beveridge and Cairns, 2012). Briefly, numerous microRNAs have been implicated in the disorder across multiple studies, including 16 microRNAs with increased and 11 microRNAs with decreased expression (Beveridge and Cairns, 2012). Of particular interest in the context of the present review is the study by Moreau et al. (2011). This reported a significant overlap between microRNAs dysregulated in human post-mortem brain tissue and microRNAs previously found to be altered in the PFC of a 22q11.2DS mouse model (Stark et al., 2008; Moreau et al., 2011). This finding supports the hypothesis that findings in 22q11.2DS might be of relevance to idiopathic schizophrenia (Brzustowicz and Bassett, 2012).

GWAS OF IDIOPATHIC SCHIZOPHRENIA

The involvement of microRNA-dependent dysregulation in schizophrenia is supported by the results of the large GWAS of schizophrenia performed by the Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium (2011). In total 17,836 patients and 33,859 controls were investigated. A single-nucleotide polymorphism (SNP) in an intron of *MIR137* was the second strongest finding (odds ratio = 1.12). Four other loci with genome-wide significance in this study contained predicted targets of *MIR137* (*TCF4*, *CACNA1C*, *CSMD1*, *C10orf26*). All four genes have recently been validated as *miR-137* targets (Kwon et al., 2013).

The miRanda database lists 5,487 genes as targets of *miR-137* (John et al., 2004). Interestingly, *ZNF804A* is listed as a validated target (Kim et al., 2012a). This gene has shown strong association with schizophrenia in previous studies (O'Donovan et al., 2008; Williams et al., 2011). Other promising targets include the ubiquitin ligase Mind bomb one (Mib1; Smrt et al., 2010) which plays an important role in neurogenesis and neurodevelopment (Itoh et al., 2003; Choe et al., 2007; Ossipova et al., 2009).

Research in post-mortem brain samples suggests that the functional effect of the *miR-137* risk allele may result in a reduced *miR-137* expression (Guella et al., 2013). Further down-stream this may be responsible for the reduced white matter integrity, smaller hippocampi, and larger lateral ventricles observed in schizophrenia patients with the *miR-137* risk genotype (Lett et al., 2013).

CONCLUSION AND OUTLOOK

Strong evidence suggests that microRNA dysregulation is implicated in the development of schizophrenia in 22q11.2DS patients. This is consistent with the growing recognition of microRNAs as important regulators of gene expression. As microRNAs integrate different intracellular signals and regulate various signaling pathways (Johnston and Hobert, 2003; Choi et al., 2007), the dysregulation of specific microRNAs could lead to the heterogenous phenotype observed in 22q11.2DS.

As summarized above, emerging evidence from expression and genetic analyses suggests that the same microRNA-regulated pathways may also play a role in idiopathic schizophrenia. However, despite a number of systematic investigations of genes in the 22q11.2DS region and the ever increasing number of GWAS data sets (Karayiorgou et al., 2010; Sullivan et al., 2012), no genetic study to date has identified common variation in *DGCR8* or *MIR185* as a risk factor for schizophrenia. This may simply reflect a lack of common functional variants at these loci. This hypothesis is supported by a recent study of genetic regulation of microRNA expression (Gamazon et al., 2012). Gamazon et al. (2012) systematically investigated the relationship between microRNA expression levels (as quantitative traits) and common genetic variation. In this study, no SNP had significant *cis* effects on *miR-185* expression. A small number of SNPs have been reported to have significant *cis* effects on *DGCR8* expression in human monocytes (Zeller et al., 2010), and fibroblasts (Dimas et al., 2009). However, these associations might be tissue-specific, since a recent study of five different human post-mortem brain regions failed to identify any SNP with significant cis effects on *DGCR8* expression (Kim et al., 2012b).

A challenge for future research will be to identify and validate the target genes that are affected by microRNA dysregulation and their respective pathways in a more comprehensive manner (Drew et al., 2011). Such research will improve our understanding of how alterations in microRNA-regulated genetic networks contribute to the pathophysiology of both 22q11.2DS-related and idiopathic schizophrenia.

Idiopathic schizophrenia is a multifactorial disorder for which both genetic and environmental factors exert an impact on disease susceptibility (Sawa and Snyder, 2002). However, very few data are available concerning the influence of environmental factors on microRNA dysregulation. Recent studies in mice showed that environmental factors such as stress resulted in alterations of microRNA expression in the frontal cortex (Rinaldi et al., 2010). Future studies are therefore warranted to investigate the extent to which environmental factors are associated with microRNA dysregulation in schizophrenia.

Further research into the precise role of microRNAs in schizophrenia is important clinically, since modification of microRNA dysregulation would represent a novel therapeutic approach to this devastating and chronic disease. MicroRNAs are excellent candidates for therapy since they regulate multiple targets in various signaling pathways, thereby minimizing the risk of resistance development or compensatory mechanisms (Soriano et al., 2013). This view is supported by several recent studies and reviews, which have highlighted microRNAs as promising pharmacological targets in the treatment of complex diseases such as psychiatric disorders (Im and Kenny, 2012), cancer (Soriano et al., 2013), and diabetes (Mao et al., 2013).

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MicroRNAs in sensorineural diseases of the ear

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Non-coding microRNAs (miRNAs) have a fundamental role in gene regulation and expression in almost every multicellular organism. Only discovered in the last decade, miRNAs are already known to play a leading role in many aspects of disease. In the vertebrate inner ear, miRNAs are essential for controlling development and survival of hair cells. Moreover, dysregulation of miRNAs has been implicated in sensorineural hearing impairment, as well as in other ear diseases such as cholesteatomas, vestibular schwannomas, and otitis media. Due to the inaccessibility of the ear in humans, animal models have provided the optimal tools to study miRNA expression and function, in particular mice and zebrafish. A major focus of current research has been to discover the targets of the miRNAs expressed in the inner ear, in order to determine the regulatory pathways of the auditory and vestibular systems. The potential for miRNAs manipulation in development of therapeutic tools for hearing impairment is as yet unexplored, paving the way for future work in the field.

Keywords: deafness, inner ear, cochlea, vestibule, microRNAs

INTRODUCTION

Hearing loss (HL) is the most prominent neurosensory disorder in humans. Congenital deafness affects at least one in 500 newborns and more than half of these cases are hereditary (National Institutes of Health, NIDCD)[1]. As HL is also age dependent, more individuals can be affected at later stages of their lives. The ear is a complex transducing organ, which consists of both exterior and interior parts. Vibrations of the middle ear's bones mirroring incoming sounds are translated into vibration of the basilar membrane, which in turn leads to mechanotransduction at the organ of Corti in specified cells, the hair cells. Mammalian auditory hair cells, surrounded by non-sensory supporting cells, are the main functional components of the cochlea. They are organized in three rows of outer hair cells (OHC) and one row of inner hair cells (IHC). Their apical actin-based microvilli are referred to as stereocilia. The mechanical stimulus sensed by the stereocilia is converted into an action potential, which in turn transfers the detected sound to the brain (Kelley, 2006). Specifically, coding of sound travels to the higher auditory systems via the brainstem, where there are synapses in the cochlear nuclei and the superior olivary complex (SOC), to the inferior colliculus of the midbrain and finally to the auditory cortex.

For many years, the conventional dogma in molecular biology defined the mammalian genome as one containing protein-coding genes and other repetitive and non-transcribed sequences. The latter was deemed to be non-essential, unless directly involved in RNA synthesis. The last decade has completely reversed this view and the field of non-coding RNAs (ncRNAs) has undergone a dramatic metamorphosis as a portion of these RNAs, microRNAs (miRNAs) are now recognized as having a vital role in gene expression and function. The first recognized miRNAs were lin-7 and let-7 in *Caenorhabditis elegans* (Lagos-Quintana et al., 2001), but since then the number of these regulatory RNAs has grown to 30,424 mature miRNA sequences in 206 species (Kozomara and Griffiths-Jones, 2011)[2]. miRNAs are the most studied and understood forms of ncRNAs, and have been shown to fulfill regulatory functions in many species, including the mammalian system.

miRNAs are small ∼23 nucleotide long RNA species. Pri-miRNAs are transcribed together with other forms of RNA by RNA polymerase II and processed through the Drosha–Dicer pathway (Carthew and Sontheimer, 2009). While still in the nucleus, pri-miRNAs are cleaved by Drosha and exported to the cytoplasm via exportin 5. The product of the cleavage pre-miRNA hairpin is composed of the main -5p and the complementary -3p (formally star) strands that are connected by the stem loop. In the cytoplasm, the pre-miRNA is cleaved by a second enzyme, Dicer, to produce the mature miRNA. miRNAs possess a seed region of 7 nt that determines its target specificity (Bartel, 2009). Upon sequence complementarity, this region will bind to sequences at the 3′ untranslated region (UTR) of target genes. In this fashion, miRNAs inhibit target mRNAs by translational repression and mRNA destabilization (Guo et al., 2010) and regulate gene expression through the RNA interference (RNAi) pathway. Another group of ncRNAs, long intervening noncoding RNAs (lincRNAs), while more elusive in their classification, are considered to have expansive roles in gene regulation (Ulitsky and Bartel, 2013).

How have ncRNAs contributed to the study of the auditory and vestibular systems? miRNAs were first described in the zebrafish inner ear in 2005 (Wienholds et al., 2005), which heralded a number of studies in the mammalian inner ear worldwide. The study of lincRNAs has not yet advanced at the same pace.

miRNAs IN THE INNER EAR

Since miRNAs have become an essential and fascinating aspect of gene regulation in the inner ear, hundreds of miRNAs have

[1]http://www.nidcd.nih.gov/health/statistics/hearing.html

[2]http://www.mirbase.org

been identified using microarrays (Weston et al., 2006; Friedman et al., 2009; Wang et al., 2010a; Elkan-Miller et al., 2011; Zhang et al., 2013). The specific expression of a fraction of these miRNAs has been determined by *in situ* hybridization in the mouse inner ear (**Figures 1** and **2**). There are still many inner ear-expressing miRNAs waiting to be further characterized, both with regards to expression, targets and mechanisms.

The miR-183 family is the most characterized miRNA cluster in the inner ear. This conserved miRNA triad, composed of miR-183, miR-182, and miR-96, is transcribed in one polycistronic transcript. In both zebrafish and the mouse, the triad co-expressed in several neurosensory organs, including the ear, nose, and eye (Wienholds et al., 2005; Weston et al., 2006; Karali et al., 2007). A study demonstrating the role of the miR-183 family in zebrafish by reducing and increasing levels of miRNAs by morpholino (MO) or miRNA injection, respectively, revealed that the miR-183 cluster is crucial for inner ear hair cell and neuronal development (Li et al., 2010). While the miRNAs overlap in their function, given the similarity in their seed regions, they may have different targets, due to the differences in resulting phenotypes following overexpression of each. In the ENU diminuendo mouse with a miR-96 mutation (Lewis et al., 2009). the expression of all three miRNAs remained intact, indicating that the mutation did not disturb the biogenesis of the triad. The mutant mouse showed rapidly progressive HL and hair cell abnormalities. In a search for miR-96 targets, 12 were predicted by miRanda with stringent filtering and five were validated by luciferase assay analysis, Aqp5, Celsr2, Myrip, Odf2, and Ryk. Since the mutation changes miR-96 seed region, the study suggests that a new seed region was created, now binding to new targets, and therefore both loss of normal targets and gain of novel targets could be responsible for the phenotype. In a microarray comparing gene expression between the wild type and the mutant *diminuendo* mouse inner ears, 96 transcripts were significantly affected. Five genes were markedly down-regulated and strongly and specifically expressed in hair cells: Slc26a5 (prestin), Ocm (oncomodulin), Pitpnm1, Gfi1, and Ptprq. None of these genes has a miR-96 binding site, suggesting these are indirect downstream targets, and their change in expression may be causing the diminuendo phenotype.

Further studies on the diminuendo mouse found that miR-96 is responsible for the maturation of the stereocilia bundle of the inner and OHC (Kuhn et al., 2011). Moreover, the synaptic morphology of the mutant mice remained immature, suggesting that miR-96 is involved in cochlear auditory nerve formation.

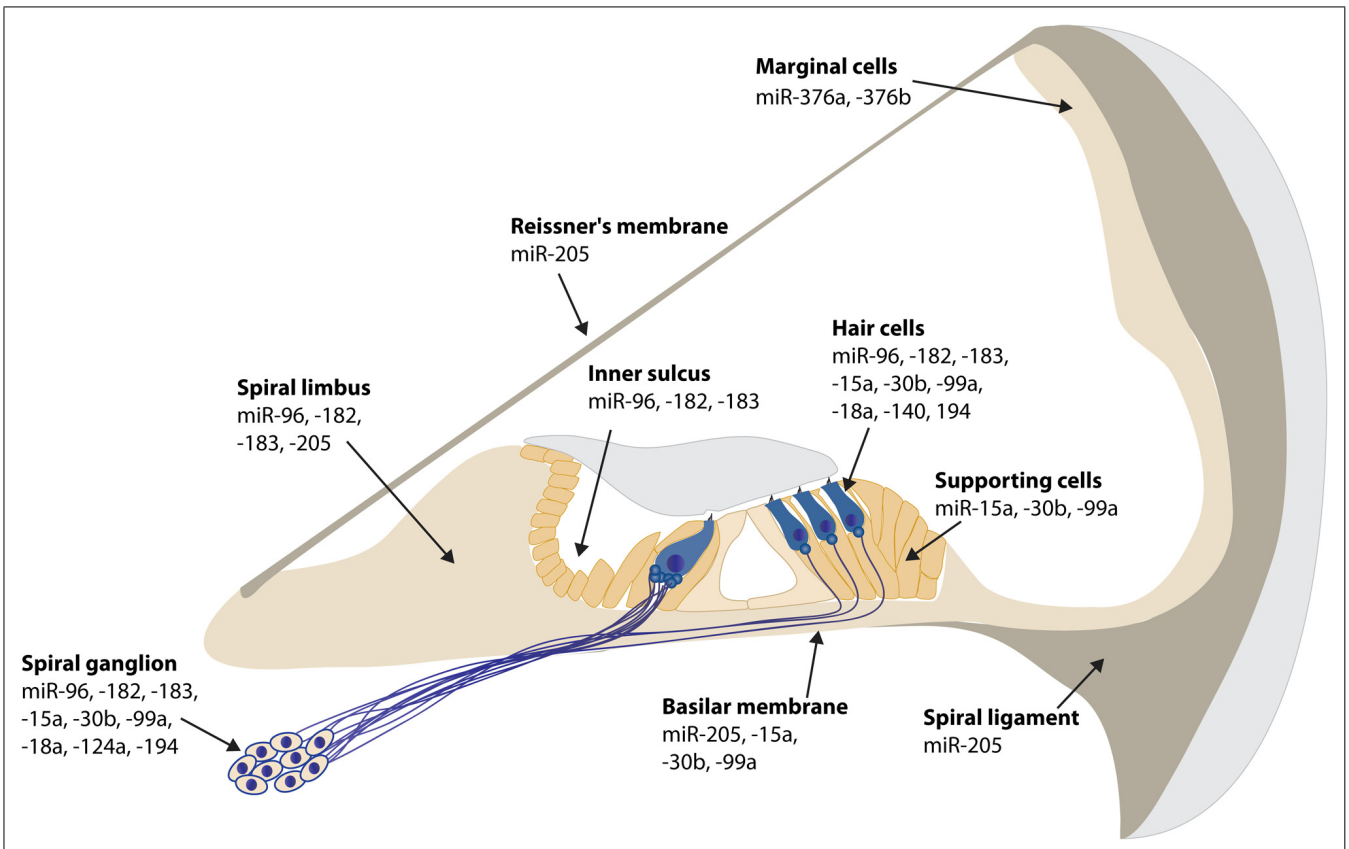

FIGURE 1 | Spatial expression patterns of miRNAs in the mouse cochlea. The expression data is based on *in situ* hybridization experiments of P0 mouse inner ear sections, except for miR-194 at E16.5, miR-140 at P1 and miR-124a and -100a at P5. miR-200b is ubiquitously expressed in all epithelial cells in the inner ear, both in the cochlea and vestibule at P0 (Weston et al., 2006; Friedman et al., 2009; Sacheli et al., 2009; Soukup et al., 2009; Wang et al., 2010a,b; Elkan-Miller et al., 2011; Hertzano et al., 2011; Yan et al., 2012).

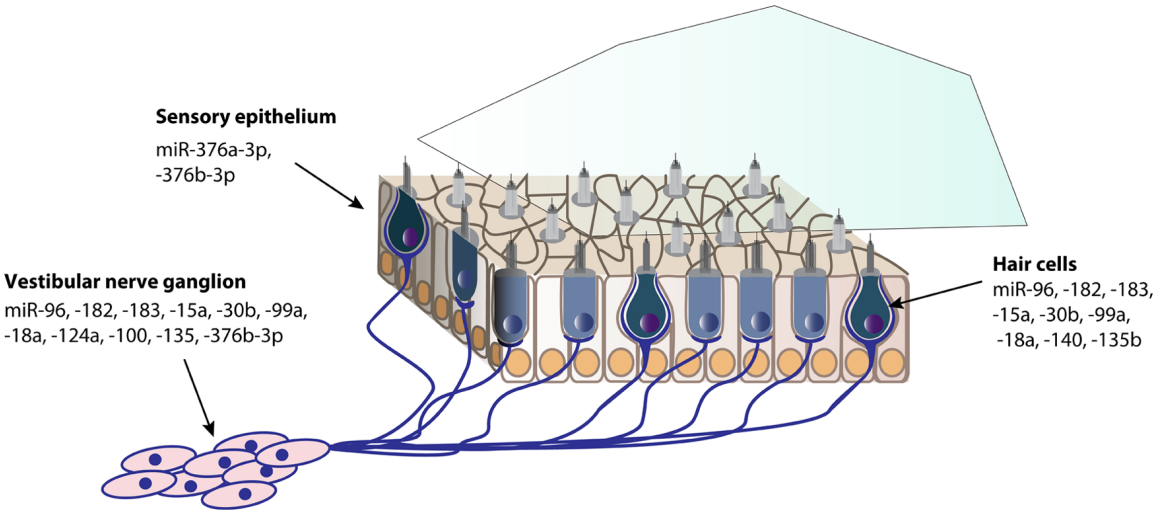

FIGURE 2 | Spatial expression patterns of miRNAs in the postnatal mouse vestibule. The expression data is based on *in situ* hybridization experiments of P0 mouse inner ear sections, except for miR-140 at P1, and miR-124a and -100a at P5 (Weston et al., 2006; Friedman et al., 2009; Wang et al., 2010a; Elkan-Miller et al., 2011; Yan et al., 2012).

Identification of targets is a key ingredient for deciphering the function of an miRNA. Several studies defined targets for members of the miR-183 triad. In a study on cells derived from mouse otocysts, miR-182 promoted differentiation of these cells to a hair cell-like fate (Wang et al., 2012). Moreover, the transcription factor Tbx1 was found to be a target of miR-182. Tbx1 is a critical gene in DiGeorge syndrome, with the phenotype of patients including ear and hearing abnormalities. Tbx1 mouse mutants exhibit severe inner ear defects. Therefore the tightly regulated transcriptional regulation of Tbx1 in the mammalian ear may be influenced in part by miR-182, providing a function in crucial inner ear developmental pathways.

Clic5, a chloride intracellular channel that is associated with stereocilia in the inner ear, was identified as a target of both miR-96 and miR-182 (Gu et al., 2013). Clic5-mutant mice stereocilia bear a resemblance to the morphology of the diminuendo ENU mouse described above, leading to an investigation of its connection to this triad. Clic5 contains a miR-96/182 binding site and its activity was confirmed by a luciferase assay. Liposome transfection of these miRNAs into auditory-cell derived HEI-OC1 led to a reduction of Clic5 at both mRNA and protein levels.

The triad clearly plays an important role in other sensory systems. Inactivation of the three miRNAs in the mouse led to multiple sensory defects, with an emphasis on the loss of this triad in the retina (Lumayag et al., 2013). Not only did the mice have progressive retinal degeneration and photoreceptor defects, but there were significant changes in overall retinal gene expression, as revealed by profiling of microarrays.

Another well-characterized and highly expressing miRNA in the brain, miR-124 (Lagos-Quintana et al., 2002), appears to have an essential role in the inner ear. miR-124 is expressed in the inner ear in neuronal cells in the spiral and vestibular ganglia (Weston et al., 2006). In a study on the differential expression of miRNAs between cochlear and vestibular sensory epithelia, miR-124 was one of the most highly differentially expressed miRNAs, with eightfold higher expression in the cochlea. This suggests a specific role and targets for miR-124 in the cochlear neurons of the inner ear (Elkan-Miller et al., 2011). A recent study, searching for miRNAs that are involved in age-related hearing loss (ARHL; see miRNAs in ARHL), compared differentially expressed miRNAs in sensory epithelia of two mouse strains, C57BL/6J and CBA/J, at several ages. miR-124 was one of four miRNAs that were significantly down regulated in both mouse strains at the age of 9 months, compared to postnatal day (P)21 (Zhang et al., 2013). While more information regarding the targets of miR-124 to elucidate its role in the inner ear is required, this miRNA should clearly have significant influence on gene regulation.

LOSS OF *DICER* IN THE INNER EAR

Dicer is a ribonuclease RNase III-like enzyme that is localized in the nucleus and functions to process double-stranded RNA (dsRNA). Dicer products then exit to the cytoplasm and are further processed into mature miRNAs. Dicer ablation is lethal in zebrafish (Wienholds et al., 2005) and produces no viable embryos in mice (Bernstein et al., 2003).

Dicer has been exploited to study miRNA function in the inner ear. Several conditional knock out (CKO) models have been generated. The first ear-specific Dicer1 CKO was generated using Pax2::Cre for specific expression in regions where Pax2 is expressed (Soukup et al., 2009). Dicer1 was ablated in the inner ear, kidneys and midbrain, resulting in embryonic lethal mice. The CKO mice showed significant loss of most inner ear structures by embryonic day (E)17.5. Although there was initial and normal formation and growth of neurons, the nerves of the CKO animals were rapidly lost after the decay in miRNA expression in the afferent neurons.

The first viable mice were the Pou4f3::Cre-Dicer CKO mice, using Dicer1 to remove miRNAs from hair cells (Friedman et al.,

2009). Pou4f3 was used to express Dicer1 specifically in these cells. The hair cells developed normally, but were degenerated by postnatal day 38. Those that survived at this stage had an aberrant morphology and were presumably dysfunctional. This was confirmed by auditory brainstem response (ABR) testing, which indicated that these mice were deaf. In addition, they showed moderate vestibular dysfunction. Scanning electron microscopy (SEM) demonstrated that the stereocilia of the auditory hair cells were either missing or fused.

Foxg1 was used for site-specific expression to generate Foxg1::Cre-Dicer CKO mice (Kersigo et al., 2011). Overall, these mice had a reduction in anterodorsal regions of the skull, leading to craniofacial abnormalities. As for the ear, it began to develop abnormally around E12.5, with a significant reduction in the size of the ear by E14.5 and in the size of the otocyst by E18.5. This change was concomitant with a reduction in the ossification of the ear and a smaller tympanic ring. miRNA-124 was found to be specifically reduced prior to loss of the neurosensory portions, suggesting this miRNA is required for normal neuronal development.

Another hair-cell specific CKO was generated using the gene responsible for hair cell agenesis, Atoh1, to create Atoh1::Cre-Dicer mice (Weston et al., 2011). Ablation of miRNAs in the hair cells resulted in a progressive loss of OHCs from the base to the apex, with OHCs being more prone to damage as compared to the IHCs. The gradient in the rigorousness of hair cell loss hints that there is also a gradient in the expression pattern of miRNAs along the cochlea.

To study the role of Dicer and subsequent loss of miRNAs in the central auditory pathway, Erg2::Cre-Dicer mice were created (Rosengauer et al., 2012). Work on this CKO demonstrated that Dicer is indispensable for the formation of the cochlear nucleus complex (CNC) and the SOC. In the same study, an additional CKO mouse, Atoh7::Cre-Dicer, was used to dissect later stages of CNC formation. The CNC was comparable to the wild-type mouse, suggesting that Dicer is not crucial for the formation of these structures during late embryonic stages.

It is important to note that when drawing conclusions about Dicer1 function at different stages of development, the tissue-specific ablation is gradual and is specific to the Cre promoter used. Therefore residual Dicer1 expression may exist, leading to a less severe phenotype than expected with removal of this essential enzyme. Furthermore, there is no specificity with respect to miRNAs and rather provide an "all or nothing effect." For specific miRNAs, the approach taken to examine loss of miR-182 in the retina is a relevant approach (Jin et al., 2009), though not yet exploited in the inner ear.

IDENTIFICATION OF miRNA-PROTEIN TARGET PAIRS

Identifying novel or known miRNAs that are involved in specific processes in the inner ear and in the auditory pathways is the relatively easy part of miRNA research. However, discerning the molecular mechanisms, or moreover, the direct targets, is considerably more tedious and challenging. This point is exemplified by the number of miRNAs that have been identified versus the number of validated miRNA targets in the inner ear (**Table 1**). Potential targets of any miRNA can be predicted via TargetScan (Lewis et al., 2005), MicroCosm (formally MirBase; Kozomara and Griffiths-Jones, 2011) and similar prediction programs. These algorithms find a match between the 7-nucleotide seed region of the miRNA and the 3′UTR target mRNAs. It is important to note that this method is based on bioinformatics and relies on sequence similarities between the miRNA and the mRNA. TargetScan and analogous programs cannot eliminate potential targets on the basis of tissue specificity.

After the initial bioinformatic analyzes, each miRNA/gene target must be validated by experimental techniques. There are several approaches for this validation. The most commonly used *in vitro* technique is the luciferase assay. This quantitative assay system was developed originally to assess promoter strength. In the miRNA field this technique is used to study whether there is a direct interaction of a miRNA and a 3′UTR of a potential target gene. Typically the miRNA is cloned into one vector and a 3′UTR is cloned in-frame with luciferase. If the gene is a "true" target, there will be no bioluminescence. If the miRNA cannot interact with the 3′UTR, luciferase will be produced continuously. If a direct interaction between the miRNA and gene target is found, one must show that the mutation in the seed region of the miRNA can abolish the binding. To demonstrate that miR-182 is a direct target of Sox2, a luciferase assay was performed both with a luciferase reporter vector with the 3′UTR of Sox2 and a mutated version of the 3′UTR at the seed region of miR-182 (Weston et al., 2011). The mutated 3′UTR could not bind miR-182 and the decrease in luciferase activity that was observed in the wild-type construct was lost.

To demonstrate an interaction in a more "*in vivo*" approach, anti-miRNAs are used. These short oligonucleotides are used to transfect either cell lines or cochlear cultures, and quench the endogenous miRNAs. The outcome of the antagonism is then probed either at the mRNA level, using qRT-PCR, or at the protein level, using western blot analysis, of the gene target. After confirming direct binding between miR-182 and the Tbx1 3′UTR by luciferase assay, degradation of the target on an mRNA level was tested (Wang et al., 2012). Isolated IHC infected with rA-miR-182 and transfected miR-182 inhibitor were collected and harvested to explore Tbx1 transcription. In the presence of miR-182, the mRNA levels of Tbx1 were restored as compared to infected cells, suggesting target inhibition. Checking the expression level of the predicted target gene by western blot can also provide evidence for miRNA-gene target interaction. Skin samples from cholesteatoma patients and control individuals were analyzed for protein levels of the putative miR-21 targets, PTEN, and PCDC4 (Friedland et al., 2009). In 75% of the cases, there was a substantial reduction in the levels of both proteins, validating the predicted targets of the miRNA.

Target recognition may be compromised as a result of a mutation, as was suggested for some of the human miR-96 mutations (Mencia et al., 2009). Given a change in the nucleotides that define the specificity of the miRNA, the miRNA might lose its ability to regulate its original targets. This hypothesis was examined with the human miRNA mutations. As two different mutations in the seed region of MIR-96, it was appealing to consider whether there are any new acquired targets. However, they could not detect any targets that are regulated by the "new" seed region of either of the two mutations.

Table 1 | Validation of miRNA-gene targets found in the inner ear.

miRNA	Gene target	Experimental system used	Reference
miR-183	TAO kinase 1 (Taok1) Early growth response 1 (Egr1) Insulin receptor substrate 1 (Irs1)	Rat cochlear organotypic cultures transfected with antisense morpholinos.	Patel et al. (2013)
miR-182	SRY-box containing transcription factor (Sox2)	*In situ* hybridization; luciferase assay in HEK293 cells	Weston et al. (2011)
miR-182	T-box 1 (Tbx1)	Luciferase assay in COS1 cells; overexpression of miR in cultured otic progenitor/stem cells.	Wang et al. (2012)
miR-96, miR-182	Chloride intracellular channel 5 (Clic5)	Co-expression in mouse auditory HEI-OC1 cells; luciferase assay in A549 cells; down-regulation of target.	Gu et al. (2013)
miR-15a	Solute carrier family 12 (sodium/potassium/chloride transporters), member 2 (Slc12a2), Claudin (Cldn12) Brain-derived neurotrophic factor (Bdnf)	*In situ* hybridization; luciferase assay in HEK-293T cells.	Friedman et al. (2009)
miR-21	Phosphatase and tensin homolog (Pten)	Down-regulation of target in cholesteatoma; inhibition of miR.	Friedland et al. (2009), Cioffi et al. (2010)
miR-21	Programmed cell death 4 (Pdcd4)	Western blot on cholesteatoma skin samples.	Friedland et al. (2009)
miR-135b	PC4 and SFRS1 interacting protein 1 (Psip1-p75)	Luciferase assay and qRT-PCR on Cal51, breast carcinoma, cells; inhibition of miR.	Elkan-Miller et al. (2011)
miR-200b	Zinc finger E-Box binding homeobox 1 (Zeb1)	Global gene expression analysis; complementary patterns of expression validated with *in situ* and immunohistochemistry	Hertzano et al. (2011)

MECHANISMS OF miRNA FUNCTION IN THE INNER EAR

Roles of miRNAs in the inner ear can be also studied through identification of the overall intracellular pathways they are involved in. As such, proof of principle methods to check the global effect of the miRNA regulation using cellular assays, such as BrdU incorporation for proliferation or nuclear condensation by propidium iodide and caspase 3 activation for apoptosis. The latter was incorporated into a study to induce HL by means of exposure to high frequency noise and aimed to assess the amount of apoptotic hair cells (Patel et al., 2013). From these experiments they learned that the amount of nuclear condensation, an explicit sign of apoptosis, probed with the DNA intercalating agent propidium iodide is comparable following noise exposure. In a different study, set to investigate whether certain miRNAs can promote proliferation of cells in the chick inner ear, basilar papilla were cultured in the presence of BrdU a cell cycle marker that is incorporated instead of thymidine during DNA synthesis (Frucht et al., 2010). Cells were transfected with pre-miRNA181a or anti-miR181a and imaged. A significant number of new hair cells could be observed, providing a role of miR-181a in the pro-proliferative process.

Possibly the most direct method to study the involvement of miRNAs in inner ear mechanisms is in a model animal. Both zebrafish and mice are used to generate knock-out model systems of a single miRNA or miRNA family. Studying these models provides a global indication of phenotypes and can provide information on the targets and the signaling networks in which these miRNAs are involved. More specifically, to study the roles of miR-15a-1 and -18a in zebrafish development, antisense-oligonucleotide MOs were injected into zebrafish 48 h post-fertilization (Friedman et al., 2009). Both morphants showed a reduction in hair cell number and different abnormalities in inner ear structure, indicating that the two miRNAs act in parallel but different pathways. Additional models may be obtained from a resource of miRNA reporter and conditional knockout mouse lines (Park et al., 2012).

EAR-RELATED PATHOLOGIES AND miRNAs

While not prevalent, a number of mutations in miRNAs have been associated with human HL. The first mutations found were in two unrelated Spanish families (Mencia et al., 2009). This discovery provided strong evidence that two different mutations in the seed region of MIR-96, +13 G>A, and +14 C>A, are sufficient to lead to dysregulation of the miRNA, with the end result of progressive HL. An additional mutation was found in MIR-96 in an Italian family during a screening for miRNA mutations in 882 patients with NSHL (Solda et al., 2012). A mutation in the seed region of miR-96-3p, +57 T>C, is associated with HL in this family with progressive HL. The +57 T>C mutation is predicted to lead to alteration of the secondary structure of the pre-miR-96 hairpin. There was considerable reduction in the expression of both miR-96-5p and -3p. The 5p of a miRNA, together with its complementary strand 3p miRNA, form the pre-miRNA, which is then cleaved by Dicer. While miR-96

transcripts were shown to be reduced in the +13 G < A miR-96 mutation (Mencia et al., 2009), but there was no change in miR-3p, suggesting that the biogenesis of the pre-miRNA is normal. While the mutated miR-96 is degraded, the mechanism is still unknown.

In an effort to determine whether the miRNA-183 cluster is further involved in deafness, predicted target genes of the miR-183 miRNA, expressed in the inner ear, were screened in 150 Americans with autosomal dominant NSHL and 576 Iranians with autosomal recessive NSHL (Hildebrand et al., 2010). A miRNA binding site was predicted in the 3′UTR of radixin, a gene associated with DFNB24 deafness. A variant was found in an Iranian family, c.*95C>A, predicted to alter the binding site of miR-96/182 and create a new miRNA binding site for miR-507 and -557. However, during the validation process, no correlation was found between either of the miRNAs and radixin. It appears that mutations affecting gene regulation of the miR-183 family are not typical causes of a deafness phenotype.

miRNAs IN AGE-RELATED HEARING LOSS

While hearing impairment does not spare any population, the aging population is hardest hit with this sensory loss. In the aging population, 43% of individuals over the age of 65–75 have a HL (National Academy on an Aging Society)[3]. ARHL has both genetic and environmental contributions. There is growing evidence that miRNAs are involved in cell senescence, death and aging (Inukai and Slack, 2013). To investigate whether miRNAs are involved in regulation of ARHL and the processes leading to it, sensory epithelia were dissected from two mouse strains at several ages, ranging from 21 days after birth (P21) to 16 months (Zhang et al., 2013). They hybridized the isolated RNA from each group on a GeneChip microarray, probing for all known miRNA genes, and differential expression of miRNAs was examined. In both strains, more miRNAs were downregulated from P21 to 9 or 16 month. Moreover, there were a few miRNAs that were differentially expressed in each one of the strains. The data verified that two miRNAs, miR-29a and -34a, which have been implicated in apoptotic pathways, are up-regulated and the two miRNAs, miR-181 and -183, which have been shown to have roles in proliferation and differentiation, are down-regulated

While it is believed that a major cause of ARHL is the death of hair cells, other age-related changes in the central auditory pathways cannot be ruled out. It would therefore be useful to examine the miRNA expression profile in the SOC of aged mice as well. In addition, with the aid of RNA-Seq techniques that have become relatively common and less expensive, it is anticipated that additional miRNAs will be found to play a role in ARHL using this technology.

miRNAs IN THE MIDDLE EAR

Otitis media (OM) is the most common cause of HL in children. OM is an inflammatory disease of the middle ear mucosa (Lieberthal et al., 2013). While OM is predicted to be multifactorial, with bacterial infections as a contributing factor, its etiology is largely unknown. The cell wall of gram-negative bacteria is partly composed of lipopolysaccharides (LPS), which upon interaction with the host, induce inflammation. Human middle ear epithelial cells (HMEECs), treated with LPS to trigger inflammation, were used to study miRNAs that are differentially expressed in this model system of OM (Song et al., 2011). A gene expression analysis using microarrays led to the identification of 15 differentially expressed miRNAs in HMEECs treated with LPS versus controls, five of which were upregulated and 10 were downregulated. mRNAs that are predicted to be targeted by the upregulated miRNAs are involved in developmental processes, response to biotic stimuli, acute inflammatory responses, and regulation of cell growth, while the downregulated miRNAs are involved in developmental processes, cell differentiation, endocytosis, cell communication, the NFkB cascade, complement activation, innate immune response and cell adhesion. This is the first study to implicate miRNA regulation in OM.

miRNAs AND APOPTOSIS IN THE INNER EAR

Reactive oxygen species (ROS) are important intercellular messengers; however, when in excess, these species underlie processes such as cell death and apoptosis by modulating the expression of many genes (Circu and Aw, 2010). ROS have shown to be involved in HL and specifically hair cell death (Kopke et al., 1999). Moreover, they have been found in human inner ear perilymph derived from patients with sensorineural HL (Ciorba et al., 2010). To explore whether miRNAs are involved in ROS production in the ear, an *in vitro* cellular model system was used. Tert-butyl hydroperoxide (t-BHP) was used to promote generation of ROS in HEI-OC1 cells derived from the organ of Corti (Wang et al., 2010b). The miRNA expression profile was determined for the t-BHP treated cells; 35 miRNAs were found to be upregulated, while 40 miRNAs were downregulated. The treatment also modulated the expression of many mRNAs, and most relevant, changes in miRNAs were associated with changes in mRNA expression of their predicted targets. Specific examples of predicted miRNA-target pairs were IGF-1, PIK3R1, and PTPN11, which were downregulated, with upregulation of miR-29a, miR-17, and miR-200c, respectively. These results suggest that as a result of oxidative stress, the IGF-1 mediated signaling was altered due to increased transcription of miRNAs in this ROS model.

Antibiotic-induced HL is a major factor in ototoxicity. The potential link between aminoglycoside toxicity and miRNA regulation and its effect on the inner ear was examined (Yu et al., 2010). Kanamycin ototoxicity was induced in mice by subcutaneous injection and inner ears were analyzed. In response to the treatment, the mice exhibited a reduced ABR response, which deteriorated as a function of time. Cell death, evaluated by the TUNEL assay, was increased in particular in the stria vascularis, supporting cells and spiral ganglion cells. Due to their previously known role in apoptosis, levels of the miR-34 family were examined in RNA extracted from cochleae of treated mice by qRT-PCR analysis. Both miR-34a and miR-34c were significantly elevated, as compared to untreated controls. This data suggested that apoptosis in the inner ear, followed by hearing damage in this model previously linked to programmed cell death, is partly mediated by members of the miR-34 family.

[3]http://www.agingsociety.org/agingsociety/

miRNAs and REGENERATION IN THE INNER EAR

An early study in miRNAs and regeneration appeared soon after the first report of miRNAs in the mammalian inner ear (Tsonis et al., 2007). The adult newt has the ability to regenerate body parts, including the cells of the inner ear, by transdifferentiation of terminally differentiate cells. In an effort to identify potential changes in gene expression during this process, miRNA profiles were examined during hair cell (and eye lens) regeneration. The level of expression of let-7 miRNAs were found to be significantly reduced. While there have been several studies on lens regeneration and miRNAs since then, no additional studies on the ear have been reported.

The avian auditory sensory epithelium, the basilar papilla, is different from the mammalian sensory epithelium not only in its structural organization, but also in its ability to regenerate following hair cell loss. As in the mammalian cochlea, in the basilar papilla, both hair cells and supporting cells can be found. Upon injury of any kind, such as noise or ototoxicity, there are new hair cells produced from de-differentiation of supporting cells (Balak et al., 1990). Supporting cells of birds that were exposed to acute noise will re-enter cell cycle and within 4–5 days of trauma, new hair cells could be found in the basilar papilla (Stone and Cotanche, 2007).

To elucidate the role of miRNAs in the intracellular signaling pathways of chick hair cell regeneration, forskolin, a compound known to induce proliferation of supporting cells to hair cells, was applied on basilar papilla cultures (Frucht et al., 2010). The miRNA expression profile was evaluated using microarray analysis. miR-181a, which was greatly enriched in the proliferating basilar papilla and as it had previously been identified to have a role in promoting proliferation in a human leukemia cell line, was selected as a hair cell proliferation candidate. Overexpression of miRNA-181a was indeed able to stimulate proliferation within the basilar papilla, with new cells labeling with the hair cell marker myosin VI. A subsequent study further explored miR-181a involvement in the pro-proliferative processes in chickens (Frucht et al., 2011). To this end, the hair cells of the basilar papilla were destroyed using streptomycin. Down-regulation of this miRNA inhibited proliferation during regeneration, rather than preventing hair cell death, providing not only its mechanism in the process, but a promising candidate for regeneration.

FUTURE OF miRNAs IN THE INNER EAR

High-throughput sequencing for RNA, dubbed RNA-seq, has facilitated the study of miRNAs dramatically (Oshlack et al., 2010). RNA-seq is being used to evaluate miRNA expression with a comparison of multiple sets of conditions. The large datasets obtained can be narrowed down to a smaller set of miRNAs to be evaluated in their role in regulation and gene expression. While RNA-seq has been used in multiple fields to identify and characterize miRNAs, this technology has still not been exploited in the inner ear field.

The field on ncRNAs in the mammalian inner ear is still very much in development. While there has been tremendous progress in the last decade, there are areas that are still in their infancy. One such area is that of lincRNAs. lincRNAs are relatively long stretches of RNA larger than 200nt (Ponting et al., 2009). They were identified relying on knowledge from protein-coding transcripts. Both coding and non-coding transcripts have particular chromatin signatures consisting of H3K4me3 and H3K36me3. By identifying K4–K36 domains that lay outside known protein-coding loci, lincRNAs could be methodically identified (Guttman et al., 2009). Unlike miRNAs, lincRNAs have no shared structural characteristics; their biogenesis and processing is unique, as well as their mode of action. Therefore it is not straightforward to identify and study them. An additional factor that hampers the research in the field of these new species is that lincRNAs are extremely cell- and tissue-specific, making their discovery particular to each system. Although there is some evolutionary conservation between species, it is much less prominent than the one observed in coding RNA transcripts, adding to the complexity of their identification. As opposed to miRNAs, the biological functions of lincRNAs are largely unknown. Moreover, some of the already described roles of the lincRNA are variable and are not necessarily mutually exclusive (Da Sacco et al., 2012). lincRNAs have been found to act as gene activators, gene suppressors, cis and trans gene expression regulators, and chromatin modificators.

LincRNAs have been shown to play a critical role in the development and regulation of the sensory systems. As for the long ncRNA species, lincRNAs have been found expressed in the mouse retina (Mustafi et al., 2013) and suggested to be associated with retinal and visual maintenance in mammals. Another study showed that the taurine upregulated gene 1 (Tug1) lincRNA is required for differentiation of the murine retina acting via regulation of the cell cycle (Young et al., 2005). Two studies have reported lincRNAs in the inner ear. MEG3, a lincRNA implicated in leukemia (Heuston et al., 2011), was examined following microarray detection experiment of enriched transcripts in a mouse inner ear library (Manji et al., 2006). Following detection of its expression in the developing otocyst, spiral ganglion, stria vascularis, Reissner's membrane, and greater epithelial ridge (GER), as well as hair and supporting cells, MEG3 was hypothesized to play a role in pattern specification and differentiation during otocyst development and maintenance terminally differentiated cochlear cells. *Rubie* was identified as an inner-ear specific lincRNA upstream of the *Bmp4* gene (Roberts et al., 2012). Rubie was predicted to be the gene mutated in epistatic circler (*Ecl*) mice, contributing to its vestibular phenotype. There is clearly room for a comprehensive investigation of lincRNAs in the inner ear.

One of the most exciting developments in the field has been the generation of a novel *in vitro* model system for the inner ear (Koehler et al., 2013). Mouse embryonic stem cells (ESC) were differentiated in a step-wise manner into a 3D culture of vestibular sensory epithelia. These cells showed characteristics of innate hair cells; they were able to take up FM1-43 dye and exhibited voltage-dependent currents. Moreover, ribbon synapses were formed between the hair cells and neighboring neurons in the 3D culture. These cells may serve as a substrate for investigating additional aspects of RNA regulation and lead to identification of additional RNA species in the inner ear.

CONCLUSION

miRNAs are being developed as therapeutics for breast cancer (Piva et al., 2013), rheumatic diseases (Pers and Jorgensen, 2013), and hepatitis C virus infection (Lindow and Kauppinen, 2012; Janssen et al., 2013), which are already involved in Phase 2 clinical trials. The major limitations in miRNA research in the inner ear are the lack of robust cell lines and the inability to gain access to human tissue in an efficient manner. Nevertheless, the identification of hundreds of miRNAs in the auditory system and the elucidation of the function of many of these miRNAs and their targets holds promise for their use in therapeutics one day. A deeper understanding of the regulatory elements involved in the diseased state of the ear, including hearing impairment, cholesteatoma, OM, and vestibular schwannomas can be reached, with miRNAs serving as a potential source of regeneration therapies and relevant pharmaceutical studies. The ability to generate stem cells may open up further avenues for RNA regulation studies.

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MicroRNA responses to focal cerebral ischemia in male and female mouse brain

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Stroke occurs with greater frequency in men than in women across diverse ethnic backgrounds and nationalities. Work from our lab and others have revealed a sex-specific sensitivity to cerebral ischemia whereby males exhibit a larger extent of brain damage resulting from an ischemic event compared to females. Previous studies revealed that microRNA (miRNA) expression is regulated by cerebral ischemia in males; however, no studies to date have examined the effect of ischemia on miRNA responses in females. Thus, we examined miRNA responses in male and female brain in response to cerebral ischemia using miRNA arrays. These studies revealed that in male and female brains, ischemia leads to both a universal miRNA response as well as a sexually distinct response to challenge. Target prediction analysis of the miRNAs increased in male or female ischemic brain reveal sex-specific differences in gene targets and protein pathways. These data support that the mechanisms underlying sexually dimorphic responses to cerebral ischemia includes distinct changes in miRNAs in male and female brain, in addition to a miRNA signature response to ischemia that is common to both.

Keywords: microRNA, cerebral ischemia, sex-differences, array analysis, qRT-PCR, pathway analysis, stroke

INTRODUCTION

Stroke occurs more frequently in men than women across diverse ethnic backgrounds and nationalities (Bushnell, 2008; Reeves et al., 2008; Saini and Shuaib, 2008; Appelros et al., 2009; Persky et al., 2010; Ovbiagele et al., 2013; Towfighi et al., 2013). Our lab and others have shown that sensitivity to cerebral ischemia, i.e., the extent of brain damage resulting from ischemic insult, is sex-specific, with female animals being less sensitive than males (Murphy et al., 2004; Koerner et al., 2007; Lang and McCullough, 2008; Reeves et al., 2008; Vagnerova et al., 2008; Cheng and Hurn, 2010; Siegel et al., 2010). Sex-specific responses are also observed in response to focal cerebral ischemia in isoflurane preconditioned mice (Kitano et al., 2007) and in immune responses to ischemia (Banerjee et al., 2013). Furthermore, sex differences in ischemic sensitivity have been extended to the cellular level as our lab and others have shown that astrocytes (Liu et al., 2007, 2008) and neurons (Li et al., 2005; Johnsen and Murphy, 2011) from male newborn rodents are more sensitive to oxygen-glucose deprivation (an *in vitro* model of ischemia) than cells from female newborn rodents. These observations suggest that the male brain exhibits a more "ischemia-sensitive" phenotype than the female brain. However, the underlying molecular mechanisms for this sexually dimorphic response to ischemia are not well understood.

We examined a role for miRNAs in ischemic responses in the male and female brain. MiRNAs are short, non-coding RNA sequences that regulate post-transcriptional gene expression via translational repression or mRNA degradation (Ambros, 2004; Murchison and Hannon, 2004; Niwa and Slack, 2007; Guarnieri and DiLeone, 2008; Chua et al., 2009). MiRNAs have been implicated in the regulation of numerous physiological and pathological processes such as brain differentiation (Feng and Feng, 2011), neurological disorders (Saugstad, 2010), ischemic preconditioning (Lusardi et al., 2010), and stroke (Rink and Khanna, 2011; Tan et al., 2011). The few studies which have examined miRNA responses to injury in brain have either focused on irradiation injury (Ilnytskyy et al., 2008; Koturbash et al., 2011), evaluated a single miRNA target of interest following brain ischemia (Siegel et al., 2011), or profiled miRNAs in male ischemic brain without linking them functionally to ischemic mechanisms and outcomes (Jeyaseelan et al., 2008; Dharap et al., 2009; Liu et al., 2010; Lusardi et al., 2010).

For these studies we focused on miRNA expression at 8 h after ischemia, based on our previous miRNA studies in rodent brain showing that this reperfusion time is optimal for robust change in miRNA expression levels. Two previous studies revealed little or no changes in miRNA expression at 2 and 4 h after treatment, robust changes 8 h after treatment, and a return to levels comparable to naïve controls by 24 h after treatment (Lusardi et al., 2010, 2012). These studies suggest that the treatments used (ischemia or glutamate activation) induced transcriptional changes in miRNA expression, or alterations in the miRNA processing pathway, that were optimally detected 8 h after the treatment. This time course would be consistent with miRNAs as early mediators of mRNA translation and protein expression that in turn lead to cellular changes that develop within 24–72 h after ischemia.

Our miRNA profiling studies revealed that there are sex-specific differences in miRNA responses to ischemia as well as a universal, ischemia-induced miRNA signature equally present in

both male and female brains. Our findings reveal a novel mechanism, namely the differential regulation of miRNA responses, for sex differences in ischemic sensitivity mediated by sex-specific miRNA pathways in male and female brain.

MATERIALS AND METHODS

EXPERIMENTAL GROUPS

Experiments were carried out in male and female C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA), 8–14 weeks of age and weighing 20–25 g. Experiments were carried out in accordance with the National Institutes of Health guidelines for research animal care and approved by the Oregon Health and Science University Animal Care and Use Committee. All mice were maintained on a 12/12 h light-dark cycles and permitted *ad libitum* access to food and water. Male and female mice were randomized to one of the following experimental groups: control (experimentally naïve), sham surgery, or transient focal cerebral ischemia.

TRANSIENT FOCAL CEREBRAL ISCHEMIA

All surgeries were conducted under aseptic conditions by a single surgeon. Transient focal cerebral ischemia was induced in male and female mice for 60 min by reversible right middle cerebral artery occlusion (MCAO) under isoflurane anesthesia, followed by 8 h of reperfusion as previously described (Chen et al., 2012). Peri-ischemic head and body temperature were controlled at 36.5 ± 1.0°C (mean ± standard deviation) with warm water pads and a heating lamp. The common carotid artery was temporarily occluded while a 6-0 nylon monofilament surgical suture (ETHICON, Inc., Somerville, NJ, USA) with a silicone-coated (Xantopren Comfort Light, Heraeus Kulzer, Germany) tip was inserted via an external carotid artery stump distal to the internal carotid artery to the origin of the middle cerebral artery. After 60 min of MCAO, the filament was withdrawn to allow for reperfusion. All incisions were the closed with 6-0 surgical sutures (ETHICON, Inc., Somerville, NJ, USA) before each mouse was awakened and recovered in a separate cage with a warm water pad. For sham surgeries, the filament was placed but not advanced to achieve MCAO. Occlusion and reperfusion were verified in each mouse by laser Doppler flowmetry (LDF) (Model DRT4, Moor Instruments Inc. Wilmington, USA). Mice were excluded if intra-ischemic LDF (% pre-ischemic LDF baseline) was greater than 25%. Neurological deficit scores were determined at 1 h of reperfusion to confirm the presence of ischemic injury using a 0–4 point scale as follows: 0, no neurological dysfunction; 1, failure to extend left forelimb fully when lifted by tail; 2, circling to the contralateral side; 3, falling to the left; and 4, no spontaneous movement or in a comatose state (Chen et al., 2012). Any animal without a deficit at 1 h of reperfusion was excluded from the study. Eight hours following either sham surgery or focal cerebral ischemia, mice were anesthetized with isoflurane and euthanized by decapitation. Experimentally naïve mice were also anesthetized with isoflurane and euthanized by decapitation. Right and left cortices were sub-dissected from each mouse brain, and the tissues were frozen in 2 methyl-2-butane on dry ice, then stored at −80°C.

RNA ISOLATION

To correlate with the right MCAO model, RNA was isolated from the right mouse brain cortex with the mirVana miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA), following the recommended protocol for total RNA isolation from frozen tissue. The RNA isolation did not include the "Enrichment Procedure for Small RNA" in the protocol provided with the kit. Total RNA was eluted with 100 μL of Elution Solution provided with the RNA isolation kit, and the RNA concentrations quantified by spectroscopic measurement of A260. RNA samples were stored at −80°C until further use.

MICRORNA ARRAY PROFILING

Mouse MicroRNA Genome V2.0 PCR Arrays (MAM-200C; SABiosciences/Qiagen, Valencia, CA) were used to quantitatively assay miRNA expression in mouse brain. The arrays consisted of the 528 most abundantly expressed and well-characterized miRNA sequences in the mouse genome, as annotated by the Sanger miRBase Release 14. For qRT-PCR array analysis we used total RNA samples representing control male and female mice, and ischemic male and female mice. The RT2 miRNA First Strand Kit (SABiosciences) was used for Reverse Transcription (RT) of the RNAs, as per the manufacturer's instructions. Total RNA from control and ischemic male and female mice ($n = 3$ mice/group) were pooled, then 2 μg of the pooled total RNA was incubated in RT buffers at 37°C for 2 h, followed by 95°C for 5 min to degrade the RNA and to inactivate the reverse transcriptase. The first-strand cDNA samples were chilled on ice then diluted with RNase-free water. The RT2 SYBR green master mix (PA-012) was used for the qRT-PCR reactions, as per the manufacturer's instructions (SABiosciences). Briefly, the diluted first-strand cDNA was combined with master mix, then 25 μL was aliquoted into each well of six 96 well plates. The six plates were briefly centrifuged then stored at −20°C. For amplification, individual plates were removed from −20°C, defrosted for 5 min at RT, briefly centrifuged and placed into a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Parameters were set to: (i) one cycle at 95°C for 10 min, (ii) 40 cycles at 95°C for 15 s, 60°C for 40 s, and 72°C for 30 s. A dissociation step set to: (iii) 95°C for 15 s; 60°C for 1 min and 95°C for 15 s was performed to ensure that all PCRs generated a single product. Normalized delta CT (ΔCt) values were calculated with respect to the average of all Ct values, and ΔΔCt was calculated as (ΔCt-Ischemic minus ΔCt-Control) for all of the male and female miRNAs.

SABIOSCIENCES qRT-PCR MICRORNA ARRAY NORMALIZATION

We calculated the ΔCt for each miRNA in a given experiment based on the average Ct value of all miRNAs in that experimental condition: $\Delta Ctmir = Ctavg - Ctmir$. We then determined the response to ischemia for males and females separately, and calculated the change in miRNA expression as $\Delta\Delta Ctmir = \Delta Ctmirischemia - \Delta Ctmircontrol$. We defined statistical significance as a multiple of a standard deviation from the mean ΔΔCt (SD).

REAL-TIME qRT-PCR DETECTION

We analyzed miRNA expression in individual mice (each group) from control ($n = 5$), sham ($n = 5$), and ischemic ($n = 4$) cortices. Sham surgery groups were added after the initial miRNA array profiling to account for any effects due to anesthesia and surgical stress. Detection of miRNAs was completed with a 2-step qRT-PCR assay, using the miScript PCR System (Qiagen, Valencia, CA). One microgram of total RNA was converted to cDNA, for RNA from each individual sample, with the miScript II RT Kit, following the standard protocol with the miScript HiSpec Buffer. Negative control samples included those with no reverse transcriptase (No RT). The qRT-PCR assays were prepared for each individual using 1 μg of total RNA in the same reverse transcription reaction protocol, omitting the RT enzyme in the reaction mix. The resulting 20 μL of RT products (or No RT controls) were diluted to a total volume of 100 μL with the addition of 80 μL RNase/ DNase Free water, and stored at −80°C before use in PCR assays. Two micro litres of cDNA was assayed in each PCR reaction well. miScript PCR Primer Assays (Qiagen) were used for detection of mature miRNA, using the miScript SYBR Green PCR Kit for qRT-PCR assays. The following primer sets were used:

miR-15b	5′ CGAAUCAUUAUUUGCUGCUCUA	(#MS00011242)
miR-125b-3p	5′ ACGGGUUAGGCUCUUGGGAGCU	(#MS00024066)
miR-296-5p	5′ AGGGCCCCCCCUCAAUCCUGU	(#MS00016436)
miR-509-3p	5′ UGAUUGACAUUUCUGUAAUGG	(#MS00012306)
miR-682	5′ CUGCAGUCACAGUGAAGUCUG	(#MS00033019)
miR-686	5′ AUUGCUUCCCAGACGGUGAAGA	(#MS00002821)
miR-883a-3p	5′ UAACUGCAACAGCUCUCAGUAU	(#MS00012845)
miR-883b-3p	5′ UAACUGCAACAUCUCUCAGUAU	(#MS00012859)
miR-1224	5′ GUGAGGACUGGGGAGGUGGAG	(#MS00011074)

Assays were performed with the standard recommended reaction mix (25 μL volume per reaction) in 96-well reaction plates, using a ViiA 7 Real Time PCR Detection System (Life Technologies). Each miRNA assay was performed in triplicate for individual samples. The Ct values were calculated using the same cycle threshold and baseline for all reactions. The ΔCtmir = Ctnon-changers − Ctmir, responses to ischemia for males and females were calculated the change in miRNA expression as ΔΔCtmir = ΔCtmirischemia − ΔCtmircontrol, and ΔCtmirischemia − ΔCtmirsham.

MICRORNA TARGET PREDICTION

We used the miRmap target prediction program (http://mirmap.ezlab.org/, accessed November 2013) to query the predicted targets of miRNAs increased by ischemia in male and female brain (Vejnar et al., 2013). We then used the predicted protein targets identified in miRmap to query the PANTHER v8.1 program (http://www.pantherdb.org/, accessed November 2013). The PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification system was designed to classify proteins (and their genes) according to: family and subfamily, molecular function, biological process, and pathway that explicitly specifies the relationships between the interacting molecules (Mi and Thomas, 2009; Mi et al., 2013).

RESULTS

STATISTICAL ANALYSIS OF MICRORNA ARRAYS

We used the Mouse MicroRNA Genome V2.0 PCR Arrays to profile the expression of mouse cortical miRNAs in both male and female brains. For the array studies, we pooled RNA samples isolated from control and ischemic male and female cortices. For array normalization, we first considered the endogenous controls included on the PCR arrays (Snord85, Snord68, Snord66, and Rnu6). We found that within a given treatment group, the endogenous controls were similar from plate-to-plate. However, across experimental groups the response of the endogenous controls was not consistent for sex or stroke groupings. We therefore calculated the ΔCt for each miRNA in a given experiment (control or ischemia) based on the average Ct value of all miRNAs in that experimental condition: ΔCtmiR = Ctavg − CtmiR, consistent with the assumption that most miRNAs would not be altered by focal ischemia as shown by linear regression analysis in **Figure 1**.

We then determined the response to ischemia for males and females separately, and calculated the change in miRNA expression as ΔΔCtmiR = ΔCtmiRischemia − ΔCtmiRcontrol. We defined significance as a multiple of a standard deviation from the mean ΔΔCt (SD), as shown in **Figure 2**. A miRNA was considered to be significantly decreased in response to ischemia if ΔΔCtmir (≤ -1.5 *SD*); the specific miRNAs are listed in **Table 1A**. A miRNA was considered to be significantly increased in response to ischemia if ΔΔCtmir (≥ 1.5 *SD*); specific miRNAs are listed in **Table 1B**. Approximately half of the 528 miRNAs did not change in response to ischemia based on the cutoff criterion (-0.5 *SD*) < ΔΔCtmir < (0.5 *SD*). An additional group of miRNAs possibly changed in response to ischemia, with a (0.5 *SD*) < |ΔΔCtmir| < (1.5 × *SD*), but did not meet the criterion for significance and were excluded from further analysis in this study.

For initial studies, miRNA was considered significantly changed if ΔΔCt was greater than 1.5 *SD* (standard deviation) from the mean ΔΔCt. The studies revealed two profiles: (1) sex-dependent responses wherein ischemia-regulated miRNAs were unique to male or female brain, and (2) a sex-independent response in which miRNAs are equally present and equally regulated in both male and female brains (the focus of another study). Of the significantly increased miRNAs, >50% were present in both male and female brain (**Table 1**).

INDIVIDUAL MICRORNA VALIDATION

Individual miRNAs representing down- and up-regulated species in male and female mice were further validated by Taqman qRT-PCR assays (Qiagen). Although the initial miRNA array studies did not include a sham surgery group, we did include sham surgery groups in the individual qRT-PCR validation studies to account for any treatment effects due to anesthesia and surgical stress. As indicated in **Table 1**, we examined miR-883b-3p that decreased in males and increased in females, miR-296-5p that decreased in males and females, miR-509-3p that decreased in females (1A). We also examined miR-682 that increased in males, miR-686 and miR-1224 that increased in males and females, and miR-883a-3p that increased in females (1B). Bold font in **Table 1** highlights the miRNAs that were chosen for individual qRT-PCR

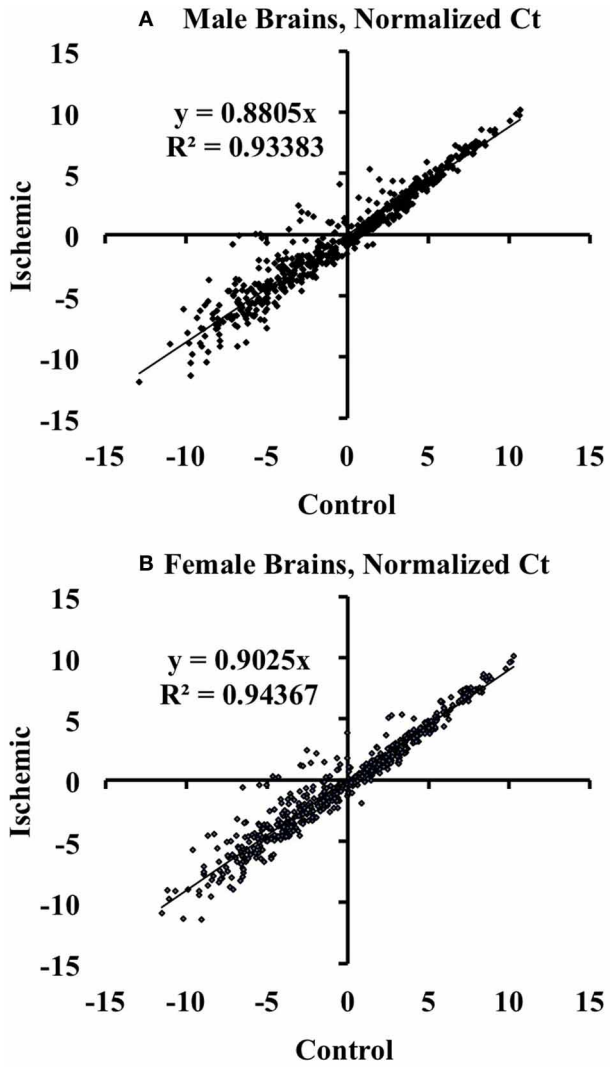

FIGURE 1 | Linear regression analysis of miRNAs altered by ischemia vs. control mouse cortices. The graphs illustrate that there are changes in miRNA expression in ischemia relative to control in male **(A)** and female **(B)** mouse cortex. The graphs also show that the majority of the assayed miRNAs are not altered by ischemia.

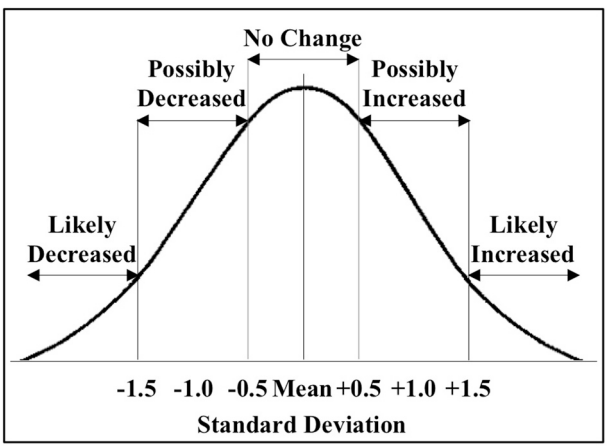

FIGURE 2 | Classification of miRNAs altered by ischemia. The figure shows the distribution of the miRNAs altered by ischemia relative to control in male and female mouse cortex. For initial studies we focused on miRNAs that were significantly decreased (<−1.5 *SD*) or increased (>1.5 *SD*) in the male and female mouse cortex.

validation, while italic font indicates the miRNAs that showed significant changes in the opposite direction in male and female brain.

We calculated the ΔΔCts using both control ($n = 5$ each male and female) and sham surgery ($n = 5$ each male and female) ΔCt values, relative to ischemia ($n = 4$ each male and female). Based on the array results, we selected miR-15b* and miR-125b-3p as candidates for a normalizing factor for our qRT-PCR validation. The rationale for this choice is as follows. We compared the raw Ct values for each of these miR across experimental conditions (control, sham, and ischemic) using a 2-Way repeated measures ANOVA (Prism, GraphPad Software, Inc. La Jolla, CA). The results showed: (1) there is no effect of the experimental condition (i.e., ischemia vs. sham vs. control) ($p = 0.1429$), there is a significant difference between miR-15b* and miR-125b-3p ($p < 0.0001$), and (3) there was no interaction between the experimental condition and the miRNAs ($p = 0.1272$). The within subject matching is significant ($p = 0.0004$), suggesting that variation across is due to individual variation, not to chance. These analyses support that neither miR-125b-1-3p nor miR-15b* is sensitive to the experimental manipulations (**Figure 3**) in males (3A) or females (3B), thus the average of both was used as a normalizing factor.

We then examined whether any of the selected miRNAs were sensitive to the sham surgery. Since the original miRNA array comparison was performed on control vs. ischemic conditions, we examined whether there was any significant influence of a surgical sham on miRNA expression. Thus, we compared the normalized Ct values (ΔCt) for each miRNA in Control and Sham experimental groups using a 2-Way repeated measures ANOVA (Prism, GraphPad Software). The results show: (1) there is no effect of the experimental group ($p = 0.1130$), (2) there is a significant effect of miRNA ($p < 0.0001$), (3) there is no interaction between miRNA and experimental group ($p = 0.1344$), and (4) there is a significant within-subject matching ($p = 0.0116$), suggesting variations are due to individual subject variation, rather than random. The conclusions from these analyses are that there is no influence of the surgery alone on any of the miRNAs examined here. Thus, **Figure 4** shows the changes in select miRNAs expressed in male (**Figure 4A**) of female (**Figure 4B**) cortex in control vs. ischemic treated cortex.

After accounting for the above, the results show that many but not all of the miRNAs under study showed changes in the same direction (up or down) in the individual qRT-PCR assays, consistent with the changes in expression detected by the pooled samples in the miRNA arrays (**Figure 5**). MiR-686 and miR-1224 both showed increased expression in males and females, as predicted by the miRNA arrays. In addition, miR-296-5p decreased

Table 1 | MicroRNAs Regulated By Ischemia.

Male	ΔΔCt		Male and Female	ΔΔCt		Female	ΔΔCt	
	M	F		M	F		M	F
(A) SIGNIFICANTLY DECREASED IN ISCHEMIA (<−1.5 *SD*)								
miR-19a*	−2.57	−0.23	**miR-296-5p**	−2.16	−2.76	***miR-509-3p***	*2.03*	−2.35
miR-145*	−2.68	−1.41				miR-543	−1.70	−1.79
miR-450b-3p	−2.32	0.22				miR-741	−0.22	−1.87
miR-883b-3p	−2.37	2.18						
(B) SIGNIFICANTLY INCREASED IN ISCHEMIA (>1.5 *SD*)								
miR-207	2.22	1.64	miR-27a*	4.06	3.94	miR-197	1.30	2.47
miR-218-2*	2.37	1.06	miR-135a*	3.95	2.56	miR-200c*	1.51	1.95
miR-327	3.17	1.57	miR-196a*	2.79	1.93	miR-466f-3p	1.58	2.03
miR-345-3p	2.26	0.76	miR-200a*	2.59	2.13	miR-466f-5p	1.06	2.08
miR-466g	2.33	1.29	miR-200b*	2.72	2.10	miR-615-3p	0.18	2.47
miR-493	4.33	1.59	miR-291a-5p	3.00	2.84	miR-875-3p	1.43	2.29
miR-509-3p	2.03	−2.35	miR-323-5p	2.01	2.94	**miR-883a-3p**	−0.83	1.86
miR-669g	2.47	0.00	miR-370	4.57	3.86	***miR-883b-3p***	−2.37	2.18
miR-675-3p	2.90	0.73	miR-466i	1.98	1.79	miR-883b-5p	0.93	2.88
miR-682	2.12	−0.41	miR-470*	3.30	2.37			
miR-697	2.27	−0.09	miR-483*	4.84	3.70			
miR-770-5p	2.10	1.33	miR-546	2.03	2.32			
miR-1187	2.16	0.20	miR-681	1.95	2.16			
miR-1190	2.66	1.12	miR-615-5p	3.40	2.22			
miR-1892	3.06	1.22	miR-654-3p	4.03	3.88			
miR-1896	2.18	0.70	miR-677	2.78	4.10			
miR-1897-5p	1.97	0.02	miR-678	2.87	2.60			
			miR-684	2.03	3.86			
			miR-685	5.74	4.60			
			miR-686	6.32	5.88			
			miR-695	2.95	1.77			
			miR-709	2.50	2.59			
			miR-712*	5.43	4.97			
			miR-743b-5p	2.17	2.42			
			miR-1188	4.67	4.11			
			miR-1195	4.07	4.15			
			miR-1196	4.98	4.56			
			miR-1199	5.41	4.95			
			miR-1224	4.17	3.67			
			miR-1895	3.58	2.46			
			miR-1897-3p	6.67	5.09			

MiRNAs significantly regulated by ischemia in male and female mouse brain.

(A) *Lists the ΔΔCts for miRNAs significantly decreased in ischemia (<− 1.5 SD) in male and female cortex.*

(B) *Lists the ΔΔCts for miRNAs significantly increased by ischemia (> 1.5 SD) in male and female cortex.*

The bold indicates the miRNAs in the table that were used for subsequent studies, the data for which is presented in ***Figure 4****. The italics indicate those miRNAs that were found to be oppositely regulated in male and female brain.*

in males and females, and miR-125b-3p showed no changes in male or female brain, consistent with the miRNA arrays. MiR-682 showed increased expression in males, consistent with the miRNA arrays, but there was increased expression in females that was opposite from the arrays. MiR-883a-3p showed increased expression in females, consistent with the miRNA arrays, but there was a slight increase in the expression in males relative to the small decrease in expression that was detected in the arrays. Also, miR-509-3p increased in the males, consistent with the miRNA arrays, but decreased significantly in the females which was opposite from the arrays.

These outcomes support that miRNAs are regulated by ischemia in male and female brain. However, they also show that the use of different array formats, or pooled vs. individual qRT-PCR samples, likely influences the expression results (Git et al., 2010). The miRNAs with the most robust changes in

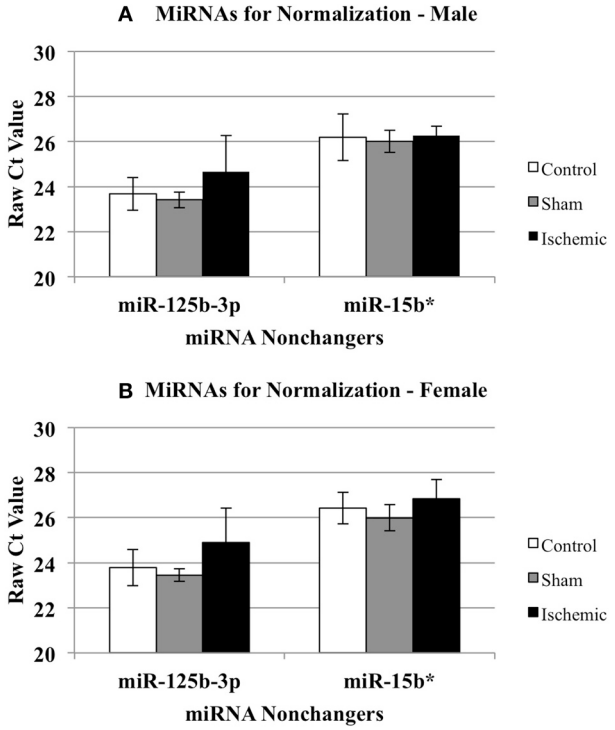

FIGURE 3 | Effect of treatment on miRNAs identified as non-changers in male and female mouse cortex. The graphs illustrate that miR-15b* and miR-125b-3p were not changed significantly in any treatment group in male **(A)** or female **(B)** mouse cortex. Control (white bar), sham (gray bar), ischemia (black bar).

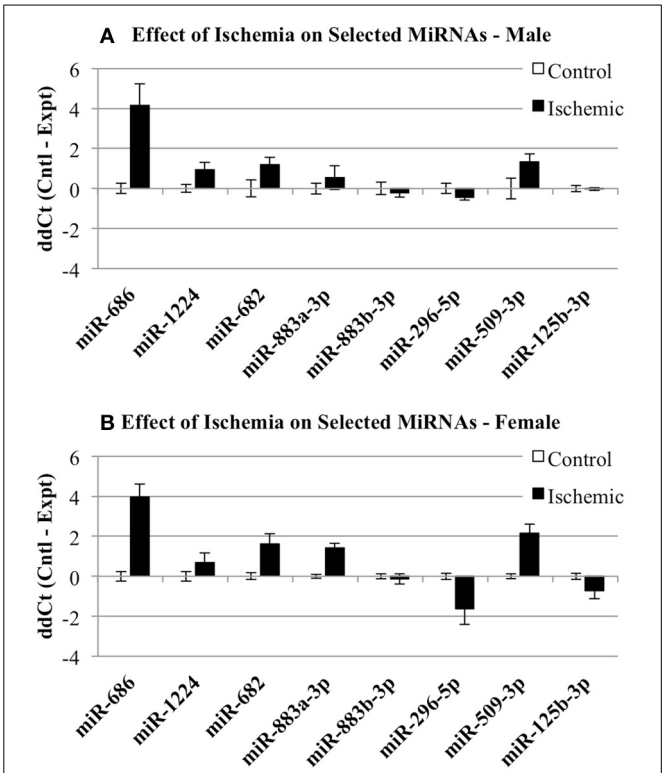

FIGURE 4 | Individual microRNAs regulated by ischemia in male and female mouse cortex. The graphs illustrate the ΔΔCt changes for specific miRNAs chosen for further validation by individual qRT-PCR. The graphs show expression of miRNAs in control (white bar) and ischemic (black bar) male **(A)** or female **(B)**.

expression in the arrays showed consistent changes in the individual arrays, particularly for those miRNAs that increased in expression. This observation suggests that decreases in miRNA expression from RNA degradation, low abundance of miRNAs, or pooling samples, may affect the array outcomes and data interpretation.

MICRORNA TARGET PREDICTION

We used the miRmap target prediction program (http://mirmap.ezlab.org/) to query the predicted targets of miRNAs increased by ischemia in male and female brain (Vejnar et al., 2013). We used miRmap to query the targets of the 17 miRNAs increased by ischemia in male brain. The results show target genes and the top number of miRNAs predicted to target this gene with a cutoff of 6 (**Table 2A**). We also used miRmap to query the targets of the 9 miRNAs increased by ischemia in female brain. The results show target genes and the top number of miRNAs predicted to target this gene with a cutoff of 4 (**Table 2B**). The results show that the miRNAs increased by ischemia in male and female brain are distinct, and only 2 targets are detected in common in the groups, CD73 and PKN2. These findings suggest that differential pathways are targeted by miRNAs increased by ischemia and that these pathways may lead to differential outcomes to ischemia may underlie the sexually dimorphic responses to ischemia, wherein females are afforded greater protection against ischemia insult than males. PANTHER target analysis revealed that there are 36 pathways of the genes targeted by miRNAs increased in male brain, and there are 69 pathways for the genes targeted by miRNAs in the female brain (Table 3—Supplemental Data). These findings support that distinct pathways are targeted by miRNA responses to ischemia, and provide an opportunity to focus studies on specific protein and protein pathways that may have been overlooked in prior studies.

DISCUSSION

Previous studies have shown that miRNAs are regulated in the brain in response to stress, including cerebral ischemia (Fasanaro et al., 2010; Saugstad, 2010; Rink and Khanna, 2011; Liu et al., 2013; Ouyang et al., 2013). However, to our knowledge, we are the first to show miRNA responses to ischemia that are sex dependent, i.e., there are differential responses in male or female brain following ischemia. For this study we focused on those miRNAs differentially expressed between males and females greater than 1.5 *SD* from the mean ΔΔCt in order to look at the most robust changers in response to ischemia. However, mRNAs can be targeted by many miRNAs (Doench and Sharp, 2004) and even small, subtle changes in miRNA levels can lead to significant changes in mRNA translation or stability. Thus, we are currently evaluating those differentially expressed miRNAs that

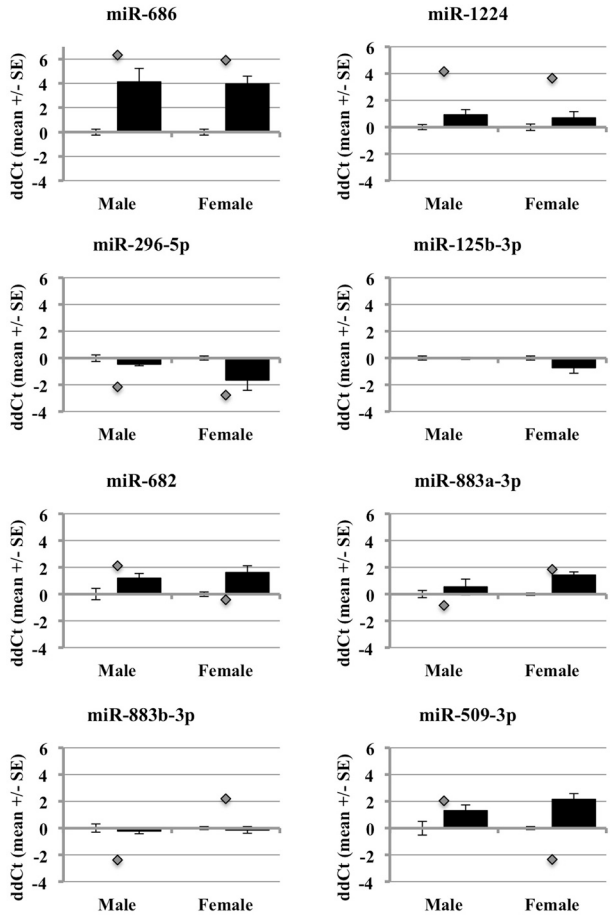

FIGURE 5 | Comparison of miRNA array expression to individual qRT-PCR assays in male and female mouse cortex. The graphs show the ΔΔCt of male and female miRNAs control (white bar) or ischemic (black bar) mouse cortex. The graphs also include the ΔΔCt for each miRNA as detected in the miRNA arrays (gray diamond).

show a change between 1.0 and 1.5 *SD* from the mean to identify additional proteins/pathways that could underlie differential responses to ischemia in male and female brain.

Our studies also revealed a signature miRNA response to ischemia that is common to both males and females. These key findings provide a mechanism, based on differential miRNA expression in male and female brain, to identify cellular or molecular targets that could underlie the sex differences in responses to ischemia. Such insight is likely to have implications for therapeutic strategies for the treatment of stroke in men vs. women. In addition, these studies also provide the ability to examine new targets that might contribute to ischemic injury in both males and females. Thus, we are focused on identifying the cellular/molecular targets of the ischemic-regulated miRNAs to determine how these miRNAs produce differential outcomes to ischemic injury. The genes identified in this study as predicted target of the miRNAs increased by ischemia in male and female ischemic cortex (**Table 2**) are quite distinct, and support that these miRNAs are likely initiating changes in distinct proteins and pathways that lead to altered phenotypes in in response to ischemia in male and female brain. PANTHER pathway analysis of the gene targets of miRNAs increased by ischemia in male and female (Table 3—Supplemental Data) support that distinct pathways are induced in each gender, and future studies are focused on clarifying the importance of these pathways in differential responses to ischemia in male and female brain.

Table 2 | Predicted Proteins Targeted By MicroRNAs Significantly Increased Following Ischemia.

(A) Male		(B) Female			
Of 17 Increased microRNAs		Of 9 Increased microRNAs			
Gene	No. of microRNAs	Gene	No. of microRNAs	Gene	No. of microRNAs
Brwd3	9	Cdc73	6	Lyvel	4
Ccr3	7	Cnrl	6	Mbtps2	4
Cdc73	7	Cadm2	5	Mklnl	4
Clintl	7	Cbln2	5	Mon2	4
Em15	7	Lcorl	5	Naa50	4
Fam55c	7	Pkn2	5	Nucksl	4
Map3k2	7	S1c30a4	5	Odzl	4
Mef2c	7	S1c6a14	5	Ogfrll	4
Ano3	6	Slitrk4	5	Prrg3	4
Atp 1 3a3	6	Strbp	5	Pura	4
Atp2c1	6	Taf4 a	5	Ragl	4
Ccdc93	6	Akap2	4	Rbpms2	4
Cnot61	6	Ap4e 1	4	Rhoa	4
Coll lal	6	Arl5b	4	Rqcdl	4
Csmdl	6	Atadl	4	Sec24a	4
Dio2	6	Atpafl	4	Serpinb7	4
Edaradd	6	Cblb	4	Slitrkl	4
Eif2c3	6	Cd200r4	4	Sox4	4
Fgfbp3	6	Clec2h	4	Sox5	4
Gabrg3	6	Cltc	4	Syngr3	4
Ghr	6	Crebzf	4	Tbc1d24	4
Gng2	6	Cxxc4	4	Tdrd5	4
Hipk3	6	Ect2	4	Tecrl	4
Hmgcsl	6	Femlb	4	Tmem236	4
Htrl a	6	Flil	4	Trip 12	4
Illrapll	6	Foxal	4	Ubrl	4
1121	6	Gas7	4	Ugp2	4
Mbn12	6	Gnal3	4	Usp38	4
Ms4a4c	6	Gngtl	4	Usp46	4
Mtmr6	6	Golga7	4	Usp9x	4
Nlgn3	6	Gopc	4	Vmn1r7	4
Oxtr	6	Gpr149	4	Xpo7	4
Pkn2	6	Grik2	4	Ywhag	4
Ppmll	6	Hipl	4	Zdbf2	4
Rora	6	Ikzf2	4	Zfand6	4
Sdpr	6				

Proteins predicted to be targeted by miRNAs differentially regulated by ischemia in male and female brain. The table lists show the gene name and the top number of miRNAs in each group predicted to target the gene (6–9 of 17 miRNAs in male, 4–6 miRNAs in female).

One limitation of the present studies is that miRNA target prediction is still evolving. However, we used the miRmap target prediction program (http://mirmap.ezlab.org/) to query the predicted targets of miRNAs increased by ischemia in male and female brain (Vejnar et al., 2013). The miRmap open source software library employs eleven predictor features, three of which are novel, as well as common features of target prediction including thermodynamic, evolutionary, probabilistic, and sequence-based features. This program allows the examination of feature correlations and comparison of their predictive power in an unbiased way using high throughput experimental data. Overall, target site accessibility appears to be the most predictive feature. Methods for identifying real miRNA targets are also evolving, such as using RISCtraps to stabilize and purify targets of specific miRNAs (Cambronne et al., 2012), but will be essential for future studies identifying the targets of the miRNAs regulated by ischemia in male and female brain. Another limitation is that we examined miRNA expression in the whole ischemic cortex, which may have diluted the expression of distinct miRNAs due to inclusion of both the core and penumbra. However, at 8 h reperfusion time, there are no apparent changes in cell death and thus no reliable method to indicate the boundary between core and penumbra. Thus, we will examine the regional/cellular changes in differentially expressed miRNAs to identify their potential role as mediators of cell death in the ischemic core.

In conclusion, we have shown that the sex-related differences in the response to ischemic insult observed in males and female mice is also characterized by a corresponding difference in expression levels of a specific set of miRNAs. Thus, current and future studies in our laboratory are focused on elucidating the roles of ischemia-regulated miRNAs that show distinct changes in male or female brain, along with those miRNAs that are regulated in a sex-independent fashion. We anticipate that these studies will clarify the mechanisms underlying responses to ischemia, both sex-related and non-sex-related. We also trust that they will provide guidance for the future design of therapeutic strategies to treat stroke specifically tailored to male and female patients. For example, a recent study of miRNAs in cardiac ischemia revealed that cardiomyocyte proliferation could be stimulated by the exogenous administration of miRNAs, and more importantly, that this treatment could restore cardiac mass and promote functional recovery after myocardial infarction in adult rats (Eulalio et al., 2012). We propose that future therapies could similarly be developed for cerebral ischemia, whereby administration of miRNAs designed specifically for males or females would promote functional recovery after stroke in humans.

AUTHOR CONTRIBUTIONS

Stephanie J. Murphy and Julie A. Saugstad conceptualized the project. Yingxin Chen performed all of the mouse middle cerebral artery occlusion surgeries and brain sub-dissections. Simon J. Thompson, Catherine M. Davis, and Jennifer M. Young isolated the RNAs and performed the SABiosciences miRNA arrays. Theresa A. Lusardi and Julie A. Saugstad analyzed the miRNA array data. Jay I. Phillips isolated RNA and performed the individual qRT-PCR assays. Theresa A. Lusardi, Julie A. Saugstad, and Jay I. Phillips analyzed the miRNA qRT-PCR data. Theresa A. Lusardi, Stephanie J. Murphy, and Julie A. Saugstad prepared the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnmol.2014.00011/abstract

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Circulating microRNAs in Alzheimer's disease: the search for novel biomarkers

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Alzheimer's disease (AD) is the most common neurodegenerative disease in the elderly. While advancements have been made in understanding the genetic and molecular basis of AD, the clinical diagnosis of AD remains difficult, and post-mortem confirmation is often required. Furthermore, the onset of neurodegeneration precedes clinical symptoms by approximately a decade. Consequently, there is a crucial need for an early and accurate diagnosis of AD, which can potentially lead to strategies that can slow down or stop the progression of neurodegeneration and dementia. Recent advances in the non-coding RNA field have shown that microRNAs (miRNAs) can function as powerful biomarkers in human diseases. Studies are emerging suggesting that circulating miRNAs in the cerebrospinal fluid and blood serum have characteristic changes in AD patients. Whether miRNAs can be used in AD diagnosis, alone or in combination with other AD biomarkers (e.g., amyloid and tau), warrants further investigation.

Keywords: microRNA, Alzheimer's disease, biomarker, diagnosis, mild cognitive impairment

INTRODUCTION

Alzheimer's disease (AD) is a prevalent, devastating, and progressive neurodegenerative disorder. Epidemiological studies predict that over 35 million people worldwide will be affected by 2050, thus significantly increasing social and economical burdens. There is no cure at hand, and only a few medications aimed at slowing down memory deficits and clinical symptoms are available, with limited benefits. Consequently, there is an urgent need for the identification of biomarkers that will allow the detection of AD at early (prodromal) stages, potentially leading to novel diagnostic or therapeutic strategies.

Pathologically, AD is characterized by the gradual, widespread loss of neurons, synapses, and neuropil, culminating in ∼40% loss of brain mass in end-stage disease (West et al., 1994; Gomez-Isla et al., 1996). There are two main AD pathological hallmarks: extracellular amyloid (senile) plaques and intracellular neurofibrillary tangles (NFTs; Hyman et al., 2012). The amyloid plaques comprise aggregated amyloid-beta (Aβ) peptides that are generated by sequential cleavage of amyloid precursor protein (APP) by β-secretase/BACE1 and the presenilin (PSEN)-containing γ-secretase complex (Kang et al., 1987; Wolfe, 2006). The NFTs result from the abnormal aggregation of hyperphosphorylated microtubule-associated protein, tau. The reason for tau aggregation into tangles remains under investigation, but may result from an imbalance in the delicate regulation of tau kinases and phosphatases. Whereas approximately 1–5% of AD cases can be explained genetically by mutations in APP or PSEN genes, the exact cause(s) of sporadic AD remains obscure. Most experts agree, however, that sporadic AD is caused by a combination of genes and environmental factors (multifactorial), perhaps exacerbated by oxidative stress and inflammation.

Biomarkers are used to measure or indicate the effects or progress of a disease or condition. A subtype of biomarkers relates to specific and traceable biochemical molecules or compounds found in body fluids. Detection of these substances may indicate disease states or allow correlations with the progression or the susceptibility to a disease or a given treatment. They can be measured in, for instance, saliva, sweat, breath, blood/serum, urine, and cerebrospinal fluid (CSF). The collection of these biological fluids is significantly less invasive than biopsies, an important and practical issue when studying neurodegenerative disorders like AD.

Accumulating evidence suggests that circulating biomarkers may be used in AD diagnosis, the most common being Aβ peptides (Aβ40 and Aβ42, the latter being more prone to aggregation) and tau/phospho-tau (Thr181 being one of the common phospho-epitopes). While this area of research continues to progress (Tarawneh and Holtzman, 2010; Holtzman, 2011), large variability exists in the literature, hampering or delaying their routine use in the clinic (Ingelson et al., 1999). Moreover, their potential use as prodromal AD biomarkers remains uncertain. Therefore, most experts agree that additional biomarkers are required for an accurate and early diagnosis of AD vs. other potential causes of dementia. In this review, we discuss recent studies suggesting that miRNAs could function as novel, non-invasive biomarkers in AD.

miRNAs AS BIOMARKERS

The miRNAs are a class of small (∼22 nt) non-protein-coding RNAs crucially involved in the post-transcriptional regulation

of gene expression. They are important for multiple biological processes such as development, proliferation, inflammation, and apoptosis (Xu et al., 2004; Pasquinelli et al., 2005; Thounaojam et al., 2013). The biogenesis and role(s) of the miRNA pathway have been recently and thoroughly reviewed by Treiber et al. (2012, and references therein). In brief, miRNAs function by binding with partial complementarity to messenger RNA (mRNA) sequences, mainly in the 3′ untranslated region (3′UTR). This targeting leads to either degradation or translational repression of the mRNA template(s), causing an overall downregulation in protein output. The miRNAs can target several disease-related genes involved in neurodegeneration (Delay et al., 2012; Abe and Bonini, 2013).

The precise mechanism(s) involved in miRNA release from cells remain largely unknown, but may involve the ceramide-dependent secretory machinery (Kosaka et al., 2010). Alternatively, there may be a passive leakage from necrotic or apoptotic cells (Zernecke et al., 2009). In any case, these small RNAs are highly stable in body fluids such as plasma and CSF (Mraz et al., 2009), making them attractive biomarkers. There are several factors involved in modulating (distant) circulating miRNAs. These small RNAs are transported in free forms, exosomes, liposomes, or high-density lipoproteins, which protect them from degradation (Vickers et al., 2011; Hu et al., 2012; the stable packaging, processing, and functionality of miRNAs in biofluids is a fascinating and important area of research mostly beyond what is addressed in the current review). While some miRNAs are ubiquitously expressed, others are present in specific cells or tissues, including the central nervous system (CNS; Landgraf et al., 2007). Furthermore, bioinformatics studies suggest that miRNA abundance is directly correlated with mRNA target activity (Dorval et al., 2012).

Interestingly, miRNAs have been described as epigenetic contributors to age-related cognitive changes (Kosik et al., 2012). It has been suggested that dysregulation of these miRNA-dependent epigenetic functions in vulnerable brain regions may lead to cognitive impairments. Accordingly, the past few years have witnessed an explosion of papers linking miRNAs to disease states, and current research efforts establish that miRNA expression profiles are altered in a variety of pathogenic conditions. This is particularly recognized in the cancer field (Sayed and Abdellatif, 2011). Interestingly, the various changes in miRNA levels are observable not only in cells/tissues directly related to disease (e.g., tumors vs. adjacent tissues), but often in the periphery or distant biological systems (e.g., tumors vs. blood). It is noteworthy that most peripheral miRNAs are also found in the brain, albeit at various levels (Hebert et al., 2013).

CIRCULATING miRNA BIOMARKERS IN AD

CEREBROSPINAL FLUID

Cerebrospinal fluid is a clear fluid that flows within the ventricles and around the surface of the brain and spinal cord. One primary function of CSF is to circulate nutrients within the CNS and, in turn, to act as a waste remover. The CSF is an attractive source of biomarkers as it is in direct and constant contact with the extracellular space of the brain, and can reflect biochemical and/or physiological changes that occur inside the brain.

In a pioneer study by Cogswell et al. (2008), the group performed a large-scale expression analysis of miRNAs in control and AD CSF. About 201 (out of 242 tested) miRNAs were detected above background levels, as measured by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) using TaqMan probes (Applied Biosystems). They identified 60 miRNAs, including let-7i, that were significantly altered in AD CSF (Braak V stage) when compared to healthy elderly controls (Braak I stage; $n = 10$ per group, $P < 0.05$). Using biological pathway enrichment algorithms, the group observed an association between misregulated miRNAs and the immune system, including pathways such as *innate immunity* (e.g., miR-146b) and *T cell activation and differentiation* (e.g., miR-181a, miR-142-5p). Putative targets for these miRNAs include IRAK1, TRAF6 (Lindsay, 2008), and Bcl-2 family members (Ouyang et al., 2012). The authors suggested that abnormally expressed miRNAs in the CSF were likely derived from immune cells. This was the first study demonstrating that miRNAs can be detected in the CSF (even when initially frozen) and are altered in neurodegenerative disease conditions.

van Harten et al. (2011) confirmed that it was technically feasible to perform genome-wide expression analyses of circulating miRNAs in control and AD CSF. The authors used two stem-loop qRT-PCR methods, including: (1) an individual miRNA TaqMan qRT-PCR and (2) a Megaplex modified microarray. Using this latter approach, the authors detected 667 miRNAs from one control and one AD subject (note that more than 2,000 human miRNAs are currently registered in the miRNA database – www.mirbase.org). The authors specifically quantified and validated changes in neuronal miR-802, a suppressor of caveolin-1 (Lin et al., 2011), in the CSF of control ($n = 8$) and AD ($n = 14$) patients. Clinical tests, combined with Aβ42, t-tau, and p-tau-181 measurements in the CSF were globally consistent with the diagnosis of either group. Whether other miRNAs were misregulated in AD conditions was not evaluated.

Only recently have two critical questions been addressed in relation to CSF miRNAs in neurodegenerative diseases. The first natural question relates to *why* miRNAs are stably present in this biofluid. After all, RNAs are notoriously unstable in solution, and yet there presence has been reliably affirmed. Thus, there is a tantalizing possibility that the miRNAs in solution – and in biochemical packaging as described above – may be playing a role in the CNS. In an elegant study, Lehmann et al. (2012) demonstrated that circulating miRNAs, and in particular let-7b, could exacerbate brain damage and neurodegeneration by binding directly to the Toll-like receptor 7 (TLR7). As measured by miRNA qRT-PCR, AD CSF ($n = 13$) contained significantly higher levels of let-7b when compared to controls ($n = 11$). Here, AD patients were selected, in part, on the basis of Aβ42 and t-tau levels. Unfortunately, no correlation between these AD markers and let-7b levels was provided. However, this study demonstrates that miRNAs in CNS are bioactive, and may have paracrine/hormonal-like functions, which, if generally true, provides a novel and potentially incredibly important context for miRNA function (and pathological impact) in the brain.

A second key question is more practical, and was addressed by Alexandrov et al. (2012): is there a correlation between Aβ peptides and miRNA levels in the CSF? In this study, the patient

groups consisted of six AD and six age-matched controls. Consistent with previous studies using enzyme-linked immunosorbent assay (ELISA), they reported a decrease in Aβ40 and Aβ42 in AD CSF, although this observation did not reach statistical significance ($P \sim 0.06$). Interestingly, the authors measured higher (greater than 100-fold) levels of total miRNAs (total mass) when compared to Aβ peptides, and this, both in control and AD CSF. Fluorescence-based miRNA microarrays indicated that the pro-inflammatory miRNAs miR-9, miR-125b, miR-146a, and miR-155 were significantly increased in AD CSF. These observations were further validated by a highly sensitive light-emitting diode (LED)-based Northern dot-blot analysis. This increase of specific miRNAs was extended to *in vitro* paradigms, where primary human neuronal/glial cells treated with AD-derived extracellular fluid lead to an increase of the same set of miRNAs. Significant negative correlations were observed between Aβ42 peptides and miR-137 ($r = -0.75$, $P = 0.003$), miR-181c ($r = -0.57$, $P = 0.037$), miR-9 ($r = -0.7$, $P = 0.007$), miR-29a ($r = -0.64$, $P = 0.01$), and miR-29b-1 ($r = -0.569$, $P = 0.037$), and this, in both control and AD patients. Based on these observations, it is tempting to speculate that miRs, alone or in combination with known AD biomarkers, could provide a better assessment of AD diagnosis.

BLOOD

Blood circulates in the principal vascular system, composed of arteries and veins, to carry oxygen to and carbon dioxide from tissues. The combination of lymphocytes, monocytes, and macrophages composes the peripheral blood mononuclear cells (PBMCs) population. These blood cells are critical components in the immune system.

Schipper (2007) assessed miRNA levels in blood mononuclear cells (BMCs) derived from sporadic AD and age-matched controls ($n = 16$ per group), using a microarray chip containing 462 human miRNAs. Several miRNAs were identified to be significantly altered in AD BMCs. A large number of miRNAs, including miR-34a, miR-181b, and let-7f, were validated by miRNA qRT-PCR. Interestingly, miR-34 targets include p53 (He et al., 2007), Notch (Bu et al., 2013), and Bcl-2 (Cole et al., 2008). The let-7 targets the oncogene Ras protein, thus promoting tumorigenesis (Johnson et al., 2005). Inversely, let-7 expression is regulated by the oncogenic Myc protein (Chang et al., 2008), suggesting a regulatory feedback loop. Together, these observations highlight the importance of these miRNAs in cell/tissue homeostasis.

Geekiyanage and Chan (2011) showed by miRNA qRT-PCR a decrease in miR-137, miR-181c, miR-9, and miR-29a/b levels in the neocortical region of controls ($n = 7$) and AD subjects ($n = 7$), which negatively correlated with Aβ42 levels in post-mortem brain tissues. In a follow-up study, using the same technical approach, the group reported that the same miRNAs were also present in the blood, albeit at lower basal levels (Geekiyanage et al., 2012). They were found to be downregulated in the blood serum of mild cognitive impairment (MCI; $n = 7$) and "probable" AD patients ($n = 7$) when compared controls ($n = 7$).

Villa et al. (2013) provided further evidence that dysregulation of peripheral miRNAs might contribute to AD development. In isolated PBMCs, they first showed that the transcription factor Sp1 was regulated at a post-transcriptional level by miR-29b. Interestingly, Sp1 regulates the expression of AD-related genes such as APP (La Fauci et al., 1989) and tau (Heicklen-Klein and Ginzburg, 2000). In a cohort of 393 AD patients and 412 healthy controls, the group observed an inverse relationship between Sp1 mRNA and miR-29b levels in PBMCs ($p = 0.002$). To our knowledge, this is the first report suggesting that changes in miRNA levels (e.g., miR-29b) and its/their target(s) (e.g., Sp1) may serve as cooperative biomarkers for AD diagnosis. Whether a genuine interaction between both molecules occurs in the blood remains to be validated.

Very recently, Bekris et al. (2013) reported in an elegant 3-phase study including post-mortem brain arrays and qRT-PCR validation that plasma miR-15a correlated with neuritic plaque score and Braak stages in AD. This particular miRNA was predicted to modulate 9 AD-relevant genes, including APP (Liu et al., 2012) and tau (Hebert et al., 2010). The authors concluded that pathologically-altered brain miRNAs might be detected in CSF or plasma during life, providing further proof of principle that miRNAs are relevant clinical biomarkers of AD pathology.

CIRCULATING miRNA BIOMARKERS IN MILD COGNITIVE IMPAIRMENT, AND CHALLENGES IN PATHOLOGICAL SPECIFICITY

Mild cognitive impairment is a term often conflated with indicating early clinical manifestation of AD, and many do indeed progress to full-blown AD clinically, although many other pathologies than AD underlie the clinical state of MCI (see below). Nevertheless, it is essential to develop tools that can accurately discriminate between normal aging, MCI, AD, and likely other cognitive disabilities. An attractive approach has recently been proposed, namely miRNA "pairs." This concept uses, following single qRT-PCR TaqMan assays, bioinformatics to analyze the ratios of all measured miRNAs, and select the most promising pair(s) of biomarkers (Sheinerman et al., 2012). In a pilot study, 13 miRNA pairs allowed to discriminate between AD and age-matched controls, as well as between MCI and age-matched controls ($n = 10$ per group), and this, with up to 90% accuracy. The proposed sets of miRNAs could detect pre-symptomatic MCI 1–5 years before the diagnosis in 70% of cases. Finally, the same pairs of miRNAs have been able to discriminate between aged and young healthy controls ($n = 20$ per group).

There are two practical issues that are important to the clinical relevance of any biomarker: sensitivity and specificity. The issue of sensitivity is basic and relates to the fact that by the time AD is manifest as MCI, it may be too late for (at least some) therapeutic interventions. The Aβ/tau CSF studies have now shown that, as expected (Nelson et al., 2009), up to one-third of non-demented subjects harbor some AD-type pathology (Nelson et al., 2012). It is increasingly appreciated that these are the patients that should be targeted for biomarker studies as well as clinical trials.

Aspects of biomarker specificity are perhaps paramount, and often under-appreciated. Although MCI is often used to indicate an early stage of AD, MCI was originally defined according to neuropsychological features (Portet et al., 2006), which have been recognized to entail "multiple sources of heterogeneity." As such, it

is quite usual for MCI to be associated with brain pathologies other than AD: dementia with Lewy bodies (DLB), vascular pathologies, hippocampal sclerosis (HS-Aging), frontotemporal lobar dementia (FTLD), and other conditions may cause or contribute to MCI, as to dementia (Jicha et al., 2006). This highlights an important aspect of AD-related biomarkers: they are not only used in predicting whom will become demented, but also for specifying which subtype of dementia will be predominant; the importance of this specificity for clinical trials is obvious. Novel insights, relevant to this consideration, were obtained by deep sequencing miRNAs from brains of individuals with multiple different pathological diseases (AD, DLB, FTLD, and HS-Aging; Hebert et al., 2013). Although not a biomarker study *per se*, this showed that some miRNAs (particularly miR-132-5p) are downregulated in neurodegenerative diseases non-specifically. In the future, it is hoped that more specific miRNA "fingerprints" may help to distinguish the individual subtypes of neurodegenerative diseases before their earliest manifestations.

CONCLUSION AND PERSPECTIVES

To date, most researchers have relied on the combination of Aβ peptides, total-tau, and phospho-tau (Thr181) ratios to provide the best discriminative values for individuals with or without AD. However, in most cases, large variability and differences between studied groups did not reach statistical significance, leaving inconsistencies. Without excluding the amyloid and tau biological markers, a combination of biomarkers may provide a better tool for AD diagnosis, therefore improving their clinical usefulness. Known examples include structural (e.g., hippocampal shrinkage), functional (e.g., glucose metabolism), and molecular imaging (e.g., fluorescent Pittsburgh compound B; Chintamaneni and Bhaskar, 2012).

Small non-coding RNAs, and in particular miRNAs, have come a long way in the past two decades. As discussed herein, circulating miRNAs provide an exciting and emerging research area in the biomarker field. As of now, long lists of miRNAs potentially misregulated in disease conditions have been reported, although finding overlaps is challenging (note that this is also the case for miRNA profiling studies in the brain). However, some AD-specific miRNAs were "consistently" identified, including some let-7 family members (let-7f, let-7b, and let-7i), miR-9, miR-181, and miR-29 (Maes et al., 2009). These miRNAs seem involved in processes previously associated with AD, that is to say inflammation and immunological response. Perhaps expectedly, several miRNAs and their functions as biomarkers have been patented or in the process thereof (see, e.g., www.freepatentsonline.com or www.patentlawlinks.com). Although very attractive, the applicability of miRNAs as diagnostic tools into the clinic for AD (or MCI) will require extensive validation and follow-up studies in larger cohorts of patients. This is important as AD is a heterogeneous, multifactorial disease, with often display overlapping pathologies (e.g., Aβ deposits and Lewy bodies; Gomperts et al., 2008) and/or co-morbid diagnoses (e.g., diabetes, stroke). Obviously, the ultimate goal is to provide a sensitive, reproducible, and accurate detection of AD neuropathological changes prior to the onset of the disease and the appearance of the clinical symptoms. To this end, future studies will require better neuropathological validations as well as, ultimately, far greater sample sizes for robust statistical power.

In conclusion, circulating miRNAs are amongst the promising next generation of biomarkers for AD, and ultimately the discrimination between neurodegenerative diseases. They may be small molecules, but miRNAs certainly provide a big potential for the diagnosis of human diseases.

Note: While this work was in progress, a report has been published with regards to a circulating miRNA signature in AD patients (Leidinger et al., 2013).

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GENERAL COMMENTARY
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Erratum: Circulating microRNAs in Alzheimer's disease: the search for novel biomarkers

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A commentary on

Circulating microRNAs in Alzheimer's disease: the search for novel biomarkers
by Dorval, V., Nelson, P. T., and Hébert, S. S. (2013). Front. Mol. Neurosci. 6:24. doi: 10.3389/fnmol.2013.00024

An error has been pointed out under the section CSF after our mini-review was published. At the end of the last paragraph, the reported correlations have been wrongly assigned to Alexandrov et al. The correct citation for this work is "Geekiyanage, H., and Chan, C. (2011). MicroRNA-137/181c regulates serine palmitoyltransferase and in turn amyloid beta, novel targets in sporadic Alzheimer's disease. *J. Neurosci.* 31, 14820–14830. doi: 10.1523/JNEUROSCI.3883-11.2011." However, these results were obtained from the study of frontal cortices of Alzheimer's patients, not the CSF. Thus, caution should be taken with regards to this work, which is unfortunately no longer in the scope of the present mini-review on circulating microRNAs as biomarkers.

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Increased microRNA-34c abundance in Alzheimer's disease circulating blood plasma

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Circulating microRNAs, present either in the cellular component, peripheral blood mononuclear cells (PBMC), or in cell-free plasma, have emerged as biomarkers for age-dependent systemic, disease-associated changes in many organs. Previously, we have shown that microRNA (miR)-34a is increased in circulating PBMC of Alzheimer's disease (AD) patients. In the present study, we show that this microRNA's sister, miR-34c, exhibits even greater increase in both cellular and plasma components of AD circulating blood samples, compared to normal age-matched controls. Statistical analysis shows the accuracy of levels of miR-34c assayed by receiver operating characteristic (ROC) analysis: the area under the curve is 0.99 ($p < 0.0001$) and the 95% confidence level extends from 0.97 to 1. Pearson correlation between miR-34c levels and mild and moderate AD, as defined by the mini-mental state examination (MMSE), shows an r-value of −0.7, suggesting a relatively strong inverse relationship between the two parameters. These data show that plasma levels of microRNA 34c are much more prominent in AD than those of its sister, miR-34a, or than its own level in PBMC. Transfection studies show that miR-34c, as does its sister miR-34a, represses the expression of several selected genes involved in cell survival and oxidative defense pathways, such as Bcl2, SIRT1, and others, in cultured cells. Taken together, our results indicate that increased levels of miR-34c in both PBMC and plasma may reflect changes in circulating blood samples in AD patients, compared to age-matched normal controls.

Keywords: peripheral blood mononuclear cells (PBMC), plasma microRNA, miR-34a, miR-34c

INTRODUCTION

Recently, microRNAs found in circulating blood, especially in cell-free plasma, have been noted functionally for inter-cellular and/or inter-organ communication. Circulating plasma microRNAs seem to be largely released from the cellular compartment, i.e., peripheral blood mononuclear cells (PBMC), either associated with specific protein or lipid molecules, or released in vesicles known as exosomes, with some portion derived from the cell debris of apoptotic bodies (Smalheiser, 2007; De Smaelea et al., 2010; Etheridge et al., 2011). Not surprisingly, differentially expressed plasma microRNAs have been noted as powerful biomarkers for several central nervous system disorders, from bipolar disorder to schizophrenia, Huntington's disease and Alzheimer's disease (AD) (Gaughwin et al., 2011; Rong et al., 2011; Suarez-Gomez et al., 2011; Geekiyanage et al., 2012; Sheinerman et al., 2012; Shi et al., 2012). Our own work has shown that lead microRNAs are differentially regulated in PBMC of AD patients compared with age-matched controls (Schipper et al., 2007; Maes et al., 2010). Therefore, unique microRNAs offer another aspect, in addition to specific transcriptome and serum protein profiling: blood biomarker discovery, for instance for AD.

Among many microRNAs, the miR-34 family, composed of three members, miR-34a, -34b, and -34c, is relatively well-understood. MicroRNA-34b, a plasma biomarker for Huntington disease (Gaughwin et al., 2011), and its sister, miR-34c, are linked as a bi-cistronic transcriptional unit (Liang et al., 2009); together with the other sister, miR-34a, they participate functionally in at least two signaling pathways: (1) Bcl2 for cell survival/apoptosis; and (2) SIRT1 deacetylase for p53 or neuroprotection signaling. SIRT1, p53, and miR-34a are involved in a positive feedback loop for miR-34a expression: acetylated p53 binds the promoter to activate this microRNA's transcription (He et al., 2007; Yamakuchi et al., 2008). Increased miR-34a suppresses SIRT1 expression, thereby diminishing its deacetylation of p53, leading to an increase in acetylated p53 transcriptional activity, resulting in the

Abbreviations: miR or miRNA, microRNA; AD, Alzheimer's disease; NEC, normal elderly control; MMSE, Mini-Mental State Examination; MMSE score (4–9), severe group; MMSE score (10–20), moderate group; MMSE score (21–24), mild group; Bcl2, B-cell lymphoma 2; SIRT1, sirtuin1; Psen1, presenilin-1.

continued up-regulation of this microRNA (Yamakuchi et al., 2008).

Many reports, including our own work in aged mice, observe parallel changes during aging between differentially regulated circulating microRNAs and changes in the central nervous system (Li et al., 2011a). During aging, accumulating oxidative stress-activated p53 may tilt the balance toward age-dependent increase of miR-34a, observed in the brains of old rats and mice (Li et al., 2011b,c); and a reduction in age-dependent increase in miR-34a in brain has been observed in calorie-restricted mice (Khanna et al., 2011). In animal models of AD, increased miR-34a levels are observed in brains of mouse models bearing both the APP^{swe} and presenilin transgenes (Wang et al., 2009). MicroRNA 34-c, a sister of miR-34a, is also observed in the hippocampal region of AD animal models to be functionally connected to cognitive decline, because inhibition of this microRNA rescues memory impairment in AD transgenic mice, with corresponding regained SIRT1 levels (Zovoilis et al., 2011). These results suggest that increased expression of miR-34a and -34c may repress SIRT1 and Bcl2 expression, one of the many underlying causes for dysregulation of oxidative defense and neuronal cell survival in the brain of AD transgenic animals.

The main objective of this study is to test the hypothesis that unique microRNA changes in AD patients, specifically those of the microRNA-34 family, can be identified in circulating blood samples in both the cellular component, PBMC, and cell-free plasma. To test this hypothesis, we have expanded beyond our previous observation of increased miR-34a in PBMC of AD patients (Schipper et al., 2007) to include also miR-34c in our study of specimens of both blood components. Here, we report that miR-34c in circulating plasma and PBMC indeed exhibits increased levels of expression in AD patients, compared with age-matched normal elderly controls (NEC). Transfection study shows that miR-34c, similar to reported findings for miR-34a, functionally represses Bcl2, SIRT1, and other proteins, all key genes whose decreases are functionally associated with weakened oxidative defense and cell survival.

MATERIALS AND METHODS

SUBJECTS AND CLINICAL EVALUATION

Informed consent was obtained from all participants, following the Institutional Review Board protocol approved by the Sir Mortimer B. Davis Jewish General Hospital (JGH) Research Ethics Committee. Blood samples were obtained from subjects at the Memory Clinic at the JGH, from 110 AD patients (age: 56–90) and 123 NEC (age: 61–90) without cognitive impairment. NEC were recruited by newspaper advertisements and public lectures, scored less than 4 on the Subjective Memory Scale of Schmand (Schmand et al., 1996), lacked other medical or neurological illnesses, and scored within 1 standard deviation (SD) of age and education means on memory screening tests. These consist of scores of 26 or more on the Montreal Cognitive Assessment (MoCA) (Nasreddine et al., 2005), a Mini-Mental State Examination (MMSE) scoring 25 or above (Folstein et al., 1975), and normal range scores on the delayed paragraph recall component of the Logical Memory Test of the WAIS-R (Wechsler, 2008). Patients with sporadic late onset AD were screened and assessed at the JGH McGill University Memory Clinic in Montréal; the diagnosis was made according to standard NINCDS-ADRDS criteria (McKhann et al., 1984), and conformed to the more recent revised criteria for probable AD as well (McKhann et al., 2011). Severity of dementia was stratified according to MMSE results: mild (21–24), moderate (10–20), or severe (4–9).

BLOOD COLLECTION AND ISOLATION AND QUALITY EVALUATION OF RNA SAMPLES

Approximately 30 ml of blood per donor was collected in EDTA vacutainers, and processed to isolate the plasma and PBMC fractions, using Ficoll-Plaque Plus (GE Healthcare, Piscataway, NJ). PBMC and plasma samples, stored in RNAlater buffer solution (Ambion, Austin, TX), were processed for RNA and protein isolation by Trizol/chloroform and centrifugation, to separate the upper RNA-containing aqueous phase from the lower proteinaceous phase, for isolation of microRNA from the plasma specimens, as previously described (Li et al., 2011a). Prior to RNA isolation, 0.625 ng of synthetic miRNA-39 from *Caenorhabditis elegans* (cel-miRNA-39) was added to the Trizol as a spike-in control for purification efficiency and cDNA synthesis quality validation during qPCR assays, as described below (Kroh et al., 2010) (Exiqon, #203203). RNA integrity was assessed using a Nanodrop spectrophotometer (Thermo Fischer Scientific, Barrington, IL) and Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany).

TAQMAN MicroRNA REAL TIME qPCR

Plasma microRNAs were used to generate cDNA using the Taqman® MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA), with specific miRNA stem-loop primers for miR-34a, -34b, -34c, and -16, by MultiScribe Reverse Transcriptase; the reactions were carried out in a GeneAmp PCR System 9700 (Applied Biosystems). These cDNA samples were processed to assess mature miRNA levels by real time polymerase chain reactions (qPCR) using the Taqman® Universal PCR Master Mix kit (Applied Biosystems). The qPCR was conducted in a 7500 real time PCR system (Applied Biosystems) under the following conditions: 95°C 10 min, 60 cycles of 95°C 15 s, and 60°C 1 min. The levels of mature cel-miR-39 mRNA were measured using individual TaqMan microRNA Assays (Applied Biosystems) according to the manufacturer's instructions (Exiqon, #203203); cel-miR-39 was used to normalize miRNA levels. A mean Ct was calculated for *C. elegans* miRNA for each sample, followed by calculating the median of all mean *C. elegans* synthetic miRNA Cts, taking all samples into consideration. Then a normalization factor was calculated for each sample by subtracting the mean *C. elegans* synthetic miRNA Ct of the sample of interest from the median value calculated earlier. This normalization factor was then integrated into the calculation of the raw Ct value obtained for each sample, which was further normalized by reference to Ct values for miR-16.

WESTERN BLOT ANALYSES

Western blot analyses were performed as previously described (Bates et al., 2010; Li et al., 2011c), using an actin band

for transfection cell lysates, to check equal loading across all lanes (Pendyala et al., 2010). The antibodies used were mouse anti-Bcl2 (1:1000, 692, Abcam Inc., Cambridge, MA), rabbit anti-Psen1 (1:500, 71181;Abcam), rabbit anti-Onecut2 (1:500, 28466;Abcam), rabbit anti-SIRT1 (1:500, 110304;Abcam), and rabbit anti-β-actin (1:1000, 8226;Abcam). Goat anti-mouse (31403, Thermo Fischer Scientific, Barrington, IL) was used for Bcl2 (1:2000), and goat anti-rabbit (31460, Thermo Scientific) for β-actin, SIRT1, Psen1, and Onecut2 (1:1000) as secondary antibodies. Intensities of antibody reactive bands were detected by Enhanced Chemiluminescence (ECL), (Pierce Biotechnology, Rockford, IL), and quantified by densitometry using ImageJ software (Public domain, NIH, USA).

CONSTRUCTION OF hsa34a AND hsa34c GFP RECOMBINANTS, AND FUNCTIONAL TARGET SUPPRESSION STUDY

Micro-34a and -34c were amplified from DNA purified from human embryonic kidney cells (HEK 293 cell line; ATCC# CRL 1573) with the following primers:

miR-34a forward 5′-tctagaGAG TCC CCT CCG GAT GCC GTG,
reverse 5′-ggatccCCA CCC ACCG TGG CGC AG, 229 bp;
miR-34c forward 5′-tctagaAGC CCC TCC ATC CAT GTA ACG GT,
reverse 5′-ggatccAAC ACC CCT CTT CCC CAC GCA, 328 bp.

Amplified PCR products were purified and cloned by the Qiagen PCR Cloning kit (Qiagen, Valencia, CA), and subcloned into the pCDH-CMV-MCS-EF1-copGFP vector (System Biosciences, Mountain View, CA), which was then used for functional assays by transfecting human embryonic kidney cells (HEK 293), as previously described (Bates et al., 2010).

STATISTICAL ANALYSES

All statistical analyses were performed using MS Excel 2010, SPSS 17.0 statistical software package (IBM), or SAS version 9.2 (SAS Institute Inc., Cary, NC). Student's *t*-test and one-way analysis of variance (ANOVA) were performed to determine significant differences for two-way or more than two groups. For multiple comparisons, Fisher's Least Significant Difference (LSD) test was used, following one-way ANOVA, in order to assign statistical significance, and then the Scheffé test was applied to reduce Type 1 errors. Levels of miR-34c, miR-34b, and miR-34a were normalized with regard to miR-16, as recommended by the manufacturer as well as a previous report of Huntington disease plasma biomarker discovery (Gaughwin et al., 2011), as a reference gene, using the comparative Delta Ct method. The ΔCt value is the difference between the Ct of the target microRNA and that of the reference microRNA (ΔCt = Ct miR34× − Ct miR16). To include the spike-in results in this calculation, the Ct values were first normalized against *Cel-39* and then against miR-16, according to the equation: normalizing factor = median ($Avg.Ct^{Celall} - Avg.Ct^{Celsample}$); normalized $Ct^{sample} = Ct^{sample}$ + normalizing factor (Livak and Schmittgen, 2001). Correlation between MMSE scores and expression levels of miR-34c and miR-34a in AD and NEC patients was calculated using the Pearson correlation coefficient (r) (Taylor, 1990); a correlation coefficient value of 0.8 or above indicates strong correlation, whereas a value around 0.5 is indicative of moderate correlation.

Receiver-Operating Characteristic (ROC) curves were used to determine the accuracy of the test differentiating AD from NEC individuals for miR-34c and miR-34a in age-matched cohorts (Zweig and Campbell, 1993; Fawcett, 2006). We calculated the sensitivity, specificity and accuracy as follows;

Sensitivity = TP/(TP + FN) = (Number of true positive assessments)/(Number of all positive assessments);
Specificity = TN/(TN + FP) = (Number of true negative assessments)/(Number of all negative assessments)
Accuracy = (TN + TP)/(TN + TP + FN + FP) = (Number of correct assessments)/(Number of all assessments)

A true positive (TP) is defined as an individual showing concordance between disease presence and a diagnostic test result, while a true negative (TN) represents disease absence with test result also negative. Contrary to these scenarios is discordance between these two categories: i.e., a false positive (FP) is defined as disease absence in an individual whose diagnostic test is positive, and a false negative (FN) characterizes disease presence in an individual whose diagnostic test is negative. All four counts, i.e., TP, TN, FP, and FN, were calculated with a cut-off point base (Li and Chung, 2013).

RESULTS

SELECTION OF SAMPLES FOR BEST RNA QUALITY, AND CONTROLS FOR CALCULATION OF miR-34a AND miR-34c LEVELS

From blood samples of 110 AD patients and 123 NEC, only 78 AD and 85 plasma specimens were selected to meet our requirements for RNA integrity: a single peak of small RNA in the 4–40 nucleotide (nt) range (Figure 1S). The age range for the AD group is between 56 and 90, with an average age of 80; the NEC cohort is between 61 and 90, with an average of 72 (**Table 1**). Our cohort's cognitive ability, assessed by the MMSE, is as reported in the literature (Sharp and Gatz, 2011), i.e., lower scores are linked with fewer years of education in AD individuals (Figure 2S). Our recruitment program for normal control individuals is limited by the fact that many are in their 60 s. In order to achieve an age-matched study design, we further selected from the AD and NEC groups such that the age range of both subcohorts lies between 76–90; the average age for the former is 82, and the latter 80 (**Table 1**).

To optimize the accuracy of quantitative PCR assays, we used two control steps: (a) quality control for the cDNA synthesis step with *C. elegans* Cel 39 spike-in; and (b) using miR-16 to standardize baseline level determination. **Figures 1A–D** shows similar levels of miR-34a and miR-34c with and without spike-in experiments in both AD and NEC plasma samples. These results suggest that the protocol established for miR-34a and miR-34c qPCR assays is stringent, providing optimal quality cDNA synthesis with minimal putative RT-RNA inhibitions. An additional standardized control is selecting appropriate microRNAs with no changes

Table 1 | Plasma specimens from the entire cohorts of Alzheimer's disease (AD) and normal elderly control (NEC) individuals, selected after RNA quality control, with age range, average age with standard deviation (SD), and sample size (N) for acceptable RNA in the entire cohort and age-matched cohorts.

	Original	Acceptable RNA			Age-matched		
	N	Age range	Avg (*SD*)	*N*	Age range	Avg (*SD*)	*N*
AD	110	56–90	80 (6.17)	78	76–90	82 (4.11)	25
NEC	123	61–90	72 (1.31)	85	76–90	80 (3.26)	27

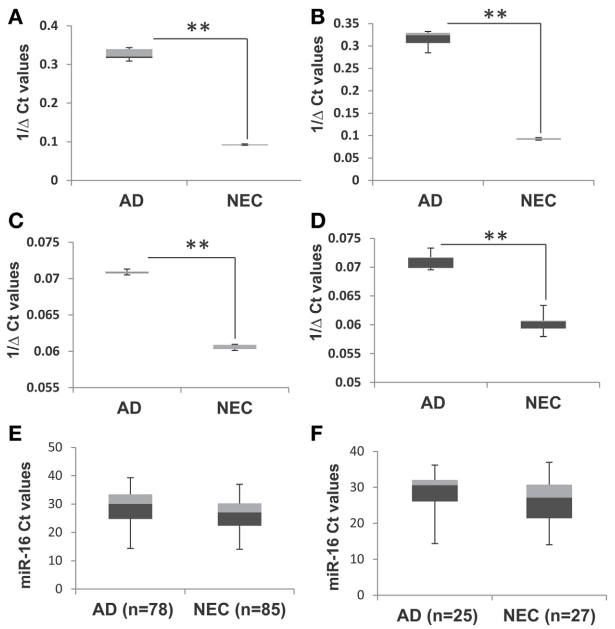

FIGURE 1 | Box plot representation of data from spike-in experiment with AD and NEC samples, miR-34c, with spike-in (A) and without spike-in (B) of *Cel-39*; and miR-34a with spike-in (C) and without spike-in (D). These figures show that levels of miR-34a and miR-34c are similar with and without spike-in experiments in both Alzheimer disease (AD) and normal elderly control (NEC) plasma samples. The Ct values of miR-16 in AD and NEC samples are represented as box plots in the whole population **(E)** and age-matched cohorts **(F)**. These figures indicate that levels of miR-34a and miR-34c are of the same range in both total and age-matched cohorts among both AD and NEC plasma samples. Student's *t*-test was used to determine statistical significance; $^{**}P < 0.01$.

between AD and control group blood samples. We followed the manufacturer's recommendation, as well as reports by Kroh et al. (2010) and Gaughwin et al. (2011) to use miR-16 as the control for each individual qPCR assay. However, since miR-16 is an abundant microRNA associated with red blood cells, even minimal hemolysis can cause unreliable qPCR results, as shown by Müller et al. (2014) in cerebrospinal fluid (CSF) qPCR assays. In this context, we evaluated miR-16 levels in all samples used in our study, as shown in **Figures 1E,F**, and found that in both total and age-matched cohorts, levels of this microRNA are similar between the AD and NEC cohorts. Thus, miR-16 levels are not differentially regulated between AD and NEC plasma samples in our study, and can serve as a standard.

EXPRESSION LEVELS OF miR-34a AND miR-34c IN PLASMA SAMPLES OF AD PATIENTS AND NORMAL ELDERLY CONTROLS (NEC)

Our initial study of quantitative reverse transcription PCR (qPCR) analyses of transcript levels of miR-34a and -34c in plasma samples of AD and NEC was performed with the entire cohorts (**Table 1**). When miR-34c levels were analyzed, NEC individuals were distributed in a close cluster around the median levels of the box plots, while AD specimens were distributed over a wider scatter span, with significantly wider SD range (**Figures 2A,B**). Similar wide spreads of AD values while NEC samples are clustered were also observed with miR-34a levels (**Figures 2C,D**). **Table 2** shows the wide distribution and high SD indices for both microRNAs in the AD group. To address the concern that higher AD microRNA values could be due to the older age group used, we next selected from our cohorts NEC individuals age-matched to AD counterparts, as listed in **Table 1**.

Figures 3A,B shows box plots and distribution of individual values for miR-34c in the two age-matched subcohorts. Clearly, the majority of AD plasma specimens in the age-matched control study exhibit higher miR-34c levels than do normal controls; **Table 3** shows 1/deltaCt values in the 0.20–0.53 range for AD, and 0.05–0.22 for NEC plasma specimens. When levels of miR-34a were analyzed, however, the range of distribution is not differentially expressed between the AD and NEC groups, with more spread-out distribution for both groups, as well as significant overlapping ranges, by both box plot and scatter plots (**Figures 3C,D**; **Table 3**). Levels of miR-34b were evaluated in the same age-matched subcohorts of the AD and NEC groups in plasma; the results show no significant difference between the two groups (Figure 3S, Table 1S). The box plot presentation of inverted ΔCt values from AD and NEC groups shows no change, indicating that the level of miR-34b does not change in the AD group compared to NEC.

Accuracy in differentiating AD ($n = 25$) and NEC ($n = 27$) individuals by miR-34c levels in plasma specimens was further tested by ROC curves (**Figure 4**). The area under the miR-34c curve is 0.99 ($p < 0.0001$), essentially 1.0, indicating perfect accuracy of the data; 95% confidence interval values fall in the range of 0.97–1.0. The ROC curve shows that miR-34c level is an excellent test for AD, with 94% accuracy, 92% sensitivity, and 96% specificity (**Table 4**). The area under the ROC curve for miR-34a is 0.81 ($p = 0.0001$), indicating a fairly good test; the 95% confidence interval lies between 0.69 and 0.93. The sensitivity is 84%, and specificity is 74% (**Figure 4**, **Table 4**). The area under the ROC curve, sensitivity, specificity and accuracy were calculated for a cut-off point chosen using the coordinates of ROC curves with balanced levels of high sensitivity and specificity.

miR-34c LEVELS IN MODERATE AND MILD AD COMPARED WITH AGE-MATCHED NORMAL ELDERLY CONTROLS

In our age-matched AD cohort, only two patients were evaluated by the MMSE in the severe stage, with scores in the range 4–9. **Figures 5A,B** show that when these two individuals' samples are excluded from our analysis, miR-34c levels in samples

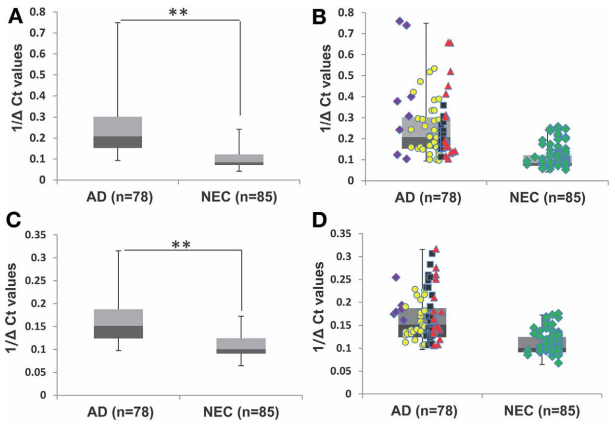

FIGURE 2 | Transcript levels of miR-34a and miR-34c in plasma samples of Alzheimer's disease (AD) patients and normal elderly controls (NEC). (A) Comparison of plasma levels of miR-34c in the whole population of AD patients ($n = 78$) with the NEC cohort ($n = 85$). **(B)** Distributions of individuals' levels of miR-34c along the box plot. NEC individuals were distributed in a close cluster around the median levels of the box plots, whereas AD specimens were distributed over a wider range with higher standard deviation. **(C)** Box plot representation of comparison in plasma levels of miR-34a in the whole population of AD patients ($n = 78$) with the NEC cohort ($n = 85$). **(D)** Individual miR-34a values along the box plot are less wide spread in the AD group as compared to those for miR-34c and a tight cluster for NEC is also observed. The data are plotted as inverse ΔCt derived from qPCR analysis. Data points of Mini-Mental Status Examination (MMSE) score ranges are: ◆ severe AD (score 4–9), ● moderate AD (score 10–20), ■ mild AD (score 21–24), ▲ AD outlier (score 25–29), ◆ NEC (score 25–30). Student's *t*-test was used to determine statistical significance; $^{**}P < 0.01$.

Table 2 | Plasma microRNA 1/deltaCt level ranges in Alzheimer disease (AD) patients and normal elderly controls (NEC) with standard deviation *(SD)* and median, for miR-34c and miR-34a in the whole cohorts.

Whole cohort	miR-34c		miR-34a	
	Range (*SD*)	Median	Range (*SD*)	Median
AD	0.09–0.75 (0.15)	0.21	0.09–0.32 (0.05)	0.15
NEC	0.04–0.24 (0.05)	0.08	0.06–0.17 (0.02)	0.1

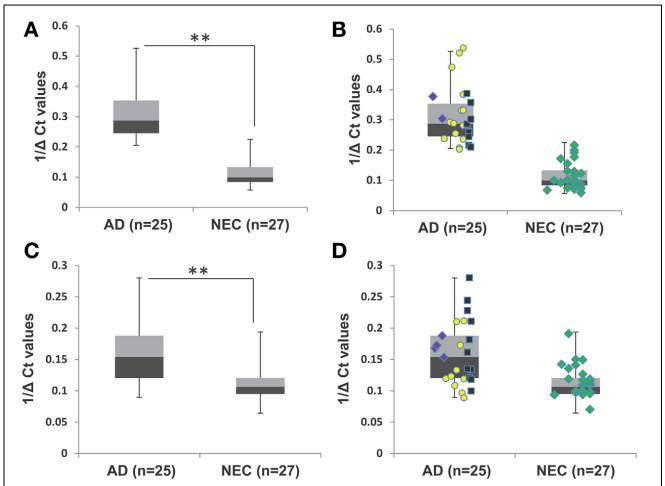

FIGURE 3 | Transcript levels of miR-34a and miR-34c in plasma samples of age-matched cohorts of Alzheimer's disease (AD) patients and normal elderly controls (NEC). (A) Plasma level comparison of miR-34c in the age-matched cohort of AD patients ($n = 25$) with the NEC cohort ($n = 27$). AD plasma samples in the age-matched cohort showed higher miR-34c levels than controls. **(B)** Distribution of individuals' levels of miR-34c along the box plot; NEC individuals were distributed in a close cluster around the median of the box plots, while AD patients were distributed over a wider range with greater standard deviation. **(C)** Comparison of plasma levels of miR-34a in age-matched cohorts of AD patients ($n = 25$) with the NEC cohort ($n = 27$). miR-34a levels were distributed in a range that is not differentially expressed between AD and NEC groups, with more spread-out distribution for both groups, as well as significant overlapping ranges, by both box and scatter plots. Distributing individuals along the box plot, NEC were distributed in a close cluster around the median levels of the box plots, whereas AD specimens were distributed over a wider range with higher standard deviation. Data points represent Mini-Mental Status Examination (MMSE) score as follows, (◆) severe AD (4–9), (●) moderate AD (10–20), (■) mild AD (21–24), and (◆) NEC (25–30). **(D)** Representation of individual miR-34a levels along the box plot for the range of distribution between AD and NEC groups. The data are plotted as inverse ΔCt derived from qPCR analysis. Student's *t*-test was used to determine statistical significance; $^{**}P < 0.01$.

Table 3 | Ranges of 1/deltaCt levels of plasma microRNAs in Alzheimer disease (AD) patients and normal elderly controls (NEC), with standard deviation *(SD)* and median, for miR-34c and miR-34a in age-matched subcohorts.

Age-matched cohort	miR-34c		miR-34a	
	Range (*SD*)	Median	Range (*SD*)	Median
AD	0.20–0.53 (0.09)	0.29	0.08–0.28 (0.05)	0.15
NEC	0.05–0.22 (0.05)	0.1	0.06–0.19 (0.03)	0.11

from moderate and mild stages, with MMSE scores in 10–20 or 21–24 ranges respectively, remain higher than NEC controls. **Table 5** shows that levels of miR-34c expression are not only distinct between the moderate and NEC groups, but also between the mild group and normal controls, with the former in the range of 0.21–0.38, while the latter shows 0.05–0.22 1/deltaCt values. Similar separation between mild AD patients and the normal control group is not observed for levels of miR-34a in this age-matched cohort study, due to overlapping ranges between these two groups (**Table 5**).

We investigated further the relationship between MMSE and miR-34c levels, by calculating Pearson's correlation *r*-value of miR-34c levels and MMSE scores for moderate and mild AD and age-matched control cohorts. **Figure 6A** shows an *r*-value of −0.72, suggesting a strong inverse correlation between the expression levels of miR-34c and the two cognitive assessment groups. This inverse correlation between MMSE scores and miR-34c levels, i.e., lower scores and higher levels, as shown for the three groups, was not observed for miR-34a expression, with an r coefficient score of −0.34 (**Figure 6B**). Likewise, when Pearson's correlation was computed for only the moderate and mild clinical groups without NEC, the *r*-value is very low. These results

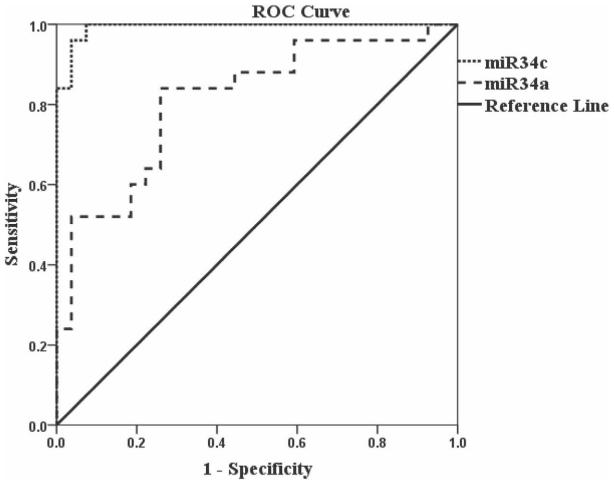

FIGURE 4 | Receiver Operation Characteristic (ROC) curves representing AD diagnostic tests by miR-34c and miR-34a in age-matched cohorts. The Receiver Operation Characteristic (ROC) curve for miR-34c (·····) is an excellent test since the area under the curve is 0.99 ($p < 0.0001$), number of samples for AD = 25 and NEC = 27. The area under the Receiver Operation Characteristic (ROC) for miR-34a (----) is 0.81 ($p = 0.0001$) which is a good/fair test, number of samples for AD = 25 and NEC = 27. Sensitivity and specificity are reported based on a cut-off point chosen using the coordinates of Receiver Operation Characteristic (ROC) curves with balanced levels of high sensitivity and specificity. The diagonal line represents a reference line showing zero sensitivity and zero specificity (—).

Table 4 | Receiver Operating Characteristic (ROC) curve parameters for plasma miR-34c and -34a among age-matched subcohorts.

ROC curve	miR-34c	miR-34a
Area under curve (AUC)	0.99 ($p < 0.0001$)	0.81 ($p = 0.0001$)
Sensitivity	0.92	0.84
Specificity	0.96	0.74
Accuracy	0.94	0.79

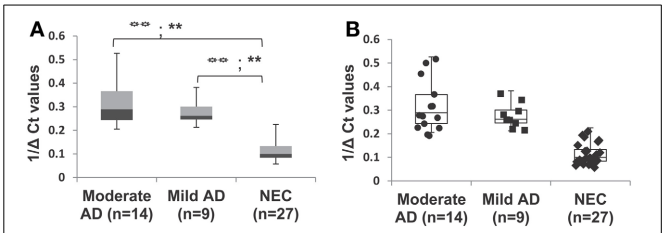

FIGURE 5 | Differential expression of miR-34c in plasma from patients with mild and moderate severity of Alzheimer's disease (AD), compared with normal elderly controls (NEC) in age-matched cohorts. AD patients were grouped by mild and moderate severity according to their Mini-Mental Status Examination (MMSE) scores. **(A)** Percentile distribution of samples within the various stages of AD and NEC, plotted against inverse ΔCt for individuals of age-matched cohorts. The levels of miR-34c are higher in samples from moderate (●) and mild (■) clinical groups, with MMSE scores in the range of 10–20 or 21–24, respectively, than NEC (◆) controls in the range of 25–30. **(B)** Data points added to illustrate the distribution of individuals within each group of the age-matched cohorts. We observe NEC to be in a close cluster around the median, whereas AD patients are scattered in a wider range in both moderate and mild groups. Both the LSD test (higher type 1 Error) and the Scheffé test (higher stringency) were used to determine statistical significance. The statistical significance level for the LSD test is represented with the symbol (⁎⁎), and for Scheffé test is represented with the symbol (⁎). ⁎⁎ $P < 0.01$, ⁎⁎ $P < 0.01$.

Table 5 | 1/deltaCt levels of plasma miR-34c and -34a by clinical category of mild and moderate Alzheimer's disease (AD) and in age-matched normal elderly controls (NEC): range with standard deviation (*SD*) and median.

	miR-34c		miR-34a	
	Range (*SD*)	Median	Range (*SD*)	Median
NEC	0.05–0.22 (0.05)	0.1	0.06–0.19 (0.02)	0.1
Mild AD	0.21–0.38 (0.06)	0.26	0.10–0.28 (0.06)	0.16
Moderate AD	0.20–0.53 (0.11)	0.29	0.09–0.21 (0.04)	0.12

suggests no relationship between MMSE scores and the levels of the two microRNA expression when comparing the moderate and mild groups between themselves (Figure 4S).

INCREASED EXPRESSION OF miR-34c IN CELLULAR COMPONENTS OF AD BLOOD SAMPLES

The observation of increased miR-34c expression in AD plasma prompted us to determine the level of expression of this microRNA in the cellular compartment, i.e., PBMCs of age-matched cohorts of AD and NEC samples. Increased miR-34c levels were observed in PBMC samples of AD blood specimens, compared to normal controls (**Figure 7A**), with a similar increase for miR-34a in AD over normal controls (**Figure 7B**). However, as shown in **Table 6**, as observed in the plasma samples, there is scant range overlap for miR-34c levels between AD and age-matched controls; this is not true for miR-34a levels between the two groups. Interestingly, the miR-34a levels assayed here as validation for our previous study (Schipper et al., 2007) show the same range of fold changes, i.e., ∼3-fold increase in AD samples beyond normal controls (Figure 5S).

FUNCTIONAL ANALYSIS OF THE REPRESSION OF TARGET PROTEINS BY OVEREXPRESSING miRs -34a AND -34c IN CULTURED CELLS

We next investigated whether miR-34c represses the same targets as reported for its sister, miR-34a (He et al., 2007; Yamakuchi et al., 2008), by transfection studies in cultures of human embryonic kidney cells (HEK 293) overexpressing these microRNAs. Only cultures with >98% of cells showing green fluorescence positivity for the green fluorescence protein gene (GFP) were used in our functional assays. Our results with these transfection experiments show repression of Psen1, Bcl2, Sirt1, and Onecut2 by miR-34a ranging from ∼11% to 27%, with the latter being the most affected (**Figures 8A–C**, **Table 7**). A more pronounced impact is observed by miR-34c, with repression ranging from ∼16 to 40% (**Figures 8A,B,D**, **Table 7**). Co-transfection with both miRNAs induced further repression of all four target proteins, from ∼32 to 46% (**Figures 8A,B,E**, **Table 7**). In all analyses, repression levels were evaluated by comparing transfected and

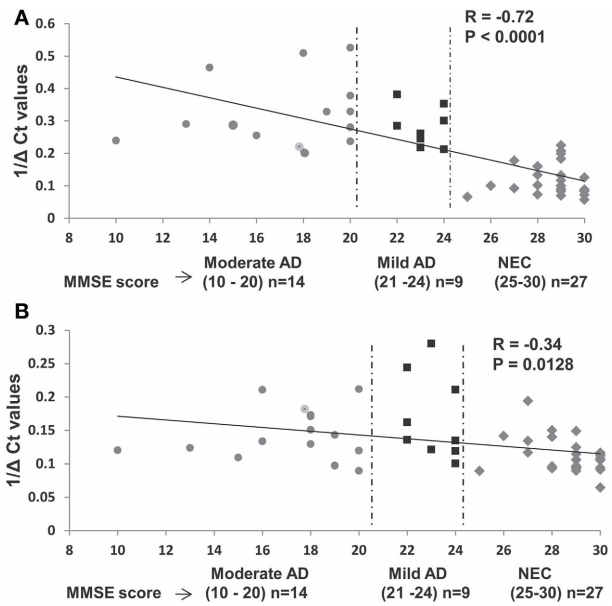

FIGURE 6 | Relationship between expression levels of miR-34c and miR-34a in plasma with Mini-Mental Status Examination (MMSE) scores in selected samples of age-matched groups. (A) Plasma expression levels of miR-34c represented by inverse ΔCt, plotted against corresponding MMSE scores. A high Pearson correlation coefficient value of −0.72 indicates a strong correlation between MMSE scores and 1/deltaCt values, $P < 0.0001$. **(B)** Expression levels of miR-34a in plasma of AD and NEC individuals represented by inverse ΔCt, plotted against corresponding MMSE scores. A low Pearson correlation coefficient value of −0.34 indicates a weak correlation between the MMSE scores and 1/deltaCt values, $P = 0.012$. Data points of Mini-Mental Status Examination (MMSE) score are shown as follows: (●) moderate AD (score 10–20), (■) mild AD (score 21–24), and (◆) NEC (score 25–30).

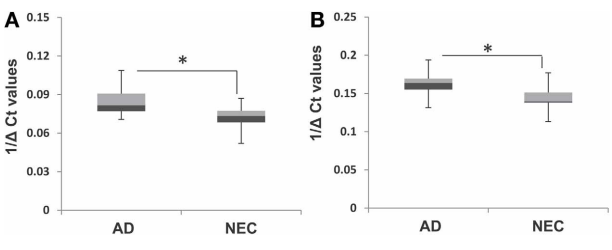

FIGURE 7 | Box plot representation of transcript levels of miR-34a and miR-34c in peripheral blood mononuclear cell (PBMC) samples from Alzheimer's disease (AD) patients and normal elderly controls (NEC). Comparison of levels of miR-34c **(A)** and miR-34a **(B)** in AD patients with that of the NEC cohort in PBMCs, showing increased transcript levels for both microRNAs in the former group, compared to normal controls. Student's *t*-test was used to determine statistical significance; $^*P < 0.05$.

scrambled control cultures, with repression of actin observed at ∼0%. These observations suggest that miR-34c is a stronger suppressor of target gene expression than miR-34a, and that Onecut2 is the most repressed among the target proteins.

DISCUSSION

Members of the microRNA-34 family are present in most tissues, including PBMC. Our previous study reported miR-34a as a lead microRNA from array profiling and validated by qPCR assays, showing a 2.5-fold increase in PBMC isolated from AD specimens (Schipper et al., 2007). The present study shows that this microRNA's sister, miR-34c, is even more prominently increased in both PBMC and plasma fractions of AD blood samples over age-matched NEC. Most importantly, the majority of miR-34c levels among mild AD patients, determined by mini-mental state examination (MMSE), are elevated beyond those of normal controls. In addition, inclusion of patients with moderate degrees of AD dementia with mild and normal counterparts in our study shows a relatively strong inverse relationship between MMSE scores and levels of this microRNA among the three groups. Levels of miR-34b, the bicistronic sister of miR-34c, are not significantly different between AD and NEC age-matched controls. Thus, our results reported here will serve as future leads to identify key microRNA abundance, such as miR-34c, as noninvasive indicators for plasma changes associated with AD.

Table 6 | 1/deltaCt level ranges, with standard deviation (SD) and median, of microRNAs in PBMC from Alzheimer's disease (AD) patients and age-matched normal elderly controls (NEC), for miR-34c and miR-34a.

PBMC	miR-34c		miR-34a	
	Range (*SD*)	Median	Range (*SD*)	Median
AD	0.07–0.11 (0.01)	0.08	0.13–0.19 (0.01)	0.16
NEC	0.05–0.08 (0.01)	0.07	0.11–0.17 (0.02)	0.14

Age-matched circulating blood microRNA studies like ours by design suffer several limitations, such as: (1) restriction of obtaining gene expression changes at a particular snap-shot time window; and (2) inability to link with changes of the same gene expression in individuals' brains. The first limitation cannot be addressed by small scale studies like ours, but rather in large consortium studies such as the Alzheimer's Disease Neuroimaging Initiative (ADNI) study of the National Institute on Aging, USA, with longitudinal blood sample collection linking plasma and CSF analysis study, and MRI imaging of brain changes in the same individuals. However, most of these studies are centered on the analysis of protein changes, with some focus on Tau and Aβ as biomarkers (O'Bryant et al., 2011; Toledo et al., 2011, 2013). Other studies such as Müller et al. (2014) have limited sample size because such samples are only available from a very few centers where longitudinal *ante mortem* blood or CSF samples are collected along with follow-up acquisition of autopsy brain specimens. Comparative studies between *ante-mortem* blood samples and *post mortem* autopsy will emerge in future studies, yielding results elucidating the relationships between plasma biomarkers and brain pathology structural changes, including but not limited to amyloid plaques and Tau-associated tangle formation.

To identify body fluid-associated microRNA biomarkers for neurodegeneration, standardization of the sample collection protocol and quality control of RNA isolation are two obvious quality control criteria in any study of this kind. Although we have established meticulous procedures for collecting blood samples and further processing to PBMC and plasma fractions, a significant portion, ∼20%, of the RNA fractions isolated from these

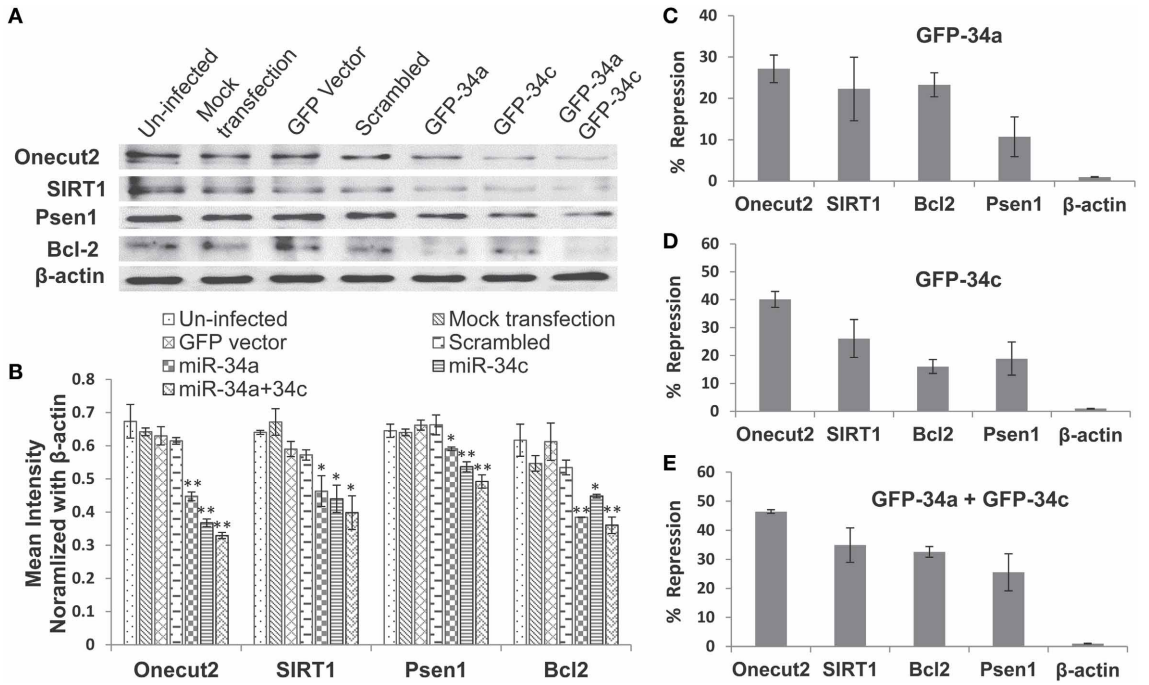

FIGURE 8 | Repression of expression of selected candidate target genes of miR-34a and miR-34c by transfection analysis. Human embryonic kidney (HEK 293) cultures were transfected with green fluorescence protein (GFP) expression vectors containing sequences encoding miR-34a, miR-34c, or a scrambled sequence. Controls included un-infected cultures, mock transfected cultures, cells transfected with either GFP vector alone or carrying a computer-generated scrambled sequence. **(A)** Western blot analysis of cell lysates probed for SIRT1, Onecut2, Presenilin-1, Bcl2, and β-actin proteins; **(B)** Graphic representation of a comparison of band intensities between the various treatments; image intensities of Western blotted bands were normalized against the β-actin band, which is constant throughout. Graphic representation showing percent of repression, estimated using scrambled controls for comparison (panels **C–E**): transfection with miR-34a **(C)**; miR-34c **(D)**; miR-34a and miR-34c **(E)**. One-way ANOVA followed by LSD test was used to determine statistical significance; $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 7 | Levels of miR-34a and miR-34c repression of four target genes' expression.

	miR-34a *vs.* scrambled	**miR-34c *vs.* scrambled**	**miR-34a + 34c *vs.* scrambled**
Protein	**Mean % repression (*SD*)**	**Mean % repression (*SD*)**	**Mean % repression (*SD*)**
Onecut2	27.15 (3.34)	40.13 (2.85)	46.42 (0.63)
SIRT1	22.28 (7.68)	26.09 (6.78)	34.89 (5.92)
Bcl2	23.27 (2.87)	16.06 (2.48)	32.57 (1.85)
Psen1	10.69 (4.8)	18.89 (5.94)	25.54 (6.39)
β-actin	0	0	0

*Percent repression of target proteins in HEK 293 cells transfected with miR-34a, miR-34c, or both miRNAs. The repression levels shown here represent protein band intensities, after normalization against β-actin band intensities, when cultures transfected by microRNA are compared with those transfected by scrambled controls. As shown in **Figure 8**, the % change of β-actin levels between cultures transfected by the microRNAs of interest and by the scrambled controls is ∼0. Mean % repression with standard deviation (SD) of each miR for each protein is tabulated.*

samples were unacceptable because of poor RNA integrity. Even among the 70–80 samples with good RNA quality, we had to narrow our study to 25 AD and 27 NEC as shown in **Table 1**, in order to satisfy age-matched criteria for our study. With samples from these two subcohorts, as in most neurodegenerative studies, before proceeding to qPCR assays of a particular microRNA of interest, we implemented quality control to insure the cDNA synthesis part of the assays by incorporating *Cel 39* spike-in in the step of RNA isolation.

Besides the above issues, selection of reference microRNAs for determination of microRNA levels of expression is crucial for studies of blood samples of neuronal disorders. We use miR-16 as our reference microRNA, following the report by Gaughwin et al. (2011), who used it as the reference microRNA to show that miR-34b is elevated in the plasma of Huntingdon mutation gene carriers, prior to disease manifestation. Interestingly, our study with this microRNA shows that miR-34b levels do not differ significantly in plasma specimens between AD patients and NEC. A recent study by Müller et al. (2014) shows that miR-16 and miR-146 are increased in AD in both hippocampus and cerebrospinal fluid (CSF). However, this report also suggests that miR-16, a major red blood cell microRNA (Kirschner et al., 2011, 2013; McDonald et al., 2011), may be contaminatedby hemolysis in CSF samples; even as little as 100 μl of erythrocytes added skews the qPCR results (Müller et al., 2014). In addition, several other microRNAs reported so far as unchanged in AD tissue have been used as references to determine levels of microRNAs of interest

(Geekiyanage et al., 2012; Sheinerman et al., 2012; Sheinerman and Umansky, 2013). Notwithstanding all these reports, no unifying approach of selecting appropriate reference controls has yet been reached. At present we are limited to the use of miR-16 as the reference, based on the rationale that we found no significant variance in it among our samples, as shown in **Figures 1E,F**.

The widespread variance in levels of both miR-34c and its sister, miR-34a in AD plasma compared with the tight NEC cluster range, shown in **Figures 1–4**, clearly suggests individual variability in the control of these microRNAs' expression. From our small age-matched cohorts, miR-34c levels may be a better candidate than miR-34a and miR-34b for future large-scale study as a blood-based biomarker, simply because its expression in most mild AD patients shows higher levels than most NEC. The ROC analyses performed for both miR-34c and miR-34a 1/deltaCt values in age-matched cohorts proved the former to be an excellent discriminant (AUC = 0.99), whereas the latter was merely fairly good (AUC = 0.81). However, these results suggest the need of further research with larger cohorts, to stringently validate our results of sensitivity using miR-34c as a diagnostic biomarker for plasma samples. Likewise, the inverse correlation, observed only when the moderate and mild groups are compared with their NEC between cognitive scoring and levels of expression of miR-34c in plasma also needs future studies with larger cohorts to define their true relationship. Nevertheless, our findings open the possibility that plasma microRNAs, along with other biomarkers, may be fertile ground for future research linking cognitive decline with changes of gene expression in blood samples.

The increased miR-34c in AD plasma may derive from PBMC, where miR-34c is also increased. As reported, microRNAs present in the circulating plasma may be found either in the encapsulated vesicles known as exosomes, or in lipid- or protein-associated free particulates (Smalheiser, 2007; Etheridge et al., 2011). Reports so far are inconclusive as to what proportion of plasma microRNAs are associated with the former vs. the latter components. Future comparative studies of PBMC, exosomal and free-form miR-34c will reveal the relationship among these three components of AD circulating blood specimens responsible for this microRNA's increased expression.

MicroRNA-34c, as reported for its sister, miR-34a (He et al., 2007; Yamakuchi et al., 2008) and as shown in **Figure 8**, represses Bcl2, SIRT1, Psen1, and Onecut2, all associated with cellular survival and oxidative defense signaling (Clotman et al., 2005; Goodall et al., 2013). This led us to suggest that increased miR-34c may be one of many factors contributing to an overall systemic weakening of stress defense and cell survival, as suggested in our model presented in **Figure 9**. Life-long cumulative oxidative stress induction of p53 up-regulation may activate the expression of this microRNA, with a steadily increasing trend during aging (Li et al., 2011b,c). In AD, this oxidative stress may be further enhanced, manifested in brain as well as perhaps system-wide, reflected in circulating blood by both PBMC and plasma (Maes et al., 2010; Goodall et al., 2013). Future culture experiments in PBMC isolated from AD patients compared with those from normal counterparts will allow us to investigate whether these cells with increased miR-34c levels are indeed prone to apoptotic death, and whether plasma from the same samples stimulates stress and apoptotic signaling in neighboring cells.

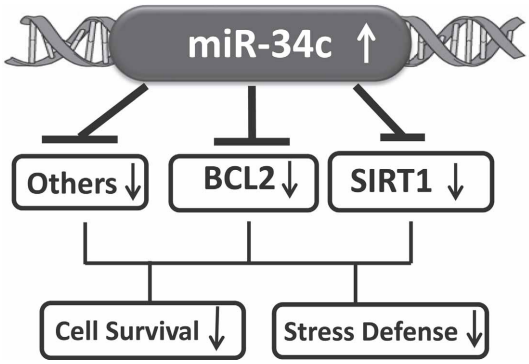

FIGURE 9 | A model suggesting that increased circulating miR-34c expression represses targets involved in oxidative stress and cell survival signaling pathways.

In conclusion, data presented here indicate that levels of miR-34c significantly increase in plasma samples of sporadic AD. Future studies with larger age-matched cohorts will validate these results, and reveal whether this microRNA change is characteristic of sporadic AD as a biomarker criterion, and further comparative studies will elucidate whether it is a common biomarker shared among various neurodegenerative disorders, associated with the decline of oxidative defense and cell survival in neuronal dysfunction.

DISCLOSURE STATEMENT

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnmol.2014.00002/abstract

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RISC in PD: the impact of microRNAs in Parkinson's disease cellular and molecular pathogenesis

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Parkinson's disease (PD) is a debilitating neurodegenerative disease characterized primarily by the selective death of dopaminergic (DA) neurons in the substantia nigra pars compacta of the midbrain. Although several genetic forms of PD have been identified, the precise molecular mechanisms underlying DA neuron loss in PD remain elusive. In recent years, microRNAs (miRNAs) have been recognized as potent post-transcriptional regulators of gene expression with fundamental roles in numerous biological processes. Although their role in PD pathogenesis is still a very active area of investigation, several seminal studies have contributed significantly to our understanding of the roles these small non-coding RNAs play in the disease process. Among these are studies which have demonstrated specific miRNAs that target and down-regulate the expression of PD-related genes as well as those demonstrating a reciprocal relationship in which PD-related genes act to regulate miRNA processing machinery. Concurrently, a wealth of knowledge has become available regarding the molecular mechanisms that unify the underlying etiology of genetic and sporadic PD pathogenesis, including dysregulated protein quality control by the ubiquitin-proteasome system and autophagy pathway, activation of programmed cell death, mitochondrial damage and aberrant DA neurodevelopment and maintenance. Following a discussion of the interactions between PD-related genes and miRNAs, this review highlights those studies which have elucidated the roles of these pathways in PD pathogenesis. We highlight the potential of miRNAs to serve a critical regulatory role in the implicated disease pathways, given their capacity to modulate the expression of entire families of related genes. Although few studies have directly linked miRNA regulation of these pathways to PD, a strong foundation for investigation has been laid and this area holds promise to reveal novel therapeutic targets for PD.

Keywords: Parkinson's disease, microRNA (miRNA), ubiquitin-proteasome system, autophagy, apoptosis, mitochondria, dopaminergic neurons, iPS cells

INTRODUCTION

PARKINSON'S DISEASE

PD is a progressive neurodegenerative disease which manifests as a debilitating movement disorder with late cognitive sequelae. Pathologically, PD is characterized by the selective loss of DA neurons in the midbrain substantia nigra pars compacta with four clinical hallmarks resulting from the loss of dopamine signaling in the striatum. These four hallmarks, which clinically define Parkinsonism, are pill-rolling tremor, cogwheel rigidity, bradykinesia/akinesia and postural instability (Savitt et al., 2006). With progression of the disease, the neurodegenerative process spreads to involve other brain regions. Most notably, the forebrain is commonly affected in late PD leading to cognitive decline and dementia (Jellinger, 2012). Despite pharmacologic therapy with dopamine replacement (Cotzias, 1968; Antonini and Cilia, 2009), and recent advances in surgical treatment with deep brain stimulation (Pereira and Aziz, 2006; Pereira et al., 2007; Farris and Giroux, 2011), there remains a void in understanding and inhibiting the underlying progressive neurodegeneration that defines PD. It has thus become a significant research focus of recent years to examine the molecular mediators underlying this process with the objective of translating this understanding to the development of new therapeutic approaches.

In recent years, researchers have identified genetic mutations which cause approximately 10% of PD cases (Klein and Westenberger, 2012). Investigations into the precise link between genetic mutation and disease, and the etiology of the 90% of sporadic PD cases, have led to the identification of molecular pathways that culminate in initial DA neuron injury and the resulting DA neuron death and progressive neurodegeneration. The primary pathways which have been implicated as mediators of the degenerative process are the protein quality control pathways, the ubiquitin-proteasome system (UPS) and autophagy pathway, as well as apoptosis, mitochondrial quality control, and DA differentiation and maintenance; the proposed role of each of these pathways in PD pathogenesis is described in detail below. Defects in these pathways may explain the underlying etiology of both genetic and sporadic PD pathogenesis. Thus, great interest has developed in understanding mechanisms of endogenous regulation of these pathways. Toward this end, the characterization

of miRNA function in PD pathogenesis has become of particular interest and the potential of these molecules to serve as pathway modifiers for therapeutic intervention in PD is increasingly appreciated.

miRNA BIOGENESIS AND FUNCTION

miRNAs are endogenous regulators of gene expression. These small non-coding RNAs (ncRNAs) can be transcribed by RNA polymerase II (RNA Pol II) from two primary genomic loci: miRNA genes and intronic sequences. In the canonical biogenesis pathway (**Figure 1A**), transcription from miRNA genes yields pri-miRNAs which are processed in the nucleus by the Drosha/DGCR8 microprocessor complex to produce pre-miRNAs. The processed pre-miRNAs are then exported to the cytoplasm by Exportin-5, where they are further cleaved by the RNase III enzyme Dicer to produce a mature miRNA duplex. The mature guide strand is 20–22 nucleotides in length and associates with Argonaute proteins, AGO 1–4, to form a functional RNA-induced silencing complex (RISC). The anti-sense strand, denoted by miRNA∗, was previously thought to be degraded; recent evidence suggests that some of these may have biological activity. The mature miRNA is then responsible for aligning the RISC to target mRNA by binding at complementary seed sequences in the 3′UTR. This association of target mRNA with the miRNA-containing RISC most commonly results in down-regulation of gene expression by translational repression and recruitment of protein complexes causing deadenylation and degradation of target mRNA. Conversely, miRNAs have also been shown to stabilize transcripts under certain cellular conditions (Melton et al., 2010).

The canonical pathway of miRNA biogenesis is also referred to as Drosha-dependent/Dicer-dependent miRNA biogenesis. In the much rarer, non-canonical Drosha-dependent/Dicer-independent miRNA biogenesis pathway, the exported pre-miRNA is processed by AGO2 in the cytoplasm to produce the mature guide strand (**Figure 1B**). miRNAs generated by another non-canonical pathway, termed Drosha-independent/Dicer-dependent miRNA biogenesis, are referred to as mirtrons (**Figure 1C**). In this pathway, mirtrons are spliced from the intronic sequences of transcribed genes, forming a lariat structure which is de-branched to form the pre-miRNA hairpin structure. After export from the nucleus, mirtrons function similarly to miRNA generated via the canonical pathway. Further discussion of miRNA biogenesis pathways can be found in detailed reviews on this topic (Bartel, 2009; Ameres and Zamore, 2013).

Our understanding of the role ncRNAs play in development and disease has expanded rapidly over the last decade. Recent insights are providing evidence that other classes of ncRNAs may contribute to transcriptome alterations in PD. For a more detailed discussion on the roles of long ncRNAs and small vault RNAs in PD, the reader is referred to the following references (Minones-Moyano et al., 2013; Wu et al., 2013). miRNAs in particular have been identified as critical regulators of gene expression in a number of normal biological and pathophysiological processes. miRNA dysregulation has been convincingly linked to a number of neurodegenerative diseases. For a discussion of the role miRNAs play in Alzheimer's disease and Huntington's disease, the reader is referred to the enclosed references (Delay et al., 2012; Sinha et al., 2012). Recently discovered relationships between PD-related genes and miRNAs and consideration of miRNA regulation of cellular and molecular pathways that have been implicated in PD pathogenesis are explored further in this review.

miRNA-BASED THERAPEUTICS

A better understanding of the role miRNAs play in the cellular and molecular pathogenesis of PD will undoubtedly contribute to the development of novel miRNA-based therapies. It is therefore, imperative to make a number of important considerations in moving miRNAs from molecular targets to viable therapeutics. First, because of their ability to modulate the expression of families of related genes that participate in common cellular and molecular pathways, miRNAs hold great potential to restore balance to dysregulated pathways at the onset and very early stages of PD (Ouellet et al., 2006). However, the ability to apply a miRNA-based therapy within this critical time period is currently limited by an inability to accurately diagnose PD at these early stages (Akhtar and Stern, 2012). Thus, the detection of biomarkers, development of sensitive imaging techniques, and discovery of clinical criteria that can discern PD in these early stages will be critical for the development of miRNA-based therapeutics with disease-altering potential (Akhtar and Stern, 2012). Additionally, given the mechanism of action of RNAi-based therapies, it is important to consider off-target effects resulting from transcriptional and translational repression of unintended miRNA targets (Rao et al., 2009). In the development of miRNA-based therapies for PD, it is also necessary to consider the route of delivery to achieve therapeutic levels of the miRNA in the brain (Boudreau et al., 2011). Although targeted delivery of small RNAs to the brain has presented a formidable challenge in the past, recent evidence suggests that exosomes may be used to deliver exogenous cargo, including nucleic acids, to the brain, providing a novel means of overcoming this current limitation to the translation of miRNA-based therapies (Alvarez-Erviti et al., 2011). Finally, an important limitation to the testing of miRNA therapeutics is the lack of an animal model that recapitulates key features of PD pathology (Beal, 2010). Although animal models have been developed that display nigostriatal degeneration, an animal model that demonstrates Lewy body pathology, a key feature of human Parkinsonism, remains to be discovered. This underscores a potential difference between the cellular and molecular mechanisms of Parkinson's symptomatology in existing models compared to that in humans, presenting a challenge for the accurate detection and application of miRNAs that would modulate these pathways for therapeutic benefit. Despite these limitations, it has been demonstrated that an integrated analysis of PD mouse models, existing human cell culture models and novel human iPSC-derived DA neuron models can provide an accurate prediction of clinical efficacy for drug treatments of PD, indicating promise for utilizing such an integrated approach for testing miRNA-based therapies. Specifically, it has been shown that a subset of compounds which were found to have pharmacologic benefit in mouse models of PD had a protective effect in MPP+-treated SH-SY5Y cells, and further that a subset of those were

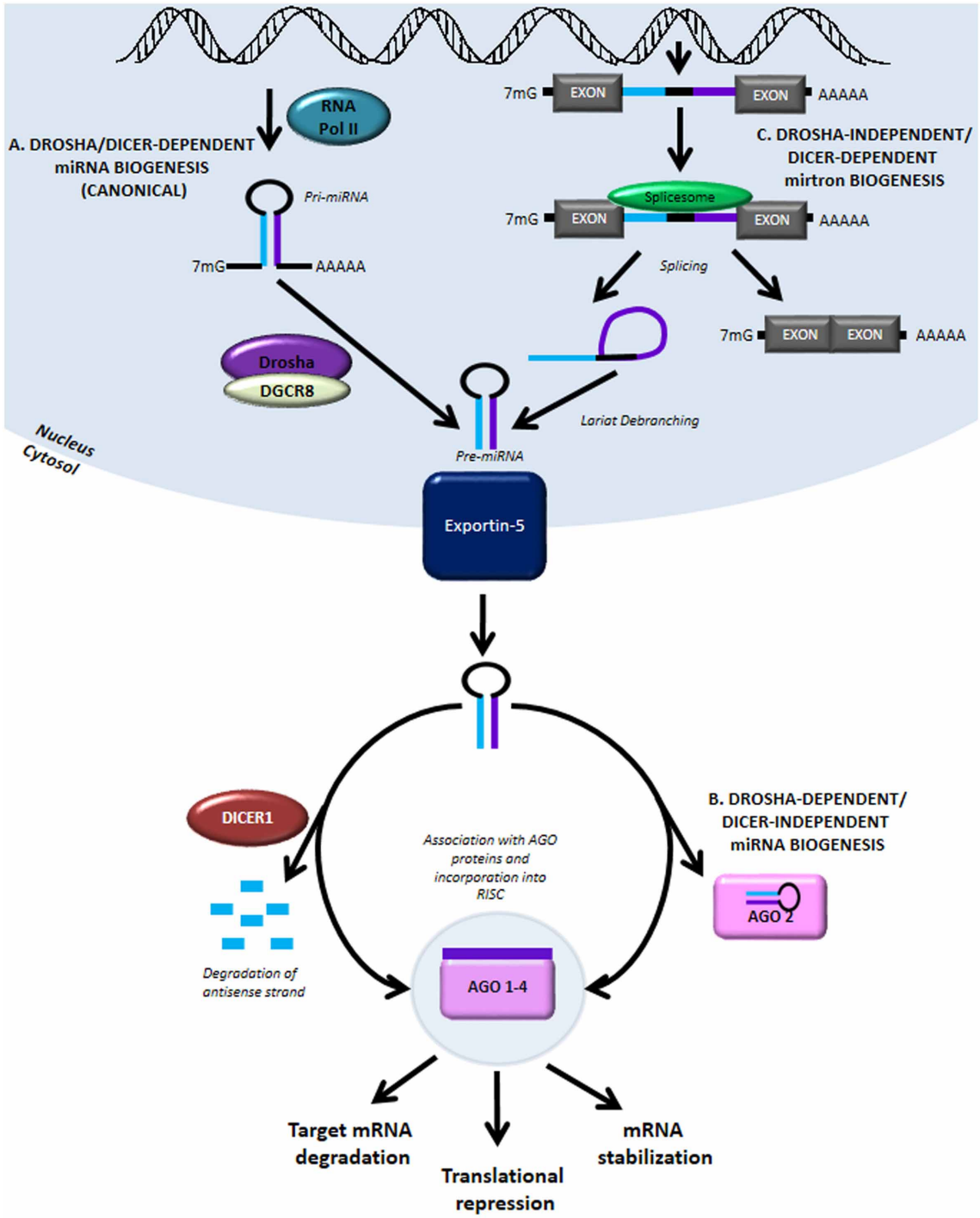

FIGURE 1 | MiRNA biogenesis and function. (A) The canonical miRNA biogenesis pathway is Drosha- and Dicer-dependent. It begins with RNA Pol II-mediated transcription of genomic loci containing miRNA genes. The primary transcript is referred to as pri-miRNA and like other RNA Pol II transcripts contains a 5′ 7-methylgunaosine cap and 3′ poly-A tail. The pri-miRNA is processed in the nucleus by Drosha and DGCR8 to form pre-miRNA. The pre-miRNA is then exported into the cytoplasm by Exportin-5 where it is further processed by the RNase III enzyme, Dicer. Interaction with Dicer leads to hairpin cleavage and degradation of the anti-sense strand of the miRNA duplex, while the mature guide strand is complexed with members of the Argonaute family of proteins to form a functional RNA-induced silencing complex (RISC). **(B)** In a Drosha-dependent and Dicer-independent non-canonical pathway of miRNA biogenesis, the cytoplasmic pri-miRNA undergoes processing by AGO2, although the precise mechanism of miRNA maturation via this pathway remains unclear. **(C)** Another non-canonical pathway involving Drosha-independent/Dicer-dependent biogenesis generates mirtrons, transcribed from intronic sequences and obtained by splicing and lariat-debranching. Mature miRNA can act via three primary methods: (1) destabilization and cleavage of target mRNA, (2) translational repression, and (3) mRNA stabilization.

protective in MPP+-treated iPSC-derived TH+ neurons (Peng et al., 2013). Importantly, those compounds which were effective in both SH-SY5Y and iPSC-derived TH+ neurons had the greatest benefit when translated to patient care (Peng et al., 2013). Similar integrative approaches using non-human primate and human iPSC culture models have been successful in predicting miRNA-based therapeutic efficacy (Chan and Kocerha, 2012). Thus, this integrative approach provides a means of thoroughly testing miRNA-based therapeutics with a readout that can provide an indication of clinical effectiveness as never previously attainable.

INTERACTION BETWEEN miRNA AND PD-RELATED GENES

PD-RELATED GENES

Of the 28 distinct chromosomal loci that have been convincingly linked to PD, only six have been demonstrated to cause heritable monogenic PD (Klein and Westenberger, 2012). **Table 1** lists these six genes, their mode of inheritance, and the cellular pathways currently thought to be affected. The following sections discuss studies which have identified miRNA interactions with these genes; these are summarized in **Figure 2**. No studies to date have identified direct miRNA interactions with PINK1 or ATP13A2.

Table 1 | PD-related genes.

PD-related gene symbol	Gene name	Mode of inheritance	Relevance in PD
PARK1/PARK4	Alpha synuclein	Autosomal dominant	Point mutations and gene multiplications cause synucleinopathy
PARK2	Parkin	Autosomal recessive	Loss of E3 ubiquitin-ligase activity leads to aberrancies of ubiquitin-proteasome system and mitophagy
PARK6	PINK1	Autosomal recessive	Dysfunction of mitochondrial quality control
PARK7	DJ-1	Autosomal recessive	Dysfunction of mitochondrial quality control
PARK8	LRRK2	Autosomal dominant	Gain of kinase activity; proposed aberrancies of membrane trafficking and cytoskeletal dynamics
PARK9	ATP13A2	Autosomal recessive	Causes Kufor-Rakeb syndrome (atypical PD); normally located in lysosomal membrane, retained in ER in disease

Mutations in these six genes have been demonstrated to cause inherited monogenic forms of PD. Several miRNAs have been identified to regulate particular genes in this list, including alpha synuclein and LRRK2. A miRNA-gene interaction has been described for DJ-1 and Parkin, however the mechanisms remain elusive. Each of these interactions is described in detail in the text. Of note, no miRNA have yet been described to interact with PINK1 or ATP13A2.

ALPHA SYNUCLEIN LEVELS ARE REGULATED BY miR-7 AND miR-153

Alpha synuclein has a long-known role in PD pathogenesis. Point mutations, duplications, and triplications in this gene are sufficient to cause the death of DA neurons via alpha synuclein aggregation and neurotoxicity (Singleton et al., 2003). Additionally, alpha synuclein is a major component of Lewy bodies, a pathologic hallmark of PD (Spillantini et al., 1997, 1998). A dose-response relationship of this gene has been described, in which individuals with alpha synuclein multiplications develop PD at an earlier onset age and with increasing severity associated with dementia (Singleton et al., 2003; Farrer et al., 2004). Where the overproduction of a gene product is the mechanism by which the gene contributes to PD pathogenesis, there is a clear implication that miRNA-mediated gene suppression might hold potential to improve the disease phenotype.

Using this as a basis for investigation, two groups independently discovered miR-7 as a regulator of alpha synuclein expression (Junn et al., 2009; Doxakis, 2010). Junn et al. demonstrated that miR-7 levels are higher in the substantia nigra and striatum of mice, compared to cerebral cortex and cerebellum.

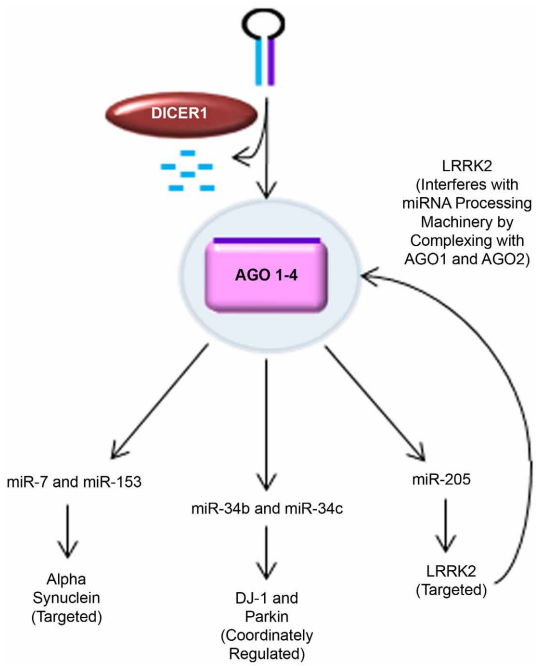

FIGURE 2 | MiRNA regulation of PD-related genes. Novel findings supporting the role of miRNA in regulating key PD-related genes are shown. Alpha synuclein was predicted to have seed sequences in the 3′ UTR for miR-7 and miR-153. This was experimentally validated by Junn et al. (miR-7) and Doxakis (miR-7 and miR-153) in several PD-relevant models, including mice challenged with MPTP and MPP+-treated SH-SY5Y cells. DJ-1 and Parkin were found to be coordinately regulated with miR-34b and miR-34c expression in PD patient brains and SH-SY5Y cells (Minones-Moyano et al., 2011). LRRK2 was putatively predicted to be regulated by miR-205 and experimentally confirmed in post-mortem PD patient brain samples, primary cultured mouse cortical neurons, and LRRK2 R1441G BAC transgenic mice (Cho et al., 2013). Interestingly, pathogenic LRRK2 has also been found to interfere with miRNA processing machinery by complexing with drosophila AGO1 and human AGO2 (Gehrke et al., 2010).

MiR-7 levels were found to be 40 times higher in neurons than in astrocytes, and as well alpha synuclein was detected in neurons but not astrocytes. This provides support for endogenous miR-7 regulation of alpha synuclein levels in neurons. To further understand the potential implications of miR-7 in PD, the authors investigated miR-7 levels in MPP+ treated SH-SY5Y cells, and MPTP-intoxicated mice, finding elevated alpha synuclein levels, and reduced miR-7 levels in both cases. This indicates that a reduction in miR-7 may contribute to nigrostriatal degeneration. Doxakis et al. independently described a similar result of alpha synuclein regulation by miR-7, as well as miR-153. Additionally, overexpression of miR-7 and miR-153 in cultured cortical neurons caused a 30–40% reduction in endogenous alpha synuclein levels. Taken together these results suggest that delivery of miR-7 and miR-153 may represent an appealing therapeutic strategy to promote neuroprotection in patients with known alpha synuclein gene multiplications.

LRRK2 IS TARGETED BY miR-205

The precise normal function of the leucine-rich repeat kinase 2 (LRRK2) has yet to be determined, although recent evidence suggests involvement in membrane trafficking (West et al., 2005; Biskup et al., 2006, 2007; Gloeckner et al., 2006; Hatano et al., 2007; Sakaguchi-Nakashima et al., 2007; Alegre-Abarrategui and Wade-Martins, 2009; Alegre-Abarrategui et al., 2009; Lee et al., 2010a,b,c; Tong et al., 2010; Vitte et al., 2010) and cytoskeletal dynamics (Jaleel et al., 2007; Gandhi et al., 2008; Gillardon, 2009; Parisiadou et al., 2009; Lin et al., 2010). Mutations in LRRK2 have been identified as the most common cause of dominantly inherited PD (Brice, 2005; Lesage et al., 2006; Ozelius et al., 2006; Healy et al., 2008) and importantly, variation in the LRRK2 gene has been implicated as a risk factor for sporadic PD (Kett and Dauer, 2012). LRRK2 is thought to contribute to PD pathogenesis through a gain-of-function mechanism (Kett and Dauer, 2012). Indeed, the most common LRRK2 mutation, a glycine to serine substitution at position 2019 (G2019S), leads to increased activity in the activation loop of the kinase domain (West et al., 2005; Greggio et al., 2006; Jaleel et al., 2007). The finding that LRRK2 inhibition blocks neurotoxicity *in vitro* and *in vivo* provides additional support for a gain-of-function mechanism (Greggio et al., 2006; Smith et al., 2006; Lee et al., 2010a).

Congruent with the notion that increased LRRK2 activity contributes to PD pathogenesis, Cho et al. demonstrated that normal LRRK2 levels are higher in the frontal cortex of sporadic PD and PD with dementia (PDD) patients compared to non-pathological controls (NPC) (Cho et al., 2013). LRRK2 mRNA levels were found to be comparable between these groups, suggesting post-transcriptional regulation, as would be mediated by miRNA. MiR-205 was identified as a putative regulator of LRRK2 by target prediction algorithms. Further investigation revealed significantly lower levels of miR-205 in the frontal cortex and striatum of PD and PDD patients, compared to NPC. In primary mouse cortical neurons, inhibition of miR-205 caused upregulation of LRRK2 protein expression whereas overexpression of miR-205 repressed LRRK2 protein expression. Strikingly, wild type mouse midbrain DA neurons displayed a high level of miR-205 and in transgenic mice overexpressing R1441G (arginine to glycine at position 1441) mutant LRRK2, miR-205 treatment rescued impairment of neurite outgrowth. This study described a novel regulatory role for miR-205 with LRRK2. Given that over-activity of LRRK2 is suggested to cause PD, therapeutic replacement of miR-205 is an attractive target for therapeutic intervention, particularly for sporadic cases in which LRRK2 levels were found to be elevated and miR-205 levels were found to be low.

It is worth noting that new insights are beginning to reveal a reciprocal role for LRRK2 in regulating miRNA biogenesis by interfering with the miRNA processing machinery through complexing with drosophila AGO1 and human AGO2 (Gehrke et al., 2010). Further investigation will be required before a precise role for LRRK2 regulation of miRNA biogenesis and translational repression can be fully appreciated.

DJ1 AND PARKIN ARE COORDINATELY REGULATED WITH miR-34B AND miR-34C

Miñones-Moyano et al. profiled the miRNA expression pattern in post-mortem tissue from PD patient brains, discovering a dysregulation of miR-34b and miR-34c (Minones-Moyano et al., 2011). The authors identified miR-34b and miR-34c downregulation at advanced stages of PD finding that miR-34 reduction compromises neuronal viability by mitochondrial dysfunction and production of reactive oxygen species in an SH-SY5Y neuroblastoma culture model. They further characterized that the miR-34b/c reduction is correlated with decreased expression of DJ1 and Parkin, noting that these proteins were indeed downregulated in PD brain tissue as well. This provides evidence that miR-34b/c downregulation may involve DJ1 and Parkin, however the precise mechanism by which this interaction occurs remains unclear.

It is an important consideration that as DA neurons degenerate throughout the life of PD patients, post-mortem tissue samples often lack the quantity and quality of DA neurons necessary to delineate whether the molecular defects observed are truly relevant to the DA neurons that produce pathology, or whether they are simply a result of the lack of the diseased neurons in the region investigated. To date, examinations of post-mortem brain tissue have provided the most physiologically relevant means of investigating the molecular mechanisms of PD. Newer models such as PD patient-specific induced pluripotent stem cell (iPSC)-derived DA neurons will aid in further interpreting and probing miRNA mediated defects in human DA neurons and delineating the precise molecular events which lead to disease.

PREDICTED miRNA TARGETING PD-RELATED GENES

Although significant progress has been made in our understanding of the role miRNA play in regulating PD-related genes, much remains to be answered. A small number of miRNA have been identified to regulate the six monogenic PD-causing genes, whereas many more which can be identified as putative targets by multiple target prediction algorithms (Enright et al., 2003; John et al., 2004; Lewis et al., 2005; Grimson et al., 2007; Betel et al., 2008, 2010; Friedman et al., 2009; Maragkakis et al., 2009a,b; Garcia et al., 2011), have not been experimentally validated (**Table 2**). Although central to discovery of miRNA targets, this underscores both the limitations of target prediction algorithms

Table 2 | Putative miRNA targeting PD-related genes.

PD-Related gene	TargetScan	miRanda		DIANA microT
Alpha synuclein (PARK1/PARK4)	**miR-7**	miR-539	miR-431	**miR-7**
	miR-7a	**miR-7**	miR-23b	**miR-153**
	miR-7b	miR-488	miR-23a	
	miR-153	miR-504	miR-425	
	miR-223	miR-487b	miR-216a	
	miR-214	miR-374b	miR-340	
	miR-761	miR-374a	miR-133b	
	miR-3619-5p	miR-144	miR-133a	
		miR-495	miR-125a-3p	
		miR-153	miR-485-5p	
		miR-129-5p	miR-17	
		miR-599	miR-106b	
		miR-223	miR-106a	
		miR-449b	miR-519d	
		miR-449a	miR-20b	
		miR-34-c-5p	miR-20a	
		miR-34a	miR-93	
		miR-148a	miR-342-3p	
		miR-152	miR-454	
		miR-148b	miR-130a	
		miR-539	miR-494	
		miR-361-5p	miR-222	
		miR-182	miR-221	
		miR-29b		
Parkin (PARK2)	***miR-181a***	miR-379	miR-488	miR-147
	miR-181b	miR-544	miR-216a	***miR-181c***
	miR-181c	miR-488	miR-203	***miR-181a***
	miR-181d	miR-590-3p	miR-187	***miR-181d***
	miR-4262	miR-708	miR-185	***miR-181b***
		miR-28-5p	miR-505	
		miR-599	miR-320d	
		miR-363	miR-320c	
		miR-367	miR-320b	
		miR-25	miR-320a	
		miR-92b	miR-140-5p	
		miR-92a	miR-876-5p	
		miR-32	miR-222	
		miR-125a-3p	miR-221	
		miR-146b-5p	miR-199b-5p	
		miR-146a	miR-199a-5p	
		miR-155	miR-200a	
		miR-758	miR-141	
		miR-181c	miR-19b	
		miR-181b	miR-19a	
		miR-181a	miR-200b	
		miR-181d	miR-200c	
		miR-543	miR-429	
PINK1 (PARK6)	miR-532-3p	miR-346		*
		miR-124		
		miR-506		
		miR-216b		
		miR-340		
		miR-217		
DJ-1 (PARK7)	*	miR-128		*
		miR-758		
		miR-539		
		miR-216b		
		miR-544		
		miR-365		
		miR-874		
LRRK2 (PARK8)	**miR-205**	miR-384	miR-103	***miR-181c***
	miR-205a	miR-590-3p	miR-708	***miR-181a***
	miR-205b	miR-185	miR-28-5p	***miR-410***
	miR-19a	**miR-205**	miR-30a	***miR-19b***
	miR-19b	miR-543	miR-30b	***miR-19a***
	miR-181a	miR-410	miR-30c	***miR-454***
	miR-181b	miR-136	miR-30d	**miR-205**
	miR-181c	miR-301b	miR-30e	***miR-181b***
	miR-181d	miR-301a	miR-328	***miR-181d***
	miR-4262	***miR-454***	miR-186	
	miR-130a	***miR-19b***	miR-429	
	miR-130c	***miR-19a***	miR-200c	
	miR-301a	miR-382	miR-200b	
	miR-301b	miR-144	miR-23b	
	miR-301b-3p	miR-384	miR-23a	
	miR-454	miR-376c	miR-340	
	miR-721	***miR-181c***	miR-129-5p	
	miR-4295	***miR-181d***	miR-32	
	miR-3666	***miR-181b***	miR-363	
		miR-181a	miR-367	
		miR-381	miR-92b	
		miR-300	miR-92a	
		miR-141	miR-25	
		miR-200a	miR-9	
		miR-107		
ATP13A2 (PARK9)	*	miR-199a-5p	miR-424	
		miR-199b-5p	miR-15a	
		miR-24	miR-15b	
		miR-299-3p	miR-497	
		miR-122	miR-433	
		miR-16	miR-873	
		miR-195		

*This table presents miRNA which are independently predicted to target PD-related genes by three different open-access target prediction algorithms. Bolded miRNA have been experimentally validated, whereas bolded and italicized miRNAs were identified by all three algorithms, but have not been experimentally confirmed. *, No targets found using the following parameters: miRNA mapped to "Conserved sites for miRNA families broadly conserved among vertebrates" or those listed as "Conserved" in TargetScan; score threshold of 7.3 in DIANA-microT 3.0.*

as well as the necessity for models which more closely recapitulate the pathophysiology of PD to delineate the specific effects of these miRNAs in the context of PD.

GLOBAL miRNA DYSREGULATION IN PD PATHOGENESIS

Many compelling lines of evidence suggest a role for global miRNA dysregulation from interference with miRNA processing machinery in neurodegeneration. Defects due to Dicer knockout are evident as early as the embryonic stage in mice, resulting in defects in cell proliferation (Murchison et al., 2005) and differentiation (Kanellopoulou et al., 2005). Importantly, mouse Dicer knockouts demonstrate lethality before neurulation (Bernstein et al., 2003). Depletion of Dicer in the developing mouse neocortex leads to reduction in cortex thickness and defective cortical layering, and the mouse dies shortly after weaning (De Pietri Tonelli et al., 2008). Several studies have demonstrated the appearance of hallmarks of neurodegeneration in mouse brains with conditional knockout of Dicer in cortical, hippocampal, cerebellar, motor and striatal neurons, as well as astroglia (Schaefer et al., 2007; Cuellar et al., 2008; Davis et al., 2008; Kawase-Koga et al., 2009; Haramati et al., 2010; Tao et al., 2011). Furthermore, loss of the DGCR8 component of the microprocessor complex has been shown to produce neuronal and behavioral defects in mice (Stark et al., 2008). Additionally, mice with lineage specific defects in miRNA processing machinery have been shown to develop defects in spinal motor neurons, reminiscent of spinal muscular atrophy (Haramati et al., 2010) as well as defects in forebrain neurons, consistent with Alzheimer's pathology (Hebert et al., 2010).

One of the first studies to demonstrate a role for miRNAs in the maintenance of midbrain DA neurons was conducted by Kim et al. (2007). Cre-mediated deletion of Dicer in ES cells at stage 4 of differentiation, when post-mitotic DA neurons first arise, resulted in complete loss of DA neuron accumulation at stage 5, while the generation of other mature neuronal classes was less affected (i.e., GABAergic neurons and TUJ1 positive neurons). Importantly, the phenotype was rescued by transfection of low molecular weight RNA species, indicating that the observation indeed results from the lack of mature small RNA species, including miRNA. Furthermore, in a rodent model, conditional knockout of Dicer under the control of the dopamine transporter (DAT) induced apoptosis in substantia nigra, and behavioral studies demonstrated dramatically reduced locomotion, reminiscent of the phenotype of human PD. This seminal study provided some of the first evidence that miRNAs have a unique role in the development and maintenance of midbrain DA neurons. We further discuss the roles of specific miRNA in developing DA neurons in section DA neuron differentiation and maintenance.

ROLE OF miRNA IN PATHWAYS IMPLICATED IN PD MOLECULAR PATHOGENESIS

In recent years, dysfunction of a number of critical pathways has been directly implicated in the pathogenesis underlying PD (**Figure 3**). Such aberrant pathways are thought to be a common effector leading to PD in both inherited and sporadic cases. Whereas the PD-related gene interactions with miRNAs discovered to date generally describe one miRNA interacting with one gene, perhaps the most impactful role of miRNAs is their ability to regulate families of related genes. In this regard, understanding the roles of these pathways in PD pathogenesis and the mechanism by which miRNA may form regulatory networks with the gene families they comprise will be essential to our ability to harness the potential of miRNA to serve as neuroprotective and disease-modifying agents in PD therapy.

PROTEIN QUALITY CONTROL

A well described cellular phenomenon observed in nearly all forms of neurodegeneration, including PD, is abnormal protein accumulation (Ross and Poirier, 2004). This protein accumulation is primarily thought to be a result of dysfunctional protein clearance, although it is worth noting that it may also be the consequence of protein overproduction, as is seen in patients with alpha synuclein gene multiplications (Singleton et al., 2003). The two primary mechanisms by which cells perform clearance of protein aggregates are the UPS and autophagy (Cook et al., 2012). Whereas the UPS is the primary mechanism by which damaged or misfolded proteins are degraded by the cell, it has limited capacity to handle protein aggregates, such as those that form the characteristic Lewy bodies of PD. In contrast, the autophagy pathway has the ability to rid the cell of large protein aggregates as well as aged and damaged organelles, including mitochondria (mitophagy). Since damaged mitochondria and protein aggregation are suggested events upon which PD pathogenesis converges, autophagy has recently been recognized as a pathway which may unify many divergent cellular etiologies of PD pathogenesis.

Ubiquitin-proteasome system

The UPS is comprised of E1 ubiquitin-activating enzymes which generate a reactive thiol ester between the E1 cysteine residues and the C-terminal glycine of ubiquitin, E2 ubiquitin-conjugating enzymes which carry ubiquitin to the protein substrate, and E3 ubiquitin ligase enzymes which catalyze the ligation of ubiquitin to the protein substrate (Hershko and Ciechanover, 1992). Whereas E1 and E2 enzymes have non-specific activity, E3 ubiquitin ligases confer target specification. After the addition of a minimum of four ubiquitin molecules, the protein substrate is carried into the 20S proteolytic core of the 26S proteasome for cleavage (Hershko and Ciechanover, 1998; Ciechanover et al., 2000; Ciechanover and Brundin, 2003).

Dysfunction of the UPS has been implicated in both genetic and sporadic forms of PD. In brains from sporadic PD patients, ubiquitinated proteins and components of the UPS appear in Lewy bodies (Lennox et al., 1989; Lowe et al., 1990; Li et al., 1997; Auluck et al., 2002; McNaught et al., 2002; Schlossmacher et al., 2002). Furthermore, Parkin has been identified as an E3 ubiquitin ligase, mutations in which have been confirmed to cause an autosomal recessively inherited form of early-onset PD (Klein and Westenberger, 2012). Interestingly, patients with these mutations tend to display a loss of DA neurons, but no Lewy body accumulation, indicating that Parkin activity may be required for LB formation (Cook et al., 2012). It is worth noting that the controversial (Maraganore et al., 2004; Healy et al., 2006)

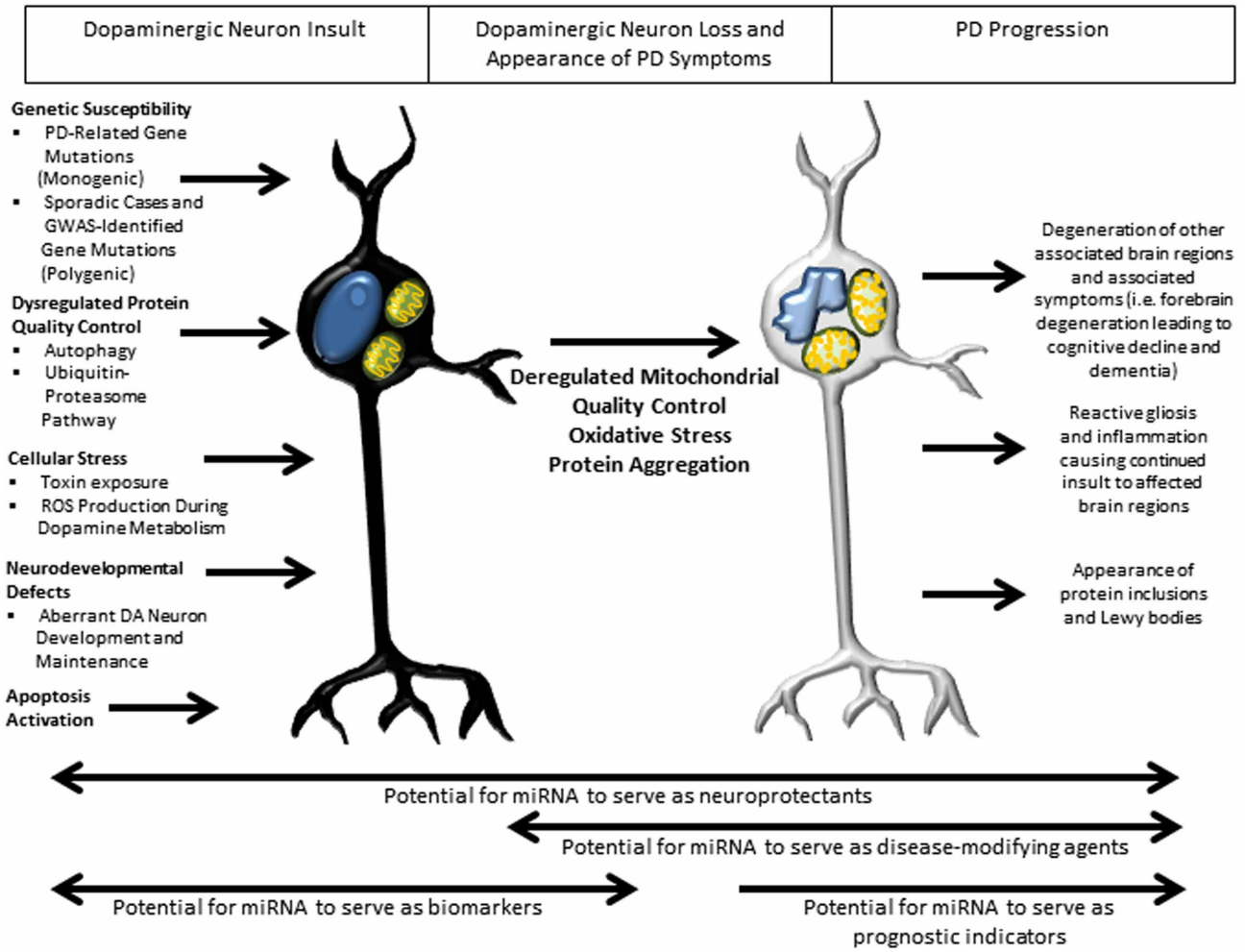

FIGURE 3 | Overview of pathways implicated in PD pathogenesis and potential roles of miRNA in therapy. Several well-studied cellular pathways have now been implicated in PD pathogenesis, with an apparent convergence on mitochondrial damage, oxidative stress and protein aggregation leading to DA neuron death and downstream sequelae of the progressive pathogenic process. In the earliest stages of insult, miRNA may have their greatest therapeutic benefit as biomarkers, with the potential to identify patients before the appearance of symptoms, allowing early therapeutic intervention. Throughout the disease course, miRNA might serve as neuroprotectants owing to their ability to regulate families of genes. Later in the disease course there is the potential for miRNA to serve as disease-modifying agents and prognostic indicators of disease progression.

ubiquitin carboxy-terminal hydrolase L1 (UCHL1/PARK5), gene acts in the UPS to hydrolyze the E1-ubiquitin bond formed by E1 ubiquitin-activating enzymes. Thus, despite uncertainty as to whether UCHL1 is a true PD susceptibility factor, its link to PD and the UPS overall provides evidence supporting a role for UPS dysfunction in PD.

The UPS has also been recently identified as a regulator of miRNA biogenesis (Smibert et al., 2013). In drosophila, interference with the UPS leads to accumulation of AGO1. Additionally, the stability of AGO2 in mouse cells is linked to miRNA availability. AGO2 is decreased with Dicer-knockout, causing a loss of miRNAs, but rescued by proteasome blockade. Together these findings implicate the UPS as an essential regulator of miRNA biogenesis by controlling levels of miRNA processing enzymes and RISC components. Although this study provides initial evidence for an interaction between the UPS and miRNA function, no studies as of yet have directly linked miRNA dysregulation of the UPS to PD.

Autophagy

Autophagy is the only known pathway by which cells can degrade organelles and protein aggregates that cannot be processed by the proteasome (Lynch-Day et al., 2012). There are three main types of autophagy, which are active to varying degrees in different cell types: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy. CMA is a process wherein misfolded proteins are carried to lysosomes by chaperone proteins and transferred across the lysosomal membrane for degradation by hydrolases. In microautophagy, the lysosome invaginates to take in misfolded proteins, protein aggregates and other cytosolic substrates. Once inside the lysosome, the vesicle contents are quickly hydrolyzed. In macroautophagy large sections of the cytosol are

engulfed by a double-membrane vesicle known as an autophagosome. The autophagosome then fuses with lysosomes, releasing lysosomal enzymes and hydrolases into its lumen to degrade its contents.

Compared to other cell types, neurons have a higher basal rate of autophagy, necessary due to the inability to dilute aged and damaged particles via mitosis (Son et al., 2012). However, aberrancies in this process have long been postulated as a source of neuronal death and neurodegeneration (Son et al., 2012). Macroautophagy is the most clearly linked to PD pathogenesis thus far (Cook et al., 2012). Several studies have described involvement of the aforementioned PD-related genes in the autophagy pathway, including LRRK2 and alpha synuclein (Bandyopadhyay and Cuervo, 2007; Alegre-Abarrategui and Wade-Martins, 2009). Cells harboring alpha synuclein mutations and gene multiplications have been found to demonstrate greater autophagic clearance of normal mitochondria. In cell culture models pre-aggregated alpha synuclein is resistant to degradation and impairs autophagy. Mechanistically these aggregates impair overall macroautophagy by reducing autophagosome clearance. This potentially could contribute to the increased cell death observed in aggregate-bearing cells (Tanik et al., 2013). Furthermore, Parkin and PINK1 have been described to act in concert to remove damaged mitochondria by promoting their degradation via mitophagy (Lee et al., 2010b; Matsuda et al., 2010; Narendra et al., 2010). It is suggested that both reduced and overactive autophagy are detrimental to the health of DA neurons, thus making miRNAs particularly compelling as therapeutic targets given their key properties of containing endogenous regulatory mechanisms and functioning to fine-tune the activity of gene families.

Regulation of miRNA homeostasis by autophagy. A recent paper by Gibbings et al. demonstrated a novel role for the autophagic pathway in miRNA homeostasis (Gibbings et al., 2012, 2013). In this study, it was found that autophagy regulates miRNA biogenesis and function via selective degradation of DICER1 and AGO2. These enzymes accumulate in cells lacking the key autophagy genes, ATG5, ATG6, ATG7, as well as the selective autophagy receptor, CALCOCO2. Interestingly, when the selective receptor CALCOCO2 is depleted, ubiquitinated AGO2 accumulates, suggesting a role for ubiquitin-mediated clearance, in addition to ubiquitin-independent recognition events. Importantly, pre-miRNA, miRNA and miRNA* strands are not degraded by autophagy. However, when autophagy is inhibited, the ability of AGO2 to bind miRNA duplexes is decreased, and this prevention of miRNA loading onto AGO2 leads to miRNA instability and decay. Ultimately, this release of translation repression leads to overexpression of proteins which are regulated by miRNA (**Figure 4**). This is the first study to demonstrate a role for autophagy in regulating miRNA homeostasis.

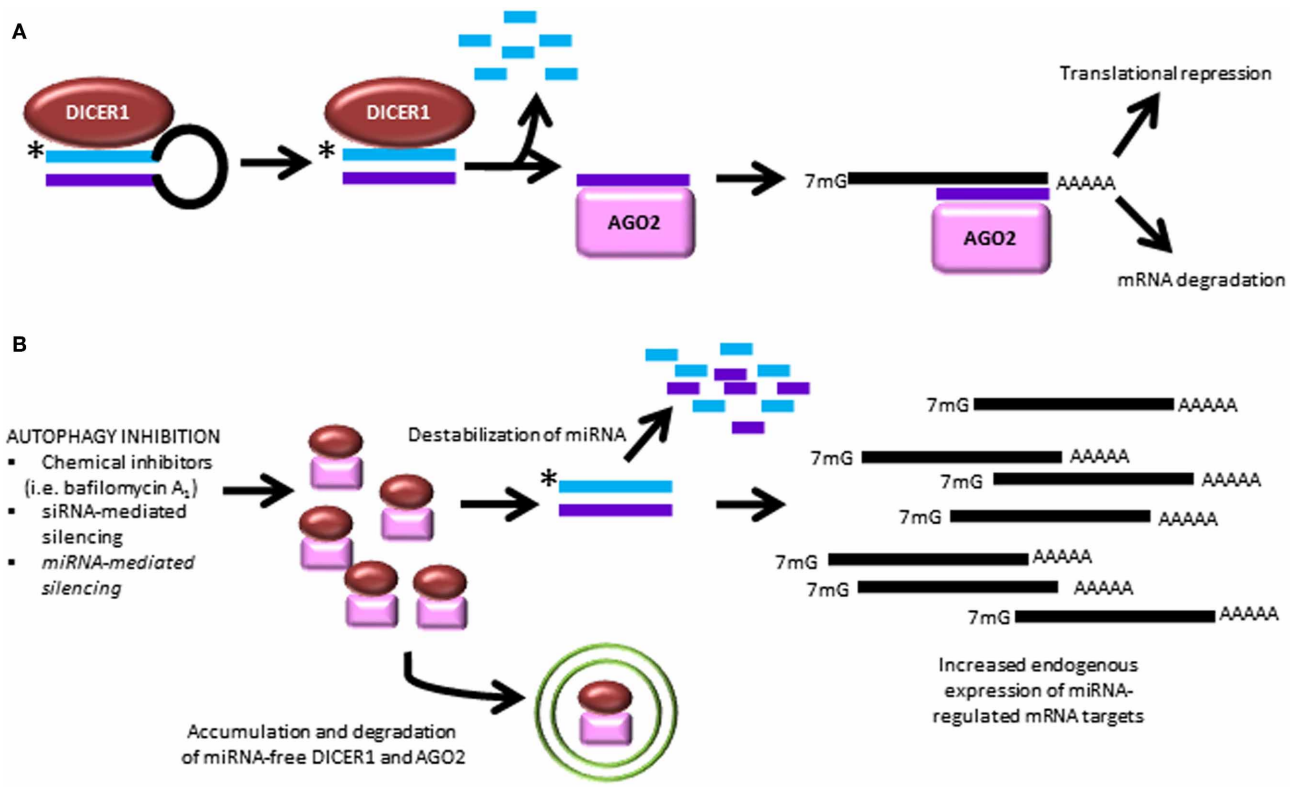

FIGURE 4 | Autophagy inhibition causes dysregulated miRNA processing and function. (A) The canonical pathway of miRNA biogenesis is shown leading to the two best characterized actions of miRNA to cause degradation of mRNA targets and interfere with mRNA translation. **(B)** Recent findings demonstrate that autophagy inhibition leads to break-down of miRNA-free Dicer and AGO2, causing downstream miRNA instability and overexpression of untargeted transcripts (Gibbings et al., 2012, 2013). *, antisense miRNA strand, which is degraded during processing.

miRNA downregulation of autophagy in PD. In another recent paper, Alvarez-Erviti et al. expanded on their previous findings that LAMP-2A and hsc70, two autophagy mediators, are low in PD brains by investigating the modulation of the CMA pathway by miRNA (Alvarez-Erviti et al., 2013). Target prediction identified four putative LAMP-2A targeting miRNAs and two putative hsc70 targeting miRNAs, which had also been previously reported to be increased in PD brains (Kim et al., 2007). MiR-106a caused a dose-dependent decrease in hsc70 3′UTR activity, as did miR-224 for LAMP-2A. Ultimately LAMP-2A was found to be regulated by four and hsc70 by three of the identified miRNAs. Strikingly, alpha synuclein levels increased in response to all of the miRNAs tested, presumably because of decreased activity of the CMA pathway. Importantly, three miRNA targeting LAMP-2A and three targeting hsc70 were significantly increased in PD brain substantia nigra pars compacta and amygdala, associated with a decrease in protein levels. Thus, this paper provides additional evidence supporting an intricate interplay between autophagy and miRNA pathways, in this case providing evidence that miRNA may be useful modulators of CMA-related dysfunction in PD.

Although we are only beginning to uncover the roles of miRNA in regulating autophagy in the context of PD, already, there are critical insights gained from studies in other systems that foreshadow investigation in PD-relevant cellular and animal models. Since it has been suggested that cancer and neurodegeneration are manifestations of opposing cellular dysfunctions (Plun-Favreau et al., 2010), studies of miRNA regulation of autophagy in cancer may provide an invaluable foundation upon which investigations of miRNA regulation of autophagy in neurodegeneration may build (Frankel and Lund, 2012).

APOPTOSIS

Many of the mutations and toxins associated with PD are linked with mitochondrial function, providing a connection between known risk factors and cellular physiology that could explain PD pathophysiology. DA neurons in particular are sensitive to mitochondrial stressors and toxins. The maintenance of ion gradients underlying neuronal excitability signifies one of the major energetic burdens for neurons. The energy is generated in mitochondria, the sites of cellular respiration, which also leads to the production of damaging superoxide and other reactive oxygen species (Surmeier et al., 2012).

Superoxide and reactive oxygen species are only a subset of the stressors that cells encounter and that damage macromolecules and organelles. In response they can either attempt to regain cellular homeostasis or, if the severity or duration of the encountered stress is too harmful, they can induce cell death. For example, when cellular clearing processes like autophagy or mitophagy fail, apoptosis can be induced. This mechanism normally guarantees the removal of cells that are not able to adapt to the encountered stress or regain cellular homeostasis. However, programmed cell death can have devastating consequences—in many neurodegenerative diseases, like PD, these dying cells will not be replaced.

Apoptosis is a critical pathway of programmed cell death in which cells undergo a well-defined series of steps that result in the ultimate fragmentation of the nucleus and blebbing of the cell membrane. A period of massive apoptosis is a necessary step in the normal development of the human brain to define functional synaptic connectivity. Just as critical however, is that this process ends at the formation of post-mitotic neurons, as these neurons must then survive throughout the life of the organism. In recent years, significant progress has been made in understanding the role of this pathway in neuronal development and differentiation, as well as the molecular mediators regulating these processes, thus paving the way for future investigations of molecular aberrations that may lead to disease. Particularly, iPSCs originating from PD patients have made it possible to study the molecular events leading to apoptosis in DA neurons. Several studies have demonstrated that DA neurons generated from iPSCs with familial mutations in LRRK2 and alpha synuclein exhibited greater sensitivity to oxidative stress and had a higher number of apoptotic neurons (Byers et al., 2011; Nguyen et al., 2011; Reinhardt et al., 2013). This increased vulnerability to oxidative stress was also observed in iPSC-derived DA neurons from idiopathic PD patients (Sanchez-Danes et al., 2012). Apoptotic markers were also detected in post-mortem brains of PD patients (Tatton et al., 1998; Hartmann et al., 2000; Mogi et al., 2000; Tatton, 2000) as well as in animal models of PD, in particular models generated by the DA neurotoxin MPTP (Tatton and Kish, 1997; Viswanath et al., 2001).

In the following section we describe how miRNAs modulate programmed cell death during development and disease. MiRNAs would be well positioned to help neurons regain homeostasis upon cellular stresses. MiRNAs can act as restorers of homeostasis by resuming normal gene expression through negative feedback loop mechanisms. When miRNAs are components of a positive feedback loop, they can induce new gene expression patterns that help overcome cellular stress. MiRNAs themselves can also become the targets of regulation and their loss can affect downstream gene expression.

Apoptosis-promoting miRNA are associated with neuronal differentiation

Aranha et al. identified three miRNAs with previously described pro-apoptotic functions to be intimately involved with the differentiation of CNS subtypes from neural stem cells, including neurogenesis and gliogenesis (Aranha et al., 2011). This study was based on recent evidence implicating pro-apoptotic molecules, such as p53, caspases and Bcl-2 in differentiation and development, and aimed to further understand the role of pro-apoptotic mediators in neural differentiation processes. The authors characterized miR-14, let7a, and miR-34a upregulation during neural stem cell differentiation, and demonstrated that their expression, particularly miR-34a, coincided with the appearance of post-mitotic immature neurons. The use of apoptosis promoting miRNAs during neuronal development is consistent with the high rate of neuronal apoptosis that occurs throughout early development during the process of pruning, a normal developmental program that facilitates the formation of efficient synaptic configurations. Interestingly, however, the increased expression of

these miRNAs was not associated with increased cell death. The authors hypothesize that the role in this context may be more to control cell cycle exit and mitotic inhibition, given the timing of expression and appearance of neuronal and glial subtypes. This study provides crucial evidence for a novel role of pro-apoptotic miRNAs in neural differentiation.

Conversely, in a Drosophila model of aging it was shown that miR-34 is up-regulated in aging brains and deletion of miR-34 leads to accelerated brain ageing, neural degeneration, defective protein folding, and a decline in survival. Rescue with miR-34 was sufficient to mitigate mutant effects, wherein inclusion formation was slowed, protein retained greater solubility and neural degeneration was suppressed. Furthermore, overexpression of miR-34 had a neuroprotective effect in a transgenic Drosophila model with genetic background that lead to overexpression of neurotoxic poly-glutamines (Liu et al., 2012).

miR-29b restricts apoptosis and promotes neuronal maturation

In 2011, Kole et al. became the first to identify a mammalian miRNA with the ability to inhibit the BH3-only family of apoptosis initiators in neurons (Kole et al., 2011). Importantly, the focus of this paper was on post-mitotic neurons, consistent with the biological condition in which it would be most important to restrict further death of neurons. MiR-29b was found to be selectively enriched in sympathetic ganglion, cerebellar and cortical neurons isolated from postnatal day 28 (P28) mice, compared to the lower expression level in postnatal day 5 (P5) neurons. MiR-29b overexpression was sufficient to inhibit apoptosis in response to three independent stimuli: nerve growth factor (NGF) deprivation, endoplasmic reticulum (ER) stress and DNA damage. NGF deprivation leads to c-Jun phosphorylation, BH3-only induction and subsequent cytochrome c release, caspase activation and cell death. It was determined that miR-29b acts downstream of c-Jun phosphorylation, but upstream of cytochrome c release, prompting the investigation of a possible interaction with BH3-only proteins. Strikingly, miR-29b was found to interact with five out of eight members of the BH3-only family of proteins. The BH3-only protein family repression was responsible for blocking apoptosis only in mature neurons, and protein expression failed to be induced on NGF deprivation in P28 neurons, while being induced in P5 neurons. The findings of this paper are particularly important because it is remarkable example of a single miRNA interacting with multiple members of the same family of proteins. These proteins have been well-characterized to serve redundant functions. Thus, it would be essential that a miRNA capable of affecting the apoptotic pathway through interactions with this family regulate many of its members. MicroRNA-29 has been previously reported to have roles in Alzheimer's disease (Hebert et al., 2008), as well as cancer (Pekarsky et al., 2006; Mott et al., 2007; Wang et al., 2008; Gebeshuber et al., 2009; Park et al., 2009; Han et al., 2010), highlighting the cell- and context-specific nature of its role in regulating apoptosis. Overall, this paper indicates miR-29b as an important miRNA with the ability to fine-tune apoptosis activation and regulation via key members of the BH3-only proteins. These findings certainly warrant further investigation in the context of PD.

Downregulation of miRNA through UPR leads to activation of apoptosis

Extensive research has made a link between accumulation of unfolded proteins and apoptosis in PD. One of the pathways activated by accumulating unfolded proteins in the ER is the unfolded protein response (UPR). UPR signaling requires IRE1α, an ER membrane localized endonuclease that upon induction of UPR cleaves the mRNA of XBP1 and this cleavage leads to a change of its open reading frame and activation of XBP1's transcription factor activity. If ER stress is too lasting, it can trigger cell death. This is mediated through the protease caspase-2 as an early apoptotic switch. Upton et al. now report that IRE1α is the ER stress sensor that activates caspase-2 and does so through a mechanism involving miRNAs (Upton et al., 2012). Upon UPR activation, the RNase activity of IRE1α cleaves selected microRNAs (miR-17, -34a, -96, -125b) that normally repress translation of caspase-2 mRNA, consequently increasing caspase-2 abundance and activating apoptosis. Whether these events occur in neurodegenerative disease with UPR activation remains to be investigated.

MITOCHONDRIAL miRNA

How mitochondrial dysfunction affects miRNAs and how mitochondrial epistasis is regulated by miRNAs is under much investigation. Some evidence came from an *in vitro* study showing that RISC loading with small duplex RNA is inefficient in the absence of ATP, which is generated by mitochondria (Yoda et al., 2010). In addition, disruption of mitochondrial ATP production in human cell lines leads to decreased RISC activity caused by failing RISC assembly (Huang et al., 2011). Therefore mitochondrial dysfunction in PD could lead to an overall weakening of miRNA pathways. Localization of the RISC complex has been shown in multivesicular bodies, like late endosomes, P-bodies, and stress granules. More recently, miRNAs have been detected in mitochondria, providing a link between mitochondrial function and miRNA regulation.

MiRNAs specifically enriched in mitochondria have received much attention. Their localization allows regulation of mitochondrial transcription and translation, but potentially could indicate that miRNAs are stored in mitochondria. In these studies miRNAs were isolated from mitochondria from a variety of tissues and cell lines including rat liver (Kren et al., 2009), mouse liver (Bian et al., 2010), a human epithelial carcinoma cell line (HeLa) (Bandiera et al., 2011), a human embryonic kidney cell line (HEK293) (Sripada et al., 2012) and human skeletal muscle cells (Barrey et al., 2011). Technically, it was crucial that the isolated mitochondria were highly purified and treated with RNase to remove mitochondria-bound cytosolic RNA prior to miRNA extraction to avoid unwanted contamination.

In 2011, Bandiera et al. provided the first comprehensive view of mitochondrial associated miRNAs in HeLa cells (Bandiera et al., 2011). After confirming the presence and activity of AGO2 localized to the mitochondria, the authors set out to identify mitochondrial associated miRNAs, which they termed mitomiRs. Comparing isolated cytosolic and mitochondrial RNA fractions, the authors identified 57 differentially expressed miRNAs, 13 of which were significantly enriched in mitochondria. Interestingly,

of the 13 miRNA identified, three (miR-1974, -1977 and -1978) map to the mitochondrial genome. It is important to note, however, that these three mitomiRs map specifically to tRNA and rRNA genes and were removed from miRBase, among other comprehensive microRNA databases, putting into question whether they are bona fide miRNAs or breakdown products of tRNAs and rRNAs. Among the notable findings from this investigation, the authors were able to ascertain that the mitomiRs had predicted targets on mitochondrial genes, including those essential for ATP synthesis coupled electron transport, translation initiation, cell cycle and mitochondrial translation.

A further study by Sripada et al. took a similar approach in fractionating cells to isolate mitochondrial RNAs (Sripada et al., 2012). In this report, however, the authors chose to investigate miRNA from two different commonly studied cell lines, HEK293 and HeLa, by next generation RNA sequencing on the Illumina HiSeq2000 platform, with enrichment for small RNA. The authors note that the same 13 miRNA identified by Bandiera et al. were represented in their data. The differences observed in the most abundant miRNAs highlight that in addition to technical variation in the cellular fractionation procedure, differences in the method of probing miRNA expression may produce significant variance. Importantly, RNA sequencing allowed the identification of several putative novel mitochondrial miRNAs. Interestingly, only 35 miRNA were similarly expressed in mitochondria of HEK293 and HeLa cells.

The overlap in the above data sets is surprisingly small and could be explained by mitochondrial isolation procedure and the miRNA profiling platform chosen. This again highlights the importance of consistent sub-fractionation procedures, consistent miRNA probing methods, but possibly also the tissue specificity of mitochondrial miRNA expression. Thus transcriptome analysis of PD post-mortem tissue, DA neurons from PD animal models, and patient-specific iPSC-derived neurons will provide an important link between the mitochondrial dysfunction in disease and changes in miRNA profiles. Several studies of miRNA function in DA neurons have already laid the path for future investigation.

DA NEURON DIFFERENTIATION AND MAINTENANCE

miR-133b regulates Pitx3

The seminal paper for its first demonstration of the role of miRNAs in midbrain DA neuron development by Kim et al. has been mentioned previously (Kim et al., 2007). The authors set out to determine specific miRNAs that mediated the findings described above (see section Global Mirna Dysregulation in PD Pathogenesis). The authors used a qPCR panel to quantify 230 miRNA precursors on samples derived from PD and control midbrain, cerebellum and cerebral cortex, finding miR-133b to be specifically deficient in PD samples. A similar reduction in miR-133b was observed in Pitx3 mutant mice. This regulation was confirmed *in vitro* by luciferase reporters, showing that Pitx3 activates the miR-133b promoter and secondly that the Pitx3 3′UTR is negatively regulated by miR-133b overexpression.

Interestingly, in primary embryonic rat midbrain cultures, overexpression of miR-133b caused decreased expression of late DA neuron markers, such as DAT, and resulted in the formation of fewer TH-positive neurons, and lower dopamine release. Inhibition of miR-133b had the opposite effect to increase the expression of late DA neuron markers. It is counterintuitive that low miR-133b expression levels are found in human Parkinsonism, as well as rodent Parkinson's models, while high expression of the miR-133b causes fewer mature DA neurons to form. However, these findings would be consistent with a role for miR-133b in a negative feedback circuit. Thus, the authors hypothesize that miR-133b normally functions to suppress Pitx3 expression post-transcriptionally, while Pitx3 induces midbrain DA gene expression and transcription of its own regulator, miR-133b (**Figures 5A,B**). In support of this hypothesis, the authors note their finding that overexpression of a Pitx3 transgene lacking

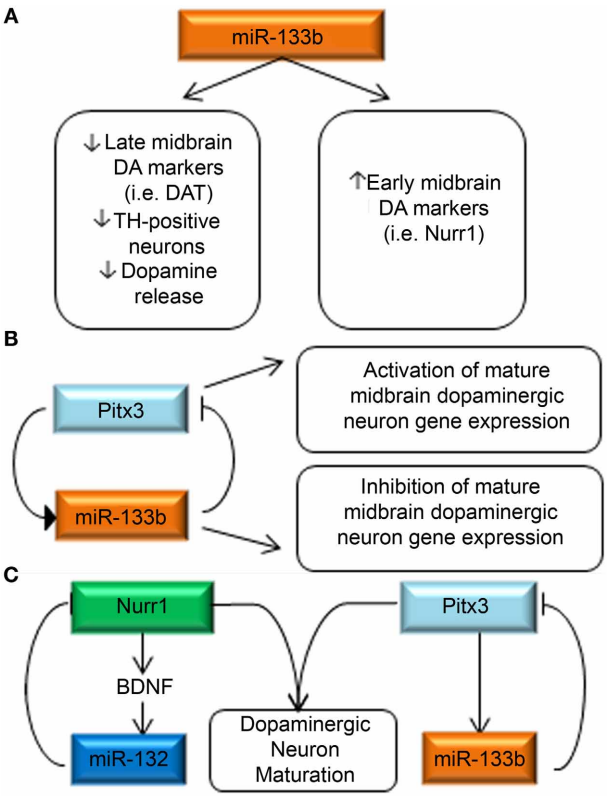

FIGURE 5 | miRNA regulation of key DA neurodevelopmental genes. (A) miR-133b decreases the expression of late DA neuron marker signatures, while increasing the expression of early DA neuron markers (Kim et al., 2007). **(B)** Pitx3 is capable of inducing miR-133b expression, while miR-133b is reciprocally capable of regulating Pitx3 expression. The authors hypothesize a negative feedback loop in which Pitx3 concurrently activates expression of DA maturation genes and miR-133b and when miR-133b levels reach a threshold consistent with maturation, they inhibit continued expression of Pitx3. **(C)** Summary of miRNA interactions with DA neuron-relevant genes. Two primary investigations have separately identified miR-132 (Yang et al., 2012) and miR-133b (Kim et al., 2007) as regulators of Nurr1 and Pitx3, respectively. It is worth noting that SNPs in both of these genes have recently been identified as potential causes of spontaneous Parkinsonism highlighting the relevance of DA neurodevelopmental events in PD pathogenesis.

the 3′UTR was capable of partially reversing miR-133b mediated DAT suppression. This finding is also in concert with growing evidence which suggests that the same miRNAs can function to fine-tune gene expression in a context-specific manner.

A recent description of a miR-133b null mouse model suggests that miR-133b does not play a significant role in midbrain DA neuron development and maintenance *in vivo*. The animals have normal numbers of midbrain DA neurons during development and aging with unchanged dopamine levels in the striatum. Further the suggested miR133b target, Pitx3, is unaffected as is the expression of DA genes tested (Heyer et al., 2012).

miR-132 regulates DA differentiation and maintenance

Another key transcription factor for midbrain DA development is also subjected to miRNA-mediated regulation and was described in 2011 by Yang et al. who profiled miRNAs in purified DA neurons (Yang et al., 2012). A mouse ES cell line expressing GFP under control of the TH promoter was generated and allowed a FACS sort for GFP positive cells after 13 days of DA differentiation. A qPCR-based array method was used to profile relative expression of miRNAs in the GFP-positive, GFP-negative and neural progenitor populations. MiR-132 was identified to be more than fivefold higher in GFP-positive cells than in neural progenitors. MiR-132 overexpression suppressed DA neuron differentiation, whereas miR-132 down-regulation promoted the differentiation of DA neurons. Bioinformatic analysis revealed Nurr1 as a putative target of miR-132, which was confirmed by luciferase reporter assay. The authors further investigated the interaction between Nurr1, BDNF and miR-132, as Nurr1 was previously known to regulate BDNF, and BDNF had been previously identified to regulate miR-132 expression. They found that Nurr1 and miR-132 expression levels were inversely correlated, while BDNF and miR-132 were coordinately expressed. They hypothesize that a homeostatic mechanism thus exists between Nurr1, BDNF and miR-132 (**Figure 5C**).

In addition to its role in DA differentiation through regulation of BDNF and Nurr1, miR-132 has been found to have a pro-survival effect which may contribute to DA neuron maintenance. A delicate balance between acetylcholine and dopamine signaling exists within the striatum (Threlfell and Cragg, 2011). It has been shown that overexpression of acetylcholinesterase (AChE), the enzyme that catabolizes acetylcholine, has a pro-apoptotic effect (Park et al., 2004). In PD, declining dopamine signaling in the striatum may contribute to an acetylcholine imbalance resulting in relative overexpression of AChE, further promoting the death of DA neurons (Llinas and Greenfield, 1987). miR-132 has been found to inhibit AChE, thus suggesting a neuroprotective role for this miRNA in DA neurons (Shaked et al., 2009). This finding remains to be investigated in human neuronal models.

Taken together these studies have identified miRNAs as significant regulators of genes necessary for the differentiation and maintenance of DA neurons. The contrasting results seen in the aforementioned studies of miR-133b highlight the importance of further investigation of these particular miRNAs and their gene-specific interactions in the context of PD patient-specific iPSC-derived DA neurons.

CONCLUSION

This review has presented recent advances in the emerging field which aims to elucidate the role of miRNAs in PD. Many of these studies which have arisen in just the last decade have provided strong evidence that dysregulated miRNA serves as an essential molecular trigger which potentiates pathogenesis in PD. Here we have highlighted studies which demonstrated a role for global miRNA dysregulation in aberrant development and maintenance of DA neurons, a finding which complements the growing notion that neurodegeneration may be a late manifestation of neurodevelopmental disease. Further, we have included a discussion of miRNAs that have been found to regulate the protein clearing pathways, the UPS and autophagy, as well as those involved in apoptosis, mitochondrial maintenance, and DA neuron differentiation, aberrancies in each of which have been previously implicated as cellular and molecular mediators of PD. Despite the dedicated work of many, and the rapidity with which advances have occurred, there remain several unanswered questions about the molecular pathogenesis of PD. The development of novel and robust technologies, such as patient-specific iPSC-derived DA neurons, will further the study of PD on cellular and molecular levels as never previously attainable. By applying evidence obtained from pathway-specific analyses with these new tools, researchers are closing the gap between our knowledge of the disease process and our desire to advance its treatment.

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RNA pathogenesis via Toll-like receptor-activated inflammation in expanded repeat neurodegenerative diseases

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Previously, we hypothesized that an RNA-based pathogenic pathway has a causal role in the dominantly inherited unstable expanded repeat neurodegenerative diseases. In support of this hypothesis we, and others, have characterized *rCAG.rCUG*$_{100}$ repeat double-strand RNA (dsRNA) as a previously unidentified agent capable of causing pathogenesis in a *Drosophila* model of neurodegenerative disease. *Dicer*, *Toll*, and autophagy pathways have distinct roles in this *Drosophila* dsRNA pathology. *Dicer* dependence is accompanied by cleavage of *rCAG.rCUG*$_{100}$ repeat dsRNA down to *r(CAG)*$_7$ 21-mers. Among the "molecular hallmarks" of this pathway that have been identified in *Drosophila*, some [i.e., *r(CAG)*$_7$ and elevated tumor necrosis factor] correlate with observations in affected people (e.g., Huntington's disease and amyotrophic lateral sclerosis) or in related animal models (i.e., autophagy). The *Toll* pathway is activated in the presence of repeat-containing dsRNA and toxicity is also dependent on this pathway. How might the endogenously expressed dsRNA mediate *Toll*-dependent toxicity in neuronal cells? Endogenous RNAs are normally shielded from *Toll* pathway activation as part of the mechanism to distinguish "self" from "non-self" RNAs. This typically involves post-transcriptional modification of the RNA. Therefore, it is likely that *rCAG.rCUG*$_{100}$ repeat dsRNA has a characteristic property that interferes with or evades this normal mechanism of shielding. We predict that repeat expansion leads to an alteration in RNA structure and/or form that perturbs RNA modification, causing the unshielded repeat RNA (in the form of its *Dicer*-cleaved products) to be recognized by *Toll*-like receptors (TLRs), with consequent activation of the *Toll* pathway leading to loss of cell function and then ultimately cell death. We hypothesize that the proximal cause of expanded repeat neurodegenerative diseases is the TLR recognition (and resultant innate inflammatory response) of repeat RNA as "non-self" due to their paucity of "self" modification.

Keywords: RNA pathogenesis, Toll-like receptor, innate inflammation, expanded repeat diseases, neurodegeneration

INTRODUCTION

Since the first discovery of trinucleotide repeat expansion as the basis for many important human genetic diseases (Kremer et al., 1991; La Spada et al., 1991; Yu et al., 1991; Richards and Sutherland, 1992), there has been a vast amount of research in this area (*PubMed* search "trinucleotide repeat disorders" gives >3,700 results). Much of this research is aimed at identifying the mechanism of pathogenesis underlying diseases caused by this form of mutation. Individual diseases can follow either dominant or recessive mode of inheritance indicating distinct pathogenic pathways. Repeat sequences that are expanded in copy number are the basis for ~20 dominantly inherited neurodegenerative diseases, including Huntington's disease (HD). Despite some of the responsible genes being identified as long as 20 years ago, the identity and nature of the disease-causing pathogenic pathway remains a gap in knowledge for these diseases, i.e., no definitive molecular pathway from the mutation to the clinical symptoms has yet been identified. For at-risk individuals in families affected with dominantly inherited late-onset neurodegenerative diseases due to expanded repeats, the majority opt not to have the definitive pre-symptomatic diagnostic test. Their preference is to live with the uncertainty of not knowing, than the certainty of getting the disease, as no treatments are yet available. Therefore, determining the pathogenic pathway and identifying therapeutic targets for intervention is an urgent priority for reducing the impact of these devastating diseases. This understanding is essential for rational approaches to delay onset, slow progression, or ultimately effect cure.

MOLECULAR PATHWAY FROM REPEAT EXPANSION TO DISEASE

There are common properties exhibited by the various repeat expansions that give rise to human disease. The vast majority of these diseases originate from an existing repeat sequence that

exhibits copy number variation in the human population. In each case, the disease alleles arise when copy number increases beyond a critical threshold. The repeat composition varies, but most are trinucleotide repeats. In some diseases, the repeat expands to the point where gene expression at the expanded repeat locus is either substantially reduced or lost altogether, resulting in loss-of-function of the repeat-harboring gene. Typically such diseases are inherited in a recessive manner. Many repeat loci, however, give rise to dominantly inherited diseases in a manner that is not gene-dose dependent (i.e., two mutant alleles are no worse, and may be even better than one – see Carroll et al., 2013). This suggests that gain-of-function is the mechanism rather than haploinsufficiency. Repeat copy number in many cases is a major determinant of age at onset of clinical symptoms (referred to as "anticipation") indicating that the repeat itself is a rate-limiting determinant of the pathogenic pathway. However, since pathology typically involves cell death and there are many ways in which cells die, identification of the disease-causing "toxic agent" has been problematic.

IS THERE A COMMON PATHOGENIC AGENT?

The unstable expanded repeat diseases (**Figure 1**) typically manifest as neurodegenerative and/or muscular diseases, some with a high degree of clinical overlap, despite affecting distinct proteins and unrelated loci. Where expanded repeats are translated, they generally code for polyglutamine; however, the proteins in which they are located are all unrelated in the remainder of their amino-acid sequence. Therefore, much attention has been focused on expanded polyglutamine as the common basis of pathology (McLeod et al., 2005; van Eyk et al., 2011, 2012). Some of these diseases, however, have repeat expansions located within untranslated RNAs and/or arise from repeat sequences that cannot encode polyglutamine (**Figure 1**; Richards, 2001; La Spada and Taylor, 2010). Despite these significant differences in the location of the repeat in this family of diseases they exhibit overlapping symptoms resulting from neuronal loss of function and/or neurodegeneration. In addition, in most cases the polyglutamine and "untranslated" diseases have similar disease allele copy number repeat thresholds (*HD* CAG > 36, *SCA17* CAG > 47, *FXTAS* CGG > 55, *HDL2* CUG > 44, *DM1* CUG > 50, *SCA12* CAG > 66). This suggests that there may be a common pathogenic agent or agents in the translated and untranslated repeat diseases.

RNA MAY BE PATHOGENIC IN TRANSLATED REPEAT DISEASES

While there is growing consensus that RNA plays a causal role in "non-coding repeat expansion disorders," its contribution when the repeat is located in coding regions (specifically polyglutamine disorders) is more controversial (Fiszer and Krzyzosiak, 2013). Yet even here there is evidence that RNA is key. For example, intermediate copy number CAG alleles of *SCA2* that are below the threshold required to encode aggregate forming polyglutamine, increase the risk of amyotrophic lateral sclerosis (ALS; Elden et al., 2010). Furthermore, interruption of CAG repeat with CAA dramatically mitigates polyglutamine toxicity in a *Drosophila* model of *SCA3* (Li et al., 2008).

It is possible that multiple pathways (at least one of which is RNA mediated) contribute to progression of expanded repeat neurodegenerative diseases. In support of this possibility, ALS and *SCA7* both appear to involve two cell types (nerve cells and glial cells; Furrer et al., 2011; Polymenidou and Cleveland, 2011). Astrocytes and glial cells have both been shown to affect their neighboring neurons in individuals with repeat expansions, leading Ilieva et al. (2009) to hypothesize that the onset of the disease is determined in the nerve cell, and the progression of the disease determined in adjacent glial(-like) cells. But

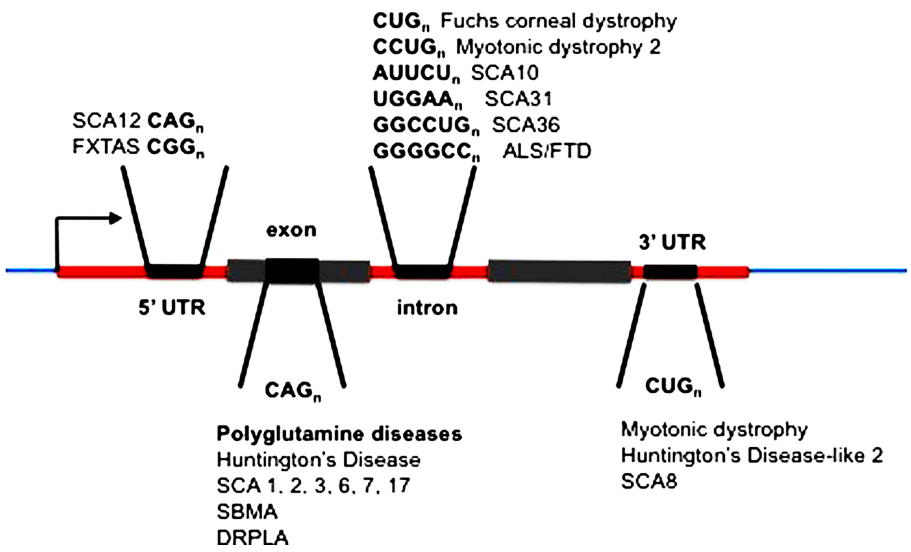

FIGURE 1 | Location of expanded repeats in disease genes. SCA, spinocerebellar ataxia (multiple loci numbered); FXTAS, fragile X tremor ataxia syndrome; ALS, amyotrophic lateral sclerosis; FTLD, frontotemporal lobar dementia; SBMA, spinobulbar muscular atrophy; DRPLA, dentatorubral-pallidoluysian atrophy.

importantly, there is consistent evidence implicating RNA as of principle importance as the originating causal event that initiates pathology.

MECHANISMS OF RNA-INITIATED PATHOLOGY

What precedents and potential mechanisms are there for RNA to initiate pathogenesis in human diseases? See **Figure 2**.

SINGLE-STRANDED RNA TOXICITY

Precedence for expanded repeat RNA being a disease-causing entity in its own right first came from the *DM1* and *DM2* repeat expansions that both give rise to myotonic dystrophy (Ranum and Day, 2004). The repeat expansions in these diseases are similar, but importantly, not identical (CUG vs. CCUG) and are located in untranslated regions (3′UTR or intron) of two otherwise unrelated genes (*DMPK* and *ZNF9*). In muscle cells, RNAs from expanded alleles of either repeat are able to bind and sequester alternative splicing factors (muscleblind and CUG-BP) and in so doing, perturb the splicing pathways of proteins for which alternative splicing is a necessary step for their complete range of functions (Mankodi et al., 2002; Ranum and Day, 2004). It is now generally accepted that RNA is the common pathogenic agent in these diseases most likely through its impact on alternative splicing, although this has recently been challenged with evidence that GSK3β mediates at least some aspects of the RNA-based pathology in myotonic dystrophy (Jones et al., 2012) and in a *Drosophila* model (van Eyk et al., 2011).

Evidence for a more widespread role for RNA in neurodegenerative diseases has been steadily accumulating. *SCA31* and *SCA36* are due to large expansions of *de novo* 5 bp TGGAA repeat and an existing 6 bp GGCCTG repeat, respectively – both located within introns of different genes (Sato et al., 2009; Kobayashi et al., 2011). An expanded GGGGCC repeat has recently been found to cause a substantial proportion of cases of ALS and frontotemporal lobar dementia (FTLD; DeJesus-Hernandez et al., 2011; Renton et al., 2011). As indicated by others (Orr, 2011) "The location of this repeat within an intron of the *C9ORF72* gene along with some evidence for alternative splicing of *C9ORF72* transcripts brings in to play a prominent aspect of non-coding repeat expansion disorders – the role of RNA metabolism in pathogenesis."

REPEAT ASSOCIATED NON-AUG TRANSLATION

The hairpin structure of expanded repeat RNA is such that it can enable the initiation of translation in the absence of the normal requirement of an AUG start codon (Zu et al., 2011). Although this mechanism involves conversion of the RNA into peptides, thereby rendering the RNA no longer "untranslated," the phenomenon can occur to RNA sequences that do not normally appear in protein-coding sequences, i.e., RNA from introns or 5′ or 3′ untranslated regions of mRNAs. The resultant translated

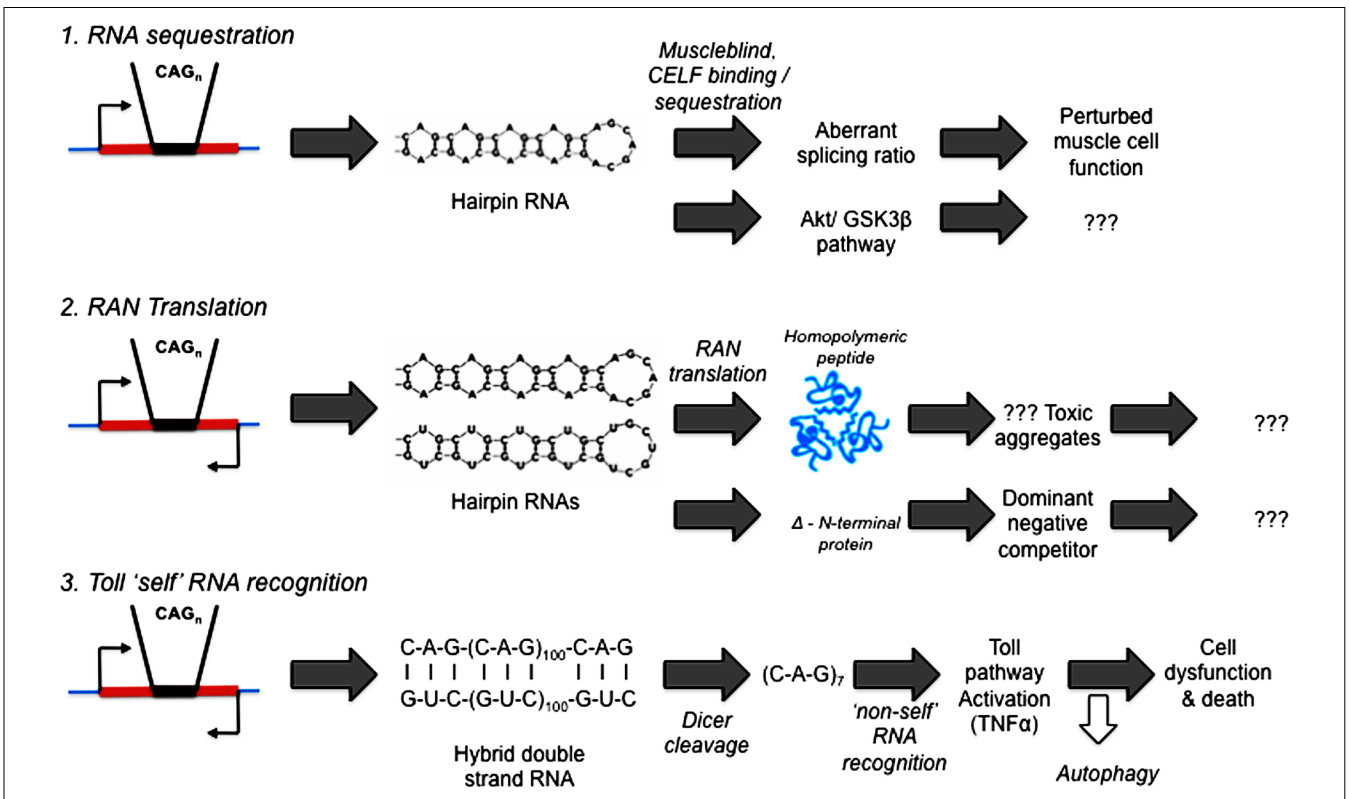

FIGURE 2 | Competing hypotheses of expanded repeat disease pathogenic pathways involving RNA. (1) RNA sequestration – via alternative splicing (Mankodi et al., 2002; Ranum and Day, 2004) or Akt/GSK3β pathway (van Eyk et al., 2011; Jones et al., 2012; Lawlor et al., 2012). (2) RAN (repeat associated non-AUG) Translation (Zu et al., 2011; Ash et al., 2013; Mori et al., 2013; Todd et al., 2013). (3) *Toll* "self" RNA recognition (Lawlor et al., 2011; Yu et al., 2011; Samaraweera et al., (2013)).

polypeptides can initiate from within the repeat sequence and in any reading frame, therefore, a single strand containing repeat RNA sequence can encode three different polypeptide sequences. Since expanded repeat sequences are typically located in regions of bi-directional transcription (Batra et al., 2010), the resultant transcripts from both strands potentially enable the production of six different peptide sequences, any of which may be toxic to the cell. Such polypeptides have now been detected in pathology specimens from individuals affected with a number of different expanded repeat diseases including DM1, fragile X syndrome (FRAXA) and ALS/FTLD (Zu et al., 2011; Ash et al., 2013; Mori et al., 2013; Todd et al., 2013). Of particular note, two recent publications (Ash et al., 2013; Mori et al., 2013) have identified repeat associated non-AUG (RAN)-translation of the GGGGCC expanded repeats that cause ALS/FTLD into polypeptides that also form aggregates in affected tissues. However, these aggregates are confined to nerve cells and are absent from adjacent glial cells that are also involved in the pathology. On the other hand, the absence of visible aggregates does not prove the absence of toxic peptides.

These RAN translation results have suggested that an aggregate polypeptide analogous to polyglutamine could be neurotoxic in diseases where the causative repeat expansion cannot encode polyglutamine. However, this is doubtful in the cases of ALS and FTLD because of the observations that mutations in either of two RNA-binding proteins, FUS and TDP-43, can also cause disease (Rutherford et al., 2008; Van Langenhove et al., 2010). In individuals affected due to these mutations, no such expanded polyGly-Pro polypeptide is evident, therefore while polyGly-Pro may lead to subtle differences in pathology (Ash et al., 2013; Mori et al., 2013), it appears to play a modifying role at most.

Furthermore, inhibition of an RNA lariat debranching enzyme has recently been shown to suppress TDP-43 toxicity in ALS disease models (Armakola et al., 2012). These observations reinforce the view that RNA has a central role to play in this disease. While the role of such polypeptides in disease pathogenesis is unclear, for example, whether their aggregation may actually be protective rather than pernicious, they are a curious set of products driven by the unusual structure of expanded repeat RNAs. One possibility is that rather than the homopolymeric polypeptides themselves being toxic, the initiation of translation within the repeats could give rise to *N*-terminal truncated proteins devoid of upstream functional domains that could then act as dominant negative competitors for the full-length functionally intact proteins.

DOUBLE-STRANDED EXPANDED REPEAT RNA IS PATHOGENIC

Drosophila models of expanded repeat diseases have been described that specifically investigate the intrinsic toxicity of both translated and untranslated expanded repeat sequences (Lawlor et al., 2011; van Eyk et al., 2011, 2012; Samaraweera et al., 2013). In one study (Lawlor et al., 2011), a single line of *Drosophila* expressing untranslated CAG was identified with a marked degenerative phenotype (whereas multiple other random insertion lines of the same transgene had no such phenotype). Upon detailed characterization, this degenerative phenotype line was found to have the repeat transgene inserted into an endogenous gene (*cheerio*) in the opposite orientation to normal transcription. Transcripts containing expanded repeats would, therefore, originate from both strands via bi-directional transcription. This finding coincided with numerous reports in the literature that expanded repeat disease loci are typically transcribed from both DNA strands (see Batra et al., 2010). Therefore, this *Drosophila* line mimicked a previously uncharacterized property of these disease genes. Bi-directional transcription was subsequently modeled in a controlled manner by co-expression from two different transgenes of expanded $rCAG_{\sim 100}$ together with $rCUG_{\sim 100}$ [giving rise to $rCAG.rCUG_{\sim 100}$ or double-strand RNA (dsRNA)] to produce repeat-containing dsRNA (Lawlor et al., 2011). Flies expressing dsRNA showed *Dicer*-dependent toxicity. Additionally dsRNA expression throughout the nervous system caused an age-dependent neurodegenerative phenotype. An abundance of $r(CAG)_7$ also implicated specific *Dicer* processing of the $rCAG.rCUG_{\sim 100}$ dsRNA as a pathogenic pathway in this model (Lawlor et al., 2011). Similar findings have also been reported in an independent *Drosophila* model (Yu et al., 2011). There are, with all animal models, caveats. In order to manifest a phenotype in the time frame of laboratory experiments, these *Drosophila* (and other animal) models employ copy numbers well in excess of those that cause pathology (after several decades) in some of these diseases. This is thought to be due to an inverse relationship between repeat copy number and age-at-onset, the basis of which could be somatic repeat instability over time (see **Figure 2** in Richards, 2001 and Swami et al., 2009). Furthermore, the level of expression of the repeat RNAs required to give an early phenotype in animal models (Lawlor et al., 2011; Yu et al., 2011) may be well in excess of that of the endogenous human disease gene. Importantly, however, examination of HD patient samples (Bañez-Coronel et al., 2012) revealed the presence of the same $r(CAG)_7$ cleavage product seen in the *Drosophila* models, providing evidence in support of the activity of this pathway in HD pathogenesis.

In an effort to identify further components of expanded repeat RNA pathogenesis in *Drosophila*, microarray analyses of *Drosophila* expressing $rCAG.rCUG_{\sim 100}$ dsRNA have been undertaken (Samaraweera et al., 2013). Changes in transcription profiles revealed candidate pathways for mediating the resultant pathogenesis. Alterations in transcripts common to several pathways were detected, including components of inflammation and innate immunity. Hallmarks of immune activation, including elevated plasma tumor necrosis factor (TNF), appear prior to clinical symptoms of dominantly inherited expanded repeat human diseases (Moreau et al., 2005; Björkqvist et al., 2008). Therefore, the *Drosophila* model expressing $rCAG.rCUG_{\sim 100}$ dsRNA was utilized to test two key elements of immune activation – the *Toll* and autophagy pathways for their contribution to expanded repeat RNA pathogenesis. *Toll* signaling pathway was identified as essential for dsRNA pathogenesis and autophagy was found to reduce toxicity in this model (Samaraweera et al., 2013). Furthermore, multiple reports implicate glial cells in the pathology of expanded repeat diseases. Neurons are dependent upon glial cell function that includes the destruction and removal of the carcasses of dead neurons. The $rCAG.rCUG_{\sim 100}$ dsRNA was found to impact nerve cell function even when exclusively expressed in glial cells (Samaraweera et al., 2013), providing evidence that dsRNA pathology in

Drosophila is, like the human expanded repeat diseases, non-cell autonomous (Ilieva et al., 2009; Furrer et al., 2011).

The requirement for *Toll* signaling pathway in this *Drosophila* model is intriguing. *Toll*-like receptors (TLRs) function in normal biology to protect an organism from infection by viruses and bacteria. They recognize foreign pathogen molecules including DNA and RNA through specific receptors (such as endosomal *TLR3*) and can distinguish these nucleic acids (as "non-self") from the endogenous nucleic acids ("self"). Therefore, while the *rCAG.rCUG*$_{\sim}$*100* dsRNA is being expressed endogenously in this *Drosophila* model, it is being recognized by the *Toll* signaling pathway as foreign or "non-self" – a recognition that then activates innate inflammatory regulatory pathways, ultimately leading to cell death.

PATHOGENIC MUTATIONS IN PROTEINS THAT FUNCTIONALLY INTERACT WITH RNA

While it can be difficult to ascribe specific functions to RNA in pathogenic pathways, there are some noteworthy instances of disease-causing mutations in proteins that functionally interact with RNAs. By implication, the RNAs that these proteins normally act upon are, therefore, likely contributors to and/or mediators of the relevant pathogenic process.

RNA-BINDING MOTIFS – THE RNAS THAT HAVE THEM AND THE PROTEINS THAT RECOGNIZE THEM

Recent discoveries regarding the importance of RNA–protein recognition in disease pathogenesis have led to a renewed interest in the role that these interactions play in biological processes. While they have long been recognized as key regulators of gene expression, only a small fraction have been functionally characterized. A recent compendium of RNA-binding motifs (Ray et al., 2013) highlighted both the significance and scope of these interactions. The human genome encodes at least 400 known or predicted RNA-binding proteins with a diverse array of RNA sequence-binding motifs. Indeed the number of such human RNA-binding proteins appears to be much higher than this, with 860 identified in HeLa cells alone (Castello et al., 2012). The scope and specificity of RNA recognition is determined both by the number and variety of RNA-binding proteins and by the number and variety of RNA-sequence motifs that they bind.

FRAGILE X SYNDROME IS DUE TO LOSS OF RNA-BINDING PROTEIN FUNCTION

Fragile X syndrome is a striking example of the role of an RNA-binding protein in human disease. FRAXA is due to the expanded CGG repeat that is responsible for the *FRAXA* rare, folate-sensitive chromosomal fragile site (Kremer et al., 1991), located in the 5′UTR of the *FMR1* gene (Verkerk et al., 1991). Expansion of the repeat beyond ∼230 copies results in inactivation of the gene and consequent loss of encoded FMRP (fragile X mental retardation protein) function (Pieretti et al., 1991). The FMRP is an RNA-binding protein with KH- and RGG-binding motifs (Ashley et al., 1993). The loss of function of this protein is responsible for the clinical symptoms as rare cases of point mutation or deletion of the *FMR1* gene have similar clinical symptoms. Indeed one of these pathogenic point mutations is at a highly conserved amino acid in a KH domain of FMRP highlighting the significance of the role of RNA interaction in FMRP function (De Boulle et al., 1993). The FMRP has an impact on the translation of the mRNAs with which it interacts (Darnell et al., 2001) and, therefore, its absence leads to the dysregulation of the translation of these specific mRNAs. This is thought to be the proximal cause of the symptoms of FRAXA.

THE INTRIGUING PATHOGENESIS OF AICARDI–GOUTIÈRES SYNDROME

Aicardi–Goutières syndrome (AGS) is a genetically heterogeneous disorder that is due (at least in a substantial proportion of cases) to the mutation of various nucleic acid-metabolizing enzymes, including various subunits of ribonuclease H2 or the RNA-editing enzyme ADAR1 (see Crow and Rehwinkel, 2009 and OMIM #225750). AGS is characterized, in its more severe forms, by severe neurological dysfunction in infancy that includes progressive microcephaly, spasticity, dystonic posturing, profound psychomotor retardation, and often death in early childhood (OMIM #225750) (**Figure 3**). In its milder forms, these neurological symptoms are diminished or even absent, but peripheral symptoms outside the nervous system are common to the phenotypic spectrum and include thrombocytopenia, hepatosplenomegaly, and elevated hepatic transaminases along with intermittent fever. Chilblains are also a typical feature. Together these symptoms demonstrate phenotypic overlap both with systemic lupus erythematosus and with the sequelae of congenital infection (Crow and Rehwinkel, 2009). The disease, therefore, appears to be due to defects in the processes that remove and/or modify endogenous nucleic acids. These endogenous unmodified nucleic acids then accumulate and are sensed as "non-self" by TLRs, that, in turn, activate innate inflammatory regulatory pathways. This bears a striking resemblance to mechanisms we have identified as responsible for dsRNA pathogenesis in the *Drosophila* model of expanded repeat neurodegenerative diseases.

HYPOTHESIS

EXPANDED REPEAT RNAS AS PATHOGENIC AGENTS BY *TOLL* "SELF" RNA RECOGNITION

Repeat RNA sequences represent a pivotal point of potential weakness in processes that utilize RNA–protein recognition, as the repeat RNA sequence will harbor either a paucity or excess of sequence-binding motifs. Expansion of repeat RNA sequences, therefore, clearly has the potential to give rise to too much or too little of an interaction that is a rate-limiting factor in a crucial biological process. RNA modification is one process that is sequence motif-dependent and known to be key to the distinction between "self" and "non-self" by components of the innate immune pathways. Indeed, it has been shown that exogenous "non-self" RNAs require *in vitro* modification in order to escape innate immune recognition and activation when transferred *in vivo* (Warren et al., 2007; Pan, 2013). The exposure of the innate immune activators to unmodified nucleic acids, including RNA, appears to be the proximal cause of AGS. We, therefore, hypothesize that this provides a clear molecular mechanism for the ability of expanded repeat RNA sequences, through their paucity of RNA modification, to initiate pathogenesis in the dominantly inherited, expanded repeat neurodegenerative diseases (**Figure 4**).

Aicardi-Goutieres Syndrome *(AGS)* **– *Mutations in RNA Metabolizing Genes in Bold***

Locus – mutated gene

AGS1 – TREX1	*– DNA 3' to 5' exonuclease, prevents autoimmunity caused by endogenous retroelements.*
AGS2 – RNASEH2B	**– subunit B of the human ribonuclease H2 enzyme complex – ribonuclease H2 cleaves ribonucleotides from RNA:DNA duplexes.**
AGS3 – RNASEH2C	**– subunit C of the human ribonuclease H2 enzyme complex.**
AGS4 – RNASEH2A	**– subunit A of the human ribonuclease H2 enzyme complex.**
AGS5 – SAMHD1	*– converts deoxynucleoside triphosphates to constituent deoxynucleoside and inorganic triphosphate. Restriction factor that renders human dendritic and myeloid cells largely refractory to HIV-1 infection.*
AGS6 – ADAR1	**– converts adenosine to inosine in double strand RNA**

FIGURE 3 | Pathogenic mutations in Aicardi–Goutières syndrome. Mutations in genes in at least six distinct loci are able give rise to the constellation of symptoms that defines Aicardi–Goutières syndrome. Four of these (AGS2, AGS3, AGS4, and AGS6) are in genes that encode RNA-metabolizing proteins. The remaining two that have been identified (AGS1 and AGS5) are also in enzymes that have roles in nucleic acid metabolism. Deficiencies in any one of these enzymes are thought to result in the accumulation of endogenous nucleic acids that are sensed as "non-self" by *Toll*-like receptors, that in turn activate innate inflammatory pathways (Crow and Rehwinkel, 2009).

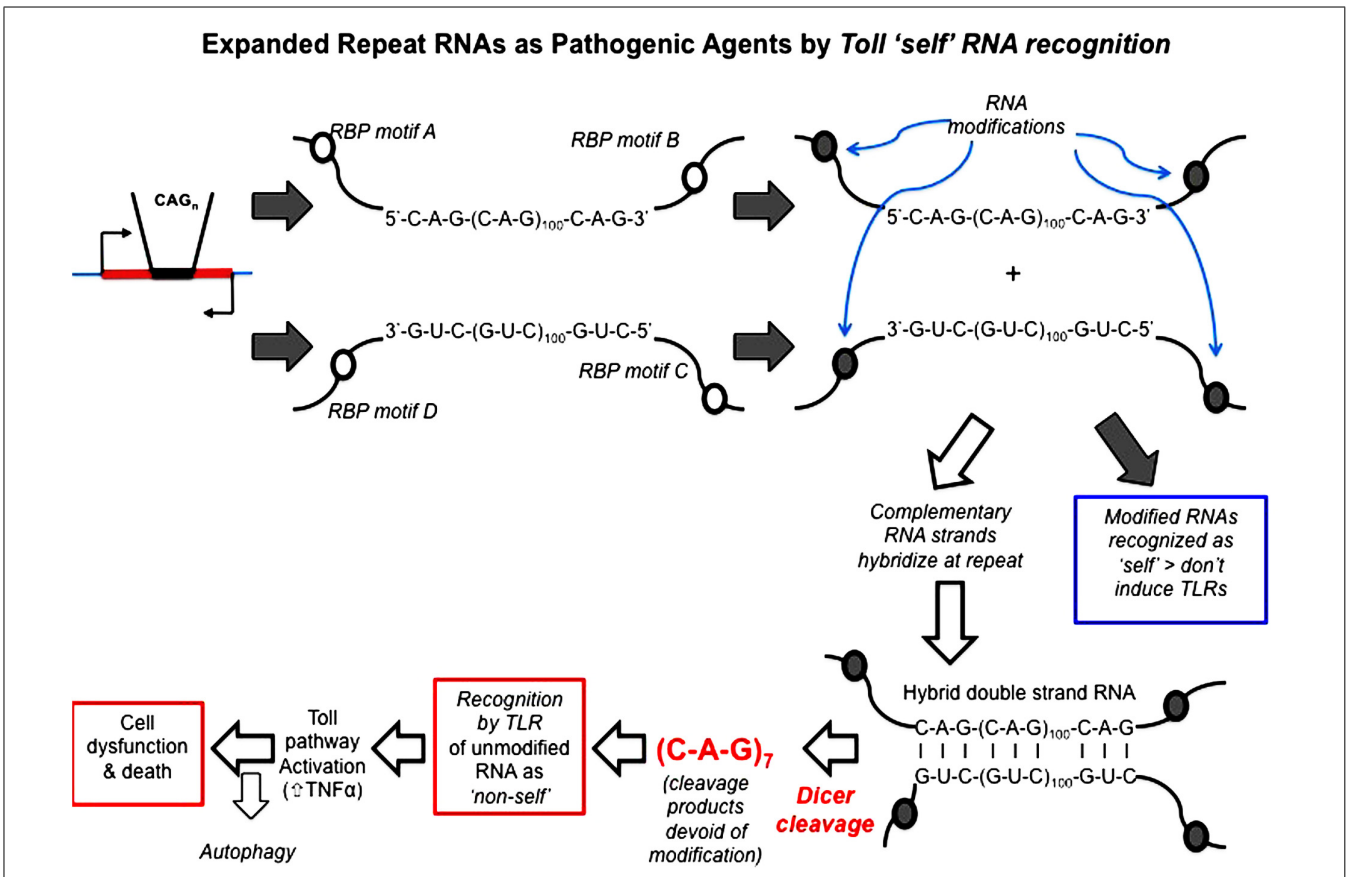

FIGURE 4 | Hypothesis: expanded repeat neurodegenerative diseases are caused by the TLR recognition (and resultant innate inflammatory response) of repeat RNA as "non-self" due to their paucity of "self" modification that is exposed upon Dicer processing of double-strand RNA. Open circles represent sequence motifs for RNA modifying proteins; filled circles represent the modification of RNA at these specific sequence motifs (e.g., by methylation or A > I editing). Dicer is required for pathology in the *Drosophila* model and cleaves long high copy number repeat RNA down to 21mers [mainly $r(CAG)_7$ mers; Lawlor et al., 2011]. These $r(CAG)_7$ mers are, therefore, unmodified and recognized by TLRs as "non-self." *Toll*-like receptor pathways (most probably the endosomal TLR3 receptor) are required for pathology (Samaraweera et al., 2013), through activation of the innate inflammatory pathway. Autophagy reduces pathology, possibly by metabolizing $r(CAG)_7$ mers.

ACTIVITY OF *TOLL* "SELF" RNA RECOGNITION IN NEURODEGENERATIVE DISEASES

Double strand expanded repeat RNA pathology has been modeled in *Drosophila*. What evidence is there that this pathway of TLR recognition of expanded repeat RNA and subsequent activation of the innate inflammatory cascade is active in the human dominantly inherited neurodegenerative diseases due to expansion of repeat sequences?

One of the key steps in dsRNA pathology is the generation of $r(CAG)_7$ 21mers from the much greater copy number double strand repeat RNA by *Dicer*. This $r(CAG)_7$ 21mer has been identified in the brain RNA of individuals affected with HD (Bañez-Coronel et al., 2012). The activity of *Dicer* is crucial to the observed pathology in the *Drosophila* model (Lawlor et al., 2011) and, therefore, it would appear that this step is a likely proximal event in the observed phenotype. The appearance of $r(CAG)_7$ 21mers in HD brain is therefore an important "molecular hallmark" of this pathway and a key indicator of its activity in the human disease. Another, albeit less direct, indicator of this pathway is seen in the increased activity of components of the innate inflammatory response mechanism in human diseases associated with expanded repeats. Elevated TNF is seen in the *Drosophila* model as one read-out of innate immune activation (Samaraweera et al., 2013) and both TNF and various interleukins (i.e., IL-4, IL-5, IL-6, IL-8, and IL-10) have been found to be elevated in people affected with the repeat expansion responsible for HD even before clinical manifestation of the disease (Björkqvist et al., 2008). Another indicator of innate immune activation in HD is the abnormal peripheral chemokine profile that has been observed in HD (Wild et al., 2011). Various reports indicate activation of innate adaptive immunity via TLR signaling in ALS (Casula et al., 2011; Sta et al., 2011) – a disease that has recently been found, at least in a proportion of instances, to be also due to an expanded repeat (DeJesus-Hernandez et al., 2011; Renton et al., 2011).

CONCLUSION

A growing body of literature indicates a consistent association between innate immunity, neuroinflammation and neurodegeneration (Shastri et al., 2013). Where there are exogenous causes (e.g., trauma or infection), activation of the TLR pathway can be attributed to an external agent (e.g., bacterial lipopolysaccharide or viral RNA); however, a causal basis for this relationship has not been clear when there is an endogenous basis to the disease, e.g., expansion of a repeat sequence beyond a pathogenic threshold. Recognition by the *Toll* receptor pathway of expanded repeat RNA as "non-self" and consequent activation of the innate immune inflammatory cascade provides a mechanism and a common pathogenic pathway for the neurodegenerative diseases due to expanded repeats. This new understanding, once proven in the relevant human diseases, will provide new targets for intervention and ultimately, we hope, therapeutic targets for drugs to delay onset and/or alleviate disease progression.

AUTHOR CONTRIBUTIONS

Robert I. Richards drafted the initial version of the manuscript, including the hypothesis, then edited in the additions and changes made by the other co-authors. Saumya E. Samaraweera and Clare L. van Eyk provided original unpublished data on which the manuscript and its hypothesis is based, as well as contributions to the development of the hypothesis, the text and figures. Louise V. O'Keefe contributed to the development of the hypothesis and additional text and revision of the manuscript. Catherine M. Suter contributed to information on RNA-binding proteins, the development of the hypothesis and content of the text and figures.

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Small non-coding RNAs add complexity to the RNA pathogenic mechanisms in trinucleotide repeat expansion diseases

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Trinucleotide-repeat expansion diseases (TREDs) are a group of inherited human genetic disorders normally involving late-onset neurological/neurodegenerative affectation. Trinucleotide-repeat expansions occur in coding and non-coding regions of unique genes that typically result in protein and RNA toxic gain of function, respectively. In polyglutamine (polyQ) disorders caused by an expanded CAG repeat in the coding region of specific genes, neuronal dysfunction has been traditionally linked to the long polyQ stretch. However, a number of evidences suggest a detrimental role of the expanded/mutant mRNA, which may contribute to cell function impairment. In this review we describe the mechanisms of RNA-induced toxicity in TREDs with special focus in small-non-coding RNA pathogenic mechanisms and we summarize and comment on translational approaches targeting the expanded trinucleotide-repeat for disease modifying therapies.

Keywords: small non-coding RNAs, trinucleotide repeat expansion, RNA-toxicity, miRNA, antisense small RNA

INTRODUCTION

In the human genome trinucleotide repeats (TNR) are especially abundant in intergenic regions, gene introns and untranslated regions, and translated segments of protein coding genes. Short tandem-TNR have generally less than 30 copies in the normal population (Ellegren, 2004). Abnormal expansions of certain types of TNR result in trinucleotide repeat expansion diseases (TREDs), a group of inherited human genetic disorders involving the nervous system (**Table 1**; Orr and Zoghbi, 2007). Expansions of triplet repeats occur in coding or non-coding regions of unrelated genes and typically result in late-onset neurological diseases. Disease severity and onset are largely dependent on the expansion length. The pathogenic mechanisms associated to TNR expansions have been an extensive field of research over the last two decades. Different studies have revealed a tremendous complexity in the pathomechanisms, with diverse detrimental effects probably coexisting in cells. This complexity lies beneath the selective affectation of specific cell types in the brain, which is characteristic in each TRED.

The largest group of inherited polyglutamine (polyQ) disorders is caused by expansions of CAG repeats in the open reading frame (ORF) of unique genes, including the Huntington's disease (HD) genes and several spinocerebellar ataxias (SCA) genes. In these diseases the predominant hypothesis has been that the expanded polyQ track confers detrimental properties to the protein, that compromise cell homeostasis. The consequences of polyQ expansion in the HTT protein have been systematically characterized, with detrimental effects in transcriptional activity, vesicle trafficking, mitochondrial function and proteasome activity (Zheng and Diamond, 2012). However, the view of a protein-based toxocity in polyQ disorders has been challenged, as recent findings point to an additional toxic effect of the expanded CAG in the exon 1 of *HTT* mRNA (Banez-Coronel et al., 2012).

TNR expansions also occur in non-translated regions of selective genes. In myotonic dystrophy (DM1) a CTG expansion in the 3′UTR of the *DMPK* gene (50–3000 repeats) leads to neuromuscular degeneration (Brook et al., 1992). A CGG expansion (above 200 repeats) in the 5′-untranslated region (5′UTR) of the *FMR1* gene produces fragile X syndrome, the most common type of mental retardation. Yet, shorter CGG expansions (55–200 repeats) are associated to different pathologies such as fragile X tremor/ataxia syndrome (FXTAS) and primary ovarian failure (POF; Verkerk et al., 1991; Hagerman and Hagerman, 2004). Expansions occurring in non-translated regions produce RNAs with a toxic gain of function, involving a number of mechanisms, described in subsequent sections.

The recent discovery of repeat associated non-ATG (RAN) translation (Zu et al., 2011) has changed the view of TREDs pathogenesis, as toxic proteins may be also produced from expanded TNR thought to be embedded in non-coding RNAs. RAN-translation from Ataxin8 Oposite Strand (*Ataxin8_OS*) with an expanded CAG has been shown in different frames, in SCA8 mouse models and in patients with SCA8 (Zu et al., 2011). The same study showed RAN-translation across DM1 transcripts, resulting in the accumulation of PolyQ expanded proteins in DM1 mice models myoblasts and cardiomyocites. A similar phenomenon has

Table 1 | Trinucleotide expansion diseases.

Disease	Repeat type (normal vs expanded)	Gene	Gene function	Repeat in coding regions of the sense transcript	Repeat in non-coding regions of the sense transcript	Bidirectional-transcription	RAN translation	Small repeated CNG biogenesis	Described pathogenic process
Dentatorubral-pallidoluysian atrophy (DRPLA)	CAG (3–36/49–88)	*ATN1*	Nuclear receptor correpressor	PolyQ	-	Yes*	Unknown	Unknown	PolyQ gain of function
Fragile X tremor/ataxia associated syndrome (FXTAS)	CGG (6–52/60–200)	*FMR1*	Translation repressor, mRNA trafficking from the nucleus to the cytoplasm	-	5′UTR	Yes (CCG expansion in the *FMR1_AS*)	Yes (PolyG)	Yes	RNA foci sequestering MBNL, hnRNP G, hnRNPA2/B1, SAM68, Pur α, lamin A/C. Chromatin changes. Altered miRNA biogenesis
Friedeich ataxia (FRDA)	GAA (6–32/>200)	*FXN*	Biosynthesis of heme and assembly and repair of iron-sulfur clusters	-	Intron	Yes *FAST-1*	Unknown	Unknown	FXN loss of function, chromatin changes
Huntington disease (HD)	CAG (6-35/36-121)	*HTT*	Transcription, intracellular signalling, trafficking, endocytosis, metabolism	PolyQ	-	Yes (CUG expansion in the *HTT_AS*)	Unknown	Yes	PolyQ gain of function, RNA foci sequestering MBNL, sCAG biogenesis and activity
Huntington's disease like 2 (HDL2)	CTG (6–28/40–59)	*JPH3*	Formation of junctional membrane complexes, which link the plasma membrane with the endoplasmic or sarcoplasmic reticulum in excitable cells	PolyL; PolyA	3′UTR	Yes (CAG expansion in the *JPH3_AS*)	Unknown	Unknown	RNA foci sequestering MBNL
Myotonic dystrophy type 1 (DM1)	CTG (5–37/50 to >3500)	*DMPK*	Regulates the expression of muscle-specific genes	-	3′-UTR	Yes (CAG expansion in the DMPK_AS)	Yes (PolyQ)	Yes	RNA foci sequestering MBNL, CUGBP1 activation chromatin changes

(Continued)

Table 1 | Continued

Disease	Repeat type (normal vs expanded)	Gene	Gene function	Repeat in coding regions of the sense transcript	Repeat in non-coding regions of the sense transcript	Bidirectional-transcription	RAN translation	Small repeated CNG biogenesis	Described pathogenic process
Spinocerebellar ataxia 1 (SCA1)	CAG (6–39/39–81)	*ATXN1*	Gene expression regulation	PolyQ	-	Yes*	Unknown	Yes	PolyQ gain of function
Spinocerebellar ataxia 2 (SCA2)	CAG (13–33/>34)	*ATXN2*	Possible role in RNA metabolism	PolyQ	-	Yes*	Unknown	Unknown	PolyQ gain of function
Spinocerebellar ataxia 3 (SCA3)	CAG (13–44/>55)	*ATXN3*	Deubiquitination, transcriptional regulation	PolyQ	-	Yes*	Unknown	Unknown	PolyQ gain of function
Spinocerebellar ataxia 6 (SCA6)	CAG (13–44/>55)	*CACNA1A*	Calcium channel controlling neurotransmitter release and calcium homeostasis	PolyQ	-	Yes*	Unknown	Unknown	Protein GOF/LOF (?)
Spinocerebellar ataxia 7 (SCA7)	CAG (4–35/37–306)	*ATXN7*	Component of TFTC/STAGA transcriptional coactivator complexes, regulates retinal gene expression	PolyQ	-	Yes (CUG expansion in SCAANT1) (Sopher et al., 2011)	Unknown	Unknown	PolyQ gain of function
Spinocerebellar ataxia 8 (SCA8)	CTG (<50/74–1300)	*ATXN8*	Unknown	Non-coding RNA	Non-coding RNA	Yes (CUG expansion in the *ATX8_OS* 3′UTR)	Yes (PolyA, PolyQ)	Unknown	PolyQ gain of function ATX8; RNA gain of function ATX8_OS
Spinocerebellar ataxia 12 (SCA12)	CAG (4–32/51–78)	*PPP2R2B*	Negative control of cell growth and division	-	5′-UTR	Yes (CUG expansion in the *PPP2R2B_AS*) (Brusco et al., 2002)	Unknown	Unknown	Unknown
Spinocerebellar ataxia 17 (SCA17)	CAG (25–42/47–63)	*TBP*	Initiation of transcritption	PolyQ	-	Yes*	Unknown	Unknown	PolyQ gain of function

**Detected according to ASSAGE sequencing in normal peripheral blood monocyteic cells (He et al., 2008). Deeper characterization of the antisense transcript is not available.*

been recently demonstrated in expanded CGG repeats in *FMR1* 5′-UTR (Todd et al., 2013). A cryptic polyglycine-containing protein (FMRpolyG) was detected accumulating in ubiquitin-positive inclusions in Drosophila, cell culture and mouse disease models, and in brains of patients with FXTAS. The relevance of this mechanism needs to be specifically addressed for each TRED.

In this review we focus on the RNA pathogenic mechanisms in TREDs. We present the existing evidences for RNA binding protein (RBP) sequestration by different expanded TNR and the linked altered biological processes. We address the possible relevance of bidirectional transcritption in TREDs loci and further discuss about the role of small non-coding RNAs in TREDs pathogenesis. Finally, we summarize the latest therapeutic strategies in TREDs, based on selective targeting of the allele with the expanded TNR.

MECHANISMS OF RNA-TOXICITY IN TREDs

Recent findings indicate that alterations of RNA sequences can lead to abnormal RNA–protein interactions, alteration of protein translation, or RNA interference (RNAi) activation, among other anomalous processes. These altered pathways contribute to disruption of normal cell function and homeostasis, eventually leading to cell degeneration.

TRINUCLEOTIDE REPEAT EXPANSIONS MODIFY ALTERNATIVE SPLICING EVENTS

In TREDs abnormal expanded TNR RNA–protein interactions results in disrupted protein conformation and inclusion formation. Sequestration of RBP by the expanded TNR leads to a loss of function of such proteins (**Figure 1**). Muscleblind-like splicing regulator 1 (MBNL1) and Elav-like family member 1 (CUGBP1 or CELF1) are regulators of mRNA splicing that present affinity for CUG and/or CAG repeats. In DM1, long CUG repeats lead to decreased MBNL1 activity and increased CELF1 activity in muscle cells, which results in mis-splicing events in different developmentally regulated genes including the insulin receptor (*IR*), the chloride channel (*CLCN1*) and the cardiac tropin T (*TNNT2*), which explain several aspects of DM1 symptomatology (Ranum and Cooper, 2006; Wheeler and Thornton, 2007).

Aberrant splicing of the bridging integrator-1 (*BIN1*) pre-mRNA has been recently described in DM1 (Fugier et al., 2011). BIN1 protein is required for the biogenesis of muscle T tubules, essential for excitation-contraction coupling. Mis-splicing of *BIN1* linked to a loss of function of MBNL1, produces an inactive form of BIN1 protein. While direct interaction of MBNL1 with the CUG repeat depletes MBNL1, increased levels of CEFL1 are the consequence of an indirect effect of CUG expansion involving PKC-pathways (Kuyumcu-Martinez et al., 2007). CELF1 hyperphosphorylation mediated by PKC, leads to its increased stability and activity (Kuyumcu-Martinez et al., 2007). Splicing alterations have been also reported in DM1 and SCA8 brains, with neuronal cells showing MBNL1 nuclear inclusions (Jiang et al., 2004; Daughters et al., 2009; Mykowska et al., 2011). Abnormal splicing of multiple exons in microtubule associated protein tau (*MAPT*), and exon 7 in the amyloid precursor protein (*APP)* and exon 5 in glutamate receptor *NMDAR1*, have been detected in brains of DM1 patients and mouse models (Jiang et al., 2004; Gomes-Pereira et al., 2007) and analogous splicing alterations have been shown

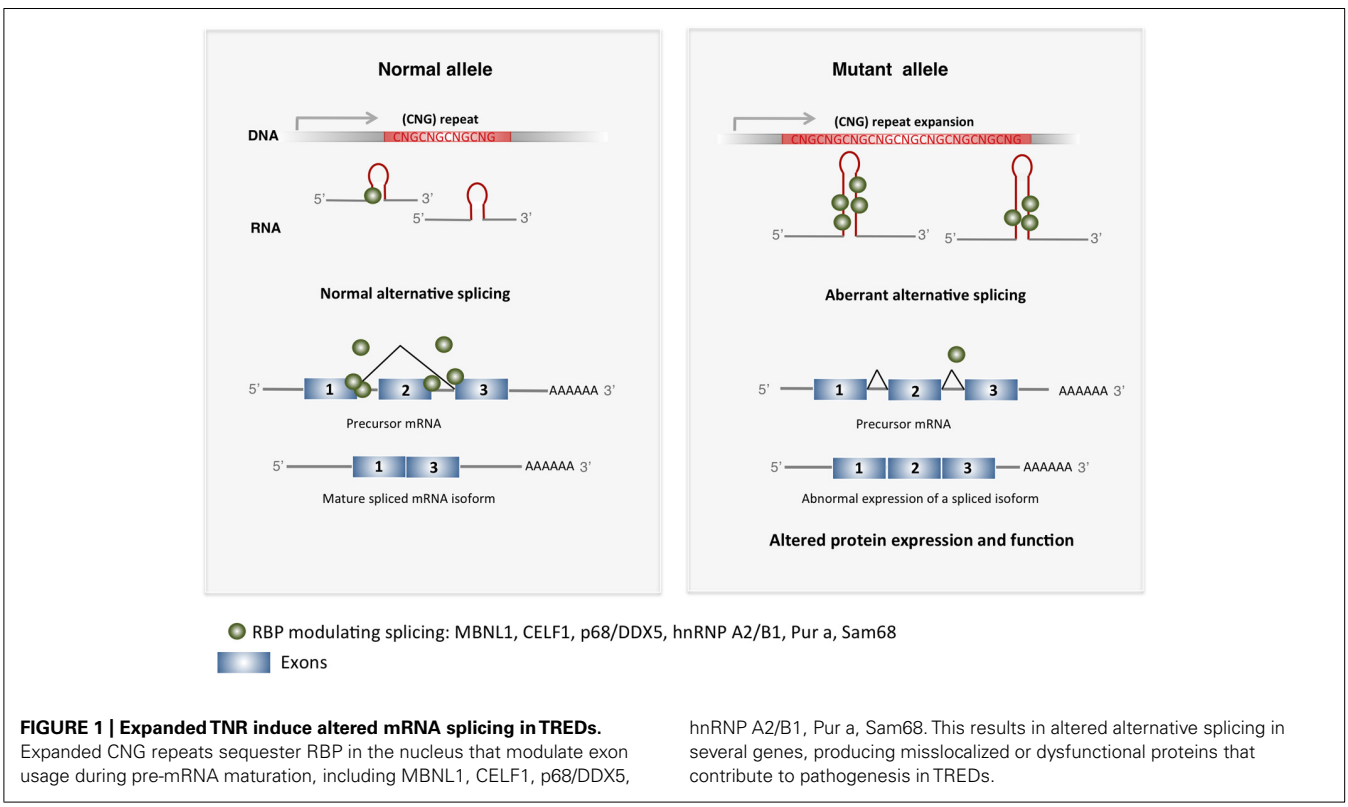

FIGURE 1 | Expanded TNR induce altered mRNA splicing in TREDs. Expanded CNG repeats sequester RBP in the nucleus that modulate exon usage during pre-mRNA maturation, including MBNL1, CELF1, p68/DDX5, hnRNP A2/B1, Pur a, Sam68. This results in altered alternative splicing in several genes, producing misslocalized or dysfunctional proteins that contribute to pathogenesis in TREDs.

in SCA8 mice (Daughters et al., 2009) which may explain neurological alterations in these diseases. The relevance of expanded RNA-MBNL1 interaction in DM1 is strengthened in a mouse model expressing an expanded CUG RNA that recapitulates DM1 phenotypes. In this model, partial recovery of the mis-splicing defects is achieved by reestablishing MBNL1 levels. Another recently described repeated-CUG interactor is the p68/DDX5 helicase, which is present in mutant *DMPK* foci in DM1 (Laurent et al., 2012). p68/DDX5 modifies of MBNL1 splicing activity and has been proposed to influence pathogenicity in DM1.

The CGG expansions in the FMR1 5′UTR causing FXTAS may also sequester MBNL1, which is accumulated as abnormal inclusions in brain of FXTAS patients (Iwahashi et al., 2006). Other RBPs with specific affinity for CGG repeats are Pur-α and hnRNP A2/B1, which have been found in inclusions in FXTAS models (Jin et al., 2007; Sofola et al., 2007). In addition, CELF1 indirect binding to the CGG repeats through the RBP hnRNP A2/B1, leads to its loss of function (Sofola et al., 2007). Loss of function of Pur-α and hnRNP A2/B1 induce neurological alterations in mice (Khalili et al., 2003), suggesting their participation in FXTAS neuropathology. Recently, loss of function of the splicing factor Sam68 through binding to expanded CGG repeats has been shown in FXTAS patients, contributing to aberrant splicing of the ATPase *ATP11B* and the Survival of Motor Neuron 2, centomeric *SMN2* genes (Sellier et al., 2010).

Together, these data indicate that loss of function of RBP that have affinity for expanded TNR is a common mechanism operating in TREDS and further suggest that full characterization of the set of RBP binding to different types of TNR expansions will provide insights into specific pathogenic processes.

TRINUCLEOTIDE-REPEAT EXPANSIONS ALTER miRNA BIOGENESIS

The most recent findings suggest that sequestration of RBP by TNR expansions has other consequences in addition to alternative splicing perturbations (**Figure 2**). MBNL1 and the RNA helicases p68 and p72 influence the activity of proteins involved in microRNA (miRNA) biogenesis (Fukuda et al., 2007; Rau et al., 2011). MBNL1 in normal conditions binds to pre-miR-1 precursor, allowing the normal production of mature miR-1. Depletion of MBNL1 in DM1 permits the activity of the processing regulator LIN28, which binds to pre-miR-1 and promotes 3′-end uridylation, thus resulting in inhibition of pre-miR-1 processing by the endonuclease Dicer. The disruption of the normal pre-miR-1 processing by MBNL1 loss of function results in increased levels of miR-1 targets, including the calcium channel CACNA1C and the gap-junction channel GJA1, which may contribute to the cardiac defects in DM1 (Rau et al., 2011). The modulatory role of p68 and p72 helicases in the miRNA-processing complex (Fukuda et al., 2007) suggest that analogous mechanisms may exist for these proteins.

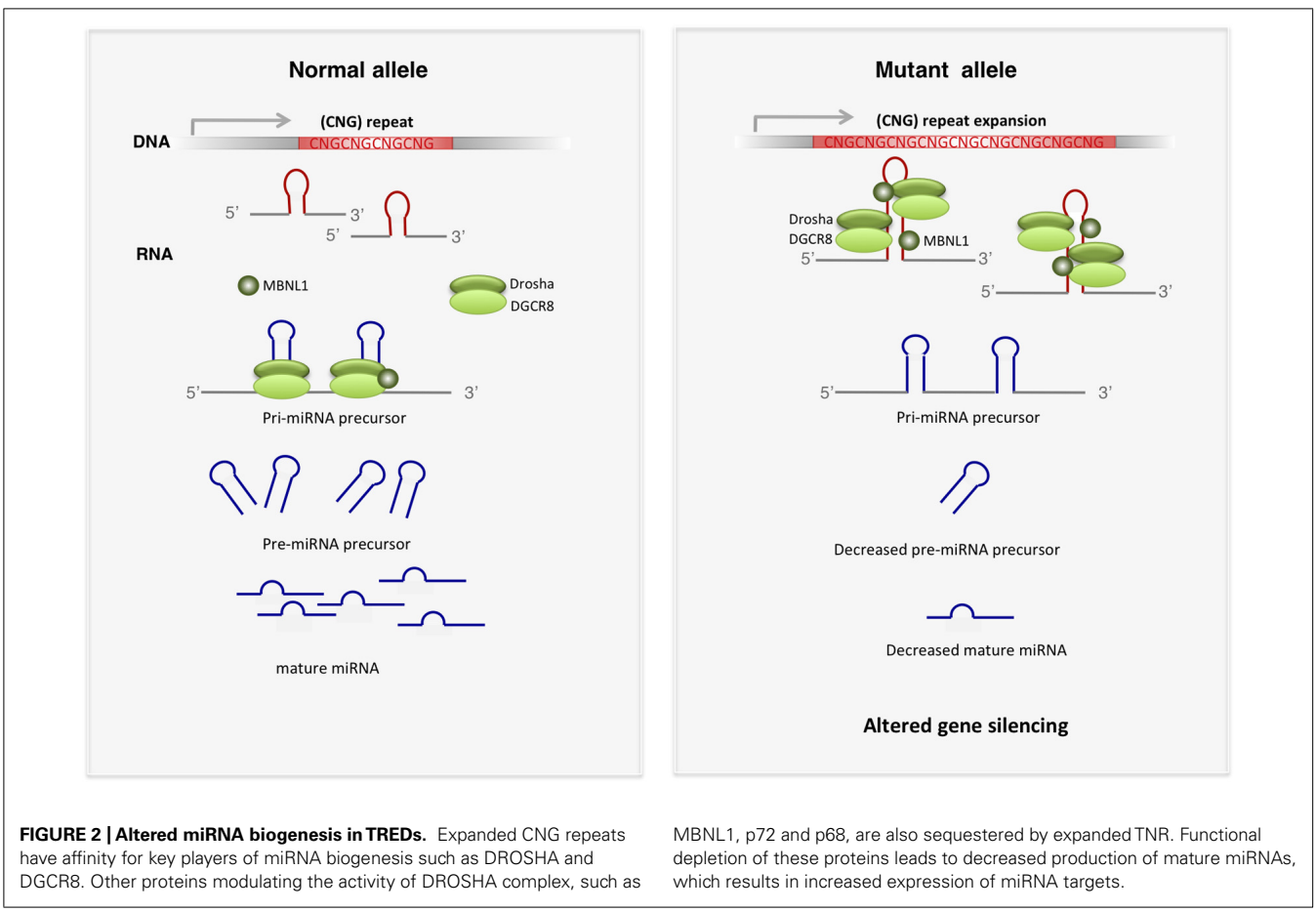

FIGURE 2 | Altered miRNA biogenesis in TREDs. Expanded CNG repeats have affinity for key players of miRNA biogenesis such as DROSHA and DGCR8. Other proteins modulating the activity of DROSHA complex, such as MBNL1, p72 and p68, are also sequestered by expanded TNR. Functional depletion of these proteins leads to decreased production of mature miRNAs, which results in increased expression of miRNA targets.

Alterations in miRNA biogenesis pathways have been also described in FXTAS (Sellier et al., 2013). The hairpin structure of the expanded CGG in the FMR1 5′UTR mRNA mimics the structure of the miRNA-precursors (pri-miRNAs). DGCR8 and its partner DROSHA, key players in miRNA-precursor processing, are sequestered by CGG-RNA repeats (**Figure 2**). Depletion of these processors compromises the biogenesis of many miRNAs, thus triggering downstream detrimental gene expression perturbations, which likely contribute to FXTAS pathogenesis. However, this mechanism may be tissue-dependent, as small RNA profiling in peripheral blood of FXTAS patients does not reveal a general miRNA downregulation (Alvarez-Mora et al., 2013).

General perturbations in miRNA biogenesis result in altered mature miRNAs expression and subsequent modifications of gene silencing, which likely contribute to disrupted cell homeostasis in TREDs.

BIOGENESIS AND ACTIVITY OF SMALL REPEATED CNG IN TREDs SENSE TRANSCRIPTS

The hairpin structure of expanded CNG repeats (Galka-Marciniak et al., 2012) constitutes a substrate for Dicer, an endonuclease involved in miRNA biogenesis that excises RNA precursors to generate the mature short miRNA (**Figure 3**). Dicer recognizes the expanded triplet and cleaves it, producing small repeated RNAs (sCNG). *In vitro* approaches demonstrated that single stranded CGG-RNA constructs are cleaved by Dicer, producing short CGG-RNAs of approximately 21 nt (sCGG; Handa et al., 2003). Recombinant Dicer also cleaves long transcripts containing other types of long CNG repeats (CAG, CUG, CCG; Krol et al., 2007). Importantly, the Dicer-dependence of sCNG biogenesis has been demonstrated in fibroblasts of patients with DM1 (sCUG), HD (sCAG) and SCA1 (sCAG; Krol et al., 2007). In this study the authors further showed that sCNG were active as transcriptional inhibitors, since they downregulated the expression of transcripts with complementary target sequences. This inhibitory activity was dependent on Ago2, a key member of the RNA silencing machinery (Krol et al., 2007).

The relevance of sCNG in TREDs pathogenesis has been recently addressed in HD (Banez-Coronel et al., 2012). This study confirmed the biogenesis of sCAG in a neuronal cell model expressing expanded *HTT* exon-1, and in brain samples of patients with HD or the R6/2 HD mouse model. Importantly, the fraction of small RNAs (sRNAs) derived from cells expressing expanded *HTT* exon-1 produced neuronal death. Both the biogenesis and the toxic activity of sCAG were dependent on Dicer. Similarly, transfection of sRNAs isolated from the striatum and cortex of patients with HD induced significant neuronal toxicity. This toxic effect was prevented by oligonucleotides complementary to short CAG, strongly suggesting a detrimental effect of sCAG. Furthermore, toxicity may depend on downstream silencing effects, as HTT exon-1-derived sCAG were loaded onto Ago2 complexes and knocking-down of Ago2 prevented damage. However, the sequence/structure requirements for effective silencing of sCAG-targets remain to be resolved, since similar moderate inhibition was detected in luciferase assays performed with targets harboring a perfect sCAG-complementary CTG repeat or a CAG repeat that offers an interrupted sCAG target site. Whether the detrimental properties of sCAG include silencing effect through mRNA degradation and/or translational repression, or perturb gene expression networks through other mechanisms, remains to be determined. Interestingly, this study shows that the effect of sCAG-RNAs differed depending on the cell type, with high toxicity detected in BDNF-differentiated neuroblastoma cells. In this scenario, sCAG activity may provide a mechanism contributing to tissue selective affectation. The relevance of sCNG biogenesis and activity in other TREDs is an interesting field for future studies.

BIDIRECTIONAL TRANSCRIPTION IN TREDs LOCI PRODUCE NEW PATHOGENIC PLAYERS

Much of the transcriptome is transcribed in both directions (Chen et al., 2004). While recent data suggest that only a small part of the sense transcript produces proteins (Derrien et al., 2012), the anti-sense transcripts, normally less abundant, are involved in the regulation of gene expression (He et al., 2008; Morris et al., 2008; Yu et al., 2008; Batra et al., 2010). Bidirectional transcription has been detected in many TREDs loci including, DM1, SCA8, FXTAS, SCA7, HDL2, and HD, suggesting a role in disease pathogenesis (**Table 1**; Cho et al., 2005; Ladd et al., 2007; Batra et al., 2010; Chung et al., 2011; Sopher et al., 2011; Wilburn et al., 2011; Seixas et al., 2012). Thus, TREDs pathogenic mechanisms typically associated with expanded toxic RNA may be complemented with those induced by abnormal expanded peptides that result from coding-antisense transcripts or by a complementary non-coding expanded RNA. For instance, in SCA8, the progressive cerebellar degeneration inducing ataxia is the consequence of a CUG expansion in the 3' end of the non-protein coding *Ataxin 8OS* mRNA (Koob et al., 1999; Day et al., 2000). This led to the conclusion that the pathogenic mechanism was related with an expanded CUG-RNA toxic gain of function. Subsequently, bidirectional transcription was demonstrated in transgenic mice expressing the entire human locus with either normal or expanded CTG allele (Moseley et al., 2006). A progressive neuronal loss was found in the lines expressing expanded CUG, with concomitant co-expression of two transcripts in opposite directions. The sense transcript produced a non-coding CUG-expanded transcript (*Ataxin 8OS*) and an anti-sense transcript resulted in a CAG expansion that was translated into a highly enriched polyQ track (*Ataxin 8*). Intranuclear inclusions immunopositive for anti-polyQ antibodies, which are typical from PolyQ diseases and CUG foci formation co-localizing with MNBL1 were detected in cerebellar cells of the mouse model and patients with SCA8 (Daughters et al., 2009). Thus, both RNA and protein toxic gain of function may account for SCA8 pathogenesis.

A similar process could account for HDL2 pathogenesis that is caused by a CUG/CAG repeat expansion at the Juctophilin-3 (JPH3) locus (Holmes et al., 2001), The alternatively spliced forms in the *JPH3* gene place the CUG expansion in the polyleucine or polyalanine ORFs or in the 3′UTR. A *JPH3* transcript with expanded CUG repeats produce RNA foci that co-localize with MNBL1 and induces cell toxicity (Rudnicki et al., 2007). The existence of an anti-sense CAG transcript in the JPH3 locus was recently demonstrated, which may account for the detected polyQ proteianceous inclusions (Wilburn et al., 2011).

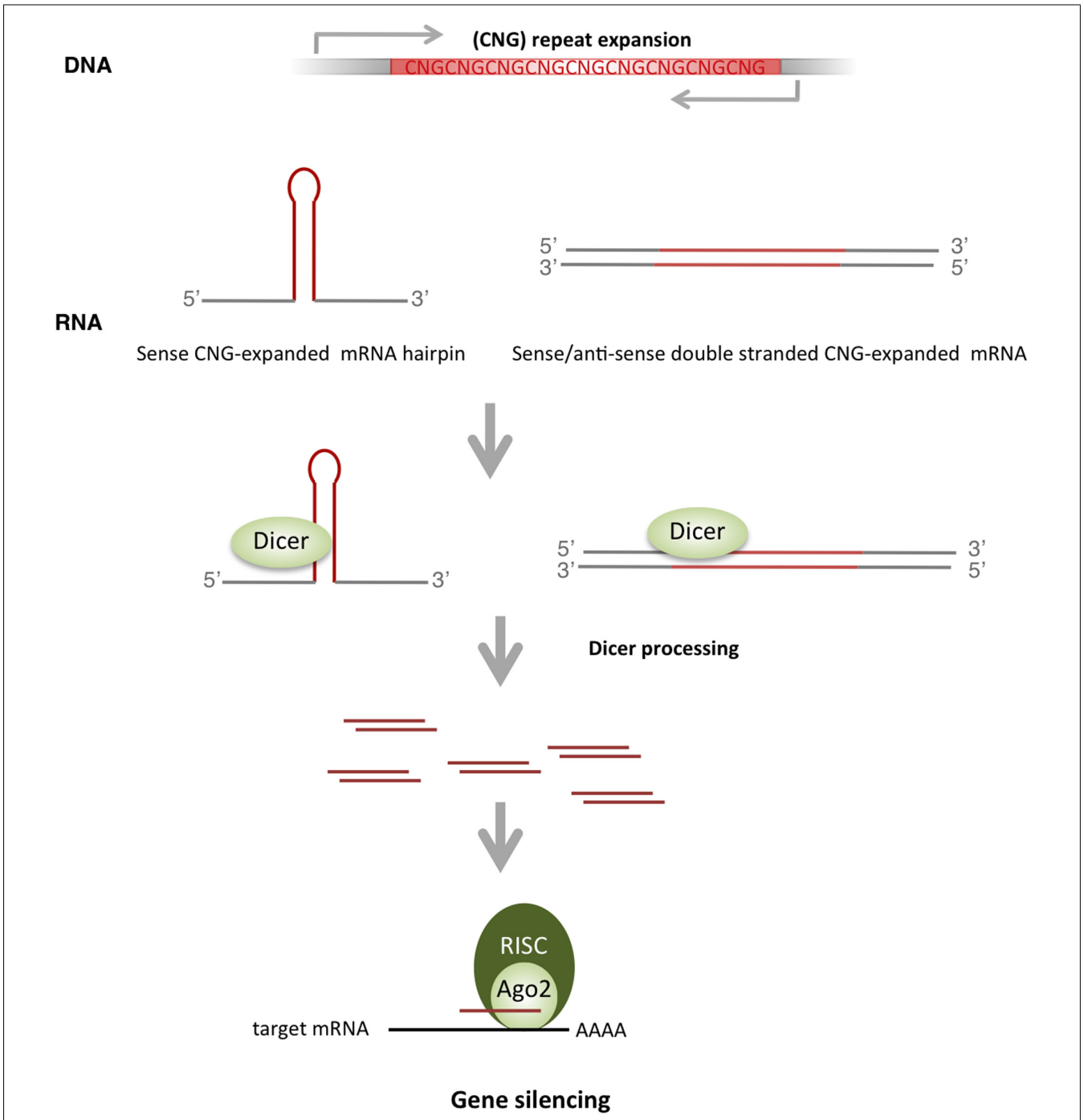

FIGURE 3 | Biogenesis and activity of sCNG in TREDs. TNR expansions produce hairpin-like structures that are recognized and cleaved by Dicer to form small RNAs with repeated TNR (sCNG). Bidirectional transcription though the expanded TNR offers another source of sCNG, where perfectly complementary double stranded mRNAs with expanded TNR are cleaved by Dicer to form sCNG. sCNG are incorporated into the RISC complex and silence genes with partial or perfect complementarity.

Anti-sense transcripts spanning the CGG repeat have been described in the FMR1 locus (*FMR1AS*) in human lymphoblastoma cells (Ladd et al., 2007). *FMR1AS* is spliced, processed and exported from the nucleus. The regulation of *FMR1AS* expression is dependent on CGG expansion size; being silenced in full CGG mutations (CGG > 200 nt), similar to the *FMR1* sense transcript. A recent study suggested that elevated expression levels of the sense and antisense expanded *FMR1* involving mitochondrial dysfunction participate in parkinsonism phenotype that is associated with CGG-repeat moderate expansions (Loesch et al., 2011).

Thus, both *FMR1* and *ASFMR1* may contribute to the variable phenotypes associated with the CGG repeat expansion.

Anti-sense transcription at the DM1 locus has also been reported (Cho et al., 2005). Both sense and antisense transcripts extending across the CAG repeat were found in independent nuclear foci in a mouse model carrying >1,000 CTG repeats in the DM1 locus and in human tissues (Huguet et al., 2012).

HTT anti-sense (*HTTAS*) transcripts have been identified that contain the repeated CAG track (Chung et al., 2011). Repeat expansion reduces *HTTAS* promoter efficiency, and therefore *HTTAS* expression is reduced in the brain of HD patients. Through knocking down the *HTTAS* transcript the authors demonstrated its regulatory activity on HTT expression. The relevance of this regulatory mechanism in HD has not been addressed. However, a possibility exists that *HTTAS* provides an expanded CUG-based pathogenic mechanism.

One interesting mechanism derived from anti-sense transcription of genes containing TNR expansions is the activation of silencing mechanisms (**Figure 3**). Complementary repeats can form double-stranded structures compatible with endonuclease Dicer slicing activity. This results in the formation of short repeated RNAs that are incorporated into the RISC complex, possibly driving downstream gene silencing with detrimental consequences. This mechanism has been proven in a DM1 Drosophila model, in which the toxic effect of an expanded CUG track was largely enhanced if co-expressed with a CAG expansion. The co-expression of sense and anti-sense transcripts lead to the formation of repeat-derived small interfering RNAs in a process dependent of Dicer-2 and Ago-2 (Yu et al., 2011). Similarly, flies models expressing CAG/CUG -100 nt double stranded RNAs (Lawlor et al., 2011) showed a Dicer 2-dependent progressive neurodegenerative phenotype.

From these results it seems clear that small double stranded RNAs are detrimental for neuronal cells. However, the relevance of bidirectional transcription derived-siRNA in human disease needs to be proven, as the expression of antisense transcripts is normally low, which may limit the formation of these products.

In summary, bidirectional transcription through repeat regions of TREDs genes likely increases the complexity of the pathogenic mechanisms underlying the disease process, including the sequestration of different RBP and the biogenesis of small repeated TNR RNAs with silencing activity.

EVIDENCES FOR RNA TOXICITY IN POLYQ DISORDERS

Although in polyQ diseases pathogenesis has been traditionally linked to altered function of the protein, a number of evidences suggest a complementary detrimental role of the expanded RNA.

In vitro structure determinations of expanded CAG repeats in the mRNA context of the *HTT, ATXN1-3, ATN1* and AR genes that cause different polyQ diseases (**Table 1**) show compatibility with double stranded hairpin formation (Galka-Marciniak et al., 2012). Biochemical studies further suggested that MBNL1 has similar affinity for RNA containing either CUG or CAG repeats (Yuan et al., 2007). Although CAG expansions in polyQ diseases occur in the protein coding sequence, nuclear RNA inclusions accumulating MBNL1 have been detected in fibroblasts of patients with ATXN3 and HD (de Mezer et al., 2011; Mykowska et al., 2011). Alternative splicing defects similar to those observed in DM1 have been shown in these cells, suggesting that splicing alterations are likely the consequence of MBNL1 sequestration.

Expanded CAG repeats were shown to induce *in vivo* toxicity at the RNA level in Drosophila, *C. elegans* and mouse models. The *in vivo* evidence for repeated CAG RNA toxicity was first obtained in a Drosophila model of SCA3 (Li et al., 2008). The expression of untranslated CAG repeats of pathogenic length led to neurodegeneration in the absence of a mutant polyQ protein. The expression of translated CAA or interrupted CAG repeats resulted in a less severe phenotype than the expression of translated pure CAG repeats, which supported the importance of RNA structure for toxicity.

The CAG repeat toxicity at the RNA level was also demonstrated in a worm system (Wang et al., 2011). Both CAG and CUG repeats of pathological length were shown to form nuclear foci, in which the mutant transcript colocalized with the nematode ortholog of MBNL1, CeMBL. The disease phenotype was partially reversed by CeMBL over-expression.

The expression of untranslated long CAG repeats (200 copies) was also shown to be deleterious in transgenic mice (Hsu et al., 2011). Mice expressing EGFP transcripts with long CAG repeats in the 3′-UTR developed electrophysiological, histological and behavioral aberrations in the muscle. Detection of nuclear RNA foci in muscle cells in this model (Hsu et al., 2011) and in the striatum of the YAC128 HD mouse model expressing full-length human *HTT* (Pouladi et al., 2012) further suggests toxicity through expanded CAG-RNA.

These data indicate that cell failure in polyQ diseases may be the result of both an abnormal function of the protein harboring the expanded glutamine and the altered properties of the expanded-CAG RNA. The secondary structure of the CAG-repeat in each gene context and the dynamic expression and activity of RBP may provide specific pathogenic scenarios for cell dysfunction.

miRNAs PERTURBTIONS IN TREDs

MicroRNAs are small RNA molecules of 20–24 nucleotides that generally inhibit the expression of target mRNA, by a mechanism involving mRNA degradation, translational inhibition or a combination of the two (reviewed in Esteller, 2011). miRNAs biogenesis involves processing of a primary transcript in the nucleus (pri-miRNAs) by the Drosha/DGCR8 microprocessor. This generates a precursor miRNA (pre-miRNA) that is exported to the cytoplasm by exoprtin-5, where the endonuclease Dicer cleaves it to release the double-stranded miRNA. One of these strands preferentially loads onto an RNA induced silencing complex (RISC), while the other strand is usually degraded. In animals, miRNAs recognize their targets through complementarity with the seed sequence (nucleotides 2–8 of the 5′ end of the miRNA). Hundreds of mRNA targets could exist per miRNA family and at least 30% of the mRNAs are targeted by miRNAs (O'Carroll and Schaefer, 2013).

miRNAs are fine-tuners of gene expression with key roles in the central nervous system function and development. The first evidences for a major role of miRNAs in neurons involved *in vitro* and *in vivo* models of loss of function of Dicer, a key limiting

endonuclease in miRNA biogenesis. Dicer depletion disrupts the development of the CNS, with clear effects on brain morphology and cell-type specification and differentiation. Conditional knocking-down of Dicer in specific neuronal populations in adult mice further suggests a role of miRNAs in postmitotic long-term neuronal maintenance (Kim et al., 2007; Schaefer et al., 2007; Haramati et al., 2010). Furthermore, conditional loss of Dicer in astrocytes and oligodendrocyes causes neuronal dysfunction and degeneration (Shin et al., 2009; Tao et al., 2011). DGCR8 is one of the genes whose heterozygous deletion results in DiGeorge syndrome (Shiohama et al., 2003) with the majority of patients showing heart defects and developmental problems. The description of DGCR8 as key component of the microprocessor (Gregory et al., 2004) highlighted that defects in miRNA biogenesis likely underlie developmental defects. Haploinsufficiency of the microprocessor member DGCR8 also compromises neuronal viability in mice (Stark et al., 2008).

Perturbations of miRNA pathways have emerged as effectors of CNS damage, contributing to impaired cell homeostasis and neuronal death. MiRNAs deregulation produces alterations in the transcriptome that impact brain function, with consequences in neurodegeneration-relevant pathways, including inflammation, oxidative stress and mitochondrial integrity. The causes of miRNA expression deregulation are diverse, including changes in the activity of transcription factors or disease-associated genes and/or alterations in miRNA biogenesis or stability (Packer et al., 2008; Martí et al., 2010). Ischemia, excitotoxicity, oxidative stress or aging are examples of harmful stimuli producing alterations in the coding and non-coding transcriptome (Persengiev et al., 2011; Xu et al., 2012).

Several studies point to an involvement of miRNAs in the pathogenicity associated to TREDs. The toxicity of Ataxin-3 is enhanced upon Dicer ablation in Drosophila and human cell models. The administration of a pool of sRNAs restored Ataxin-3 toxic effect, suggesting a protective role of miRNAs (Bilen et al., 2006). Supporting a protective role of miRNAs in polyQ diseases, miR-34b was shown to mitigate the toxicity of Ataxin-3 in a Drosophila model (Liu et al., 2012).

The activity of disease-associated genes has emerged as one of the causes for miRNA deregulation in TREDs. Mutant HTT protein interacts with Ago2 in the P-bodies, and HTT depletion impairs miRNA mediated gene silencing (Savas et al., 2008). Strong miRNA deregulation has been detected in HD that may be in part associated with altered activity of the RE1-silencing transcription factor (REST). The expanded HTT polyQ track impedes sequestration of REST by wild-type HTT in the cytoplasm, therefore allowing its translocation to the nucleus. Mislocalization in the nucleus permits REST binding to RE1 repressor sequences thus decreasing neuronal gene expression, which triggers neuronal dysfunction. Several miRNAs with RE1 upstream binding sites are down-regulated in HD, including miR-9/miR-9* (Packer et al., 2008). A negative feed-back loop was proposed to occur in HD, involving the activity of REST-silencing complex that is regulated through the effect of miR9 and miR-9* on REST and Co-REST, respectively (Packer et al., 2008). High-throughput sequencing analysis has revealed strong miRNA expression deregulation in the striatum and frontal cortex of patients with HD (Martí et al., 2010). A significant enrichment of down-regulated miRNAs harboring upstream RE1 or P53 binding sites was also reported in this study, suggesting a major role of these transcriptional modulators in miRNA deregulation.

In another example, Ataxin-2 has been recently identified as a component of the miRNA pathway to regulate synapse-specific long-term-plasticity. This targets the putative relevance of ataxin-2/miRNAs in spinocerebellar ataxia neurodegeneration (McCann et al., 2011).

Significant miRNA deregulation has been also detected in pre-symptomatic versus symptomatic SCA1 mouse model cerebellum, which suggests a role of miRNAs in the evolution of the disease (Rodriguez-Lebron et al., 2013). In addition, the miRNA transcriptome has been also characterized in the muscle of a Drosophila model of DM1, expressing CTG repeats alone (Fernandez-Costa et al., 2013). Among the downregulated miRNAs, miR-1, miR-7, and miR-10 were confirmed in muscle of patients with DM1. Interestingly, over-expression of miR-10 extended the lifespan of CUG-expressing flies, suggesting a role in the disease.

Deregulation of miRNA specifically targeting dosage-sensitive disease genes may highlight their relevance as pathogenic biomarkers, which could be selectively targeted in therapeutic strategies. Dentatorubral-pallidoluysian atrophy (DRPLA) is caused by a CAG/polyQ expansion in DRPLA gene/protein, respectively. miR-200b and miR-429 target *REPRE* mRNA, whose protein product binds to DRPLA protein. Overexpression of REPRE induces DRPLA mislocalization. Thus, expression levels of miR-200b and miR-429 could potentially contribute to DRPLA (Yanagisawa et al., 2000; Karres et al., 2007). In another example, miR-886-3p targets the frataxin gene (*FXN*) that carries an intronic GAA. TTC triplet repeat expansion in Friedeich ataxia (FRDA; Mahishi et al., 2012). *FXN* mRNA and protein are decreased in FRDA. The authors found increased levels of miR-886-3p in blood and cells of patients with FRDA and further demonstrated that inhibition of miR-886-3p resulted in increases of *FXN* mRNA and protein. In addition, miR-19, miR-101 and miR-130 regulate Ataxin 1 (*ATXN1*) that causes spinocerebellar ataxia 1 (SCA1), when presenting a CAG expansion (Lee et al., 2008). The authors showed that inhibition of the activity of these miRNAs enhanced the cytotoxic activity of ATXN1 with an expanded polyQ in human cells, suggesting a miRNA mechanism modulating pathogenesis. More recently, it has been shown that, miR-144 and miR-101 play a central role in modulating the levels of *ATXN1* (Persengiev et al., 2011). In SCA1 patients and aging the levels of these miRNAs are increased, suggesting a role in neurodegeneration. Finally, the 3′UTR of the *FMR1* mRNA is targeted by miR-101, miR-129-5p, and miR-221 (Zongaro et al., 2013). Downregulation of miRNAs has been generally detected in the brain of patients with FXTAs (Sellier et al., 2013) and miR-221 is also downregulated in peripheral blood of males with FXTAS (Alvarez-Mora et al., 2013). Thus, deregulation of certain miRNAs may contribute to upregulation of expanded *FMR1*, which has been shown to participate in FXTAS pathogenesis.

These data indicate that altered expression of specific miRNAs may contribute to TREDs pathogenesis, directly perturbing the expression of dosage-sensitive genes that are essential in the

maintenance of cell homeostasis. The dynamics of miRNA alterations may define the relevance of miRNA-pathways in disease evolution.

THERAPEUTIC APPROACHES TARGETTING RNA-TOXICICITY

Several therapeutic targets in polyQ diseases such as HD involve the intervention of pathways perturbed by mutant polyQ proteins, including histone acetylation, excitotoxicity and oxidative stress (Clabough, 2013). Mutant HTT inhibits acetyltransferases, resulting in reduced levels of acetylated histones (Gray, 2010) Inhibition of histone deacetylase activity (HDACs) has been proposed as a therapeutic approach, alleviating altered gene expression produced by diminished acetyltransferase activity (Steffan et al., 2001; Gray, 2010). In addition, mitochondrial impairment and excitotoxicity have been involved in neuronal death in HD. Administration of antioxidants such as Coenzyme Q10 slow striatal atrophy in mouse models of HD (Beal, 2002) and delivery of growth factors and cytokines modify neuronal degeneration and prevents excitotoxic deficits in murine HD models (Mittoux et al., 2000; Perez-Navarro et al., 2000; de Almeida et al., 2001). Other strategies involve lowering the amount of mutant polyQ protein by reducing its production (see below) or enhancing its clearance. In this line, increased mutant HTT protein turnover in a mouse model improves disease outcome (Southwell et al., 2009).

Although targeting the mutant/expanded polyQ protein or its downstream pathogenic effectors improves disease readouts, these strategies may not impede expanded TNR RNA toxicity. Approaches aimed at blocking protein and/or RNA toxicity include the use of antisense oligonucleotides (ASOs) or RNAi (short hairpin RNAs, shRNA, double stranded siRNA or modified single stranded siRNAs), targeting the expression of the mutant gene (Sah and Aronin, 2011; Watts and Corey, 2012; **Figure 4**). RNAi using shRNA against mutant TREDs gene mRNA and protein have been successfully used *in vivo*. Intrastriatal adenoviral-delivery shRNA targeting mutant human HTT resulted in improved neuropathology and behavioral deficits in HD mouse models (Harper et al., 2005; Rodriguez-Lebron et al., 2005). Davidson's lab later showed that miRNA expression systems to inhibit *HTT* were more efficient, overcoming unspecific toxic effects induced by shRNA expressing vectors (McBride et al., 2008; Boudreau et al., 2011). The therapeutic potential of 2′-O-(2-methoxy)ethyl modified ASOs targeting human *HTT* has been recently addressed (Kordasiewicz et al., 2012). The intracerebral transient infusion of these ASOs resulted in the RNAse H mediated degradation of the human *HTT* mRNA, in transgenic mouse models of HD (Kordasiewicz et al., 2012). Importantly, transient *HTT* reduction resulted in sustained motor and histopathological phenotypic reversal in the HD rodent models.

Because multiple studies suggest that reducing the expression of the wild-type allele may have deleterious consequences, selective targeting of the expanded allele should be optimal (Omi et al., 2005; Godin et al., 2010; Huang et al., 2011). In polyQ diseases, single-nucleotide polymorphisms (SNPs) linked to repeat expansions that distinguish wild-type from mutant alleles offer possibilities for specific targeting by RNAi. SNP targeting has been shown in HD (Schwarz et al., 2006; Carroll et al., 2011), SCA3 (Li et al., 2004) and SCA7 (Scholefield et al., 2009). Low frequency of allele-distinguishing SNPs in the human population limits this strategy. However, several authors have shown that targeting several specific SNPs can be applied to the majority of HD patients (Lombardi et al., 2009; Pfister et al., 2009; Warby et al., 2009).

Other potential therapeutic strategies in polyQ diseases, are based on the use of several types of modified single stranded ASOs that target the CAG expansion in the mutant *HTT* or *ATXN3* mRNA, while preserving the normal function of the wild type allele (Hu et al., 2009a,b,c; Gagnon et al., 2010; Fiszer et al., 2012; Yu et al., 2012). The longer CAG track in the mutant allele offers more binding sites for the complementary ASOs (**Figure 4**). In addition, the RNA structures of the expanded CAG that differ from those of the wild type allele (de Mezer et al., 2011; Krzyzosiak et al., 2012), may offer distinctive feature for preferential recognition of the mutant allele. In these studies, single-stranded ASOs containing locked nucleic acid (LNA) or peptide nucleic acid have been shown to selectively block the expression of mutant HTT at the protein level. This effect was not associated with extensive *HTT* mRNA degradation and LNA ASOs were shown to form stable structures with the target RNA. Although expanded RNA toxicity in HD has not been addressed, the formation of stable LNA ASOs : RNA duplexes has also the potential to block expanded CAG toxic effects in *HTT* mRNA.

Several studies have demonstrated a similar therapeutic potential of a CAG-repeat antisense or morpholino targeted to expanded-CUG in the *DMPK* mRNA (Mulders et al., 2009). In cell myoblast-myotube and patient cell models a 2′-O-methyl-phosphorothioate-modified (CAG)7 ASO silenced *DMPK* expression and reduced the ribonuclear aggregates. Intramuscular administration of these ASO in a DM1 mouse model further reduced expanded RNA toxicity (Mulders et al., 2009). In another study, a morpholino anti-sense (CAG)25 was shown to block the interaction of MBNL1 with the expansion in a mouse model, further dispersing nuclear RNA foci, preventing alterations in alternative splicing and preventing RNA toxicity. More recently, it has been shown that systemic administration of ASOs effectively knocked down the expression of nuclear retained-transcripts containing expanded CUG in the muscle, thus correcting the physiological, histopathological and transcriptional alterations associated to this DM1 model (Wheeler et al., 2012).

Together, these data suggest that in TREDs, targeting the expression and activity of the expanded allele both at the RNA and protein levels is a promising therapeutic strategy.

CONCLUSION

RNA toxicity is a process underlying pathogenicity in TREDs, with TNR expansions occurring in both coding and non-coding regions of specific genes. Sequestration of transcriptionally active RBP, and RBP participating in miRNA biogenesis result in direct and indirect perturbations of the coding-transcriptome, which likely contribute to cell dysfunction. Full characterization of the repertoire of RBP in different types of TNR expansions is essential to understand common detrimental pathways in etiologically diverse neurological disorders. Studies that take into consideration the gene context may provide hints to understand disease specific aspects.

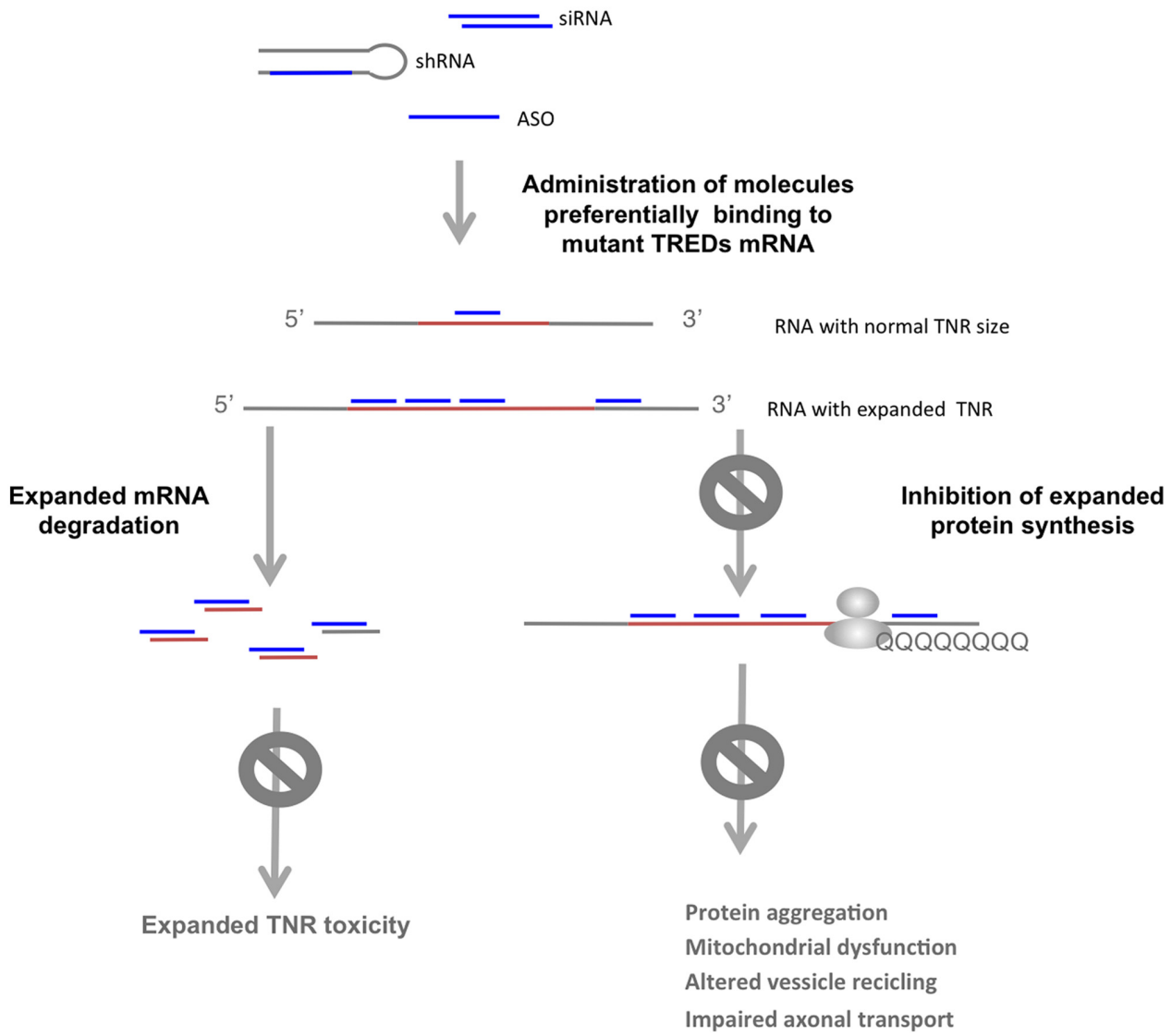

FIGURE 4 | Selective targeting of the expanded allele in TREDs. shRNA, modified ASO or siRNA directed against the expanded allele results in degradation of mutant mRNA or blockage of the mutant protein synthesis, which impedes the detrimental activities of the expanded TNR in the mRNA and the mutant protein. With this strategy the normal allele is not targeted, allowing its function.

The biogenesis and activity of sCNG may contribute to TREDs pathogenesis. The gene silencing activity of these species likely trigger downstream detrimental effects, which may differ, depending on the cell type. This mechanism may complement the damaging activity of expanded protein and/or expanded RNA. Whether sCNG mechanisms are only related with gene silencing or present other activities and the real importance of sCNG mechanisms in each TRED remain to be determined.

In each TRED, the mechanistic bases for tissue specificity, with particular affectation of selective neuronal types, remain largely unknown. The different RNA-based pathogenic processes provide a number of scenarios that may underlie this specificity. These include the dynamic expression and cellular and subcellular localization of RBP, the amount of expression of the sense and anti-sense transcripts spanning the expanded TNR in each cell context, the regulation of the biogenesis and activity of sCNG in different brain areas and/or the temporal and spatial primary and secondary perturbations of the miRNA transcriptome.

The mechanistic complexity in TREDs stresses the need of additional studies to dissect the relative relevance of expanded protein-RNA-, and/or sRNAs-mechanisms in each disease. In this context, the use of modified ASOs or siRNA directed to the expanded TNR has the potential to block deleterious effects of expanded full-length RNA and derived sCNG, with concomitant inhibition of expanded protein expression.

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