Regional-dependent, comprehensive characterization of miRNA signatures in sporadic Creutzfeldt-Jakob Disease and early Alzheimer's Disease-type neuropathology

Doctoral Thesis

In partial fulfillment of the requirements for the degree
"Doctor rerum naturalium (Dr. rer. nat.)"
in the Molecular Medicine Study Program
at the Georg-August University Göttingen

submitted by
Katrin Thüne
born in Heilbad Heiligenstadt

Göttingen, 2018

Instructor and Group Leader:

Prof. Dr. Inga Zerr

Department of Neurology
University Medical Center Göttingen

Members of the Thesis Committee:

First member: Prof. Dr. Mikael Simons

German Center for Neurodegenerative Diseases
Technical University of Munich

Second member: Prof. Dr. Silvio Rizzoli

Department of Neuro- and Sensory Physiology
University Medical Center Göttingen

Third member and working supervisor: Dr. Franc Llorens

Department of Neurology
University Medical Center Göttingen

Date of Disputation: 11th October 2018

Table of Contents

List of Figures

List of Tables

1. Acknowledgement

I am grateful to all of those with whom I had the pleasure to work with during the period of my thesis and who contributed to the work presented in this dissertation and other related projects.

I would especially like to thank our group leader and supervisor **Prof. Inga Zerr** for her support. I am very grateful for her scientific guidance, constant optimism and encouragement throughout this research work.

My deep gratitude goes to my PhD supervisor **Dr. Franc Llorens**, a truly dedicated mentor, who taught me more than I could ever give him credit for here. My PhD has been a wonderful experience and I thank Franc wholeheartedly, not only for his tremendous academic support, but also for promoting so many great opportunities such as research stays in internationally-renowned laboratories and attendance to high-level scientific conferences.

I would like to express my thanks to **Prof. Isidre Ferrer**, who invited and warmly hosted me at his department for two research stays in Barcelona. Without his enduring support, ideas and encouragement this project would not have been completed and I am highly appreciative for this. I owe a very special thank to **Margarita Carmona**, **Pol Andrés-Benito** and **Belén Ansoleaga** for their indispensable contributions in scientific work.

I am also very grateful for the scientific discussions and fruitful collaboration with **Dr. Eirini Kanata**. This work has greatly benefited from her thoroughness ideas and constructive feedback.

I am very thankful to **Prof. Uwe Michel**, **Prof. Thomas Meyer** and **Prof. Ira Milosevic** for their crucial scientific guidance and for providing the possibilities to use their excellent equipment and microscopes.

I would like to extend my heartfelt thanks and appreciation to all my colleagues and labmates for their help and the cooperative atmosphere in our lab. Very special thanks go to **Waqas Tahir**, **Nadine Gotzmann** and **Silja Köchy** for their precious help, to **Ângela** and **Susana** for their motivation skills and spreading Portuguese sunshine, and to **Aman** for always having a back-up plan.

This research was supported by the *German Center for Neurodegenerative Diseases (DZNE)*, the *University Medical Center Göttingen (UMG)* and grants from *the Felgenhauer-Stieftung* and *Boehringer Ingelheim Fonds*. The financial support enabled me to conduct two research stays in Barcelona in order to perform experimental analysis. I am very grateful for that. Also, I received a grant from the Unibund Göttingen, which gave me the opportunity to attend conferences abroad where I presented this work and received scientific feedback from experts of this field. I am very grateful for this support.

Finally, I would like to thank my family, especially my parents and sisters, for their limitless love, support and understanding throughout my life and career.

2. List of Abbreviations

Aβ	Amyloid beta peptide
AD	Alzheimer's disease
Ago	Argonaute protein
ALS	Amyotrophic lateral scleroses
APP	Amyloid precursor protein
BSE	Bovine spongiform encephalopathy
CSF	Cerebrospinal fluid
DGCR8	DiGeorge Critical Region 8 protein
DLB	Dementia with Lewy Bodies
DNA	Desoxyribonucleic acid
dpi	days post inoculation
EC	Entorhinal cortex
EEG	Electroencephalogram
ER	Endoplasmic reticulum
fAD	early-onset familial Alzheimer's disease
FFI	Fatal familia insomnia
GABA	*Gamma*-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GSS	Gerstmann-Sträussler-Scheinker syndrome
GPI	Glycosylphosphatidyl inositol
Hsp90	Heat-shock protein 90
IKK-NF-κB	"NF-κB kinase/nuclear factor kappa-light-chain-enhancer of activated B cells"-pathway
JAK-STAT	Janus kinase/signal transducer and activator of transcription
M	Methionine
MAPK/ERK	"mitogen-activated protein kinases/extracellular signal-regulated kinases"-pathway
MCI	mild cognitive impairment
mRNA	messenger RNA
miRNA	micro RNA
MS	Multiples sclerosis
ND	Neurodegenerative Diseases
NfL	Neurofilament light
NFT	Neurofibrillary tangles
nt	Nucleotide
PART	primary age-related tauopathy
PrP	Prion protein
PRNP	Prion protein gene
$Prnp^{0/0}$	Prion protein gene-knockout
PrPc	Cellular prion protein
PrPsc	Scrapie prion protein
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PD	Parkinson's disease
Tg340-PRNP	Human prion protein gene transgenic mice

RISC	miRNA-mediated silencing complex
RNA	Ribonucleic acid
RNAseq	RNA sequencing
rpAD	rapidly progressive Alzheimer's Disease
sCJD	sporadic Creutzfeldt-Jakob Disease
sCJD MM1	sporadic Creutzfeldt-Jakob Disease patient with methionine homozygosity at codon 129 of the *PRNP* gene and PrPsc type 1
sCJD VV2	sporadic Creutzfeldt-Jakob Disease patient with valine homozygosity at codon 129 of the *PRNP* gene and PrPsc type 2
Tau	Microtubule-associated protein Tau
Tau	Microtubule-associated protein Tau gene
p-Tau	phosphorylated microtubule-associated protein Tau
t-Tau	total levels of microtubule-associated protein Tau
qPCR	quantitative polymerase chain reaction
IBA1	Ionized calcium binding adaptor molecule 1
V	Valine
XPO5	Exportin-5
3'UTR	3 prime untranslated region

3. Abstract

The pathogenic mechanisms and the origins of neurodegenerative diseases remain unclear on molecular level. Increasing evidences postulate alterations in miRNA signatures as critical factor in pathogenesis, progression and prognosis of neurodegenerative disorders. miRNAs are a class of small non-coding RNAs shaping gene expression post-transcriptionally. As a regulator of cellular functions and homeostasis, miRNAs maintain brain cell integrity and their dysregulation contributes to neuropathological conditions.

In our studies, we demonstrated strong evidences for disease-associated miRNA expression changes during critical disease stages in brain regions vulnerable to neurodegeneration. In **publication I**, we gained a comprehensive picture of global miRNA expression changes in frontal cortex and cerebellum during clinical stage of sporadic Creutzfeldt–Jakob disease, the most prevalent human prion disease. We observed marked miRNA expression alterations highly changed in a regional and disease subtype-dependent manner in sporadic Creutzfeldt–Jakob disease. We additionally revealed complex impairments of key proteins involved in the miRNA silencing machinery and biogenesis that might underlie miRNA dysregulation in sporadic Creutzfeldt–Jakob disease. We clearly validated the miRNA expression signatures observed in sporadic Creutzfeldt–Jakob disease, in a mouse model highly relevant to human pathology. In cross-disease validation studies, we detected that specific sporadic Creutzfeldt–Jakob disease-regulated miRNAs are commonly altered in alternative neurodegenerative disease shedding light into potential common miRNA-related mechanisms in the neurodegenerative conditions.

In **publication II**, we provided crucial insights into temporal miRNA expression alterations in brain regions vulnerable to early Alzheimer's disease-type pathology. Therefore, we performed targeted expression profiling of miRNAs implicated in Alzheimer's disease pathogenesis in the locus coeruleus, entorhinal cortex, hippocampal CA1 region and dentate gyrus during early and mid-stages of Braak neurofibrillary tangles pathology. In this way, we provided for the first time insights into crucial miRNA expression alterations in the locus coeruleus during early Alzheimer's disease-type pathology before disease-associated neuronal death is evident.

By a combination of different miRNA quantification approaches we provided a state-of-art methodology for a comprehensive screening and reliable validation of disease-associated miRNA signatures. Thereby, we emphasized the importance of a more holistic understanding in temporal, spatial and cellular aspects of miRNA alterations during neurodegenerative conditions, which will help to unravel biological consequences of miRNA dysregulation in disease.

4. Introduction

Neurodegenerative diseases (NDs) represent a heterogeneous group of incurable and debilitating conditions that are characterized by the progressive degeneration of the structures and function of the central and/or peripheral nervous system. In NDs, pathogenic changes in disease-specific brain areas lead to dysfunction and/or loss of specific neuronal populations that arise from often unknown or insufficiently characterized molecular and cellular risk factors and causes. The clinical manifestations depend on the particular brain regions involved during disease progression. Neurodegenerative conditions represent distinct disease entities; however, their pathogenesis shares many common features including a disturbed protein homeostasis, the aggregation and deposition of pathological altered proteins, progressive neuroinflammation, and neuronal dysfunction and loss (Dugger and Dickson 2017; Ramanan and Saykin 2013; Armstrong, Lantos, and Cairns 2005; Rudy J. Castellani, Perry, and Smith 2004).

Prion diseases are progressive neurodegenerative conditions characterized by a rapid neuronal loss in specific areas of the brain corresponding to a high heterogeneity in their pathological features and clinical presentation ranging from rapidly progressive dementia to cerebellar ataxia (Appleby et al. 2009). The changes in the metabolism of the cellular prion protein PrPc and its formation into the abnormal isoform PrPsc, spongiform degeneration, neuronal loss and neuroinflammation are attributed to the pathogenesis of prion diseases (Colby and Prusiner 2011).

Alzheimer's disease (AD) is the most common form of neurodegenerative dementia characterized by gradual cognitive decline and impaired memory. AD is featured by a complex and multifactorial pathogenesis and the disease hallmarks include the deposition of protein aggregates consisting of extracellular amyloid plaques and intracellular generation of neurofibrillary tangles (NFTs) as well as brain atrophy, neuronal loss, vascular damage and neuroinflammation (De Strooper and Karran 2016; Wadsworth and Collinge 2011)
The pathogenesis of prion diseases and AD shares the impairment of fundamental cellular machineries associated to an abnormal processing and aggregation of proteins, impaired lysosomal function and clearance mechanisms, proteopathic stress on different brain cells of disease-specific brain regions, impaired metabolisms, mitochondrial dysfunction as well as uncontrolled glial activation, which lead to an irreversible dys-homeostasis of the brain (De Strooper and Karran 2016; Puoti et al. 2012a; Castellani, Perry, and Smith 2004). The protein aggregation into amyloid conformations is proposed to play a causative role in the initiation of neurodegenerative cascades leading to disease pathology in prion disease as well as in AD (Selkoe and Hardy 2016; Colby and Prusiner 2011). However, the underlying pathogenic mechanisms and triggering factors, which

favour the development and progression of these neurodegenerative conditions, remain unclear.

NDs, including prion disease and AD, are thought to develop over a clinically silent phase followed by a progressive worsening of clinical symptoms over time associated to a cumulative loss of neurons. The lack of knowledge on the ND-specific molecular and cellular events, as well as key molecules driving disease progression hampers the development of therapeutic and diagnostic strategies. Studying a broad spectrum of fundamental cellular machineries in ND affected brain regions and cells during critical disease stages is essential for understanding the complex pathogenic mechanisms in a spatial, temporal and cellular dependent manner.

Recently, straightforward links have been established between NDs and miRNA deregulated patterns suggesting miRNAs as contributing factor in neurodegeneration (Abe and Bonini 2013a; Hébert and De Strooper 2009a). miRNAs are a class of short, non-conding RNAs that represent a complex layer of gene expression regulation involved in cellular function and survival (Bartel 2009). miRNAs control protein expression on transcriptional level through the binding of mRNA targets, which is leading to translation repression and/or mRNA degradation dependent on sequence complementarity (Jonas and Izaurralde 2015b). With their ability to regulate complex networks of genes, dysregulated miRNA networks have the potential to be involved in virtually all pathophysiological mechanism in NDs (Goodall et al. 2013). Thereby, deregulated miRNA networks can be involved in early molecular alterations in initial disease affected brain regions before manifestation of clinical signs, as well as throughout clinical disease progression in NDs.

The present work provides comprehensive characterizations of miRNA signatures in the brain of patients afflicted with sporadic Creutzfeldt-Jakob disease (sCJD), the most common human prion disease, and AD, revealing crucial insights in the role of miRNA deregulation during critical stages of NDs.

Our studies revealed marked alterations of the miRNA network and strong indications for complex impairments of the miRNA machinery with sCJD disease progression. A miRNA expression time course analysis from a sCJD mouse model provided a link between regional pathology and stage of disease and validated main observations from human tissue regarding miRNA expression changes. Functional miRNA explorations in sCJD were revealed by a selective cell death induction through the transfection of sCJD associated miRNAs in cell cultures.

Using the expertise of characterizing miRNA signatures in sCJD by the usage of different analytical techniques and methodologies, the studies were extended to expression profiling of selected miRNAs reported to be involved in AD pathology. The analyses of miRNA expression in the locus coeruleus (LC), entorhinal cortex (EC) and hippocampus at early and mid-stages of AD revealed alterations of

disease-associated miRNAs during early AD. Moreover, a neuronal cellular environment has been identified for the altered miRNAs in early AD affected brain regions, suggesting the involvement of those miRNAs in early AD pathology.

4.1 Human Prion Disease

Prion diseases, also known as transmissible spongiform encephalopathies, are a group of rapidly progressive neurodegenerative conditions present in human and in a wide range of animal species. The key neuropathological findings shared by prion diseases are widespread neuronal loss, spongiform vacuolation, gliosis, as well as the accumulation of the disease-associated isoform of the prion protein in the brain, sometimes accompanied by the formation of amyloid plaques (Fig 1A) (C. Soto and Satani 2011a; Appleby et al. 2009; Prusiner 1998).
The main causative pathogenic event in prion diseases is attributed to the changes in the structural conformation of the cellular prion protein PrPc, and its formation into the abnormal, insoluble, pathogenic isoform, PrPsc, in a self-propagating manner (Prusiner 1998).

PrPc is an evolutionarily conserved protein predominantly expressed in the brain, and particularly enriched in neurons (Stahl et al. 1987). A large body of knowledge supports that PrPc expression and its misfolding into the pathological form PrPsc are necessary for the development of prion disease (Prusiner 1998; Aguzzi 2006). In this regard, it is suggested that loss of PrPc signaling and/or gain of PrPsc-mediated toxic signaling can be involved in neuronal dysfunction and death (Winklhofer, Tatzelt, and Haass 2008).
During its biosynthesis in the endoplasmic reticulum (ER) and Golgi apparatus, PrPc undergoes a range of post-translational modification, including glycosylation that result in di-, mono-, or unglycosylated forms (Ermonval et al. 2003).
Whereby most PrPc is exposed on the cell surface as a membrane-bound, glycosylphosphatidyl inositol (GPI) –anchored glycoprotein, there are also transmembrane PrPc forms that traffic in the endosomal system (S. J. Kim 2002). PrPc has been supposed to cycle continuously between the plasma membrane and endocytic compartments, and thereby, likely involved in complex cellular mechanisms (Prado et al. 2004). Multiple biological functions have been proposed for PrPc including roles in neuroprotection, protection against oxidative stress, circadian rhythm, memory and cognition, metal homeostasis, controlling proliferation and differentiation of neural cells (De Mario et al. 2017; Castle and Gill 2017; Wulf, Senatore, and Aguzzi 2017; Steele et al. 2006). As an extracellular scaffolding protein, PrPsc is suggested to be involved in transmembrane signaling and to serve as a platform for different signaling molecules (Linden 2017).
It is hypothesized that PrPc may spontaneously misfold into a pathogenic prion protein form, PrPsc, which can occur as a stochastic event or be favored by a somatic mutation. A failure in protein clearance mechanisms associated to cellular senescence might lead to an accumulation of pathological PrPsc species resulting in an irreversible cellular dysfunction. Thereby, PrPsc is proposed to act as an infectious agent that recruits endogenous PrPc to replicate itself in a self-propagating manner, which leads to neuronal injury and death (Gabor G. Kovacs and Budka 2008; Colby and Prusiner 2011; Prusiner 1998).

The abnormally conformed PrPsc possess several distinct attributes that include the propensity to form from α-helical- into a ß-sheet-rich configuration, which is associated to the formation of amyloidogenic structures developing various degrees of protease-resistance, and tendency to polymerize and aggregate into insoluble deposits (Fig 1B, C, D). Moreover, PrPsc is featured by distinct conformational protein structure arrangements, which result in specific protease cleavage sites and subsequently distinct sizes of protease-resistant PrPsc core fragments (Fig 1D). It has been observed that oligomeric PrPsc species represent the main toxic and infective agent in prion disease models (Puoti et al. 2012; Parchi et al. 1999; C. Soto and Satani 2011b; Colby and Prusiner 2011; Silveira et al. 2005).

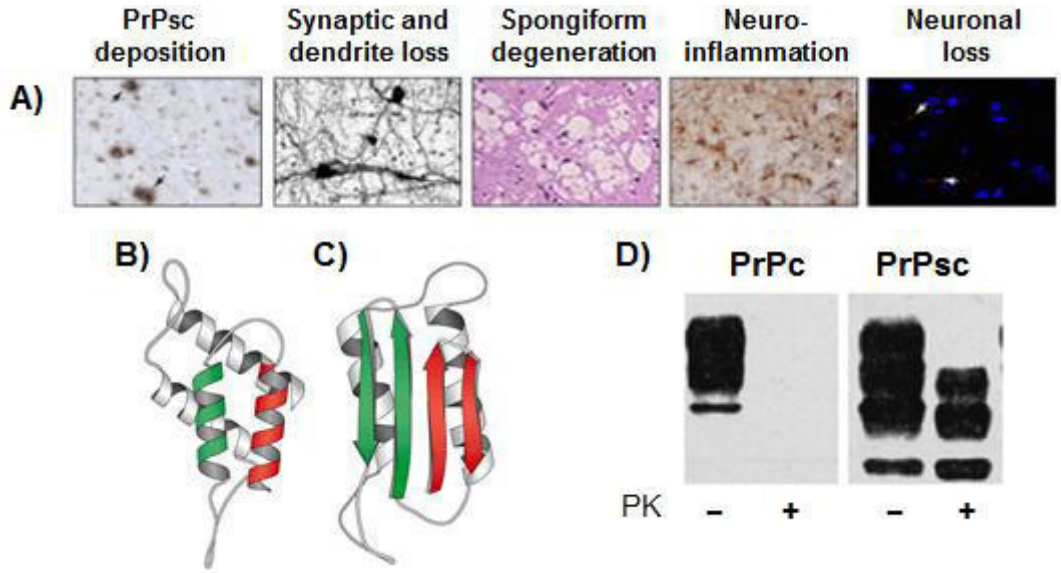


Figure 1: **Prion disease hallmarks and PrPsc characteristic:** A) Main neuropathological findings in prion disease brain are PrPsc deposition, synaptic and dendrite loss, spongiform degeneration, neuroinflammation and neuronal loss. B) Physiological PrPc structure with high α-helical content. C) Pathological PrPsc structure with high ß-sheet. D) Physiological PrPc (variable glycolysated) is sensitive towards proteinase K (PK) digestion, whereas distinct pathological PrPsc structure arrangements lead to specific protease cleavage sites and subsequently distinct sizes of PK-resistant PrPsc core fragments.
Modified after Soto and Satani, 2011. *Trends in Molecular Medicine.*

Prion diseases appear in three principal forms: 1) as sporadic forms manifesting as sCJD, 2) as inherited forms manifesting as genetic CJD (gCJD), fatal familial insomnia (FFI) and Gerstmann-Sträussler-Scheinker syndrome (GSS), and 3) as forms acquired by infection including iatrogenic CJD, kuru and variant CJD.
Human prion diseases represent a wide-ranging clinical heterogeneity including rapidly progressive dementia, motor dysfunction, cerebral ataxia, myoclonus and insomnia (Geschwind 2015; Imran and Mahmood 2011; Sikorska and Liberski 2012).

The high variability in the clinical presentations and neuropathological patterns are associated to disease-causing mutations and polymorphisms in the prion protein gene (*PRNP*), as well as, different conformational protein arrangements of the pathological PrPsc (Parchi et al. 2012; Hill et al. 2003; Gambetti et al. 2003). Several polymorphisms in the prion protein gene have been described, with particular importance of the methionine (M) /valine (V) polymorphism at the codon 129 of *PRNP,* which appears to modify disease phenotype and influence neuropathological signatures (Jeong and Kim 2014).

The majority of human prion disease occurs sporadic, whereas the cases of prion diseases that are inherited or acquired by infection are comparatively rare. The inherited forms account for around 15% of human prion diseases, which are associated to autosomal, dominant mutations of the *PRNP* gene. The most prevalent genetic prion disease are those associated to P102L mutation (manifesting as GSS), D178N mutation and methionine on the mutated allele at codon 129 of the *PRNP* gene (manifesting as fatal familial insomnia) and E200K, (manifesting as familial or genetic CJD (f/gCJD)). In those cases, somatic mutations in the *PRNP* gene favour the spontaneous formation of PrPsc (Mastrianni 2010; Takada et al. 2017; Wadsworth and Collinge 2011).

The very rare cases of acquired human prion disease are caused by exposure to exogenous PrPsc through: the consumption of bovine spongiform encephalopathy (BSE) contaminated food, cannibalism (kuru), use of contaminated surgical instruments or intra-cerebral electroencephalogram (EEG) electrodes, dura mater and corneal grafts and hormone administration (Will 2003; Collinge et al. 2006).

4.1.1 Sporadic human prion diseases: sCJD

sCJD is the most prevalent sporadic prion disease, accounting for the majority (85%) of all human prion diseases. The disease presents as a rapidly progressive dementia and neurologic signs leading to death in a rapid, progressive course, usually, in less than 1 years of disease onset. In fact, the clinical manifestation of sCJD is highly variable and might present as purely cognitive deficits, including commonly rapid progressive dementia, behavioural and personality changes, cerebellar symptoms, vision changes or any combination of these symptoms (Johnson 2005; Kovács et al. 2005; Heinemann et al. 2007).
The variable phenotypes in sCJD can be distinguished by the usage of the polymorphism at the codon 129 in the *PRNP* gene encoding either for M or V (MM, MV, and VV) in combination with the occurrence of two PrPsc types (type 1 or 2), which differ in their conformational protein structure arrangements resulting in different protease cleavage sites, and subsequently, different sizes of protease-resistant PrPsc core fragment. The resulting six phenotypes are used to classify molecular disease subtypes (MM1, MM2, MV1, MV2, VV1, VV2), which

differentiate clearly in clinical and neuropathological features such early symptoms, disease duration and distribution of lesion in the brain including spongiform degeneration, neuroinflammation, neuronal loss and amyloid plaque depositions (Gambetti et al. 2003; Parchi et al. 2012; Hill et al. 2003; Puoti et al. 2012).
Several cerebrospinal fluid (CSF)–based proteins have been demonstrated to mirror neuropathological processes in the brain of sCJD. Among them, the 14-3-3 protein, Tau, ratio phosphor-Tau/Tau, α-synuclein and the detection of PrPsc via real-time quaking-induced conversion (RT-QuIC) assay achieve the highest diagnostic accuracy in the differential diagnose of sCJD (Atarashi et al. 2011; Llorens et al. 2018; 2016; Sanchez-Juan et al. 2006; Collins et al. 2006). However, the clinical–pathological variations among the sCJD subtypes have a strong influence on the sensitivity of various diagnostic tests (Llorens et al. 2016; Castellani et al. 2004; Collins et al. 2006).

The clinical overlap of sCJD with potentially treatable disorders manifesting as rapid progressive dementia, highlights the need for reliable tools for early differential diagnosis of sCJD (Rosenbloom and Atri 2011; Geschwind et al. 2008). Indeed, a definitive diagnosis of sCJD is currently only obtained by histopathologic diagnosis via brain biopsy or autopsy (Paterson et al. 2012).

4.1.1.1 sCJD subtypes MM1 and VV2

sCJD MM1 and VV2 are the most prevalent disease subtypes which occur in patients with a methionine or valine homozygosity at codon 129 of the *PRNP* gene and PrPsc type 1 or 2, respectively. Both subtypes are characterized by rapidly progressive neurological syndromes with marked brain damage and neurodegeneration especially with severe affection of the frontal cortex and cerebellum. However, the subtypes show distinct differences in their clinical course, early symptoms and neuropathological features with a more prominent cortical involvement in MM1 and a more cerebellar involvement in VV2 patients (Fig 2) (Parchi et al. 1999; Parchi et al. 2009; Llorens et al. 2013; Gambetti et al. 2003).

The most common sCJD subtype MM1 (70%), also termed as “classical” CJD type, presents predominately a rapid progressive decline occasionally accompanied by cortical visual symptoms, usually followed by ataxia, myoclonus and pyramidal signs at disease progression. The peak age of onset in sCJD MM1 is 65 years with a range of 42-91 years. The MM1 subtype is featured by a short clinical duration of 4 months ranging between 1-18 months representing, in compare to the other subtypes, the shortest disease duration (Appleby et al. 2009; Snowden, Mann, and Neary 2002; Parchi et al. 1999; Gambetti et al. 2003).

The second most common form after MM1 is the sCJD subtype VV2 (20%), also termed ataxic type. sCJD VV2 presents as predominant early symptoms rapid progressive ataxia, whereas cognitive decline usually follows later during disease progression, followed by myoclonus and extrapyramidal signs (Parchi et al. 1999). In sCJD VV2 patients show a mean age of onset of about 60 years with a range of 41-81 years and a mean survival of 6 months ranging between 3-18 months (Baiardi et al. 2017).

4.1.1.1.1 Neuropathological features of the sCJD subtypes MM1 and VV2

The principal neuropathological features in prion diseases are rapid and massive neuronal loss leading to spongiform degeneration, the presence of PrPsc deposition and intense microglial and astrocytic activation. However, the histopathological findings in sCJD including severity and distribution of neuronal loss, the morphology and distribution of spongiform changes, the presence or absence of amyloid plaques and microglial activation vary among molecular sCJD subtype and brain region (Gambetti et al. 2003; Parchi et al. 1999; Parchi et al. 2009; Puoti et al. 2012; Franceschini et al. 2018).

In the brain of sCJD MM1 patients, PrPsc is found as synaptic-like depositions in the cerebellar cortex, cerebellum and as perivascular PrPsc depositions in brain regions containing spongiform change (Fig 2). Whereas in sCJD VV2, PrPsc deposition occur as synaptic-like pattern along with occasional small plaques and perineuronal PrPsc deposits in the cerebral cortex (Fig 2). In contrast to MM1, the cerebellum of sCJD VV2 shows additionally to synaptic-like depositions, widespread plaque-like PrPsc depositions involving the molecular and granular layers, as well as in the white matter (Fig 2) (Kovács, Kalev, and Budka 2004; Budka 2003; Gambetti et al. 2003; Llorens et al. 2013).

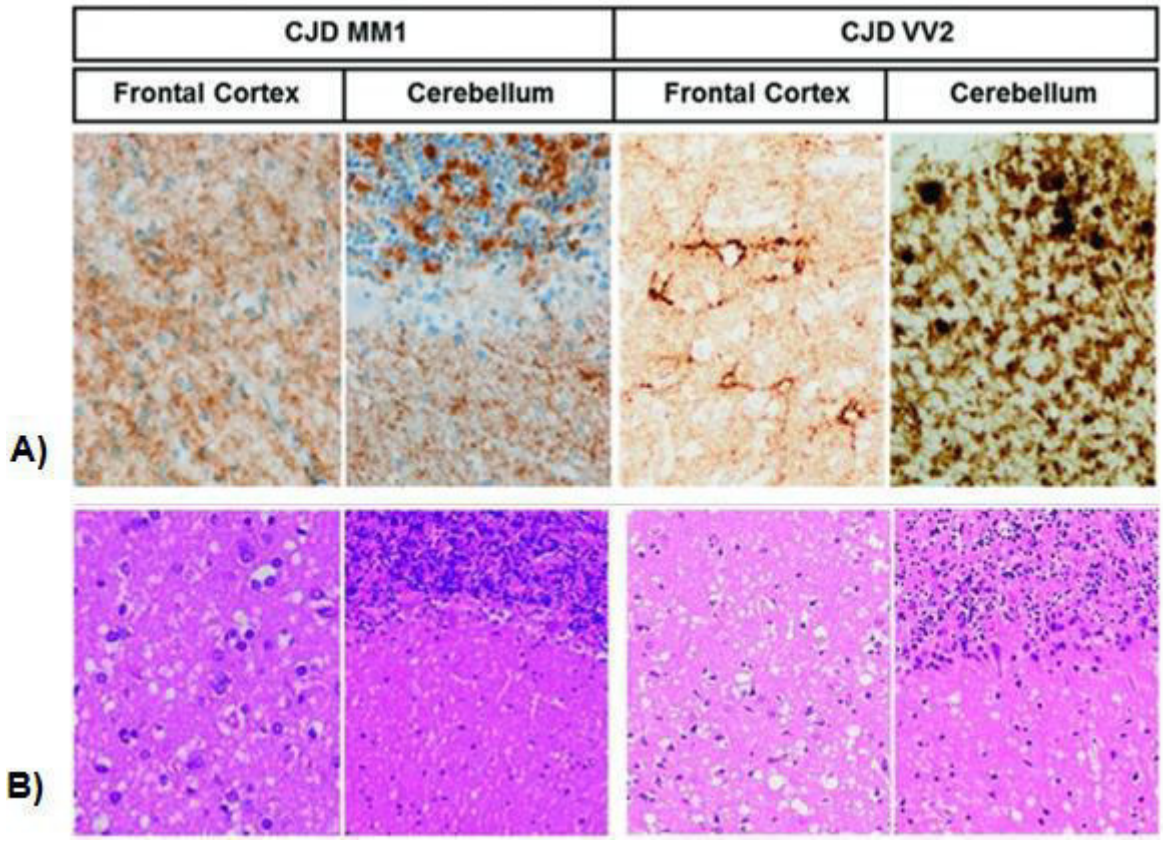


Figure 2: **Regional and subtype dependent histopathological changes in the brain of sCJD:** A) Immunohistochemical analyses of PrPsc revealed differential PrPsc depositions in frontal cortex and cerebellum in sCJD MM1 and sCJD VV2 cases. In sCJD MM1, PrPsc is found as synaptic-like depositions in the frontal cortex and molecular layer of the cerebellum. In sCJD VV2, PrPsc occurred additionally to synaptic-like depositions also as plaque-like depositions, severely in the cerebellum. B) Differential morphology and distribution of spongiform degeneration in sCJD MM1 and sCJD VV2 observed via haematoxylin and eosin staining.
Modified after Llorens et al., 2013. *Prion.*

sCJD MM1 exhibits a widespread, microvascular spongiform degeneration accompanied by extensive neuronal loss in the cerebral cortex (Fig 2, table 1). The basal ganglia, thalamus, hippocampus and cerebellum are involved less severely with preserved Purkinje and granule cell layers, whereas the brain stem and hippocampus appear relatively spared. The cerebral cortex in sCJD VV2 appears relatively spared and microvacuolar spongiform change occurs in a laminar distribution that usually affects deep layers, particularly more severe in the frontal and temporal cortex. The spongiform degeneration is often more severe in the basal ganglia, thalamus and hippocampus. The cerebellum of sCJD VV2 is often affected severely with spongiform degeneration in the molecular and granular layer with marked loss of Purkinje cells (Fig 2,table 1) (Yang et al. 1999; Llorens et al. 2013; Gambetti et al. 2003). Specific neuronal populations appear to be vulnerable in prion disease and *gamma*-aminobutyric acid-ergic (GABAergic) neurons seem to be the main target of neuronal loss in sCJD and prion disease

models (Guentchev et al. 1997; Kovács, Kalev, and Budka 2004; Sikorska and Liberski 2012).

	sCJD MM1		sCJD VV2	
	Frontal cortex	**Cerebellum**	**Frontal cortex**	**Cerebellum**
		Neuropathological hallmarks		
Spongiform degeneration	1–2 small vacuoles	1–2 small vacuoles	2 small vacuoles	2–3 medium vacuoles
Purkinje loss	-	-1	-	1–2
Granule cell loss	-	0–1	-	2
Neuronal loss (frontal cortex)	1–2	-	1–2	-
Astrogliosis	2–3	1–2	2	2
Microgliosis	2–3	2	2	2–3

Table 1: **Overview of regional and subtype dependent neuropathology in sCJD:** Parameters were scored as 0 = absent, 1 = mild, 2 = moderate, and 3 = severe. Modified after Llorens et al., 2013. *Prion.*

The sCJD brain is characterized by subtype- and regional-specific neuroinflammation (Franc Llorens et al. 2014; Parchi et al. 1999) (Fig 3). In this context, the extent of microglia activation and its regional distribution have been shown to relate significantly to sCJD subtypes (Llorens et al. 2014; Franceschini et al. 2018; Puoti et al. 2005). sCJD MM1 and VV2 exhibit distinct regional profiles of activated microglia, which is resembled by microglial morphology changes and expression of activation markers, such as the ionized calcium binding adaptor molecule 1 (IBA1) (Fig 3A) (Llorens et al. 2014; Franceschini et al. 2018). In MM1 brains, activated microglia is mainly dominating in the cerebral cortices, the thalamus and cerebellum, whereas in VV2 brains, activated microglia are found prominently in the subcortical areas (Franceschini et al. 2018). Similar, a widespread activation of astrocytes can be observed in the sCJD brain showing subtype-specific and region-specific changes, which is resembled by a reactive protoplasmic and fibrillary phenotype, as well as the expression of astrocytic activation markers, such as the Chitinase 3-like protein 1 (YKL-40) or the glial fibrillary acidic protein (GFAP) (Fig 3B) (Llorens et al. 2014; 2017; Parchi et al. 1999).

As activated inflammatory-mediating cell signaling pathways in sCJD have been the “janus kinase/signal transducer and activator of transcription” (JAK-STAT) and the “NF-κB kinase/nuclear factor kappa-light-chain-enhancer of activated B cells” (IKK-NF-κB) identified (Llorens et al. 2014).

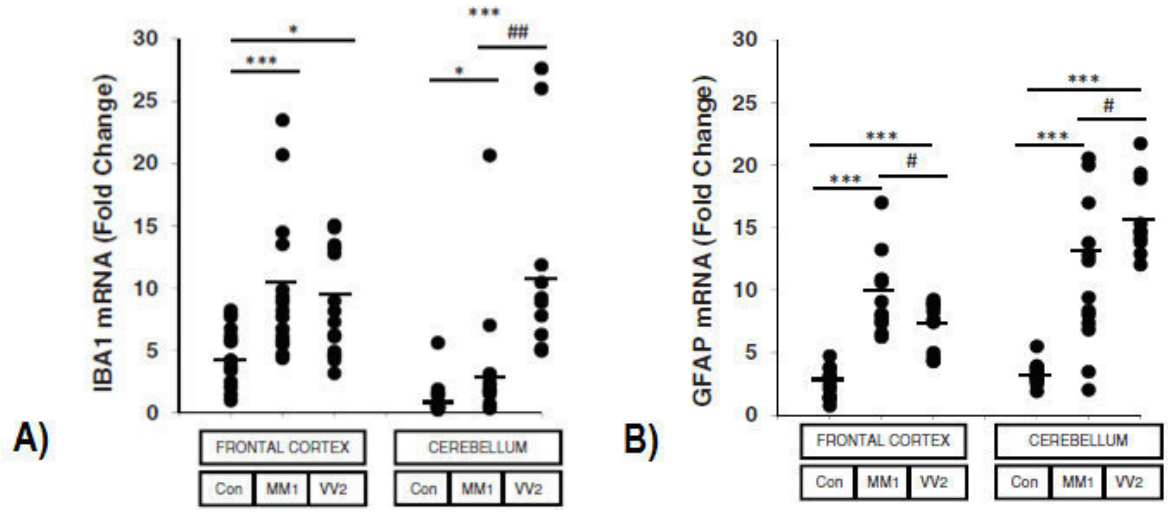


Figure 3: **Subtype dependent molecular neuropathology of glial changes in sCJD:** Gene expression analyses of glial activation marker in the frontal cortex and cerebellum of age-matched control brains, and sCJD subtype MM1 and VV2 was performed via qPCR-analysis. The gene expression of A) IBA1 and B) GFAP resemble microglial activation and astrocytic gliosis in sCJD, respectively.
Modified after Llorens et al., 2014. *Front Aging Neuroscience*

Whether aggregates of PrPsc in the brain reflect neurotoxicity in prion disease is not clear. Observations on the relationship between PrPsc deposition and neuronal damage in sCJD revealed no strong associations between PrPsc load and spongiform degeneration (Llorens et al. 2013; Piccardo et al. 2007) with exception of the cerebellum in sCJD VV2 (Llorens et al. 2013; Faucheux et al. 2009). Moreover, it has been suggested that the synaptic-like deposition of PrPsc is associated to neuronal loss and activation as well as proliferation of astrocytes and microglia, whereas large plaque-like PrPsc deposition might represent a neuroprotective mechanisms through the sequestering of neurotoxic PrP species (Faucheux et al. 2009). In this context, soluble PrP oligomers have been proposed as highly neurotoxic and infectious PrP species in prion disease (Simoneau et al. 2007; Huang et al. 2013).

The regional- and subtype-specific neuropathological patterns in sCJD have been linked with progressive cellular and molecular alterations with disease progression, which include neuronal dysfunction and death (Gabor G. Kovacs and Budka 2010), changes in gene expression networks and RNA editing profiles (Erini Kanata et al. 2018), progressive neuroinflammatory and oxidative stress responses (Llorens et al. 2014; 2017; Tahir et al. 2016), cellular alterations in the Ca^{2+} homeostasis and activation of Calpain-Cathepsin axis with disease progression (Llorens, Thüne, Sikorska et al. 2017), changes in PrP metabolism (Llorens et al. 2013).

4.1.1.1.2 sCJD biomarkers

The clinical diagnosis of sCJD is based on the symptomatic presentation, characteristic abnormalities on EEG and/or magnetic resonance imaging, and CSF testing for the presence of protein prion biomarkers (I. Zerr et al. 2009).
CSF biomarkers have been demonstrated to reflect the neuropathological processes in the sCJD brain in a subtype specific manner, potentially enabling the identification of sCJD subtypes in differential diagnostic context (Llorens et al. 2015; Lattanzio et al. 2017; Castellani et al. 2004; Gmitterová et al. 2016; Hamlin et al. 2012; Sanchez-Juan et al. 2006). In particular, brain-derived CSF proteins, which serve as biomarkers for neuronal damage, display sCJD subtype dependent differences (Zerr et al. 1998). In sCJD MM1 and VV2, 14-3-3 has been found to be elevated in the CSF, whereas 14-3-3 levels were higher in the CSF of VV2 patients in compare MM1 cases (Llorens et al. 2015). Similarly, total (t)-Tau and phosphorylated (p)-Tau are found to be elevated in the CSF of sCJD, whereas homozygous patients (MM/VV) show higher CSF Tau levels than heterozygous cases (MV) (Fig 4) (Llorens et al. 2015; Abu-Rumeileh et al. 2018). Moreover, VV2 shows higher CSF Tau and p-Tau levels than MM1 (Lattanzio et al. 2017). Equally, the concentration of CSF neurofilament light (NfL), a general marker for white matter damage, has been demonstrated to display higher levels in VV2 in compare to MM1 followed by heterozygous cases, which has been proposed to reflect subtype dependent degree of white matter damage (Fig 4) (Franc Llorens et al. 2018; Abu-Rumeileh et al. 2018).

CJD	MM(V)1	VV2	MV2
Disease duration (months)	4	6.5	16
Cortical pathology	++	+	++(+)
Subcortical pathology	+(+)	++(+)	++(+)
CSF NfL	↑	↑↑(↑)	↑↑
CSF t-tau	↑↑(↑)	↑↑↑	↑

Figure 4: **sCJD subtype dependent clinical progression and pathology is associated to heterogeneity in CSF biomarker levels.** CSF NfL and total (t)-tau protein levels reflect rate of clinical progression, degree of neuropathological affection and subcortical involvement.
Modified after Abu-Rumeileh et al., 2018. *Alzheimer's Research and Therapy.*

4.1.2 Genetic human prion disease: Fatal familial Insomnia

More than 20 disease-causing mutations in the *PRNP* gene have been described (Castilla, Hetz, and Soto 2004; Kovács et al. 2002). Along with the E200K mutation in the *PRNP* gene, which develops in a clinico-neuropathological manner as sCJD, the most prevalent genetic prion disease is FFI.

FFI is caused by a single point mutation, D178N, in the *PRNP* gene accompanied by the presence of methionine at codon 129 on the mutated allele of the same gene (Goldfarb et al. 1992). Clinically, FFI manifest predominantly as progressive insomnia, autonomic and neuroendocrine dysfunction, usually followed by apathy, cognitive and motor problems with disease progression. The mean age at onset is 51 years of age, and clinical duration is typically 8–72 months (Krasnianski et al. 2008; Cortelli et al. 1999; Gallassi et al. 1996).

Neuropathological alterations include predominantly marked astrocytic gliosis and neuronal loss of the mediodorsal and anterior thalamic nuclei, whereas temporal cortex, hippocampal CA1 region, cingulate cortex and other cortical areas are also affected often. Although severely damaged, the thalamus shows no PrPsc deposits or spongiosis. Moreover, limited microglia activation is evident in the FFI brain (Llorens, Thüne, et al. 2016; Llorens, Zarranz, Fischer, et al. 2017; Cracco, Appleby, and Gambetti 2018).

A diagnosis of FFI requires the presence of a probable sCJD diagnosis in combination with the disease-specific gene mutation (Llorens, Zarranz, Fischer, et al. 2017). CSF biomarkers used for the clinical diagnostic of sCJD, provide less clinical value for FFI (Ladogana et al. 2009). Marker for neuronal damage, such as 14-3-3 and α-synuclein, show less or no sensitivity, whereby CSF tau is significant higher in FFI, but lower when compared to sCJD (Frau-Méndez et al. 2017; Llorens, Schmitz, et al. 2016).

Structural analysis of the mutated PrP D178N revealed that no major alterations affect the overall protein structure, however, slight alterations occur in the intermolecular contacts between PrP molecules that might be responsible for structural instability and increased aggregation propensity (S. Lee et al. 2010; Swietnicki et al. 1998). The reason for the spontaneous misfolding of PrP D178N and the mechanisms underlying the selective vulnerability of specific brain regions during FFI progression are unknown. The impairment of fundamental cellular processes including translational and mitochondrial dysfunction and increased oxidative stress has been implicated in the pathophysiology of FFI (Llorens, Thüne, et al. 2016; Llorens, Zarranz, Fischer, et al. 2017; Frau-Méndez et al. 2017).

4.2 Tauopathies

Tauopathies are a heterogeneous group of incurable neurodegenerative conditions that are pathologically characterized by intracellular accumulation of abnormal tau in the brain. Clinically, tauopathies manifest highly diverse and can contain both cognitive/behavioral- and movement-disorders including, but not limited to AD, primary age-related tauopathy (PART), progressive supranuclear palsy, corticobasal degeneration, Pick's disease, some frontotemporal dementias, chronic traumatic encephalopathy and parkinsonism linked to chromosome 17 (Irwin 2016; Orr, Sullivan, and Frost 2017; Gabor G. Kovacs 2017; Williams 2006; Ferrer et al. 2014).

The most well described role of tau is that as a microtubule-associated protein involved in microtubule assembly and stabilization, particularly of neuronal axons (Holtzman et al. 2016). In disease states, pathological tau forms are featured by aberrant phosphorylation, truncation and aggregation into neurotoxic oligomers and insoluble filaments (Arendt, Stieler, and Holzer 2016; Mair et al. 2016; Y. Wang and Mandelkow 2016; Orr, Sullivan, and Frost 2017). The cellular mechanisms and drivers involved in pathogenic tau-induced neuronal dysfunction and death are still illusive. The development of therapeutic approaches faces significant challenges due to the inability to diagnose early and to discriminate clearly between distinct tauopathy entities. It is supposed that pathophysiological changes in tauopathies occur decades before earliest observed cognitive and behavioral impairments (Arendt, Stieler, and Holzer 2016; Orr, Sullivan, and Frost 2017).

4.2.1 Alzheimer's disease

AD is a progressive, neurodegenerative disorder and the most common form of senile dementia affecting more than 20% of the population over 80 years of age (Prince et al. 2015). Clinically, AD manifests as gradual cognitive decline and impaired memory. The neuropathological hallmarks in AD are extracellular deposition of amyloid beta peptides (Aβ) within senile plaques and the intracellular generation of NFTs. These neuropathological changes are accompanied by a multifaceted disease pathology including accelerated synaptic and neuronal loss, vascular lesions (angiopathy), atrophy and neuroinflammation in specific AD associated brain region (De Strooper and Karran 2016). The majority of AD cases occurs sporadically in an age-dependent manner with disease onset of usually older than 65 years and mean disease duration of 8 years (Mucke 2009; Zetterberg and Mattsson 2014). However, due to a wide pathological and clinical heterogeneity, AD is featured by the occurrence of subphenotypes (Lam et al. 2013).

Rapidly progressive AD (rpAD), a variant of the disease, is characterized by rapid decline in cognition and short disease duration of less than 2 years (Sona, Ellis, and Ames 2013; M. E. Soto et al. 2008; Schmidt et al. 2011; 2012). rpAD can mimic the clinical signs of other diseases with rapidly progressive neurological syndromes, such as sCJD. Furthermore, up to now, classical CSF biomarker used for AD diagnosis reflecting pathophysiological processes in the brain, associated to Aβ plaques and NTFs pathology, are not able to discriminate between AD and rpAD (Tapiola et al. 1997; 2009; Llorens, Schmitz, Knipper, et al. 2017).

The mechanisms which contribute to AD heterogeneity in clinical and pathological signs are still illusive. Moreover, factors underlying the selective vulnerability of specific brain regions during disease progression are unclear. Elucidating the molecular drivers that initiate AD at its earliest stages is essential for the understanding of triggering factors leading to the development and progression of this multifactorial disease.

The progressive build up and deposition of the Aβ peptide in the brain parenchyma, with plaques first appearing in cortical regions, is considered as a key event in the AD pathology (Hardy and Higgins 1992; Karran, Mercken, and Strooper 2011). According to the Aβ cascade hypothesis an imbalance between Aβ production and clearance is an early event in AD initiating a neurotoxic cascade that ultimately leads to the development of pathological hallmarks of AD, including neurodegeneration and NFT formation, as downstream effects (Selkoe and Hardy 2016). Aβ peptides are derived from the amyloid precursor protein (APP) which is a transmembrane protein that is predominantly located at the synapses and constitutively cleaved into peptides during cell metabolism. Through an amyloidogenic processing of APP aggregation prone Aβ peptides are generated via enzymatic cleavage (γ-secretases and β-secretases). The presence of excessive Aβ amounts promoting its aggregation into extracellular oligomers, fibrils, and eventually into senile and neuritic plaques. Especially soluble oligomers of Aβ have been suggested as early neurotoxic species triggering neurodegeneration in AD (Ferreira et al. 2015; Viola and Klein 2015). Less than 5% of AD cases are associated with autosomal-dominant mutations in genes involved in the Aβ metabolism causing early-onset familial AD (fAD), which includes mutations occurring either in the APP, presenting the substrate of Aβ, or in the protease presenilin (PSEN1 and PSEN2) that generates Aβ (L. Wu et al. 2012).

However, the causative role of Aβ aggregation and its importance in the pathogenesis of AD is not clear and still under debated (Reitz 2012; H. G. Lee et al. 2004; Zetterberg and Mattsson 2014). No strong associations between the amount of Aβ neuritic plaque pathology and the degree of atrophy as well as clinical dementia have been observed in AD (Josephs et al. 2008; Serrano-Pozo et al. 2011). Frequently, Aβ deposits are present in the brain of cognitively healthy

older people (Rodrigue et al. 2012). AD-associated neurodegeneration has been observed to occur independently of Aβ pathology (Fjell and Walhovd 2012).

4.2.1.1 Neuropathological alterations in early AD

One critical pathological event in early AD is the aggregation of the microtubule protein tau into neurofibrillary tangles (NFT) in neurons (Iqbal et al. 2005; Serrano-Pozo et al. 2011). Tau is the main microtubule associated protein in mature neurons that regulates and stabiles microtubule assembly essential for the maintenance of neuronal structure, axonal transport, and neuronal plasticity. Various regulatory mechanisms, such as phosphatases and kinases, are responsible for the associating and dissociating of tau from microtubules (T. Guo, Noble, and Hanger 2017). In AD, the abnormal hyperphosphorylation of tau results in its dysfunction and mislocalization followed by tau polymerization and its aggregation into NFTs in the cell body of neurons, which is associated to neuronal dysfunction and death (Iqbal, Liu, and Gong 2016). In fact, it has been shown that the density of hyperphosphorylated tau correlates closely with the presence of neurodegeneration, brain atrophy and cognitive deficit in AD (Bejanin et al. 2017).

The NFT pathology preferentially affects specific brain regions and spreads along distinct neural pathways in a hierarchical manner, whereas other brain regions remain relatively spared. Based on the stepwise progression of neuropathological alterations in the AD brain, the NFT pathology is categorized in six stages (Fig 5) (H. Braak and Braak 1991; Murayama and Saito 2004). During AD progression, NFT pathology first appears in parts of the brainstem, especially the LC, as well as entorhinal regions (stages I-II), followed by hippocampal input regions and projection zones including region CA1 and subiculum as well as the outer molecular layer of dentate gyrus and advancing to temporal regions (stages III-IV) and finally followed by large parts of the neocortex (stages V-VI) (H. Braak and Braak 1991; Goedert 2015) (Fig 5). The clinical status of AD has been linked to Braak stages: during preclinical and early clinical stages of AD, the brain pathology has been advanced to Braak stages II and III, while Braak stages V and VI are associated to a fully developed AD dementia. During AD, the development of tau pathology often occurs before Aβ accumulation or may also occur in the absence of Aβ pathology (Heiko Braak et al. 2011; H. Braak and Braak 1997; Heiko Braak and Del Tredici 2015). The development of Aβ accumulation is considerable different from NFT pathology spreading, with plaques initially appearing in cortical regions (Thal et al. 2002).

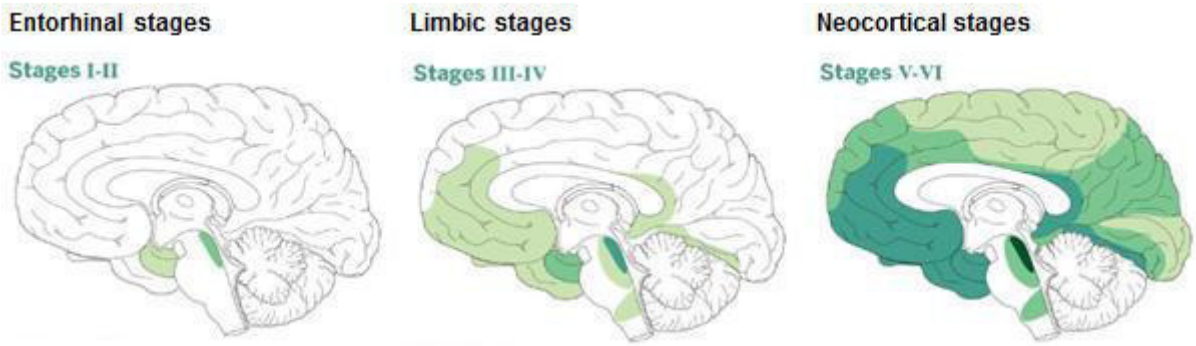


Figure 5: **Staging of NFT pathology in AD:** Tau pathology initially develops in the locus coeruleus, as well as transentorhinal and entorhinal regions (stages I-II), followed by the hippocampal formation and partially also the neocortex (stages III-IV), and finally followed by large parts of the neocortex (stages V-VI).
Modified after Goedert, 2015. *Science.*

The LC is a brain stem nucleus which serves as a main source of noradrenergic innervation projecting widespread throughout the whole forebrain, including the EC, hippocampus, amygdala, and cerebral cortex, which are AD pathology associated regions (Šimić et al. 2017; Nagai et al. 1981). Through its wide projections, the LC modulates a variety of functions such as sleep-waking cycles, cognition, learning and memory, neuroinflammation, and neuronal survival (Kayama and Koyama 2003; M. T. Heneka et al. 2010; Szabadi 2013; Counts and Mufson 2012; Sara 2009; Weinshenker 2008).

The LC is one of the earliest regions affected by consistent and severe neuronal dysfunction and loss during early AD stages, which has been linked to noradrenalin depletion of the projection areas in the forebrain region (Adolfsson et al. 1979; Mravec, Lejavova, and Cubinkova 2014). During large-scale screens in non-selected brains, the formation of aberrant tau species within the LC has been observed in the absence of Tau-related pathology in the trans-entorhinal region, suggesting the LC as an initial site of tau pathology (Heiko Braak et al. 2011; Giorgi et al. 2017). It has been demonstrated that the degeneration of LC neurons correlates with AD pathology including the presence of Aβ plaques and NFT pathology as well as cognitive impairment (Andrés-Benito et al. 2017; Giorgi et al. 2017; Weinshenker 2008). Therefore, the LC degeneration is proposed to play a significant role in the AD pathogenesis influencing multiple facets of disease progression, including aberrant neuronal metabolism, synaptic dysfunction and progressive neuroinflammation. (M. T. Heneka et al. 2010; Weinshenker 2008).

Strong evidences suggest that aberrant tau species accumulate in LC neurons for decades before LC degeneration occurs in AD which might present a potential window for early diagnostic and therapeutic intervention (Chalermpalanupap, Weinshenker, and Rorabaugh 2017). However, if the buildup of aberrant tau itself mediates neurotoxicity is not yet clear and the pathological mechanisms ultimately leading to LC dysfunction and degeneration are still elusive. Identifying intrinsic

factors and cell-specific, molecular properties of LC neurons during early AD pathogenesis are crucial for the understanding of early disease mechanisms. One of the main objectives to understand tau pathology associated to AD is to identify molecular drivers that could favor the aberrant tau phosphorylation.

4.2.2 Primary age-related tauopathy

PART is common pathologic entity almost universally observed in the brain of aged individuals. Neuropathologically, PART is featured by AD-type neurofibrillary changes corresponding to Braak stages I-IV, with minimal or without Aβ depositions (Reas 2017; Crary et al. 2014). Symptoms in individuals with PART range from normal to mild cognitive changes, whereas profound impairment occurs rarely (Jefferson-George et al. 2017; Besser et al. 2017).

Upon neuropathological assessment, brains with PART often appear indistinguishable from those with early AD pathology during the absence of Aβ plaques (Reas 2017; Crary et al. 2014). Due to these common pathological features, PART has been suggested to be a subset or an early stage of AD (Heiko Braak and Del Tredici 2011). On the hand, there might be distinct clinical and pathological features that differ between PART and AD. In comparison to AD, PART shows often limited impact on cognition and progress without development of Aβ deposits (Santa-Maria et al. 2012), whereas neuropathological AD diagnosis requires the significant presence of Aβ depositions (Murayama and Saito 2004; Hyman et al. 2016). At present time, PART or/and early AD evolution cannot be reliable identified and differentiate *premortem,* and thus, clear systematic characterizations for the clinical-pathological spectrum of these pathological entities are needed. Improved biomarkers and imagine techniques might help to detect and to prognosticate early NFT-associated pathological changes (Reas 2017; Crary et al. 2014).

4.3 Neurodegeneration disease models: Mice model for studying prion diseases of human

Reliable model systems are crucial to examine prion induced neurodegenerative mechanisms and to gain clues for relevant therapy and biomarker approaches (Watts and Prusiner 2014; Brandner and Jaunmuktane 2017; Delay and Hébert 2011). By taking advantage of the infectious nature of prions, the intracerebral inoculation of mice with prions represents an essential prion bioassay to induce the formation and propagation of prions in the host (Brandner and Jaunmuktane 2017; Watts and Prusiner 2014). Prion isolates, as infectious agent, are known to induce highly diverse clinical and pathological outcomes which are supposed to be determined and encoded in the biochemical and conformational features of the infectious particle PrPsc. This effect is also termed as prion strain phenomenon, whereby distinct “prion strains” are hypothesized to propagate their biochemical properties in the host by the induction of strain-specific conformational changes in the endogenous PrPc resulting in distinct disease phenotypes (Schoch et al. 2005; Morales 2017; Moira E. Bruce 2003; M. E. Bruce 1993). Biochemical characteristic of PrPsc used to identify distinct strains include protease resistance, glycosylation profile and electrophoretic mobility. *In vivo*, prion bioassays in mice enable the analysis of prion strain specific induction of distinct disease phenotypes including incubation time, clinical signs and types and patterns of brain lesions (Morales 2017; Morales, Abid, and Soto 2007; Watts and Prusiner 2014).

In the last decades the main animal model for prion biology is a mouse model infected with scrapie, a prion disease mostly affecting sheep and goats (Watts and Prusiner 2014; M. E. Bruce 1993). Classical scrapie strains often used in murine bioassays, such as RML, 22A, 139A, Me7, S15, are relevant tools to study differences among prion strains *in vivo* (Di Bari, Nonno, and Agrimi 2012). However, mice model for scrapie represent a suboptimal model for prion diseases affecting humans (Watts and Prusiner 2014).

In order to use a disease model highly relevant to human prion pathology, the investigations in the present thesis are based on a humanized mice model inoculated with sCJD prions (Padilla et al. 2011; Llorens et al. 2014). By expressing human PrP on the background of murine *Prnp*$^{0/0}$, this transgenic mice model is susceptible to prions relevant to humans, particularly those causing sCJD, and thereby enabling an efficient transmission and propagation of human PrPsc in the host without the interference by the presence of the endogenous murine PrP homolog (Watts and Prusiner 2014; Padilla et al. 2011; Telling 2011). Importantly, the transgenic human *PRNP* gene carries the polygenic genotype (M or V) at codon 129 which is associated to the susceptibly to distinct human prion diseases. The inoculation of humanized mice model with sCJD brain isolate leads to a faithful recapitulation of the distinct sCJD subtype specific neuropathological patterns and biochemical changes observed in human brain (Padilla et al. 2011;

Bishop, Will, and Manson 2010; Giles et al. 2010; Asante et al. 2002). In fact, through their reliable resembling of prion disease hallmarks in human brain, these mice models are superior to those of major NDs, such as AD (Watts and Prusiner 2014; Brandner and Jaunmuktane 2017). To date, AD mouse models reflect a range of AD-related pathologies. However, none of those models resembles fully the complexity of the human disease (Elder, Gama Sosa, and De Gasperi 2010). Tau-based disease models associated to mutations in *tau* are not leading to the development of AD, but mimics features of human tauopathies. Transgenic mouse models carrying human *tau* with a P301L mutation, which is causing familial frontotemporal dementia, develop motor and behavior deficits and NFT pathology in an age-dependent manner (Lewis et al. 2000). On the other hand, amyloid-based animal models combine genetic mutations associated to an impaired Aβ metabolism leading to early-onset fAD and develop cerebral amyloid plaque depositions, neuronal loss and cognitive decline, but do not show tangle pathology seen in the human AD brain (Oakley et al. 2006).

The CJD mouse model Tg340-PRNP(129MM), on which investigations are focused in the present thesis, is expressing 4-fold human PrP with a methionine homozygosity at the codon 129 in the *PRNP* gene on a $Prnp^{0/0}$ mouse background (Padilla et al. 2011). Transgenic mice that overexpress PrP display an accelerated disease process with the development of clinical signs of prion disease upon a comparatively short and stable incubation time. Inoculated with human brain isolates of sCJD subtype MM1, the mice model resembles the human pathology in a brain regional and disease subtype specific manner. In particular, the mice model recapitulates the patterns of neuropathological affections observed in the brain of sCJD MM1 patients including neuronal loss and spongiform change, progressive neuroinflammation as well as accumulation of pathogenic PrPsc in the brain with disease progression (Padilla et al. 2011; Llorens, Thüne, Sikorska, et al. 2017; 2014). Moreover, alterations in molecular mechanisms and cellular pathways have been described to be resembled in the sCJD MM1 mouse model involving changes in gene expression networks and RNA editing profiles (Erini Kanata et al. 2018), as well as calcium dyshomeostasis and activation of Calpain-Cathepsin axis with disease progression (Llorens, Thüne, Sikorska, et al. 2017).

4.4 Function and biogenesis of miRNAs in the brain

miRNAs are a class of small regulatory RNA molecules, which constitute fundamental regulators of gene expression through the binding of mRNA targets, which is leading to translation repression and/or mRNA degradation dependent on sequence complementarity (Jonas and Izaurralde 2015b). Through their flexible, rapid and reversible functions, miRNAs enable a precise, temporal and spatial gene expression regulation, which is essential for correct function and integrity of the brain (Davis, Haas, and Pocock 2015). Moreover, the human brain contains on average the longest mRNAs in compare to other human tissues, suggesting a high density of regulatory motifs, including miRNA seed sequences, which underlie complex temporal and spatial translational programs of the neural cells (Miura et al. 2013; L. Wang and Yi 2014).

miRNAs are ubiquitously and highly cell-type specifically expressed in the CNS and thereby involved in a broad range of biological processes including brain development, function as well as in the majority of cellular activities such as metabolism, proliferation, differentiation, cell fate, survival, synapse formation and plasticity (O'Carroll and Schaefer 2013a; Jovičić et al. 2013). Mature miRNAs are localized in the cytoplasm and also in multiple subcellular compartments associated to various physiological functions such as local miRNA-mediated control of gene expression, miRNA storage or degradation (Fig 6) (Anthony K.L. Leung 2015). For instance, processing bodies and stress granules are dynamic cytoplasmic sites that contain distinct subsets of proteins and other regulatory factors involved in translational regulation including miRNAs. Thereby, processing bodies are linked to mRNA turnover and storage, whereas stress granules are described to inhibit translation initiation under various stress conditions (Chantarachot and Bailey-Serres 2017). Especially in neuronal cells, miRNAs have been proposed to be involved in local, autonomously regulated protein translation occurring in dendrites, axons and at active synapses (Ye et al. 2016; Schratt 2009; O'Carroll and Schaefer 2013a; Hu and Li 2017; Sambandan et al. 2017).

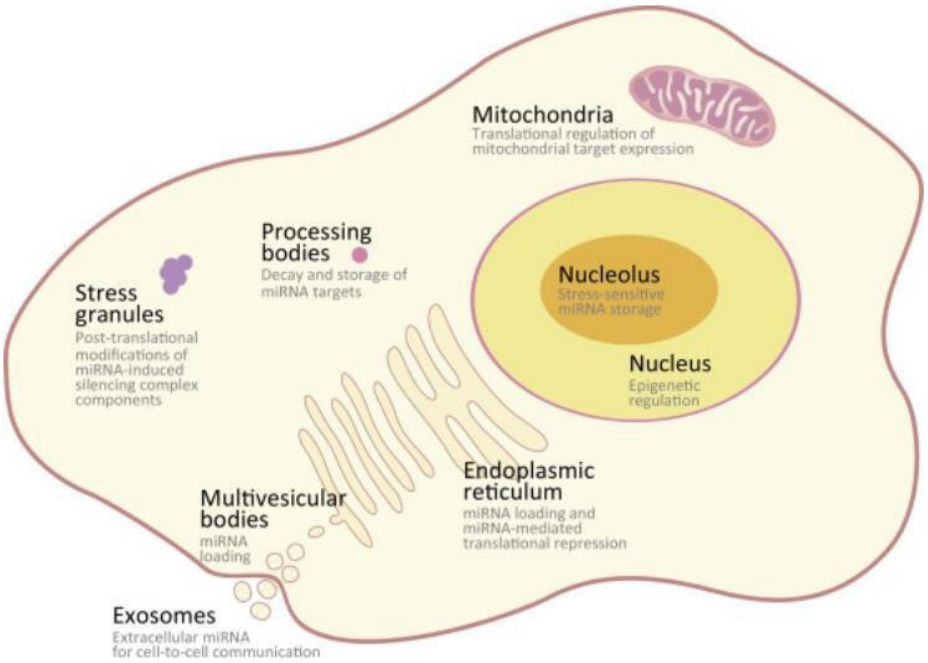


Figure 6: **Schematic overview of subcellular location-specific miRNA functions.** Subcellular miRNA localization affects miRNA function.
Leung, 2015. *Trends in Cell Biology.*

miRNAs have the ability to regulate several genes involved in one pathway or multiple cross-talking pathways through complex miRNA-mRNA interactions, in which each miRNA can regulate the translation of hundreds of distinct mRNA targets and each mRNA transcript can possess multiple miRNA targeting sites (Fig7) (Davis, Haas, and Pocock 2015; Jonas and Izaurralde 2015b).
miRNA networks modulate gene expression by different mechanisms that are associated to specific biological outcomes of miRNA-mRNA interactions. One way of miRNA-mediated gene expression modulation is through ‘expression tuning’, by which miRNAs tune or reset the mean of the expression level of their target genes often in a rapid and flexible way associated to genetic switches (Tsang, Zhu, and van Oudenaarden 2007; Wu, Shen, and Tang 2009). For instance, specific miRNAs play a role in neuroinflammatory regulation, whereby pro-inflammatory (miR-155, miR-27b, miR-326) and anti-inflammatory (miR-124, miR-146a, miR-21, miR-223) miRNAs inhibit the translation of corresponding proteins and thereby frame neuroinflammatory responses in a coordinated and dynamic manner (Gaudet et al. 2017). Uncontrolled neuroinflammation is associated to pathological conditions especially during neurodegeneration (Michael T. Heneka et al. 2015; Dorothée 2018).

Another way how miRNA networks mediate gene expression regulation is through ‘gene expression buffering’, by which miRNAs reduce target gene variances by establishing thresholds, which helps to maintain a dynamic homeostasis and stabilization for the transcriptome against input noises (Tsang, Zhu, and van Oudenaarden 2007; C. I. Wu, Shen, and Tang 2009). For instance, the constant

expression of specific miRNAs contributes to the maintenance of the cellular identity of various cell types. In this regard, specific neuronal-enriched miRNAs (miR-124, miR-9) have been demonstrated to maintain the neuronal phenotype by suppressing non-neuronal genes, which is necessary for neuronal function and survival (Jovičić et al. 2013; Neo et al. 2014; Yoo et al. 2011).

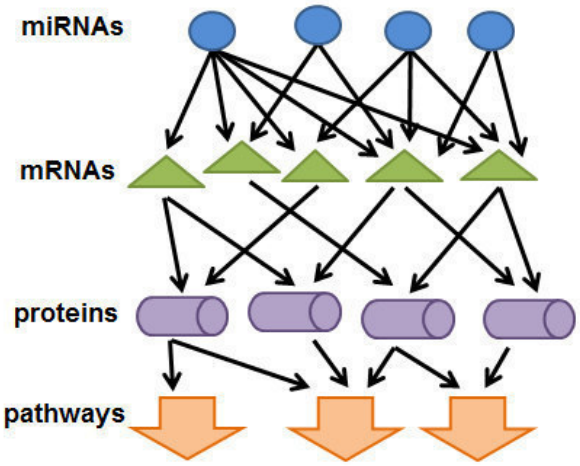


Figure 7: **Model of miRNA-mediated gene regulatory networks:** miRNAs modulate gene expression through complex miRNA-mRNA network interactions, leading to mRNA cleavage and/or translational inhibition, and thereby miRNA networks exert regulatory effects on protein expression and biological pathways.

The miRNA biogenesis pathway consists of various processing steps that take place inside the nucleus and the cytoplasm involving multiple miRNA maturating proteins, which represents a plethora of regulatory options affecting miRNA processing efficiency and activity (Fig 8) (O'Carroll and Schaefer 2013a; M. Ha and Kim 2014).
miRNA genes are encoded within various genomic contexts. In human, the majority of miRNAs are located in introns of coding or non-coding transcripts, whereas some miRNAs are encoded in exonic regions. Often, miRNA genes are found to be clustered and organized into a polycistronic transcription unit enabling a co-transcription of miRNAs located in the same cluster (Schanen and Li 2011a). Some miRNAs genes appear to be embedded in the introns of protein-coding genes and, thus, share the promotor of the host gene, often presenting complex, regulatory feedback loops between miRNAs and its host genes (Boivin, Deschamps-Francoeur, and Scott 2017). However, various regulatory mechanisms including multiple miRNA gene transcription start sites, alternative promoters or epigenetic mechanisms, such as DNA methylation and histone modifications contribute to miRNA expression regulation (O'Carroll and Schaefer 2013a; M. Ha and Kim 2014).

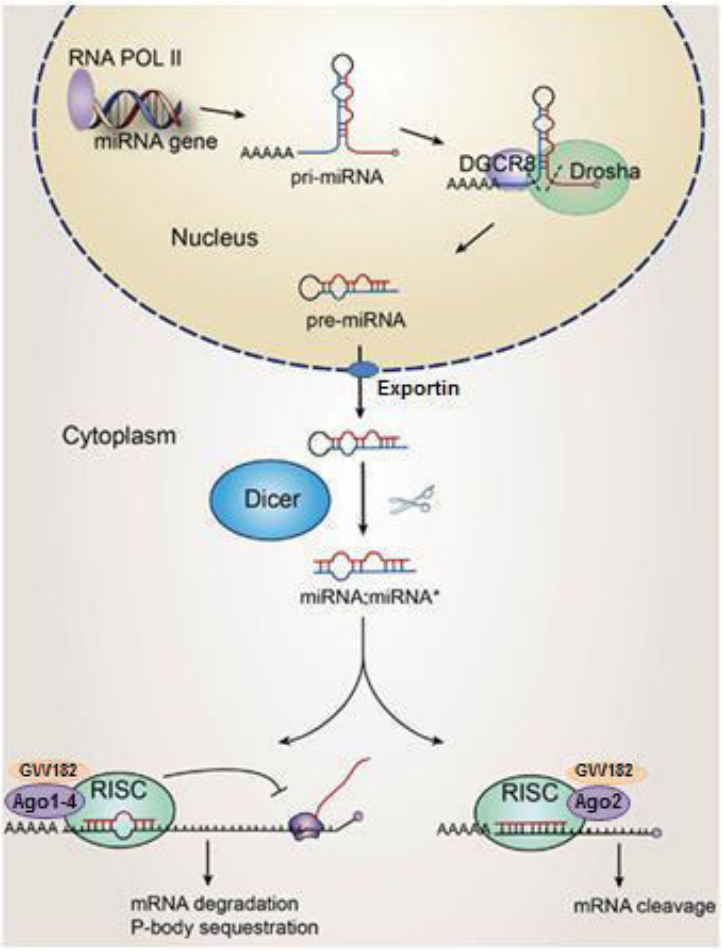


Figure 8: **miRNA biogenesis and gene expression control:** miRNAs are usually transcribed by the RNA polymerase II as long, primary miRNA transcripts following a series of cleavage events. In the nucleus, primary miRNAs are processed by the nuclear microprocessor complex containing the ribonuclease Drosha. The processed precursor miRNAs are exported into cytoplasm by Exportin-5 and further cleaved by the ribonuclease Dicer into mature miRNAs, which interact with Argonaute proteins to form the miRNA-induced silencing complex. This complex mediates translational inhibition or mRNA cleavage according to miRNA-mRNA sequence complementarity. A plethora of regulatory mechanisms affect miRNAs processing efficiency and activity.
Modified after Barca-Mayo and Lu, 2012. *Frontiers in Neuroscience.*

In the nucleus, the majority of miRNAs are transcribed by the RNA polymerase II as long, primary-(pri)miRNAs transcripts forming hairpin structures that undergo 5'-capping, polyadenylation at the 3'-end as well as editing and splicing processing (Schanen and Li 2011b; Barca-Mayo and Richard Lu 2012). The pri-miRNA is next processed by the nuclear microprocessor complex composed of Drosha, an nuclear ribonuclease III (Rnase III) enzyme, and its cofactor DGCR8 (DiGeorge Critical Region 8) protein. By its double-stranded RNA binding domains, DGCR8 mediates the site-specific binding of microprocessor complex, while Drosha cleaves the stem-loop pri-miRNA structure by its endonuclease activity releasing a small hairpin-shaped precursor-(pre)miRNA of ~70-100 nucleotides in length (Conrad et al. 2014). The nuclear processed pre-miRNA presents a defined 3'-

overhang, allowing a specific recognition and export of the pre-miRNA into the cytoplasm by Exportin-5 (XPO5) via a guanosine triphosphate-depended mechanism (Jonas and Izaurralde 2015a; O'Carroll and Schaefer 2013b).

Alternatively, several classes of miRNAs have been identified to be generated using a microprocessor complex independent route through bypassing Drosha-mediated processing (Y.-K. Kim, Kim, and Kim 2016). The sources for alternative precursor miRNAs can be short spliced-out RNA transcripts originated from introns of protein-coding genes (Curtis, Sibley, and Wood 2012), small nucleolar RNAs (Falaleeva and Stamm 2013), or transfer RNAs-derived RNA fragments (Shigematsu, Honda, and Kirino 2014; Keam and Hutvagner 2015).

In the cytoplasm, pre-miRNAs are recognized and cleaved by an Rnase III-type endonuclease termed Dicer producing small, intermediate RNA duplexes with a very precise size of 21-24 nucleotides. Dicer is a large (~200 kDa) multi-domain enzyme interacting with various proteins and cofactors, which can influence the specificity of its action (Song and Rossi 2017). The Dicer-processed small RNA duplexes are later separated, whereby one of those strands is determined by various strand selection mechanisms and loaded onto an Argonaute (Ago1 to 4 isofoms) protein. Ago proteins represent the core components of the miRNA silencing complex (RISC) that form associated with miRNAs the active functional unit of the miRNA-induced silencing complex (Meister 2013a; Jonas and Izaurralde 2015a).

Ago proteins, as RISC effector components, mediate the mRNA degradation, destabilization or translational inhibition according to the base-pairing of the Ago-incorporated miRNA with its complementary site in the 3' untranslated regions (3'UTR) of mRNA-target. Perfectly complementary mRNA targets are cleaved by catalytically active Ago proteins, whereas in human only Ago2 possess catalytically activity (Meister 2013a; Jonas and Izaurralde 2015a). However, in animals the majority of miRNA-mediated gene silencing takes place by partially complementary miRNA binding with the mRNA target, leading to translation repression and/or mRNA destabilization by Ago-mediated recruitment of additional protein partners (Ipsaro and Joshua-Tor 2015). One of those critical Ago-partners are GW182 proteins that function as flexible scaffolds to bridge the interaction between Ago and downstream gene silencing effector complexes, such as cytoplasmatic deadenylase complexes (Braun, Huntzinger, and Izaurralde 2013).

4.4.1 The role of miRNAs in neurodegeneration

In the past decade, increasing links have been established between NDs and dysregulated miRNA expression patterns. Experimental evidences suggest that miRNA dysregulation is implicated in the etiology of several ND, including AD, Parkinson's disease (PD), amyotrophic lateral scleroses (ALS), Huntington's disease and prion diseases (Abe and Bonini 2013b; Hébert and De Strooper 2009b). Even though these diseases represent distinct entities, their pathogenesis shares the impairment of fundamental cellular pathways leading to disturbed protein homeostasis, an overload of protein clearance pathways, the aggregation of pathological altered proteins, and the dysfunction and/or loss of specific neuronal populations. With their ability to regulate complex networks of genes, dysregulated miRNA networks have the potential to be involved in virtually all pathophysiological mechanism in ND (Goodall et al. 2013; Delay, Mandemakers, and Hébert 2012; Abe and Bonini 2013a; Femminella, Ferrara, and Rengo 2015; Tan, Yu, and Tan 2015; Walter J. Lukiw et al. 2013)

4.4.1.1 miRNAs in prion disease

To date, evaluation of miRNA alterations in human prion diseases are restricted to targeted studies in the brain of sCJD (Montag et al. 2012; Lukiw et al. 2011) and GSS patients (Lukiw et al. 2011). By performing targeted miRNA screens, the upregulation of miR-146a-5p (n=5) and miR-342-2p (n=2) has been observed in the brain of prion disease patients (Montag et al. 2012; Lukiw et al. 2011). However, no data about the global miRNA expression patterns in the brain of human prion disease are available so far. Alterations of miRNA expression in prion disease have been investigated in animal and cell culture models. miRNA analysis in animal models focus on murine models intracerebrally inoculated with various scrapie strains (Saba et al. 2008; Montag et al. 2012; Majer et al. 2012; Boese et al. 2016; Gao et al. 2016) and BSE-infected macaques (Montag et al. 2009). In cell culture models, the expression of miRNAs was analyzed in mouse cell lines infected with different prion strains (Gao et al. 2016; Bellingham, Coleman, and Hill 2012; Bellingham and Hill 2017; Montag et al. 2012).These miRNA analyses revealed temporally distinct and dynamic miRNA alterations during preclinical and clinical prion disease stages.

Significant altered miRNA profiles were observed in the brain of prion disease animal models after the onset of clinical symptoms including few common miRNA alterations reported in different disease models including the upregulation of miR-146a-5p (Gao et al. 2016a; Lukiw et al. 2011; Saba et al. 2008), the upregulation miR-26a-5p (Montag et al. 2009), the upregulation of 342-3p (Saba et al. 2008), wich was also found to be upregulated in the brain sCJD patients (Montag et al.

2009). Majer et al. performed temporal miRNA expression analysis in microdissected hippocampal CA1 neurons of prion infected mice revealing dynamic miRNA alteration patterns of miR-124-3p, miR-16-5p, miR-26a-5p, miR-29a-3p, miR-132-3p, miR-140-5p and miR-146a-5p suggesting a potential disease-related miRNA mediated effect during early prion disease response. Interestingly, all analysed miRNAs showed similar, temporally distinct expression patterns during disease progression. While the neuronal miRNAs appear to be upregulated during pre-clinical disease stages, their expression profiles returned to basal level during disease progression and were found to be downregulated in clinical disease stage. Whereas the expression of miR-146a-5p was found to be upregulated throughout disease progression, with highest levels during preclinical stages displaying distinct miR-146a-5p expression profiles during prion disease progression (Majer et al. 2012).

Boese et al. revealed changes in the abundance of miRNAs in synaptoneurosomes from the brain of scrapie infected mice with a small number of miRNAs which were found to be changed during preclinical prion disease including increased levels of miR-124a-3p, miR-136-5p, miR-376a-3p, miR-345-3p, miR-361-5p and miR-212-3p, while miR-141-3p, miR-183-5p and miR-200c-3p were reduced. The miRNA abundance in the synaptoneurosomes has been observed to change with disease progression and increases of miRNAs which have been associated to prion diseases in previous studies (Majer et al. 2012; Saba et al. 2008; Montag et al. 2009; 2012) were detected during clinical disease stages including miR-146a-5p, miR-142-3p, miR-143-3p, miR-145a-5p, miR-451a, let-7b-5p, miR-320-3p and miR-150-5p (Boese et al. 2016).

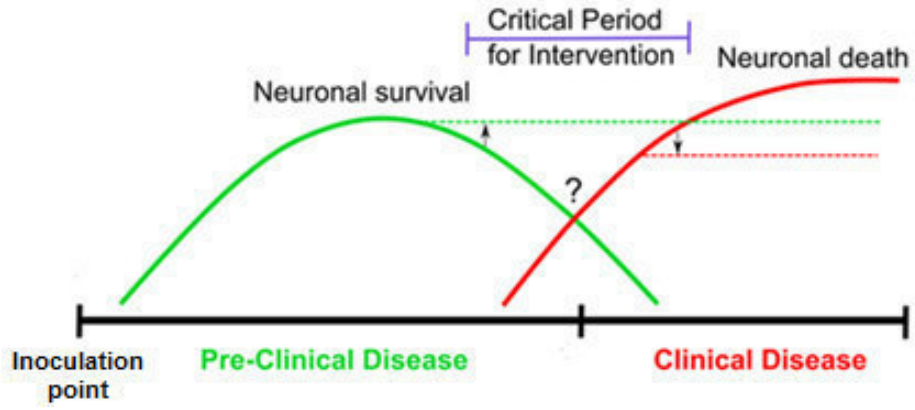


Figure 9: **Molecular alterations during prion disease response in neurons:** Majer et al. proposed the induction of a bi-phasic gene expression program upon prion infection (inoculation point) in neurons. During preclinical disease, genes involved in neuroprotective pathways are upregulated, while after an unknown switch, genes involved in cell death and apoptosis. Especially, a set of profiled miRNAs showed a dynamic and disease-stage specific expression patterns during prion disease progression.
Modified after Majer et al., 2012. *PLOS Pathogens.*

Whether the altered miRNAs are part of compensatory mechanisms in order to help restore homeostasis upon early prion propagation or if these miRNAs might be involved in early prion-meditated neuropathology for instance impaired dendrite and synapse formation deserves further investigation. However, Majer et al. evidenced a bi-phasic gene expression program in which neuroprotective mechanism are upregulated as an early prion disease response while these protective mechanisms are diminished during late disease stages (Fig 9) (Majer et al. 2012).
It needs to be stressed that beside analyses in prion disease models, there are no preclinical insights into miRNA alterations in human prion disease available. Therefore, there is an urgent need for the analyses of potential disease-associated miRNA expression patterns in human prion disease and their validation in relevant model system reflecting *in vivo* disease processes, in order to gain crucial insights into preclinical molecular alterations.

4.4.1.2 miRNAs in Alzheimer's disease

In the past years, a growing number of studies report dysregulated miRNA expression in the brain of AD patients suggesting miRNA dysregulation as a contributing factor in pathophysiological signaling in AD (Maoz, Garfinkel, and Soreq 2017; Putteeraj, Yahaya, and Teoh 2017). Transcriptome-wide studies uncovering global miRNA expression profiles in disease-context are from great importance for the understanding of the role of miRNA dysregulation in AD. Several groups performed high-throughput assays to identify miRNA signatures in the brain of AD patients revealing altered miRNA networks which are associated to brain regional and disease stage-dependent features of AD pathology (Lau et al. 2013; Cogswell et al. 2008; Hébert and De Strooper 2009a; Müller et al. 2014; Delay, Mandemakers, and Hébert 2012). The most comprehensive study was performed so far from Lau et al. in which temporal miRNA expression analyses were conducted in three different AD brain areas including hippocampus by a combination of different methodology for RNA quantification in a cellular- and tissue-specific context. The study demonstrated regional and Braak stage specific deregulated miRNA expression patterns reflecting miRNA associated disease related processes (Lau et al. 2013).

Experimental evidences have linked specific miRNA changes to multiply pathological aspects of AD including misregulated protein homeostasis and the occurrence of pathological forms of Aβ peptides and tau protein, aberrant lipid metabolism and neuroinflammation (Femminella, Ferrara, and Rengo 2015; Lukiw et al. 2013; Basavaraju and De Lencastre 2016).

4.4.1.2.1 miRNAs involved in the regulation of tau metabolism

In regard to tau pathology, miRNA dysregulation in AD could be potentially involved in various impaired processes regulating tau metabolism, including tau expression, transcriptional- and posttranscriptional modifications and/or cellular localization. In particular, the abnormal tau hyperphosphorylation in AD could be associated to misregulated tau kinases, phosphatases and/or other aberrant tau protein modifications in which miRNA dysfunction might play a role (Ballatore, Lee, and Trojanowski 2007; Schonrock and Götz 2012).

Indeed, several studies have identified disease-specific miRNA changes associated to tau pathology in AD. The miR-132-3p has been found downregulated in several brain areas in AD (Lau et al. 2013; Cogswell et al. 2008; Wong et al. 2013; Hebert et al. 2008; Salta et al. 2016) including the hippocampus in which the miRNA was constantly diminished already in early Braak stages (Lau et al. 2013). The chronic miR-132-3p downregulation in AD has been linked to the dysregulation of tau which is directly targeted by the miRNA as well as through miR-132-3p-regulated kinases which are involved in aberrant tau phosphorylation (Salta and De Strooper 2017) and impaired APP metabolism (Smith et al. 2011). Smith et al. demonstrated that a deletion of miR-132-3p in a transgenic AD mice model is leading to increased tau expression, phosphorylation, and aggregation accompanied with an enhanced tau and Aβ pathology (Smith et al. 2015). On the other hand, miR-132-3p overexpressing restored tau pathology and cognitive function in AD mice (Hernandez-Rapp et al. 2016). Further miRNA dysregulation suggested to be involved in tau hyperphosphorylation are altered levels of miR-15, miR-26a, miR-125b in AD brain since these miRNAs were identified to target tau kinases (Hébert et al. 2010; Cai, Zhao, and Zhao 2012; Banzhaf-Strathmann et al. 2014). Another example for a tau pathology associated miRNA deregulation is miR-219 which has been found downregulated in AD brain. Santa-Maria et al. showed that miR-219 directly regulate tau as the reduction of this miRNA in a Drosophila model expressing human tau enhanced tau toxicity, whereas miR-219 overexpression diminished neurotoxic effects (Santa-Maria et al. 2015).

Alternative splicing of the primary tau mRNA transcripts results in tau isoforms and the occurrence of unbalanced ratios of tau isoforms have been causally linked to neurodegeneration (Liu and Gong 2008). Smith et al. 2011 identified several miRNAs including miR-132, miR-124 and miR-9 which are involved in the regulation of tau isoform levels by targeting regulatory and splicing factors suggesting that the dysregulation of those miRNAs could be a contributing factor in the presence of aberrant tau isoforms in AD (Smith et al. 2011).

4.4.1.2.2 miRNAs involved in the regulation of neuroinflammation

Neuroinflammation is a central event in the AD pathophysiology (Michael T. Heneka et al. 2015) and miRNAs dysregulation has been proposed to be implicated in neuroinflammatory mechanism in AD (Gaudet et al. 2017). Some miRNA alterations have been suggested to play an immune-mediating role in AD including the upregulation of miR-7, miR-9, miR-34a, miR-125b, miR-146a and miR-155. *In vitro* studies revealed that these miRNAs can be induced by a wide variety of inflammation-linked stressors and environmental stimuli such us pro-inflammatory cytokines and chemokines, or Aβ peptides (Zhao, Pogue, and Lukiw 2015). Further, the expression of all miRNAs can be triggered by the inflammation-inducing NF-kB signaling pathway (Devier, Lovera, and Lukiw 2015; Lukiw et al. 2011; 2012) and the putative gene targets of these miRNAs seem to be involved in underlying AD pathomechanisms. In this regard, Zhao et al. demonstrated that miR-34a is upregulated in the hippocampal C1 region in AD brain which was linked to impaired microglia responses in AD. In vitro studies in murine microglia cell cultures revealed that miR-34a modulates the microglia receptor TREM2, a critical player in the phagocytic Aβ peptide clearance which was found to be downregulated in AD (Zhao et al. 2013; Bhattacharjee et al. 2016).

5. List of Publications

Publication I

Title:

Regional and subtype-dependent miRNA signatures in sporadic Creutzfeldt-Jakob disease are accompanied by alterations in miRNA silencing machinery and biogenesis.

PLOS Pathogenes 2018. 14(1): e1006802. doi: 10.1371/journal.ppat.1006802. **Journal Impact: 6.158**

Authors:
Franc Llorens*, Katrin Thüne*, Eulàlia Martí, Eirini Kanata, Dimitra Dafou, Daniela Díaz-Lucena, Ana Vivancos, Orr Shomroni, Saima Zafar, Matthias Schmitz, Uwe Michel, Natalia Fernández-Borges, Olivier Andréoletti, José Antonio del Río, Juana Díez, André Fischer, Stefan Bonn, Theodoros, Sklaviadis, Juan Maria Torres, Isidre Ferrer#, Inga Zerr#

*These authors contributed equally to this work.
#These authors share senior authorship.

Publication II

Title:

MicroRNA Expression in the Locus Coeruleus, Entorhinal Cortex, and Hippocampus at Early and Middle Stages of Braak Neurofibrillary Tangle Pathology.

Journal of Molecular Neuroscience 2017. 63(2):206-215. doi: 10.1007/s12031-017-0971-4. **Journal Impact: 2.454**

Authors:
Franc Llorens*, Katrin Thüne*, Pol Andrés-Benito*, Waqas Tahir, Belén Ansoleaga, Karina Hernández-Ortega, Eulàlia Martí, Inga Zerr#, Isidro Ferrer#

*These authors contributed equally to this work.
#These authors share senior authorship

5.1 Statement on original contribution

Publication I

The study was conceived, directed and coordinated by Dr. Franc Llorens, Prof. Isidre Ferrer and Prof. Inga Zerr and, as working supervisor and principle instructors, they provided conceptual and technical guidance in all aspects of the project.

With the guidance of my working supervisor Dr. Llorens, I acquainted myself with the project and became an integral part of the study. After a short initial training, I was able to learn all processes, techniques and methods and I took part in the continuously development and optimization of the project (table 2). I was able to develop the project significantly and to assess the results critically. I participated in writing, reviewing and editing of the original draft.

Experimental work:	Contributions:
Fig. 1: small RNAseq analysis and miRNA expression levels in the frontal cortex and cerebellum of sCJD **(A)** Relative quantitation of regulated miRNAs in the brain of sCJD compared to age and gender matched controls by small RNA-Seq **(B)** Venn diagram of subtype-dependent altered miRNA in the brain of sCJD by small RNA-Seq	The small RNAseq was perfomed by the collaborating partner Prof. Isidre Ferrer and Dr. Franc Llorens. RNAseq data analyses: Franc Llorens, Ana Vivancos, Orr Shomroni and Eulàlia Martí. Figures were done by Franc Llorens and me.
Fig. 2: **Validation of small RNAseq signature** **(A)** Of selected miRNAs in the frontal cortex and cerebellum of controls, sCJD MM1 and VV2 brains **(B)** Detection of sCJD-related miRNAs in Argonaut-protein (Ago) immunoprecipitates from the frontal cortex of controls and sCJD MM1 brain homogenates **(C)** Western blot analyses of Ago-2 in the frontal cortex and cerebellum	Experimental design and setup was done by me upon consultation with Dr. Llorens **my contribution:** • brain sample collection, RNA isolation and tissue lysate preparation, selection of relevant miRNAs • carrying out experiments (qPCR, Ago-Immunoprecipitation, Western Blot) • analyses (densitometric analyses of Western blot, delta CT analysis of qPCR + application of statistical tests • evaluation + technical validation of results • data interpretation • creating figures
Fig. 3: Alterations in the levels and distribution of the miRNA silencing machinery in sCJD. **(A)** Western-blot analysis for Ago-2 immunodetection (upper panel) and densitometry (lower panels) of the chromatography gel filtration fractions from the frontal cortex of control and sCJD MM1 cases.	Experimental design and setup was done by Dr. Llorens and me **my contribution:** • carrying out experiments (brain tissue preparations, gel filtration chromatography, Western Blot) • analyses + application of statistical test • evaluation + technical validation of results • data interpretation • creating figures

(B) Representative fluorescence photomicrographs of Ago immunoreactivity in the frontal cortex of control and sCJD cases **(D)** Representative fluorescence photomicrographs of eIF3, Tia-1, p54/rck and dcp1a in the frontal cortex of control, sCJD cases.	The protein analyses in sCJD were performed during a research stay in the Dept. of Neuropathology, IDIBELL, Barcelona, which provided expertise and human brain samples. **my contribution:** • successfully application of research grants (granted by Boehringer-Ingelheim-Fonds) • carrying out experiments during research (brain tissue sectioning, immunohistochemistry staining) • imaging + analyses • data interpretation • creating figures
(C) RT-QuIC analysis of Ago-2 immunoprecipitates obtained from brain homogenates of control and sCJD cases.	Experimental design, setup and figures were done by Dr. Llorens.
(E) Western-blot analyses of eIF3, p54/rck and dcp1a in the frontal cortex of control and sCJD cases.	Experimental design and setup was done by me upon consultation with Dr. Llorens **my contribution:** • carrying out experiments (Western Blot analyses) • densitometry analyses + application of statistical test • creating figures
Fig. 4: Altered expression levels of miRNA biogenesis components in sCJD. **(A)** Gene expression levels of Drosha, DGCR8 and Dicer in the frontal cortex and cerebellum of controls, sCJD cases by qPCR. **(B)** Protein levels of Drosha, DGCR8, Dicer in the frontal cortex and cerebellum of controls, sCJD cases.	Experimental design and setup was done by me and Dr. Llorens. **my contribution:** • carrying out experiments (brain sample collection, RNA isolation and tissue lysate preparation, Western Blot) • analyses (densitometry analyses of Western blot, delta CT analysis of qPCR) + application of statistical tests • technical validation of results • data interpretation • creating figures
Fig. 5: Neural-type miRNA profiling. **(A)** Heat map analysis of the neurons, microglia and astrocyte enriched miRNAs, whose levels were found to be changed in the brain of sCJD cases.	miRNA profiling data analysis + figures was done by Dr. Llorens.
(B) In situ hybridization of miRNAs: 124-3p, 26a-5p and 146a-5p in control and sCJD brain tissue. **(C)** Quantification of miRNA-124-3p intensity in neurons of control and sCJD brain tissue.	The miRNA in situ hybridization assays in human brains were performed during a second research stay in the Dept. of Neuropathology, IDIBELL, Barcelona, in which the in situ hybridization technic was implemented and optimized by me under the supervision of Prof. Ferrer. **my contribution:** • successfully application of research grants (granted by Felgenhauer Stiftung) • carrying out experiments (brain tissue sectioning, miRNA in situ hybridization) • imaging + analyses • creating figures

Fig. 6: Analysis of common altered miRNAs in sCJD, AD, DLB and FFI. **(A)** Venn diagrams of the comparison between altered miRNAs in AD and sCJD. **(B)** qPCR analysis for miRNA: 146a-5p, 195-5p, 342-5p, 877-5p, 323a-5p, 5701 in the frontal cortex of controls, AD and rpAD **(C)** qPCR analysis for miRNA: 146a-5p, 195-5p, 342-5p, 877-5p, 323a-5p, 5701 in the frontal cortex of controls and DLB **(D)** qPCR analysis for miRNA: 342-5p, 146a-5p, 195-5p, 5701 in the frontal cortex and cerebellum of controls and FFI	Experimental design and setup was done by me and Dr. Llorens. The relevant miRNAs for the cross-disease analyses were selected by me and Dr. Llorens. The Venn diagram was designed by Dr. Llorens. **my contribution:** • carrying out experiments (brain sample collection, RNA isolation, qPCR) • analyses (delta CT) + application of statistical tests • creating figures
Fig. 7: Regional and temporal-dependent neuropathological characteristics and miRNA signatures in sCJD MM1 mouse model tg340-PRNP129MM. **(A)** tg340 mice inoculated with control or sCJD MM1 brain homogenates.	The miRNA expression analyses were expanded on a sCJD mice model. A detailed and comprehensive characterization of the neuropathological hallmarks of the tg340 mouse model was designed and coordinated by Dr. Llorens and me. The tg340 mice model was developed and inoculated with human brain homogenates by Prof. Olivier Andréoletti, INRA, Toulouse, France.
(B) PET-blot analysis for the detection of PrP^{SC} in the cortex and cerebellum of sCJD MM1 inoculated tg340 mice.	The PET-blot analyses of the mice brain section were done by Prof. Olivier Andréoletti, INRA, Toulouse.
(C) Densitometric analysis of western blots developed for PSD-95 and synaptophysin in the cortex and cerebellum of tg340 mice samples at different disease stages.	Western blot analyses were done by Dr. Llorens. **my contribution:** Densitometry analysis + figures were done by me.
(D) Heat map analysis of key inflammatory mediators and cytokines measured by qPCR analysis in the cortex and cerebellum of control and tg340 mice at different disease stages.	**my contribution:** The qPCR and double delta CT analyses + figure were done by me.
(E) Hematoxylin-eosin staining in the cortex and cerebellum of control and sCJD MM1 infected tg340 animals.	The histochemistry analysis + figures of mice brain tissue were performed during my first research stay in the neuropathological laboratory of Prof. Ferrer, IDIBELL, Barcelona.
(F) qPCR analysis of the miRNAs validated in human sCJD tissue in the cortex and cerebellum of control and tg340 mice at different disease stage	**my contribution:** The qPCR and double delta CT analyses + figures were done by me.
Fig. 8: miRNA profiling in the CSF of control and sCJD cases **(A)** RT-qPCR analysis of the housekeeping U6 and **(B)** miRNA-378a-3p, miRNA-26a-5p and miRNA-204-5p in the CSF of control and sCJD cases	Experimental design and setup was done by me and Dr. Llorens. **my contribution:** • CSF sample collection, isolation of RNA from CSF, miRNA profiling in the CSF via qPCR analyses • creating figures + statistical analyses
Supplementary Fig 1: MA plots from small RNAseq in the frontal cortex and cerebellum of control, sCJD MM1 and VV2 cases	miRNA data analyses and plots were done by Dr. Eulàlia Martí.
Supplementary Fig 2: Distribution of the different types of isomiRs in control and sCJD	miRNA data analyses and plots were done by Prof. Eulàlia Martí, Centre of Genomic Regulation, Barcelona.

Supplementary Fig 3: Expression of GW182 in sCJD brain tissue	**my contribution:** qPCR, delta CT analyses + figures were done by me. Immunohistochemistry + phase contrast microscopy and imagine were done by Dr. Daniela Diaz-Lucena.
Supplementary Fig 4: Analysis of gel filtration chromatography fractions from sCJD cases	**my contribution:** Western Blot (A, D), RT-QuIC (B), Dot Blot (C) + figures.
Supplementary Fig 5: Subcellular localization of Ago-2 in sCJD brain tissue	Immunohistochemistry + phase contrast microscopy and imagine were done by Dr. Daniela Diaz-Lucena.
Supplementary Fig 6: Expression of Exportin 5 in sCJD brain tissue	**my contribution**: Analyses of Exportin 5 via qPCR and Western blot + figures were done by me.
Supplementary Fig 7: Synaptic loss in the sCJD MM1 mouse model tg340-PRNP129MM	**my contribution:** western blot analyses were done by me
Supplementary Fig 8: Temporal-dependent expression of validated miRNAs in the sCJD MM1 mouse model tg340-PRNP129MM	**my contribution:** qPCR analyses + figures were done by me
Supplementary Fig 9: Selective cell death induction by sCJD miRNAs transfection in cell cultures	Functional miRNA experiment was designed by me upon feedback from Dr. Llorens. **my contribution:** RNA isolation from human brain, gel purification of miRNA from total RNA, miRNA transfection of two different cell cultures, qPCR analyses + technical validation + figures

Table 2: **Statements on original contribution to publication I.**

Publication II

The study was conceived, directed and coordinated by Prof. Isidre Ferrer, Dr. Franc Llorens and Prof. Inga Zerr and, as working supervisor and principle instructors, they provided conceptual and technical guidance in all aspects of the project.

I acquainted myself with the project and became an integral part of the study (table 3). By analyzing the cellular environment of mRNAs during early AD brain pathology, I provided key experimental work through which the project developed significantly. The main body of experimental work in this study was done by me, whereas experiments were supplemented and enhanced by Dr. Belén Ansoleaga and Pol Andrés-Benito. The manuscript was written by Prof. Ferrer and Dr. Llorens, whereas I contributed partially in writing:

- chapter: In Situ Hybridization (Material and Methods), page 209
- chapter: Discussion, page 211-212, literature research + partially written by me

Experimental work:	Contributions:
Fig. 1: miRNA expression analysis in early AD: miRNAs: 27a-3p, 124-3p, 132-3p, and 143-3p expression levels in the locus coeruleus, entorhinal cortex, CA1 region, and dentate gyrus in middle-aged and stages I-II and III-IV of NFT pathology as revealed by RT-qPCR.	The qPCR analyses of miRNAs and figures were done by Pol Andrés-Benito and Dr. Belén Ansoleaga und supervision of Dr. Franc Llorens.
Fig. 2: **Validation of small RNAseq signature:** **(a, d, g)** Localization of miRNA-27a-3p, miRNA-124-3p, and miRNA-143-3p in the dentate gyrus in cases with NFT pathology at stage I-II revealed by in situ hybridization **(b, e, h)** Localization of miRNA-27a-3p, miRNA-124-3p, andmiRNA-143-3p in CA1 region of the hippocampus in cases with NFT pathology at stage I-II revealed by in situ hybridization. **(c, f, i)** Localization of miRNA-27a-3p, miRNA-124-3p, and miRNA-143-3p in entorhinal cortex in cases with NFT pathology at stage I-II revealed by in situ hybridization.	Experimental design and setup was done by me upon consultation with Prof. Ferrer and Dr. Llorens. **my contribution:** • carrying out experiments (brain sample collection and tissue sectioning, miRNA in situ hybridization) • imaging + analyses • creating figures

Table 3: **Statements on original contribution to publication II.**

6. Publication I

PLOS | PATHOGENS

RESEARCH ARTICLE

Regional and subtype-dependent miRNA signatures in sporadic Creutzfeldt-Jakob disease are accompanied by alterations in miRNA silencing machinery and biogenesis

Franc Llorens[1,2☯]*, Katrin Thüne[1,3☯], Eulàlia Martí[4], Eirini Kanata[5], Dimitra Dafou[6], Daniela Díaz-Lucena[2], Ana Vivancos[7], Orr Shomroni[8], Saima Zafar[1,3], Matthias Schmitz[1,3], Uwe Michel[1], Natalia Fernández-Borges[9], Olivier Andréoletti[10], José Antonio del Río[2,11,12], Juana Díez[13], Andre Fischer[14], Stefan Bonn[8,15,16], Theodoros Sklaviadis[5], Juan Maria Torres[9], Isidre Ferrer[2,17‡], Inga Zerr[1,3‡]

1 Department of Neurology, University Medical School, Göttingen, Germany, **2** Center for Networked Biomedical Research on Neurodegenerative Diseases (CIBERNED), Barcelona, Spain, **3** German Center for Neurodegenerative Diseases (DZNE), Translational Studies and Biomarkers, Göttingen, Germany, **4** Centre for Genomic Regulation, Barcelona, Spain, **5** Prion Diseases Research Group, School of Health Sciences, Department Of Pharmacy, Aristotle University of Thessaloniki, Thessaloniki, Greece, **6** Department of Genetics, Development and Molecular Biology, School of Biology, Aristotle University of Thessaloniki, Thessaloniki, Greece, **7** Vall d'Hebron Institute of Oncology (VHIO), Barcelona, Spain, **8** German Center for Neurodegenerative Diseases (DZNE), Computational Systems Biology, Göttingen, Germany, **9** Centro de Investigación en Sanidad Animal (CISA-INIA), Madrid, Spain, **10** Institut National de la Recherche Agronomique/Ecole Nationale Vétérinaire, Toulouse, France, **11** Molecular and Cellular Neurobiotechnology, Catalonian Institute for Bioengineering (IBEC), Parc Científic de Barcelona, Barcelona, Spain, **12** Department of Cell Biology, University of Barcelona, Barcelona, Spain, **13** Molecular Virology group, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain, **14** German Center for Neurodegenerative Diseases (DZNE), Epigenetics and Systems Medicine in Neurodegenerative Diseases, Göttingen, Germany, **15** German Center for Neurodegenerative Diseases (DZNE), Tuebingen, Germany, **16** Center for Molecular Neurobiology University Medical Center Hamburg-Eppendorf, Hamburg, Germany, **17** Senior consultant, Bellvitge University Hospital-IDIBELL, Department of Pathology and Experimental Therapeutics, University of Barcelona, Hospitalet de Llobregat, Barcelona, Spain

☯ These authors contributed equally to this work.
‡ IF and IZ share senior authorship of this work.
* Franc.llorens@gmail.com

OPEN ACCESS

Citation: Llorens F, Thüne K, Martí E, Kanata E, Dafou D, Díaz-Lucena D, et al. (2018) Regional and subtype-dependent miRNA signatures in sporadic Creutzfeldt-Jakob disease are accompanied by alterations in miRNA silencing machinery and biogenesis. PLoS Pathog 14(1): e1006802. https://doi.org/10.1371/journal.ppat.1006802

Editor: Jason C. Bartz, Creighton University, UNITED STATES

Received: June 22, 2017

Accepted: December 11, 2017

Published: January 22, 2018

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by the Robert Koch Institute through funds from the Federal Ministry of Health (grant no. 1369-341) to IZ, by the Seventh Framework Program of the European Commission (Grant No. 278486 [DEVELAGE project]), by the Spanish Ministry of Health, Instituto Carlos III (Fondo de Investigación

Abstract

Increasing evidence indicates that microRNAs (miRNAs) are contributing factors to neurodegeneration. Alterations in miRNA signatures have been reported in several neurodegenerative dementias, but data in prion diseases are restricted to ex vivo and animal models. The present study identified significant miRNA expression pattern alterations in the frontal cortex and cerebellum of sporadic Creutzfeldt-Jakob disease (sCJD) patients. These changes display a highly regional and disease subtype-dependent regulation that correlates with brain pathology. We demonstrate that selected miRNAs are enriched in sCJD isolated Argonaute(Ago)-binding complexes in disease, indicating their incorporation into RNA-induced silencing complexes, and further suggesting their contribution to disease-associated gene expression changes. Alterations in the miRNA-mRNA regulatory machinery and perturbed levels of miRNA biogenesis key components in sCJD brain samples reported here further implicate miRNAs in sCJD gene expression (de)regulation. We also show that a

Sanitaria [FIS] PI1100968, FIS PI14/00757 and by CIBERNED (Network center for biomedical research of neurodegenerative diseases) project BESAD-P to IF, by the Spanish Ministry of Health, Instituto Carlos III-Fondo Social Europeo (CP16/00041) to FL, by the Red Nacional de priones (AGL2015-71764-REDT- MINECO) to JADR, FL, IZ, JMT and IF, by the bilateral IKYDA project (ID 57260006) to FL, IZ and TS, by the Spanish Ministry of Economy and Competitiveness (BFU2016-80039-R) (AEI/MINEICO/FEDER, UE) and the "Maria de Maeztu" Program for Units of Excellence in R&D (MDM-2014-0370) to JD, by the Spanish Ministry of Economy and Competitiveness (SAF2014-60551-R) to EM and by the Spanish Ministry of Economy, Industry and Competitiveness (MEICO) (BFU2015-67777-R), the Generalitat de Catalunya (SGR2014-1218), CIBERNED (PRY-2016-2, MFDEND), La Caixa Obra Social Foundation and La Marató de TV3 to JADR. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist."

subset of sCJD-altered miRNAs are commonly changed in Alzheimer's disease, dementia with Lewy bodies and fatal familial insomnia, suggesting potential common mechanisms underlying these neurodegenerative processes. Additionally, we report no correlation between brain and cerebrospinal fluid (CSF) miRNA-profiles in sCJD, indicating that CSF-miRNA profiles do not faithfully mirror miRNA alterations detected in brain tissue of human prion diseases. Finally, utilizing a sCJD MM1 mouse model, we analyzed the miRNA deregulation patterns observed in sCJD in a temporal manner. While fourteen sCJD-related miRNAs were validated at clinical stages, only two of those were changed at early symptomatic phase, suggesting that the miRNAs altered in sCJD may contribute to later pathogenic processes. Altogether, the present work identifies alterations in the miRNA network, biogenesis and miRNA-mRNA silencing machinery in sCJD, whereby contributions to disease mechanisms deserve further investigation.

Author summary

miRNAs are small non-coding RNAs that regulate gene expression through complementary binding to their mRNA targets. Specific miRNA signatures have been proposed for several neurodegenerative diseases supporting the idea that miRNA deregulation is a common disease hallmark. Here we present the comprehensive miRNA signature in sporadic Creutzfeldt-Jakob disease (sCJD). Our study unravels the complex network of regional and disease-subtype miRNA alterations, and the presence of a disturbed miRNA biogenesis pathway and miRNA-mRNA silencing machinery. We also highlight the existence of time-dependent miRNA profiles and identify commonly regulated miRNAs between several dementias with cortical pathology sharing a partial clinical overlap and pathological involvement with sCJD. The present data shed light on the potential role of miRNAs as a contributing factor of pathogenic molecular traits associated with sCJD.

Introduction

Creutzfeldt-Jakob disease (CJD) is a human transmissible spongiform encephalopathy characterized by behavior changes, progressive dementia, loss of coordination and myoclonus. At the molecular level, CJD is associated with the conversion of the normal, cellular prion protein (PrPC) to an abnormal conformation (PrPSc) and further accumulation of PrPSc in the brain in the form of protein aggregates [1]. Despite the established role of PrPC in several neuronal functions such as synaptic plasticity, neurotransmission and neuronal development, the molecular mechanisms triggering the PrPC to PrPSc conversion and the cellular pathways unchained by prion infection leading to neuronal damage and cell death remain elusive.

Sporadic CJD (sCJD) is the most common human prion disease, presenting a high degree of heterogeneity. sCJD is classified into six subtypes, based on variations at codon 129 of the prion protein gene (*PRNP* Met or Val) and on the size of protease resistant PrPSc (type 1 or 2). Among these subtypes MM1 and VV2 are the most prevalent [2,3] and give rise to unique clinical and neuropathological features, such as specific gliosis, neuroinflammation, spongiosis and synaptic loss signatures [2,4–7]. A widespread regional and subtype-specific mRNA and protein deregulation leading to the alteration of multiple biological functions and signaling pathways is also associated with sCJD [5,8–10].

Transcriptomic and proteomic patterns are regulated by several factors including miRNAs; these have been recognized as key regulators of gene expression. miRNAs are small (21–25 nucleotides long), non-coding RNAs, that regulate gene expression through partial complementary binding to their mRNA targets in the RNA-induced silencing complex (RISC). This miRNA-mRNA interaction usually leads to gene silencing through a variety of forms, including mRNA cleavage, translational repression and de-adenylation [11,12].

Several miRNAs are selectively expressed in the central nervous system (CNS) and have been reported to be involved in CNS development, function and pathogenesis [13,14]. In addition, specific miRNA signatures have been proposed for Alzheimer's (AD), Parkinson's (PD) and Huntington's (HD) disease, as well as for Fronto-temporal dementia (FTD) [15–20], supporting the idea that miRNA deregulation is a common hallmark of neurodegenerative diseases. While the study of miRNAs in relation to prion pathogenesis has gained experimental momentum since several miRNAs were found to be altered in *in vivo* and *ex vivo* models of prion diseases [21–25], the miRNA signature in sCJD has not been reported so far.

A potential link between miRNAs and prion diseases has been suggested based on the co-localization of PrPC within RISC components in endosomes and multivesicular bodies. Binding of PrPC to the type III RNase Dicer (Dicer) and Argonaute (Ago) proteins, which represent essential components of the RISC loading complex, has been proposed as a requirement for effective repression of several miRNA targets [26]. Hence, miRNA deregulation could be triggered by many aspects of sCJD pathology, including replacement of the physiological PrP forms with pathological ones. Simultaneously, miRNA deregulation may have drastic consequences in sCJD gene expression patterns and may act as a contributing factor in the cascade of events leading to fast disease progression.

In order to increase our understanding of the miRNA contribution to sCJD pathogenesis, we performed small RNA-Sequencing (small RNA-Seq) in the two most affected brain regions in sCJD, frontal cortex (FC) and cerebellum (CB), in the two most prevalent sCJD subtypes (MM1 and VV2), which are linked to region specific clinical and pathological outcomes [2,27].

We demonstrate a strong regional and subtype-specific alteration of miRNA expression in sCJD and molecular alterations in miRNA biogenesis and silencing machinery; we further show that a subset of sCJD enriched miRNAs are actively incorporated in the RISC complex. Additionally, we detected the presence of commonly changed miRNAs in other neurodegenerative dementias such as AD, dementia with Lewy bodies (DLB) and fatal familial insomnia (FFI). Further, sCJD-related miRNA alterations were studied in a temporal manner, utilizing a sCJD mouse model; sCJD miRNA profiles were validated in the utilized animal model at clinical disease stages, whereas most of those miRNAs were not found to be regulated at earlier disease points suggesting diverse and dynamic miRNA expression programs during disease progression. Finally, we profiled selected miRNAs in the CSF of sCJD cases, which ruled out the presence of a major correlation between miRNA levels in CSF and brain tissue.

Altogether, our results show a significant deregulation of miRNA expression, activity and biogenesis in sCJD and they highlight the potential role of miRNAs in the pathology of prion diseases and alternative neurodegenerative conditions.

Results

Altered miRNA signatures in the FC and CB of sCJD MM1 and VV2 cases

miRNA expression signatures were determined by small RNA sequencing in the frontal cortex (FC) and in the cerebellum (CB) of sCJD MM1 and VV2 and in age and gender matched controls. We selected these brain regions, because they are strongly affected in sCJD and display differential neuropathological patterns between MM1 and VV2 subtypes [2, 27]. Obtained

sequences were annotated based on the overlap with publicly available genome annotations, including miRNAs, tRNAs, rRNAs, other small RNAs and genomic repeats. miRNAs represented an average of 27% of total counts (S1 Table). Total number of reads on the FC and CB mapping onto miRNAs with at least 2 counts in a given sample are shown (S2 Table). Two independent pipelines were used for the analysis of the differential miRNA expression, Seqbuster [28] and OASIS [29]. Both pipelines showed a high level of agreement in the detection of differentially expressed miRNAs (89%). Seqbuster analysis revealed the presence of 70 miRNAs with altered expression in the FC of sCJD MM1 and 27 in sCJD VV2 compared to controls (Fig 1A, S3 Table). In the CB, 22 miRNAs were changed in sCJD MM1 and 69 in sCJD VV2 compared to controls (Fig 1A, S3 Table). The majority of the differentially altered miRNAs were expressed in both tissues, suggesting that the changes on their levels are tissue specific (S2 and S3 Tables).

The miRNA signature in sCJD was highly dependent on the brain region and sCJD subtype (Fig 1B and S3 Table). Regarding sCJD subtype alterations, a high percentage of miRNAs were commonly regulated between both subtypes in the FC (31%) (Fig 1B). In the CB, the percentage of commonly altered miRNAs between subtypes was lower (10%) (Fig 1B).

miRNA variability is ubiquitous in the brain of control and sCJD cases

Isoforms of a mature miRNA have been referred as isomiRs [30]. They are functionally active and highly abundant in brain tissue, both in control and in neurodegenerative diseases [15,31,32]. In the present study, 2883 and 4075 different isomiRs were found in the CB and FC, respectively (S4 Table). Furthermore, most of the sequences mapping onto miRNA database (reference miRNAs and IsomiRs) showed 3–50 counts. No major differences were detected between control and sCJD subtype cases regarding their isomiRs profiles (S2 Fig), suggesting that isomiR processing is not significantly altered in sCJD.

sCJD-related miRNA validation and enrichment in RISC complexes

A subset of miRNAs found to display altered expression in sCJD based on small RNA-Seq analysis was further validated by qPCR analysis. miRNAs were selected according to number of counts and fold change alterations in the RNA-seq analysis and/or their previous association with prion disease pathogenesis and/or other neurodegenerative diseases [21,23,33,34]. A total of 18 miRNAs were analyzed in both regions. The alterations in the levels of 15 and 10 miRNAs were validated in a regional specific manner in the FC and CB, respectively (Fig 2A). In sCJD FC, miRNAs 29b-3p, 342-3p, 146a-5p, 154-5p, 195-5p, 26a-5p, 16-5p, 449a, 142-3p, let7i-5p and 135a-5p were increased, while miRNAs 124-3p, 331-3p, 877-5p and 125a-5p were decreased compared to controls, in agreement with RNA-seq data. miRNAs 378a-3p and 5701, which expression was only altered in CB did not present changes in the FC. In CB, miRNAs 146a-5p, 154-5p, 26a-5p, 378a-3p, 449a, 142-3p, let7i-3p and 5701 were increased, and miRNAs 124-3p and 877-5p were decreased in sCJD, in agreement with RNA-seq data. The rest of miRNAs, which expression was only altered in FC did not present changes in the CB. Finally, miRNA-204-5p, a miRNA presenting no alterations in the FC and CB of sCJD by RNA-seq analysis, was used as negative control showing no changes among groups at qPCR level. Therefore, while most of the qPCR validated miRNAs were altered in both disease subtypes, a subset of them presented subtype-specific changes, which were in agreement with small RNA-Seq data (S3 Table).

To confirm that upregulated miRNAs in sCJD brain tissue were functionally active, we performed RISC immunoprecipitations in the FC of control and sCJD MM1 cases using two different Argonaute (Ago) antibodies detecting Ago-2 (11A9) and Ago1-4 family members

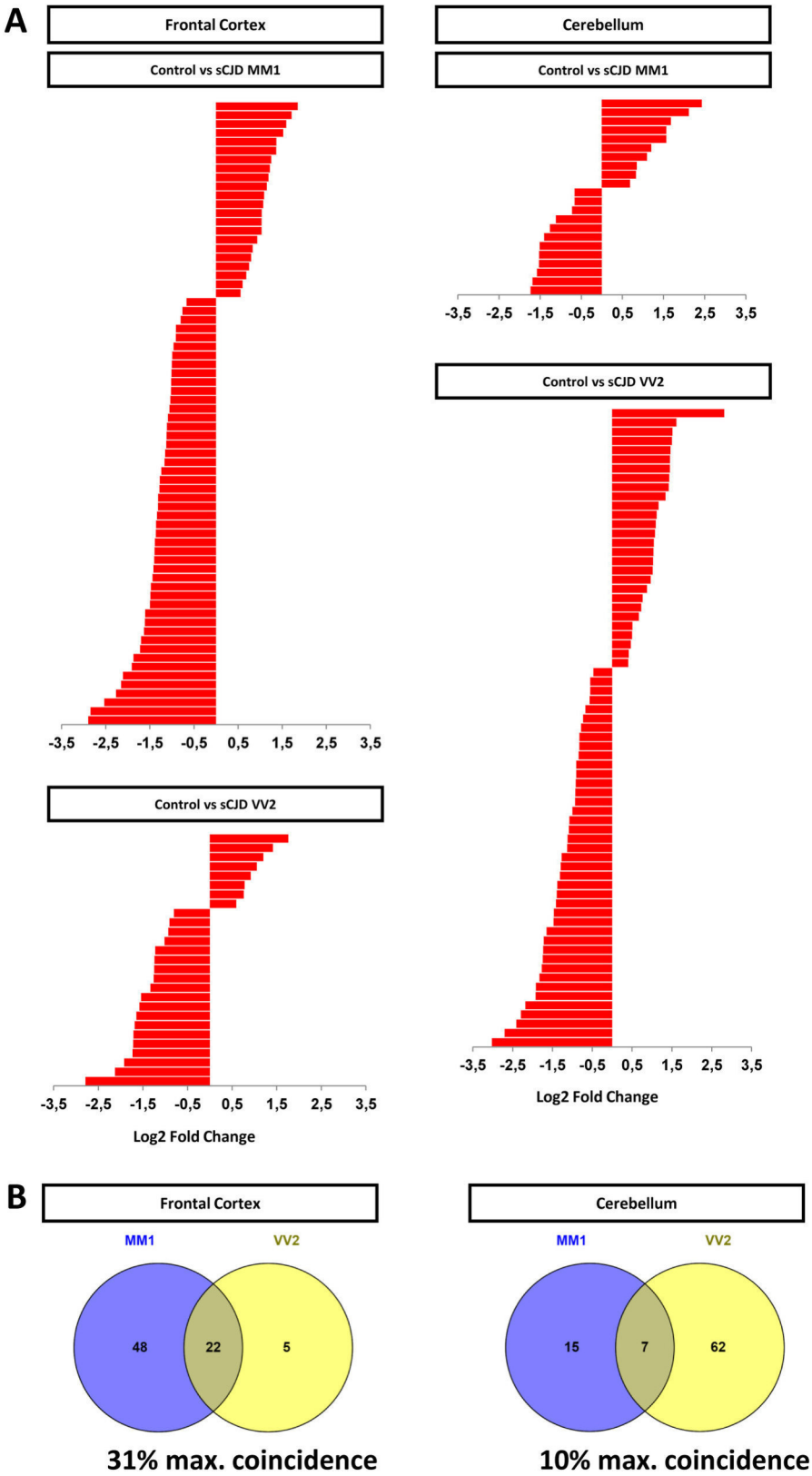


Fig 1. Small RNA-Seq analysis and miRNA expression levels in the FC and CB of sCJD. (A) Relative quantitation of regulated miRNAs in the FC and CB of sCJD MM1 and VV2 subtypes compared to age and gender matched controls

by small RNA-Seq. For FC 13 controls, 11 sCJD MM1 and 13 sCJD VV2 were analyzed. For CB: 12 controls, 12 sCJD MM1 and 9 sCJD VV2 were analyzed. Differential expression in miRNA sequencing data was evaluated with the DESeq2 tool and significantly differently expressed miRNAs were detected according to an adjusted p value of <0.05. Data represent log2 fold change for each comparison. log2 fold change was >0.5 for upregulated miRNAs and <-0.5 for downregulated miRNAs. MA plots are also supplied for data visualization (S1 Fig). (B) Venn diagram of subtype-dependent altered miRNA in the FC and CB of sCJD by small RNA-Seq. Percentage of maximal coincidence (percentage of maximal number of miRNAs that can be coincident between groups) is indicated.

https://doi.org/10.1371/journal.ppat.1006802.g001

(H-300). These antibodies were able to immunoprecipitate Ago-containing miRNA complexes from brain tissue (Fig 2B). RNA extraction from immunoprecipitates and further qPCR

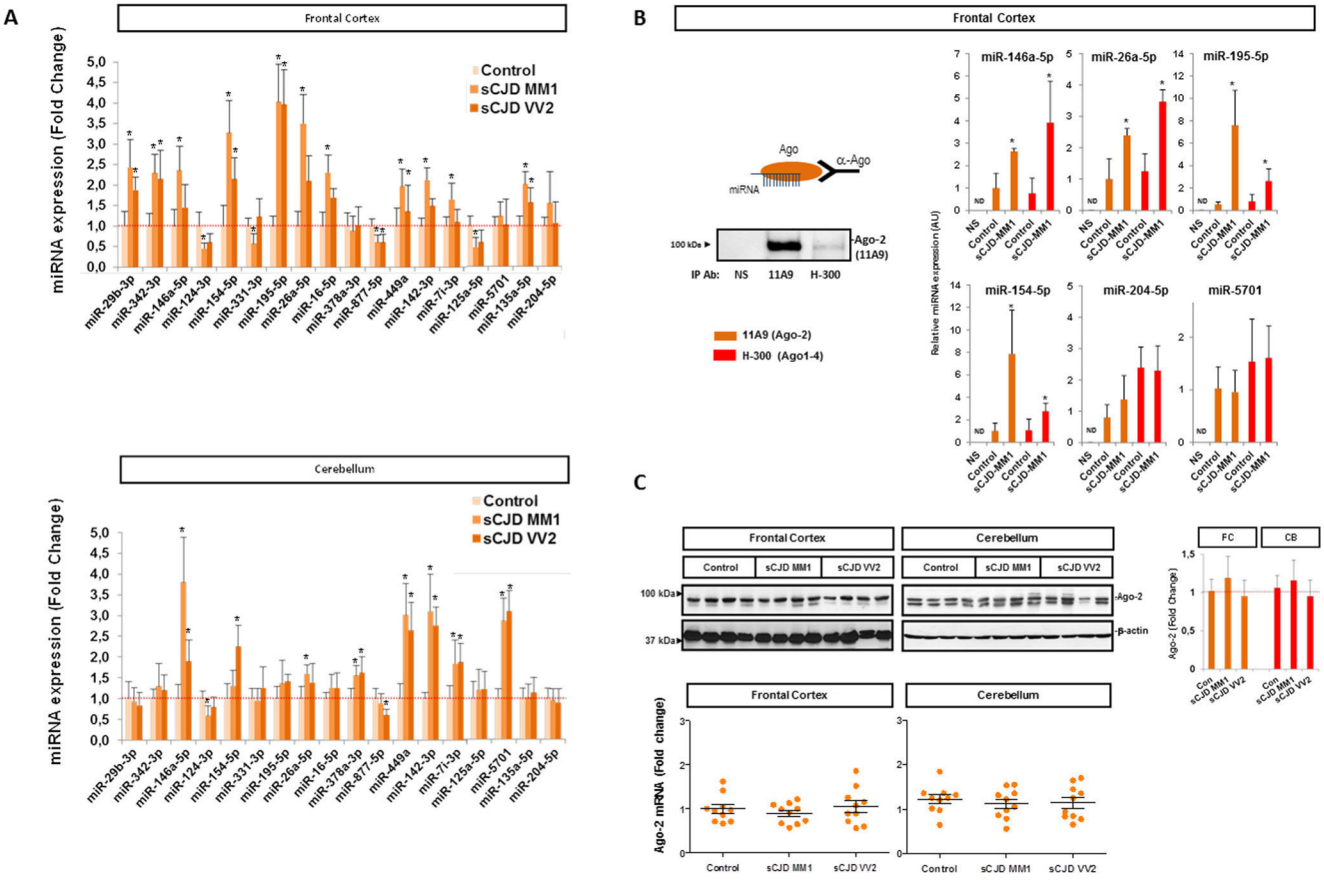


Fig 2. Regional and subtype-dependent miRNA expression confirmation and representative miRNA incorporation into functional complexes. (A) Validation of the small RNA-Seq signature in selected miRNAs by RT-qPCR analysis in the FC (upper panel) and CB (lower panel) of controls, sCJD MM1 and VV2. Results were normalized to the housekeeping genes U6 snRNA (figure) and RNU5 expression, which showed similar results in the expression analysis of deregulated miRNAs between control and sCJD cases. Housekeeping gene levels remained unaltered between groups. Normalization was performed relative to controls. Error bars indicate SD. (B) Detection of sCJD-related miRNAs by RT-qPCR in Ago immunoprecipitates from the FC of controls (n = 3) and sCJD MM1 brain homogenates (n = 3). The two Ago antibodies used (11A9 and H-300) were able to immunoprecipitate Ago-2 from brain tissue (left panel) and reported similar results in the specific enrichment of sCJD-related miRNAs (146a-5p, 26a-5p, 195-5p, 154-5p, 204-5p and 5701) in the FC of sCJD MM1 cases (right panels). miRNA-204-5p was selected as negative control, since no changes were detected between control and sCJD cases with small RNA-Seq and RT-qPCR analysis. Non-specific immunoglobulins (NS) were used as control antibody for the immunoprecipitation. Error bars indicate SD. (C) Upper panel: western blot analysis of Ago-2 (11A9 antibody) in the FC and CB control, sCJD MM1 and VV2 cases. Four representative cases per diagnostic group and brain region are shown in the western blot. Quantifications derived from densitometry analysis were performed in 15 cases per diagnostic group (n = 15/group). β-actin was used as loading control. Densitometries of the western blots are shown. Normalization was performed relative to controls. Error bars indicate SD. Bottom panel: Gene expression levels of Ago-2 in the FC and CB of controls, sCJD MM1 and VV2 cases by RT-qPCR. Results were normalized to housekeeping genes GAPDH (figure) and GUSB with similar results. Housekeeping levels remained unaltered between groups. 10 cases per diagnostic group (n = 10/group) were analyzed. In all cases, statistical significance (compared to controls) was set at $^{*}p<0.05$.

https://doi.org/10.1371/journal.ppat.1006802.g002

analysis allowed us to detect miRNA enrichment for a subset of miRNAs with increased expression in sCJD MM1 brain tissue according to qPCR analysis (miRNA-146a-5p, miRNA-26a-5p, miRNA-195-5p and miRNA-154-5p). As negative controls, miRNA-204-5p and miRNA-5701 were tested. According to RNA-seq and qPCR data, miRNA-204-5p was regulated neither in the FC nor in the CB of sCJD, while miRNA-5701 was upregulated only in the CB of sCJD cases. In agreement with this, miRNA-204-5p and miRNA-5701 levels were unchanged in RISC immunoprecipitates between control and sCJD cases (Fig 2B). To rule out the possibility that the differences in RISC-miRNA enrichment were due to alterations in Ago-2 expression between controls and sCJD, Ago-2 levels were analyzed by qPCR and western blot. No alterations were found in Ago-2 protein and mRNA levels between controls and sCJD cases, either in the FC or in the CB (Fig 2C). Similarly, no changes on the expression levels of GW182, an Ago binding protein essential for miRNA-mediated gene silencing [35] were detected between control and sCJD cases (S3A and S3B Fig).

Alterations in miRNA-mRNA silencing complexes in sCJD

Next, we investigated potential alterations in the miRNA-mRNA silencing complexes that could explain the alterations in sCJD miRNA signatures previously detected. As a first step, we performed gel filtration chromatographic assays of control and sCJD brain homogenates. Ten fractions containing different proteomic patterns according to their molecular weight were obtained. PrP levels were homogeneously distributed along the chromatographic fractions as described before [36,37] (S4A and S4B Fig). Western blot analysis revealed the presence of Ago-2 in higher molecular weight fractions in sCJD compared to control samples, suggesting that Ago-2 in sCJD is interacting with a different subset of partners (Fig 3A). This is in agreement with a different subcellular localization of Ago in sCJD brain tissue as revealed by immunohistochemistry analysis. Indeed, using two different antibodies, we detected an increased nuclear localization of Ago in the FC of sCJD MM1 and VV2 cases, in contrast to controls, where staining was mainly detected in the cytoplasmic compartment (Fig 3B and S5A and S5B Fig). While Ago-2 expression was mainly detected in neurons, both in controls and sCJD cases, double immunofluorescence analysis revealed the presence of Ago-2 positive microglial cells (S5 Fig). In contrast, no differences on subcellular localization were detected for GW182 between control and sCJD cases (S3 Fig).

PrP and Ago-2 are interacting proteins in physiological conditions [26], but nothing is known about the potential role of PrPSc in the RISC complex. Since the endosomal compartment, in which RISC assembly and turnover occurs, has been proposed as a site of prion conversion [38] we aimed to investigate the presence of PrPSc in Ago-2 complexes, which could be one of the contributors to their altered chromatographic Ago-2 patterns in sCJD. To this end, Ago-2 immunoprecipitates from the FC of controls and sCJD MM1 were subjected to RT-QuIC analysis. Positive signal was detected in immunoprecipitates from sCJD samples, but not from controls (Fig 3C), indicating the presence of pathogenic PrP in Ago-2 complexes, in agreement with the presence of RT-QuIC signal and protein oligomers in Ago-2 containing chromatographic fractions (S4C and S4D Fig).

The alteration of RISC components in sCJD and the well-known presence of reticulum stress in models of prion disease [39–41] raised the possibility that stress granules (SG), which are normally transient structures, form stable complexes in sCJD. Immunohistochemical and immunoblot analysis of the SG markers eukaryotic initiation factor 3 (eIF3) and T-cell-restricted intracellular antigen-1 (Tia-1) revealed that, in sCJD, neither their subcellular localization nor their expression levels were altered (Fig 3D and 3E). In agreement with this, we did not detect hyper-phosphorylation of the SG activator eIF2α in sCJD (Fig 3D). Similarly, levels

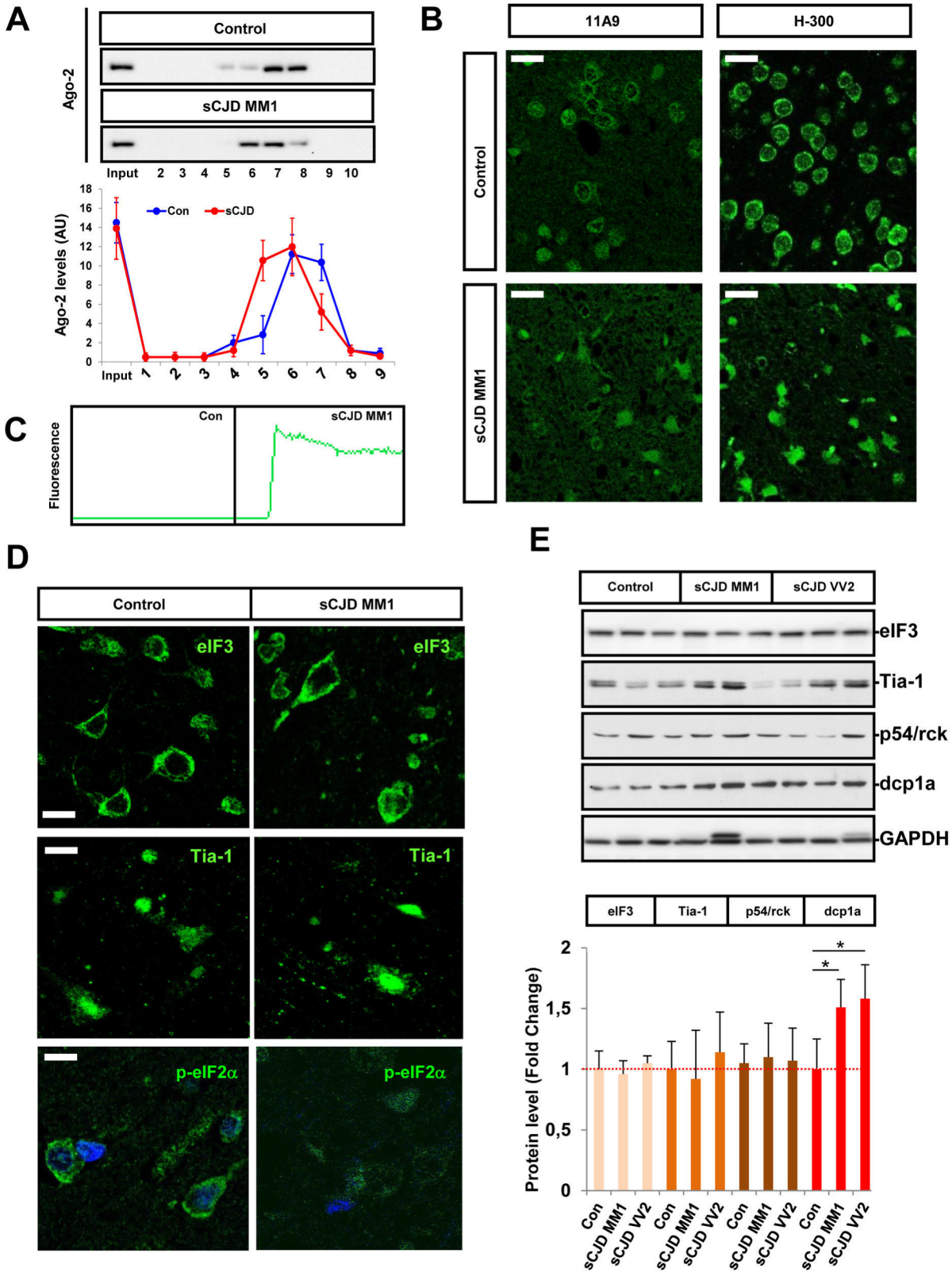


Fig 3. Alterations in the levels and distribution of the miRNA silencing machinery in sCJD. (A) Western blot analysis for Ago-2 immunodetection (upper panel) and densitometry (lower panel) of the chromatography gel filtration fractions from the FC of control (n = 3) and sCJD MM1 (n = 3) cases. Western blot analysis shows a representative image of a control and a sCJD case, while densitometry shows

mean values and SD of all cases studied. (B) Representative fluorescence photomicrographs of Ago immunoreactivity in the FC of control and sCJD MM1 cases. The two antibodies used (11A9 and H-300) showed similar Ago distribution between control and sCJD MM1 cases. Scale bar = 25μm (C) RT-QuIC analysis of Ago-2 immunoprecipitates (11A9) obtained from the FC of brain homogenates of control (n = 3) and sCJD MM1 (n = 3) cases. RT-QuIC assays were run in triplicate for each sample. A representative curve for each condition is shown. (D) Representative fluorescence photomicrographs of eIF3, Tia-1 and p-eIF2α(Ser51) immunoreactivity in the FC of control and sCJD MM1 cases. Scale bar for eIF3 = 10 μm and Tia-1 = 20μm, for p-eIF2α(Ser51) = 10μm (E) Western blot analysis of eIF3, Tia-1, p54/rck and dcp1a in the FC of control, sCJD MM1 and VV2 cases. Three representative cases per diagnostic group and brain region are shown in the western blot. Quantifications derived from densitometry analysis were performed in 15 cases per diagnostic group (n = 15/group). GAPDH was used as a loading control. Densitometries of the western blots are shown. Normalization was performed relative to controls. Error bars indicate SD. In all cases, statistical significance (compared to controls) was set at $^{*}p<0.05$.

https://doi.org/10.1371/journal.ppat.1006802.g003

of the SG and p-bodies marker DEAD-Box Helicase 6 (p54/rck) showed no alterations between controls and sCJD. However, increased expression levels of the specific p-bodies marker decapping mRNA 1a (dcp1a) was detected in sCJD MM1 and VV2 (Fig 3E). Altogether, our findings suggest the presence of alterations in the p-bodies-dependent mRNA decay mechanisms without the activation of stress granule responses.

Altered miRNA biogenesis machinery in sCJD

Disruption of the miRNA biogenesis pathway components might cause alteration of miRNA homeostasis and neurodegeneration [42,43]. miRNA alterations in sCJD prompted us to consider possible alterations in the miRNA biogenesis pathway. The expression levels of three key components of miRNA biogenesis, the ribonucleases Dicer and Drosha and the microprocessor complex DGCR8, a cofactor of Drosha, were studied in sCJD brain samples. mRNA expression analysis revealed decreased DGCR8 levels in the CB of sCJD cases (Fig 4A). At the protein level, Drosha levels were significantly lower in the FC of sCJD MM1 and in the CB of sCJD VV2, resembling the regional and subtype pathological involvement of the disease. Decreased Dicer levels were detected in the FC of sCJD MM1, while reduced levels of DGCR8 were found in the CB of sCJD VV2 (Fig 4B). The absence of direct regional and/or subtype-specific alterations among the main components of the miRNA biogenesis pathway suggests the presence of a complex impairment of the miRNA biogenesis machinery in sCJD. Additionally, we investigated the expression levels of Exportin 5, a RanGTP-dependent dsRNA-binding protein mediating pre-miRNAs nuclear export [44,45], which expression is deregulated in AD, but not in PD or Down's syndrome dementia [46]. Exportin 5 levels in sCJD were altered neither at mRNA nor at protein levels compared to controls (S6 Fig).

Neural-type miRNA expression profiling in sCJD

A prominent hallmark in sCJD pathogenesis is the concomitant increase of neuronal loss and gliosis [2,47]. Since cell-type specific miRNA signatures have been described in neural populations [34,48,49], we aimed to investigate the neural-type miRNA expression profiling in sCJD. First, sCJD-regulated miRNAs were compared to those reported to be enriched in neurons, microglia and astrocytes [34]. The expression of neuron-enriched miRNAs was not significantly altered in sCJD (Fig 5A), indicating that the sCJD-related miRNA signature is not a mere consequence of neuronal death. On the other hand, most microglia and astrocyte-enriched miRNAs presented deregulated levels in sCJD, most likely as a result of glial proliferation and activation. To gain insight into subcellular and neural-type localization of sCJD-associated miRNAs in human brain tissue, *in situ* hybridizations were performed for the following miRNAs: miRNA-124-3p, miRNA-26-5p and miRNA-146a-5p (Fig 5B and 5C). The three miRNAs were localized in the cytoplasm of neurons. Additionally, miRNA-146a-5p labeling was also detected in capillary walls and in some small cells compatible with glial morphology.

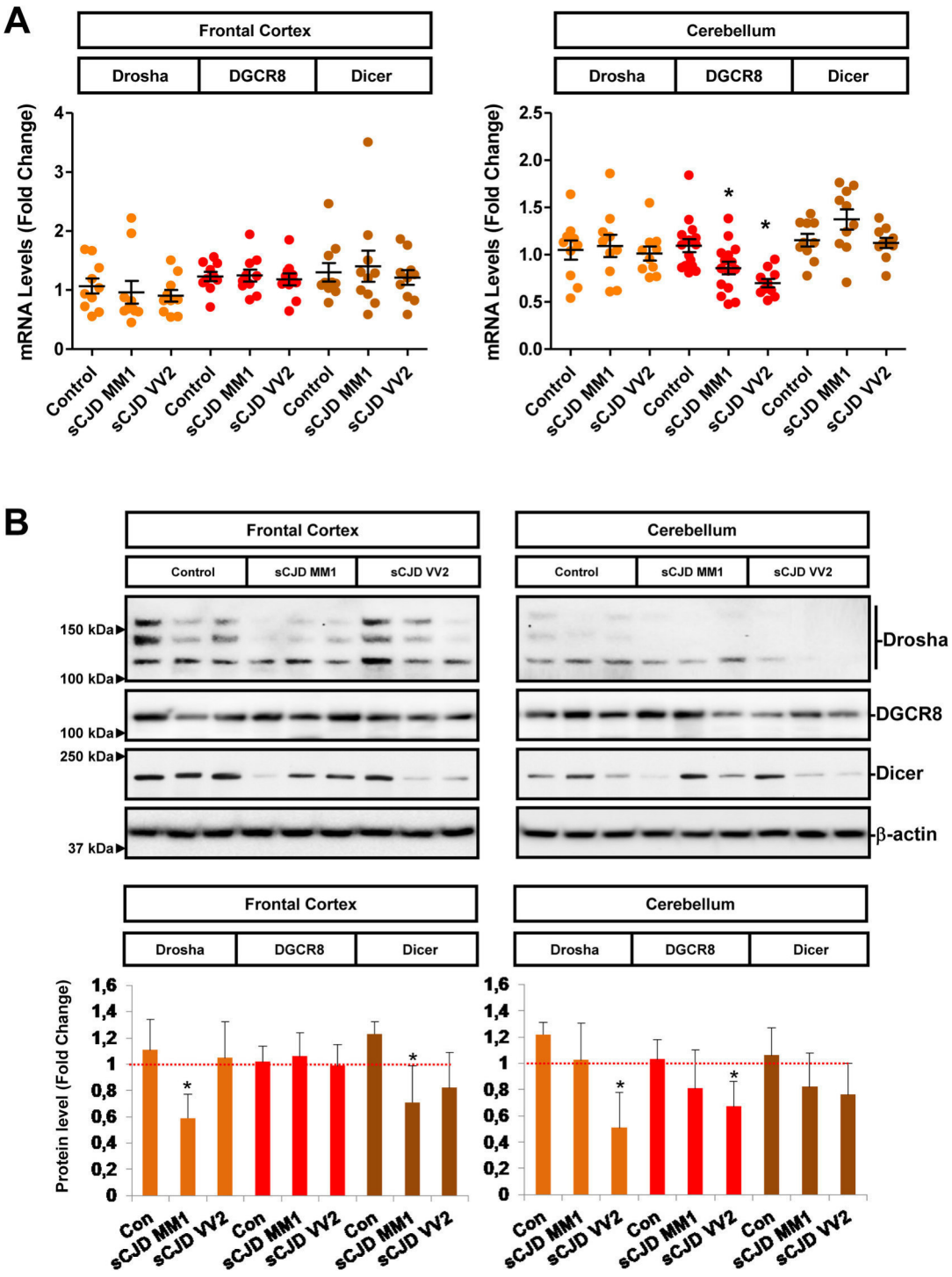


Fig 4. Altered expression levels of miRNA biogenesis components in sCJD. (A) Gene expression levels of Drosha (n = 10), DGCR8 (n = 10) and Dicer (n = 10) in the FC and CB of controls, sCJD MM1 and VV2 cases by RT-qPCR. Results were normalized to housekeeping genes GAPDH (figure) and GUSB with similar results. Housekeeping levels remained unaltered between groups. (B) Protein levels of Drosha, DGCR8, Dicer in the FC and CB of controls, sCJD MM1 and VV2 cases, by western blot analysis. Three representative cases per diagnostic group and brain region are shown in the western blot. Quantifications derived from densitometry analysis were performed in 15 cases per diagnostic group (n = 15/group). β-actin was used as a loading control. Densitometries of the western blots are shown. Normalization was performed relative to controls. Error bars indicate SD. In all cases, statistical significance (compared to controls) was set at $^{*}p<0.05$.

https://doi.org/10.1371/journal.ppat.1006802.g004

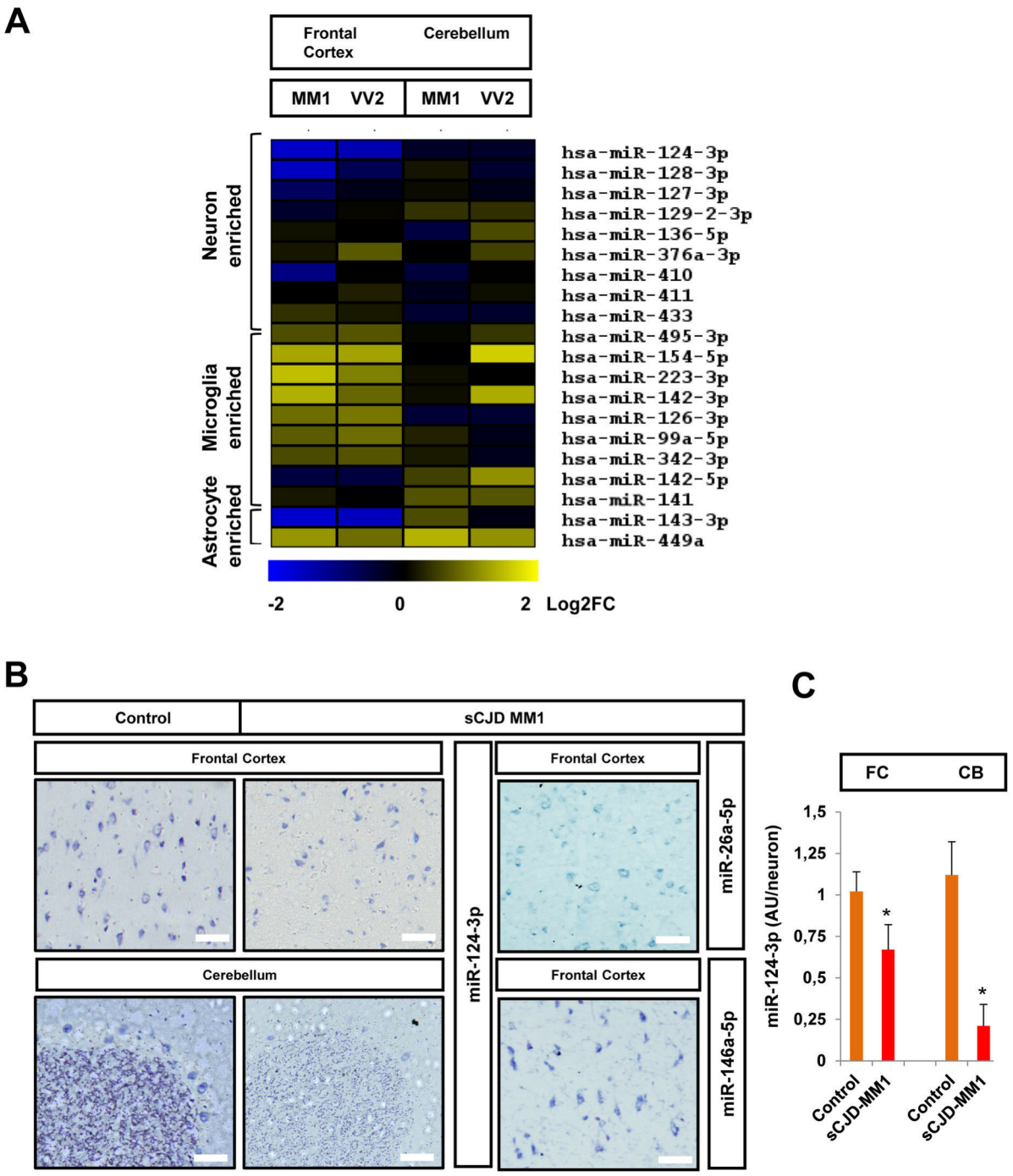


Fig 5. Neural-type miRNA profiling in sCJD. (A) Heat map analysis of the neuron, microglia and astrocyte enriched miRNAs, whose levels were changed in the FC and CB of sCJD MM1 and VV2 cases by RNA-seq analysis. Neural-type enriched miRNAs were reported in the bibliography based on neural-type enrichment analysis (49). (B) In situ hybridization of miRNA-124-3p in the FC and CB of control and sCJD MM1 brain tissue, and of miRNAs 26a-5p and 146a-5p in the FC of sCJD MM1 brain tissue. (C) Quantification of miRNA-124-3p intensity in the FC and CB of control and sCJD MM1 neurons. >100 neurons in total were quantified for each group. Normalization was performed relative to controls. AU/neuron indicates arbitrary units quantified in the densitometry analysis for each neuron. Error bars indicate SD. Scale bar in FC = 30μm and in CB = 50μm. In all cases, statistical significance (compared to controls) was set at $^{*}p<0.05$.

https://doi.org/10.1371/journal.ppat.1006802.g005

Decreased miRNA-124-3p staining detectable in sCJD was associated with a reduced number of neurons. However, we also detected less signal intensity in surviving sCJD neurons, both in the FC and in the CB (Purkinje and granular cells) regions (Fig 5B and 5C), in agreement with the idea that lower expression of neuronal-related miRNAs in sCJD is not exclusively associated with neuronal loss.

Cross-validation of sCJD-miRNA signature in alternative neurodegenerative diseases

Several neurodegenerative disorders share pathological hallmarks such as accumulation of protein aggregates and self-propagation, and common pathways seem to contribute to the neurodegenerative mechanisms in different diseases [50–52]. Thus, we speculated that an overlap between miRNA sCJD profiling and other dementia-related conditions could exist. In order to select the most appropriate miRNAs we compared the miRNA signatures in the FC of sCJD obtained from the small RNA-seq analysis from this study with the one reported in the pre-frontal cortex (PFC) of AD cases by Lau et al. [20]. Eight (22.8% max. coincidence) and seven miRNAs (14% max. coincidence) were commonly increased and decreased respectively in both datasets (Fig 6A). Among these, miRNA-195-5p, 877-5p and 323a-5p were previously validated in sCJD from our small RNA-Seq dataset and miRNA-195-5p and 877-5p were validated by qPCR (Fig 2A).

To perform a cross-validation study with the corresponding sCJD brain regions and methodologies we extracted RNA from the FC of AD and DLB cases and age-matched controls. Six miRNAs were selected: i) miRNA-195-5p, 877-5p and 323a-5p, commonly regulated in the FC of sCJD and in the PFC of AD, ii) miRNA-146a-5p and miRNA-342-3p, reported to be altered in AD and prion disease models [22,23,53,54] and iii) miRNA-5701. The latter was used as a negative control due to its exclusive enrichment in the CB of sCJD. In AD samples, we detected coincident gene expression regulations with sCJD for miRNA-195-5p (increased) and for miRNA-877-5p and 323a-5p (decreased) (Fig 6B). Rapid progressive forms of AD (rpAD) mimicking the disease progression and cognitive decline of sCJD [55,56] were included in our study. No significant differences in the expression levels of the six analyzed miRNAs were detected between AD and rpAD (Fig 6B). In DLB, we detected coincident gene expression regulations with sCJD for miRNA-877-5p and miRNA-323a-5p (both with decreased expression levels) (Fig 6C).

Finally, we extended our analysis to cases of fatal familial insomnia (FFI), a genetic prion disease presenting mild cortical and cerebellar affection [57,58]. A subset of four sCJD-regulated miRNAs in the FC and CB were tested. Only miRNA-195-5p showed common expression profiles in sCJD and FFI, with increased expression in both brain regions compared to age-matched control (Fig 6D) indicating a lack of complete specificity of miRNA patterns between neurodegenerative diseases from same etiology.

Temporal dependent sCJD miRNA profiling

To gain insights into the temporal-dependent sCJD miRNA profiles we took advantage of the sCJD MM1 mouse model tg340-PRNP129MM (tg340) inoculated with sCJD MM1 brain homogenate. Mice were sacrificed at pre-clinical (120 dpi), early clinical (160 dpi) and clinical (180 dpi and 210 dpi) disease stages. Survival time was 199 ± 7.5 days (Fig 7A). To confirm the disease-specific regional and subtype neuropathological and biochemical alterations in the tg340 mice, several prion hallmarks were assessed. Increased PrPSc deposition (Fig 7B), synaptic damage (Fig 7C and S7 Fig), neuroinflammation (Fig 7D) and spongiform degeneration (Fig 7E) were detected in the cortex compared to the CB of the tg340 infected mice. These data

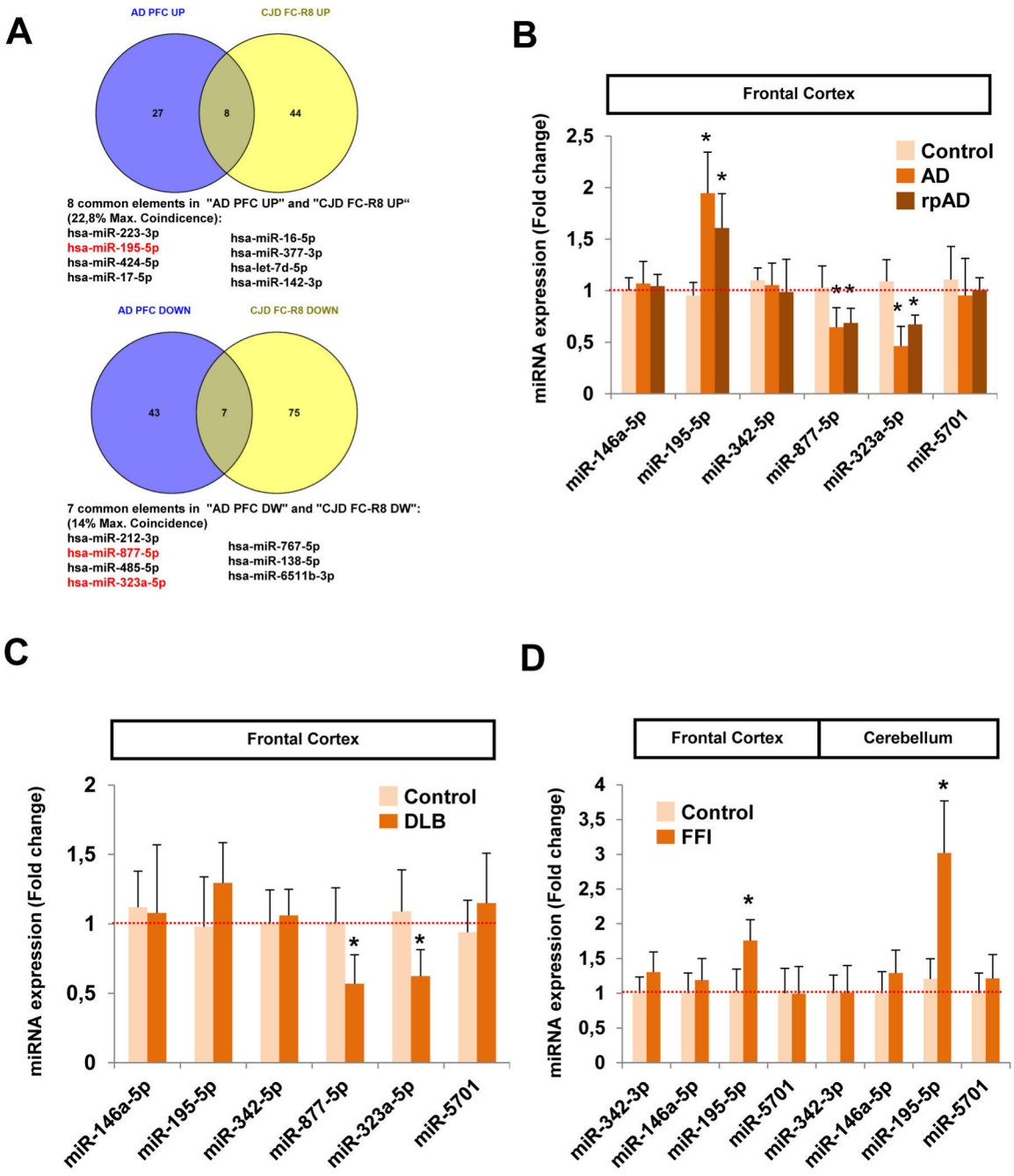


Fig 6. Analysis of commonly altered miRNAs in sCJD, AD, DLB and FFI. (A) Venn diagrams of the comparison between altered miRNAs in AD and sCJD. Commonly deregulated miRNAs in the PFC of AD cases obtained from small RNA-seq analysis (20) (blue circles) and in the FC of sCJD cases obtained from small RNA-seq analysis in the present work (yellow circles). Common elements and percentage of maximal coincidence between groups are shown. Among these, the deregulated expression levels of miRNA-195-5p, miRNA-877-5p and miRNA-323a-5p (marked in red) were previously validated by qPCR in the FC of sCJD cases (Fig 2). (B) RT-qPCR analysis for miRNA-146a-5p, miRNA-195-5p, miRNA-342-5p, miRNA-877-5p, miRNA-323a-5p and miRNA-5701 in the FC of controls (n = 5), AD (n = 8) and rpAD (n = 6) cases. (C) RT-qPCR analysis for miRNA-146a-5p, miRNA-195-5p, miRNA-342-5p, miRNA-877-5p, miRNA-323a-5p and miRNA-5701 in the FC of controls (n = 5) and DLB (n = 5) cases. (D) RT-qPCR analysis for miRNA-342-5p, miRNA-146a-5p, miRNA-195-5p and miRNA-5701 in the FC and CB of controls (n = 3) and FFI cases (n = 3). Results were normalized to the housekeeping U6 snRNA expression, which remained unaltered between groups. Normalization was performed relative to controls. Error bars indicate SD. In all cases, statistical significance (compared to controls) was set at $^{*}p<0.05$.

https://doi.org/10.1371/journal.ppat.1006802.g006

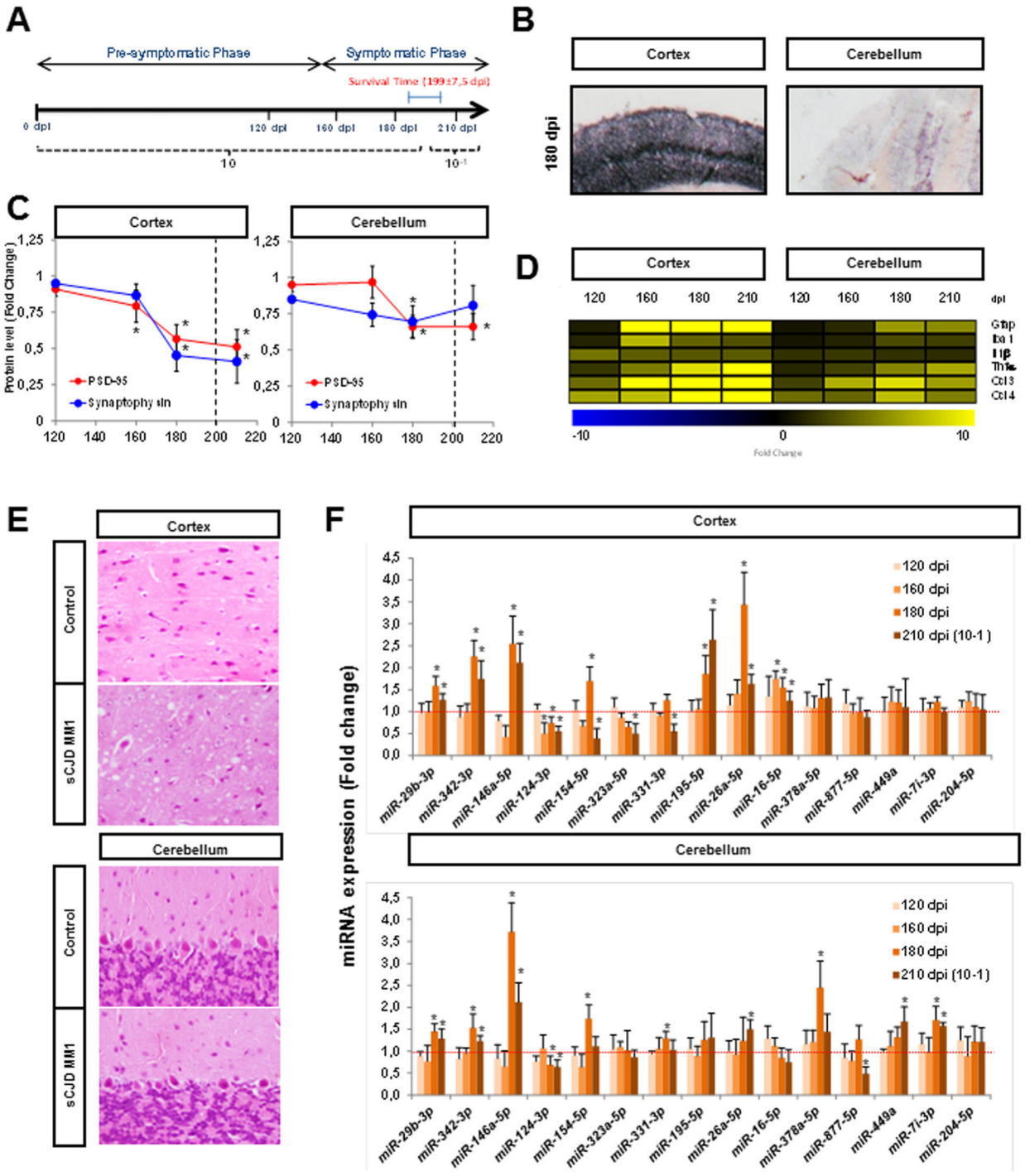


Fig 7. Regional and temporal-dependent neuropathological characteristics and miRNA signatures in the sCJD MM1 mouse model tg340-PRNP129MM. (A) tg340 mice were inoculated with control or sCJD MM1 homogenates; cortex and CB samples were collected at different time points: 120 dpi for pre-symptomatic phase and 160 dpi, 180 dpi and 210 dpi for symptomatic phase (n = 4–5 per group). Animals sacrificed at 210 dpi were inoculated with a 10–1 inoculum dilution. (B) PET-blot analysis for the detection of PrPSc in the cortex and CB of sCJD MM1 inoculated tg340 mice at clinical disease stage. (C) Densitometric analysis of western blots developed for PSD-95 and synaptophysin in the cortex and CB of tg340 samples at different disease stages. Significant alterations on PSD-95 and synaptophysin levels between control and sCJD inoculated animals is indicated. Statistical significance was set at $^{*}p<0.05$, (n = 4–5 per group). (D) Heat map analysis of key inflammatory mediators and cytokines measured with RT-qPCR analysis in the cortex and CB of control and sCJD MM1 inoculated tg340 mice at different stages of the disease. Fold change between sCJD MM1 infected and control animals is shown. (E) Hematoxylin-eosin staining in the cortex and CB of control and sCJD MM1 infected tg340 animals. (F) RT-qPCR analysis of the miRNAs validated in human sCJD tissue in the cortex and CB

of tg340 mice. Samples from different time points of disease progression were analyzed. Fold change between sCJD MM1 infected and control animals is shown. Results were normalized to the housekeeping gene U6 snRNA expression. U6 levels remained unaltered between groups. Normalization was performed relative to controls. Error bars indicate SD. In all cases, statistical significance (compared to controls) was set at $^{*}p<0.05$.

https://doi.org/10.1371/journal.ppat.1006802.g007

confirmed the region specific alterations of sCJD MM1 subtype in the tg340, resembling the most prominent cortical pathology in human sCJD MM1 [5,59]. Next, the expression levels of the qPCR-validated miRNAs in sCJD were analyzed in a temporal manner (Fig 7F). Ten and eleven miRNAs were validated in the cortex and in the CB at one of the clinical stages (180 and/or 210 dpi), respectively. Among these, seven were commonly changed in both regions, in agreement with data from sCJD MM1. An interesting observation from our qPCR panel was the temporal specific alterations of the sCJD-related miRNAs, since only miRNA-16a-5p (increased) and miRNA-124-3p (decreased) showed altered levels at early clinical stages of the disease in the cortex (Fig 7F and S8 Fig). This indicates that qPCR validated miRNAs are reflecting late pathogenic alterations, while miRNA-16a-5p and miRNA-124-3p may also participate in early pathogenic mechanisms. In agreement with this, functional enrichment analysis from small RNA-Seq indicates that the main common functions related to the sCJD-regulated miRNAs are cell death and survival (S5 Table).

These results suggested that diverse deregulated miRNA, rather than a specific miRNA deregulation, could contribute to the pathological mechanisms in sCJD. Therefore, we highly purified miRNAs from the FC of control, sCJD MM1 and VV2 brains and transfected them into neuroglioma (H4) and differentiated neuroblastoma (SH-SY5Y) cells. Five miRNAs were analyzed with qPCR in both cell lines, resembling the disease subtype profiling in human sCJD brain (S9A Fig). Overexpression of sCJD-MM1 purified miRNAs lead to an increased cell death in neuroblastoma, but not in neuroglia cells (S9B Fig), indicating that the overexpression of the sCJD regulated miRNAs is able to induce subtype specific cell death in neuron-like cells. Finally, to gain insight into the potential upstream regulators of differential miRNA expression in sCJD a motif enrichment analysis was performed for data generated from FC of sCJD MM1 sequencing. Among the known sCJD related pathways, our analysis revealed a significant enrichment of a STAT3-binding motif for miRNAs with increased expression in sCJD (S6 Table). This suggests that the expression of sCJD-specific miRNAs is under STAT3 regulation, which has been described as activated not only in experimental models of prion diseases [60,61], but also in sCJD post-mortem tissue [5].

Differential miRNA expression levels of abundant CSF miRNAs in sCJD

CSF miRNAs have been suggested as a source of biomarkers in neurodegenerative disease mirroring alterations in the brain tissue [62,63]. Thus, we aimed to investigate whether the detected miRNA alterations in the brain tissue of sCJD patients could be reflected in the CSF. CSF RNA was extracted from twelve control and twelve sCJD cases and was subjected to qPCR analysis for the following miRNAs: 154-5p, 204-5p, 378a-3p, 331-3p, 26a-5p, 195-5p, 124-3p, 7i-3p, 143-3p, 449a and 5701. For normalization we used the non-coding small nuclear RNA U6, which showed stable levels between control and sCJD cases (Fig 8A). Detectable signal (35<Cts) was obtained for miRNAs 378a-5p, 26a-5p and 204-5p, with miRNA-204-5p showing significantly decreased levels in sCJD compared to controls (Fig 8B).

Discussion

In the present study, we report the first systematic analysis of miRNA populations in two brain areas and two disease subtypes of sCJD cases, utilizing small RNA-seq analysis. We detected

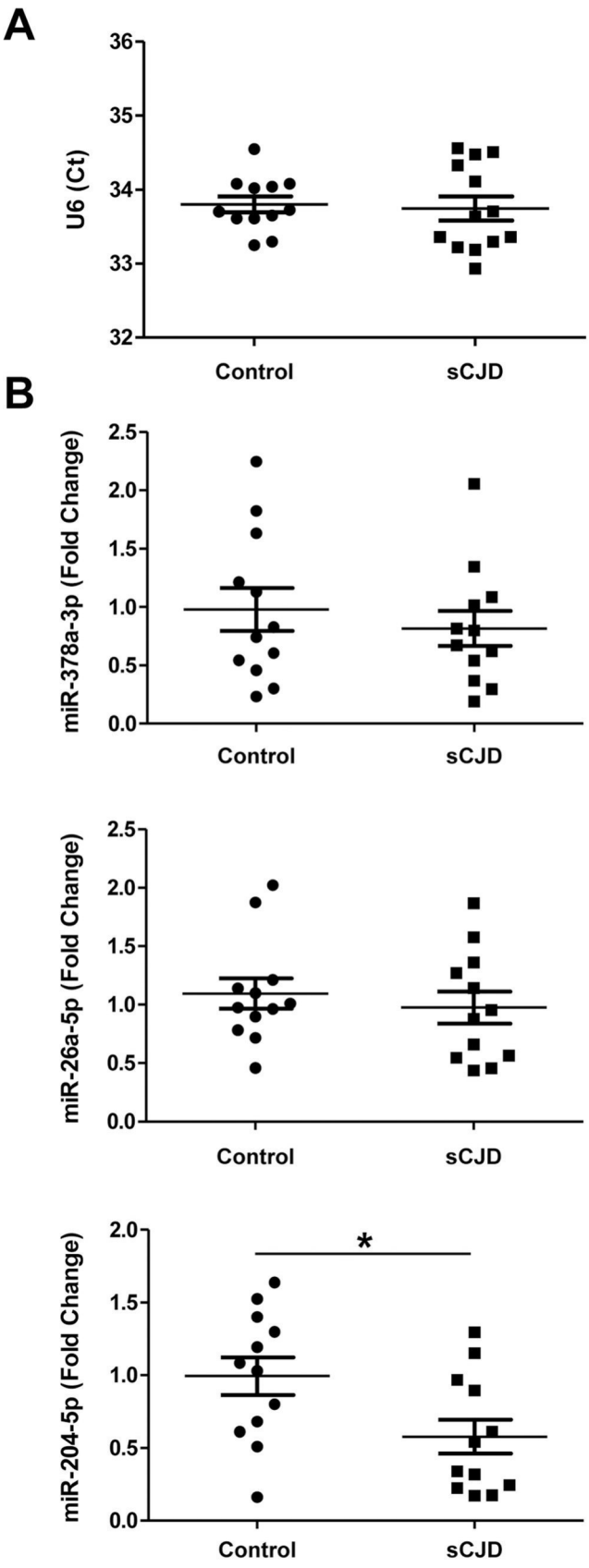

A
U6 (Ct)
36
35
34
33
32
Control
sCJD
B
miR-378a-3p (Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
Control
sCJD
miR-26a-5p (Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
Control
sCJD
miR-204-5p (Fold Change)
2.0
1.5
1.0
0.5
0.0
*
Control
sCJD

Fig 8. miRNA profiling in the CSF of control and sCJD cases. RT-qPCR analysis of the housekeeping U6 snRNA (A) and miRNA-378a-3p, miRNA-26a-5p and miRNA-204-5p (B) in the CSF of control (n = 12) and sCJD cases (n = 12). Samples were normalized by the relative expression of the housekeeping small nuclear RNA U6, which showed stable Ct values between the studied groups. Statistical significance was set at $^{*}p<0.05$.

https://doi.org/10.1371/journal.ppat.1006802.g008

marked alterations in miRNA patterns with the presence of regional and sCJD-subtype specific signatures, with highest deregulations in the FC of MM1 and in the CB of VV2 cases, two brain regions and subtypes showing high pathological affection in sCJD [1]. In this regard, the low overlap between sCJD subtypes altered miRNAs in CB and FC could be explained by the singular pathology of sCJD VV2 in CB, where characteristic PrPSc aggregates (synaptic and plaque-like) and degree of spongiform degeneration, neuronal loss and neuroinflammation profiling are detected [5–7], following classical well-known sCJD regional and subtype-dependent molecular neuropathology [2,47,64].

Small RNA-seq provides a blind and unbiased approach to the study of the small RNA transcriptome. However, a limitation of this technique is the presence of potentially biased fold changes when number of counts is low. Therefore, confirmatory analysis by qPCR is indispensable to consistently validate the regulation of selected targets. The enrichment of regulated miRNAs in Ago-containing complexes, as well as a severe, global reduction of miRNA expression levels in sCJD compared to controls described herein, supports the idea that alterations in expression levels are translated into the functional miRNA silencing machinery. Small RNA-seq also revealed length and sequence heterogeneity for the vast majority of miRNAs. However, the fact that the proportion of different miRNA variants detected by isomiR profiling was similar in all cases indicates that the molecular mechanisms involved in isomiR generation are not altered in sCJD, similarly to the situation previously reported for HD [15].

Data on miRNA alterations in sCJD are limited to two targeted studies, including very small cohorts of cases. Upregulation of miRNA-146a-5p in the neocortex of sCJD cases (n = 3 sCJD, n = 3 controls) [65] and upregulation of miRNA-342-3p in sCJD brain tissue (n = 2 sCJD, n = 1 control) [23] are in agreement with our observations. In contrast, several studies have been devoted to analysing the miRNAome in prion animal models. A lack of major correlation between regulated miRNAs in sCJD and scrapie-infected mice was detected in high-throughput studies. However, miRNAs: 146a-5p, 342-3p, 142-3p, 26a-5p, 124a-3p (RNA-seq altered and qPCR validated in sCJD) and 338-5p, 18a-3p, 455-5p, 182-5p (RNA-seq altered in sCJD) are commonly altered in at least one of the studies where scrapie miRNA profiles have been investigated [21–23,25,33,66]. Additionally, two miRNAs upregulated in the *basis pontis* of bovine spongiform encephalopathy-infected macaques, miRNA-342-3p and miRNA-494-3p [23], were also enriched in sCJD, but exclusively in the FC region. Although low co-occurrence on miRNA profiles may be due to different methodologies, the absence of detailed regional studies in mouse models and the specific prion-related pathology in humans may explain this divergence. Altogether, these data highlight the importance of detailed regional and disease-subtype studies in prion diseases. Yet, intra-species comparisons are now achievable through the study of humanized *PRNP* mouse models, which not only fully recapitulate pathological hallmarks of human disease [5,59] but also, as reported in the present study, resemble human miRNA profiling. In this regard, alterations in miRNA signatures in tg340 at clinical stages are not detectable at pre-clinical stages. This finding, along with the presence of enriched miRNA-target genes related to cell death and survival, indicates that sCJD-regulated miRNAs may play a role in the molecular mechanisms related to the neurodegenerative process and that a different population of miRNAs, would be responsible for the primary causative events of the disease.

Relative decreased mature miRNA levels in sCJD are consistent with decreased expression levels of Dicer, Drosha and DGCR8. The miRNA biogenesis pathway is highly conserved and

its disruption is a well-reported cause of neurodegeneration. Loss of Dicer levels provokes neuronal dysfunction in Purkinje cells [67], dopaminergic neurons [68] and motor neurons [69], and increased excitability of CA1 pyramidal neurons [70] while compromising axonal integrity in Schwan cells [71]. In addition, Dicer protein levels have been found to be decreased in temporal lobe epilepsy patients with hippocampal cell loss, with about half of the miRNAs in the tissue displaying reduced levels [72]. Finally, similar to our observations, impaired miRNA biogenesis at Dicer level associated with downregulation of miRNA levels and reorganization of Dicer and Ago-2 complexes has recently been described in amyotrophic lateral sclerosis [73] suggesting that miRNA malfunction could be a contributor to pathogenesis associated with protein-misfolding associated diseases. Interestingly, lack of expression changes in Exportin 5 suggests that changes detected in the miRNA expression profiles in sCJD are most likely not due to alterations in the nuclear export of pre-miRNA.

Whether alterations of miRNA biogenesis and homeostasis in sCJD are primary factors in the neurodegenerative phenotype of the disease due to dysfunctional miRNA maturation, or are a consequence of the pathology, deserves further studies.

Besides highlighting alterations in miRNA biogenesis and network, our results also reveal a remodelling of the miRNA-mRNA silencing complexes in sCJD. This is sustained by partial re-distribution of Ago-2 to higher molecular weight chromatographic fractions, increased Ago nuclear reactivity, presence of Ago-2 positive microglial cells and increased expression of p-body marker dcp1 in sCJD brain tissue. Ago-2 and RISC components have recently been found in the nucleus of humans and Drosophila and associated in multi-protein complexes with functional silencing activity over nuclear targets [74,75], as well as with additional functions in pre-mRNA splicing and transcriptional repression [75]. The role of Ago proteins, especially Ago-2, in prion pathology deserves attention not only due to its differential localization in sCJD, potentially altering its physiological functions, but also because PrPC has been described as an Ago-2 interacting partner, promoting the accumulation of miRNA-RISC effector complexes [26]. PrPC is internalised into the endocytic recycling pathway and most of the molecules are recycled intact to the cell surface [76]. Since late endosomes and/or multivesicular bodies are the main site for intracellular conversion of PrPC to PrPSc, and RISC formation and/or turnover depends on the endosomal pathway [77,78]; the detection of PrPSc in Ago-2 complexes provides a link between RNA silencing and membrane trafficking in sCJD pathogenesis. Additionally, a translocation of Ago-2 from cytoplasm to nuclear fractions in sCJD would alter, and potentially impair, the silencing of cytoplasmic mRNA targets by the RISC complex.

As additional modifiers of the miRNA-mRNA silencing complexes in sCJD we investigated the presence of SG, which appear in the cell under stress conditions such as oxidative and endoplasmic reticulum stress [79,80], two well-known sCJD hallmarks [41,81]. Although both p-bodies and SG may support overlapping cellular functions and share components, they are not equivalent and they are spatially distinct. SG are thought to be responsible for mRNA storage as these sites lack the decapping enzyme [82], and their formation is mediated through phosphorylation of eIF2α and aggregation of Tia-1 [83,84]. Neither increased eIF2α phosphorylation nor altered levels of SG markers were detected, suggesting that SG are not specific structures in sCJD.

A surprising finding of our study was the absence of massive reduction of neuronal-enriched miRNAs levels, indicating high miRNA stability in brain after neuronal death. An exception to this was the decreased levels of miRNA-124-3p, also decreased in experimental models of prion diseases [21,22,66]. *In situ* hybridization supports the idea that miRNA-124-3p is not merely decreased as a consequence of neuronal death, since surviving neurons express less miRNA-124-3p compared to those from controls, pointing towards a specific role

for this miRNA in sCJD pathology. miRNA-124-3p is the most abundant miRNA in the brain and promotes neuronal differentiation and maintenance of neuronal identity [85]. In AD, its expression is decreased in the anterior temporal cortex [86], decreased in the dentate gyrus, and upregulated in the locus coeruleus [87]. In an *ex vivo* model of PD, miRNA-124-3p is decreased regulating apoptosis and impaired autophagy [88]. This plethora of evidence denotes a functional role of miRNA-124-3p in neurodegeneration.

In fact, our study details the existence of common miRNA traits in the cortical region of AD, DLB and sCJD. There is virtually no information on the functions of the two commonly regulated miRNAs in the brain of the three dementias (miRNA-877-5p and miRNA-323a-5p). On the contrary, miRNA-195-5p, elevated in AD and sCJD, downregulates Aβ production by targeting APP and BACE1, and protects against chronic brain hypoperfusion-mediated dementia [89]. Its overexpression also led to reduced BACE1 and decreased Aβ levels in an independent study [90]. Although the precise role of miRNA-195-5p in prion diseases is unknown, we also detected increased levels in the FC of fatal familial insomnia (FFI), a genetic prion disease with moderate cortical involvement [91].

Based on the conception that one miRNA can target multiple mRNAs and one mRNA can be targeted by multiple miRNAs, our results support multiple lines of evidence indicating that the result of the intricate alterations in miRNA networks and clusters, rather than representing a change in the expression of a single miRNA, are responsible for pathological phenotypes [92–94]. Thus, the precise miRNA homeostasis in sCJD brain would underlie the spectrum of molecular and phenotypic cues, in agreement with the acquisition of a disease-related phenotype by a neuronal-like cell line upon transfection with the sCJD-associated miRNA transcriptome.

CSF miRNAs may reflect alterations in brain pathology of neurodegenerative diseases. Indeed, miRNA profiling in AD and PD correlates with disease status and pathological features [63,95] but less is known about the levels of brain-regulated miRNAs in the CSF. Our targeted study revealed that only miRNAs with reported high CSF expression levels were detectable [96]. Of these, only miRNA-204-5p displayed decreased levels in sCJD cases. Interestingly, this miRNA was not statistically regulated in sCJD brain. Our findings are in line with those reported in AD, where no obvious relationship between altered miRNAs in CSF and pathologically affected brain regions was found [17,95]. As the reason for this lack of correlation is unknown, it is tempting to speculate that different disease stages between CSF and brain sample collection (time of diagnosis for CSF versus post-mortem for brain tissue) may contribute to these differences. Additionally, as the origin of CSF miRNAs is not well understood, CSF miRNAs may originate not only from brain, but also from extracraneal tissues.

In summary, our study presents, for the first time, comprehensive miRNA signatures in human prion diseases and unravels the complex network of regional and disease-subtype miRNA alterations in sCJD, as well as revealing the presence of a disturbed miRNA biogenesis pathway and miRNA-mRNA silencing machinery. It also highlights the existence of time-dependent miRNA profiles along disease duration and identifies commonly altered miRNAs between several dementias sharing a partial clinical overlap. Taken together, the present data shed light on the potential role of miRNAs as a contributing factor and/or transmitters of pathogenic molecular traits in sCJD.

Materials and methods

Reagents

List of Taqman probes assays, Exiqon primer sets and antibodies is given in S7 Table. Lipofectamine 2000 was from Thermo Fisher Scientific. Thioflavin and Propidium Iodide were from

Sigma. WST-1 was from Roche. TruSeq Small RNA Sample Preparation Kit was from Illumina. CHROMA SPIN-200 spin columns were from Clontech and Protein G magnetic beads were from Invitrogen.

Cases and general processing

Brain tissue processing has been described before [5–7,97]. Mean ages and gender for studied control and sCJD cases, for RNA-seq, qPCR and western blot analysis are detailed in S8 Table. Information on the mean ages and gender for the AD, DLB and FFI samples that were analysed with RT-qPCR in the present study are as below: For AD analysis: Control = 75 (3M/2F), AD = 76 (4M/4F) and rapid progression AD (rpAD) = 77 (3M/3F). rpAD cases were AD cases with disease duration shorter than 2 years. For DLB analysis: Control = 71 (3M/2F), DLB = 75 (3M/2F). For FFI analysis: Control = 58 (2M/1F), FFI = 52 (2M/1F). Biochemical studies including sCJD and FFI cases were performed in biosafety rooms (S3 level). mRNA and miRNA levels were associated neither to RNA integrity number (RIN) values nor to post-mortem time. Protein levels were not associated to post-mortem time.

CSF samples were obtained from an unrelated series of patients with sCJD and from controls. sCJD patients diagnosed with probable or definite sCJD according to established criteria were considered for the sCJD group [98]. The control group was composed of patients suffering from neurological conditions (S9 Table). The presence of neurodegenerative diseases in the control cohort was excluded in the follow-up clinical diagnostic, and CSF neurodegenerative biomarkers (14-3-3, tau, p-tau and Aβ42) were negative at the time of diagnosis.

CJD subtype characterization

PRNP codon 129 genotyping (Met or Val) was performed after genomic DNA isolation from blood samples according to standard methods [99]. Western blot PrPSc profile was classified as type 1 (un-glycosylated PrPSc at 20 kDa) or type 2 (un-glycosylated PrPSc at 19 kDa), based on electrophoretic mobility after proteinase K (PK) digestion [4,64].

RNA extraction and sequencing

The purification of RNA from FC and CB of CJD and age-matched controls was performed using the mirVana isolation kit (Ambion, US) according to the manufacturer's instructions. After purification, samples were treated with the RNase-free DNase set (Ambion, US) for 30 min to avoid carry over and subsequent amplification of genomic DNA. The concentration of each sample was determined using the NanoDrop 2000 spectrophotometer (Thermo Scientific, US). RNA integrity number (RIN) was verified with the Agilent 2100 Bioanalyzer (Agilent, US). The threshold for further sample selection was set to RIN value equal to or greater than 5.5. Starting from 1 μg of total RNA, libraries were prepared following the TruSeq Small RNASample Preparation Guide protocol from Illumina (Part # 15004197 Rev. E). Library quality was assessed on the Agilent Technologies 2100 Bioanalyzer. DNA was loaded into a lane of a single-read flow cell at a concentration of 3–3.5 pM for cluster generation using a single-read cluster generation kit (Illumina). From 13 to 15 barcoded samples were sequenced per lane. The sequencing primer (5′-CGACAGGTTCAGAGTTCTACAGTCC GACGATC-3′) was annealed to the clusters and the flow cell was then mounted on a Hiseq 2000 instrument (Illumina) for sequencing, and 36–41 sequencing cycles were performed. A PhiX control lane loaded at a concentration of 2 pM was used to monitor run quality. Image processing and base calling was performed using Illumina sequencing analysis pipelines v0.3.0 or v1.3.2. A total of 72 samples were analyzed by small RNA-seq: For FC 13 controls, 13 sCJD MM1 and 13 sCJD VV2 were analyzed for CB: 12 controls, 12 sCJD MM1 and 9 sCJD VV2.

Small RNA data processing and mapping

Reads were trimmed to 36 nt and ligation adapters were removed using the adrec.jar program from the seqBuster suite (http://github.com/lpantano/seqbuster) [28] with the following options: java -jar adrec.jar 1 8 0.3. Sequences were mapped to the hg19 genome with the command line: bowtie -f -v 1 –a–best–strata. For summing up miRNA read counts we mapped the reads against miRBase version 21 hairpins with the miraligner.jar tool with these options: java–jar miraligner.jar 1 3 3.

Out of a total of several million reads, we discarded any reads without a minimum of 10-nt linker subsequence directly adjoining the insert, showing two or less mismatches. Then sequences were mapped to human pre-miRNA and mature miRNA databases provided in the miRBase (http://miRNA.sanger.ac.uk/sequences/, Release 14), as well as mRNA, ncRNA, repeats and genome databases available at (http://hgdownload.cse.ucsc.edu/goldenPath/hg18/bogZips/), using Mega BLAST.

For motif discovery, deregulated miRNAs were searched for the genomic coordinates of their primary miRNAs. Upstream regions of 1kb size from each miRNA were extracted and exported to BED files and the script findMotifsGenome.pl in HOMER suite was used to find transcription factor binding motifs within promoter regions using genome assembly hg38 [100].

For differential expression analysis we used DESeq2 analysis and log2 transformation of the count data. Padj value was <0.15 and nominal p values were in all cases <0.05. We used the count matrix generated by Seqbuster. Only miRNAs consistently expressed (counts > 10) in at least 10 samples out of the 21–26 were included in each analysis (controls versus MM1 or VV2 cases).

Analysis of miRNA variability (IsomiRs)

IsomiRs were annotated and analyzed using the SeqBuster tool [28]. For miRNA annotation the following parameters were configured: one mismatch, 3 nts in the 3' or 5'-trimming variants, 3 nts in the 3'-addition variants. These options permitted annotations of the following types of alignment: (i) perfect match, where the sequence is completely identical to the reference sequence; (ii) trimming at the 3'-end of the reference miRNA sequence, which is an miRNA variant several nucleotides shorter or longer that matches to the mature or precursor reference sequence, respectively; (iii) trimming at the 5'-end of the sequence, an analogous case as to (ii) but focused on the 5'-end of the miRNA; (iv) nucleotide additions at the 3'-end of the sequence and (v) nucleotide substitutions, showing nucleotide changes with respect to the reference sequence. The parameters for the alignment to the mRNA and genome databases allowed up to one mismatch and up to three nucleotide additions in the 3'-terminus.

For deep characterization of IsomiRs we applied several filters. First, the sequences considered in the analysis presented a frequency above 3. Second, 10 was chosen as the 'Contribution Cut-Off' parameter, meaning that every isomiR considered in the analysis contributes by more than 10% to the total number of variants annotated in the same miRNA locus. Third, we applied the Z-score option to exclude sequencing errors as the possible cause of the nucleotide changes observed in some variants.

miRNA purification for transfection experiments

RNA samples extracted with the mirVANA isolation kit (Ambion) using the specific protocol for small RNA isolation were run on a 6% Urea–PAGE. The bands containing miRNAs (15–30 nt) were excised from the gel and incubated for 1 hour with 250 mM NaCl and 1 mM EDTA. miRNAs were then precipitated with 2.5 volumes of 100% ethanol (v/v) over night at −80˚C, washed twice with 70% ethanol and re-suspended in nuclease-free water.

Cell cultures and transfection

H4 and SH-SY5Y cells (American Type Culture Collection) were cultured at 37˚C in a 95%/5% Air/CO2 water-saturated atmosphere in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific) containing 10% heat inactivated fetal bovine serum (FBS, Thermo Fisher Scientific), 2 mM L-glutamine and 100U/ml Penicillin/streptomycin (Gibco). SH-SY5Y cells were differentiated with DMEM containing 3% FBS and 10 μM all-trans retinoic acid (RA, Sigma) for 72 hours. Differentiation medium was replenished after 48 hours. Cells were transfected with 250 ng of highly purified miRNAs with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

Gel filtration

Analysis of protein fractions according to their molecular weight was performed as described before [36] using CHROMA SPIN-200 (Clontech, USA) spin columns. Columns were pre-spun at 200xg for 3 min to remove storage buffer. Buffer exchange was made by the addition of 500 μl lysis buffer followed by centrifugation at 200 xg for 3 min. This step was then repeated. 75 μl of 1% brain homogenates were applied to the gel bed. After spinning at 120 xg for 2 min elution fractions were collected, and 40 μl of extraction buffer was added after each centrifugation step.

sCJD MM1 mice–tg340 PRNP129MM

The tg340 mouse line expressing about 4-fold level of the human PrP M129 on a mouse PrP null background was generated as described elsewhere [59]. Control and sCJD MM1 brain tissues (10% (w/v) homogenates) were used as inocula. Individually identified 6–10 week-old mice were anesthetized and inoculated in the right parietal lobe using a 25-gauge disposable hypodermic needle. Additionally, MM1 inoculum dilutions were performed to study prolonged disease times; animals were sacrificed at 210 dpi (10–1 dilution). Mice were observed daily and their neurological status was assessed weekly. The animals were euthanized at pre-symptomatic (pre-clinical: 120 dpi) and symptomatic (early clinical: 160 dpi and clinical: 180 dpi) stages and the brain was removed. A part of the brain was fixed by immersion in 10% buffered formalin, to quantify spongiform degeneration and perform immunohistological analysis. The other part was frozen at −80˚C, for extraction of protein and RNA. Paraffin-embedded tissue blots from tg340 mice samples was carried out as described previously [5]. For each tissue sample, serial sections, 4 mm thick for PET blot, were collected on membranes. Serial sections were stained with hematoxylin and eosin. SHa31 antibody was used for PrP immunodetection.

RT-qPCR

In order to confirm the direction of the miRNA alterations detected by RNA-seq in sCJD cases (increased or decreased levels compared to controls) by an independent methodology qPCR analysis of selected miRNAs was performed. Quantitative real time PCR for miRNAs was performed using the miRCURY LNAmiRNA PCR System (Exiqon) following Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines. RNA was extracted with the mirVana isolation kit (Ambion) following the manufacturer's instructions. PCR amplification and detection were performed with the Roche LightCycler 480 detector, using 2x SYBR GREEN Master Mix. The reaction profile was: Polymerase Activation/Denaturation (95˚C for 10 min) followed by 40 amplification cycles (95˚C-10 sec, 60˚C-20 sec). miRNA levels were calculated using the LightCycler 480 software. Samples were normalized

by the relative expression of the housekeeping small nuclear RNAs U6 and U5. Housekeeping genes showed no variability between analyzed groups. CT values obtained from the miRNA qPCR analysis ranged from 18 to 31.

Quantitative real time PCR for mRNAs was performed using Taqman probes (Life Technologies) on total RNA extracted with mirVana's isolation kit (Ambion) following the manufacturer's instructions. PCR assays were conducted in duplicate using cDNA samples obtained from the retrotranscription reaction and diluted 1:15 in 384-well optical plates. PCR amplification and detection were performed with the Roche LightCycler 480 detector, using Taqman Universal PCR Master Mix, following the manufacturer's instructions. The reaction profile was as follows: denaturation–activation cycle (95˚C for 10 min) followed by 40 cycles of denaturation–annealing–extension (95˚C, 10 min; 72˚C, 1 min; 98˚C, continuous). mRNA levels were calculated using the LightCycler 480 software. Samples were normalized based on the relative expression of a housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]). The housekeeping gene showed no variability between analyzed groups.

Western blot

Human tissues were lysed in lysis buffer: 100 mM Tris pH 7, 100 mM NaCl, 10 mM EDTA, 0.5% NP-40 and 0.5% sodium deoxycolate plus protease and phosphatase inhibitors. After centrifugation at 14,000g for 20 min at 4˚C, supernatants were quantified for protein concentration (BCA, Pierce), mixed with SDS-PAGE sample buffer, boiled, and subjected to 8–15% SDS-PAGE. Gels were transferred onto nitrocellulose membranes and processed for specific immunodetection by chemiluminescence (ECL Amersham, US) using the indicated antibodies. Densitometries were carried out with the ImageJ software and values were normalized using β-actin levels.

RISC immunoprecipitation

Protein G magnetic beads were pre-equilibrated in lysis buffer (150 mM NaCl, 50 mM Tris, 0.5% NP40, protease and phosphatase inhibitors) and mixed with 1 mg of human FC brain homogenate from either control or sCJD MM1 cases and with 4 μg of Ago antibodies 11A9 or H-300. As a control, 4 μg of an unspecific IgG antibody was used. Complexes were incubated overnight at 4˚C with gentle end-to-end shaking. To extract the immunoprecipitated RNA, beads were washed three times in lysis buffer and resuspended in phenol-chloroform. The RNA in the aqueous phase was precipitated for 1 h at -80˚C after addition of 2.5-fold volume ethanol and 0.1-fold volume NaAc (3mol/l); precipitated RNA was pelleted by centrifugation for 25 min at 4˚C at 20,000xg. After washing in cold 70% ethanol, centrifugation and air drying, RNA was re-suspended in 10 μl of RNase-free water. The miRCURY LNA Universal RT miRNA PCR kit (Exiqon) was used for miRNA reverse transcription. For this, 6.5 μl of re-suspended RNA was applied in a total RT reaction volume of 10 μl (2μl 5x reaction buffer, 1μl enzyme mix, 0.5μl nuclease free water). cDNA was synthesized as described before for miRNA RT. A 1:80 cDNA dilution was used for miRNA quantification via real-time PCR amplification and miRNA LNA primer sets.

miRNA in situ hybridization

For miRNA recognition locked nucleic acid (LNA) modified probes combined with signal amplification technology using enzyme-labeled immunoassay were obtained from Exiqon (Vedbaek, Denmark). The following double digoxigenin (DIG)-labelled sequences of the LNA probes were used: hsa-miRNA-124: 5'-DIG/ggcattcaccgcgtgcctta/DIG-3', hsa-miRNA-146a 5'-DIG/aacccatggaattcagttctca/DIG-3', has-miRNA-26a 5'-DIG/agcctatcctggattacttgaa/DIG-3'.

The sequence of the U6 snRNA positive control probe was: 5'-DIG/cacgaatttgcgtgtcatcctt/-3'. 6 μM-thick brain tissue sections were deparaffinised, deproteinized with Proteinase K (15μg/ml) at 37˚C for 10 min, washed in PBS and dehydrated in increasing concentrations of ethanol. Probe hybridization was performed over night at 55˚C with 100 nM (hsa-miRNA-146a, hsa-miRNA-26a), 40 nM (miRNA-124), or 1 nM (U6 snRNA) of LNA probe diluted in hybridization mix. After stringent washing in salt sodium citrate (SSC) buffer and blocking with 2% sheep serum/1% bovine serum albumin, probe-target complex was visualized immunologically with anti-DIG antibody (Roche, 1:800) conjugated to alkaline phosphatase acting on the chromogen NBT/BCIP. In some cases, slides were counterstained with nuclear fast red (Vector laboraties). For quantification of miRNA-124-3p, 3 controls and 2 sCJD MM1 cases were used.

Immunofluorescence

For immunofluorescence analysis in brain tissues, de-waxed sections, 4 microns thick, were stained with a saturated solution of Sudan black B (Merck, DE) for 15 min, to block the autofluorescence of lipofuscin granules present in cell bodies, and then rinsed in 70% ethanol and washed in distilled water. The sections were boiled in citrate buffer to enhance antigenicity and blocked for 30 min at room temperature with 10% fetal bovine serum diluted in PBS. Then, the sections were incubated at 4˚C overnight with primary antibodies. After washing, the sections were incubated with Alexa488 or Alexa546 (1:400, Molecular Probes, US) fluorescence secondary antibodies against the corresponding host species. The sections were mounted in Immuno-Fluore mounting medium (ICN Biomedicals, US), sealed, and dried overnight. Sections were examined with a Leica TCS-SL confocal microscope.

Real time quaking induced conversion (RT-QuIC)

RT-QuIC was performed as previously described [101] with minor modifications. Briefly, recombinant PrP (10 μg) was seeded with 15 μl of Ago-2-Immunoprecipitates in 85 μl of reaction buffer. Reaction was set in a final volume of 100 μl and placed in a 96-well black optical bottom plate (Fisher Scientific). Each sample was run in duplicate. Prepared plates were sealed and incubated in a FLUO Star OPTIMA plate reader (BMG Labtech Ortenberg, GE) at 42˚C for 80 h, with intermittent shaking cycles consisting of 1 min double orbital shaking at the highest speed (600 rpm) followed by a 1 min break.

CSF analysis

Lumbar punctures were performed for diagnostic purposes at the time point of the first diagnostic work-up and samples were stored at −80˚C until analysis. 14-3-3 protein was analyzed as described previously [102] and total tau was quantitatively measured using the enzyme-linked immunosorbent assay kits INNOTEST-hTAU-Ag from Fujirebio according to the manufacturer's instructions. RT-QuIC analysis was performed as described before [101]. RNA purifications from CSF were performed using miRCURY RNA Isolation Kit–Biofluids (Exiqon) following manufacturer-provided protocol with minor modifications. 200 μl CSF input volume was used and treated with 2 μg/μl Proteinase K in order to optimize the RNA yield. As an inert RNA carrier 2 μg Glycogen per CSF sample was added. The miRCURY LNA Universal RT miRNA PCR kit (Exiqon) was used for miRNA reverse transcription. For this, 4 μl of re-suspended RNA was applied as input in a total RT reaction volume of 10 μl (2 μl 5x reaction buffer, 1 μl enzyme mix, 3 μl nuclease free water) and cDNA was synthesized as described before for miRNA RT. A 1:80 cDNA dilution was used for miRNA quantification via real-time PCR amplification and miRNA LNA primer sets. The small RNA U6 revealed to be stable

in CSF samples from sCJD and control samples and was used as reference gene for miRNA quantification.

Statistical analysis

For comparisons of the two groups, the Mann-Whitney test was used. In multiple comparisons, the Kruskal-Wallis test was used. Dunn's multiple comparison test was used for *post hoc* analysis. Statistical analyses and calculations were carried out using GraphPad Prism 5 software. Statistical significance was set at $^{*}p<0.05$.

Ethics

Brain tissue samples were obtained from the Institute of Neuropathology Brain Bank (HUB-ICO-IDIBELL Biobank) and the Biobank of Hospital Clinic-IDIBAPS, following pertinent guidelines of the Spanish legislation and the local ethics committee. The present study was conducted according to the revised Declaration of Helsinki and Good Clinical Practice guidelines and was approved by the local ethics committees (University of Göttingen -No. 9/6/08, 19/11/09 and 18/8/15). Informed written consent was given by all study participants or their legal representative. All participants were adults, and samples were anonymized.

For animal investigation, principles of laboratory animal care (NIH publication No. 86–23, revised 1985) were followed. All animal experiments were performed in compliance with the French, national guidelines, in accordance with the European Community Council Directive 86/609/EEC. The protocols comply with the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines. The experimental protocol was approved by the INRA Toulouse/ENVT ethics committee (Permit number: 310955547).

Supporting information

S1 Fig. MA plots from small RNA-Seq in the FC and CB of control, sCJD MM1 and VV2 cases. Plots represent the log2 fold change over the mean expression of mean normalized counts for each group comparison.
(TIF)

S2 Fig. Distribution of the different types of isomiRs in control and sCJD. IsomiR distribution in control individuals (red) and MM1 or VV2 samples (blue), in the FC (A-B) and the CB (C-D). The size of the dots shows the relative abundance of the different types of isomiRs. The Y-axis shows the fraction of unique sequences affecting specific nucleotides along the miRNAs, indicated in the X-axis. In the X-axis the nucleotide changes at diverse positions defining the isomiR are indicated with respect the reference miRNA (the miRbase sequence). For the trimming variants positions with a minus refer positions upstream of the reference miRNA.
(TIF)

S3 Fig. Expression of GW182 in sCJD brain tissue. (A) Gene expression levels of GW182 (n = 10) in the FC and CB of controls, sCJD MM1 and VV2 cases by RT-qPCR. Results were normalized to housekeeping genes GAPDH (figure) and GUSB with similar results. Housekeeping levels remained unaltered between groups. (B) Protein levels of GW182 in the FC of controls, sCJD MM1 and VV2 cases, by western blot analysis. Three representative cases per diagnostic group and brain region are shown in the western blot. Quantifications derived from densitometry analysis were performed in 15 cases per diagnostic group (n = 15/group). β-actin was used as a loading control. Densitometries of the western blots (n = 15 cases/group) are shown. Normalization was performed relative to controls. Error bars indicate SD. In all cases, statistical significance (compared to controls) was set at $^{*}p<0.05$. (C) Representative fluorescence photomicrographs of

GW182 immunoreactivity in the FC of control, sCJD MM1 and sCJD VV2 cases. Phase contrast and merge images are shown. Scale bar = 25μm.
(TIF)

S4 Fig. Analysis of gel filtration chromatography fractions from sCJD cases. (A) Coomassie-blue staining of the chromatographic gel filtration fractions obtained from the FC of a sCJD brain homogenate. A representative image of three independent experiments is shown. Molecular weight and fraction numbers are indicated (B) Western blot analysis of the different gel filtration fractions using the Prion protein SAF70 antibody. A representative control and sCJD case is shown. (C) RT-QuIC analysis of the input and chromatographic gel filtration fractions 2 and 7 obtained from the FC of a control and of sCJD brain homogenate. Positive signal was only detected in sCJD fractions. Higher signal, as detected by shorter lag phase was detected in the input, fraction 2 and fraction 7, respectively. A representative image of three independent experiments is shown. (D) Dot-blot analysis developed against the oligomer antibody 11A of the input and chromatographic gel filtration fractions 2 and 7 obtained from the FC of a control and sCJD brain homogenate. Positive signal was only detectable in sCJD fractions. A representative image of three independent experiments is shown.
(TIF)

S5 Fig. Subcellular localization of Ago-2 in sCJD brain tissue. (A) Representative fluorescence photomicrographs of Ago-2 immunoreactivity (11A9 antibody) in the FC of control, sCJD MM1 and sCJD VV2 cases. Phase contrast and merge images are shown. Scale bar = 25μm. Representative fluorescence photomicrographs of Ago-2 (11A9 antibody, green channel) and Iba-1 (red channel) immunoreactivity in the FC of control and sCJD MM1 cases. Arrowheads indicate absence of Ago-2 expression in microglial cells in control cases. In contrast, in sCJD cases some microglial cells are double stained with Ago-2 antibody. Merge images are shown. Scale bar = 25μm (C).
(TIF)

S6 Fig. Expression of Exportin 5 in sCJD brain tissue. (A) Gene expression levels of Exportin 5 (n = 10) in the FC and CB of controls, sCJD MM1 and VV2 cases by RT-qPCR. Results were normalized to housekeeping genes GAPDH (figure) and GUSB with similar results. Housekeeping levels remained unaltered between groups. (B) Protein levels of Exportin 5 in the FC of controls, sCJD MM1 and VV2 cases, by western blot analysis. Two representative cases per diagnostic group and brain region are shown in the western blot. Quantifications derived from densitometry analysis were performed in 15 cases per diagnostic group (n = 15/group). β-actin was used as a loading control. Densitometries of the western blot (n = 15 cases/group) are shown. Normalization was performed relative to controls. Error bars indicate SD. In all cases, statistical significance (compared to controls) was set at $^{*}p<0.05$.
(TIF)

S7 Fig. Synaptic loss in the sCJD MM1 mouse model tg340-PRNP129MM. Western blot analysis of PSD-95 and synaptophysin in the cortex and CB of control and sCJD MM1 inoculated mice at different disease stages. GAPDH was used as a loading control.
(TIF)

S8 Fig. Temporal-dependent expression of validated miRNAs in the sCJD MM1 mouse model tg340-PRNP129MM. Representation of the miRNAs with regulated expression levels in the cortex (A) and CB (B) of the tg340 mice by RT-qPCR analysis at any of the different stages of the disease. Data is represented as the fold change between sCJD MM1 and control

inoculated animals and each colored line represents a miRNA.
(TIF)

S9 Fig. Selective cell death induction by sCJD miRNAs transfection in cell cultures. (A) miRNAs from Control, sCJD MM1 and sCJD VV2 cases (FC region) were highly purified using commercial RNA extraction kits followed by in-gel purification and transfection into SH-SY5Y RA-differentiated cells and glioblastoma H4 cells. RNA was extracted from the cells and selected sCJD-related miRNAs were quantified by qPCR. NT: non-transfected. Statistical differences of comparison with non-transfected cells are indicated; $p<0.05$. (B) Cell toxicity assay, using Iodide Propidium staining (PI) on H4 and SY-SY5Y cells at 48h post-transfection with highly purified miRNAs derived from Control, sCJD MM1 and sCJD VV2 cases (FC region). (C) Cell viability, using WST-1 on SY-SY5Y cells at 48h post-transfection transfected with highly purified miRNAs derived from Control, sCJD MM1 and sCJD VV2 cases (FC region). Statistical differences are referred to controls. Statistical significance was set at $^{*}p<0.05$.
(TIF)

S1 Table. Excel file including the total RNA-sequence reads in each case (online).
(XLSX)

S2 Table. Excel file including the total number of reads mapping onto miRNAs. miRNAs with at least 2 counts in a given sample (online).
(XLSX)

S3 Table. Excel file including regulated miRNAs detected in this study (online).
(XLSX)

S4 Table. Excel file displaying isomiRs detected in this study. Base mean > or equal to 10 (online).
(XLSX)

S5 Table. Table providing information on the functional enrichment analysis of miRNAs identified based on small RNA-Seq in this study.
(TIF)

S6 Table. Top transcription factor binding sites enriched for promoter regions of target downregulated (A) and upregulated (B) miRNAs in the FC of sCJD MM1 cases.
(TIF)

S7 Table. List of Taqman probes, miRCURY LNA primers and antibodies used in this study.
(TIF)

S8 Table. Brain samples used in the present study. Diagnostic, sCJD subtype, brain region, age, sex and the type of experiment for which each case was used (RNA-seq, miRNA qPCR, mRNA qPCR and western blot) is indicated.
(TIF)

S9 Table. Demographics and biomarker profiling for the CSF samples analyzed in this study. NA = Non-analyzed, + = positive according to sCJD cut-off, − = negative according to sCJD cut-off. M = male, F = female.
(TIF)

Acknowledgments

We thank Nadine Gotzmann for technical assistance.

Author Contributions

Conceptualization: Franc Llorens, Katrin Thüne, Isidre Ferrer, Inga Zerr.

Data curation: Franc Llorens, Katrin Thüne, Eulàlia Martí, Ana Vivancos, Orr Shomroni, Matthias Schmitz, Stefan Bonn.

Formal analysis: Franc Llorens, Katrin Thüne, Eulàlia Martí, Eirini Kanata, Ana Vivancos, Orr Shomroni, Stefan Bonn.

Funding acquisition: Franc Llorens, Eulàlia Martí, José Antonio del Río, Juana Díez, Juan Maria Torres, Isidre Ferrer, Inga Zerr.

Investigation: Franc Llorens, Katrin Thüne, Eulàlia Martí, Eirini Kanata, Dimitra Dafou, Daniela Díaz-Lucena, Matthias Schmitz, Natalia Fernández-Borges, Isidre Ferrer.

Methodology: Uwe Michel, Natalia Fernández-Borges, Olivier Andréoletti, Juan Maria Torres.

Project administration: Franc Llorens, Katrin Thüne, Isidre Ferrer, Inga Zerr.

Resources: Uwe Michel, José Antonio del Río, Juana Díez, Andre Fischer, Theodoros Sklaviadis, Juan Maria Torres, Isidre Ferrer, Inga Zerr.

Software: Eulàlia Martí, Orr Shomroni, Stefan Bonn.

Supervision: Franc Llorens.

Writing – original draft: Franc Llorens, Katrin Thüne.

Writing – review & editing: Franc Llorens, Katrin Thüne, Eulàlia Martí, Eirini Kanata, Dimitra Dafou, Daniela Díaz-Lucena, Ana Vivancos, Orr Shomroni, Saima Zafar, Matthias Schmitz, Uwe Michel, Natalia Fernández-Borges, Olivier Andréoletti, José Antonio del Río, Juana Díez, Andre Fischer, Stefan Bonn, Theodoros Sklaviadis, Juan Maria Torres, Isidre Ferrer, Inga Zerr.

References

1. Puoti G, Bizzi A, Forloni G, Safar JG, Tagliavini F, Gambetti P. Sporadic human prion diseases: Molecular insights and diagnosis. Lancet Neurol. 2012; 11: 618–628. https://doi.org/10.1016/S1474-4422(12)70063-7 PMID: 22710755
2. Gambetti P, Kong Q, Zou W, Parchi P, Chen SG. Sporadic and familial CJD: Classification and characterisation. British Medical Bulletin. 2003. pp. 213–239. https://doi.org/10.1093/bmb/66.1.213 PMID: 14522861
3. Parchi P, De Boni L, Saverioni D, Cohen ML, Ferrer I, Gambetti P, et al. Consensus classification of human prion disease histotypes allows reliable identification of molecular subtypes: An inter-rater study among surveillance centres in Europe and USA. Acta Neuropathol. 2012; 124: 517–529. https://doi.org/10.1007/s00401-012-1002-8 PMID: 22744790
4. Parchi P, Giese a, Capellari S, Brown P, Schulz-Schaeffer W, Windl O, et al. Classification of sporadic Creutzfeldt-Jakob disease based on molecular and phenotypic analysis of 300 subjects. Ann Neurol. 1999; 46: 224–233. https://doi.org/10.1002/1531-8249(199908)46:2<224::AID-ANA12>3.0.CO;2-W PMID: 10443888
5. Llorens F, Lopez-Gonzalez I, Thune K, Carmona M, Zafar S, Andeoletti O, et al. Subtype and regional-specific neuroinflammation in sporadic creutzfeldt-jakob disease. Front Aging Neurosci. 2014; 6. https://doi.org/10.3389/fnagi.2014.00198 PMID: 25136317
6. Llorens F, Zafar S, Ansoleaga B, Shafiq M, Blanco R, Carmona M, et al. Subtype and regional regulation of prion biomarkers in sporadic Creutzfeldt-Jakob disease. Neuropathol Appl Neurobiol. 2015; 41: 631–645. https://doi.org/10.1111/nan.12175 PMID: 25134744
7. Llorens F, Ansoleaga B, Garcia-Esparcia P, Zafar S, Grau-Rivera O, López-González I, et al. PrP mRNA and protein expression in brain and PrP(c) in CSF in Creutzfeldt-Jakob disease MM1 and VV2. Prion. 2013; 7: 383–93. https://doi.org/10.4161/pri.26416 PMID: 24047819

8. Gawinecka J, Cardone F, Asif AR, De Pascalis A, Wemheuer WM, Schulz-Schaeffer WJ, et al. Sporadic Creutzfeldt-Jakob disease subtype-specific alterations of the brain proteome: Impact on Rab3a recycling. Proteomics. 2012; 12: 3610–3620. https://doi.org/10.1002/pmic.201200201 PMID: 23070823
9. Ansoleaga B, Garcia-Esparcia P, Llorens F, Moreno J, Aso E, Ferrer I. Dysregulation of brain olfactory and taste receptors in AD, PSP and CJD, and AD-related model. Neuroscience. 2013; 248: 369–382. https://doi.org/10.1016/j.neuroscience.2013.06.034 PMID: 23817016
10. Gawinecka J, Nowak M, Carimalo J, Cardone F, Asif AR, Wemheuer WM, et al. Subtype-specific synaptic proteome alterations in sporadic creutzfeldt-jakob disease. J Alzheimer's Dis. 2013; 37: 51–61. https://doi.org/10.3233/JAD-130455 PMID: 23780662
11. Jonas S, Izaurralde E. Towards a molecular understanding of microRNA-mediated gene silencing. Nat Rev Genet. 2015; 16: 421–433. https://doi.org/10.1038/nrg3965 PMID: 26077373
12. Fabian MR, Sonenberg N. The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC. Nat Struct Mol Biol. 2012; 19: 586–593. https://doi.org/10.1038/nsmb.2296 PMID: 22664986
13. Kosik KS. The neuronal microRNA system. Nat Rev Neurosci. 2006; 7: 911–920. https://doi.org/10.1038/nrn2037 PMID: 17115073
14. Bhalala OG, Srikanth M, Kessler JA. The emerging roles of microRNAs in CNS injuries. Nat Rev Neurol. 2013; 9: 328–39. https://doi.org/10.1038/nrneurol.2013.67 PMID: 23588363
15. Martí E, Pantano L, Bañez-Coronel M, Llorens F, Miñones-Moyano E, Porta S, et al. A myriad of miRNA variants in control and Huntington's disease brain regions detected by massively parallel sequencing. Nucleic Acids Res. 2010; 38: 7219–7235. https://doi.org/10.1093/nar/gkq575 PMID: 20591823
16. Miñones-Moyano E, Porta S, Escaramís G, Rabionet R, Iraola S, Kagerbauer B, et al. MicroRNA profiling of Parkinson's disease brains identifies early downregulation of miR-34b/c which modulate mitochondrial function. Hum Mol Genet. 2011; 20: 3067–3078. https://doi.org/10.1093/hmg/ddr210 PMID: 21558425
17. Cogswell JP, Ward J, Taylor I a, Waters M, Shi Y, Cannon B, et al. Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways. J Alzheimers Dis. 2008; 14: 27–41. https://doi.org/10.1016/j.jalz.2008.05.420 PMID: 18525125
18. Hebert S, Wang W, Zhu Q, Nelson P. A Study of Small RNAs from Cerebral Neocortex of Pathology-Verified Alzheimer's Disease, Dementia with Lewy Bodies, Hippocampal Sclerosis, Frontotemporal Lobar Dementia, and Non-Demented Human Controls. J Alzheimer's Dis. 2013; 35: 335–348. https://doi.org/10.3233/JAD-122350.A
19. Kocerha J, Kouri N, Baker M, Finch N, DeJesus-Hernandez M, Gonzalez J, et al. Altered microRNA expression in frontotemporal lobar degeneration with TDP-43 pathology caused by progranulin mutations. BMC Genomics. 2011; 12: 527. https://doi.org/10.1186/1471-2164-12-527 PMID: 22032330
20. Lau P, Bossers K, Janky R, Salta E, Frigerio CS, Barbash S, et al. Alteration of the microRNA network during the progression of Alzheimer's disease. EMBO Mol Med. 2013; 5: 1613–1634. https://doi.org/10.1002/emmm.201201974 PMID: 24014289
21. Majer A, Medina SJ, Niu Y, Abrenica B, Manguiat KJ, Frost KL, et al. Early Mechanisms of Pathobiology Are Revealed by Transcriptional Temporal Dynamics in Hippocampal CA1 Neurons of Prion Infected Mice. PLoS Pathog. 2012; 8. https://doi.org/10.1371/journal.ppat.1003002 PMID: 23144617
22. Saba R, Goodman CD, Huzarewich RLCH, Robertson C, Booth SA. A miRNA signature of prion induced neurodegeneration. PLoS One. 2008; 3. https://doi.org/10.1371/journal.pone.0003652 PMID: 18987751
23. Montag J, Hitt R, Opitz L, Schulz-Schaeffer WJ, Hunsmann G, Motzkus D. Upregulation of miRNA hsa-miR-342-3p in experimental and idiopathic prion disease. Mol Neurodegener. 2009; 4: 36. https://doi.org/10.1186/1750-1326-4-36 PMID: 19712440
24. Bellingham SA, Coleman BM, Hill AF. Small RNA deep sequencing reveals a distinct miRNA signature released in exosomes from prion-infected neuronal cells. Nucleic Acids Res. 2012; 40: 10937–10949. https://doi.org/10.1093/nar/gks832 PMID: 22965126
25. Gao C, Wei J, Zhang B-Y, Shi Q, Chen C, Wang J, et al. MiRNA expression profiles in the brains of mice infected with scrapie agents 139A, ME7 and S15. Emerg Microbes Infect. Nature Publishing Group; 2016; 5: e115. https://doi.org/10.1038/emi.2016.120 PMID: 27826142
26. Gibbings D, Leblanc P, Jay F, Pontier D, Michel F, Schwab Y, et al. Human prion protein binds Argonaute and promotes accumulation of microRNA effector complexes. Nat Struct Mol Biol. 2012; 19: 517–24, S1. https://doi.org/10.1038/nsmb.2273 PMID: 22484317

27. Kovacs GG, Budka H. Molecular pathology of human prion diseases. Int J Mol Sci. 2009; 10: 976–999. https://doi.org/10.3390/ijms10030976 PMID: 19399233

28. Pantano L, Estivill X, Martí E. SeqBuster, a bioinformatic tool for the processing and analysis of small RNAs datasets, reveals ubiquitous miRNA modifications in human embryonic cells. Nucleic Acids Res. 2009; 38. https://doi.org/10.1093/nar/gkp1127 PMID: 20008100

29. Capece V, Garcia Vizcaino JC, Vidal R, Rahman R-U, Pena Centeno T, Shomroni O, et al. Oasis: online analysis of small RNA deep sequencing data. Bioinformatics. 2015; 31: 2205–7. https://doi.org/10.1093/bioinformatics/btv113 PMID: 25701573

30. Morin RD, O'Connor MD, Griffith M, Kuchenbauer F, Delaney A, Prabhu AL, et al. Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells. Genome Res. 2008; 18: 610–621. https://doi.org/10.1101/gr.7179508 PMID: 18285502

31. Llorens F, Bañez-Coronel M, Pantano L, del Río JA, Ferrer I, Estivill X, et al. A highly expressed miR-101 isomiR is a functional silencing small RNA. BMC Genomics. 2013; 14: 104. https://doi.org/10.1186/1471-2164-14-104 PMID: 23414127

32. Tan GC, Chan E, Molnar A, Sarkar R, Alexieva D, Isa IM, et al. 5′ isomiR variation is of functional and evolutionary importance. Nucleic Acids Res. 2014; 42: 9424–9435. https://doi.org/10.1093/nar/gku656 PMID: 25056318

33. Saba R, Gushue S, Huzarewich RLCH, Manguiat K, Medina S, Robertson C, et al. MicroRNA 146a (miR-146a) is over-expressed during prion disease and modulates the innate immune response and the microglial activation state. PLoS One. 2012; 7. https://doi.org/10.1371/journal.pone.0030832 PMID: 22363497

34. Jovičić A, Roshan R, Moisoi N, Pradervand S, Moser R, Pillai B, et al. Comprehensive expression analyses of neural cell-type-specific miRNAs identify new determinants of the specification and maintenance of neuronal phenotypes. Ann Intern Med. 2013; 158: 5127–5137. https://doi.org/10.1523/JNEUROSCI.0600-12.2013

35. Braun JE, Huntzinger E, Izaurralde E. The role of GW182 proteins in miRNA-mediated gene silencing. Adv Exp Med Biol. 2013; 768: 147–163. https://doi.org/10.1007/978-1-4614-5107-5_9 PMID: 23224969

36. Sasaki K, Minaki H, Iwaki T. Development of oligomeric prion-protein aggregates in a mouse model of prion disease. J Pathol. 2009; 219: 123–130. https://doi.org/10.1002/path.2576 PMID: 19479969

37. Minaki H, Sasaki K, Honda H, Iwaki T. Prion protein oligomers in Creutzfeldt-Jakob disease detected by gel-filtration centrifuge columns: Original Article. Neuropathology. 2009; 29: 536–542. https://doi.org/10.1111/j.1440-1789.2009.01007.x PMID: 19389076

38. Marijanovic Z, Caputo A, Campana V, Zurzolo C. Identification of an intracellular site of prion conversion. PLoS Pathog. 2009; 5. https://doi.org/10.1371/journal.ppat.1000426 PMID: 19424437

39. Nunziante M, Ackermann K, Dietrich K, Wolf H, Gädtke L, Gilch S, et al. Proteasomal dysfunction and endoplasmic reticulum stress enhance trafficking of prion protein aggregates through the secretory pathway and increase accumulation of pathologic prion protein. J Biol Chem. 2011; 286: 33942–33953. https://doi.org/10.1074/jbc.M111.272617 PMID: 21835918

40. Torres M, Encina G, Soto C, Hetz C. Abnormal calcium homeostasis and protein folding stress at the ER: A common factor in familial and infectious prion disorders. Commun Integr Biol. 2011; 4: 258–261. https://doi.org/10.4161/cib.4.3.15019 PMID: 21980554

41. Xu K, Zhu X-P. Endoplasmic reticulum stress and prion diseases. Rev Neurosci. 2012; 23: 79–84. https://doi.org/10.1515/rns.2011.062 PMID: 22718614

42. Abe M, Bonini NM. MicroRNAs and neurodegeneration: Role and impact. Trends Cell Biol. 2013; 23: 30–36. https://doi.org/10.1016/j.tcb.2012.08.013 PMID: 23026030

43. Maciotta S, Meregalli M, Torrente Y. The involvement of microRNAs in neurodegenerative diseases. Front Cell Neurosci. 2013; 7: 265. https://doi.org/10.3389/fncel.2013.00265 PMID: 24391543

44. BOHNSACK MT. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA. 2004; 10: 185–191. https://doi.org/10.1261/rna.5167604 PMID: 14730017

45. Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. 2003; 17: 3011–3016. https://doi.org/10.1101/gad.1158803 PMID: 14681208

46. Nuovo G, Amann V, Williams J, Vandiver P, Quinonez M, Fadda P, et al. Increased expression of importin-β, exportin-5 and nuclear transportable proteins in Alzheimer's disease aids anatomic pathologists in its diagnosis. Ann Diagn Pathol. 2018; 32: 10–16. https://doi.org/10.1016/j.anndiagpath.2017.08.003

47. Budka H. Neuropathology of prion diseases. British Medical Bulletin. 2003. pp. 121–130. https://doi.org/10.1093/bmb/66.1.121

48. Butovsky O, Jedrychowski MP, Moore CS, Cialic R, Lanser AJ, Gabriely G, et al. Identification of a unique TGF-β-dependent molecular and functional signature in microglia. Nat Neurosci. 2014; 17: 131–43. https://doi.org/10.1038/nn.3599 PMID: 24316888

49. Sasaki Y, Gross C, Xing L, Goshima Y, Bassell GJ. Identification of axon-enriched MicroRNAs localized to growth cones of cortical neurons. Dev Neurobiol. 2014; 74: 397–406. https://doi.org/10.1002/dneu.22113 PMID: 23897634

50. Finkbeiner S, Cuervo AM, Morimoto RI, Muchowski PJ. Disease-Modifying Pathways in Neurodegeneration. J Neurosci. 2006; 26: 10349–10357. https://doi.org/10.1523/JNEUROSCI.3829-06.2006 PMID: 17035516

51. Ahmed RM, Devenney EM, Irish M, Ittner A, Naismith S, Ittner LM, et al. Neuronal network disintegration: common pathways linking neurodegenerative diseases. J Neurol Neurosurg Psychiatry. 2016; jnnp-2014-308350. https://doi.org/10.1136/jnnp-2014-308350 PMID: 27172939

52. Jucker M, Walker LC. Neurodegeneration: Amyloid-β pathology induced in humans. Nature. 2015; 525: 193–4. https://doi.org/10.1038/525193a PMID: 26354478

53. Müller M, Kuiperij HB, Claassen JA, Küsters B, Verbeek MM. MicroRNAs in Alzheimer's disease: Differential expression in hippocampus and cell-free cerebrospinal fluid. Neurobiol Aging. 2014; 35: 152–158. https://doi.org/10.1016/j.neurobiolaging.2013.07.005 PMID: 23962497

54. Sethi P, Lukiw WJ. Micro-RNA abundance and stability in human brain: Specific alterations in Alzheimer's disease temporal lobe neocortex. Neurosci Lett. 2009; 459: 100–104. https://doi.org/10.1016/j.neulet.2009.04.052 PMID: 19406203

55. Schmidt C, Wolff M, Weitz M, Bartlau T, Korth C, Zerr I. Rapidly progressive Alzheimer disease. Arch Neurol. 2011; 68: 1124–1130. https://doi.org/10.1001/archneurol.2011.189 PMID: 21911694

56. Grau-Rivera O, Gelpi E, Nos C, Gaig C, Ferrer I, Saiz A, et al. Clinicopathological Correlations and Concomitant Pathologies in Rapidly Progressive Dementia: A Brain Bank Series. Neurodegener Dis. 2015; 15: 350–360. https://doi.org/10.1159/000439251 PMID: 26523804

57. Montagna P, Gambetti P, Cortelli P, Lugaresi E. Familial and sporadic fatal insomnia. Lancet Neurol. 2003; 2: 167–176. https://doi.org/10.1016/S1474-4422(03)00323-5 PMID: 12849238

58. Parchi P, Castellani R, Cortelli P, Montagna P, Chen SG, Petersen RB, et al. Regional distribution of protease???resistant prion protein in fatal familial insomnia. Ann Neurol. 1995; 38: 21–29. https://doi.org/10.1002/ana.410380107 PMID: 7611720

59. Padilla D, Béringue V, Espinosa JC, Andreoletti O, Jaumain E, Reine F, et al. Sheep and goat BSE propagate more efficiently than cattle BSE in human PrP transgenic mice. PLoS Pathog. 2011; 7. https://doi.org/10.1371/journal.ppat.1001319 PMID: 21445238

60. Na YJ, Jin JK, Kim J Il, Choi EK, Carp RI, Kim YS. JAK-STAT signaling pathway mediates astrogliosis in brains of scrapie-infected mice. J Neurochem. 2007; 103: 637–649. https://doi.org/10.1111/j.1471-4159.2007.04769.x PMID: 17897356

61. Carroll JA, Striebel JF, Race B, Phillips K, Chesebro B. Prion infection of mouse brain reveals multiple new upregulated genes involved in neuroinflammation or signal transduction. J Virol. 2015; 89: 2388–2404. https://doi.org/10.1128/JVI.02952-14 PMID: 25505076

62. Rao P, Benito E, Fischer A. MicroRNAs as biomarkers for CNS disease. Front Mol Neurosci. 2013; 6: 1–13. https://doi.org/10.3389/fnmol.2013.00039 PMID: 24324397

63. Burgos K, Malenica I, Metpally R, Courtright A, Rakela B, Beach T, et al. Profiles of extracellular miRNA in cerebrospinal fluid and serum from patients with Alzheimer's and Parkinson's diseases correlate with disease status and features of pathology. PLoS One. 2014; 9. https://doi.org/10.1371/journal.pone.0094839 PMID: 24797360

64. Parchi P, Saverioni D. Molecular pathology, classification, and diagnosis of sporadic human prion disease variants. Folia Neuropathol. 2012; 50: 20–45. PMID: 22505361

65. Lukiw WJ, Dua P, Pogue AI, Eicken C, Hill JM. Upregulation of micro RNA-146a (miRNA-146a), a marker for inflammatory neurodegeneration, in sporadic Creutzfeldt-Jakob disease (sCJD) and Gerstmann-Straussler-Scheinker (GSS) syndrome. J Toxicol Environ Health A. 2011; 74: 1460–8. https://doi.org/10.1080/15287394.2011.618973 PMID: 22043907

66. Boese AS, Saba R, Campbell K, Majer A, Medina S, Burton L, et al. MicroRNA abundance is altered in synaptoneurosomes during prion disease. Mol Cell Neurosci. 2016; 71: 13–24. https://doi.org/10.1016/j.mcn.2015.12.001 PMID: 26658803

67. Schaefer A, O'Carroll D, Tan CL, Hillman D, Sugimori M, Llinas R, et al. Cerebellar neurodegeneration in the absence of microRNAs. J Exp Med. 2007; 204: 1553–8. https://doi.org/10.1084/jem.20070823 PMID: 17606634

68. Kim J, Inoue K, Ishii J, Vanti WB, Voronov S V, Murchison E, et al. A MicroRNA feedback circuit in midbrain dopamine neurons. Science. 2007; 317: 1220–4. https://doi.org/10.1126/science.1140481 PMID: 17761882

69. Haramati S, Chapnik E, Sztainberg Y, Eilam R, Zwang R, Gershoni N, et al. miRNA malfunction causes spinal motor neuron disease. Proc Natl Acad Sci. 2010; 107: 13111–13116. https://doi.org/10.1073/pnas.1006151107 PMID: 20616011

70. Fiorenza A, Lopez-Atalaya JP, Rovira V, Scandaglia M, Geijo-Barrientos E, Barco A. Blocking miRNA Biogenesis in Adult Forebrain Neurons Enhances Seizure Susceptibility, Fear Memory, and Food Intake by Increasing Neuronal Responsiveness. Cereb Cortex. 2016; 26: 1619–1633. https://doi.org/10.1093/cercor/bhu332 PMID: 25595182

71. Pereira J a, Baumann R, Norrmén C, Somandin C, Miehe M, Jacob C, et al. Dicer in Schwann cells is required for myelination and axonal integrity. J Neurosci. 2010; 30: 6763–6775. https://doi.org/10.1523/JNEUROSCI.0801-10.2010 PMID: 20463238

72. McKiernan RC, Jimenez-Mateos EM, Sano T, Bray I, Stallings RL, Simon RP, et al. Expression profiling the microRNA response to epileptic preconditioning identifies miR-184 as a modulator of seizure-induced neuronal death. Exp Neurol. 2012; 237: 346–354. https://doi.org/10.1016/j.expneurol.2012.06.029 PMID: 22771761

73. Emde A, Eitan C, Liou L-L, Libby RT, Rivkin N, Magen I, et al. Dysregulated miRNA biogenesis downstream of cellular stress and ALS-causing mutations: a new mechanism for ALS. EMBO J. 2015; 34: 2633–2651. https://doi.org/10.15252/embj.201490493 PMID: 26330466

74. Gagnon KT, Li L, Chu Y, Janowski BA, Corey DR. RNAi factors are present and active in human cell nuclei. Cell Rep. 2014; 6: 211–221. https://doi.org/10.1016/j.celrep.2013.12.013 PMID: 24388755

75. Taliaferro JM, Aspden JL, Bradley T, Marwha D, Blanchette M, Rio DC. Two new and distinct roles for Drosophila Argonaute-2 in the nucleus: Alternative pre-mRNA splicing and transcriptional repression. Genes Dev. 2013; 27: 378–389. https://doi.org/10.1101/gad.210708.112 PMID: 23392611

76. Biasini E, Turnbaugh JA, Unterberger U, Harris DA. Prion protein at the crossroads of physiology and disease. Trends in Neurosciences. 2012. pp. 92–103. https://doi.org/10.1016/j.tins.2011.10.002 PMID: 22137337

77. Gibbings DJ, Ciaudo C, Erhardt M, Voinnet O. Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. TL—11. Nat Cell Biol. 2009; 11 VN-r: 1143–1149. https://doi.org/10.1038/ncb1929 PMID: 19684575

78. Lee YS, Pressman S, Andress AP, Kim K, White JL, Cassidy JJ, et al. Silencing by small RNAs is linked to endosomal trafficking. Nat Cell Biol. 2009; 11: 1150–6. https://doi.org/10.1038/ncb1930 PMID: 19684574

79. Anderson P, Kedersha N. RNA granules: post-transcriptional and epigenetic modulators of gene expression. Nat Rev Mol Cell Biol. 2009; 10: 430–436. https://doi.org/10.1038/nrm2694 PMID: 19461665

80. Buchan JR, Parker R. Eukaryotic Stress Granules: The Ins and Outs of Translation. Molecular Cell. 2009. pp. 932–941. https://doi.org/10.1016/j.molcel.2009.11.020 PMID: 20064460

81. Pamplona R, Naudí A, Gavín R, Pastrana MA, Sajnani G, Ilieva E V., et al. Increased oxidation, glycoxidation, and lipoxidation of brain proteins in prion disease. Free Radic Biol Med. 2008; 45: 1159–1166. https://doi.org/10.1016/j.freeradbiomed.2008.07.009 PMID: 18703134

82. Hoyle NP, Castelli LM, Campbell SG, Holmes LEA, Ashe MP. Stress-dependent relocalization of translationally primed mRNPs to cytoplasmic granules that are kinetically and spatially distinct from P-bodies. J Cell Biol. 2007; 179: 65–74. https://doi.org/10.1083/jcb.200707010 PMID: 17908917

83. Kedersha NL, Gupta M, Li W, Miller I, Anderson P. RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2?? to the assembly of mammalian stress granules. J Cell Biol. 1999; 147: 1431–1441. https://doi.org/10.1083/jcb.147.7.1431 PMID: 10613902

84. Mazroui R, Di Marco S, Kaufman RJ, Gallouzi I-E. Inhibition of the Ubiquitin-Proteasome System Induces Stress Granule Formation □ D. Mol Biol Cell. 2007; 18: 2603–2618. https://doi.org/10.1091/mbc.E06-12-1079 PMID: 17475769

85. Sun Y, Luo Z-M, Guo X-M, Su D-F, Liu X. An updated role of microRNA-124 in central nervous system disorders: a review. Front Cell Neurosci. 2015; 9: 193. https://doi.org/10.3389/fncel.2015.00193 PMID: 26041995

86. Smith P, Al Hashimi A, Girard J, Delay C, Hébert SS. In vivo regulation of amyloid precursor protein neuronal splicing by microRNAs. J Neurochem. 2011; 116: 240–247. https://doi.org/10.1111/j.1471-4159.2010.07097.x PMID: 21062284

87. Llorens F, Thüne K, Andrés-Benito P, Tahir W, Ansoleaga B, Hernández-Ortega K, et al. MicroRNA Expression in the Locus Coeruleus, Entorhinal Cortex, and Hippocampus at Early and Middle Stages

of Braak Neurofibrillary Tangle Pathology. Journal of Molecular Neuroscience. 2017: 1–10. https://doi.org/10.1007/s12031-017-0971-4 PMID: 28871468

88. Wang H, Ye Y, Zhu Z, Mo L, Lin C, Wang Q, et al. MiR-124 regulates apoptosis and autophagy process in MPTP model of Parkinson's disease by targeting to bim. Brain Pathol. 2016; 26: 167–176. https://doi.org/10.1111/bpa.12267 PMID: 25976060

89. Ai J, Sun L-H, Che H, Zhang R, Zhang T-Z, Wu W-C, et al. MicroRNA-195 protects against dementia induced by chronic brain hypoperfusion via its anti-amyloidogenic effect in rats. J Neurosci. 2013; 33: 3989–4001. https://doi.org/10.1523/JNEUROSCI.1997-12.2013 PMID: 23447608

90. Zhu HC, Wang LM, Wang M, Song B, Tan S, Teng JF, et al. MicroRNA-195 downregulates Alzheimer's disease amyloid-β production by targeting BACE1. Brain Res Bull. 2012; 88: 596–601. https://doi.org/10.1016/j.brainresbull.2012.05.018 PMID: 22721728

91. Parchi P, Petersen RB, Chen SG, Autilio-Gambetti L, Capellari S, Monari L, et al. Molecular pathology of fatal familial insomnia. Brain Pathol. 1998; 8: 539–548. https://doi.org/10.1111/j.1750-3639.1998.tb00176.x PMID: 9669705

92. Schratt G. Fine-tuning neural gene expression with microRNAs. Current Opinion in Neurobiology. 2009. pp. 213–219. https://doi.org/10.1016/j.conb.2009.05.015 PMID: 19539460

93. Lai X, Wolkenhauer O, Vera J. Understanding microRNA-mediated gene regulatory networks through mathematical modelling. Nucleic Acids Res. 2016; 44: 6019–6035. https://doi.org/10.1093/nar/gkw550 PMID: 27317695

94. Bracken CP, Scott HS, Goodall GJ. A network-biology perspective of microRNA function and dysfunction in cancer. Nat Rev Genet. 2016; 17: 719–732. https://doi.org/10.1038/nrg.2016.134 PMID: 27795564

95. Bekris LM, Lutz F, Montine TJ, Yu CE, Tsuang D, Peskind ER, et al. MicroRNA in Alzheimer's disease: an exploratory study in brain, cerebrospinal fluid and plasma. Biomarkers. 2013; 18: 455–66. https://doi.org/10.3109/1354750X.2013.814073 PMID: 23822153

96. Burgos KL, Javaherian A, Bomprezzi R, Ghaffari L, Rhodes S, Courtright A, et al. Identification of extracellular miRNA in human cerebrospinal fluid by next-generation sequencing. RNA. 2013; 19: 712–722. https://doi.org/10.1261/rna.036863.112 PMID: 23525801

97. Lopez-Gonzalez I, Schluter A, Aso E, Garcia-Esparcia P, Ansoleaga B, F LL, et al. Neuroinflammatory Signals in Alzheimer Disease and APP/PS1 Transgenic Mice: Correlations With Plaques, Tangles, and Oligomeric Species. J Neuropathol Exp Neurol. 2015; 74: 319–344. https://doi.org/10.1097/NEN.0000000000000176 PMID: 25756590

98. Zerr I, Kallenberg K, Summers DM, Romero C, Taratuto A, Heinemann U, et al. Updated clinical diagnostic criteria for sporadic Creutzfeldt-Jakob disease. Brain. 2009; 132: 2659–68. https://doi.org/10.1093/brain/awp191 PMID: 19773352

99. Windl O, Giese A, Schulz-Schaeffer W, Zerr I, Skworc K, Arendt S, et al. Molecular genetics of human prion diseases in Germany. Hum Genet. 1999; 105: 244–252. https://doi.org/10.1007/s004399900124 PMID: 10987652

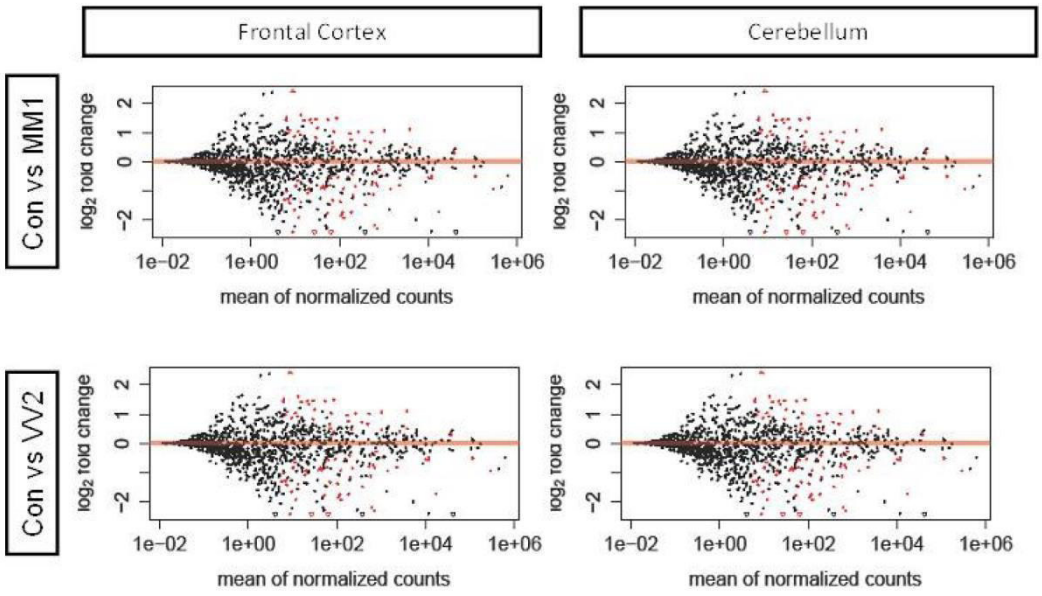
100. Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, et al. Simple Combinations of Lineage-Determining Transcription Factors Prime cis-Regulatory Elements Required for Macrophage and B Cell Identities. Mol Cell. 2010; 38: 576–589. https://doi.org/10.1016/j.molcel.2010.05.004 PMID: 20513432

101. Schmitz M, Cramm M, Llorens F, Collins S, Atarashi R, Satoh K, et al. The real-time quaking-induced conversion assay for detection of human prion disease and study of other protein misfolding diseases. Nat Protoc. 2016; 11: 2233–2242. https://doi.org/10.1038/nprot.2016.120 PMID: 27735933

102. Schmitz M, Ebert E, Stoeck K, Karch A, Collins S, Calero M, et al. Validation of 14-3-3 Protein as a Marker in Sporadic Creutzfeldt-Jakob Disease Diagnostic. Mol Neurobiol. 2015; https://doi.org/10.1007/s12035-015-9167-5 PMID: 25947081

Supporting Information

S1 Fig



S1 Fig. MA plots from small RNA-Seq in the FC and CB of control, sCJD MM1 and VV2 cases.

Plots represent the log2 fold change over the mean expression of mean normalized counts for each group comparison.

https://doi.org/10.1371/journal.ppat.1006802.s001

(TIF)

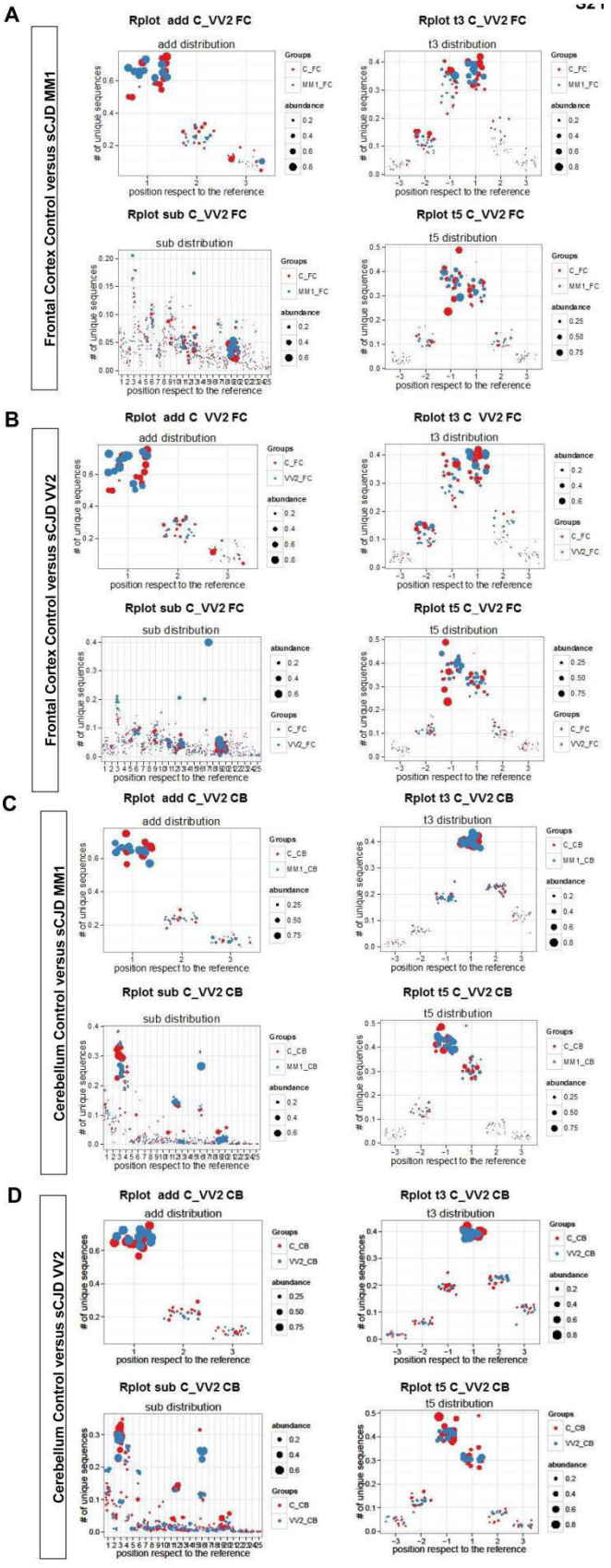
S2 Fig

S2 Fig. Distribution of the different types of isomiRs in control and sCJD.

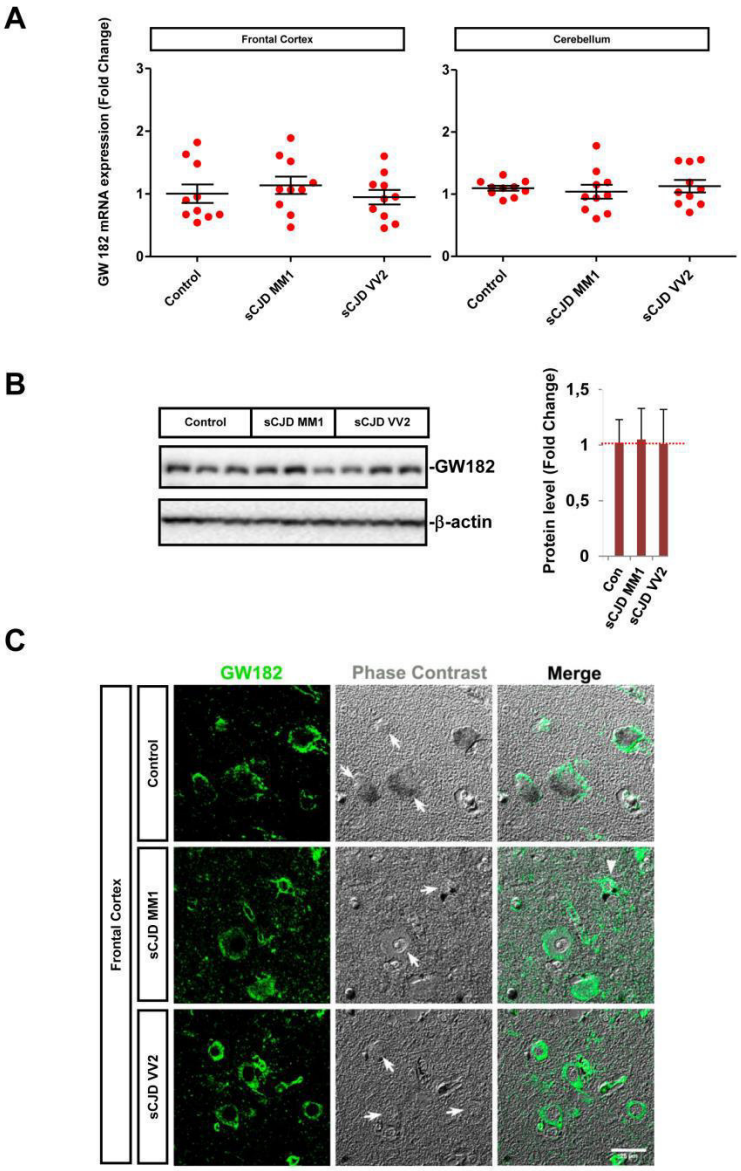
IsomiR distribution in control individuals (red) and MM1 or VV2 samples (blue), in the FC (A-B) and the CB (C-D). The size of the dots shows the relative abundance of the different types of isomiRs. The Y-axis shows the fraction of unique sequences affecting specific nucleotides along the miRNAs, indicated in the X-axis. In the X-axis the nucleotide changes at diverse positions defining the isomiR are indicated with respect the reference miRNA (the miRbase sequence). For the trimming variants positions with a minus refer positions upstream of the reference miRNA.

https://doi.org/10.1371/journal.ppat.1006802.s002

(TIF)



S3 Fig



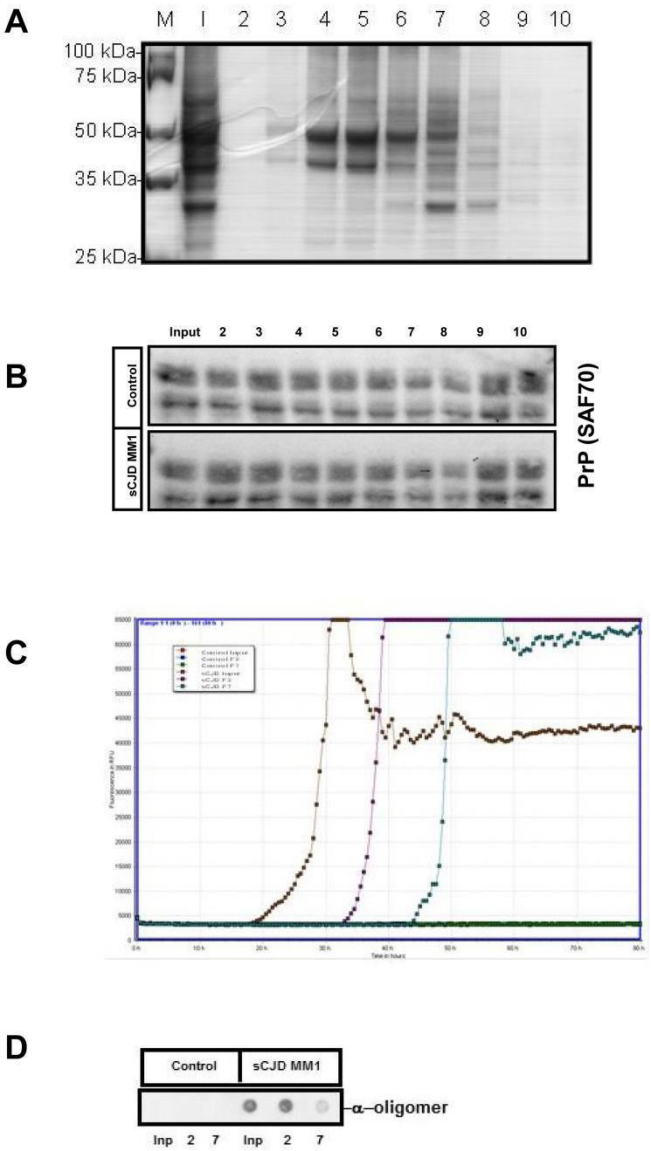
S3 Fig. Expression of GW182 in sCJD brain tissue.

(A) Gene expression levels of GW182 (n = 10) in the FC and CB of controls, sCJD MM1 and VV2 cases by RT-qPCR. Results were normalized to housekeeping genes GAPDH (figure) and GUSB with similar results. Housekeeping levels remained unaltered between groups. (B) Protein levels of GW182 in the FC of controls, sCJD MM1 and VV2 cases, by western blot analysis. Three representative cases per diagnostic group and brain region are shown in the western blot. Quantifications derived from densitometry analysis were performed in 15 cases per diagnostic group (n = 15/group). β-actin was used as a loading control. Densitometries of the western blots (n = 15 cases/group) are shown. Normalization was performed relative to controls. Error bars indicate SD. In all cases, statistical significance (compared to controls) was set at *p<0.05. (C) Representative fluorescence photomicrographs of GW182 immunoreactivity in the FC of control, sCJD MM1 and sCJD VV2 cases. Phase contrast and merge images are shown. Scale bar = 25μm.

https://doi.org/10.1371/journal.ppat.1006802.s003

(TIF)

S4 Fig



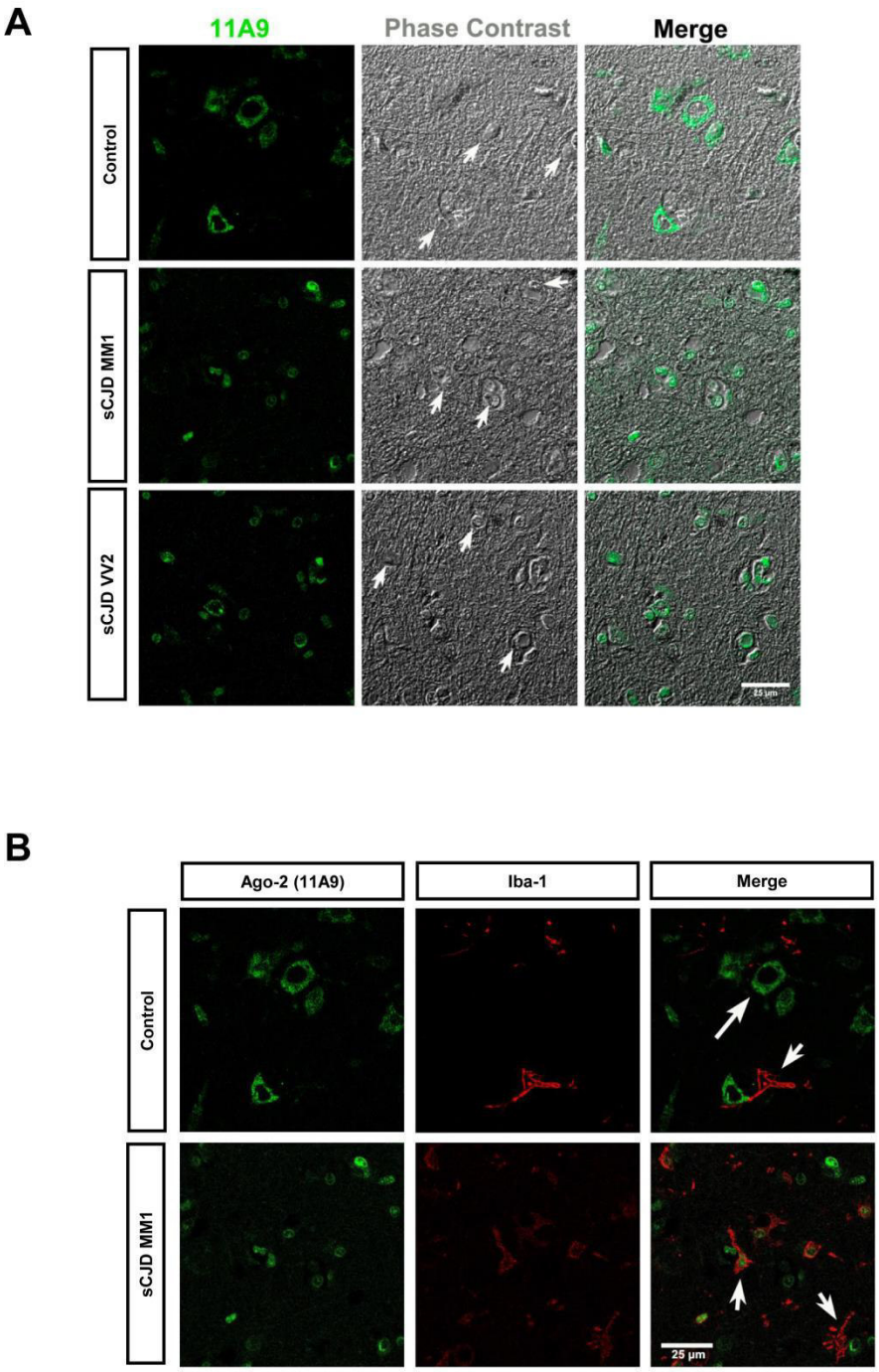
S4 Fig. Analysis of gel filtration chromatography fractions from sCJD cases.

(A) Coomassie-blue staining of the chromatographic gel filtration fractions obtained from the FC of a sCJD brain homogenate. A representative image of three independent experiments is shown. Molecular weight and fraction numbers are indicated (B) Western blot analysis of the different gel filtration fractions using the Prion protein SAF70 antibody. A representative control and sCJD case is shown. (C) RT-QuIC analysis of the input and chromatographic gel filtration fractions 2 and 7 obtained from the FC of a control and of sCJD brain homogenate. Positive signal was only detected in sCJD fractions. Higher signal, as detected by shorter lag phase was detected in the input, fraction 2 and fraction 7, respectively. A representative image of three independent experiments is shown. (D) Dot-blot analysis developed against the oligomer antibody 11A of the input and chromatographic gel filtration fractions 2 and 7 obtained from the FC of a control and sCJD brain homogenate. Positive signal was only detectable in sCJD fractions. A representative image of three independent experiments is shown.

https://doi.org/10.1371/journal.ppat.1006802.s004

(TIF)

S5 Fig



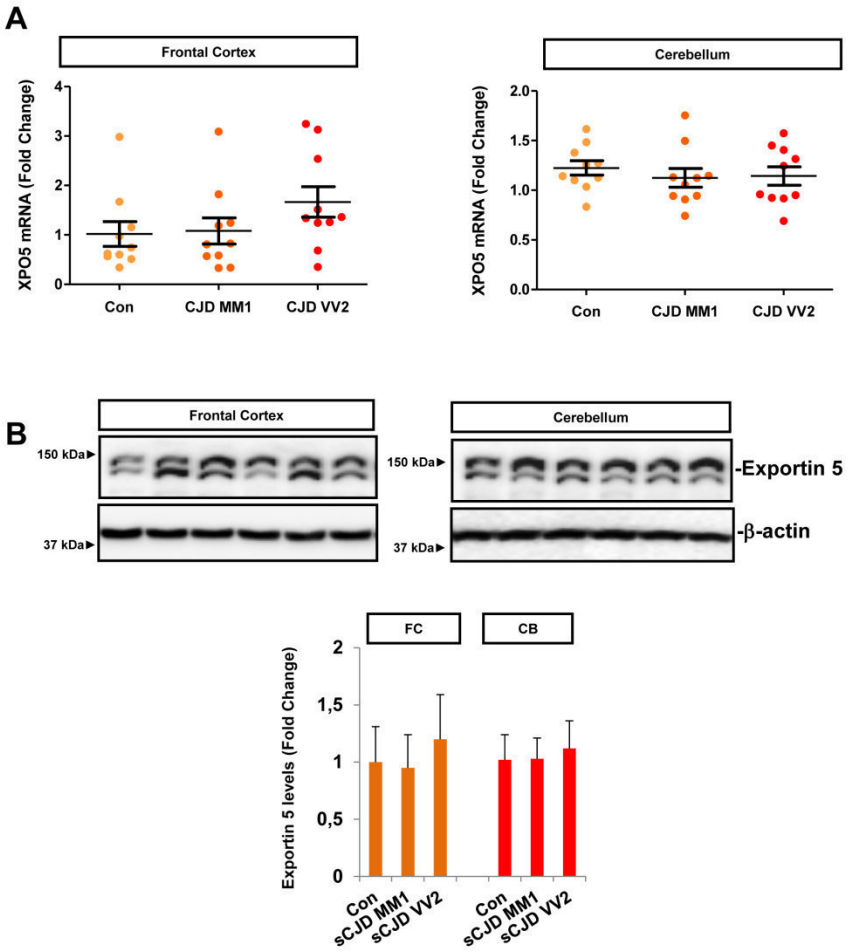
S5 Fig. Subcellular localization of Ago-2 in sCJD brain tissue.

(A) Representative fluorescence photomicrographs of Ago-2 immunoreactivity (11A9 antibody) in the FC of control, sCJD MM1 and sCJD VV2 cases. Phase contrast and merge images are shown. Scale bar = 25μm. Representative fluorescence photomicrographs of Ago-2 (11A9 antibody, green channel) and Iba-1 (red channel) immunoreactivity in the FC of control and sCJD MM1 cases. Arrowheads indicate absence of Ago-2 expression in microglial cells in control cases. In contrast, in sCJD cases some microglial cells are double stained with Ago-2 antibody. Merge images are shown. Scale bar = 25μm (C).

https://doi.org/10.1371/journal.ppat.1006802.s005

(TIF)

S6 Fig



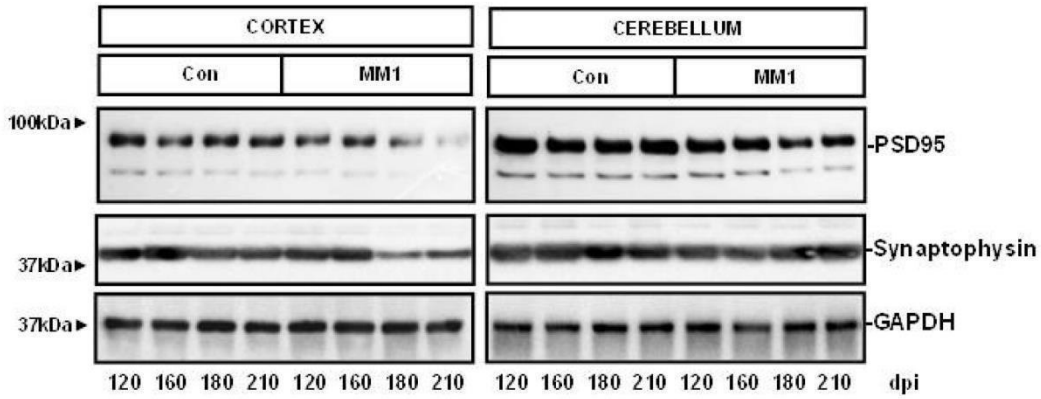
S6 Fig. Expression of Exportin 5 in sCJD brain tissue.

(A) Gene expression levels of Exportin 5 (n = 10) in the FC and CB of controls, sCJD MM1 and VV2 cases by RT-qPCR. Results were normalized to housekeeping genes GAPDH (figure) and GUSB with similar results. Housekeeping levels remained unaltered between groups. (B) Protein levels of Exportin 5 in the FC of controls, sCJD MM1 and VV2 cases, by western blot analysis. Two representative cases per diagnostic group and brain region are shown in the western blot. Quantifications derived from densitometry analysis were performed in 15 cases per diagnostic group (n = 15/group). β-actin was used as a loading control. Densitometries of the western blot (n = 15 cases/group) are shown. Normalization was performed relative to controls. Error bars indicate SD. In all cases, statistical significance (compared to controls) was set at *p<0.05.

https://doi.org/10.1371/journal.ppat.1006802.s006

(TIF)

S7 Fig



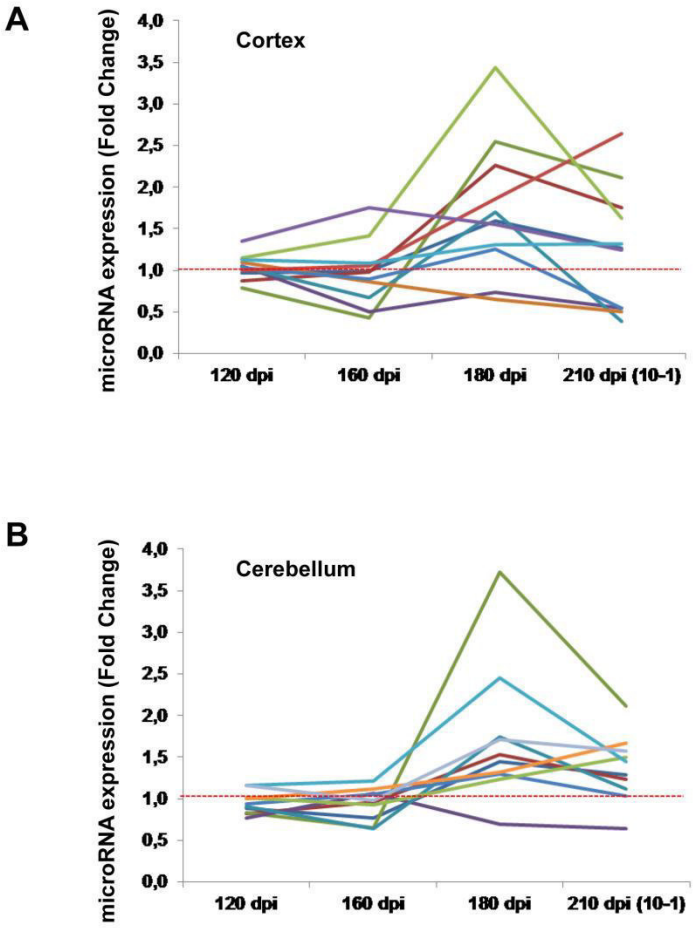
S7 Fig. Synaptic loss in the sCJD MM1 mouse model tg340-PRNP129MM.

Western blot analysis of PSD-95 and synaptophysin in the cortex and CB of control and sCJD MM1 inoculated mice at different disease stages. GAPDH was used as a loading control.

https://doi.org/10.1371/journal.ppat.1006802.s007

(TIF)

S8 Fig



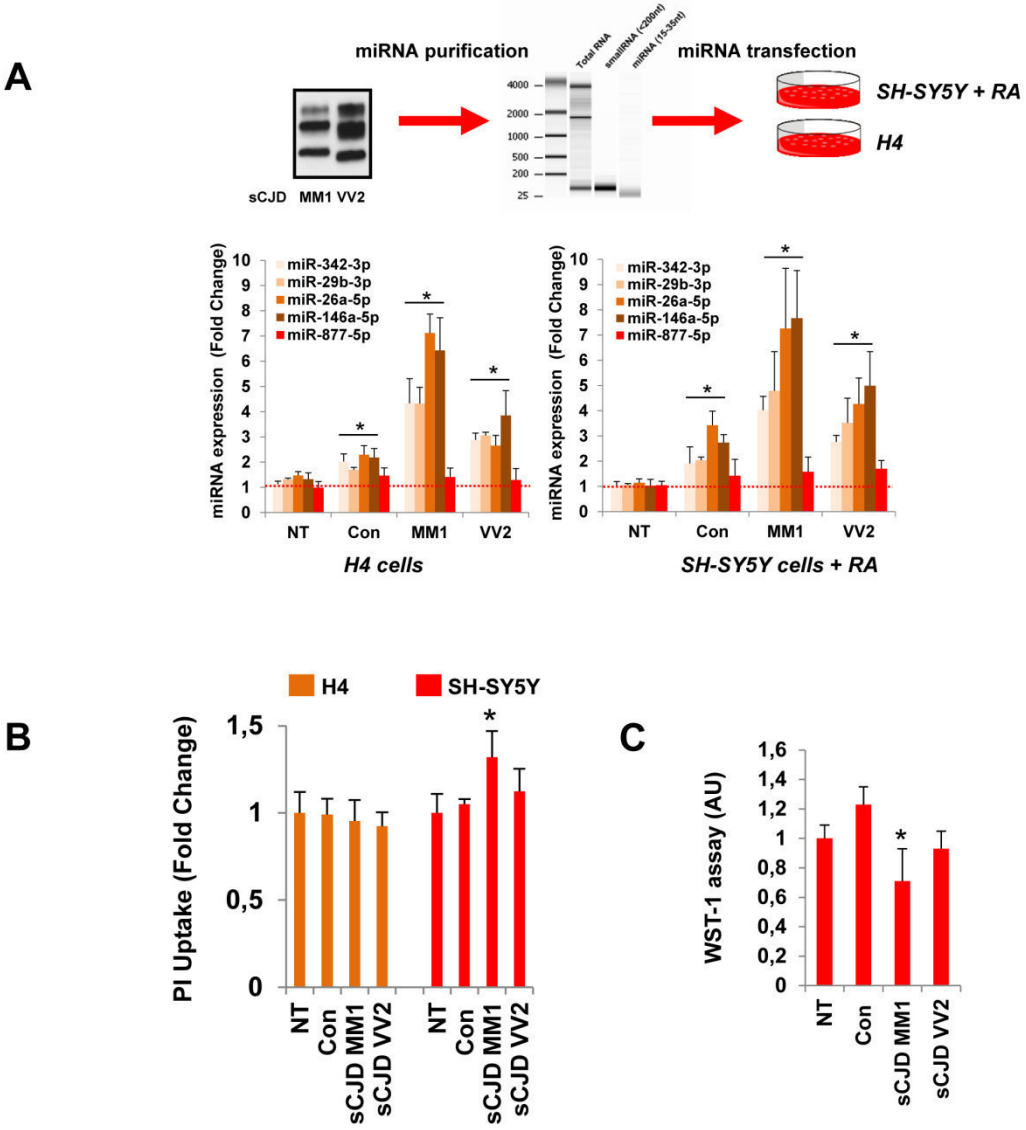
S8 Fig. Temporal-dependent expression of validated miRNAs in the sCJD MM1 mouse model tg340-PRNP129MM.

Representation of the miRNAs with regulated expression levels in the cortex (A) and CB (B) of the tg340 mice by RT-qPCR analysis at any of the different stages of the disease. Data is represented as the fold change between sCJD MM1 and control inoculated animals and each colored line represents a miRNA.

https://doi.org/10.1371/journal.ppat.1006802.s008

(TIF)

S9 Fig



S9 Fig. Selective cell death induction by sCJD miRNAs transfection in cell cultures.

(A) miRNAs from Control, sCJD MM1 and sCJD VV2 cases (FC region) were highly purified using commercial RNA extraction kits followed by in-gel purification and transfection into SH-SY5Y RA-differentiated cells and glioblastoma H4 cells. RNA was extracted from the cells and selected sCJD-related miRNAs were quantified by qPCR. NT: non-transfected. Statistical differences of comparison with non-transfected cells are indicated; $p<0.05$. (B) Cell toxicity assay, using Iodide Propidium staining (PI) on H4 and SY-SY5Y cells at 48h post-transfection with highly purified miRNAs derived from Control, sCJD MM1 and sCJD VV2 cases (FC region). (C) Cell viability, using WST-1 on SY-SY5Y cells at 48h post-transfection transfected with highly purified miRNAs derived from Control, sCJD MM1 and sCJD VV2 cases (FC region). Statistical differences are referred to controls. Statistical significance was set at *$p<0.05$.

https://doi.org/10.1371/journal.ppat.1006802.s009

(TIF)

S1 Table. Excel file including the total RNA-sequence reads in each case (online).

https://doi.org/10.1371/journal.ppat.1006802.s010

(XLSX)

S2 Table. Excel file including the total number of reads mapping onto miRNAs.

miRNAs with at least 2 counts in a given sample (online).

https://doi.org/10.1371/journal.ppat.1006802.s011

(XLSX)

S3 Table. Excel file including regulated miRNAs detected in this study (online).

https://doi.org/10.1371/journal.ppat.1006802.s012

(XLSX)

S4 Table. Excel file displaying isomiRs detected in this study.

Base mean > or equal to 10 (online).

https://doi.org/10.1371/journal.ppat.1006802.s013

(XLSX)

S5 Table

ID	Score	Focus Molecules	Top Diseases and Functions
MM1 Frontal Cortex			
1	37	33	**Cellular Development**, Nervous System Development and Function, Metabolic Disease
2	37	32	**Cell Death and Survival,** Cell Cycle, DNA Replication, Recombination, and Repair
3	37	32	**Cell Death and Survival, Cellular Development,** Cellular Growth and Proliferation
MM1 Cerebellum			
1	48	33	Cancer, **Cell Death and Survival, Cellular Development**
2	48	34	**Cell Death and Survival,** Cancer, Cell Cycle
3	18	22	Cancer, Cellular Movement, Nervous System Development and Function
VV2 Frontal Cortex			
1	42	35	Cancer, **Cell Death and Survival**, Tumor Morphology
2	42	35	**Cellular Development,** Cellular Growth and Proliferation, Connective Tissue Development and Function
3	42	35	**Cellular Development**, Tissue Development, Gene Expression
VV2 Cerebellum			
1	45	32	**Cell Death and Survival**, Cell Signaling, Small Molecule Biochemistry
2	45	33	Cell Cycle, Cancer, DNA Replication, Recombination, and Repair
3	45	33	Cancer, Cellular Growth and Proliferation, Tissue Development

S5 Table. Table providing information on the functional enrichment analysis of miRNAs identified based on small RNA-Seq in this study.

https://doi.org/10.1371/journal.ppat.1006802.s014

(TIF)

S6 Table

A

Motif	P-value	% of Targets	% of Background	Best Match/Details
	1.00E-10	15.91%	0.23%	Hoxb4(Homeobox)
	1.00E-10	25.00%	1.52%	Oct4:Sox17
	1.00E-10	15.91%	0.29%	PB0154.1_Osr1_2

Motif	P-value	% of Targets	% of Background	Best Match/Details
	1.00E-09	17.86%	0.11%	PH0162.1_Six2
	1.00E-09	17.86%	0.11%	JunD(bZIP)
	1.00E-09	17.86%	0.15%	Stat3+il21(Stat)

S6 Table. Top transcription factor binding sites enriched for promoter regions of target downregulated (A) and upregulated (B) miRNAs in the FC of sCJD MM1 cases.

https://doi.org/10.1371/journal.ppat.1006802.s015

(TIF)

S7 Table

qPCR probe	Probe ID
human	
Drosha	Hs00203008_m1
Dicer	Hs00229023_m1
DGCR8	Hs00987085_m1
GAPDH	Hs03929097_g1
XPO5	Hs00382453_m1
TNRC6A	Hs00379422_m1
AGO2	Hs01085579_m1
mice	
Aif1/Iba1	Mm00479862_g1
Gfap	Mm01253033_m1
Gapdh	Mm99999915_g1
Hprt	Mm00446968_m1
Il1ß	Mm00434228_m1
Tnfα	Mm99999068_m1
Ccl3	Mm00441258_m1
Ccl4	Mm00443111_m1

miRCURY LNA PCR primer	Primer mix ID
hsa-miR-29b-3p	204679
hsa-miR-342-3p	205625
hsa-miR-146-5p	204688
hsa-miR-124-3p	206026
hsa-miR-154-5p	204518
hsa-miR-331-3p	204156
hsa-miR-195-5p	205869
hsa-miR-26a-5p	205905
hsa-miR-16-5p	205702
hsa-miR-378a-3p	204179
hsa-miR-877-5p	205626
hsa-miR-449a	204481
hsa-miR-142-3p	204291
let-7i-3p	204247
let-7d-5p	204124
hsa-miR-494-3p	204579
hsa-miR-125a-5p	204339
hsa-miR-5701	206999
hsa-miR-135a-5p	204762
hsa-miR-323a-5p	204085
hsa-miR-204-5p	205708
U6	203907
RNU5G	203908

Antibodies	Commercial source	Reference
anti-ß-Actin	Sigma	A5316
anti-GAPDH	Abcam	9484
anti-Ago2 (11A9)	EMD Millipore	MABE253
anti-eIF2C (H-300)/Ago1-4	Santa Cruz	sc-32877
anti-Dicer (H-212)	Santa Cruz	sc-30226
anti-Drosha (E-19)	Santa Cruz	sc-31159
anti-DGCR8 (D78E4)	Cell Signalling	6914
anti-PSD-95	Cell Signalling	2507
anti-synaptophysin	Novocastra	NCL-L-SYNAP-299
anti-eIF3	Santa Cruz	sc-16377
anti-Tia-1	Santa Cruz	sc-1751
anti-p54/rcK	MBL international	PD009
anti-dcpb1	Abnova	H00055802-M06
anti-oligomer (11A)	Thermo Fisher	AHB0052
anti-GW182	Novus Biological	NBP1-28751
anti-XPO5	ABclonal	A6790
anti-PrP	Bertin Pharma	A03206

S7 Table. List of Taqman probes, miRCURY LNA primers and antibodies used in this study.

https://doi.org/10.1371/journal.ppat.1006802.s016

(TIF)

S8 Table. Brain samples used in the present study.

Diagnostic, sCJD subtype, brain region, age, sex and the type of experiment for which each case was used (RNA-seq, miRNA qPCR, mRNA qPCR and western blot) is indicated.

https://doi.org/10.1371/journal.ppat.1006802.s017

(TIF)

S9 Table

Controls			
ID	*Age*	*Gender*	*Diagnostic*
Control 1	53	M	Infection
Control 2	83	M	Facial paralysis
Control 3	71	M	Neurodegenerative process
Control 4	57	F	Vasculitis
Control 5	72	M	Dementia
Control 6	59	F	Infection
Control 7	54	F	Dementia
Control 8	88	F	Encephalitis
Control 9	65	F	Encephalitis
Control 10	75	M	Polyneuropathy
Control 11	65	M	Unknown (incresed cell counts)
Control 12	57	F	Encephalitis

sCJD					
ID	*Age*	*Gender*	*tau*	*14–3–3*	*RT-QuiC*
sCJD 1	81	M	NA	+	+
sCJD 2	59	F	NA	+	+
sCJD 3	75	F	+	+	+
sCJD 4	64	M	NA	+	+
sCJD 5	77	M	NA	+	+
sCJD 6	64	M	+	+	+
sCJD 7	65	M	+	-	+
sCJD 8	72	F	+	+	-
sCJD 9	84	M	+	+	-
sCJD 10	65	M	+	+	NA
sCJD 11	60	F	+	+	NA
sCJD 12	65	F	+	+	+

S9 Table. Demographics and biomarker profiling for the CSF samples analyzed in this study.

NA = Non-analyzed, + = positive according to sCJD cut-off, − = negative according to sCJD cut-off. M = male, F = female.

https://doi.org/10.1371/journal.ppat.1006802.s018

(TIF)

7. Publication II

J Mol Neurosci (2017) 63:206–215
DOI 10.1007/s12031-017-0971-4

MicroRNA Expression in the Locus Coeruleus, Entorhinal Cortex, and Hippocampus at Early and Middle Stages of Braak Neurofibrillary Tangle Pathology

Franc Llorens[1] · Katrin Thüne[2,3] · Pol Andrés-Benito[4] · Waqas Tahir[1] · Belén Ansoleaga[4] · Karina Hernández-Ortega[4] · Eulàlia Martí[5] · Inga Zerr[1,3] · Isidro Ferrer[1,4,6,7]

Received: 11 August 2017 / Accepted: 24 August 2017 / Published online: 5 September 2017

Abstract The present study analyzes by RT-qPCR the expression of microRNA (miRNA)-27a-3p, miRNA-124-3p, miRNA-132-3p, and miRNA-143-3p in the locus coeruleus (LC), entorhinal cortex (EC), CA1 region of the hippocampus (CA1), and dentate gyrus (DG) of middle-aged (MA) individuals with no brain lesions and of cases at Braak and Braak stages I-II and II-IV of neurofibrillary tangle (NFT) pathology. The most affected region is the LC in which miRNA-27a-3p, miRNA-124-3p, and miRNA-143-3p show a trend to increase at stages I-II and are significantly up-regulated at stages III-IV when compared with MA. Only miRNA-143-3p is up-regulated in the EC at stages III-IV when compared with MA and with stages I-II. No modifications in the expression levels of miRNA-27a-3p, miRNA-124-3p, miRNA-132-3p, and miRNA-143-3p are found in CA1 at any stage, whereas miRNA-124-3p is significantly down-regulated in DG at stages I-II. Accompanying in situ hybridization reveals miRNA-27a-3p, miRNA-124-3p, and miRNA-143-3 localization in neurons, indicating that changes in miRNA expression are not a direct effect of changes in the numbers of neurons and glial cells. Present observations show for the first time important miRNA de-regulation in the LC at the first stages of NFT. Since the LC is the main noradrenergic input to the cerebral cortex, key regulator of mood and depression, and one of the first nuclei affected in aging and Alzheimer's disease (AD), these findings provide insights for additional study of the LC in aging and AD.

Keywords MicroRNA · Neurofibrillary tangle pathology · Alzheimer disease · Locus coeruleus · Entorhinal cortex · Hippocampus

Franc Llorens, Katrin Thüne, and Pol Andrés-Benito have equal contribution.

✉ Isidro Ferrer
8082ifa@gmail.com

1 CIBERNED (Network Centre for Biomedical Research of Neurodegenerative Diseases), Institute Carlos III, Ministry of Health, Madrid, Spain

2 Department of Neurology, University Medical School, Göttingen, Germany

3 German Centre for Neurodegenerative Diseases (DZNE), Göttingen, Germany

4 Institute of Neuropathology, IDIBELL-University Hospital Bellvitge, Hospitalet de Llobregat, Spain

5 Centre for Genomic Regulation, Barcelona, Spain

6 Department of Pathology and Experimental Therapeutics, Faculty of Medicine, University of Barcelona, c/Feixa Llarga sn, 08907 Hospitalet de Llobregat, Spain

7 Institute of Neurosciences, University of Barcelona, Barcelona, Spain

Introduction

MicroRNAs (miRNAs) are genome-encoded small RNAs that primarily regulate gene expression at the post-transcriptional level, thus controlling diverse cellular functions. miRNA genes can be located in intergenic regions or within protein-coding genes; they are transcribed as precursor miRNAs which are processed into its final ~ 21–23 nucleotide mature forms. Incorporated in the RNA-induced silencing complex (RISC), miRNAs imperfectly bind to specific target mRNA and direct gene expression repression (Meister 2007; Eulalio et al. 2009). Importantly, a single miRNA can target several mRNAs, and a single mRNA can be targeted by different miRNAs, thus leading to complex regulatory mechanisms geared to fine tuning

gene expression (Krek et al. 2005; Friedman et al. 2009). miRNAs are involved in a broad range of biological processes, presenting specific expression patterns in the central nervous system, where they regulate several functions including neuronal differentiation, circadian clock, synaptic plasticity, and neurite outgrowth (Follert et al. 2014). In this regard, miRNA levels do not necessarily reflect their expression efficacy; rather, these values represent a dynamic equilibrium between the synthesis and degradation rates, which may be disease-specific and different in particular brain regions compared to others (Krol et al. 2010b). For instance, miRNAs with high decay and increased transcription rates, such as miR-211 (Krol et al. 2010a), have been recently involved in the pathological mechanisms associated to several neurological conditions (Fan et al. 2016; Bekenstein et al. 2017).

Alzheimer's disease (AD) is a common age-related neurodegenerative process characterized neuropathologically by the presence of neurofibrillary tangles (NFTs) composed of hyper-phosphorylated tau and β-amyloid diffuse and senile plaques (Duyckaerts and Dickson 2011). NFT pathology is categorized into stages I-II with involvement of the entorhinal, transentorhinal cortex, and part of the hippocampus; stage III-IV with additional involvement of the limbic system; and stages V-VI with widespread NFTs in the neocortex. NFTs are also present in selected nuclei of the brain stem, as the locus coeruleus (LC), at early stages of the disease (Braak and Braak 1991; Rub et al. 2001; Simic et al. 2009; Arendt 2012; Ferrer 2012; Arendt et al. 2015; Braak and Del Tredici 2015; Andrés-Benito et al. 2017). It is worth noting that stages I-II and even stages III-IV are asymptomatic, although some individuals at stage IV may have cognitive impairment; classical dementia of AD type appears at stages V-VI. Stages I-IV, usually, are incidentally encountered in the routine post-mortem neuropathological examination.

miRNA expression in AD shows high region- and stage-dependent regulation (Cogswell et al. 2008; Hébert et al. 2008; Delay et al. 2012; Lau et al. 2013; Femminella et al. 2015). In this regard, Lau et al. showed down-regulation of 20 miRNAs and up-regulation of 15 miRNAs in the total hippocampus of 41 AD cases when compared to 23 controls. Temporal analysis was also performed in the pre-frontal cortex in a second cohort of 49 cases of AD at stages III-IV and V-VI (Lau et al. 2013). Here, we selected four miRNAs which have been implicated in pathological aspects of AD such as miRNA-27a-3p, miRNA-124-3p, and miRNA-132-3p (Cogswell et al. 2008; Hébert et al. 2008; Smith et al. 2011; Delay et al. 2012; Fang et al. 2012; Lau et al. 2013; Wong et al. 2013; Salta et al. 2016), or its expression is altered in blood as miRNA-143-3p (Cheng et al. 2014). Our focus was on the early stages of NFT pathology (stages I-II and III-IV) and in those regions which are first affected by NFT pathology in aging and AD. Expression levels of selected miRNAs were assessed by RT-qPCR in LC, entorhinal cortex, CA1 region of the hippocampus, and dentate gyrus. This was accompanied by in situ hybridization in tissue sections of a limited number of cases just to identify cellular localization.

Material and Methods

Human Cases

Human brain tissue was obtained from the Institute of Neuropathology Brain Bank (HUB-ICO-IDIBELL Biobank, Barcelona, Spain) following the guidelines of Spanish legislation and the approval of the local ethics committee.

One hemisphere was immediately cut into 1-cm-thick coronal sections, and selected areas of the encephalon were rapidly dissected, frozen on metal plates over dry ice, placed in individual air-tight plastic bags, numbered with water-resistant ink, and stored at − 80 °C. The other hemisphere was fixed by immersion in 4% buffered formalin for 3 weeks for morphologic studies. Neuropathological categorization was performed according to the Braak and Braak classification adapted for paraffin sections (Braak et al. 2006). Early-onset familial AD cases were not included. Since first stages of NFT pathology are not associated with β-amyloid deposits, we cannot exclude the possibility that several cases in the present series could be classified as primary age-related tauopathy (PART) (Crary et al. 2014). Cases with associated neurodegenerative processes (i.e., Lewy body disease, TDP43 proteinopathy, and other tauopathies such as argyrophilic grain disease and aging-related tau astrogliopathy (ARTAG)), vascular diseases, and metabolic syndrome were not included. Importantly, cases with systemic inflammatory, autoimmune, and infectious diseases were rejected. Special care was also taken to not include cases with hypoxia and prolonged agonal state (patients subjected to intensive care). Cases with minor changes consistent with small blood vessel disease were acceptable for the present purpose. Middle-aged (MA) cases with no neurological disease and no lesions at post-mortem neuropathological examination were used as controls. A total of 114 human samples of four different regions—locus coeruleus (LC), entorhinal cortex (EC), CA1 region of the hippocampus, and dentate gyrus (DG)—from 75 cases were selected for this study including 19 middle-aged (50.5 ± 7.3 years), 31 stage I-II (69.2 ± 9.1 years), and 25 stage III-IV (78.6 ± 7.1 years) cases. Not all regions were available in every case. The summary of cases and samples analyzed is shown in Table 1.

RNA Purification

Purification of RNA from LC, EC, CA1, and DG was carried out with RNeasy Lipid Tissue Mini Kit (Qiagen GmbH, Hilden, Germany) following the protocol provided by the manufacturer. Quality of isolated RNA was first measured with BioAnalyzer Assay (Agilent Technologies, Palo Alto,

Table 1 Summary of control (middle-aged: MA) and cases at Braak and Braak stages I-II and III-IV used for RT-qPCR analysis

Number	Braak &Braak	Age	Sex	PM	RIN			
					LC	EC	CA1	DG
1	I-II	61	M	03 h 40 min		6.9		
2	I-II	64	F	02 h 15 min		7.3		
3	I-II	58	M	08 h 00 min				6.7
4	I-II	79	F	03 h 35 min		7.3		
5	I-II	79	F	06 h 25 min		7.1		
6	I-II	64	M	04 h 35 min	6.4			
7	I-II	66	M	09 h 45 min		6.6		
8	I-II	73	M	07 h 05 min	6.4			
9	I-II	56	F	08 h 00 min	6.7			
10	I-II	67	M	14 h 40 min	6.6			
11	I-II	70	M	02 h 00 min	7.9			
12	I-II	61	M	04 h 30 min	6.6			
13	I-II	65	M	05 h 00 min		6.9		
14	I-II	77	F	03 h 15 min		7.2		
15	I-II	79	M	04 h 15 min		7.2		
16	I-II	83	M	04 h 30 min		8.0		
17	I-II	71	M	05 h 15 min		6.6	7.1	
18	I-II	86	F	04 h 15 min		7.4		
19	I-II	85	M	03 h 45 min		8.2		
20	I-II	66	M	05 h 45 min			6.7	6.6
21	I-II	77	M	02 h 55 min		7.0		
22	I-II	55	F	09 h 30 min			6.9	
23	I-II	66	M	14 h 00 min			6.4	
24	I-II	68	F	04 h 30 min	7.6		7.3	7.1
25	I-II	55	M	09 h 45 min		6.5	7.5	6.6
26	I-II	57	M	04 h 30 min	7.6	8.0		
27	I-II	69	M	03 h 45 min			7.0	8.0
28	I-II	80	M	03 h 30 min		7.3		
29	I-II	78	M	12 h 00 min			6.5	7.2
30	I-II	72	F	04 h 00 min				7.4
31	I-II	60	M	02 h 35 min	5.9			
32	III-IV	83	M	18 h 00 min		6.8		
33	III-IV	77	F	11 h 30 min		6.7		
34	III-IV	81	M	04 h 00 min		6.5		
35	III-IV	78	F	06 h 00 min	7.0			
36	III-IV	82	M	03 h 45 min		6.8		
37	III-IV	77	M	04 h 10 min			6.3	7.3
38	III-IV	65	F	04 h 10 min			6.8	7.2
39	III-IV	69	M	13 h 10 min		7.7		
40	III-IV	80	M	04 h 20 min		6.7	7.2	7.8
41	III-IV	67	F	06 h 10 min		6.6		
42	III-IV	90	F	04 h 00 min	7,2	6.9	7.1	7.5
43	III-IV	79	F	03 h 35 min		7.7	6.9	
44	III-IV	73	M	04 h 15 min	8.1			
45	III-IV	77	M	05 h 40 min		6.4		
46	III-IV	76	F	03 h 50 min			6.5	
47	III-IV	82	F	04 h 50 min		6.4		
48	III-IV	79	F	03 h 40 min			6.5	6.9
49	III-IV	82	F	03 h 05 min		6.4	6.8	6.4
50	III-IV	75	M	03 h 25 min	6.3	7.3		
51	III-IV	76	M	06 h 00 min	6.0			
52	III-IV	74	M	04 h 45 min	6.6			
53	III-IV	99	F	05 h 00 min			7.3	7.1
54	III-IV	79	M	04 h 15 min		7.0		
55	III-IV	89	M	03 h 20 min	7.5		6.4	
56	III-IV	77	M	19 h 00 min	8.1			
57	MA	66	F	04 h 15 min		6.9		
58	MA	40	M	05 h 10 min			6.3	
59	MA	44	M	06 h 40 min		7.0		
60	MA	52	M	03 h 00 min	6.50		7.10	7.2
61	MA	50	F	14 h 30 min				6.6
62	MA	50	M	17 h 15 min		6.6		
63	MA	48	F	04 h 00 min		6.7		8.1
64	MA	54	F	06 h 45 min			6.3	6.4

Table 1 (continued)

Number	Braak &Braak	Age	Sex	PM	RIN			
					LC	EC	CA1	DG
65	MA	52	M	04 h 40 min	7.0	6.7	7.2	
66	MA	52	F	05 h 45 min	6.1	6.6	6.2	6.7
67	MA	57	M	05 h 20 min		7.3		7.2
68	MA	41	M	11 h 35 min	5.9		6.8	6.9
69	MA	60	F	11 h 30 min			6.5	
70	MA	47	M	10 h 25 min		6.4		
71	MA	35	M	17 h 00 min			6.4	
72	MA	59	M	08 h 30 min	6.6		6.2	
73	MA	51	F	04 h 00 min		8.1	7.7	8.3
74	MA	54	M	08 h 45 min		6.8	6.8	
75	MA	48	F	14 h 30 min	6.0			

F female, *M* male, *PM* post-mortem delay, *RIN* RNA integrity number, *LC* locus coeruleus, *EC* entorhinal cortex, *CA1* region of the hippocampus, *DG* dentate gyrus

CA). RIN values in human cases are indicated in Table 1. The concentration of each sample was obtained from A260 measurements with Nanodrop 1000 (Thermo Scientific, Wilmington, DE). RNA integrity was tested using the Agilent 2100 BioAnalyzer (Agilent). No association between post-mortem delay and RNA integrity was found.

Retrotranscription and Quantitative PCR (RT-qPCR)

miRNAs were reverse-transcribed and individually quantified via miRNA-specific primers using the TaqMan® microRNA RT kit (Applied Biosystems, USA) and the associated microRNA-specific stem-loop primers (TaqMan® microRNA assay kit). Primer IDs were as follows: 001182 (miRNA-124-3p), 000408 (miRNA-27a-3p), 000457 (miRNA-132-3p), and 002249 (miRNA-143-3p). RT product was mixed with TaqMan® Universal PCR Master Mix and TaqMan® microRNA assay and run using an Applied Biosystems 7900HT real-time PCR instrument (Applied Biosystems). PCR reactions for each sample were run in duplicate, including blank controls without cDNA. Normalization, which showed no expression alteration between the analyzed groups of samples, was performed using RNU44. Shapiro-Wilk normality test was applied. After evaluation of non-parametric data distribution, Kruskal-Wallis test was used to evaluate statistical differences between groups. Dunn's multiple test comparison was used for *post hoc* analysis. Values of $p < 0.05$ were considered significant ($* p < 0.05$; $** p < 0.01$; $*** p < 0.001$). Since alterations between RNA expression profiles and RNA quality, as well as with the neuropathological state of the brain tissue/cognitive status, have been recently reported (Vermeulen et al. 2011; Barbash et al. 2017), correlation analyses were performed in order to determine potential associations between RIN values and post-mortem timing with miRNA expression levels. However, no associations were detected (Spearman rank correlations $p > 0.05$).

In Situ Hybridization

Fixation lasted for no more than 4 weeks, paraffin embedding was performed at 57 °C, and tissue sections 6 μm thick were obtained with a sliding microtome, mounted on glass slices and stored at 4 °C until use. All these procedures were carried out in RNase-free medium. Three cases were used for each one of the groups: MA, stages I-II, and stages III-IV. For miRNA detection, locked nucleic acid (LNA)-modified probes, which achieve high sequence specificity, low secondary structure, and minimal self-annealing, combined with signal amplification technology using enzyme-labeled immunoassay were obtained from Exiqon (Vedbaek, Denmark). The following double-digoxigenin (DIG)-labeled sequences of LNA probes were used: hsa-microRNA-124-3p: 5′-DIG/ggcattcaccgcgtgcctta/DIG-3′, hsa-microRNA-143-3p: 5′-DIG/gagctacagtgcttcatctca/DIG-3′, and hsa-microRNA-27a: 5′-DIG/gcggaacttagccactgtgaa/DIG-3′. The sequence for the U6 snRNA was 5′-DIG/cacgaatttgcgtgtcatcctt/-3′. U6 was used as positive control of specific nuclear staining. Six-micrometer-thick human tissue sections were de-waxed, deproteinized with 15 μg/ml protease K at 37 °C for 10 min, washed in PBS, and dehydrated in increasing concentrations of ethanol. The probe hybridization was performed overnight at 55 °C (miRNA-143, miRNA-27a-3p, U6 snRNA) or at 59 °C (miRNA-124-3p) with 100 nM for miRNA-143-3p and miRNA-27a, 40 nM for miRNA-124-3p, and 1 nM for U6 snRNA of LNA probe diluted in hybridization mix. After stringent washing in salt sodium citrate (SSC) buffer and blocking with 2% sheep serum/1% bovine serum albumin, probe-target complex was visualized immunologically with anti-DIG antibody (Roche, 1:800) conjugated to alkaline phosphatase acting on the chromogen NBT/BCIP. In some cases, slices were counterstained with nuclear fast red (Vector Laboratories, Burlingame, CA, USA). In situ hybridization for miRNA-132-3p was unsuccessful in our hands.

Results

Analysis of miRNA expression levels obtained by RT-qPCR using Taqman-specific primers showed marked regional differences (Fig. 1). Major alterations were observed in the LC, as miRNA-27a-3p, miRNA-124-3p, and miRNA-143-3p showed a trend to increase or a significant increase at stages I-II ($p = 0.07$, $p = 0.12$, and $p = 0.02$, respectively) and were significantly up-regulated at stages III-IV ($p = 0.01$, $p = 0.03$, and $p = 0.001$, respectively) when compared to MA. miRNA-132-3p showed a significant increase at stages I-II ($p = 0.01$).

In contrast, only miRNA-143-3p was up-regulated in the EC at stages III-IV when compared with MA ($p = 0.001$) and has a tendency to increase at stages I-II ($p = 0.1$). Expression levels of miRNA-27a-3p, miRNA-124-3p, and miR132-p were not modified.

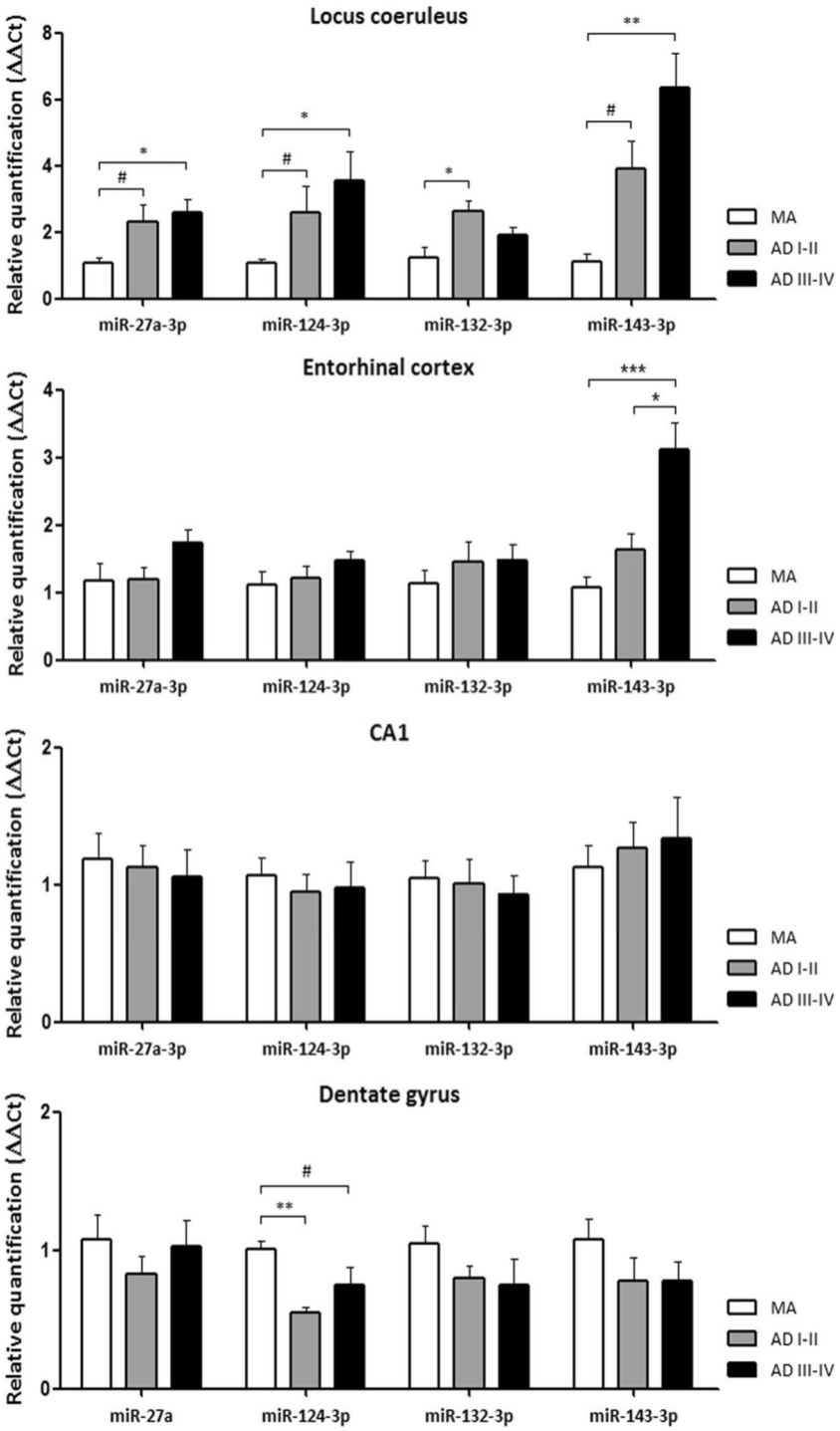


Fig. 1 miR-27a-3p, miR-124-3p, miR-132-3p, and miR-143-3p expression levels in the locus coeruleus, entorhinal cortex, CA1 region, and dentate gyrus in middle-aged (MA) and stages I-II and III-IV of BFT pathology as revealed by RT-qPCR. Expression levels are normalized with RNU44. Comparisons between groups are assessed with the Kruskal-Wallis test followed by Dunn's multiple test comparison for post-hoc analysis; #trend with no significant difference. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

No modifications in the expression levels of miRNA-27a-3p, miRNA-124-3p, miRNA-132-3p, and miRNA-143-3p were observed in the CA1 region at any stage.

Finally, miRNA-124-3p was significantly down-regulated ($p = 0.0001$) in the DG at stages I-II and showed a trend to decrease at stages III-IV ($p = 0.09$). Expression levels of miRNA-27a-3p, miRNA-132-3p, and miRNA-143-3p were not modified in the DG at any stage.

No modifications in the expression levels of miRNA-27a-3p, miRNA-124-3p, miRNA-132-3p, and miRNA-143-3p were observed in the CA1 region at any stage.

Gender differences have been reported in the prevalence and cognitive function in AD. After stratification of RT-qPCR results, absence of gender-dependent differences on miRNA was detected ($p > 0.05$).

In situ hybridization of miRNA-27a-3p, miRNA-124-3p, and miRNA-143-3 was carried out in a few slices containing the EC, CA1, and DG (Fig. 2). miRNA-143-3p was expressed in the cytoplasm of neurons in every case whereas miRNA-27a-3p staining was in the cytoplasm and nucleus of neurons. U6 small nuclear RNA was used as a positive control of specific nuclear staining (data not shown). miRNA-124-3p was expressed in the cytoplasm of neurons. In situ hybridization of miRNA-124-3p and miRNA-143-3p in the LC revealed expression localized in the cytoplasm of neurons (Fig. 3). Because of the low number of cases in every group, no attempt was made to quantify the in situ hybridization signal in tissue sections.

Discussion

The present findings support the concept that marked differences exist among miRNAs, brain regions, and age-related Braak and Braak stages of NFT pathology. Since MA cases are younger than those with NFT pathology, it cannot be established as a working hypothesis that modifications in miRNA expression are partly due to aging. In this line, it is worth stressing that about 85% of individuals aged 65 years have at least stages I-II of NFT pathology (Ferrer 2012). Whether such early stages at this age are normal aging or first stages of a degenerative process named AD or PART is not solved. A limitation of the study is the modest cohort of cases analyzed due to the low availability of neuropathologically well-characterized AD samples from the selected brain regions.

miRNA-27a-3p was previously reported up-regulated in the cerebellum, hippocampus, middle-frontal gyrus, and pre-frontal cortex in AD (Cogswell et al. 2008; Hébert et al. 2008; Delay et al. 2012; Lau et al. 2013). The present findings do not support these data in the EC, CA1, and DG, at least at stages I-II and III-IV of NFT pathology. However, miRNA-27a-3p is up-regulated in the LC at these stages. In situ hybridization shows miRNA-27a-3p localization in the cytoplasm and nuclei of neurons. Mature forms of miRNA-92a, miRNA-25, miRNA-27a, and miRNA-92b have been found in nuclear structures (Politz et al. 2009) and nuclear-enriched fractions of primary cultured neurons (Khudayberdiev et al. 2013). Although the precise function of nuclear miRNAs and other RISC components is unknown, some studies suggest roles in post-transcriptional gene silencing/activation, miRNA storage, alternative splicing, and chromatin structure (Liang et al. 2013; Roberts 2014).

miRNA-124-3p expression has been reported as unaltered in pre-frontal cortex (Lau et al. 2013), moderately decreased in the anterior temporal cortex (Smith et al. 2011), and decreased in the hippocampus (Lau et al. 2013) in AD. miRNA-124-3p is up-regulated in the LC at stages I-II and III-IV, not modified in EC and CA1, and reduced in the DG at stages I-II, in the present series. miRNA-124-3p is expressed in neurons and regulates a broad range of genes involved in synaptic plasticity, memory functions, and neuronal development, among others (Jovičić et al. 2013; Sun et al. 2015; Gu et al. 2016; Li and Ling 2017). miR-124 has been reported to play a protective role in β-amyloid-mediated toxicity by targeting BACE 1 (Fang et al. 2012). Yet, no relation is found between lack of β-amyloid deposition in these structures at early stages of NFT pathology and miRNA-124-3p de-regulation. miRNA-124 also induces protection after focal ischemia and in experimental models of Parkinson's disease (Hamzei Taj et al. 2016; Saraiva et al. 2016).

On the other hand, miRNA-132 is a highly conserved and stress-responding miRNA (Haviv et al. 2017) involved in a broad range of neuronal functions such as neuronal plasticity and activity (Magill et al. 2010; Hansen et al. 2010) in agreement with its specific neuronal localization detected in our in situ hybridizations. miRNA-132 regulates differentiation of dopaminergic neurons (Yang et al. 2012), is a major regulator of the cholinergic system (Maharshak et al. 2013), and seems to play a role in AD pathogenesis as its decrease augments β-amyloid deposition in transgenic mice (Hernandez-Rapp et al. 2016; Salta and De Strooper 2017). miRNA-132-3p expression is down-regulated in several brain regions, including the hippocampus and prefrontal cortex in AD brains (Cogswell et al. 2008; Smith et al. 2011; Salta et al. 2016). Down-regulation of miRNA-132-3p in AD brain has been associated with neuronal tau hyper-phosphorylation (Lau et al. 2013; Salta et al. 2016) and with β-amyloid (Smith et al. 2011). Yet, the present findings show no changes in EC, CA1, and DG but miRNA-132-3p up-regulation in the LC. In this regard, it is worth noting that tau-hyper-phosphorylation is present in the LC, EC, and CA1 whereas no tau pathology is found in the DG; β-amyloid is absent in the LC and only scarce in the hippocampus at the assessed stages of NFT pathology. It can be argued that miRNAs may

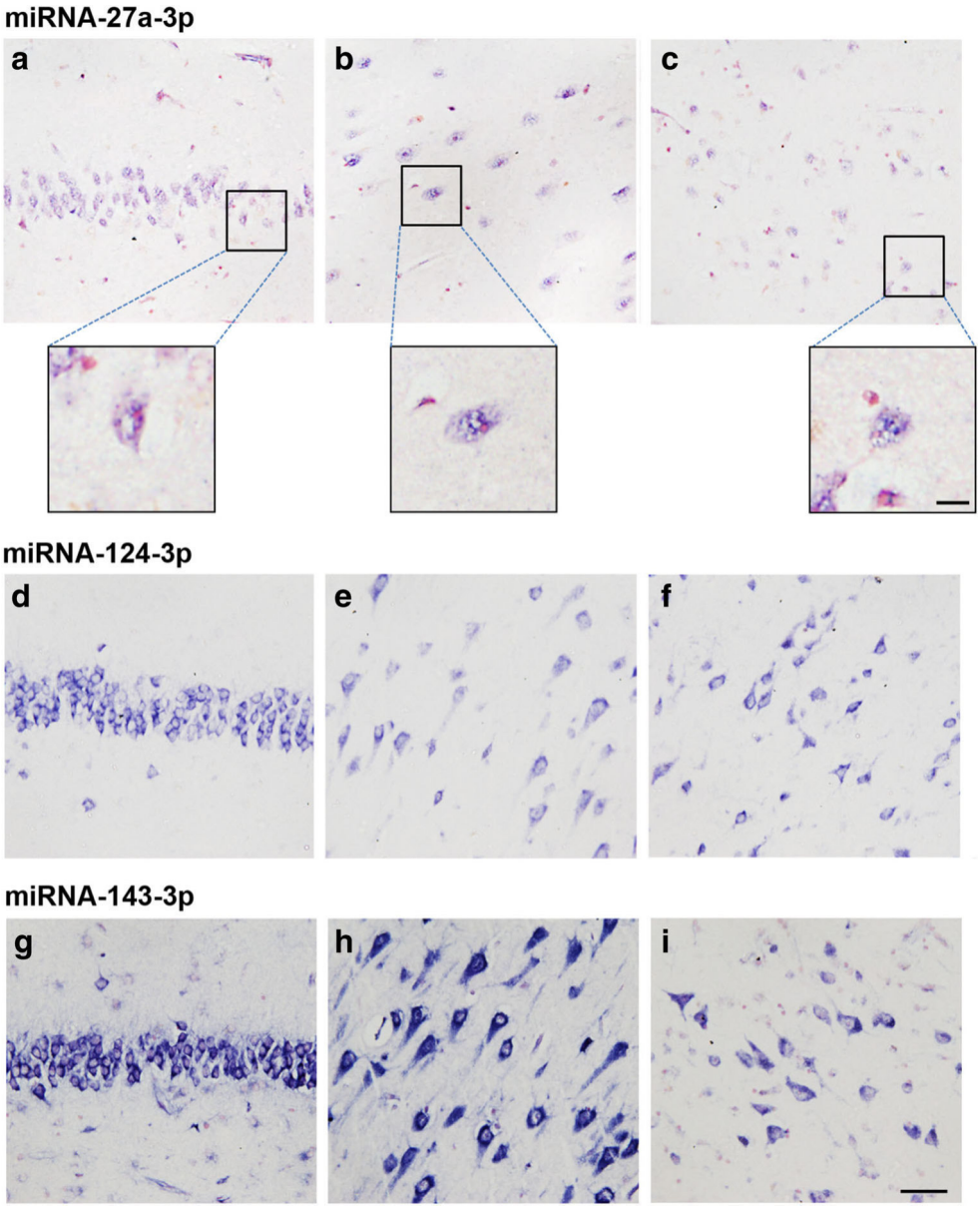


Fig. 2 Localization of miRNA-27a-3p, miRNA-124-3p, and miRNA-143-3p in the dentate gyrus (**a**, **d**, **g**), CA1 region of the hippocampus (**b**, **e**, **h**), and entorhinal cortex (**c**, **f**, **i**) in a case with NFT pathology at stage I-II as revealed by in situ hybridization. miRNA-27a-3p is localized in the cytoplasm and nuclei of neurons whereas miRNA-124-3p and miRNA-143-3p are localized only in the cytoplasm of neurons. Paraffin sections without counterstaining, bar = 100 μm; bar inserts = 10 μm

have different functions in different settings/regions and when acting in combination with other miRNAs, but this suggestion must be sustained by more robust experimental data.

miRNA-143-3p expression is elevated in blood in AD (Cheng et al. 2014). Interestingly, miRNA-143-3p is significantly up-regulated in the LC and EC in the present series. In situ hybridization has shown localization in neurons; therefore, it cannot be established that increased miRNA-143-3p expression in EC is due to increased numbers of astrocytes. miRNA-143-3p has been linked to muscle aging and altered muscle regeneration (Soriano-Arroquia et al. 2016). However, no information is available, as far as we know, regarding effects of miR-143-p regulation in brain.

The focus of the present study is on the LC, in which up-regulation of four miRNAs is found with disease progression at early stages of NFT pathology. It is important to stress that the LC is the main source of noradrenergic innervations of the cerebral cortex and that LC is the main noradrenergic nucleus affected in AD (Zarow et al. 2003). Moreover, LC is one the first regions of the brain showing NFTs in the context of AD-related pathology (Rub et al. 2001; Simic et al. 2009; Attems et al. 2012; Andrés-Benito et al. 2017; Šimić et al. 2017). De-regulation of molecular pathways in LC has important implications in the control of arousal, attention, sleep-awake cycles, emotional states, cognition, memory, and learning, among others, all of which are altered in AD (Aston-Jones and Cohen 2005; Szabadi 2013; Šimić et al. 2017).

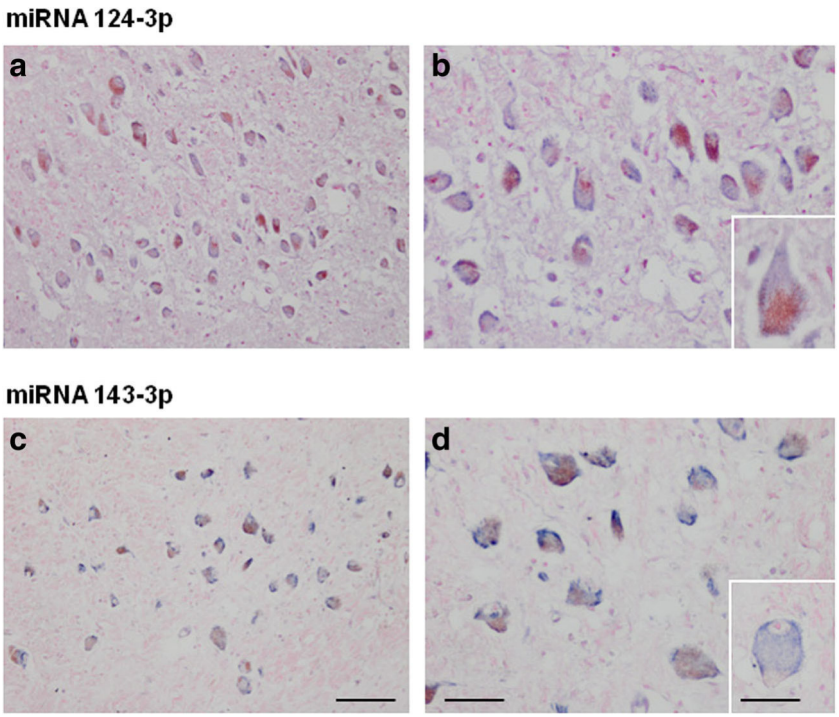


Fig. 3 Localization of miRNA-124-3p (**a**, **b**) and miRNA-143-3p (**c**, **d**) in the locus coeruleus of a case with NFT pathology at stage I-II as revealed by in situ hybridization. miRNA-124-3p and miRNA-143-3p are localized only in the cytoplasm of neurons. Paraffin sections without counterstaining. **a**, **c** Bar in A = 120 μm; **b**, **d** Bar in D = 60 μm; bar inserts = 28 μm

Conclusion

Our working hypothesis was that similar alterations might be found in EC (at least) and LC because these structures are first targets of NFT pathology with age. Present findings indicate that miRNA changes are independent of tau-phosphorylation and are likely not related to neuron loss as no neuronal loss is found at these stages of NFT pathology in LC (Rodríguez et al. 2012; Arendt et al. 2015). However, involvement of the LC at early stages of NFT pathology has important functional implications: loss of synapses in spite of preserved tyrosine hydroxylase (TH) immunoreactivity is found in NFT-containing neurons of LC, but decreased TH and increased α_{2A} adrenergic receptor protein levels occur in the hippocampus and the amygdala which primary targets noradrenergic innervations (Andrés-Benito et al. 2017). These observations shift the focus of the present observations to the LC and to its participation in non-cognitive symptoms in aging and AD including altered mood and depression. In this line, miRNA-124-3p has been postulated as a putative epigenetic signature of major depressive disorder (Roy et al. 2016). In short, they suggest that certain miRNAs may participate in the development of non-cognitive symptoms in aging and early stages of AD by modulating the function of the LC.

Acknowledgements This study was supported by grants from CIBERNED and Instituto de Salud Carlos III (Ministerio de Economía y Competitividad) co-funded by FEDER funds/European Regional Development Fund (ERDF)—a way to build Europe: PIE14/00034 and PI14/00757 to IF, by the Spanish Ministry of Health—Instituto Carlos III (Miguel Servet - CP16/00041) to FL and IFI15/00035 fellowship to PA-B; and by the Alzheimer-Forschungs-Initiative e.V. (AFI 12851) to IZ. We thank T. Yohannan for editorial help.

Author's Contribution FL, KT, and WT carried out RT-qPCR and in situ hybridization; PA and BA performed RT-qPCR; KHO contributed to the selection of samples and dissection of tissues; FL, IZ, and EM designed the microRNA study; and IF designed the study, supervised all the results, and wrote the manuscript which was circulated among the authors. All the authors contributed to the final discussion and approval of the manuscript.

Compliance with Ethical Standards Human brain tissue was obtained from the Institute of Neuropathology Brain Bank (HUB-ICO-IDIBELL Biobank, Barcelona, Spain) following the guidelines of Spanish legislation and the approval of the local ethics committee.

Competing Interests The authors declare that they have no competing interests.

References

Andrés-Benito P, Fernández-Dueñas V, Carmona M, Escobar LA, Torrejón-Escribano B, Aso E, Ciruela F, Ferrer I (2017) Locus coeruleus at asymptomatic early and middle Braak stages of neurofibrillary tangle pathology. Neuropathol Appl Neurobiol 43:373–392. https://www.ncbi.nlm.nih.gov/pubmed/28117912

Arendt T (2012) Cell cycle activation and aneuploid neurons in Alzheimer's disease. Mol Neurobiol 46:125–135. https://doi.org/10.1007/s12035-012-8262-0

Arendt T, Bruckner MK, Morawski M et al (2015) Early neurone loss in Alzheimer's disease: cortical or subcortical? Acta Neuropathol Commun 3:10. https://doi.org/10.1186/s40478-015-0187-1

Aston-Jones G, Cohen JD (2005) An integrative theory of locus coeruleus-norepinephrine function: adaptive gain and optimal

performance. Annu Rev Neurosci 28:403–450. https://doi.org/10.1146/annurev.neuro.28.061604.135709

Attems J, Thal DR, Jellinger KA (2012) The relationship between subcortical tau pathology and Alzheimer's disease. Biochem Soc Trans 40:711–715. https://doi.org/10.1042/BST20120034

Barbash S, Garfinkel BP, Maoz R et al (2017) Alzheimer's brains show inter-related changes in RNA and lipid metabolism. Neurobiol Dis 106:1–13. https://doi.org/10.1016/j.nbd.2017.06.008

Bekenstein U, Mishra N, Milikovsky DZ et al (2017) Dynamic changes in murine forebrain miR-211 expression associate with cholinergic imbalances and epileptiform activity. Proc Natl Acad Sci. https://doi.org/10.1073/pnas.1701201114

Braak H, Braak E (1991) Acta H â€™ pathologica neuropathological stageing of Alzheimer-related changes. Acta Neuropathol 82:239–259. https://doi.org/10.1007/BF00308809

Braak H, Del Tredici K (2015) The preclinical phase of the pathological process underlying sporadic Alzheimer's disease. Brain 138:2814–2833. https://doi.org/10.1093/brain/awv236

Braak H, Alafuzoff I, Arzberger T et al (2006) Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. Acta Neuropathol 112:389–404. https://doi.org/10.1007/s00401-006-0127-z

Cheng L, Doecke J, Sharples R et al (2014) Prognostic serum miRNA biomarkers associated with Alzheimer's disease shows concordance with neuropsychological and neuroimaging assessment. Mol Psychiatry 20:1–9. https://doi.org/10.1038/mp.2014.127

Cogswell JP, Ward J, Taylor IA et al (2008) Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways. J Alzheimers Dis 14:27–41. https://doi.org/10.1016/j.jalz.2008.05.420

Crary JF, Trojanowski JQ, Schneider JA et al (2014) Primary age-related tauopathy (PART): a common pathology associated with human aging. Acta Neuropathol 128:755–766. https://doi.org/10.1007/s00401-014-1349-0

Delay C, Mandemakers W, Hébert SS (2012) MicroRNAs in Alzheimer's disease. Neurobiol Dis 46:285–290

Duyckaerts C, Dickson D (2011) Neuropathology of Alzheimer's disease and its variants. In: Dickson DW, Weller RO (eds) Neurodegeneration, the molecular pathology of dementia and movement disorders, 2nd edn. Wiley-Blackwell, Oxford, pp 62–91

Eulalio A, Tritschler F, Izaurralde E (2009) The GW182 protein family in animal cells: new insights into domains required for miRNA-mediated gene silencing. RNA 15:1433–1442. https://doi.org/10.1261/rna.1703809

Fan C, Wu Q, Ye X et al (2016) Role of miR-211 in neuronal differentiation and viability: implications to pathogenesis of Alzheimer's disease. Front Aging Neurosci. https://doi.org/10.3389/fnagi.2016.00166

Fang M, Wang J, Zhang X et al (2012) The miR-124 regulates the expression of BACE1/??-secretase correlated with cell death in Alzheimer's disease. Toxicol Lett 209:94–105. https://doi.org/10.1016/j.toxlet.2011.11.032

Femminella GD, Ferrara N, Rengo G (2015) The emerging role of microRNAs in Alzheimer's disease. Front Physiol 6

Ferrer I (2012) Defining Alzheimer as a common age-related neurodegenerative process not inevitably leading to dementia. Prog Neurobiol 97:38–51

Follert P, Cremer H, Béclin C (2014) MicroRNAs in brain development and function: a matter of flexibility and stability. Front Mol Neurosci 7:5. https://doi.org/10.3389/fnmol.2014.00005

Friedman RC, Farh KKH, Burge CB, Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 19:92–105. https://doi.org/10.1101/gr.082701.108

Gu X, Li A, Liu S et al (2016) MicroRNA124 regulated neurite elongation by targeting OSBP. Mol Neurobiol 53:6388–6396. https://doi.org/10.1007/s12035-015-9540-4

Hamzei Taj S, Kho W, Riou A et al (2016) MiRNA-124 induces neuroprotection and functional improvement after focal cerebral ischemia. Biomaterials 91:151–165. https://doi.org/10.1016/j.biomaterials.2016.03.025

Hansen KF, Sakamoto K, Wayman GA, Impey S, Obrietan K (2010) Transgenic miR132 alters neuronal spine density and impairs novel object recognition memory. PLoS One 5: e15497. https://www.ncbi.nlm.nih.gov/pubmed/21124738

Haviv R, Oz E, Soreq H (2017) The stress-responding miR-132-3p shows evolutionarily conserved pathway interactions. Cell Mol Neurobiol. https://doi.org/10.1007/s10571-017-0515-z

Hébert SS, Horré K, Nicolaï L et al (2008) Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/beta-secretase expression. Proc Natl Acad Sci U S A 105:6415–6420. https://doi.org/10.1073/pnas.0710263105

Hernandez-Rapp J, Rainone S, Goupil C et al (2016) microRNA-132/212 deficiency enhances Aβ production and senile plaque deposition in Alzheimer's disease triple transgenic mice. Sci Rep 6:30953. https://doi.org/10.1038/srep30953

Jovičić A, Roshan R, Moisoi N et al (2013) Comprehensive expression analyses of neural cell-type-specific miRNAs identify new determinants of the specification and maintenance of neuronal phenotypes. Ann Intern Med 158:5127–5137. https://doi.org/10.1523/JNEUROSCI.0600-12.2013

Khudayberdiev SA, Zampa F, Rajman M, Schratt G (2013) A comprehensive characterization of the nuclear microRNA repertoire of post-mitotic neurons. Front Mol Neurosci 6:43. https://doi.org/10.3389/fnmol.2013.00043

Krek A, Grün D, Poy MN et al (2005) Combinatorial microRNA target predictions. Nat Genet 37:495–500. https://doi.org/10.1038/ng1536

Krol J, Busskamp V, Markiewicz I et al (2010a) Characterizing light-regulated retinal microRNAs reveals rapid turnover as a common property of neuronal MicroRNAs. Cell 141:618–631. https://doi.org/10.1016/j.cell.2010.03.039

Krol J, Loedige I, Filipowicz W (2010b) The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 11:597–610. https://doi.org/10.1038/nrg2843

Lau P, Bossers K, Janky R et al (2013) Alteration of the microRNA network during the progression of Alzheimer's disease. EMBO Mol Med 5:1613–1634. https://doi.org/10.1002/emmm.201201974

Li G, Ling S (2017) MiR-124 promotes newborn olfactory bulb neuron dendritic morphogenesis and spine density. J Mol Neurosci 61:159–168. https://doi.org/10.1007/s12031-016-0873-x

Liang H, Zhang J, Zen K et al (2013) Nuclear microRNAs and their unconventional role in regulating non-coding RNAs. Protein Cell 4:325–330

Maharshak N, Shenhar-Tsarfaty S, Aroyo N et al (2013) MicroRNA-132 modulates cholinergic signaling and inflammation in human inflammatory bowel disease. Inflamm Bowel Dis 19:1346–1353. https://doi.org/10.1097/MIB.0b013e318281f47d

Magill ST, Cambronne XA, Luikart BW, Lioy DT, Leighton BH, Westbrook GL, Mandel G, Goodman RH (2010) microRNA-132 regulates dendritic growth and arborization of newborn neurons in the adult hippocampus. Proc Natl Acad Sci U S A 107:20382–20387. https://www.ncbi.nlm.nih.gov/pubmed/?term=magill%2C+2010%2C+microRNA

Meister G (2007) miRNAs get an early start on translational silencing. Cell 131:25–28

Politz JCR, Hogan EM, Pederson T (2009) MicroRNAs with a nucleolar location. RNA 15:1705–1715. https://doi.org/10.1261/rna.1470409

Roberts TC (2014) The microRNA biology of the mammalian nucleus. Mol Ther Nucleic Acids 3:e188. https://doi.org/10.1038/mtna.2014.40

Rodríguez JJ, Noristani HN, Verkhratsky A (2012) The serotonergic system in ageing and Alzheimer's disease. Prog Neurobiol 99:15–41

Roy B, Dunbar M, Shelton RC, Dwivedi Y (2016) Identification of MicroRNA-124-3p as a putative epigenetic signature of major depressive disorder. Neuropsychopharmacology:1–38. https://doi.org/10.1038/npp.2016.175

Rub U, Del TK, Schultz C et al (2001) The autonomic higher order processing nuclei of the lower brain stem are among the early targets of the Alzheimer's disease-related cytoskeletal pathology. Acta Neuropathol 101:555–564. https://doi.org/10.1007/s004010000320

Salta E, De Strooper B (2017) MicroRNA-132: a key noncoding RNA operating in the cellular phase of Alzheimer's disease. FASEB J 31: 424–433. https://doi.org/10.1096/fj.201601308

Salta E, Sierksma A, Vanden Eynden E, De Strooper B (2016) miR-132 loss de-represses ITPKB and aggravates amyloid and TAU pathology in Alzheimer's brain. EMBO Mol Med 8:1005–1018. 10.15252/emmm.201606520

Saraiva C, Paiva J, Santos T et al (2016) MicroRNA-124 loaded nanoparticles enhance brain repair in Parkinson's disease. J Control Release 235:291–305. https://doi.org/10.1016/j.jconrel.2016.06.005

Simic G, Stanic G, Mladinov M et al (2009) Does Alzheimer's disease begin in the brainstem?: annotation. Neuropathol Appl Neurobiol 35:532–554

Šimić G, Babić Leko M, Wray S et al (2017) Monoaminergic neuropathology in Alzheimer's disease. Prog Neurobiol 151:101–138

Smith P, Al Hashimi A, Girard J et al (2011) In vivo regulation of amyloid precursor protein neuronal splicing by microRNAs. J Neurochem 116:240–247. https://doi.org/10.1111/j.1471-4159.2010.07097.x

Soriano-Arroquia A, McCormick R, Molloy AP et al (2016) Age-related changes in miR-143-3p:Igfbp5 interactions affect muscle regeneration. Aging Cell. https://doi.org/10.1111/acel.12442

Sun Y, Luo Z-M, Guo X-M et al (2015) An updated role of microRNA-124 in central nervous system disorders: a review. Front Cell Neurosci 9:193. https://doi.org/10.3389/fncel.2015.00193

Szabadi E (2013) Functional neuroanatomy of the central noradrenergic system. J Psychopharmacol 27:659–693. https://doi.org/10.1177/0269881113490326

Vermeulen J, De Preter K, Lefever S et al (2011) Measurable impact of RNA quality on gene expression results from quantitative PCR. Nucleic Acids Res. https://doi.org/10.1093/nar/gkr065

Wong HKA, Veremeyko T, Patel N et al (2013) De-repression of FOXO3a death axis by microRNA-132 and -212 causes neuronal apoptosis in Alzheimer's disease. Hum Mol Genet 22:3077–3092. https://doi.org/10.1093/hmg/ddt164

Yang D, Li T, Wang YY et al (2012) miR-132 regulates the differentiation of dopamine neurons by directly targeting Nurr1 expression. J Cell Sci 125:1673–1682. https://doi.org/10.1242/jcs.086421

Zarow C, Lyness SA, Mortimer JA, Chui HC (2003) Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. Arch Neurol 60:337–341. https://doi.org/10.1001/archneur.60.3.337

8. Discussion

8.1 miRNA research in human prion diseases and Alzheimer's disease

Increasing evidences postulate alterations in miRNA signatures in NDs as critical factor in pathogenesis, progression and prognosis of diseases (Abe and Bonini 2013a; Hébert and De Strooper 2009a; Femminella, Ferrara, and Rengo 2015; Bellingham and Hill 2017; Tan, Yu, and Tan 2015). However, different levels of progress and insights in miRNA expression patterns and miRNA-regulated gene networks have been achieved in the brain of patients afflicted with neurodegenerative conditions. The presented publications provided comprehensive characterizations of miRNA signatures in sCJD and during early AD-type NFT pathology, revealing crucial insights in the role of miRNA deregulation in NDs. The studies complement previous research and develop significantly the understanding of miRNA dysregulation in ND.

8.1.1 Current status and progress in miRNA research in human prion diseases

Evaluation of miRNA alterations in human prion diseases are limited to targeted studies in the brain of sCJD (Montag et al. 2009; Lukiw et al. 2011) and GSS patients (Lukiw et al. 2011). When identifying miRNA alterations during disease, the major issues in these studies are the small number of patients, controls and different brain regions analysed. Additionally, the miRNAs screens were performed to identify the expression of specific brain miRNAs suggested to be involved in prion diseases. Comprehensive and temporally analysis of miRNAs expression in human prion diseases are clearly needed to determine global expression signatures in disease affected brain regions in order to understand which miRNA-controlled molecular networks are impaired during disease. In order to capture fully miRNA changes, it is necessary to conduct miRNA quantifications in a large number of patients by a combination of different analytical techniques and methodologies.

In the presented **publication I**, we identified for the first time global miRNA expression alterations in the frontal cortex and cerebellum of sCJD. We demonstrated that the miRNA expression patterns in two sCJD subtypes, MM1 and VV2, are highly changed in a regional and disease subtype-dependent manner correlating with neuropathological affection, which suggests specific functional roles of the miRNA network in sCJD pathology as potential driving factors in disease etiology. In order to characterize the cellular environment of

miRNA dysregulation, a neural-type specificity of altered miRNAs was unveiled by *in situ* assays.
Potential underlying aberrant mechanisms leading to massive alteration of the miRNA patterns in sCJD were investigated. By analyzing the key proteins involved in miRNA biogenesis, we observed reduced levels of the miRNA-processing RNases Drosha and Dicer as well as the microprocessor complex subunit DGCR8 in frontal cortex and cerebellum in sCJD, pointing out strong evidences for impaired miRNA biogenesis machinery in the late stages of sCJD.
A sCJD mouse model that recapitulates faithfully sCJD neuropathology in the brain (Padilla et al. 2011; Llorens et al. 2014), provided insights into pathological processes during preclinical phases of the sCJD progression. The observed miRNA signature of the sCJD mouse model resembled the miRNA expression patterns in sCJD brain. Temporal miRNA expression analysis in the sCJD mouse model revealed a link between regional pathology and miRNA dysregulation during disease progression, indicating functional implications of the deregulated miRNA network in sCJD pathology.
In cross-disease validation studies, we detected that specific sCJD-regulated miRNAs are commonly altered in alternative NDs such as AD, dementia with Lewy bodies (DLB) and FFI, shedding light shedding light into potential common miRNA-related mechanisms in the neurodegenerative processes.

It has been demonstrated that miRNA expression levels, biogenesis, cellular abundance and miRNA function are differentially regulated in tissue of origin (Gulyaeva and Kushlinskiy 2016; Schanen and Li 2011a). Therefore systematic characterizations of putative regulators of cellular- and tissue-specific miRNA expression and function are essential for the understanding of miRNA function in pathophysiological processes during human prion disease. This entails, for instance, the context-specific characterisation of miRNA biogenesis machinery, formation of silencing complex RISC, P-bodies and stress granules, transcription factors, RNA-binding proteins, isomeric miRNA sequences, and the cellular environment of miRNAs during disease. Under consideration of context-specific factors, different experimantal procedures in relevant tissue or cell types modelling disease conditions are essential to investigate how miRNAs regulate complex gene networks in disease. Experimental disease models resembling cellular environment and gene networks including the expression of disease-associated miRNAs patterns and tissue specific target genes are needed to determining what genes are regulated by a miRNAs during disease (Akbari Moqadam, Pieters, and Den Boer 2013).

Gaining a complete picture of the global disease-associated miRNA signatures, context-specific factors and the neural cell types in which miRNA dysregulation occurs will be the first step to open new research lines for identification of miRNA targets and complex miRNA-regulated networks during disease.

8.1.2 Current status and progress in miRNA research in AD

Global and temporally alterations of miRNA signatures in the brain of AD patients have been identified revealing altered miRNA networks which are associated to brain regional and disease stage-dependent features of AD pathology (Lau et al. 2013; Cogswell et al. 2008; Hebert et al. 2008; Müller et al. 2014; Delay, Mandemakers, and Hébert 2012). Moreover, experimental evidences have linked specific miRNA changes to AD pathogenesis (Delay and Hébert 2011; Cai, Zhao, and Zhao 2012; Banzhaf-Strathmann et al. 2014; Smith et al. 2015; Hernandez-Rapp et al. 2016). However, less information are available about miRNA alterations during initial stages of AD. The LC is one of the earliest regions affected by consistent and severe neuronal dysfunction and loss during early AD stages, which has been linked to noradrenalin depletion of the projection areas in the forebrain region (Andrés-Benito et al. 2017; Weinshenker 2008; Giorgi et al. 2017).

In the presented **publication II**, we performed miRNA expression profiling in the LC, EC, hippocampal CA1 region and dentate gyrus at early and mid-stages of Braak NFT pathology. The profiled miRNAs were selected according to their described implications in pathological aspects in AD. Using the expertise of characterization miRNA signatures in sCJD by the use of different analytical techniques and methodologies, we aimed to extent our studies by analyzing the expression of selected miRNAs reported to be involved in Alzheimer's disease pathology.

We provided for the first time insights into crucial miRNA expression alterations in the LC during early NFT pathology before AD-associated neuronal death is evident. In the LC, the miRNA-27a-3p, miRNA-124-3p, and miRNA-143-3p showed a trend to increase their expression at early stages (Braak I-II) and they were found significantly upregulated at mid-stages (Braak III-IV) of disease. Only miRNA-143-3p has been found upregulated in EC at mid-stages of disease. None of the profiled miRNAs revealed expression alterations in the hippocampal CA1 region, while miRNA-124-3p was downregulated in DG at early stages of NFT pathology.
The characterization of the cellular miRNA environment via *in situ* hybridization assays revealed that the deregulated miRNAs are localized in the cytoplasm of neurons during NFT pathology progression. Additionally, miRNA-27a-3p has been shown to be localized in the nucleus.

We provided strong evidences that the observed miRNA alterations are involved in the early AD pathology. Weather the altered miRNAs are involved in the selective neuronal degeneration in early AD stages, or representing neuroprotective compensatory pathways during early disease evolution is not clear.

8.2 Profiling disease-associated miRNA signatures in sCJD and early AD-type neuropathology

We aimed to identify disease-associated miRNA expression patterns during critical disease stages in the brain of sCJD and during early AD-type NFT neuropathology. In publication I, we were able to gain a comprehensive picture of global miRNA expression changes in the frontal cortex and cerebellum in clinical stages of sCJD. In publication II, we successfully performed miRNA expression analyses during the first stages of NFT formations in the LC, EC, hippocampal CA1 region and dentate gyrus. Thereby, we intended to perform a targeted expression profiling of miRNAs that have been suggested to be implicated in AD pathogenesis in brain regions vulnerable to early AD-type NFT pathology.

8.2.1 Spatial and cellular miRNA profiling in the brain of sCJD patients

For the first time, we provided comprehensive miRNA expression analysis in *postmortem* human sCJD brains revealing regional- and subtype-specific miRNA signatures. We used small RNA sequencing (RNAseq) analysis and quantitative polymerase chain reaction (qPCR) validation to profile the miRNA expression changes in two brain areas of two sCJD subtypes in a sufficiently large cohort of sCJD patients and controls. Our work clearly demonstrates that the miRNA expression is altered in sCJD brain, with 70 miRNAs significantly altered in the frontal cortex of sCJD MM1 and 27 in sCJD VV2 compared to controls. In the cerebellum, 22 miRNAs were found altered in sCJD MM1 and 69 in sCJD VV2 compared to controls. Through the use of independent pipelines for the differential miRNA expression analyses, Seqbuster (Pantano, Estivill, and Martí 2009) and OASIS (Capece et al. 2015), we aimed to achieve reproducible and accurate representations of the transcriptional miRNA expression changes in sCJD. Indeed a high degree of coincidence between both pipelines (~ 89%) in detecting differentially expressed miRNAs was achieved. RNAseq provides the opportunity of high-throughput small RNA quantification on single-nucleotide level, however, the technology can introduce bias, for instance during small RNA library preparation method, or while detecting low copy number transcripts resulting in less sensitive RNAseq results (S. M. Smith and Murray 2012; Pritchard, Cheng, and Tewari 2012). In order to confirm the RNAseq data and to ensure an accurately quantification of miRNA transcripts in the brain samples, we successfully validated the observed differential expression of a subset of miRNAs in sCJD via qPCR analysis.

Our results revealed miRNA alterations that are highly changed in a regional and disease subtype-dependent manner, while the most of the recorded expression changes show low overlap in the brain of the sCJD subtypes MM1 and VV2. In

particular, the differing degree of pathological severity reported between sCJD subtypes in the frontal cortex and cerebellum, appears to be reflected in the degree of miRNA alterations in number and fold change. The neuropathological patterns in sCJD show in general a more prominent cortical involvement in MM1, while a more cerebellar involvement is evident in the brain of VV2 patients (Parchi et al. 1999; Parchi et al. 2009; Llorens et al. 2013; Gambetti et al. 2003). The underlying molecular mechanisms leading to the selective vulnerability and neuropathological phenotypes of different brain regions among sCJD subtypes are still elusive (Jackson 2014). The observed miRNA dysregulation in sCJD could be a contributing factor in favoring neurodegenerative processes in disease affected brain regions. More precisely, the miRNA alterations have the potential to be involved in the diverse sCJD pathogenesis at multiply molecular level including neuronal dysfunction and death (Gabor G. Kovacs and Budka 2010), changes in gene expression networks (Kanata et al. 2018), dysregulation of neuroinflammatory response (Franc Llorens et al. 2014; Franc Llorens, Schmitz, Knipper, Schmidt, et al. 2017), endoplasmic and oxidative stress (Tahir et al. 2016), cellular alterations in the Ca^{2+} homeostasis and activation of Calpain-Cathepsin axis (Llorens, Thüne, Sikorska, et al. 2017), changes in PrP metabolism (Llorens et al. 2013).

To understand the contribution of miRNA dysregulation to disease, it is essential to integrate the reported miRNA alterations into the complex cellular context of the brain. It is supposed that different brain cell types including neuronal networks, as well as microglia and astrocytes contribute to neurodegenerative processes in a cell-type specific and temporal manner (Majer et al. 2012; F. Llorens et al. 2013; Aguzzi and Liu 2017; Franceschini et al. 2018). Our miRNA expression profiling via RNAseq and qPCR validation in sCJD brain was performed on bulk tissue, which implies challenges for the interpretation of miRNA expression signatures in unique cells and cell types. The RNAseq data provides limited information, weather a miRNA and its putative target is coexpressed in the same cell. Moreover, miRNAs that are expressed only in a subgroup of cells or cell compartments might be mask during RNAseq analyses of brain tissue due to the effect of averaging the number of miRNA transcripts over the entire pool of cells (Koshiol et al. 2010; Chugh and Dittmer 2013). Especially, the heterogeneous nature of brain cells and their complex cellular reactions in sCJD needs to be considered in order to gain insights in the role of miRNA dysregulation in disease.

In order to gain cellular resolution of reported miRNA expression changes in sCJD, we successfully performed miRNA *in situ* hybridization assays of miR-124-3p, miR-26a-5p and miR-146a-5p in human brain tissue of sCJD MM1 and age-matched control brains. We demonstrated that the miRNAs, which we found altered in sCJD, are mainly localized in neuronal cytoplasm. Additionally, miR-146a-5p was found in small cells compatible to glial cells. While analyzing the cellular context of miR-124-3p, we observed that the overall miRNA signal was reduced and decreased numbers of neurons were detected in sCJD compared to

control brains, which stands in agreement with RNAseq and qPCR data. The provided insights into the cellular miRNA context via semiquantitative *in situ* hybridization analysis, represents an important complement to the miRNA expression profiling on bulk tissue level.

The brain-enriched, NF-kB-sensitive miR-146a-5p has been suggested to mediate inflammatory microglial responses in prion diseases (W. J. Lukiw et al. 2011; Saba et al. 2008). Interestingly, we demonstrated strong evidences for a neuronal miR-146a-5p expression, additionally to glial cells, in sCJD brain. This observations stands in agreement with neuronal miR-146a-5p upregulation in animal prion disease models (Majer et al. 2012; Boese et al. 2016), suggesting a miR-146a-5p upregulation in neurons induced by potential pathological pro-inflammatory and/or neurotoxic stimuli during prion disease progression. miR-146a-5p overexpression was also reported in AD brain (Müller et al. 2014; Sethi and Lukiw 2009), associated to different brain cell type context including neurons (Wang et al. 2016) and astrocytes (Arena et al. 2017; Cui et al. 2010). *In vitro* studies suggest that variable miR-146a-5p expression in the different brain cell context, contributes to cell-type specific behavior during neuroinflammation (Li et al. 2011).
Beside its role in neurogenesis and neuronal development, the neuron-enriched miR-124-3p has been described to be constitutively expressed in differentiated neurons. In this context, miR-124-3p is proposed to target non-neuronal transcripts, and thereby, maintaining neuronal integrity and function (Lim et al. 2005; Neo et al. 2014). Thus, overall miR-124-3p downregulation could reflect neuronal loss in sCJD. Additionally, the observation of less miR-124-3p signal detectable in surviving neurons, suggest a physiopathological roll of the miRNA in sCJD pathology.

8.2.1.1 Comparison of sCJD-associated miRNA signatures with miRNA alterations in prion disease

While comparing our observed miRNA signatures in sCJD with studies on miRNA alterations in human prion disease and animal models, few miRNA candidates appear to be commonly changed in prion disease (Table 4), reviewed in (Kanata, Thüne et al. 2018). The upregulation of miR-146a-5p that was reported in sCJD by us, was also demonstrated in sCJD and GSS (Lukiw et al. 2011), in mice infected with scrapie prion strains: 139A (Gao et al. 2016; Lukiw et al. 2011), Me7, S15 (Gao et al. 2016), and 22A (Saba et al. 2008), as well as in CA1 neurons (Majer et al. 2012) and forebrain synaptoneurosomes (Boese et al. 2016) from scrapie RML infected mice. Additionally, miR-146a-5p showed a trend towards upregulation in a scrapie infected neuroblastoma cell linen (Montag et al. 2012).

The downregulation of miR-124-3p in sCJD observed by us, was also reported in mircodissected CA1 neurons from scrapie RML strain infected mice during clinical

stages of disease (Majer et al. 2012). Interestingly, during pre-clinical disease stages, miR-124-3p appeared to be upregulated in neurons and synaptoneurosomes of scrapie infected mice (Majer et al. 2012; Boese et al. 2016). Similar, the upregulation of miR-16p-5p observed in sCJD by us, was also reported in pre-clinical disease stages of scrapie RML strain infected mice followed by downregulation during clinical stages (Majer et al. 2012). When considering these observations, the upregulation of miR-16p-5p and miR-124-3p could be a common feature in preclinical prion diseases. The upregulation of miR-26a-5p in sCJD observed by us was also reported in BSE-infected macaques (Montag et al. 2009). Our detected upregulation of miR-342-3p in sCJD was also found in scrapie strain 22A infected mice (Saba et al. 2008).

Common miRNA signatures might indicate common miRNA-regulated disease mechanisms in prion disease and the functional role of reported miRNA candidates deserve to be dissected in future studies (Kanata, Thüne et al. 2018). Overall, little overlap in differential expressed miRNAs was observed between different studies in prion disease (Kanata, Thüne et al. 2018). This is likely explained by the multitude of factors that vary between analyzed tissues or cells as source for RNA isolation (bulk tissue, neurons, synaptoneurosomes, etc.) and different miRNA profiling platforms. When comparing different profiling platforms, reproducibility of results is often not fully achieved between different miRNA detection technologies and even variations within the same platforms have been described (Chugh and Dittmer 2013). Standardized methodology for miRNA detection will improve miRNA signature accuracy (Pritchard, Cheng, and Tewari 2012).

Importantly, in our study, we successfully profiled miRNA expression patterns in two brain region in sCJD by a combination of different RNA quantification methodology obtaining high validation rates. Via RNAseq and qPCR validation, a total of 14 miRNAs in the frontal cortex and 18 miRNAs in cerebellum were validated in sCJD MM1 (n=15) and VV2 (n=10) in compare to controls (n=15). For a set of altered miRNAs in sCJD, the miRNA expression profiling on bulk tissue level was complemented with the identification of the cellular miRNA environment in sCJD via miRNA *in situ* hybridization. In this way, the expression changes of miR-124-3p have been successfully validated in sCJD in a cellular- and tissue-specific context by the usage of three different methodology approaches.

	Human prion diseases			*In vivo* prion models						
				sCJD	BSE	scrapie				
miRNA	**sCJD**	**FFI**	**GSS**	**sCJD-MM1-tg340 mice**	**BSE-macaques**	**139A-mice**	**Me7-mice**	**S15-mice**	**22A-mice**	**RML-mice**
miR-146a-5p	↑a, b, d, e ([1]; [2])		↑e ([2])	↑f, g ([1])		↑h ([3]; [2])	↑h ([3])	↑h ([3])	↑j ([4])	↑k, l ([5]; [6])
miR-26a-5p	↑a, b ([1])			↑f, g ([1])	↑i ([7])					↓k ([5])
miR-195-5p	↑a, c ([1])	↑f, g ([1])		↑f ([1])						
miR-342-3p	↑a, c ([1];[7])			↑f, g ([1])	↑i ([7])				↑j ([4])	
miR-16-5p	↑a ([1])			↑f ([1])						
miR-29b-3p	↑a, c ([1])			↑f, g ([1])						
Let-7i-3p	↑a, b, d ([1])			↑g ([1])						
miR-378a-3p	↑ b, d ([1])			↑g ([1])						
miR-449a	↑a, b, c, d ([1])			↑g ([1])						
miR-154-5p	↑a, c, d ([1])			↑f, g ([1])						
miR-341-3p						↑h ([3])	↑h ([3])	↑h ([3])		
miR-3470a						↑h ([3])	↑h ([3])	↑h ([3])		
miR-3473a						↑h ([3])	↑h ([3])	↑h ([3])		
miR-3473b						↑h ([3])	↑h ([3])	↑h ([3])		
miR-879-5p						↑h ([3])	↑h ([3])	↑h ([3])		
miR-200b-5p						↑h ([3])	↑h ([3])			
miR-124-3p	↓a, b ([1])			↓f, g ([1])						↓k ([5])
miR-141-3p						↓h ([3])	↓h ([3])	↓h ([3])		↓l ([6])
miR-182-5p						↓h ([3])	↓h ([3])	↓h ([3])		
miR-200a-3p						↓h ([3])	↓h ([3])	↓h ([3])		
miR-96-5p						↓h ([3])	↓h ([3])	↓h ([3])		
miR-200b-3p						↓h ([3])	↓h ([3])			

Table 4: **miRNA alterations in prion disease**: The table summarizes miRNA alterations during clinical disease stages that have been commonly reported in more than one human prion disease and/or prion disease model. Common miRNA alterations that were observed in independent studies are indicated in bold.
a: MM1 frontal cortex. b: MM1 cerebellum. c: VV2 frontal cortex. d: VV2 cerebellum. e: Temporal lobe. f: frontal cortex. g: cerebellum. h: Cortex. i: basis pontis. j: whole brain. k: CA1 hippocampus neurons. l: Synaptoneurosomes forebrain.
[1]: Llorens et al. (2018). [2]: Lukiw et al. (2011). [3]: Gao et al. (2016). [4]: Saba et al. (2008). [5]: Majer et al. (2012). [6]: Boese et al. (2016). [7]: Montag et al. (2009).
Kanata and Thüne et al., 2018. Frontiers in Aging Neuroscience.

8.2.1.2 Common miRNA alterations in sCJD and alternative NDs

We successfully demonstrated that specific sCJD-regulated miRNAs are commonly altered in alternative NDs, including AD, DLB and FFI (Fig. 10). Considering the need for standardized miRNA profiling procedures, we analyzed the miRNA expression in corresponding brain regions with uniform usage of methodology and normalization technique to ensure miRNA signature accuracy (Pritchard, Cheng, and Tewari 2012). By qPCR-based profiling of six miRNAs that was found altered in sCJD, we observed a common regulation of miR-877-5p and miR-323a-5p (downregulated) and miR-195-5p (upregulated) in the frontal cortex of sCJD and rpAD/AD, common regulation of miR-877-5p and miR-323a-5p (downregulated) was detected commonly in the frontal cortex of sCJD and DLB, and common regulation of miR-195-5p (upregulated) was found in the frontal cortex and cerebellum in sCJD and FFI.

Our results proofed that although the analysed neurodegenerative conditions represent distinct disease entities, with marked differences in affected neurons, pathology and clinical phenotype, there might be common miRNA-regulated pathways. The pathogenesis of these NDs shares many common features including disturbed protein homeostasis, the aggregation and deposition of pathological altered proteins, progressive neuroinflammation, and neuronal dysfunction and loss. In this context, our results shedding light into potential common miRNA-related mechanisms in the neurodegenerative processes (Dugger and Dickson 2017; Ramanan and Saykin 2013; Armstrong, Lantos, and Cairns 2005; R. J. Castellani et al. 2004).

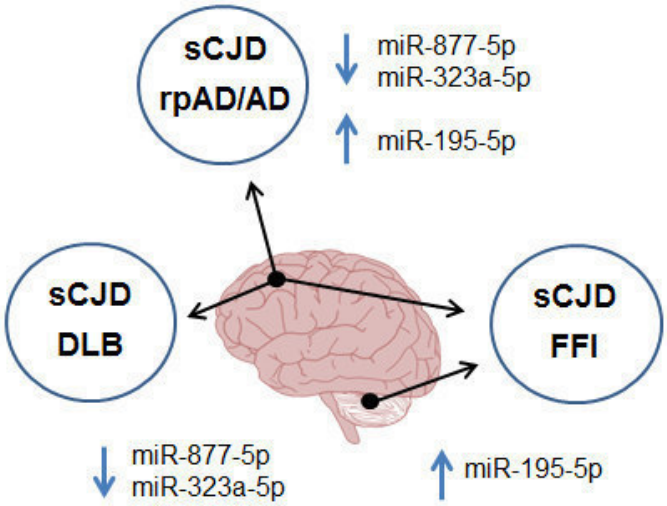


Figure 10: **Cross-validation of sCJD-miRNA signatures in alternative NDs:** A panel of six miRNAs that was found altered in sCJD, was profiled in alternative NDs via qPCR. miR-877-5p and miR-323a-5p (downregulated) and miR-195-5p (upregulated) appeared commonly in the frontal cortex of sCJD and rpAD/AD. Downregulation of miR-877-5p and miR-323a-5p was detected commonly in the frontal cortex of sCJD and DLB. Upregulation of miR-195-5p in was found in the frontal cortex and cerebellum in sCJD and FFI.

Our observed common differential miRNA expression patterns in sCJD and rpAD/AD (downregulation of miR-877-5p, miR-323a-5p and upregulation of miR-195-5p) were also reported by Lau et al. in the prefrontal cortex of late-stage AD supporting the robustness of our observation (Lau et al. 2013). We provided strong evidence for meaningful common changes in miRNA expression during NDs, which deserve clearly further functional exploration to unravel potential common miRNA-mediated molecular pathways that are relevant to these conditions.

8.2.2 Temporal- and cellular resolution of miRNA alterations in early stages of NFT pathology in AD

Genome-wide studies, uncovering global gene expression profiles in AD, provide a vast amount of expression data, which are used to understand the complex impairments of biological pathways and key regulators driving disease. However, experimental validations of miRNA dysregulation and their biological consequences based on high-scale transcriptome analyses are mostly lacking for AD. In order to understand the disease, it is essential to consider the behavior of different brain cell types over different Braak stages in relevant AD-affected brain regions (De Strooper and Karran 2016). With our work, we attempted to dissect to what extend miRNA alterations accompanied early AD-associated pathology.

We clearly demonstrated that a set of profiled miRNAs is altered in the first Braak NFT stages in a brain regional- and stage-dependent manner, whereby these miRNAs were identified by *in situ* hybridization technic to be localized in neurons. These results suggest that the observed miRNA changes are associated to regional- and time-dependent characteristics of early (I-II) and middle (III-IV) Braak NFT stages in AD and ageing. Braak stage dependent miRNA alterations were also observed by Lau et al., who performed temporal analysis of global miRNA expression, revealing major changes between Braak stage II and III in the prefrontal cortex during early AD (Lau et al. 2013; De Strooper and Karran 2016).

We provided temporally miRNA expression analysis in neuropathological characterized brain regions including LC, EC, hippocampus and dentate gyrus at early and middle stages of NFT pathology. We used qPCR-technic to profile the miRNAs: miR-27a-3p, miR-124-3p, miR-132-3p and miR-143-3p in four brain regions in a sufficiently large cohort of human samples grouped by Braak stages, which included 31 cases at Braak stage I-II (69,2 ± 9,1 years), 25 cases at Braak stage III-IV (78,6 ± 7,1 years) and 19 middle-aged control cases (50,5 ± 7,3 years). Due to limited available age-matched cases with no age- or disease-related Tau-pathology, we included younger age cases in our control group. Therefore, age-connected processes as well as early AD evolution have to be considered as contributing factors for the observed miRNA expression changes. However, no correlation between profiled miRNA expression and age was

observed among control group (data not shown). The age-associated brain pathology, termed as PART, includes formation of NFTs (low NFT Braak stages I-III), atrophy, and no or minimal Aβ depositions. PART is almost universal detectable in elderly individuals independently of AD pathology and might progress to an “only tangles-dementia” when advanced (Reas 2017; Crary et al. 2014). During neuropathological characterization, brains with NFT pathology associated to PART appear often indistinguishable to early AD related NFT pathology. Moreover, neuropathological classification of PART and early AD is mostly not accompanied by symptomatic impairment (Jefferson-George et al. 2017; Besser et al. 2017; Heiko Braak and Del Tredici 2011). Therefore, we cannot state whether the analyzed individuals in our study would have developed towards AD, however, tau-pathology occurring in the LC, EC and hippocampal region is an invariant feature of AD and its development (Duyckaerts et al. 2015).

We experimentally complement the regional miRNA expression profiling with miRNA *in situ* hybridization assays to gain insights in the cellular context of miRNA alterations in AD. Our observations indicate that neurons from different brain regions, including LC, EC and DG, display distinct miRNA expression changes during initial AD stages (Braak I-IV) before disease-associated major neuronal loss becomes evident, since no significant neuronal loss occurs in the LC until Braak stage III (Theofilas et al. 2016).
The qPCR technic is a reliable method for the relative quantification of miRNA transcripts complementing RNAseq analyses. However, our qPCR results revealed miRNA expression changes from a pool of different brain cells, whereby information regarding the cellular context of miRNA changes and cell variably in miRNA expression remain undetected. Therefore, we extended our miRNA expression profiling in AD with miRNA *in situ* hybridization assays in human brain tissue enabling crucial insights in the cellular localization of miRNAs found to be altered in early NFT pathology. This technic is a low-throughput approach, in which miRNAs were detected with lesser sensitivity and specificity than qPCR analyses, and therefore it was not used to quantify miRNA expression.
We attempted to obtain appropriate methodology to map systematically the cellular miRNA alterations in early AD progression by the combination of miRNA quantification via qPCR and the cellular identification of miRNA alterations via *in situ* hybridization assays.

8.3 Dysregulated miRNA patterns and their functional implications in sCJD and early AD

In order to unravel the biological role of miRNA dysfunction and to develop miRNA-based drug therapeutics it is essential to understand the contribution of specific miRNA – mRNA target interactions as well as complex miRNA-controlled gene networks involved in the dysregulation of biological processes during NDs.

However, the identification and functional characterization of miRNA dysregulation during disease progression remains challenging. The discovery of specific miRNA-gene target interactions, contributing to disease mechanisms, requires a broad range of experimental methods that are able to reflect *in vivo* disease processes, as well as allowing unbiased functional miRNA investigations (Seitz 2017; Akbari Moqadam, Pieters, and Den Boer 2013). Thereby, it is important to identify disease-associated miRNA signatures and to investigate the cause-consequence relationship between miRNA dysfunction and disease pathology in order to unravel the role of specific miRNA-controlled pathways in disease.
Multiple miRNAs can mediate their biological function cooperatively by targeting the same gene, and on the other hand, each miRNA has the potential to target and repress the expression of up to hundred mRNAs (Davis, Haas, and Pocock 2015; Jonas and Izaurralde 2015b). In this regard, integrative network analysis of the miRNA–mRNA interactome are necessary to understand the biological effects of miRNA dysfunction in disease context considering not only a pure miRNA perspective, but rather a systemic view including target genes and pathways.

Computational prediction tools can provide a way to identify putative miRNA targets, which is usually based on sequence complementarity and on the selection of evolutionary conserved miRNA binding sites (Akbari Moqadam, Pieters, and Den Boer 2013; Riffo-Campos, Riquelme, and Brebi-Mieville 2016). In this regard, a functional miRNA enrichment analysis was performed in publication I predicting biological functions, such as cell death/survival and cellular development, are potentially affected by reported differential expressed miRNAs in sCJD. A further possible approach to identify miRNA targets are correlation studies between miRNA and mRNA/protein expression levels (Akbari Moqadam, Pieters, and Den Boer 2013). Using computational approaches, we could not detect obvious anticorrelations between miRNA and mRNA/protein expression signatures in sCJD. These observations could be explained by the fact that most genes have multiple regulators and their expression levels are functions of multiple inputs (Naumova et al. 2013). Moreover, it needs to be stressed that computational predicted miRNA targets often appear biologically irrelevant (Pinzón et al. 2017). Therefore, true miRNA targets can only be validated by different experimental procedures resembling disease conditions.

8.3.1 Profiling functionally active miRNAs in sCJD

One important question related to the present work is to what extent the recorded miRNA alterations in sCJD reflect disease related processes. The observed regional and disease subtype-dependent miRNA signatures have the potential to be directly involved in the molecular mechanisms underlying disease etiology. Thereby, the distinct miRNA patterns might influence the molecular pathways leading to the selective vulnerability of different brain regions in sCJD MM1 and VV2 connected to the phenotypic differences of clinical and neuropathological features in sCJD. On the other hand, the observed miRNA alterations might also result as a secondary effect of disease pathology such as the modifications of cellular composition, neuronal damage and/or uncontrolled neuroinflammation in the affected sCJD brain regions.

We attempted to characterize the observed miRNA signatures in sCJD by different experimental methods, which will lead to a greater understanding of the contributions of miRNA dysregulation in sCJD and other NDs diseases. Our work provided for the first time an approach towards profiling functionally active miRNAs in sCJD. In this regard, we performed RISC immunoprecipitations in the frontal cortex of control and sCJD MM1 cases by using two different antibodies targeting Ago proteins (Ago-2 and Ago1-4). We analyzed the presence of miRNAs in the immunoprecipated Ago-containing complex and we observed that globally upregulated miRNAs in sCJD are also found to be enriched at their RISC complex, which included miR-146-5p, miR-26a-5p, miR-195-5p and miR-154-5p. We successfully provided a proof that alterations of miRNA signatures in sCJD are also leading to changed binding of mature miRNA their silencing complex. In follow-up studies, the purifications of functional active miRNA-mRNA complexes from brains can be analyzed via gene expression assays or deep sequence, which would enable the discovery of possible, multiple miRNAs bound to their mRNA target transcripts, that can be regulated by both mRNA degradation and translational inhibition (Akbari Moqadam, Pieters, and Den Boer 2013).

It needs to be stressed that the used immunoprecipitation approach might not necessarily reflect the *in vivo* miRNA-Ago interactions in sCJD afflicted brain, since non-specific and artificial miRNA-RISC associations might be formed upon tissue lysis (Beitzinger et al. 2007). Furthermore, a productive miRNA-mRNA binding does not mean that the protein level changes substantially, since various cellular mechanisms are able to compensate the effects of miRNA targeting, for instance through an increased mRNA expression. Moreover, the biological outcome of miRNA–mRNA interactions can get affected by multiple factors, such as RISC assembly, the presence of p-bodies or stress granules can affect the miRNA-mediated gene silencing (Gulyaeva and Kushlinskiy 2016; Schanen and Li 2011a). In publication I, we observed strong evidence for an impaired miRNA biogenesis in sCJD brains. Ago-proteins appeared to change partially their subcellular distribution and localization during disease.

Our work highly suggests that upregulated miRNAs in sCJD are effectively incorporated into the functional miRNA-regulated gene silencing complex and thereby likely participate in the disease associated gene expression landscape. We provided important impulses for the field of miRNA research in NDs by pointing out the crucial need for profiling miRNA-mRNA interactions in their functionally active silencing complexes instead of the analyses of mere expression levels profiled in bulk tissue. Moreover, by our observations of partially translocated Ago that likely affects Ago functionality in sCJD, we proofed the importance of studying miRNA function in a well-defined context.

Recently, other studies have performed screenings for functionally active miRNAs and miRNA targets by their co-immunoprecipitation with Ago proteins in different human tissue samples during healthy and disease states and in disease models such as for cancer (Chiyomaru et al. 2014; Fan et al. 2013; Kanematsu et al. 2014; Dzikiewicz-Krawczyk et al. 2018), cardiovascular diseases (Ling et al. 2017; Dangwal, Bang, and Thum 2012), diabetes (Ofori et al. 2017) or neurological conditions (Burak et al. 2018; Gross et al. 2016). Combined with high-throughput RNAseq analyses, Ago immunoprecipitation assays can provide a way to identify the global miRNA-mRNA targetome in complex neurological diseases (Boudreau et al. 2014; Londin et al. 2015). For instance, the global miRNA regulatory network was identified upon ischemia injury in mouse brain revealing changes of over one thousand miRNA-mRNA interactions involved in mediation of neuroprotective responses upon stroke injury (Kobayashi et al. 2018). Until today, most screenings for functional active miRNAs in human diseases were performed in *in vitro* models. We provided the first mapping of functional miRNAs in the context of NDs in human.

8.3.2 Temporal miRNA expression analysis in a CJD mouse model

miRNA alterations in prion disease models represents a unique tool to investigate the cause-consequence relationships of miRNA dysregulation in prion disease pathology, and to evaluate the use of miRNAs in diagnosis as biomarkers (Watts and Prusiner 2014; Brandner and Jaunmuktane 2017). We successfully validated the miRNA expression patterns observed in sCJD MM1, in a CJD MM1 mouse model, which resembles the neuropathological, biochemical, and gene expression alterations reported in sCJD in a region- and subtype-specific manner (Padilla et al. 2011; Llorens et al. 2014; Llorens, Thüne, Tahir, et al. 2017; Kanata et al. 2018). We validated expression changes of ten and eleven miRNAs via qPCR technic in the cortex and in the cerebellum of the CJD mouse model at one of the clinical stages (180 and/or 210 days post inoculation (dpi)), respectively. Temporal miRNA expression analysis from the CJD mouse revealed that the alterations mainly occur with the onset of symptoms, suggesting that the pool of analyzed

miRNAs specifically alters during clinical disease stages, which provides a link between regional pathology and stage of disease (Fig 11).

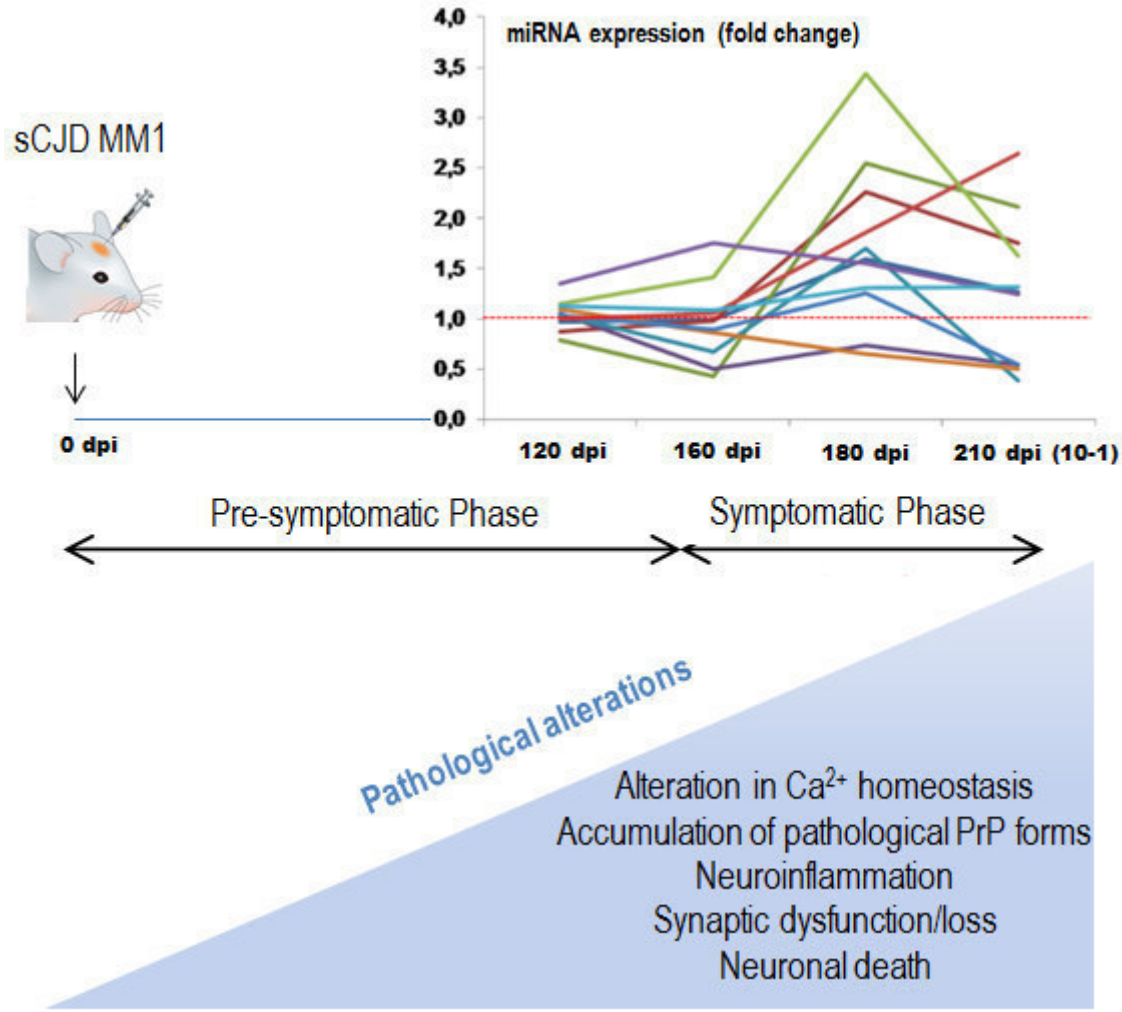


Figure 11: **Temporal profile of miRNA expression in CJD MM1 mouse model:** miRNA expression time course analyses performed via qPCR in cortex of tg340-PRNP129MM mouse model at 120 (pre-clinical), 160 (early-clinical), 180 (clinical) and 210 (late-clinical) days post inoculation with sCJD MM1 brain homogenates in compare with control inoculated mice. Profiled miRNAs seem to change their expression with disease onset, suggesting that miRNA deregulation has the potential to shape molecular phenotypes during clinical disease stages. Key events in the pathogenesis of prion disease in CJD MM1 mice are: alterations in Ca^{2+} homeostasis and accumulation of pathological PrP forms evident during pre-clinical stages (Llorens, Thüne, Sikorska, et al. 2017), neuroinflammatory processes reported as pre-clinical and early-clinical events (Llorens et al. 2014; Llorens, Thüne, Tahir, et al. 2017), synaptic loss and neuronal death at clinical stages.

Distinct prion disease phases have been suggested, in which brain cells respond with beneficial mechanisms upon early pathological stimuli, while during disease progression sustained detrimental conditions may trigger neurodegenerative pathways (De Strooper and Karran 2016; Majer et al. 2012). The underlying molecular mechanisms involved in the transition from neuroprotective signalling towards neuronal dysfunction and death during prion disease are still elusive. We successfully proofed that miRNA alterations are resembled in a CJD model system

that reflects faithfully *in vivo* disease processes and thereby providing highly valuable approaches to dissect the causative involvement and consequential effects of miRNA dysregulation in prion disease progression. Interestingly, among the pool of profiled miRNAs found to be altered in clinical sCJD, miR-124-3p (downregulated) and miR-16-5p (upregulated) change their expression during early symptomatic phase (160 dpi) in CJD mice, suggesting the contribution of miR-124-3p and miR-16-5p in pre- and early clinical prion disease mechanisms. Upregulation of miR-16-5p was also found in hippocampal neurons in a further prion disease animal model, a scrapie-infected mouse, during pre-clinical disease stages (Majer et al. 2012). In a recent publication, Burak et al. aimed to study the biological effects of miR-16-5p overexpression in a prion disease cell culture model. Lentiviral-based miR-16-5p upregulation in cultured hippocampal neurons, mimicking miR-16-5p increase observed in *in vivo* conditions, was inducing decreased neurite length and branching. Co-immunoprecipitation of miR-16-5p enriched Ago complexes with their associated mRNA transcripts revealed the presence of genes involved in the signaling pathway “mitogen-activated protein kinases/extracellular signal-regulated kinases” (MAPK/ERK) as miR-16-5p targets indicating that a miRNA-mediated mechanism might induce neuronal degeneration through MAPK/ERK pathway targeting (Burak et al. 2018).

Certain factors need to be considered while studying and interpreting miRNA dysregulation in the transgenic mouse model. CJD MM1 mice are the result of non-physiological 4-fold PrPc overexpression (Padilla et al. 2011), which might lead to overexpression artifacts that not fully corresponding to the human situation (Brandner and Jaunmuktane 2017). In sCJD brain, PrP metabolism is impaired and highly variable PrP protein levels have been observed among sCJD subtypes and brain regions likely linked to disease-specific mechanisms. In sCJD MM1 brain, PrP is decreased on mRNA and total protein levels, while high amounts of PrPsc accumulate (Llorens et al. 2013). A further challenging aspect when using the transgenic mouse model is their inoculation with brain homogenates at early ages (6-10 weeks) and development of symptoms at middle ages (around 160 dpi) (Padilla et al. 2011). The major risk factor of prion diseases is age and cellular senescence is supposed to promote neurodegenerative processes (G G Kovacs and Budka 2002). Brain aging is featured by decreased neuronal and synaptic density (Petralia, Mattson, and Yao 2014), increased numbers and reactivity of microglia and astrocytes (Spittau 2017; Clarke et al. 2018), and transcriptional changes with higher expression profiles for genes linked to inflammation, oxidative stress, DNA repair as well as decreased gene expression for pathways involved in neuronal function (Mohan et al. 2016). In order to consider the aging component while studying miRNA dysregulation in prion disease models, aged animals or mouse inbred strains with accelerated senescence can be useful (Shimada and Hasegawa-Ishii 2011).

However, with our work, we clearly demonstrated that beside highly resembled neuropathological features between sCJD and CJD mouse model, also alterations

in disease associated molecular signatures were validated between human disease and mouse model (Llorens et al. 2014; Llorens, Thüne, Tahir, et al. 2017; Kanata et al. 2018; Llorens, Thüne, Sikorska, et al. 2017).

8.3.3 Ex vivo models to study biological functions of miRNA alterations observed in sCJD

Neurodegenerative phenotypes in sCJD reflect complex, spatial- and time-dependent molecular processes and analysis of detailed molecular disease mechanisms is necessary for the identification of therapeutic targets. Modeling reported miRNA alterations in *in vitro* disease models may enable the investigation of miRNA-depend events and their biological effects during sCJD mechanism in a site specific-manner.

Many studies have investigated miRNA functions in neurodegenerative processes by mimicking expression alterations of one or few miRNAs in cell-based assays and thereby focused on the role of individual genes and pathways possibly regulated by individual miRNAs (Krützfeldt, Poy, and Stoffel 2006). In contrast, we aimed to investigate the biological consequences of genome-wide changes in miRNA signatures observed in sCJD. Therefore, we transfected the miRNAome of sCJD patients into neuronal-like and glial-like cell lines to measure cell-type specific, biological effects of sCJD-connected miRNA changes and their putative targets. We observed that the neuronal-like cell line reacts much more sensitive on the presence of the sCJD connected miRNAome, than the glial-like cell line. Our experiment indicated that disease-associated abnormalities in the miRNAome might act as molecular driver triggering detrimental effects leading to neuronal degeneration in sCJD. However, the transfected RNA from sCJD brains was isolated from bulk tissues and misexpression of miRNAs can occur in non-physiological context. Additionally, through the manipulation of miRNA expression in an additive manner, the observed downregulation of miRNAs in sCJD is not resembled in this ex vivo model.

8.3.4 Molecular alterations in the locus coeruleus during early and middle Braak stages of NFT pathology

There is a critical need for the identification of early AD-associated changes preceding symptomatic disease in order to define potential therapeutic interventions, in which neural tissue remains intact and more likely responsive to treatment. How the switch towards irreversible AD processes is characterized and which molecular mechanisms define the transitional phase from early pathological

alterations towards mild cognitive impairment (MCI) and/or AD evolution remains elusive (Vickers et al. 2016).

The LC is one brain region vulnerable for the appearance of NFTs and it has been suggested as an initial site for NFT formation during ageing and AD (Grudzien et al. 2007). Our work provided crucial insights into molecular alterations during the progression of early stages of NFT formation, especially in the LC, in which we observed the upregulation of four profiled miRNAs: miR-27a-3p, 124-3p, 132-3p, 143-3p. The altered miRNAs were indicated to be expressed in LC neurons by *in situ* hybridization analyses, suggesting that these miRNAs have the potential to shape the molecular environment in LC neurons during transitional processes from normal aging into early AD (Fig 12).

Andrés-Benito et al. analyzed molecular characteristics accompanying NFT formation in the LC revealing complex abnormalities during asymptomatic early stages of NFT that include: (1) activated tau kinases (2) truncated tau forms (3) increased oxidative stress and reduced stress response (4) microglia activation and proliferation (5) reduced mitochondria (6) upregulation of genes linked to protein folding, chaperone binding, ATP metabolism, (7) downregulation of genes coding for DNA-binding proteins and small nuclear RNAs with disease progression (Andrés-Benito et al. 2017).

It can be speculated that our reported miRNA upregulations in LC during first Braak stages are linked to disease processes itself rather than being secondary effects of disease pathology, such as changes in cellular composition in disease-affected brain regions, formation of amyloid plaques or neuronal loss. It also can be considered that the reported miRNA changes could be involved in compensatory mechanisms during early AD evolution and/or aging (Reddy et al. 2017; WANG et al. 2014). However, in order to understand to what extent the recorded miRNA alterations reflect probable early AD-associated processes in LC neurons during early stages of NFT formation, it is necessary to dissect their functional role by a range of experimental methods.

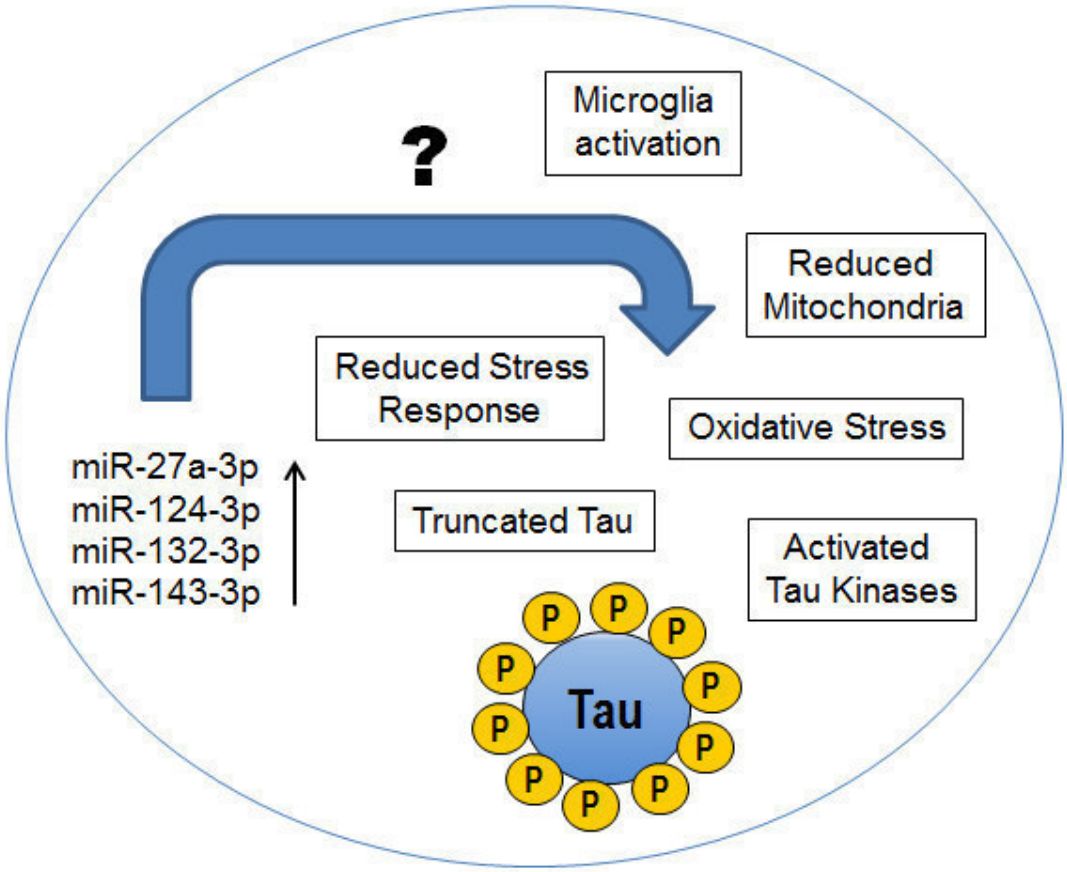


Figure 12: **Schematic overview of molecular alterations in locus coeruleus neurons during first NFT pathology stages:** The presents of hyperphosphorylated, misfolded and truncated tau in LC neurons is accompanied by cellular abnormalities at early and middle Braak NFT stages (Andrés-Benito et al. 2017). Aberrant molecular features, such as activated tau kinases, increased oxidative stress, reduced stress responses, reduced mitochondria and increased microglial activation might represent the molecular environment during transitional phase from early pathological alterations towards MCI and/or AD. We observed strong evidences for the upregulation of the miRNAs: miR-27a-3p, 124-3p, 132-3p, 143-3p in LC neurons during these early NFT stages, suggesting the miRNA alterations underlying early AD-associated mechanisms. Weather the observed miRNA alterations are involved in neuroprotective compensatory pathways or in early disease evolution is not clear and need to be dissected by functional miRNA analysis.

miR-132-3p has been supposed to target directly tau, as well as kinases involved in aberrant tau phosphorylation (Salta and De Strooper 2017). Smith et al. demonstrated that a deletion of miR-132-3p in a transgenic AD mice model is leading to increased tau expression, phosphorylation, and aggregation accompanied with an enhanced tau pathology (Smith et al. 2015). Overexpressing of miR-132-3p restored tau pathology and cognitive function in AD mice (Hernandez-Rapp et al. 2016).

Similar, miR-124 was also implicated in the regulation of tau isoform levels by targeting regulatory and splicing factors (Smith et al. 2011). Additionally, miR-124-3p has been shown to target Caveolin-1 in an *in vitro* AD model, and thereby, inhibiting aberrant tau hyperphosphorylation (Q. Kang et al. 2017).

These observations indicate that observed miRNA upregulations may play a neuroprotective role in AD. However, these functional miRNA implications could be biased towards targeted analyses of key proteins involved in AD.

Comprehensive exploratory analyses of miRNAs and their targets in the context of early NFT formation are necessary to understand their true biological effects.

8.4 Mechanisms of altered miRNA signatures in NDs

The reported miRNA alterations during critical disease stages have the potential to be involved in initiation and progression of sCJD and AD. However, a clear understanding of the underlying mechanisms, involved in miRNA deregulation in NDs, will contribute greatly to the identification of relevant targets for successful disease modifying therapies (Cummings 2017). We focused on a comprehensive characterization of the miRNA biogenesis machinery during late stages of sCJD, revealing strong evidences for complex impairments of key proteins involved in miRNA biogenesis and function, which may contribute to miRNA dysregulation in sCJD (Fig 13).

Reduced levels of the miRNA maturing proteins Drosha, its cofactor DGCR8, and Dicer were observed on mRNA and protein level in sCJD brains. These observations can be an explanation for the reported decreased miRNA levels in sCJD brain, although, more detailed cellular- and spatial resolution of observed miRNA signatures and status of biogenesis machinery are necessary to draw solid conclusions.
Analyses of the subcellular contribution of Ago proteins in sCJD via gel-filtration chromatography and immunohistochemical staining revealed a switch of Ago-2 containing protein complexes to higher molecular forms. Strong evidences for an enriched subcellular relocalization of Ago proteins to the nucleus in sCJD when compared with age-matched controls were shown.
Subcellular localization and posttranslational modifications of Ago-proteins have been described to influence miRNA-mediated gene silencing, and thereby, coordinating gene expression (Meister 2013b; Jonas and Izaurralde 2015b; Detzer et al. 2011). One possible consequence of a nuclear increase of Ago proteins could be a lack of miRNA-mediated gene regulation of cytoplasmic mRNA targets in sCJD. Mature miRNAs and components of the miRNA biogenesis machinery have been described to be capable shuffling between cytoplasm and nucleus (Roberts 2014). In this context, observed nuclear located Ago-proteins in sCJD could be involved in gene regulation of nuclear transcripts or induction of epigenetic alterations (Leung 2015). Upon translational stress condition, Ago-proteins are often recruited to stress granules, in order to enhance miRNA-regulated gene silencing (Leung, Calabrese, and Sharp 2006). By observing stress granule markers, we could not find evidences for the formation of stress granules in sCJD. The heat heat-shock protein (Hsp) 90, a chaperone involved in protein quality control, is proposed as one crucial factor regulating the recruitment of Ago-proteins to stress granules and processing bodies (Pare et al. 2009).

Dysfunctional Hsp90 has been linked to sCJD pathogenesis (Gawinecka et al. 2012), and could underlie aberrant subcellular localization of Ago-proteins.

It is worth mentioning that PrPc has been demonstrated to associate with Ago proteins under physiological conditions, stabilizing RISC assembly and function (Gibbings et al. 2012). In prion disease, most pathological PrPsc aggregates as oligomers with typically high molecular weight (Silveira et al. 2005; Tzaban et al. 2002). Therefore, a potential interaction of PrPsc with Ago could be an explanation for the observed switch of Ago-2 containing protein complexes to higher molecular forms.
It seems likely that the presence of pathological PrPsc, as well as, the loss of PrPc function might directly contribute to miRNA dysregulation in sCJD, which clearly deserves further investigations (Fig 13).

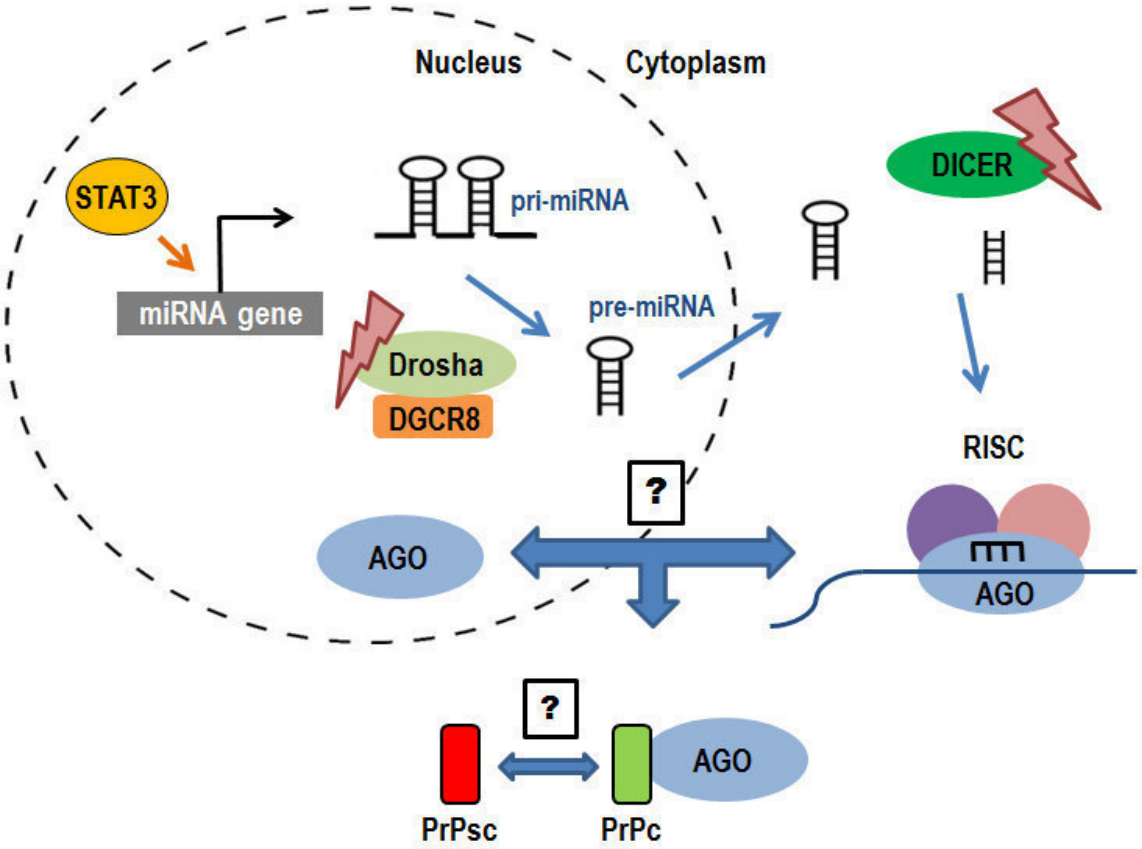


Figure 13: **Schematic overview of impaired miRNA machinery in sCJD.** Our observations showed strong indications for complex impairments of the miRNA machinery in sCJD, pointing out strong links between impaired miRNA regulatory pathways and altered miRNA patterns. Upregulation and activation of the transcription factor STAT3 might induce miRNA expression. Decreased expression of Drosha, DGCR8 and Dicer may impair miRNA biogenesis. Abnormal subcellular localization of Ago-proteins can affect gene silencing efficiency. Co-localization of PrPc with Ago has been suggested for an effective miRNA-mediated gene silencing. Weather the presence of pathological PrPsc forms and PrPc loss-of-function have an impact on miRNA-mediated gene regulation is not clear (Gibbings et al. 2012). The absence of stress granules could reduce cellular clearance ability in sCJD.

Deregulated miRNA biogenesis also appeared during other forms of ND conditions (Tan, Yu, and Tan 2015); upregulation of Drosha, DGCR8 and Dicer was observed in the brain of multiples sclerosis (MS) patients (Jafari et al. 2015), as well as, aberrant subcellular distribution of Drosha (Porta et al. 2015) and Dicer malfunction in the brain of amyotrophic lateral sclerosis (ALS) patients (Emde et al. 2015). It has been suggested that stress and stress granule formation can initiate alterations and dysfunction of miRNA biogenesis machinery, affecting neuronal integrity and driving ND processes (Emde et al. 2015; Olejniczak, Kotowska-Zimmer, and Krzyzosiak 2018). By analyzing the subcellular localization and expression levels of stress granule markers eIF3 and Tia1, as well as, phosphorylation status of stress granule activator eIF2α, we could not find evidences for the formation of stress granules in sCJD brain. The ER stress sensor PERK can phosphorylate and activate eIF2α in order to reduce translational initiation of the majority of cellular proteins (McQuiston and Diehl 2017). The activation of PERK and the mediation of an untranslated protein response appeared to be absent in human prion disease (Unterberger et al. 2006; Wiersma et al. 2016), which could underlie a lack of stress granules observed in sCJD.

The occurrences of intracellular neuronal calcium overload and ER stress have been implicated in prion disease pathogenesis (Franc Llorens, Thüne, Sikorska, et al. 2017; Ferreiro, Oliveira, and Pereira 2008). Approaches in different cellular context revealed that ER stress can mediate alterations in miRNA biogenesis machinery and subsequently modify expression of miRNAs (Maurel and Chevet 2013). In this context, it can be speculated that the accumulation of pathological PrPsc forms, accompanied by the failure of protein quality control machinery, could lead to deregulation of intracellular calcium and ER stress, which might trigger sCJD pathogenesis at multiple molecular levels including miRNA biogenesis dysfunction and miRNA expression changes. However, dissecting the cascade of pathological events in sCJD remains necessary in order to identify potential targets for therapeutic intervention.

Beside miRNA regulation at the level of their biogenesis, miRNA expression and cellular abundance can be influenced by multiply regulatory mechanisms, such as genetic and epigenetic factors, RNA editing, transcription factors, miRNA turnover and subcellular miRNA localization (Bronevetsky and Ansel 2013; Gulyaeva and Kushlinskiy 2016; Schanen and Li 2011a; Tomaselli et al. 2015).

We observed strong evidences that a pool of miRNAs, upregulated in sCJD brain, are potentially under transcriptional control of the transcription factor STAT3, which was found upregulated and activated in prion disease (Llorens et al. 2014; Carroll et al. 2015), indicating a STAT3-dependent mechanism of miRNA deregulation in sCJD. Nerveless, the potential interplay between miRNAs and

STAT3 need to be sustained by information according cellular context and characterization of *in vivo* STAT3 binding sites.

8.5 Circulating miRNAs as potential biomarkers for human prion disease and early AD

Peripheral circulating miRNAs have the potential to reflect brain abnormalities during disease states, including neurodegenerative dementias (Sheinerman et al. 2017; Grasso et al. 2014; Zendjabil 2018). Our presented studies revealing miRNA alterations during critical disease stages during two types of neurodegenerative conditions, sCJD and early Braak NFT stages, suggest the possibility of pathology-associated differences in circulating miRNA levels that may serve as non-invasive biomarker for prediction, differential diagnosis and monitoring of these diseases and drug response.

Extracellular miRNAs fit a number of criteria as promising biomarker for NDs: they are highly stable in biofluids, easy accessible through non-invasive approaches, cost efficient and reliable diagnostic assays enabling miRNA identification, such as microarray or qPCR, and miRNA signatures can be potential indicator for preclinical disease stages as well as disease progression or therapeutic response (Wang, Chen, and Sen 2016; Viswambharan et al. 2017).

miRNAs can be released by cells through active and passive processes, entering the circulation by various pathways. Cellular release mechanisms include miRNA secretion (1) via encapsulated microvesicles, including exosomes, (2) vesicle-free miRNAs bound to proteins, such as Ago or lipoprotein complexes, and (3) upon apoptotic or necrotic cell death. Beside their role as biomarker for NDs, extracellular miRNAs are suggested to mediate intercellular communication over short and/or long distance within the nervous system or peripheral tissue, and can act both in physiological context as well as in diseases (Scott 2017). For instance, glia-to-neuron transfer of miRNAs has been proposed as a molecular mechanism underlying inflammation-induced alterations of synaptic integrity during brain inflammation (Prada et al. 2018).

CSF as potential source of disease-specific miRNA signatures provides the advantage of the proximity to the extracellular brain space and regions of pathology (Gallego et al. 2012). The CSF is enriched with central nervous system-specific miRNAs that might represent highly valid biomarkers for brain pathologies. miRNAs derived from blood, either plasma or serum fractions, are easier accessible and non-invasive, however, blood contains high numbers of miRNAs from peripheral tissues (Sorensen, Nygaard, and Christensen 2016). Investigating exosomal miRNAs in biofluids provides an appealing approach for the identification of disease-specific diagnostic signatures, since the cargo contained in exosomes includes distinct miRNA pools (Soria et al. 2017).

8.5.1 Profiling CSF-based miRNAs in sCJD

Due to the rapidly evolving disease, high heterogeneity, and lack of a consistent geometrical spreading of neuropathology, biomarker research in prion disease faces significant challenges (Wadsworth and Collinge 2011; Gambetti et al. 2003; Parchi et al. 2012). Up to date, quantitative biomarkers that are able to diagnose and quantify disease progression are missing and hamper early prion disease diagnose (Canas et al. 2018). Disease-specific miRNA expression profiles provide the potential to be used as diagnostic, prognostic and therapeutic markers for prion disease.

To date, no data on miRNA alterations in biofluids as potential disease biomarker in human prion disease are available. We provided the first study investigating the presence of disease-associated miRNAs in the CSF derived from sCJD and age-matched control patients, revealing important information regarding the types of miRNAs detectable in cell-free CSF. Therefore, miRNAs that were found deregulated in sCJD brain, were analyzed in CSF of 12 sCJD patients and 12 age-matched controls via qPCR technic. In our preliminary targeted CSF-based miRNA characterization we revealed robust detectable CSF miRNA expression of three miRNAs (miR-26a-5p, miR-204-5p, miR-378a-3p) out of eleven profiled candidates. Also the small nuclear RNA RNU6 was stable detectable via qPCR in the analyzed CSF samples. One out of three detectable CSF miRNAs (miR-204-5p) appeared differentially expressed in sCJD in compare with control patients. In this regard, our results suggest that qPCR-based profiling of highly expressed CSF miRNAs could provide a potential diagnostic platform for sCJD. The detected CSF miRNAs in our study (miR-26a-5p, miR-204-5p, miR-378a-3p) have been described to be CSF enriched (Burgos et al. 2013).

Overall, no correspondence between altered miRNAs in sCJD brain and CSF was observed. miRNAs, which we found increased in sCJD brain, such as miR-26a-5p, miR-195-5p, let-7i-3p, miR-378a-3p, miR-449a, miR-124-3p and miR-154-5p, did not show significant differences in sCJD CSF samples compared to control patients. On the other hand, miR-204-5p, which expression remained unchanged in sCJD brain, was found significantly diminished in the CSF of sCJD patients, suggesting this miRNA as a possible CSF biomarker discriminating sCJD from age-matched controls.

The secretion of miRNAs into the CSF occurs in a selective manner and miRNA alterations in the brain are not necessarily mirrored in the CSF (Ng et al. 2013; Grasso et al. 2014). The source of miR-204-5p in the CSF and weather its reduced levels in the CSF derived from sCJD patients is associated to sCJD pathogenesis is unknown and needs further investigations. miR-204 has been reported to be expressed in various human cell types (Wang et al. 2010), including brain tissue, where it is found highly abundant in neuronal axons (Natera-Naranjo et al. 2010; Wang and Bao 2017). miR-204 has been also found highly abundant

in CSF, and therefore, suggested as a CSF enriched miRNA (Burgos et al. 2013; Yagi et al. 2017). In this regard, there is the possibility that miR-204 downregulation in the CSF might result upon neuronal dysfunction in sCJD, which could lead to less released miRNA levels into CSF. The miR-204 expression in the brain, investigated by RNAseq and qPCR, remained unchanged in analyzed brain regions in sCJD, suggesting altered peripheral miRNA secretion mechanisms in sCJD as possible source for CSF miR-204 downregulation. However, specific expression changes of compartmented axonal miRNAs might not have been captured via RNAseq and qPCR of analyzed bulk tissue (Koshiol et al. 2010; Chugh and Dittmer 2013). In fact, the source of miR-204-5p could be diverse and might be derived from different brain cells (such as neurons or glia cells), blood cells or peripheral sources (Bekris et al. 2013). Up to now, CSF-based miR-204-5p downregulation has been also reported in genetic FTD patients, whereby profiled miRNAs were isolated from exosomes (Schneider et al. 2018).

We aimed to establish a preliminary CSF miRNA screen for disease-specific miRNA candidates profiled in brain as a suitable tool for clinical approaches. Although peripheral circulating exosomal miRNAs might represent disease-specific signatures in sCJD (Soria et al. 2017; Hartmann et al. 2017), we focused on the isolation of total, cell-free miRNAs in the CSF. Ultracentrifugation-based exosome isolation methods can be time-consuming, requires specialized laboratory equipment and might result in low exosome yield, providing difficulties for miRNA quantification in routine diagnostic. Moreover, higher amounts of CSF starting material are necessary for the isolation of exosomal miRNAs in compare to total CSF miRNAs (Lin et al. 2015; Chen et al. 2017).

Variability in CSF miRNA profiles in inter/intra-cohort studies has been described leading to conflicting data. Potential sources for miRNA fluctuations can be connected to different postmortem intervals, age, gender, or physiologic factors such as circadian rhythm, medication, exercise, etc. (Yoon et al. 2017). Associated to the high fluctuation of miRNAs in the CSF, the selection of endogenous reference genes is a critical step for the identification of true miRNA alterations between disease and control conditions. No universal invariant CSF miRNA calibrator for relative normalization purposes exist and the best combination of reference genes must be determined (Moldovan et al. 2014). The amounts of profiled miRNAs in our study were normalized relative to the amount of the small nuclear RNA RNU6, which we detected uniformly expressed in disease and control groups. RNU6 belongs to another RNA class with different biochemical and metabolic features, which might lead to bias during CSF miRNA quantification (Schwarzenbach et al. 2015; Gee et al. 2011). Further helpful normalization approaches could include the adding of synthetic miRNA spike-ins, the usage of global mean normalization of all profiled miRNAs, or empirical tools to select stable miRNA candidates as normalizer (Yoon et al. 2017). However, we found RNU6 stable abundant in the CSF of sCJD and control samples justifying its usage as reference gene for normalization procedure.

Our study is particularly important in this context, since we identified robust and reproducible miRNA candidates in the CSF. Despite relative low CSF miRNA concentrations and the small number of analyzed patients, we were able to detect uniform levels of miRNA candidates in CSF by qPCR, providing resources for future miRNA biomarker studies. If miRNAs reveal as reliable CSF biomarker for practical clinical applications in sCJD still needs to determine. Future studies should complemented our observations with 1) unbiased global approaches for miRNA profiling, such as RNAseq, to determine absolute miRNA expression profiles in the CSF, 2) introduction of standardized strategies of miRNA detection in clinical studies, 3) large-scale clinical validation studies.

8.5.1.1 Altered miRNAs in biofluids as potential diagnostic tools in prion diseases

miRNA alterations in body fluids of animal prion diseases and disease model systems offer opportunities for miRNA biomarker research that cannot be provided by examining disease in humans (Kanata, Thüne et al. 2018). Current progress in circulating miRNAs research in prion disease is summarized in Fig 14.

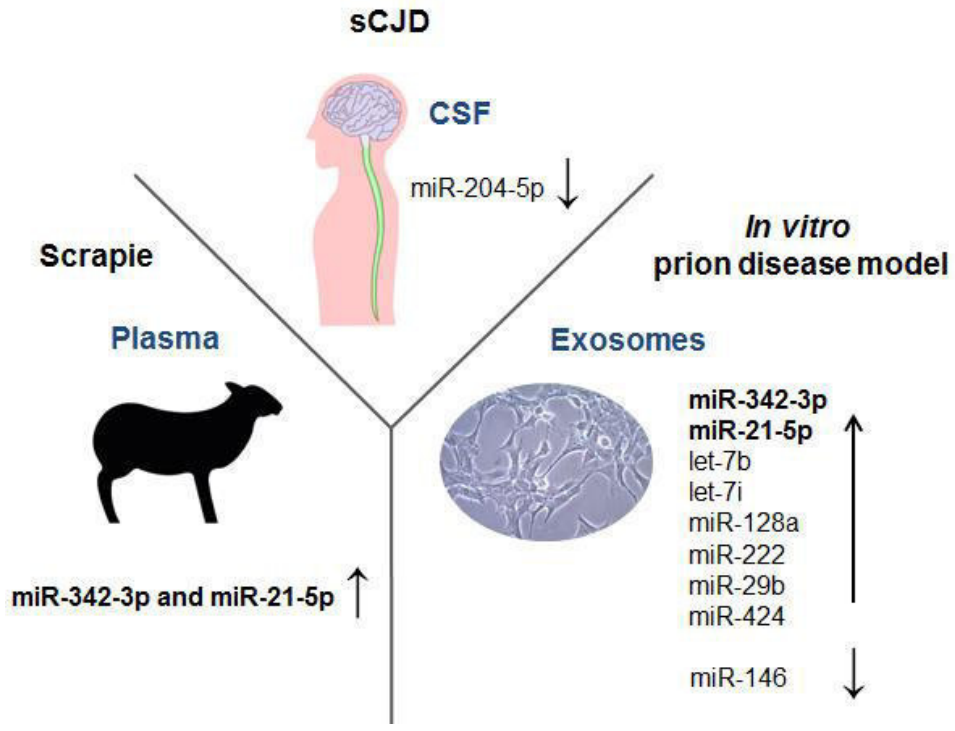


Figure 14: **Overview of studies on circulating miRNA alterations in prion diseases:** With a preliminary CSF miRNA screen in sCJD, we observed reduced CSF miR-204-5p levels in sCJD and provided the first study revealing a differential expressed miRNA candidate in human prion disease biofluids. Upregulation of miR-342-3p and miR-21-5p in plasma was demonstrated in sheep affected by scrapie (Rubio et al. 2017). The presence of a distinct miRNA pool has been shown in exosomes released *in vitro* by prion-infected neuronal cells (Bellingham, Coleman, and Hill 2012). Upregulation of circulating miR-342-3p and miR-21-5p was described in two prion disease studies.

Elevated levels of blood-based miR-342-3p and miR-21-5p during pre-clinical disease stages in sheep naturally affected by scrapie were detected, suggesting these miRNAs as potential plasma-based biomarker in animal and human prion diseases (Rubio et al. 2017). However, no data are available on blood-based miRNA signatures in human prion disease. Interestingly, increased amounts of miR-342-3p and miR-21-5p were also reported in exosomes secreted by a hypothalamic neuronal cell line infected with a human GSS prion strain (GSS-GT1-7) (Bellingham, Coleman, and Hill 2012). Moreover, among analyzed miRNA changes, the upregulation of miR-322-5p and miR-21-5p in exosomes released *in vitro* by prion-infected neuronal cells appeared exclusively in prion disease model (Bellingham, Coleman, and Hill 2012) and, up to now, were not described in other NDs, including AD (Nagaraj et al. 2017; Kiko et al. 2014; R. Guo et al. 2017; Zeng et al. 2017; Dong et al. 2015; Geekiyanage et al. 2012), MS (Vistbakka et al. 2017), PD (Ma et al. 2016; Ding et al. 2016; Gao et al. 2016) and ALS (Freischmidt et al. 2013). On the other hand, common exosomal miRNAs alterations, including let-7i-5p, miR-128-3p (upregulated), and miR-146a-5p (downregulated), have been observed in prion disease cell culture model (Bellingham, Coleman, and Hill 2012), blood-based exosomes isolated from: MS patients (let-7i-5p, (Kimura et al. 2018)), primary progressive MS (miR-128-3p (Vistbakka et al. 2017)), AD (miR-146a-5p, (Kiko et al. 2014; Dong et al. 2015)), and PD (miR-146a-5p, (Ma et al. 2016)).

8.5.2 Circulating miRNAs as potentially early AD biomarker

There is the critical need for AD biomarkers that are able to identify pre-clinical disease-associated changes, and at the same time, to predict weather pathological alterations will progress towards MCI and/or AD. However, when the irreversible AD processes begin is unknown and the underlying mechanisms of the transitional phase from early pathological alterations towards MCI and/or AD evolution remains elusive (Fiandaca et al. 2014).

We demonstrated miRNA alterations during the progression of first Braak NFT stages in compare to cases with no neuropathological lesions. One of four upregulated miRNAs in analyzed brains, miR-143-3p, was also found upregulated in exosomes isolated from blood in MCI and AD patients (Cheng et al. 2015). The observed upregulation of miR-143-3p in LC and EC during NFT Braak stage I-IV could be an explanation for elevated exosomal miRNA levels in AD blood, suggesting miR-143-3p as a possible pre-clinical and prognostic AD biomarker. Our work strongly suggests that the establishment of a miRNA screen in brain could help to identify miRNAs in biological fluids, which may indicate cognitively intact individuals at risk for AD, providing the possibility for the detection of therapeutic window for disease prevention.

The focus of our work was the assessment of miRNA alterations during early AD-associated neuropathological changes unattached from neurophysiological assessments. However, it needs to be stressed that NFT stages I-II and even stages III-IV are asymptomatic, although individuals at stage IV might represent cognitive impairment (Braak and Braak 1991; Murayama and Saito 2004).

Increasing number of miRNA biomarker studies have been focused on AD, suggesting circulating miRNAs in CSF and blood as promising disease-specific markers (Zendjabil 2018; Galimberti et al. 2014; Kumar and Reddy 2016; Dangla-Valls et al. 2017; McKeever et al. 2018). Cogswell et al. identified 60 CSF-based miRNAs in AD patients, which were also detected in AD brain, showing regional- and stage-specific expression patterns (Cogswell et al. 2008). CSF-based miRNA changes have been demonstrated to be able to differentiate AD from age-matched controls, such as miR-222 and miR-125b upregulation in AD CSF (Dangla-Valls et al. 2017). A blood-based signature of 12 miRNAs was able to differentiate AD patients from healthy controls, as well as, AD patients from other neurological disorders with high diagnostic accuracy (Leidinger et al. 2013). Tan et al. profiled the miRNA expression in sera of 158 patients and 155 controls, revealing 6 miRNAs (miR-98-5p, miR-885-5p, miR-483-3p, miR-342-3p, miR-191-5p, and miR-let-7d-5p) that present significantly different expressed miRNA signatures in AD patients in compare to controls (Tan et al. 2014). Lugli et al. revealed a panel of 7 exosomal miRNAs in the plasma fraction identifying AD patients with an accuracy of 83 to 89% (Lugli et al. 2015). However, when comparing circulating miRNA profiling data in different studies, it needs to be stressed that there is little overlap in miRNA signatures (Zendjabil 2018). The implementation of robust and standardized methods for miRNA detection in clinical application still remains challenging.

8.6 Using miRNAs to treat NDs

Up to now, NDs, including prion disease and AD, remain incurable and successful therapies to delay or stop progression of these conditions still represent the most challenging goals. With their ability to regulate complex networks of genes and pathways simultaneously, miRNAs provide a promising potential as therapeutic applications (Ouellet et al. 2006). miRNAs could help to restore dysregulated pathways within critical disease stages, and in the same manner, targeting multiply aspects of multifactorial disease pathogenesis, such as imbalance in production and clearance of misfolded proteins, neuroinflammation and imbalance in degenerative and protective signaling (Goodall et al. 2013).

Experimental evidences have shown that the build up and aggregation of misfolded amylogenic proteins during NDs processes could be mediated via miRNAs. Cell culture experiments showed that treatment with synthetic miRNAs, targeting PrPc, was capable to reduce PrPC expression, and thereby, inhibiting prion propagation in neuroblastoma cells (Kang et al. 2011) and in primary neuronal cultures (Kang et al. 2018; Pfeifer et al. 2006). With help of lentivector-mediated RNA interference, endogenous PrPc expression was suppressed in scrapie-infected mice delaying disease progression (Pfeifer et al. 2006; Ridolfi and Abdel-Haq 2018). In context of miRNAs as therapeutic target in AD, miR-16 mimics has been shown to downregulate the expression of tau, APP and Beta-secretase 1 in cell culture and mice (Parsi et al. 2015). Pereira et al. showed that a treatment with pre-miR-29b resulted in reduced Beta-secretase 1 protein expression and Aβ levels in a neuronal cell line expressing human APP (Pereira et al. 2016). Additionally, rescuing impaired miRNA biogenesis, by enhancing DICER activity in disease model, has been suggested has potential therapeutic intervention for ALS (Emde et al. 2015).

One approach that has been suggested for the delivery of miRNAs into the brain and into targeted cells vulnerable to NDs is via exosomes, which represent a delivery mechanism that is able to cross the blood brain barrier (Ha, Yang, and Nadithe 2016; Chen et al. 2017). In this context, a successful exosome-based delivery of miR-124 has been demonstrated in a Huntington's disease mouse model resulting in the downregulation of miRNA targets in disease-affected brain regions (Lee et al. 2017). Moreover, exosome-mediated delivery of miRNA has been proposed as therapeutic treatment in other neurological conditions such as ischemic brain injury (Xin, Li, and Chopp 2014). In this context, the targeted delivery of miRNAs via exosomes has been shown to promote neurogenesis in ischemic regions in post-stroke mouse models (Chopp and Zhang 2015; Yang et al. 2017)

Due to the complex nature of miRNA-mediated gene regulation, one major hurdle of miRNA-based therapies is the considerable risk of unspecific gene silencing due to off-target effects (Dubois et al. 2016). Therefore, a clear characterization of

miRNAs, their targets and functional outcome during spatial- and time-dependent molecular disease processes is necessary to achieve potential and reliable application of miRNA blockers or mimics as orchestrated and specific therapeutic applications in NDs.

9. Summary and Conclusions

A major goal of our studies was to dissect to what extent miRNA changes accompany critical stages and progression of NDs in distinct brain regions vulnerable to disease. Thereby, we emphasized the importance of extensive characterizations of disease-associated miRNA signatures as a first indispensable approach for the development of future translational studies. For this purpose, we used a combination of different miRNA quantification approaches for a comprehensive screening and reliable validation of disease-associated miRNA signatures. We aimed to gain holistic insights in temporal, spatial and cellular aspects of miRNA alterations, as well as, to define factors potentially influencing miRNA biogenesis and function during neurodegenerative conditions, which will help to unravel biological consequences of miRNA dysregulation in disease.

In publication I, we identified global miRNA expression alterations in the frontal cortex and cerebellum of sCJD. In particular, we demonstrated that the miRNA expression patterns in two sCJD subtypes, MM1 and VV2, are highly changed in a regional and disease subtype-dependent manner correlating with differential neuropathological affections. Our work strongly suggests that the observed miRNA alterations could shape the molecular disease phenotypes, and thereby, being directly involved in the molecular mechanisms underlying sCJD etiology.

We aimed to emphasize that profiling of effectively incorporated miRNAs into their functional gene silencing complex is crucial in order to understand their contributions to the disease associated gene expression landscape. In this regard, the analyses of miRNAs in immunoprecipated Ago-containing complexes in sCJD brain revealed that globally upregulated miRNAs are also found enriched in their RISC complex. Thereby, we successfully proofed that alterations of miRNA signatures in sCJD are also leading to changed binding of mature miRNA their silencing complex and likely contribute in shaping gene expression. With our work, we provide innovative impulses for the field of miRNA research in NDs by pointing out the crucial need for profiling miRNA-mRNA interactions in their functionally active silencing complexes instead of interpreting mere expression levels profiled in brain tissue lysates. Additionally, we provided a way to identify the cellular environment of miRNAs, found to be altered in sCJD, via *in situ* hybridization assays in brain tissue, which essentially complement miRNA expression profiling on bulk tissue level.

Clear characterization of potential underlying mechanisms and factors affecting miRNA mediated gene regulation in disease context is essential to dissect the contributions of miRNA dysregulation in sCJD pathology. By analyzing key proteins involved in miRNA biogenesis, we observed reduced levels of the miRNA-processing RNases Dicer, Drosha and its cofactor DGCR8 in frontal cortex and cerebellum in sCJD. Additionally, we showed that Ago-proteins appeared to

change partially their subcellular distribution and localization during disease likely affecting Ago function, and thereby the miRNA-mRNA silencing machinery.

In order to use a disease model highly relevant to human prion pathology, we investigated the miRNA expression patterns in a humanized mice model inoculated with sCJD prions. We clearly validated the miRNA expression signatures that were observed in sCJD, in the CJD mouse model. In this way, we demonstrated that beside highly resembled neuropathological features between sCJD and CJD mouse model, also alterations in disease associated molecular signatures are resembled between human disease and the corresponding mouse model.

We provided the first investigations on the presence of disease-associated miRNAs in the CSF derived from sCJD and age-matched control patients, revealing important information regarding the types of miRNAs detectable in cell-free CSF and provided evidences for miR-204 as a potential CSF prion biomarker.

In cross-disease validation studies, we detected that specific sCJD-regulated miRNAs are commonly altered in alternative NDs including AD, DLB, and FFI shedding light shedding light into potential common miRNA-related mechanisms in the NDs.

In publication II, we provided crucial insights into temporal miRNA expression alterations in brain regions vulnerable to early AD. Therefore, we performed targeted miRNA expression profiling in the LC, EC, hippocampal CA1 region and dentate gyrus during early and mid-stages of Braak NFT pathology. The profiled miRNAs were selected according to their described implications in pathological aspects in AD. With our work, we complemented knowledge about global miRNA expression analyses in AD brain performed in other studies, with targeted miRNA expression profiling in a temporal and cellular manner in brain regions vulnerable to early AD.

In this way, we showed for the first time evidences for meaningful miRNA expression alterations in the LC during early NFT pathology before AD-associated neuronal death is evident. In the LC, the miRNA-27a-3p, miRNA-124-3p, and miRNA-143-3p showed a trend to increase their expression at early stages (Braak I-II) and they were found significantly upregulated at mid-stages (Braak III-IV) of disease. Among profiled miRNAs, only miRNA-143-3p was found upregulated in EC at mid-stages of disease. None of the profiled miRNAs revealed expression alterations in the hippocampal CA1 region, while miRNA-124-3p was downregulated in DG at early stages of NFT pathology.
The characterization of the cellular miRNA environment via *in situ* hybridization assays revealed that the deregulated miRNAs are localized in the cytoplasm of neurons during NFT pathology progression. Additionally, miRNA-27a-3p has been shown to be localized in the nucleus.

Our work supplemented the current level of scientific knowledge on miRNA dysfunction in human prion disease and early AD. Additionally, we provided new important impulses that emphasize the screening for the effective miRNA interactome in the complex cellular context of the brain during NDs. We observed strong evidences that altered miRNA signatures, as well as, a dysfunctional miRNA biogenesis and silencing machinery are involved in the pathogenesis of NDs constituting one important factor in the complex network of multiply regulatory layers shaping gene expression.

10. Literature

Abe, Masashi, and Nancy M. Bonini. 2013a. “MicroRNAs and Neurodegeneration: Role and Impact.” *Trends in Cell Biology* 23 (1): 30–36. doi:10.1016/j.tcb.2012.08.013.

Abu-Rumeileh, Samir, Sabina Capellari, Michelangelo Stanzani-Maserati, Barbara Polischi, Paolo Martinelli, Paola Caroppo, Anna Ladogana, and Piero Parchi. 2018. “The CSF Neurofilament Light Signature in Rapidly Progressive Neurodegenerative Dementias.” *Alzheimer’s Research and Therapy* 10 (1). doi:10.1186/s13195-017-0331-1.

Adolfsson, R, C G Gottfries, B E Roos, and B Winblad. 1979. “Changes in the Brain Catecholamines in Patients with Dementia of Alzheimer Type.” *The British Journal of Psychiatry : The Journal of Mental Science* 135: 216–23. http://www.ncbi.nlm.nih.gov/pubmed/486847.

Aguzzi, Adriano. 2006. “Prion Diseases of Humans and Farm Animals: Epidemiology, Genetics, and Pathogenesis.” *Journal of Neurochemistry*. doi:10.1111/j.1471-4159.2006.03909.x.

Aguzzi, Adriano, and Yingjun Liu. 2017. “A Role for Astroglia in Prion Diseases.” *The Journal of Experimental Medicine* 214 (12): 3477–79. doi:10.1084/jem.20172045.

Akbari Moqadam, F., R. Pieters, and M. L. Den Boer. 2013. “The Hunting of Targets: Challenge in MiRNA Research.” *Leukemia*. doi:10.1038/leu.2012.179.

Andrés-Benito, P., V. Fernández-Dueñas, M. Carmona, L. A. Escobar, B. Torrejón-Escribano, E. Aso, F. Ciruela, and I. Ferrer. 2017. “Locus Coeruleus at Asymptomatic Early and Middle Braak Stages of Neurofibrillary Tangle Pathology.” *Neuropathology and Applied Neurobiology* 43 (5): 373–92. doi:10.1111/nan.12386.

Appleby, Brian S., Kristin K. Appleby, Barbara J. Crain, Chiadi U. Onyike, Mitchell T. Wallin, and Peter V. Rabins. 2009. “Characteristics of Established and Proposed Sporadic Creutzfeldt-Jakob Disease Variants.” *Archives of Neurology* 66 (2): 208–15. doi:10.1001/archneurol.2008.533.

Arena, A., A. M. Iyer, I. Milenkovic, G. G. Kovács, I. Ferrer, M. Perluigi, and E. Aronica. 2017. “Developmental Expression and Dysregulation of MiR-146a and MiR-155 in Down’s Syndrome and Mouse Models of Down’s Syndrome and Alzheimer’s Disease.” *Current Alzheimer Research* 14. doi:10.2174/1567205014666170706112701.

Arendt, Thomas, Jens T. Stieler, and Max Holzer. 2016. “Tau and Tauopathies.” *Brain Research Bulletin* 126: 238–92. doi:10.1016/j.brainresbull.2016.08.018.

Armstrong, Richard A., Peter L. Lantos, and Nigel J. Cairns. 2005. “Overlap between Neurodegenerative Disorders.” *Neuropathology* 25 (2): 111–24. doi:10.1111/j.1440-1789.2005.00605.x.

Asante, Emmanuel A., Jacqueline M. Linehan, Melanie Desbruslais, Susan Joiner, Ian Gowland, Andrew L. Wood, Julie Welch, et al. 2002. “BSE Prions Propagate as Either Variant CJD-like or Sporadic CJD-like Prion Strains in Transgenic Mice Expressing Human Prion Protein.” *EMBO Journal* 21 (23): 6358–66. doi:10.1093/emboj/cdf653.

Atarashi, Ryuichiro, Kazunori Sano, Katsuya Satoh, and Noriyuki Nishida. 2011. “Real-Time

Quaking-Induced Conversion." *Prion* 5 (3): 150–53. doi:10.4161/pri.5.3.16893.

Baiardi, Simone, Anna Magherini, Sabina Capellari, Veronica Redaelli, Anna Ladogana, Marcello Rossi, Fabrizio Tagliavini, Maurizio Pocchiari, Giorgio Giaccone, and Piero Parchi. 2017. "Towards an Early Clinical Diagnosis of Sporadic CJD VV2 (Ataxic Type)." *Journal of Neurology, Neurosurgery and Psychiatry* 88 (9): 764–72. doi:10.1136/jnnp-2017-315942.

Ballatore, Carlo, Virginia M.-Y. Lee, and John Q. Trojanowski. 2007. "Tau-Mediated Neurodegeneration in Alzheimer's Disease and Related Disorders." *Nature Reviews Neuroscience* 8 (9): 663–72. doi:10.1038/nrn2194.

Banzhaf-Strathmann, Julia, Eva Benito, Stephanie May, Thomas Arzberger, Sabina Tahirovic, Hans Kretzschmar, André Fischer, and Dieter Edbauer. 2014. "MicroRNA-125b Induces Tau Hyperphosphorylation and Cognitive Deficits in Alzheimer's Disease." *The EMBO Journal* 33 (15): 1667–80. doi:10.15252/embj.201387576.

Barca-Mayo, Olga, and Q. Richard Lu. 2012. "Fine-Tuning Oligodendrocyte Development by Micrornas." *Frontiers in Neuroscience*. doi:10.3389/fnins.2012.00013.

Bari, Michele A. Di, Romolo Nonno, and Umberto Agrimi. 2012. "The Mouse Model for Scrapie: Inoculation, Clinical Scoring, and Histopathological Techniques." *Methods in Molecular Biology* 849: 453–71. doi:10.1007/978-1-61779-551-0_31.

Bartel, David P. 2009. "MicroRNAs: Target Recognition and Regulatory Functions." *Cell* 136 (2): 215–33. doi:10.1016/j.cell.2009.01.002.

Basavaraju, Manasa, and Alexandre De Lencastre. 2016. "Alzheimer's Disease: Presence and Role of MicroRNAs." *Biomolecular Concepts* 7 (4): 241–52. doi:10.1515/bmc-2016-0014.

Beitzinger, Michaela, Lasse Peters, Jia Yun Zhu, Elisabeth Kremmer, and Gunter Meister. 2007. "Identification of Human MicroRNA Targets from Isolated Argonaute Protein Complexes." *RNA Biology* 4 (2): 76–84. doi:10.4161/rna.4.2.4640.

Bejanin, Alexandre, Daniel R. Schonhaut, Renaud La Joie, Joel H. Kramer, Suzanne L. Baker, Natasha Sosa, Nagehan Ayakta, et al. 2017. "Tau Pathology and Neurodegeneration Contribute to Cognitive Impairment in Alzheimer's Disease." *Brain* 140 (12): 3286–3300. doi:10.1093/brain/awx243.

Bekris, Lynn M, Franziska Lutz, Thomas J Montine, Chang En Yu, Debby Tsuang, Elaine R Peskind, and James B Leverenz. 2013. "MicroRNA in Alzheimer's Disease: An Exploratory Study in Brain, Cerebrospinal Fluid and Plasma." *Biomarkers : Biochemical Indicators of Exposure, Response, and Susceptibility to Chemicals* 18 (5): 455–66. doi:10.3109/1354750X.2013.814073.

Bellingham, Shayne A., Bradley M. Coleman, and Andrew F. Hill. 2012. "Small RNA Deep Sequencing Reveals a Distinct MiRNA Signature Released in Exosomes from Prion-Infected Neuronal Cells." *Nucleic Acids Research* 40 (21): 10937–49. doi:10.1093/nar/gks832.

Bellingham, Shayne A., and Andrew F. Hill. 2017. "Analysis of MiRNA Signatures in Neurodegenerative Prion Disease." *Methods in Molecular Biology* 1658: 67–80. doi:10.1007/978-1-4939-7244-9_6.

Besser, Lilah M., John F. Crary, Charles Mock, and Walter A. Kukull. 2017. "Comparison of Symptomatic and Asymptomatic Persons with Primary Age-Related Tauopathy." *Neurology*

89 (16): 1707–15. doi:10.1212/WNL.0000000000004521.

Bhattacharjee, Surjyadipta, Yuhai Zhao, Prerna Dua, Evgeny I. Rogaev, and Walter J. Lukiw. 2016. “MicroRNA-34α-Mediated down-Regulation of the Microglial-Enriched Triggering Receptor and Phagocytosis-Sensor TREM2 in Age-Related Macular Degeneration.” *PLoS ONE* 11 (3). doi:10.1371/journal.pone.0150211.

Bishop, M. T., R. G. Will, and J. C. Manson. 2010. “Defining Sporadic Creutzfeldt-Jakob Disease Strains and Their Transmission Properties.” *Proceedings of the National Academy of Sciences* 107 (26): 12005–10. doi:10.1073/pnas.1004688107.

Boese, Amrit S., Reuben Saba, Kristyn Campbell, Anna Majer, Sarah Medina, Lynn Burton, Timothy F. Booth, et al. 2016. “MicroRNA Abundance Is Altered in Synaptoneurosomes during Prion Disease.” *Molecular and Cellular Neuroscience* 71: 13–24. doi:10.1016/j.mcn.2015.12.001.

Boivin, Vincent, Gabrielle Deschamps-Francoeur, and Michelle S. Scott. 2017. “Protein Coding Genes as Hosts for Noncoding RNA Expression.” *Seminars in Cell and Developmental Biology*. doi:10.1016/j.semcdb.2017.08.016.

Boudreau, Ryan L., Peng Jiang, Brian L. Gilmore, Ryan M. Spengler, Rebecca Tirabassi, Jay A. Nelson, Christopher A. Ross, Yi Xing, and Beverly L. Davidson. 2014. “Transcriptome-Wide Discovery of MicroRNA Binding Sites in Human Brain.” *Neuron*. doi:10.1016/j.neuron.2013.10.062.

Braak, H., and E. Braak. 1991. “Neuropathological Stageing of Alzheimer-Related Changes.” *Acta Neuropathologica* 82 (4): 239–59. doi:10.1007/BF00308809.

———. 1997. “Frequency of Stages of Alzheimer-Related Lesions in Different Age Categories.” *Neurobiology of Aging* 18 (4): 351–57. doi:10.1016/S0197-4580(97)00056-0.

Braak, Heiko, Dietmar R. Thal, Estifanos Ghebremedhin, and Kelly Del Tredici. 2011. “Stages of the Pathologic Process in Alzheimer Disease: Age Categories from 1 to 100 Years.” *Journal of Neuropathology and Experimental Neurology* 70 (11): 960–69. doi:10.1097/NEN.0b013e318232a379.

Braak, Heiko, and Kelly Del Tredici. 2011. “The Pathological Process Underlying Alzheimer’s Disease in Individuals under Thirty.” *Acta Neuropathologica* 121 (2): 171–81. doi:10.1007/s00401-010-0789-4.

———. 2015. “The Preclinical Phase of the Pathological Process Underlying Sporadic Alzheimer’s Disease.” *Brain* 138 (10): 2814–33. doi:10.1093/brain/awv236.

Brandner, Sebastian, and Zane Jaunmuktane. 2017. “Prion Disease: Experimental Models and Reality.” *Acta Neuropathologica* 133 (2): 197–222. doi:10.1007/s00401-017-1670-5.

Braun, Joerg E., Eric Huntzinger, and Elisa Izaurralde. 2013. “The Role of GW182 Proteins in MiRNA-Mediated Gene Silencing.” *Advances in Experimental Medicine and Biology* 768: 147–63. doi:10.1007/978-1-4614-5107-5-9.

Bronevetsky, Yelena, and K. Mark Ansel. 2013. “Regulation of MiRNA Biogenesis and Turnover in the Immune System.” *Immunological Reviews*. doi:10.1111/imr.12059.

Bruce, M. E. 1993. “Scrapie Strain Variation and Mutation.” *British Medical Bulletin* 49 (4): 822–38. doi:10.1093/oxfordjournals.bmb.a072649.

Bruce, Moira E. 2003. “TSE Strain Variation.” *British Medical Bulletin* 66: 99–108. doi:10.1093/bmb/66.1.99.

Budka, Herbert. 2003. “Neuropathology of Prion Diseases.” *British Medical Bulletin* 66: 121–30. doi:10.1093/bmb/66.1.121.

Burak, Kristyn, Lise Lamoureux, Amrit Boese, Anna Majer, Reuben Saba, Yulian Niu, Kathy Frost, and Stephanie A. Booth. 2018. “MicroRNA-16 Targets MRNA Involved in Neurite Extension and Branching in Hippocampal Neurons during Presymptomatic Prion Disease.” *Neurobiology of Disease* 112: 1–13. doi:10.1016/j.nbd.2017.12.011.

Burgos, Kasandra Lovette, Ashkan Javaherian, Roberto Bomprezzi, Layla Ghaffari, Susan Rhodes, Amanda Courtright, Waibhav Tembe, Seungchan Kim, Raghu Metpally, and Kendall Van Keuren-Jensen. 2013. “Identification of Extracellular MiRNA in Human Cerebrospinal Fluid by Next-Generation Sequencing.” *RNA* 19 (5): 712–22. doi:10.1261/rna.036863.112.

Cai, Zhiyou, Yu Zhao, and Bin Zhao. 2012. “Roles of Glycogen Synthase Kinase 3 in Alzheimer’s Disease.” *Current Alzheimer Research* 9 (7): 864–79. doi:10.2174/156720512802455386.

Canas, L.S., B. Yvernault, C. Sudre, E. De Vita, M.J. Cardoso, J. Thornton, F. Barkhof, S. Ourselin, S. Mead, and M. Modat. 2018. “Imaging Biomarkers for the Diagnosis of Prion Disease.” *Progress in Biomedical Optics and Imaging - Proceedings of SPIE* 10574. doi:10.1117/12.2293676.

Capece, V., J. C. Garcia Vizcaino, R. Vidal, R.-U. Rahman, T. Pena Centeno, O. Shomroni, I. Suberviola, a. Fischer, and S. Bonn. 2015. “Oasis: Online Analysis of Small RNA Deep Sequencing Data.” *Bioinformatics* 31 (February): 1–3. doi:10.1093/bioinformatics/btv113.

Carroll, James A, James F Striebel, Brent Race, Katie Phillips, and Bruce Chesebro. 2015. “Prion Infection of Mouse Brain Reveals Multiple New Upregulated Genes Involved in Neuroinflammation or Signal Transduction.” *J Virol* 89 (4): 2388–2404. doi:10.1128/JVI.02952-14.

Castellani, R. J., M. Colucci, Z. Xie, W. Zou, C. Li, P. Parchi, S. Capellari, et al. 2004. “Sensitivity of 14-3-3 Protein Test Varies in Subtypes of Sporadic Creutzfeldt-Jakob Disease.” *Neurology* 63 (3): 436–42. doi:10.1212/01.WNL.0000135153.96325.3B.

Castellani, Rudy J., George Perry, and Mark A. Smith. 2004. “Prion Disease and Alzheimer’s Disease: Pathogenic Overlap.” *Acta Neurobiologiae Experimentalis* 64 (1): 11–17.

Castilla, Joaquin, Claudio Hetz, and Claudio Soto. 2004. “Molecular Mechanisms of Neurotoxicity of Pathological Prion Protein.” *Current Molecular Medicine* 4 (4): 397–403. doi:10.2174/1566524043360654.

Castle, Andrew R., and Andrew C. Gill. 2017. “Physiological Functions of the Cellular Prion Protein.” *Frontiers in Molecular Biosciences* 4. doi:10.3389/fmolb.2017.00019.

Chalermpalanupap, Termpanit, David Weinshenker, and Jacki M. Rorabaugh. 2017. “Down but Not Out: The Consequences of Pretangle Tau in the Locus Coeruleus.” *Neural Plasticity* 2017. doi:10.1155/2017/7829507.

Chantarachot, Thanin, and Julia Bailey-Serres. 2017. “Polysomes, Stress Granules and Processing Bodies: A Dynamic Triumvirate Controlling Cytoplasmic MRNA Fate and Function.” *Plant Physiology*, pp.01468.2017. doi:10.1104/pp.17.01468.

Chen, Jian Jiao, Bin Zhao, Jie Zhao, and Shao Li. 2017. “Potential Roles of Exosomal MicroRNAs as Diagnostic Biomarkers and Therapeutic Application in Alzheimer’s Disease.” *Neural Plasticity* 2017. doi:10.1155/2017/7027380.

Cheng, L., J. D. Doecke, R. A. Sharples, V. L. Villemagne, C. J. Fowler, A. Rembach, R. N. Martins, et al. 2015. “Prognostic Serum MiRNA Biomarkers Associated with Alzheimer’s Disease Shows Concordance with Neuropsychological and Neuroimaging Assessment.” *Molecular Psychiatry* 20 (10): 1188–96. doi:10.1038/mp.2014.127.

Chiyomaru, Takeshi, Shinichiro Fukuhara, Sharanjot Saini, Shahana Majid, Guoren Deng, Varahram Shahryari, Inik Chang, et al. 2014. “Long Non-Coding RNA Hotair Is Targeted and Regulated by MIR-141 in Human Cancer Cells.” *Journal of Biological Chemistry*. doi:10.1074/jbc.M113.488593.

Chopp, Michael, and Zheng Gang Zhang. 2015. “Emerging Potential of Exosomes and Noncoding MicroRNAs for the Treatment of Neurological Injury/Diseases.” *Expert Opinion on Emerging Drugs*. doi:10.1517/14728214.2015.1061993.

Chugh, Pauline, and Dirk P Dittmer. 2013. “Potential Pitfalls in MicroRNA Profiling.” *Wiley Interdisciplinary Reviews. RNA* 3 (5): 17–36. doi:10.1002/wrna.1120.Potential.

Clarke, Laura E., Shane A. Liddelow, Chandrani Chakraborty, Alexandra E. Münch, Myriam Heiman, and Ben A. Barres. 2018. “Normal Aging Induces A1-like Astrocyte Reactivity.” *Proceedings of the National Academy of Sciences* 115 (8): E1896–1905. doi:10.1073/pnas.1800165115.

Cogswell, John P., James Ward, Ian A. Taylor, Michelle Waters, Yunling Shi, Brian Cannon, Kevin Kelnar, et al. 2008. “Identification of MiRNA Changes in Alzheimer’s Disease Brain and CSF Yields Putative Biomarkers and Insights into Disease Pathways.” *Journal of Alzheimer’s Disease* 14 (1): 27–41. doi:10.3233/JAD-2008-14103.

Colby, David W., and Stanley B. Prusiner. 2011. “Prions.” *Cold Spring Harbor Perspectives in Biology* 3 (1): 1–22. doi:10.1101/cshperspect.a006833.

Collinge, John, Jerome Whitfield, Edward McKintosh, John Beck, Simon Mead, Dafydd J. Thomas, and Michael P. Alpers. 2006. “Kuru in the 21st Century-an Acquired Human Prion Disease with Very Long Incubation Periods.” *Lancet* 367 (9528): 2068–74. doi:10.1016/S0140-6736(06)68930-7.

Collins, S. J., P. Sanchez-Juan, C. L. Masters, G. M. Klug, C. Van Duijn, A. Poleggi, M. Pocchiari, et al. 2006. “Determinants of Diagnostic Investigation Sensitivities across the Clinical Spectrum of Sporadic Creutzfeldt-Jakob Disease.” *Brain* 129 (9): 2278–87. doi:10.1093/brain/awl159.

Conrad, Thomas, Annalisa Marsico, Maja Gehre, and Ulf Andersson Ørom. 2014. “Microprocessor Activity Controls Differential MiRNA Biogenesis in Vivo.” *Cell Reports* 9 (2): 542–54. doi:10.1016/j.celrep.2014.09.007.

Cortelli, Pietro, Pierluigi Gambetti, Pasquale Montagna, and Elio Lugaresi. 1999. “Fatal Familial Insomnia: Clinical Features and Molecular Genetics.” *Journal of Sleep Research* 8 (SUPPL. 1): 23–29. doi:10.1046/j.1365-2869.1999.00005.x.

Counts, Scott E., and Elliott J. Mufson. 2012. “Locus Coeruleus.” *The Human Nervous System*,

425–38. doi:10.1016/B978-0-12-374236-0.10012-4.

Cracco, Laura, Brian S. Appleby, and Pierluigi Gambetti. 2018. “Fatal Familial Insomnia and Sporadic Fatal Insomnia.” *Handbook of Clinical Neurology* 153: 271–99. doi:10.1016/B978-0-444-63945-5.00015-5.

Crary, John F., John Q. Trojanowski, Julie A. Schneider, Jose F. Abisambra, Erin L. Abner, Irina Alafuzoff, Steven E. Arnold, et al. 2014. “Primary Age-Related Tauopathy (PART): A Common Pathology Associated with Human Aging.” *Acta Neuropathologica* 128 (6): 755–66. doi:10.1007/s00401-014-1349-0.

Cui, Jian Guo, Yuan Yuan Li, Yuhai Zhao, Surjyadipta Bhattacharjee, and Walter J. Lukiw. 2010. “Differential Regulation of Interleukin-1 Receptor-Associated Kinase-1 (IRAK-1) and IRAK-2 by MicroRNA-146a and NF-KB in Stressed Human Astroglial Cells and in Alzheimer Disease.” *Journal of Biological Chemistry* 285 (50): 38951–60. doi:10.1074/jbc.M110.178848.

Cummings, Jeffrey. 2017. “Disease Modification and Neuroprotection in Neurodegenerative Disorders.” *Translational Neurodegeneration* 6 (1). doi:10.1186/s40035-017-0096-2.

Curtis, Helen J., Christopher R. Sibley, and Matthew J.A. Wood. 2012. “Mirtrons, an Emerging Class of Atypical MiRNA.” *Wiley Interdisciplinary Reviews: RNA*. doi:10.1002/wrna.1122.

Dangla-Valls, A., J.L. Molinuevo, J. Altirriba, R. Sánchez-Valle, D. Alcolea, J. Fortea, L. Rami, et al. 2017. “CSF MicroRNA Profiling in Alzheimer’s Disease: A Screening and Validation Study.” *Molecular Neurobiology* 54 (9). doi:10.1007/s12035-016-0106-x.

Dangwal, S., C. Bang, and T. Thum. 2012. “Novel Techniques and Targets in Cardiovascular MicroRNA Research.” *Cardiovascular Research*. doi:10.1093/cvr/cvr297.

Davis, Gregory M., Matilda A. Haas, and Roger Pocock. 2015. “MicroRNAs: Not ‘Fine-Tuners’ but Key Regulators of Neuronal Development and Function.” *Frontiers in Neurology* 6 (NOV). doi:10.3389/fneur.2015.00245.

Delay, Charlotte, and Sébastien S. Hébert. 2011. “MicroRNAs and Alzheimer’s Disease Mouse Models: Current Insights and Future Research Avenues.” *International Journal of Alzheimer’s Disease* 2011: 1–6. doi:10.4061/2011/894938.

Delay, Charlotte, Wim Mandemakers, and Sébastien S. Hébert. 2012. “MicroRNAs in Alzheimer’s Disease.” *Neurobiology of Disease* 46 (2): 285–90. doi:10.1016/j.nbd.2012.01.003.

Detzer, Anke, Christina Engel, Winfried Wünsche, and Georg Sczakiel. 2011. “Cell Stress Is Related to Re-Localization of Argonaute 2 and to Decreased RNA Interference in Human Cells.” *Nucleic Acids Research* 39 (7): 2727–41. doi:10.1093/nar/gkq1216.

Devier, Deidre J., Jesus F. Lovera, and Walter J. Lukiw. 2015. “Increase in NF-KB-Sensitive MiRNA-146a and MiRNA-155 in Multiple Sclerosis (MS) and pro-Inflammatory Neurodegeneration.” *Frontiers in Molecular Neuroscience* 8 (March): 1–5. doi:10.3389/fnmol.2015.00005.

Ding, Haixia, Zhen Huang, Mengjie Chen, Cheng Wang, Xi Chen, Jiangning Chen, and Junfeng Zhang. 2016. “Identification of a Panel of Five Serum MiRNAs as a Biomarker for Parkinson’s Disease.” *Parkinsonism and Related Disorders* 22: 68–73. doi:10.1016/j.parkreldis.2015.11.014.

Dong, Hui, Jialu Li, Lei Huang, Xi Chen, Donghai Li, Tao Wang, Caiyou Hu, et al. 2015. “Serum

MicroRNA Profiles Serve as Novel Biomarkers for the Diagnosis of Alzheimer's Disease." *Disease Markers* 2015. doi:10.1155/2015/625659.

Dorothée, Guillaume. 2018. "Neuroinflammation in Neurodegeneration: Role in Pathophysiology, Therapeutic Opportunities and Clinical Perspectives." *Journal of Neural Transmission*. doi:10.1007/s00702-018-1880-6.

Dubois, Bruno, Alessandro Padovani, Philip Scheltens, Andrea Rossi, and Grazia Dell Agnello. 2016. "Timely Diagnosis for Alzheimer's Disease: A Literature Review on Benefits and Challenges." *Journal of Alzheimer's Disease* 49 (3): 617–31. doi:10.3233/JAD-150692.

Dugger, Brittany N., and Dennis W. Dickson. 2017. "Pathology of Neurodegenerative Diseases." *Cold Spring Harbor Perspectives in Biology* 9 (7). doi:10.1101/cshperspect.a028035.

Duyckaerts, Charles, Heiko Braak, Jean-Pierre Brion, Luc Buée, Kelly Del Tredici, Michel Goedert, Glenda Halliday, et al. 2015. "PART Is Part of Alzheimer Disease." *Acta Neuropathologica* 129 (5): 749–56. doi:10.1007/s00401-015-1390-7.

Dzikiewicz-Krawczyk, Agnieszka, Arjan Diepstra, Bea Rutgers, Gertrud Kortman, Debora de Jong, Jasper Koerts, Marian Bulthuis, et al. 2018. "Argonaute 2 RNA Immunoprecipitation Reveals Distinct MiRNA Targetomes of Primary Burkitt Lymphoma Tumors and Normal B Cells." *American Journal of Pathology*. doi:10.1016/j.ajpath.2018.01.018.

Elder, Gregory A, Miguel A Gama Sosa, and Rita De Gasperi. 2010. "Transgenic Mouse Models of Alzheimer's Disease." *The Mount Sinai Journal of Medicine, New York* 77 (1): 69–81. doi:10.1002/msj.20159.

Emde, A., C. Eitan, L.-L. Liou, R. T. Libby, N. Rivkin, I. Magen, I. Reichenstein, et al. 2015. "Dysregulated MiRNA Biogenesis Downstream of Cellular Stress and ALS-Causing Mutations: A New Mechanism for ALS." *The EMBO Journal* 34 (21): 2633–51. doi:10.15252/embj.201490493.

Ermonval, M., S. Mouillet-Richard, P. Codogno, O. Kellermann, and J. Botti. 2003. "Evolving Views in Prion Glycosylation: Functional and Pathological Implications." *Biochimie*. doi:10.1016/S0300-9084(03)00040-3.

Falaleeva, Marina, and Stefan Stamm. 2013. "Processing of SnoRNAs as a New Source of Regulatory Non-Coding RNAs: SnoRNA Fragments Form a New Class of Functional RNAs." *BioEssays* 35 (1): 46–54. doi:10.1002/bies.201200117.

Fan, Meiyun, Raisa Krutilina, Jing Sun, Aarti Sethuraman, Chuan He Yang, Zhao-Hui Wu, Junming Yue, and Lawrence M Pfeffer. 2013. "Comprehensive Analysis of MicroRNA (MiRNA) Targets in Breast Cancer Cells." *The Journal of Biological Chemistry*. doi:10.1074/jbc.M113.491803.

Faucheux, Baptiste A., Nicolas Privat, Jean Philippe Brandel, Véronique Sazdovitch, Jean Louis Laplanche, Claude Alain Maurage, Jean Jacques Hauw, and Stéphane Haïk. 2009. "Loss of Cerebellar Granule Neurons Is Associated with Punctate but Not with Large Focal Deposits of Prion Protein in Creutzfeldt-Jakob Disease." *Journal of Neuropathology and Experimental Neurology* 68 (8): 892–901. doi:10.1097/NEN.0b013e3181af7f23.

Femminella, Grazia D., Nicola Ferrara, and Giuseppe Rengo. 2015. "The Emerging Role of MicroRNAs in Alzheimer's Disease." *Frontiers in Physiology* 6 (FEB). doi:10.3389/fphys.2015.00040.

Ferreira, Sergio T., Mychael V. Lourenco, Mauricio M. Oliveira, and Fernanda G. De Felice. 2015. “Soluble Amyloid-Î² Oligomers as Synaptotoxins Leading to Cognitive Impairment in Alzheimerâ€™s Disease.” *Frontiers in Cellular Neuroscience* 9. doi:10.3389/fncel.2015.00191.

Ferreiro, Elisabete, Catarina R. Oliveira, and Cláudia M.F. Pereira. 2008. “The Release of Calcium from the Endoplasmic Reticulum Induced by Amyloid-Beta and Prion Peptides Activates the Mitochondrial Apoptotic Pathway.” *Neurobiology of Disease* 30 (3): 331–42. doi:10.1016/j.nbd.2008.02.003.

Ferrer, Isidre, Irene López-González, Margarita Carmona, Laura Arregui, Esther Dalfó, Benjamin Torrejón-Escribano, Roberta Diehl, and Gabor G. Kovacs. 2014. “Glial and Neuronal Tau Pathology in Tauopathies: Characterization of Disease-Specific Phenotypes and Tau Pathology Progression.” *Journal of Neuropathology and Experimental Neurology* 73 (1): 81–97. doi:10.1097/NEN.0000000000000030.

Fiandaca, Massimo S., Mark E. Mapstone, Amrita K. Cheema, and Howard J. Federoff. 2014. “The Critical Need for Defining Preclinical Biomarkers in Alzheimer’s Disease.” *Alzheimer’s and Dementia* 10 (3 SUPPL.). doi:10.1016/j.jalz.2014.04.015.

Fjell, Anders M., and Kristine B. Walhovd. 2012. “Neuroimaging Results Impose New Views on Alzheimer’s Disease-the Role of Amyloid Revised.” *Molecular Neurobiology* 45 (1): 153–72. doi:10.1007/s12035-011-8228-7.

Franceschini, A., R. Strammiello, S. Capellari, A. Giese, and P. Parchi. 2018. “Regional Pattern of Microgliosis in Sporadic Creutzfeldt-Jakob Disease in Relation to Phenotypic Variants and Disease Progression.” *Neuropathology and Applied Neurobiology*. doi:10.1111/nan.12461.

Frau-Méndez, Margalida A., Iván Fernández-Vega, Belén Ansoleaga, Rosa Blanco Tech, Margarita Carmona Tech, Jose Antonio del Rio, Inga Zerr, Franc Llorens, Juan José Zarranz, and Isidro Ferrer. 2017. “Fatal Familial Insomnia: Mitochondrial and Protein Synthesis Machinery Decline in the Mediodorsal Thalamus.” *Brain Pathology* 27 (1): 95–106. doi:10.1111/bpa.12408.

Freischmidt, Axel, Kathrin Müller, Albert C. Ludolph, and Jochen H. Weishaupt. 2013. “Systemic Dysregulation of TDP-43 Binding MicroRNAs in Amyotrophic Lateral Sclerosis.” *Acta Neuropathologica Communications* 1 (1): 42. doi:10.1186/2051-5960-1-42.

Galimberti, Daniela, Chiara Villa, Chiara Fenoglio, Maria Serpente, Laura Ghezzi, Sara M.G. Cioffi, Andrea Arighi, Giorgio Fumagalli, and Elio Scarpini. 2014. “Circulating MiRNAs as Potential Biomarkers in Alzheimer’s Disease.” *Journal of Alzheimer’s Disease* 42 (4): 1261–67. doi:10.3233/JAD-140756.

Gallassi, R., A. Morreale, P. Montagna, P. Cortelli, P. Avoni, R. Castellani, P. Gambetti, and E. Lugaresi. 1996. “Fatal Familial Insomnia: Behavioral and Cognitive Features.” *Neurology* 46 (4): 935–39. doi:10.1212/WNL.46.4.935.

Gallego, Juan A, Marc L Gordon, Kierstyn Claycomb, Mahima Bhatt, Todd Lencz, and Anil K Malhotra. 2012. “In Vivo MicroRNA Detection and Quantitation in Cerebrospinal Fluid.” *Journal of Molecular Neuroscience : MN* 47 (2): 243–48. doi:10.1007/s12031-012-9731-7.

Gambetti, Pierluigi, Qingzhong Kong, Wenquan Zou, Piero Parchi, and Shu G. Chen. 2003.

"Sporadic and Familial CJD: Classification and Characterisation." *British Medical Bulletin* 66: 213–39. doi:10.1093/bmb/66.1.213.

Gao, Chen, Jing Wei, Bao Yun Zhang, Qiang Shi, Cao Chen, Jing Wang, Qi Shi, and Xiao Ping Dong. 2016. "MiRNA Expression Profiles in the Brains of Mice Infected with Scrapie Agents 139A, ME7 and S15." *Emerging Microbes and Infections* 5 (11). doi:10.1038/emi.2016.120.

Gaudet, Andrew D., Laura K. Fonken, Linda R. Watkins, Randy J. Nelson, and Phillip G. Popovich. 2017. "MicroRNAs: Roles in Regulating Neuroinflammation." *The Neuroscientist*, 107385841772115. doi:10.1177/1073858417721150.

Gawinecka, Joanna, Franco Cardone, Abdul R. Asif, Angela De Pascalis, Wiebke M. Wemheuer, Walter J. Schulz-Schaeffer, Maurizio Pocchiari, and Inga Zerr. 2012. "Sporadic Creutzfeldt-Jakob Disease Subtype-Specific Alterations of the Brain Proteome: Impact on Rab3a Recycling." *Proteomics* 12 (23–24): 3610–20. doi:10.1002/pmic.201200201.

Gee, H. E., F. M. Buffa, C. Camps, A. Ramachandran, R. Leek, M. Taylor, M. Patil, et al. 2011. "The Small-Nucleolar RNAs Commonly Used for MicroRNA Normalisation Correlate with Tumour Pathology and Prognosis." *British Journal of Cancer* 104 (7): 1168–77. doi:10.1038/sj.bjc.6606076.

Geekiyanage, Hirosha, Gregory A. Jicha, Peter T. Nelson, and Christina Chan. 2012. "Blood Serum MiRNA: Non-Invasive Biomarkers for Alzheimer's Disease." *Experimental Neurology* 235 (2): 491–96. doi:10.1016/j.expneurol.2011.11.026.

Geschwind, Michael D., Huidy Shu, Aissa Haman, James J. Sejvar, and Bruce L. Miller. 2008. "Rapidly Progressive Dementia." *Annals of Neurology* 64 (1): 97–108. doi:10.1002/ana.21430.

Geschwind, Michael D. 2015. "Prion Diseases." *Continuum (Minneapolis, Minn.)* 21 (6 Neuroinfectious Disease): 1612–38. doi:10.1212/CON.0000000000000251.

Gibbings, D, P Leblanc, F Jay, D Pontier, F Michel, Y Schwab, S Alais, T Lagrange, and O Voinnet. 2012. "Human Prion Protein Binds Argonaute and Promotes Accumulation of MicroRNA Effector Complexes." *Nat Struct Mol Biol* 19 (5): 517–24, S1. doi:10.1038/nsmb.2273.

Giles, Kurt, David V. Glidden, Smita Patel, Carsten Korth, Darlene Groth, Azucena Lemus, Stephen J. DeArmond, and Stanley B. Prusiner. 2010. "Human Prion Strain Selection in Transgenic Mice." *Annals of Neurology* 68 (2): 151–61. doi:10.1002/ana.22104.

Giorgi, Filippo S., Larisa Ryskalin, Riccardo Ruffoli, Francesca Biagioni, Fiona Limanaqi, Michela Ferrucci, Carla L. Busceti, Ubaldo Bonuccelli, and Francesco Fornai. 2017. "The Neuroanatomy of the Reticular Nucleus Locus Coeruleus in Alzheimer's Disease." *Frontiers in Neuroanatomy* 11. doi:10.3389/fnana.2017.00080.

Gmitterová, K., U. Heinemann, A. Krasnianski, J. Gawinecka, and I. Zerr. 2016. "Cerebrospinal Fluid Markers in the Differentiation of Molecular Subtypes of Sporadic Creutzfeldt-Jakob Disease." *European Journal of Neurology* 23 (6): 1126–33. doi:10.1111/ene.12991.

Goedert, Michel. 2015. "Alzheimer's and Parkinson's Diseases: The Prion Concept in Relation to Assembled Aβ, Tau, and α-Synuclein." *Science* 349 (6248). doi:10.1126/science.1255555.

Goldfarb, Lev G., Robert B. Petersen, Massimo Tabaton, Paul Brown, Andréa C. LeBlanc, Pasquale Montagna, Pietro Cortelli, et al. 1992. "Fatal Familial Insomnia and Familial Creutzfeldt-Jakob Disease: Disease Phenotype Determined by a DNA Polymorphism."

Science 258 (5083): 806–8. doi:10.1126/science.1439789.

Goodall, Emily F., Paul R. Heath, Oliver Bandmann, Janine Kirby, and Pamela J. Shaw. 2013. "Neuronal Dark Matter: The Emerging Role of MicroRNAs in Neurodegeneration." *Frontiers in Cellular Neuroscience* 7. doi:10.3389/fncel.2013.00178.

Grasso, Margherita, Paola Piscopo, Annamaria Confaloni, and Michela A Denti. 2014. "Circulating MiRNAs as Biomarkers for Neurodegenerative Disorders. TL - 19." *Molecules (Basel, Switzerland)* 19 VN-r (5): 6891–6910. doi:10.3390/molecules19056891.

Gross, Christina, Xiaodi Yao, Tobias Engel, Durgesh Tiwari, Lei Xing, Shane Rowley, Scott W. Danielson, et al. 2016. "MicroRNA-Mediated Downregulation of the Potassium Channel Kv4.2 Contributes to Seizure Onset." *Cell Reports*. doi:10.1016/j.celrep.2016.08.074.

Grudzien, A, P Shaw, S Weintraub, E Bigio, D C Mash, and M M Mesulam. 2007. "Locus Coeruleus Neurofibrillary Degeneration in Aging, Mild Cognitive Impairment and Early Alzheimer's Disease." *Neurobiol Aging* 28 (3): 327–35. doi:S0197-4580(06)00045-5 [pii]\r10.1016/j.neurobiolaging.2006.02.007.

Guentchev, M, J A Hainfellner, G R Trabattoni, and H Budka. 1997. "Distribution of Parvalbumin-Immunoreactive Neurons in Brain Correlates with Hippocampal and Temporal Cortical Pathology in Creutzfeldt-Jakob Disease." *J. Neuropathol. Exp. Neurol* 56 (10): 1119–24. http://www.ncbi.nlm.nih.gov/pubmed/9329455.

Gulyaeva, Lyudmila F., and Nicolay E. Kushlinskiy. 2016. "Regulatory Mechanisms of MicroRNA Expression." *Journal of Translational Medicine* 14 (1). doi:10.1186/s12967-016-0893-x.

Guo, Rui, Gang Fan, Jian Zhang, Chunxiao Wu, Yifeng Du, Hui Ye, Zhang Li, et al. 2017. "A 9-MicroRNA Signature in Serum Serves as a Noninvasive Biomarker in Early Diagnosis of Alzheimer's Disease." *Journal of Alzheimer's Disease* 60 (4): 1365–77. doi:10.3233/JAD-170343.

Guo, Tong, Wendy Noble, and Diane P. Hanger. 2017. "Roles of Tau Protein in Health and Disease." *Acta Neuropathologica* 133 (5): 665–704. doi:10.1007/s00401-017-1707-9.

Ha, Dinh, Ningning Yang, and Venkatareddy Nadithe. 2016. "Exosomes as Therapeutic Drug Carriers and Delivery Vehicles across Biological Membranes: Current Perspectives and Future Challenges." *Acta Pharmaceutica Sinica B* 6 (4): 287–96. doi:10.1016/j.apsb.2016.02.001.

Ha, Minju, and V. Narry Kim. 2014. "Regulation of MicroRNA Biogenesis." *Nature Reviews Molecular Cell Biology* 15 (8): 509–24. doi:10.1038/nrm3838.

Hamlin, Clive, Gianfranco Puoti, Sally Berri, Elliott Sting, Carrie Harris, Mark Cohen, Charles Spear, Alberto Bizzi, Sara M. Debanne, and Douglas Y. Rowland. 2012. "A Comparison of Tau and 14-3-3 Protein in the Diagnosis of Creutzfeldt-Jakob Disease." *Neurology* 79 (6): 547–52. doi:10.1212/WNL.0b013e318263565f.

Hardy, J., and G. Higgins. 1992. "Alzheimer's Disease: The Amyloid Cascade Hypothesis." *Science* 256 (5054): 184–85. doi:10.1126/science.1566067.

Hartmann, Alexander, Christiane Muth, Oliver Dabrowski, Susanne Krasemann, and Markus Glatzel. 2017. "Exosomes and the Prion Protein: More than One Truth." *Frontiers in Neuroscience* 11 (APR). doi:10.3389/fnins.2017.00194.

Hebert, S. S., K. Horre, L. Nicolai, A. S. Papadopoulou, W. Mandemakers, A. N. Silahtaroglu, S. Kauppinen, A. Delacourte, and B. De Strooper. 2008. “Loss of MicroRNA Cluster MiR-29a/b-1 in Sporadic Alzheimer’s Disease Correlates with Increased BACE1/ -Secretase Expression.” *Proceedings of the National Academy of Sciences* 105 (17): 6415–20. doi:10.1073/pnas.0710263105.

Hébert, Sébastien S., Aikaterini S. Papadopoulou, Pascal Smith, Marie Christine Galas, Emmanuel Planel, Asli N. Silahtaroglu, Nicolas Sergeant, Luc Buée, and Bart de Strooper. 2010. “Genetic Ablation of Dicer in Adult Forebrain Neurons Results in Abnormal Tau Hyperphosphorylation and Neurodegeneration.” *Human Molecular Genetics* 19 (20): 3959–69. doi:10.1093/hmg/ddq311.

Hébert, Sébastien S., and Bart De Strooper. 2009a. “Alterations of the MicroRNA Network Cause Neurodegenerative Disease.” *Trends in Neurosciences* 32 (4): 199–206. doi:10.1016/j.tins.2008.12.003.

Heinemann, U., A. Krasnianski, B. Meissner, D. Varges, K. Kallenberg, W. J. Schulz-Schaeffer, B. J. Steinhoff, E. M. Grasbon-Frodl, H. A. Kretzschmar, and I. Zerr. 2007. “Creutzfeldt-Jakob Disease in Germany: A Prospective 12-Year Surveillance.” *Brain* 130 (5): 1350–59. doi:10.1093/brain/awm063.

Heneka, M. T., F. Nadrigny, T. Regen, A. Martinez-Hernandez, L. Dumitrescu-Ozimek, D. Terwel, D. Jardanhazi-Kurutz, et al. 2010. “Locus Ceruleus Controls Alzheimer’s Disease Pathology by Modulating Microglial Functions through Norepinephrine.” *Proceedings of the National Academy of Sciences* 107 (13): 6058–63. doi:10.1073/pnas.0909586107.

Heneka, Michael T., Monica J. Carson, Joseph El Khoury, Gary E. Landreth, Frederic Brosseron, Douglas L. Feinstein, Andreas H. Jacobs, et al. 2015. “Neuroinflammation in Alzheimer’s Disease.” *The Lancet Neurology* 14 (4): 388–405. doi:10.1016/S1474-4422(15)70016-5.

Hernandez-Rapp, Julia, Sara Rainone, Claudia Goupil, Véronique Dorval, Pascal Y. Smith, Martine Saint-Pierre, Maxime Vallée, et al. 2016. “MicroRNA-132/212 Deficiency Enhances Aβ Production and Senile Plaque Deposition in Alzheimer’s Disease Triple Transgenic Mice.” *Scientific Reports* 6. doi:10.1038/srep30953.

Hill, Andrew F., Susan Joiner, Jonathan D F Wadsworth, Katie C L Sidle, Jeanne E. Bell, Herbert Budka, James W. Ironside, and John Collinge. 2003. “Molecular Classification of Sporadic Creutzfeldt-Jakob Disease.” *Brain* 126 (6): 1333–46. doi:10.1093/brain/awg125.

Holtzman, David M, Maria C Carrillo, James A Hendrix, Lisa J Bain, Ana M Catafau, Laura M Gault, Michel Goedert, et al. 2016. “Tau: From Research to Clinical Development.” *Alzheimer’s & Dementia : The Journal of the Alzheimer’s Association* 12 (10): 1033–39. doi:10.1016/j.jalz.2016.03.018.

Hu, Zhonghua, and Zheng Li. 2017. “MiRNAs in Synapse Development and Synaptic Plasticity.” *Current Opinion in Neurobiology* 45: 24–31. doi:10.1016/j.conb.2017.02.014.

Huang, Pei, Fulin Lian, Yi Wen, Chenyun Guo, and Donghai Lin. 2013. “Prion Protein Oligomer and Its Neurotoxicity.” *Acta Biochimica et Biophysica Sinica* 45 (6): 442–51. doi:10.1093/abbs/gmt037.

Hyman, Bradley T, Creighton H Phelps, Thomas G Beach, Eileen H Bigio, Nigel J Cairns, Maria C

Carrillo, Dennis W Dickson, et al. 2016. “National Institute on Aging–Alzheimer’s Association Guidelines for the Neuropathologic Assessment of Alzheimer’s Disease.” *Alzheimer’s & Dementia: The Journal of the Alzheimer’s Association* 8 (1): 1–13. doi:10.1016/j.jalz.2011.10.007.

Imran, Muhammad, and Saqib Mahmood. 2011. “An Overview of Human Prion Diseases.” *Virology Journal* 8. doi:10.1186/1743-422X-8-559.

Ipsaro, Jonathan J., and Leemor Joshua-Tor. 2015. “From Guide to Target: Molecular Insights into Eukaryotic RNA-Interference Machinery.” *Nature Structural and Molecular Biology*. doi:10.1038/nsmb.2931.

Iqbal, Khalid, Alejandra Del C. Alonso, She Chen, M. Omar Chohan, Ezzat El-Akkad, Cheng Xin Gong, Sabiha Khatoon, et al. 2005. “Tau Pathology in Alzheimer Disease and Other Tauopathies.” *Biochimica et Biophysica Acta - Molecular Basis of Disease* 1739 (2): 198–210. doi:10.1016/j.bbadis.2004.09.008.

Iqbal, Khalid, Fei Liu, and Cheng Xin Gong. 2016. “Tau and Neurodegenerative Disease: The Story so Far.” *Nature Reviews Neurology* 12 (1): 15–27. doi:10.1038/nrneurol.2015.225.

Irwin, David J. 2016. “Tauopathies as Clinicopathological Entities.” *Parkinsonism and Related Disorders* 22: S29–33. doi:10.1016/j.parkreldis.2015.09.020.

Jackson, W. S. 2014. “Selective Vulnerability to Neurodegenerative Disease: The Curious Case of Prion Protein.” *Disease Models & Mechanisms* 7 (1): 21–29. doi:10.1242/dmm.012146.

Jafari, Naser, Hassan Shaghaghi, Davood Mahmoodi, Zohreh Shirzad, Fatemeh Alibeiki, Shahab Bohlooli, and Hadi Peeri Dogaheh. 2015. “Overexpression of MicroRNA Biogenesis Machinery: Drosha, DGCR8 and Dicer in Multiple Sclerosis Patients.” *Journal of Clinical Neuroscience* 22 (1): 200–203. doi:10.1016/j.jocn.2014.06.106.

Jefferson-George, Kyra S., David A. Wolk, Edward B. Lee, and Corey T. McMillan. 2017. “CoJefferson-George, Kyra S., David A. Wolk, Edward B. Lee, and Corey T. McMillan. ‘Cognitive Decline Associated with Pathological Burden in Primary Age-Related Tauopathy.’ Alzheimer’s and Dementia 13, No. 9 (2017): 1048–53. Doi:10.1016/j.Jalz.2017.01.028.” *Alzheimer’s and Dementia* 13 (9): 1048–53. doi:10.1016/j.jalz.2017.01.028.

Jeong, Byung-Hoon, and Yong-Sun Kim. 2014. “Genetic Studies in Human Prion Diseases.” *Journal of Korean Medical Science* 29 (5): 623. doi:10.3346/jkms.2014.29.5.623.

Johnson, Richard T. 2005. “Prion Diseases.” *The Lancet. Neurology* 4 (10): 635–42. doi:10.1016/S1474-4422(05)70192-7.

Jonas, Stefanie, and Elisa Izaurralde. 2015a. “Towards a Molecular Understanding of MicroRNA-Mediated Gene Silencing.” *Nature Reviews. Genetics* 16 (7): 421–33. doi:10.1038/nrg3965.

Josephs, Keith a., Jennifer L. Whitwell, Zeshan Ahmed, Maria M. Shiung, Stephen D. Weigand, David S. Knopman, Bradley F. Boeve, et al. 2008. “Beta-Amyloid Burden Is Not Associated With Rates of Brain Atrophy.” *Annals of Neurology* 63 (2): 204–12. doi:10.1002/ana.21223.

Jovičić, Ana, Reema Roshan, Nicoleta Moisoi, Sylvain Pradervand, Roger Moser, Beena Pillai, and Ruth Luthi-Carter. 2013. “Comprehensive Expression Analyses of Neural Cell-Type-Specific MiRNAs Identify New Determinants of the Specification and Maintenance of Neuronal Phenotypes.” *Annals of Internal Medicine* 158 (6): 5127–37. doi:10.1523/JNEUROSCI.0600-

12.2013.

Kanata, Eirini, Katrin Thüne, Konstantinos Xanthopoulos, Isidre Ferrer, Dimitra Dafou, Inga Zerr, Theodoros Sklaviadis, and Franc Llorens. 2018. “MicroRNA Alterations in the Brain and Body Fluids of Humans and Animal Prion Disease Models: Current Status and Perspectives.” *Frontiers in Aging Neuroscience*. doi:10.3389/fnagi.2018.00220.

Kanata, Erini, Franc Llorens, Katrin Thüne, Dimitra Dafou, Athanasios Dimitriadis, Konstantinos Xanthopoulos, Juan Carlos Espinosa, et al. 2018. “Epitranscriptomic Alterations Define Disease Manifestations in Prion Diseases.” *Pnas*.

Kanematsu, Sotaro, Kousuke Tanimoto, Yutaka Suzuki, and Sumio Sugano. 2014. “Screening for Possible MiRNA-MRNA Associations in a Colon Cancer Cell Line.” *Gene*. doi:10.1016/j.gene.2013.08.005.

Kang, Qingmei, Yue Xiang, Dan Li, Jie Liang, Xiong Zhang, Fanlin Zhou, Mengyuan Qiao, et al. 2017. “MiR-124-3p Attenuates Hyperphosphorylation of Tau Protein-Induced Apoptosis via Caveolin-1-PI3K/Akt/GSK3β Pathway in N2a/APP695swe Cells.” *Oncotarget* 8 (15): 24314–26. doi:10.18632/oncotarget.15149.

Kang, Sang Gyun, Chiye Kim, Judd Aiken, Han Sang Yoo, and Debbie McKenzie. 2018. “Dual MicroRNA to Cellular Prion Protein Inhibits Propagation of Pathogenic Prion Protein in Cultured Cells.” *Molecular Neurobiology* 55 (3): 2384–96. doi:10.1007/s12035-017-0495-5.

Kang, Sang Gyun, Yu Mi Roh, Agnes Lau, David Westaway, Debbie McKenzie, Judd Aiken, Yong Sun Kim, and Han Sang Yoo. 2011. “Establishment and Characterization of Prnp Knock-down Neuroblastoma Cells Using Dual MicroRNA-Mediated RNA Interference.” *Prion* 5 (2): 93–102. doi:10.4161/pri.5.2.15621.

Karran, Eric, Marc Mercken, and Bart De Strooper. 2011. “The Amyloid Cascade Hypothesis for Alzheimer’s Disease: An Appraisal for the Development of Therapeutics.” *Nature Reviews Drug Discovery* 10 (9): 698–712. doi:10.1038/nrd3505.

Kayama, Y, and Y Koyama. 2003. “Control of Sleep and Wakefulness by Brainstem Monoaminergic and Cholinergic Neurons.” *Acta Neurochirurgica. Supplement* 87: 3–6.

Keam, Simon, and Gyorgy Hutvagner. 2015. “TRNA-Derived Fragments (TRFs): Emerging New Roles for an Ancient RNA in the Regulation of Gene Expression.” *Life* 5 (4): 1638–51. doi:10.3390/life5041638.

Kiko, Takehiro, Kiyotaka Nakagawa, Tsuyoshi Tsuduki, Katsutoshi Furukawa, Hiroyuki Arai, and Teruo Miyazawa. 2014. “MicroRNAs in Plasma and Cerebrospinal Fluid as Potential Markers for Alzheimer’s Disease.” *Journal of Alzheimer’s Disease* 39 (2): 253–59. doi:10.3233/JAD-130932.

Kim, S. J. 2002. “Cotranslational Partitioning of Nascent Prion Protein into Multiple Populations at the Translocation Channel.” *Molecular Biology of the Cell* 13 (11): 3775–86. doi:10.1091/mbc.E02-05-0293.

Kim, Young-Kook, Boseon Kim, and V Narry Kim. 2016. “Re-Evaluation of the Roles of DROSHA, Exportin 5, and DICER in MicroRNA Biogenesis.” *Proceedings of the National Academy of Sciences of the United States of America* 113 (13): E1881-1889. doi:10.1073/pnas.1602532113.

Kimura, Kimitoshi, Hirohiko Hohjoh, Masashi Fukuoka, Wakiro Sato, Shinji Oki, Chiharu Tomi, Hiromi Yamaguchi, Takayuki Kondo, Ryosuke Takahashi, and Takashi Yamamura. 2018. "Circulating Exosomes Suppress the Induction of Regulatory T Cells via Let-7i in Multiple Sclerosis." *Nature Communications* 9 (1). doi:10.1038/s41467-017-02406-2.

Kobayashi, Mariko, Corey Anderson, Corinne Benakis, Michael J Moore, Aldo Mele, John J Fak, Christopher Y Park, et al. 2018. "A Master Regulatory Network Restoring Brain Glutamate Homeostasis Is Coordinately Activated in Stroke." *BioRxiv*. doi:10.1101/245928.

Koshiol, Jill, Ena Wang, Yingdong Zhao, Francesco Marincola, and Maria Teresa Landi. 2010. "Strengths and Limitations of Laboratory Procedures for MicroRNA Detection." *Cancer Epidemiology, Biomarkers & Prevention : A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology* 19 (4): 907–11. doi:10.1158/1055-9965.EPI-10-0071.

Kovacs, G G, and H Budka. 2002. "Aging, the Brain and Human Prion Disease." *Exp Gerontol* 37 (4): 603–5. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11830364.

Kovacs, Gabor G. 2017. "Tauopathies." *Handbook of Clinical Neurology* 145: 355–68. doi:10.1016/B978-0-12-802395-2.00025-0.

Kovacs, Gabor G., and Herbert Budka. 2008. "Prion Diseases: From Protein to Cell Pathology." *American Journal of Pathology*. doi:10.2353/ajpath.2008.070442.

———. 2010. "Distribution of Apoptosis-Related Proteins in Sporadic Creutzfeldt-Jakob Disease." *Brain Research* 1323: 192–99. doi:10.1016/j.brainres.2010.01.089.

Kovács, Gábor G., Maria Puopolo, Anna Ladogana, Maurizio Pocchiari, Herbert Budka, Cornelia van Duijn, Steven J. Collins, et al. 2005. "Genetic Prion Disease: The EUROCJD Experience." *Human Genetics* 118 (2): 166–74. doi:10.1007/s00439-005-0020-1.

Kovács, Gábor G., Gianriccardo Trabattoni, Johannes A. Hainfellner, James W. Ironside, Richard S G Knight, and Herbert Budka. 2002. "Mutations of the Prion Protein Gene: Phenotypic Spectrum." *Journal of Neurology* 249 (11): 1567–82. doi:10.1007/s00415-002-0896-9.

Kovács, Gábor G, Ognian Kalev, and Herbert Budka. 2004. "Contribution of Neuropathology to the Understanding of Human Prion Disease." *Folia Neuropathol* 42 Suppl A: 69–76.

Krasnianski, Anna, Mario Bartl, Pascual J. Sanchez Juan, Uta Heinemann, Bettina Meissner, Daniela Varges, Ulf Schulze-Sturm, Haus A. Kretzschmar, Walter J. Schulz-Schaeffer, and Inga Zerr. 2008. "Fatal Familial Insomnia: Clinical Features and Early Identification." *Annals of Neurology* 63 (5): 658–61. doi:10.1002/ana.21358.

Krützfeldt, Jan, Matthew N. Poy, and Markus Stoffel. 2006. "Strategies to Determine the Biological Function of Micrornas." *Nature Genetics* 38 (6S): S14. doi:10.1038/ng1799.

Kumar, Subodh, and P. Hemachandra Reddy. 2016. "Are Circulating MicroRNAs Peripheral Biomarkers for Alzheimer's Disease?" *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 1862 (9): 1617–27. doi:10.1016/j.bbadis.2016.06.001.

Ladogana, Anna, Pascual Sanchez-Juan, Eva Mitrová, Alison Green, Natividad Cuadrado-Corrales, Raquel Sánchez-Valle, Silvia Koscova, et al. 2009. "Cerebrospinal Fluid Biomarkers

in Human Genetic Transmissible Spongiform Encephalopathies." *Journal of Neurology* 256 (10): 1620–28. doi:10.1007/s00415-009-5163-x.

Lam, Benjamin, Mario Masellis, Morris Freedman, Donald T. Stuss, and Sandra E. Black. 2013. "Clinical, Imaging, and Pathological Heterogeneity of the Alzheimer's Disease Syndrome." *Alzheimer's Research and Therapy* 5 (1). doi:10.1186/alzrt155.

Lattanzio, Francesca, Samir Abu-Rumeileh, Alessia Franceschini, Hideaki Kai, Giulia Amore, Ilaria Poggiolini, Marcello Rossi, et al. 2017. "Prion-Specific and Surrogate CSF Biomarkers in Creutzfeldt-Jakob Disease: Diagnostic Accuracy in Relation to Molecular Subtypes and Analysis of Neuropathological Correlates of p-Tau and Aβ42 Levels." *Acta Neuropathologica* 133 (4): 559–78. doi:10.1007/s00401-017-1683-0.

Lau, Pierre, Koen Bossers, Rekin's Janky, Evgenia Salta, Carlo Sala Frigerio, Shahar Barbash, Roy Rothman, et al. 2013. "Alteration of the MicroRNA Network during the Progression of Alzheimer's Disease." *EMBO Molecular Medicine* 5 (10): 1613–34. doi:10.1002/emmm.201201974.

Lee, Hyoung Gon, Gemma Casadesus, Xiongwei Zhu, Atsushi Takeda, George Perry, and Mark A. Smith. 2004. "Challenging the Amyloid Cascade Hypothesis: Senile Plaques and Amyloid-β as Protective Adaptations to Alzheimer Disease." *Annals of the New York Academy of Sciences* 1019: 1–4. doi:10.1196/annals.1297.001.

Lee, Seungjoo, Lizamma Antony, Rune Hartmann, Karen J. Knaus, Krystyna Surewicz, Witold K. Surewicz, and Vivien C. Yee. 2010. "Conformational Diversity in Prion Protein Variants Influences Intermolecular ??-Sheet Formation." *EMBO Journal* 29 (1): 251–62. doi:10.1038/emboj.2009.333.

Lee, Soon-Tae, Wooseok Im, Jae-Jun Ban, Mijung Lee, Keun-Hwa Jung, Sang Kun Lee, Kon Chu, and Manho Kim. 2017. "Exosome-Based Delivery of MiR-124 in a Huntington's Disease Model." *Journal of Movement Disorders*. doi:10.14802/jmd.16054.

Leidinger, Petra, Christina Backes, Stephanie Deutscher, Katja Schmitt, Sabine C. Mueller, Karen Frese, Jan Haas, et al. 2013. "A Blood Based 12-MiRNA Signature of Alzheimer Disease Patients." *Genome Biology* 14 (7). doi:10.1186/gb-2013-14-7-r78.

Leung, A. K. L., J. M. Calabrese, and P. A. Sharp. 2006. "Quantitative Analysis of Argonaute Protein Reveals MicroRNA-Dependent Localization to Stress Granules." *Proceedings of the National Academy of Sciences* 103 (48): 18125–30. doi:10.1073/pnas.0608845103.

Leung, Anthony K.L. 2015. "The Whereabouts of MicroRNA Actions: Cytoplasm and Beyond." *Trends in Cell Biology* 25 (10): 601–10. doi:10.1016/j.tcb.2015.07.005.

Lewis, Jada, Eileen McGowan, Julia Rockwood, Heather Melrose, Parimala Nacharaju, Marjon Van Slegtenhorst, Katrina Gwinn-Hardy, et al. 2000. "Neurofibrillary Tangles, Amyotrophy and Progressive Motor Disturbance in Mice Expressing Mutant (P301L)Tau Protein." *Nature Genetics* 25 (4): 402–5. doi:10.1038/78078.

Li, Yuan Yuan, Jian Guo Cui, Prerna Dua, Aileen I. Pogue, Surjyadipta Bhattacharjee, and Walter J. Lukiw. 2011. "Differential Expression of MiRNA-146a-Regulated Inflammatory Genes in Human Primary Neural, Astroglial and Microglial Cells." *Neuroscience Letters* 499 (2): 109–13. doi:10.1016/j.neulet.2011.05.044.

Lim, Lee P., Nelson C. Lau, Philip Garrett-Engele, Andrew Grimson, Janell M. Schelter, John Castle, David P. Bartel, Peter S. Linsley, and Jason M. Johnson. 2005. “Microarray Analysis Shows That Some MicroRNAs Downregulate Large Numbers of Target MRNAs.” *Nature* 433 (7027): 769–73. doi:10.1038/nature03315.

Lin, Jin, Jing Li, Bo Huang, Jing Liu, Xin Chen, Xi-Min Chen, Yan-Mei Xu, Lin-Feng Huang, and Xiao-Zhong Wang. 2015. “Exosomes: Novel Biomarkers for Clinical Diagnosis.” *The Scientific World Journal* 2015: 1–8. doi:10.1155/2015/657086.

Linden, Rafael. 2017. “The Biological Function of the Prion Protein: A Cell Surface Scaffold of Signaling Modules.” *Frontiers in Molecular Neuroscience* 10. doi:10.3389/fnmol.2017.00077.

Ling, Tian You, Xiao Li Wang, Qiang Chai, Tong Lu, John M. Stulak, Lyle D. Joyce, Richard C. Daly, et al. 2017. “Regulation of Cardiac CACNB2 by MicroRNA-499: Potential Role in Atrial Fibrillation.” *BBA Clinical*. doi:10.1016/j.bbacli.2017.02.002.

Liu, Fei, and Cheng Xin Gong. 2008. “Tau Exon 10 Alternative Splicing and Tauopathies.” *Molecular Neurodegeneration* 3 (1). doi:10.1186/1750-1326-3-8.

Llorens, F., B. Ansoleaga, P. Garcia-Esparcia, S. Zafar, O. Grau-Rivera, I. López-González, R. Blanco, et al. 2013. “PrP MRNa and Protein Expression in Brain and PrP^{c} in CSF in Creutzfeldt-Jakob Disease MM1 and VV2.” *Prion* 7 (5). doi:10.4161/pri.26416.

Llorens, Franc, Niels Kruse, André Karch, Matthias Schmitz, Saima Zafar, Nadine Gotzmann, Ting Sun, et al. 2018. “Validation of α-Synuclein as a CSF Biomarker for Sporadic Creutzfeldt-Jakob Disease.” *Molecular Neurobiology* 55 (3): 2249–57. doi:10.1007/s12035-017-0479-5.

Llorens, Franc, Irene Lopez-Gonzalez, Katrin Thune, Margarita Carmona, Saima Zafar, Olivier Andeoletti, Inga Zerr, and Isidre Ferrer. 2014. “Subtype and Regional-Specific Neuroinflammation in Sporadic Creutzfeldt-Jakob Disease.” *Frontiers in Aging Neuroscience* 6 (JUL). doi:10.3389/fnagi.2014.00198.

Llorens, Franc, Matthias Schmitz, André Karch, Maria Cramm, Peter Lange, Kerim Gherib, Daniela Varges, Christian Schmidt, Inga Zerr, and Katharina Stoeck. 2016. “Comparative Analysis of Cerebrospinal Fluid Biomarkers in the Differential Diagnosis of Neurodegenerative Dementia.” *Alzheimer’s and Dementia* 12 (5): 577–89. doi:10.1016/j.jalz.2015.10.009.

Llorens, Franc, Matthias Schmitz, Tobias Knipper, Christian Schmidt, Peter Lange, Andre Fischer, Peter Hermann, and Inga Zerr. 2017. “Cerebrospinal Fluid Biomarkers of Alzheimer’s Disease Show Different but Partially Overlapping Profile Compared to Vascular Dementia.” *Frontiers in Aging Neuroscience* 9 (SEP). doi:10.3389/fnagi.2017.00289.

Llorens, Franc, Katrin Thüne, Matthias Schmitz, Belén Ansoleaga, Margalida A. Frau-Méndez, Maria Cramm, Waqas Tahir, et al. 2016. “Identification of New Molecular Alterations in Fatal Familial Insomnia.” *Human Molecular Genetics*, ddw108. doi:10.1093/hmg/ddw108.

Llorens, Franc, Katrin Thüne, Beata Sikorska, Matthias Schmitz, Waqas Tahir, Natalia Fernández-Borges, Maria Cramm, et al. 2017. “Altered Ca2+ Homeostasis Induces Calpain-Cathepsin Axis Activation in Sporadic Creutzfeldt-Jakob Disease.” *Acta Neuropathologica Communications* 5 (1): 35. doi:10.1186/s40478-017-0431-y.

Llorens, Franc, Katrin Thüne, Waqas Tahir, Eirini Kanata, Daniela Diaz-Lucena, Konstantinos Xanthopoulos, Eleni Kovatsi, et al. 2017. “YKL-40 in the Brain and Cerebrospinal Fluid of

Neurodegenerative Dementias." *Molecular Neurodegeneration* 12 (1). doi:10.1186/s13024-017-0226-4.

Llorens, Franc, Saima Zafar, Belén Ansoleaga, Mohsin Shafiq, Rosi Blanco, Marga Carmona, Oriol Grau-Rivera, et al. 2015. "Subtype and Regional Regulation of Prion Biomarkers in Sporadic Creutzfeldt-Jakob Disease." *Neuropathology and Applied Neurobiology* 41 (5): 631–45. doi:10.1111/nan.12175.

Llorens, Franc, Juan José Zarranz, Andre Fischer, Inga Zerr, and Isidro Ferrer. 2017. "Fatal Familial Insomnia: Clinical Aspects and Molecular Alterations." *Current Neurology and Neuroscience Reports* 17 (4). doi:10.1007/s11910-017-0743-0.

Londin, Eric, Phillipe Loher, Aristeidis G. Telonis, Kevin Quann, Peter Clark, Yi Jing, Eleftheria Hatzimichael, et al. 2015. "Analysis of 13 Cell Types Reveals Evidence for the Expression of Numerous Novel Primate- and Tissue-Specific MicroRNAs." *Proceedings of the National Academy of Sciences*. doi:10.1073/pnas.1420955112.

Lugli, Giovanni, Aaron M. Cohen, David A. Bennett, Raj C. Shah, Christopher J. Fields, Alvaro G. Hernandez, and Neil R. Smalheiser. 2015. "Plasma Exosomal MiRNAs in Persons with and without Alzheimer Disease: Altered Expression and Prospects for Biomarkers." *PLoS ONE* 10 (10). doi:10.1371/journal.pone.0139233.

Lukiw, W. J., P. Dua, A. I. Pogue, C. Eicken, and J. M. Hill. 2011. "Upregulation of Micro RNA-146a (MiRNA-146a), a Marker for Inflammatory Neurodegeneration, in Sporadic Creutzfeldt-Jakob Disease (SCJD) and Gerstmann-Straussler-Scheinker (GSS) Syndrome." *Journal of Toxicology and Environmental Health - Part A: Current Issues* 74 (22–24): 1460–68. doi:10.1080/15287394.2011.618973.

Lukiw, W J. 2012. "NF-Kappa B-Regulated, Proinflammatory MiRNAs in Alzheimer's Disease." *Alzheimers Research & Therapy* 4 (6). doi:Artn 47\nDoi 10.1186/Alzrt150.

Lukiw, Walter J., Tatiana V. Andreeva, Anastasia P. Grigorenko, and Evgeny I. Rogaev. 2013. "Studying Micro RNA Function and Dysfunction in Alzheimer's Disease." *Frontiers in Genetics* 3 (FEB). doi:10.3389/fgene.2012.00327.

Ma, Wenbin, Yingying Li, Chao Wang, Fan Xu, Meiling Wang, and Yiming Liu. 2016. "Serum MiR-221 Serves as a Biomarker for Parkinson's Disease." *Cell Biochemistry and Function* 34 (7): 511–15. doi:10.1002/cbf.3224.

Mair, Waltraud, Jan Muntel, Katharina Tepper, Shaojun Tang, Jacek Biernat, William W. Seeley, Kenneth S. Kosik, Eckhard Mandelkow, Hanno Steen, and Judith A. Steen. 2016. "FLEXITau: Quantifying Post-Translational Modifications of Tau Protein in Vitro and in Human Disease." *Analytical Chemistry* 88 (7): 3704–14. doi:10.1021/acs.analchem.5b04509.

Majer, Anna, Sarah J. Medina, Yulian Niu, Bernard Abrenica, Kathy J. Manguiat, Kathy L. Frost, Clark S. Philipson, Debra L. Sorensen, and Stephanie A. Booth. 2012. "Early Mechanisms of Pathobiology Are Revealed by Transcriptional Temporal Dynamics in Hippocampal CA1 Neurons of Prion Infected Mice." *PLoS Pathogens* 8 (11). doi:10.1371/journal.ppat.1003002.

Maoz, Rotem, Benjamin P. Garfinkel, and Hermona Soreq. 2017. "Alzheimer's Disease and NcRNAs." *Advances in Experimental Medicine and Biology* 978: 337–61. doi:10.1007/978-3-319-53889-1_18.

Mario, Agnese De, Caterina Peggion, Maria Lina Massimino, Francesca Viviani, Angela Castellani, Marta Giacomello, Dmitry Lim, Alessandro Bertoli, and Maria Catia Sorgato. 2017. “The Prion Protein Regulates Glutamate-Mediated Ca(2+) Entry and Mitochondrial Ca(2+) Accumulation in Neurons.” *Journal of Cell Science* 130 (16): 2736–46. doi:10.1242/jcs.196972.

Mastrianni, James A. 2010. “The Genetics of Prion Diseases.” *Genetics in Medicine* 12 (4): 187–95. doi:10.1097/GIM.0b013e3181cd7374.

Maurel, Marion, and Eric Chevet. 2013. “Endoplasmic Reticulum Stress Signaling: The MicroRNA Connection.” *American Journal of Physiology. Cell Physiology* 304 (12): C1117-26. doi:10.1152/ajpcell.00061.2013.

McKeever, P M, R Schneider, F Taghdiri, A Weichert, N Multani, R A Brown, A L Boxer, et al. 2018. “MicroRNA Expression Levels Are Altered in the Cerebrospinal Fluid of Patients with Young-Onset Alzheimer’s Disease.” *Mol Neurobiol*. doi:10.1007/s12035-018-1032-x.

McQuiston, Alexander, and J Alan Diehl. 2017. “Recent Insights into PERK-Dependent Signaling from the Stressed Endoplasmic Reticulum.” *F1000Research* 6: 1897. doi:10.12688/f1000research.12138.1.

Meister, Gunter. 2013a. “Argonaute Proteins: Functional Insights and Emerging Roles.” *Nature Reviews Genetics*. doi:10.1038/nrg3462.

Miura, Pedro, Sol Shenker, Celia Andreu-Agullo, Jakub O. Westholm, and Eric C. Lai. 2013. “Widespread and Extensive Lengthening of 39 UTRs in the Mammalian Brain.” *Genome Research* 23 (5): 812–25. doi:10.1101/gr.146886.112.

Mohan, A, K A Mather, A Thalamuthu, B T Baune, and P S Sachdev. 2016. “Gene Expression in the Aging Human Brain: An Overview.” *Current Opinion in Psychiatry* 29 (2): 159–67. doi:10.1097/YCO.0000000000000238.

Moldovan, Leni, Kara E. Batte, Joanne Trgovcich, Jon Wisler, Clay B. Marsh, and Melissa Piper. 2014. “Methodological Challenges in Utilizing MiRNAs as Circulating Biomarkers.” *Journal of Cellular and Molecular Medicine* 18 (3): 371–90. doi:10.1111/jcmm.12236.

Montag, Judith, Markus Brameier, Ann Christin Schmädicke, Sabine Gilch, Hermann M. Schätzl, and Dirk Motzkus. 2012. “A Genome-Wide Survey for Prion-Regulated MiRNAs Associated with Cholesterol Homeostasis.” *BMC Genomics* 13 (1). doi:10.1186/1471-2164-13-486.

Montag, Judith, Reiner Hitt, Lennart Opitz, Walter J. Schulz-Schaeffer, Gerhard Hunsmann, and Dirk Motzkus. 2009. “Upregulation of MiRNA Hsa-MiR-342-3p in Experimental and Idiopathic Prion Disease.” *Molecular Neurodegeneration* 4 (1). doi:10.1186/1750-1326-4-36.

Morales, Rodrigo. 2017. “Prion Strains in Mammals: Different Conformations Leading to Disease.” *PLoS Pathogens* 13 (7). doi:10.1371/journal.ppat.1006323.

Morales, Rodrigo, Karim Abid, and Claudio Soto. 2007. “The Prion Strain Phenomenon: Molecular Basis and Unprecedented Features.” *Biochimica et Biophysica Acta - Molecular Basis of Disease* 1772 (6): 681–91. doi:10.1016/j.bbadis.2006.12.006.

Mravec, Boris, Katarina Lejavova, and Veronika Cubinkova. 2014. “Locus (Coeruleus) Minoris Resistentiae in Pathogenesis of Alzheimer’s Disease.” *Current Alzheimer Research* 11 (10): 992–1001. doi:10.2174/1567205011666141107130505.

Mucke, Lennart. 2009. “Alzheimer’s Disease.” *Nature* 461 (15): 895–97. doi:10.1038/461895a.

Müller, Mareike, H. Bea Kuiperij, Jurgen A. Claassen, Benno Küsters, and Marcel M. Verbeek. 2014. “MicroRNAs in Alzheimer’s Disease: Differential Expression in Hippocampus and Cell-Free Cerebrospinal Fluid.” *Neurobiology of Aging* 35 (1): 152–58. doi:10.1016/j.neurobiolaging.2013.07.005.

Murayama, Shigeo, and Yuko Saito. 2004. “Neuropathological Diagnostic Criteria for Alzheimer’s Disease.” *Neuropathology* 24 (3): 254–60. doi:10.1111/j.1440-1789.2004.00571.x.

Nagai, T., K. Satoh, K. Imamoto, and T. Maeda. 1981. “Divergent Projections of Catecholamine Neurons of the Locus Coeruleus as Revealed by Fluorescent Retrograde Double Labeling Technique.” *Neuroscience Letters* 23 (2): 117–23. doi:10.1016/0304-3940(81)90027-6.

Nagaraj, Siranjeevi, Katarzyna Laskowska-Kaszub, Konrad J. Dębski, Joanna Wojsiat, Michał Dąbrowski, Tomasz Gabryelewicz, Jacek Kuźnicki, and Urszula Wojda. 2017. “Profile of 6 MicroRNA in Blood Plasma Distinguish Early Stage Alzheimer’s Disease Patients from Non-Demented Subjects.” *Oncotarget*. doi:10.18632/oncotarget.15109.

Natera-Naranjo, Orlangie, Armaz Aschrafi, Anthony E. Gioio, and Barry B. Kaplan. 2010. “Identification and Quantitative Analyses of MicroRNAs Located in the Distal Axons of Sympathetic Neurons.” *RNA* 16 (8): 1516–29. doi:10.1261/rna.1833310.

Naumova, Oksana Yu, Maria Lee, Sergei Yu Rychkov, Natalia V. Vlasova, and Elena L. Grigorenko. 2013. “Gene Expression in the Human Brain: The Current State of the Study of Specificity and Spatiotemporal Dynamics.” *Child Development* 84 (1): 76–88. doi:10.1111/cdev.12014.

Neo, Wen Hao, Karen Yap, Suet Hoay Lee, Liang Sheng Looi, Piyush Khandelia, Sheng Xiong Neo, Eugene V. Makeyev, and I. Hsin Su. 2014. “MicroRNA MiR-124 Controls the Choice between Neuronal and Astrocyte Differentiation by Fine-Tuning Ezh2 Expression.” *Journal of Biological Chemistry* 289 (30): 20788–801. doi:10.1074/jbc.M113.525493.

Ng, Enders K. O., Rufina Li, Vivian Y. Shin, Hong Chuan Jin, Candy P. H. Leung, Edmond S. K. Ma, Roberta Pang, et al. 2013. “Circulating MicroRNAs as Specific Biomarkers for Breast Cancer Detection.” *PLoS ONE* 8 (1): e53141. doi:10.1371/journal.pone.0053141.

O’Carroll, Dónal, and Anne Schaefer. 2013a. “General Principals of MiRNA Biogenesis and Regulation in the Brain.” *Neuropsychopharmacology* 38 (1): 39–54. doi:10.1038/npp.2012.87.

Oakley, H., S. L. Cole, S. Logan, E. Maus, P. Shao, J. Craft, A. Guillozet-Bongaarts, et al. 2006. “Intraneuronal Beta-Amyloid Aggregates, Neurodegeneration, and Neuron Loss in Transgenic Mice with Five Familial Alzheimer’s Disease Mutations: Potential Factors in Amyloid Plaque Formation.” *Journal of Neuroscience* 26 (40): 10129–40. doi:10.1523/JNEUROSCI.1202-06.2006.

Ofori, Jones K., Vishal A. Salunkhe, Annika Bagge, Neelanjan Vishnu, Mototsugu Nagao, Hindrik Mulder, Claes B. Wollheim, Lena Eliasson, and Jonathan L.S. Esguerra. 2017. “Elevated MiR-130a/MiR130b/MiR-152 Expression Reduces Intracellular ATP Levels in the Pancreatic Beta Cell.” *Scientific Reports*. doi:10.1038/srep44986.

Olejniczak, Marta, Anna Kotowska-Zimmer, and Wlodzimierz Krzyzosiak. 2018. “Stress-Induced Changes in MiRNA Biogenesis and Functioning.” *Cellular and Molecular Life Sciences* 75 (2): 177–91. doi:10.1007/s00018-017-2591-0.

Orr, Miranda E., A. Campbell Sullivan, and Bess Frost. 2017. “A Brief Overview of Tauopathy: Causes, Consequences, and Therapeutic Strategies.” *Trends in Pharmacological Sciences* 38 (7): 637–48. doi:10.1016/j.tips.2017.03.011.

Ouellet, Dominique L., Marjorie P. Perron, Lise Andrée Gobeil, Pierre Plante, and Patrick Provost. 2006. “MicroRNAs in Gene Regulation: When the Smallest Governs It All.” *Journal of Biomedicine and Biotechnology* 2006. doi:10.1155/JBB/2006/69616.

Padilla, Danielle, Vincent Béringue, Juan Carlos Espinosa, Olivier Andreoletti, Emilie Jaumain, Fabienne Reine, Laetitia Herzog, et al. 2011. “Sheep and Goat BSE Propagate More Efficiently than Cattle BSE in Human PrP Transgenic Mice.” *PLoS Pathogens* 7 (3). doi:10.1371/journal.ppat.1001319.

Pantano, Lorena, Xavier Estivill, and Eulàlia Martí. 2009. “SeqBuster, a Bioinformatic Tool for the Processing and Analysis of Small RNAs Datasets, Reveals Ubiquitous MiRNA Modifications in Human Embryonic Cells.” *Nucleic Acids Research* 38 (5). doi:10.1093/nar/gkp1127.

Parchi, Piero, Laura De Boni, Daniela Saverioni, Mark L. Cohen, Isidro Ferrer, Pierluigi Gambetti, Ellen Gelpi, et al. 2012. “Consensus Classification of Human Prion Disease Histotypes Allows Reliable Identification of Molecular Subtypes: An Inter-Rater Study among Surveillance Centres in Europe and USA.” *Acta Neuropathologica* 124 (4): 517–29. doi:10.1007/s00401-012-1002-8.

Parchi, Piero, Armin Giese, Sabina Capellari, Paul Brown, Walter Schulz-Schaeffer, Otto Windl, Inga Zerr, et al. 1999. “Classification of Sporadic Creutzfeldt-Jakob Disease Based on Molecular and Phenotypic Analysis of 300 Subjects.” *Ann Neurol* 46: 224–33. doi:10.1002/1531-8249(199908)46:2<224::AID-ANA12>3.0.CO;2-W.

Parchi, Piero, Rosaria Strammiello, Silvio Notari, Armin Giese, Jan P.M. Langeveld, Anna Ladogana, Inga Zerr, et al. 2009. “Incidence and Spectrum of Sporadic Creutzfeldt-Jakob Disease Variants with Mixed Phenotype and Co-Occurrence of PrPSctypes: An Updated Classification.” *Acta Neuropathologica*. doi:10.1007/s00401-009-0585-1.

Pare, J. M., N. Tahbaz, J. Lopez-Orozco, P. LaPointe, P. Lasko, and T. C. Hobman. 2009. “Hsp90 Regulates the Function of Argonaute 2 and Its Recruitment to Stress Granules and P-Bodies.” *Molecular Biology of the Cell* 20 (14): 3273–84. doi:10.1091/mbc.E09-01-0082.

Parsi, Sepideh, Pascal Y. Smith, Claudia Goupil, Véronique Dorval, and Sébastien S. Hébert. 2015. “Preclinical Evaluation of MiR-15/107 Family Members as Multifactorial Drug Targets for Alzheimer’s Disease.” *Molecular Therapy - Nucleic Acids* 4. doi:10.1038/mtna.2015.33.

Paterson, Ross W., Charles C. Torres-Chae, Amy L. Kuo, Tim Ando, Elizabeth A. Nguyen, Katherine Wong, Stephen J. DeArmond, et al. 2012. “Differential Diagnosis of Jakob-Creutzfeldt Disease.” *Archives of Neurology* 69 (12): 1578–82. doi:10.1001/2013.jamaneurol.79.

Pereira, Patrícia A., Joana F. Tomás, João A. Queiroz, Ana R. Figueiras, and Fani Sousa. 2016. “Recombinant Pre-MiR-29b for Alzheimer’s Disease Therapeutics.” *Scientific Reports*. doi:10.1038/srep19946.

Petralia, Ronald S., Mark P. Mattson, and Pamela J. Yao. 2014. “Communication Breakdown: The Impact of Ageing on Synapse Structure.” *Ageing Research Reviews* 14 (1): 31–42.

doi:10.1016/j.arr.2014.01.003.

Pfeifer, Alexander, Sabina Eigenbrod, Saba Al-Khadra, Andreas Hofmann, Gerda Mitteregger, Markus Moser, Uwe Bertsch, and Hans Kretzschmar. 2006. “Lentivector-Mediated RNAi Efficiently Suppresses Prion Protein and Prolongs Survival of Scrapie-Infected Mice.” *Journal of Clinical Investigation* 116 (12): 3204–10. doi:10.1172/JCI29236.

Piccardo, P., J. C. Manson, D. King, B. Ghetti, and R. M. Barron. 2007. “Accumulation of Prion Protein in the Brain That Is Not Associated with Transmissible Disease.” *Proceedings of the National Academy of Sciences* 104 (11): 4712–17. doi:10.1073/pnas.0609241104.

Pinzï¿½n, Natalia, Blaise Li, Laura Martinez, Anna Sergeeva, Jessy Presumey, Florence Apparailly, and Hervï¿½ Seitz. 2017. “MicroRNA Target Prediction Programs Predict Many False Positives.” *Genome Research* 27 (2): 234–45. doi:10.1101/gr.205146.116.

Porta, Sílvia, Linda K. Kwong, John Q. Trojanowski, and Virginia M.Y. Lee. 2015. “Drosha Inclusions Are New Components of Dipeptide-Repeat Protein Aggregates in FTLD-TDP and ALS C9orf72 Expansion Cases.” *Journal of Neuropathology and Experimental Neurology* 74 (4): 380–87. doi:10.1097/NEN.0000000000000182.

Prada, Ilaria, Martina Gabrielli, Elena Turola, Alessia Iorio, Giulia D’Arrigo, Roberta Parolisi, Mariacristina De Luca, et al. 2018. “Glia-to-Neuron Transfer of MiRNAs via Extracellular Vesicles: A New Mechanism Underlying Inflammation-Induced Synaptic Alterations.” *Acta Neuropathologica* 135 (4): 529–50. doi:10.1007/s00401-017-1803-x.

Prado, Marco A. M., Juliana Alves-Silva, Ana C. Magalhães, Vania F. Prado, Rafael Linden, Vilma Regina Martins, and Ricardo Renzo Brentani. 2004. “PrPc on the Road: Trafficking of the Cellular Prion Protein.” *Journal of Neurochemistry* 88 (4): 769–81. doi:10.1046/j.1471-4159.2003.02199.x.

Prince, Martin, Anders Wimo, Maëlenn Guerchet, Ali Gemma-Claire, Yu-Tzu Wu, and Matthew Prina. 2015. “World Alzheimer Report 2015: The Global Impact of Dementia - An Analysis of Prevalence, Incidence, Cost and Trends.” *Alzheimer’s Disease International*, 84. doi:10.1111/j.0963-7214.2004.00293.x.

Pritchard, Colin C., Heather H. Cheng, and Muneesh Tewari. 2012. “MicroRNA Profiling: Approaches and Considerations.” *Nature Reviews Genetics* 13 (5): 358–69. doi:10.1038/nrg3198.

Prusiner, Stanley B. 1998. “Prions.” *Proceedings of the National Academy of Sciences* 95 (23): 13363–83. doi:10.1073/pnas.95.23.13363.

Puoti, Gianfranco, Alberto Bizzi, Gianluigi Forloni, Jiri G. Safar, Fabrizio Tagliavini, and Pierluigi Gambetti. 2012. “Sporadic Human Prion Diseases: Molecular Insights and Diagnosis.” *The Lancet Neurology* 11 (7): 618–28. doi:10.1016/S1474-4422(12)70063-7.

Puoti, Gianfranco, Giorgio Giaccone, Michela Mangieri, Lucia Limido, Paolo Fociani, Pietro Zerbi, Silvia Suardi, et al. 2005. “Sporadic Creutzfeldt-Jakob Disease: The Extent of Microglia Activation Is Dependent on the Biochemical Type of PrPSc.” *Journal of Neuropathology and Experimental Neurology* 64 (10): 902–9. doi:10.1097/01.jnen.0000183346.19447.55.

Putteeraj, Manish, Mohamad Fairuz Yahaya, and Seong Lin Teoh. 2017. “MicroRNA Dysregulation in Alzheimer’s Disease.” *CNS & Neurological Disorders Drug Targets* 16.

doi:10.2174/1871527316666170807142311.

Ramanan, Vijay K, and Andrew J Saykin. 2013. “Pathways to Neurodegeneration: Mechanistic Insights from GWAS in Alzheimer’s Disease, Parkinson’s Disease, and Related Disorders.” *Am J Neurodegener Dis* 2 (3): 145–75. www.AJND.us.

Reas, Emilie T. 2017. “Amyloid and Tau Pathology in Normal Cognitive Aging.” *J Neurosci* 37 (32): 7561–7563. doi:10.1523/JNEUROSCI.1388-17.2017.

Reddy, P. Hemachandra, Sahil Tonk, Subodh Kumar, Murali Vijayan, Ramesh Kandimalla, Chandra Sekhar Kuruva, and Arubala P. Reddy. 2017. “A Critical Evaluation of Neuroprotective and Neurodegenerative MicroRNAs in Alzheimer’s Disease.” *Biochemical and Biophysical Research Communications* 483 (4): 1156–65. doi:10.1016/j.bbrc.2016.08.067.

Reitz, Christiane. 2012. “Alzheimer’s Disease and the Amyloid Cascade Hypothesis: A Critical Review.” *International Journal of Alzheimer’s Disease*. doi:10.1155/2012/369808.

Ridolfi, Barbara, and Hanin Abdel-Haq. 2018. “Neurodegenerative Disorders Treatment: The MicroRNA Role.” *Current Gene Therapy* 18. doi:10.2174/1566523218666180119120726.

Riffo-Campos, Ángela L., Ismael Riquelme, and Priscilla Brebi-Mieville. 2016. “Tools for Sequence-Based MiRNA Target Prediction: What to Choose?” *International Journal of Molecular Sciences* 17 (12). doi:10.3390/ijms17121987.

Roberts, Thomas C. 2014. “The MicroRNA Biology of the Mammalian Nucleus.” *Molecular Therapy - Nucleic Acids* 3. doi:10.1038/mtna.2014.40.

Rodrigue, K. M., K. M. Kennedy, M. D. Devous, J. R. Rieck, A. C. Hebrank, R. Diaz-Arrastia, D. Mathews, and D. C. Park. 2012. “β-Amyloid Burden in Healthy Aging: Regional Distribution and Cognitive Consequences.” *Neurology* 78 (6): 387–95. doi:10.1212/WNL.0b013e318245d295.

Rosenbloom, Michael Henry, and Alireza Atri. 2011. “The Evaluation of Rapidly Progressive Dementia.” *The Neurologist* 17 (2): 67–74. doi:10.1097/NRL.0b013e31820ba5e3.

Rubio, David Sanz, Óscar López-Pérez, Álvaro De Andrés Pablo, Rosa Bolea, Rosario Osta, Juan J. Badiola, Pilar Zaragoza, Inmaculada Martín-Burriel, and Janne M. Toivonen. 2017. “Increased Circulating MicroRNAs MiR-342-3p and MiR-21-5p in Natural Sheep Prion Disease.” *Journal of General Virology* 98 (2): 305–10. doi:10.1099/jgv.0.000685.

Saba, Reuben, Chelsey D. Goodman, Rhiannon L.C.H. Huzarewich, Catherine Robertson, and Stephanie A. Booth. 2008. “A MiRNA Signature of Prion Induced Neurodegeneration.” *PLoS ONE* 3 (11). doi:10.1371/journal.pone.0003652.

Salta, Evgenia, Annerieke Sierksma, Elke Vanden Eynden, and Bart De Strooper. 2016. “MiR-132 Loss De-represses ITPKB and Aggravates Amyloid and TAU Pathology in Alzheimer’s Brain.” *EMBO Molecular Medicine* 8 (9): 1005–18. doi:10.15252/emmm.201606520.

Salta, Evgenia, and Bart De Strooper. 2017. “MicroRNA-132: A Key Noncoding RNA Operating in the Cellular Phase of Alzheimer’s Disease.” *FASEB Journal* 31 (2): 424–33. doi:10.1096/fj.201601308.

Sambandan, Sivakumar, Gï¿½ney Akbalik, Lisa Kochen, Jennifer Rinne, Josefine Kahlstatt, Caspar Glock, Georgi Tushev, Beatriz Alvarez-Castelao, Alexander Heckel, and Erin M.

Schuman. 2017. “Activity-Dependent Spatially Localized MiRNA Maturation in Neuronal Dendrites.” *Science* 355 (6325): 634–37. doi:10.1126/science.aaf8995.

Sanchez-Juan, P., A. Green, A. Ladogana, N. Cuadrado-Corrales, R. Sáanchez-Valle, E. Mitrováa, K. Stoeck, et al. 2006. “CSF Tests in the Differential Diagnosis of Creutzfeldt-Jakob Disease.” *Neurology* 67 (4): 637–43. doi:10.1212/01.wnl.0000230159.67128.00.

Santa-Maria, Ismael, Maria E. Alaniz, Neil Renwick, Carolina Cela, Tudor A. Fulga, David Van Vactor, Thomas Tuschl, et al. 2015. “Dysregulation of MicroRNA-219 Promotes Neurodegeneration through Post-Transcriptional Regulation of Tau.” *Journal of Clinical Investigation* 125 (2): 681–86. doi:10.1172/JCI78421.

Santa-Maria, Ismael, Aya Haggiagi, Xinmin Liu, Jessica Wasserscheid, Peter T. Nelson, Ken Dewar, Lorraine N. Clark, and John F. Crary. 2012. “The MAPT H1 Haplotype Is Associated with Tangle-Predominant Dementia.” *Acta Neuropathologica* 124 (5): 693–704. doi:10.1007/s00401-012-1017-1.

Sara, Susan J. 2009. “The Locus Coeruleus and Noradrenergic Modulation of Cognition.” *Nature Reviews Neuroscience* 10 (3): 211–23. doi:10.1038/nrn2573.

Schanen, Brian C., and Xiaoman Li. 2011a. “Transcriptional Regulation of Mammalian MiRNA Genes.” *Genomics* 97 (1): 1–6. doi:10.1016/j.ygeno.2010.10.005.

Schmidt, Christian, Stephane Haïk, Katsuya Satoh, Alberto Rábano, Pablo Martinez-Martin, Sigrun Roeber, Jean-Philippe Brandel, et al. 2012. “Rapidly Progressive Alzheimer’s Disease: A Multicenter Update.” *Journal of Alzheimer’s Disease : JAD* 30 (4): 751–56. doi:10.3233/JAD-2012-120007.

Schmidt, Christian, Martin Wolff, Michael Weitz, Thomas Bartlau, Carsten Korth, and Inga Zerr. 2011. “Rapidly Progressive Alzheimer Disease.” *Archives of Neurology*. doi:10.1001/archneurol.2011.189.

Schneider, Raphael, Paul McKeever, TaeHyung Kim, Caroline Graff, John Cornelis van Swieten, Anna Karydas, Adam Boxer, et al. 2018. “Downregulation of Exosomal MiR-204-5p and MiR-632 as a Biomarker for FTD: A GENFI Study.” *Journal of Neurology, Neurosurgery & Psychiatry*, jnnp-2017-317492. doi:10.1136/jnnp-2017-317492.

Schoch, Gaby, Harald Seeger, Julien Bogousslavsky, Markus Tolnay, Robert Charles Janzer, Adriano Aguzzi, and Markus Glatzel. 2005. “Analysis of Prion Strains by PrPSc Profiling in Sporadic Creutzfeldt–Jakob Disease.” *PLoS Medicine* 3 (2): e14. doi:10.1371/journal.pmed.0030014.

Schonrock, Nicole, and Jürgen Götz. 2012. “Decoding the Non-Coding RNAs in Alzheimer’s Disease.” *Cellular and Molecular Life Sciences* 69 (21): 3543–59. doi:10.1007/s00018-012-1125-z.

Schratt, Gerhard. 2009. “MicroRNAs at the Synapse.” *Nature Reviews Neuroscience* 10 (12): 842–49. doi:10.1038/nrn2763.

Schwarzenbach, Heidi, Andreia Machado Da Silva, George Calin, and Klaus Pantel. 2015. “Data Normalization Strategies for MicroRNA Quantification.” *Clinical Chemistry* 61 (11): 1333–42. doi:10.1373/clinchem.2015.239459.

Scott, Hannah. 2017. “Extracellular MicroRNAs as Messengers in the Central and Peripheral

Nervous System." *Neuronal Signaling* 1 (4): NS20170112. doi:10.1042/NS20170112.

Seitz, Hervé. 2017. "Issues in Current MicroRNA Target Identification Methods." *RNA Biology* 14 (7): 831–34. doi:10.1080/15476286.2017.1320469.

Selkoe, Dennis J, and John Hardy. 2016. "The Amyloid Hypothesis of Alzheimer's Disease at 25 Years." *EMBO Molecular Medicine* 8 (6): 595–608. doi:10.15252/emmm.201606210.

Serrano-Pozo, Alberto, Matthew P. Frosch, Eliezer Masliah, and Bradley T. Hyman. 2011. "Neuropathological Alterations in Alzheimer Disease." *Cold Spring Harbor Perspectives in Medicine* 1 (1). doi:10.1101/cshperspect.a006189.

Sethi, Prerna, and Walter J. Lukiw. 2009. "Micro-RNA Abundance and Stability in Human Brain: Specific Alterations in Alzheimer's Disease Temporal Lobe Neocortex." *Neuroscience Letters* 459 (2): 100–104. doi:10.1016/j.neulet.2009.04.052.

Sheinerman, Kira S., Jon B. Toledo, Vladimir G. Tsivinsky, David Irwin, Murray Grossman, Daniel Weintraub, Howard I. Hurtig, et al. 2017. "Circulating Brain-Enriched MicroRNAs as Novel Biomarkers for Detection and Differentiation of Neurodegenerative Diseases." *Alzheimer's Research & Therapy* 9 (1): 89. doi:10.1186/s13195-017-0316-0.

Shigematsu, Megumi, Shozo Honda, and Yohei Kirino. 2014. "Transfer RNA as a Source of Small Functional RNA." *Journal of Molecular Biology and Molecular Imaging* 1 (2): 1–15. doi:10.1161/STROKEAHA.113.002813.Oxidative.

Shimada, Atsuyoshi, and Sanae Hasegawa-Ishii. 2011. "Senescence-Accelerated Mice (SAMs) as a Model for Brain Aging and Immunosenescence." *Aging and Disease* 2 (5): 414–35. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3295080&tool=pmcentrez&rendertype=abstract.

Sikorska, Beata, and Pawel P Liberski. 2012. "Human Prion Diseases: From Kuru to Variant Creutzfeldt-Jakob Disease." *Sub-Cellular Biochemistry* 65: 457–96. doi:10.1007/978-94-007-5416-4_17.

Silveira, Jay R., Gregory J. Raymond, Andrew G. Hughson, Richard E. Race, Valerie L. Sim, Stanley F. Hayes, and Byron Caughey. 2005. "The Most Infectious Prion Protein Particles." *Nature* 437 (7056): 257–61. doi:10.1038/nature03989.

Šimić, Goran, Mirjana Babić Leko, Selina Wray, Charles R. Harrington, Ivana Delalle, Nataša Jovanov-Milošević, Danira Bažadona, et al. 2017. "Monoaminergic Neuropathology in Alzheimer's Disease." *Progress in Neurobiology* 151: 101–38. doi:10.1016/j.pneurobio.2016.04.001.

Simoneau, Steve, Human Rezaei, Nicole Salès, Gunnar Kaiser-Schulz, Maxime Lefebvre-Roque, Catherine Vidal, Jean Guy Fournier, et al. 2007. "In Vitro and in Vivo Neurotoxicity of Prion Protein Oligomers." *PLoS Pathogens* 3 (8): 1175–86. doi:10.1371/journal.ppat.0030125.

Smith, Pascal, Amelle Al Hashimi, Johanne Girard, Charlotte Delay, and Sébastien S. Hébert. 2011. "In Vivo Regulation of Amyloid Precursor Protein Neuronal Splicing by MicroRNAs." *Journal of Neurochemistry* 116 (2): 240–47. doi:10.1111/j.1471-4159.2010.07097.x.

Smith, Pascal Y., Julia Hernandez-Rapp, Francis Jolivette, Cynthia Lecours, Kanchan Bisht, Claudia Goupil, Veronique Dorval, et al. 2015. "MiR-132/212 Deficiency Impairs Tau Metabolism and Promotes Pathological Aggregation in Vivo." *Human Molecular Genetics* 24

(23): 6721–35. doi:10.1093/hmg/ddv377.

Smith, Sinéad M., and David W. Murray. 2012. “An Overview of MicroRNA Methods: Expression Profiling and Target Identification.” *Methods in Molecular Biology* 823: 119–38. doi:10.1007/978-1-60327-216-2_9.

Snowden, Julie S., D. M A Mann, and D. Neary. 2002. “Distinct Neuropsychological Characteristics in Creutzfeldt-Jakob Disease.” *Journal of Neurology Neurosurgery and Psychiatry* 73 (6): 686–94. doi:10.1136/jnnp.73.6.686.

Sona, Alessandro, Kathryn A. Ellis, and David Ames. 2013. “Rapid Cognitive Decline in Alzheimer’s Disease: A Literature Review.” *International Review of Psychiatry* 25 (6): 650–58. doi:10.3109/09540261.2013.859128.

Song, Min-Sun, and John J. Rossi. 2017. “Molecular Mechanisms of Dicer: Endonuclease and Enzymatic Activity.” *Biochemical Journal* 474 (10): 1603–18. doi:10.1042/BCJ20160759.

Sorensen, S S, A B Nygaard, and T Christensen. 2016. “MiRNA Expression Profiles in Cerebrospinal Fluid and Blood of Patients with Alzheimer’s Disease and Other Types of Dementia - an Exploratory Study.” *Transl Neurodegener* 5: 6. doi:10.1186/s40035-016-0053-5.

Soria, Federico N., Olatz Pampliega, Mathieu Bourdenx, Wassilios G. Meissner, Erwan Bezard, and Benjamin Dehay. 2017. “Exosomes, an Unmasked Culprit in Neurodegenerative Diseases.” *Frontiers in Neuroscience* 11 (JAN). doi:10.3389/fnins.2017.00026.

Soto, Claudio, and Nikunj Satani. 2011a. “The Intricate Mechanisms of Neurodegeneration in Prion Diseases.” *Trends in Molecular Medicine* 17 (1): 14–24. doi:10.1016/j.molmed.2010.09.001.

Soto, M E, S Andrieu, C Arbus, M Ceccaldi, P Couratier, T Dantoine, J-F Dartigues, et al. 2008. “Rapid Cognitive Decline in Alzheimer’s Disease. Consensus Paper.” *The Journal of Nutrition, Health & Aging* 12 (10): 703–13. doi:10.1007/BF03028618.

Spittau, Björn. 2017. “Aging Microglia-Phenotypes, Functions and Implications for Age-Related Neurodegenerative Diseases.” *Frontiers in Aging Neuroscience* 9 (JUN). doi:10.3389/fnagi.2017.00194.

Stahl, N, D R Borchelt, K Hsiao, and S B Prusiner. 1987. “Scrapie Prion Protein Contains a Phosphatidylinositol Glycolipid.” *Cell* 51 (2): 229–40. doi:10.1016/0092-8674(87)90150-4.

Steele, A. D., J. G. Emsley, P. H. Ozdinler, S. Lindquist, and J. D. Macklis. 2006. “Prion Protein (PrPc) Positively Regulates Neural Precursor Proliferation during Developmental and Adult Mammalian Neurogenesis.” *Proceedings of the National Academy of Sciences* 103 (9): 3416–21. doi:10.1073/pnas.0511290103.

Strooper, Bart De, and Eric Karran. 2016. “The Cellular Phase of Alzheimer’s Disease.” *Cell* 164 (4): 603–15. doi:10.1016/j.cell.2015.12.056.

Swietnicki, W, R B Petersen, P Gambetti, and W K Surewicz. 1998. “Familial Mutations and the Thermodynamic Stability of the Recombinant Human Prion Protein.” *The Journal of Biological Chemistry* 273 (47): 31048–52. doi:10.1074/jbc.273.47.31048.

Szabadi, Elemer. 2013. “Functional Neuroanatomy of the Central Noradrenergic System.” *Journal of Psychopharmacology (Oxford, England)* 27 (8): 659–93. doi:10.1177/0269881113490326.

Tahir, Waqas, Saima Zafar, Franc Llorens, Amandeep Singh Arora, Katrin Thüne, Matthias

Schmitz, Nadine Gotzmann, et al. 2016. “Molecular Alterations in the Cerebellum of Sporadic Creutzfeldt–Jakob Disease Subtypes with DJ-1 as a Key Regulator of Oxidative Stress.” *Molecular Neurobiology*, 1–21. doi:10.1007/s12035-016-0294-4.

Takada, Leonel T., Mee-Ohk Kim, Ross W. Cleveland, Katherine Wong, Sven A. Forner, Ignacio Illán Gala, Jamie C. Fong, and Michael D. Geschwind. 2017. “Genetic Prion Disease: Experience of a Rapidly Progressive Dementia Center in the United States and a Review of the Literature.” *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics* 174 (1): 36–69. doi:10.1002/ajmg.b.32505.

Tan, Lin, Jin-Tai Yu, Meng-Shan Tan, Qiu-Yan Liu, Hui-Fu Wang, Wei Zhang, Teng Jiang, and Lan Tan. 2014. “Genome-Wide Serum MicroRNA Expression Profiling Identifies Serum Biomarkers for Alzheimer’s Disease.” *Journal of Alzheimer’s Disease* 40 (4): 1017–27. doi:10.3233/JAD-132144.

Tan, Lin, Jin Tai Yu, and Lan Tan. 2015. “Causes and Consequences of MicroRNA Dysregulation in Neurodegenerative Diseases.” *Molecular Neurobiology* 51 (3): 1249–62. doi:10.1007/s12035-014-8803-9.

Tapiola, T, M Overmyer, M Lehtovirta, S Helisalmi, J Ramberg, I Alafuzoff, P Riekkinen, and H Soininen. 1997. “The Level of Cerebrospinal Fluid Tau Correlates with Neurofibrillary Tangles in Alzheimer’s Disease.” *Neuroreport* 8 (18): 3961–63. doi:10.1097/00001756-199712220-00022.

Tapiola, Tero, Irina Alafuzoff, Sanna Kaisa Herukka, Laura Parkkinen, Päivi Hartikainen, Hilkka Soininen, and Tuula Pirttilä. 2009. “Cerebrospinal Fluid β-Amyloid 42 and Tau Proteins as Biomarkers of Alzheimer-Type Pathologic Changes in the Brain.” *Archives of Neurology* 66 (3): 382–89. doi:10.1001/archneurol.2008.596.

Telling, Glenn C. 2011. “Transgenic Mouse Models and Prion Strains.” *Topics in Current Chemistry* 305: 79–100. doi:10.1007/128_2011_166.

Thal, D. R., U. Rub, M. Orantes, and H. Braak. 2002. “Phases of A -Deposition in the Human Brain and Its Relevance for the Development of AD.” *Neurology* 58 (12): 1791–1800. doi:10.1212/WNL.58.12.1791.

Theofilas, Panos, Alexander J Ehrenberg, Sara Dunlop, Ana T Alho, Austin Nguy, Renata Elaine Paraizo Leite, Roberta Diehl Rodriguez, et al. 2016. “Locus Coeruleus Volume and Cell Population Changes during Alzheimer’s Disease Progression: A Stereological Study in Human Postmortem Brains with Potential Implication for Early-Stage Biomarker Discovery.” *Alzheimer’s & Dementia* 13 (3): 236–46. doi:10.1016/j.jalz.2016.06.2362.

Tomaselli, Sara, Federica Galeano, Shahar Alon, Susanna Raho, Silvia Galardi, Assunta A. Polito, Carlo Presutti, et al. 2015. “Modulation of MicroRNA Editing, Expression and Processing by ADAR2 Deaminase in Glioblastoma.” *Genome Biology*. doi:10.1186/s13059-014-0575-z.

Tsang, John, Jun Zhu, and Alexander van Oudenaarden. 2007. “MicroRNA-Mediated Feedback and Feedforward Loops Are Recurrent Network Motifs in Mammals.” *Molecular Cell* 26 (5): 753–67. doi:10.1016/j.molcel.2007.05.018.

Tzaban, Salit, Gilgi Friedlander, Oshrat Schonberger, Lior Horonchik, Yifat Yedidia, Gideon Shaked, Ruth Gabizon, and Albert Taraboulos. 2002. “Protease-Sensitive Scrapie Prion

Protein in Aggregates of Heterogeneous Sizes." *Biochemistry* 41 (42): 12868–75. doi:10.1021/bi025958g.

Unterberger, Ursula, Romana Höftberger, Ellen Gelpi, Helga Flicker, Herbert Budka, and Till Voigtländer. 2006. "Endoplasmic Reticulum Stress Features Are Prominent in Alzheimer Disease but Not in Prion Diseases in Vivo." *Journal of Neuropathology and Experimental Neurology* 65 (4): 348–57. doi:10.1097/01.jnen.0000218445.30535.6f.

Vickers, James, Stan Mitew, Adele Woodhouse, Carmen Fernandez-Martos, Mathew Kirkcaldie, Alison Canty, Graeme McCormack, and Anna King. 2016. "Defining the Earliest Pathological Changes of Alzheimer's Disease." *Current Alzheimer Research* 13 (3): 281–87. doi:10.2174/1567205013666151218150322.

Viola, Kirsten L., and William L. Klein. 2015. "Amyloid β Oligomers in Alzheimer's Disease Pathogenesis, Treatment, and Diagnosis." *Acta Neuropathologica*. doi:10.1007/s00401-015-1386-3.

Vistbakka, Julia, Irina Elovaara, Terho Lehtimäki, and Sanna Hagman. 2017. "Circulating MicroRNAs as Biomarkers in Progressive Multiple Sclerosis." *Multiple Sclerosis Journal* 23 (3): 403–12. doi:10.1177/1352458516651141.

Viswambharan, Vijitha, Ismail Thanseem, Mahesh M. Vasu, Suresh A. Poovathinal, and Ayyappan Anitha. 2017. "MiRNAs as Biomarkers of Neurodegenerative Disorders." *Biomarkers in Medicine*. doi:10.2217/bmm-2016-0242.

Wadsworth, Jonathan D. F., and John Collinge. 2011. "Molecular Pathology of Human Prion Disease." *Acta Neuropathologica* 121 (1): 69–77. doi:10.1007/s00401-010-0735-5.

Wang, Bin, and Lan Bao. 2017. "Axonal MicroRNAs: Localization, Function and Regulatory Mechanism during Axon Development." *Journal of Molecular Cell Biology* 9 (2): 82–90. doi:10.1093/jmcb/mjw050.

WANG, CHUNMEI, BINGYUAN JI, BAOHUA CHENG, JING CHEN, and BO BAI. 2014. "Neuroprotection of MicroRNA in Neurological Disorders (Review)." *Biomedical Reports* 2 (5): 611–19. doi:10.3892/br.2014.297.

Wang, F. E., C. Zhang, A. Maminishkis, L. Dong, C. Zhi, R. Li, J. Zhao, et al. 2010. "MicroRNA-204/211 Alters Epithelial Physiology." *The FASEB Journal* 24 (5): 1552–71. doi:10.1096/fj.08-125856.

Wang, Gang, Yue Huang, Li Ling Wang, Yong Fang Zhang, Jing Xu, Yi Zhou, Guinevere F. Lourenco, et al. 2016. "MicroRNA-146a Suppresses ROCK1 Allowing Hyperphosphorylation of Tau in Alzheimer's Disease." *Scientific Reports* 6. doi:10.1038/srep26697.

Wang, Jin, Jinyun Chen, and Subrata Sen. 2016. "MicroRNA as Biomarkers and Diagnostics." *Journal of Cellular Physiology*. doi:10.1002/jcp.25056.

Wang, Li, and Rui Yi. 2014. "3′UTRs Take a Long Shot in the Brain." *BioEssays* 36 (1): 39–45. doi:10.1002/bies.201300100.

Wang, Yipeng, and Eckhard Mandelkow. 2016. "Tau in Physiology and Pathology." *Nature Reviews Neuroscience* 17 (1): 5–21. doi:10.1038/nrn.2015.1.

Watts, Joel C., and Stanley B. Prusiner. 2014. "Mouse Models for Studying the Formation and Propagation of Prions." *Journal of Biological Chemistry* 289 (29): 19841–49.

doi:10.1074/jbc.R114.550707.

Weinshenker, David. 2008. “Functional Consequences of Locus Coeruleus Degeneration in Alzheimer’s Disease.” *Current Alzheimer Research* 5 (3): 342–45. doi:10.2174/156720508784533286.

Wiersma, Vera I., Wim van Hecke, Wiep Scheper, Martijn A.J. van Osch, Will J.M. Hermsen, Annemieke J.M. Rozemuller, and Jeroen J.M. Hoozemans. 2016. “Activation of the Unfolded Protein Response and Granulovacuolar Degeneration Are Not Common Features of Human Prion Pathology.” *Acta Neuropathologica Communications* 4 (1): 113. doi:10.1186/s40478-016-0383-7.

Will, Robert G. 2003. “Acquired Prion Disease: Iatrogenic CJD, Variant CJD, Kuru.” *British Medical Bulletin* 66: 255–65. doi:10.1093/bmb/66.1.255.

Williams, D. R. 2006. “Tauopathies: Classification and Clinical Update on Neurodegenerative Diseases Associated with Microtubule-Associated Protein Tau.” *Internal Medicine Journal* 36 (10): 652–60. doi:10.1111/j.1445-5994.2006.01153.x.

Winklhofer, Konstanze F., Jörg Tatzelt, and Christian Haass. 2008. “The Two Faces of Protein Misfolding: Gain- and Loss-of-Function in Neurodegenerative Diseases.” *EMBO Journal*. doi:10.1038/sj.emboj.7601930.

Wong, Hon Kit Andus, Tatiana Veremeyko, Nehal Patel, Cynthia A. Lemere, Dominic M. Walsh, Christine Esau, Charles Vanderburg, and Anna M. Krichevsky. 2013. “De-Repression of FOXO3a Death Axis by MicroRNA-132 and -212 Causes Neuronal Apoptosis in Alzheimer’s Disease.” *Human Molecular Genetics* 22 (15): 3077–92. doi:10.1093/hmg/ddt164.

Wu, Chung I., Yang Shen, and Tian Tang. 2009. “Evolution under Canalization and the Dual Roles of MicroRNAs - A Hypothesis.” *Genome Research* 19 (5): 734–43. doi:10.1101/gr.084640.108.

Wu, Liyong, Pedro Rosa-Neto, Ging Yuek R. Hsiung, A. Dessa Sadovnick, Mario Masellis, Sandra E. Black, Jianping Jia, and Serge Gauthier. 2012. “Early-Onset Familial Alzheimer’s Disease (EOFAD).” *Canadian Journal of Neurological Sciences* 39 (4): 436–45. doi:10.1017/S0317167100013949.

Wulf, Marie-Angela, Assunta Senatore, and Adriano Aguzzi. 2017. “The Biological Function of the Cellular Prion Protein: An Update.” *BMC Biology* 15 (1): 34. doi:10.1186/s12915-017-0375-5.

Xin, Hongqi, Yi Li, and Michael Chopp. 2014. “Exosomes/MiRNAs as Mediating Cell-Based Therapy of Stroke.” *Frontiers in Cellular Neuroscience*. doi:10.3389/fncel.2014.00377.

Yagi, Yohsuke, Takuya Ohkubo, Hideya Kawaji, Akira Machida, Haruka Miyata, Saori Goda, Sugata Roy, Yoshihide Hayashizaki, Harukazu Suzuki, and Takanori Yokota. 2017. “Next-Generation Sequencing-Based Small RNA Profiling of Cerebrospinal Fluid Exosomes.” *Neuroscience Letters* 636: 48–57. doi:10.1016/j.neulet.2016.10.042.

Yang, Jialei, Xiufen Zhang, Xiangjie Chen, Lei Wang, and Guodong Yang. 2017. “Exosome Mediated Delivery of MiR-124 Promotes Neurogenesis after Ischemia.” *Molecular Therapy - Nucleic Acids* 7: 278–87. doi:10.1016/j.omtn.2017.04.010.

Yang, Qing, Yoshio Hashizume, Mari Yoshida, and Yin Wang. 1999. “Neuropathological Study of Cerebellar Degeneration in Prion Disease.” *Neuropathology* 19 (1): 33–39.

doi:10.1046/j.1440-1789.1999.00212.x.

Ye, Yuqin, Hongyu Xu, Xinhong Su, and Xiaosheng He. 2016. “Role of MicroRNA in Governing Synaptic Plasticity.” *Neural Plasticity* 2016. doi:10.1155/2016/4959523.

Yoo, Andrew S., Alfred X. Sun, Li Li, Aleksandr Shcheglovitov, Thomas Portmann, Yulong Li, Chris Lee-Messer, Ricardo E. Dolmetsch, Richard W. Tsien, and Gerald R. Crabtree. 2011. “MicroRNA-Mediated Conversion of Human Fibroblasts to Neurons.” *Nature* 476 (7359): 228–31. doi:10.1038/nature10323.

Yoon, Hyejin, Krystal C. Belmonte, Tom Kasten, Randall Bateman, and Jungsu Kim. 2017. “Intra- and Inter-Individual Variability of MicroRNA Levels in Human Cerebrospinal Fluid: Critical Implications for Biomarker Discovery.” *Scientific Reports* 7 (1). doi:10.1038/s41598-017-13031-w.

Zendjabil, Mustapha. 2018. “Circulating MicroRNAs as Novel Biomarkers of Alzheimer’s Disease.” *Clinica Chimica Acta* 484: 99–104. doi:10.1016/j.cca.2018.05.039.

Zeng, Qinghong, Linqing Zou, Li Qian, Fang Zhou, Hongxia Nie, Shanhua Yu, Jiandong Jiang, Aixia Zhuang, Chuanqi Wang, and Haojiang Zhang. 2017. “Expression of MicroRNA-222 in Serum of Patients with Alzheimer’s Disease.” *Molecular Medicine Reports* 16 (4): 5575–79. doi:10.3892/mmr.2017.7301.

Zerr, I., K. Kallenberg, D. M. Summers, C. Romero, A. Taratuto, U. Heinemann, M. Breithaupt, et al. 2009. “Updated Clinical Diagnostic Criteria for Sporadic Creutzfeldt-Jakob Disease.” *Brain* 132 (10): 2659–68. doi:10.1093/brain/awp191.

Zerr, Inga, Monika Bodemer, Olaf Gefeller, Markus Otto, Sigrid Poser, Jens Wiltfang, Otto Windl, Hans A. Kretzschmar, and Thomas Weber. 1998. “Detection of 14-3-3 Protein in the Cerebrospinal Fluid Supports the Diagnosis of Creutzfeldt-Jakob Disease.” *Annals of Neurology* 43 (1): 32–40. doi:10.1002/ana.410430109.

Zetterberg, Henrik, and Niklas Mattsson. 2014. “Understanding the Cause of Sporadic Alzheimer’s Disease.” *Expert Review of Neurotherapeutics* 14 (6): 621–30. doi:10.1586/14737175.2014.915740.

Zhao, Yuhai, Surjyadipta Bhattacharjee, Brandon M. Jones, Prerna Dua, Peter N. Alexandrov, James M. Hill, and Walter J. Lukiw. 2013. “Regulation of TREM2 Expression by an NF-KB-Sensitive MiRNA-34a.” *NeuroReport* 24 (6): 318–23. doi:10.1097/WNR.0b013e32835fb6b0.

Zhao, Yuhai, Aileen I. Pogue, and Walter J. Lukiw. 2015. “MicroRNA (MiRNA) Signaling in the Human CNS in Sporadic Alzheimer’s Disease (AD)-Novel and Unique Pathological Features.” *International Journal of Molecular Sciences* 16 (12): 30105–16. doi:10.3390/ijms161226223.