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Current advances in our understanding of circular RNA (circRNA) in Alzheimer's disease (AD); the potential utilization of synthetic circRNAs as a therapeutic strategy in the clinical management of AD

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Introduction

Circular RNAs (circRNAs) represent a large, naturally occurring family of extremely stable single-stranded RNAs (ssRNAs) with covalently linked 3′ and 5′ ends. Traditional methods for the detection of ssRNA species including microRNA (miRNA) and messenger RNA (mRNA) and requiring a free 3′ or 5′ ribonucleotide terminus appear to have significantly underestimated circRNA abundance and significance in eukaryotic cells (Hansen et al., 2013; Lukiw et al., 2013; Memczak et al., 2013; Rybak-Wolf and Plass, 2021). CircRNAs are currently recognized as being very highly represented signaling molecules in the transcriptome and are especially abundant in the human brain and central nervous system (CNS; Hansen et al., 2013; Lukiw et al., 2013; Memczak et al., 2013; Lu and Xu, 2016; Zhao et al., 2016). The first ~1,400 nucleotide (nt) circRNA described (ciRS-7) was discovered in the human hippocampal CA1 formation and was found to contain ~70 selectively conserved anti-miRNA-7 binding sites. CiRS-7 was subsequently shown to act as an endogenous, competing, anti-complementary miRNA-7 "sponge" to adsorb and hence quench normal miRNA-7 activities (Salzman et al., 2012; Hansen et al., 2013; Memczak et al., 2013). Down-regulated ciRS-7 in Alzheimer's disease (AD) was next demonstrated to contribute to an up-regulation of free miRNA-7 abundance and signaling in AD hippocampus, including a targeted down-regulation of the mRNA encoding ubiquitin protein ligase A (UBE2A), an autophagic, phagocytic protein essential in the clearance of neurotoxic amyloid-beta (Aβ) peptides (Salzman et al., 2013; Zhao et al., 2016; Walgrave et al., 2021; Sinha et al., 2022; Zhang et al., 2022). Because miRNA- and

mRNA-mediated gene expression appears to be significantly altered in AD brain and CNS, our understanding of the natural organization, configuration and composition of circRNA molecules is necessitated because these ssRNAs are highly stable, can persist in pathophysiological environments for extended periods, and therapeutically can be designed and synthesized to carry multiple ssRNA-mediated regulatory signals. Based on these and other very recent findings this "Opinion" paper will: 1) address our current understanding of the emerging role of circRNAs in neurodegeneration with special reference to AD; and 2) discuss the intriguing possibility of using synthetic circRNAs containing multiple inserted miRNA, anti-miRNA (AM; antagomir), mRNA, anti-mRNA and/or other ssRNA sequences in tandem combination with a "personalized medicine" approach as an innovative therapeutic strategy for the potential treatment of AD and related progressive neurological disorders of the aging CNS.

Overview

Our appreciation and understanding of the structure and biological function of single-stranded RNAs (ssRNAs) in the regulation of gene expression in the human brain and central nervous system (CNS) continues to evolve. One remarkable finding has been the relatively recent discovery of circular RNA (circRNA) containing both coding and/or non-coding ssRNA regulatory elements ranging in size from several hundred to several thousand nucleotides (nt). Due to a lack of free 3′ or 5′ ends, circRNAs are not directly targeted and inactivated by abundant cellular endo- and exonuclases and as such are remarkably stable information-rich ssRNA entities (Memczak et al., 2013; Guo et al., 2014; Zhao et al., 2016; Rybak-Wolf and Plass 2021). Abundant evidence has accumulated that circRNAs possess several biological functions including miRNA sponging, roles in transcriptional regulation, the specific splicing of heterogeneous nuclear RNA (hnRNA) and have capability to act as polypeptide-generating templates (Zhao et al., 2016; Lauretti et al., 2021; Nisar et al., 2021; Rybak-Wolf and Plass 2021; Sharma et al., 2022; Sinha et al., 2022; Wang et al., 2022; Zhang et al., 2022). Emerging data further indicates that circRNAs play important and determinant roles in both health and disease ranging from acute CNS injuries including ischemic stroke and traumatic brain injury to cancer and AD (Salzman et al., 2012; Hansen et al., 2013; Lukiw 2013; Memczak et al., 2013; Lukiw 2022; Sharma et al., 2022; Sinha et al., 2022; Zhang et al., 2022). Although circRNAs may take many forms that include coding and non-coding RNA (ncRNA) sequences and vary widely in individual abundance, speciation, size and complexity this current paper will focus on circRNAs containing miRNA- and anti-miRNA-type regulatory sequences for which a relatively large amount of basic research has already been completed. It should be possible to design and engineer custom synthetic circRNAs containing multiple types of RNA sequences linked in tandem combination, and together with a "personalized medicine" approach (see below) could serve as an innovative therapeutic strategy for the potential treatment of progressive neurodegeneration in the aging CNS.

The nature of circular RNA

Circular RNAs (circRNAs) represent both an intriguing topological isoform of ssRNA and a novel class of ncRNA. Compared to linear ssRNA, circRNAs are produced differentially by back-splicing exons or lariat introns from immature pre-microRNA (pre-miRNA) or pre-messenger RNA (mRNA) templates (Hansen et al., 2013; Lukiw 2013; Memczak et al., 2013; Salzman et al., 2013; Enuka et al., 2016; Lauretti et al., 2021; Nisar et al., 2021; Zhang et al., 2022). This biologically heterogeneous circRNA exhibiting cell- and tissue-specific expression patterns suggest multifaceted circRNA roles: 1) in the assembly of RNA binding protein (RBP) and ribonucleprotein (RNP) complexes; 2) as circular templates for potentially rapid translation, splicing and translation-event regulators; 3) occasionally acting as polypeptide-producing templates; and 4) as modulators of RNA transcriptomic function in part as miRNA 'sponging' leading to the inactivation and/or availability of free miRNA species (Lauretti et al., 2021; Nisar et al., 2021; Sharma et al., 2022; Zhang et al., 2022). To date the best characterized circRNA functions are associated with miRNA regulation (Salzman et al., 2012; Hansen et al., 2013; Lukiw 2013; Memczak et al., 2013; Salzman et al., 2013; Jeck and Sharpless 2014; Zhao et al., 2016; Akhter 2018; Nisar et al., 2021; Rybak-Wolf and Plass, 2021; Sharma et al., 2022; Sinha et al., 2022; Zhang et al., 2022). CircRNAs have been implicated in many incapacitating human diseases due to their aberrant expression under different pathological conditions. Their functional versatility also enables them to serve as potential diagnostic, prognostic or predictive outcome biomarkers for various diseases. It has been suggested that the mis-regulation of circRNA signaling is involved in angiogenesis, the inhibition of apoptosis, pro-inflammatory signaling, disruption of the process of autophagy and the blood-brain barrier system and altered cognitive function in both acute and chronic CNS injury *via* different signaling pathways involving miRNA, NF-κB, phosphatidylinositol-4,5-bisphosphate-3-kinase/protein kinase (PI3K/AKT), Notch1 and ten-eleven translocation (TET)-mediated methylcytosine dioxygenase activities (Zhao et al., 2016; Lauretti et al., 2021; Zhang et al., 2022).

miRNA abundance, complexity, and speciation in AD

AD is the progressive, age-related and ultimately lethal neurodegeneration of the limbic system of the human brain primarily involving the neocortex, hippocampal formation and related anatomical regions involved in synaptic formations and structural and functional neuroplasticity (Alzheimer et al., 1995; Weerasinghe-Mudiyanselage et al., 2022; https://www.alz.org/media/documents/alzheimers-facts-and-figures.pdf; last accessed 18 July 2022). As a complex mixed proteinopathy, AD is characterized in large part by the dysregulation of gene expression patterns as detected by Northern blots, RT-PCR, DNA array-based analysis, RNA deep-sequencing, Western immunoassay, bioinformatics and statistical analysis (Alzheimer et al., 1995; Loring et al., 2001; Colangelo et al., 2002; Ginsberg et al., 2012; Jaber et al., 2017; Rybak-Wolf and Plass 2021). Many AD-relevant mRNA levels in brain anatomical areas affected by the AD process are significantly reduced in mean abundance caused directly by the up-regulation of a small family of NF-kB (p50/p65)-sensitive miRNAs (Jaber et al., 2017; Jaber et al., 2019; Zhao et al., 2020; Pogue et al., 2022; Roy et al., 2022; Yoon et al., 2022). For example miRNA-34a-mediated deficits in mRNA encoding the triggering receptor expressed in myeloid/microglial cells 2 (TREM2) results in an inability of cells of the CNS to effectively clear Aβ peptides and other waste products from brain cells, leading to amyloid build-up and pathological amyloidogenesis (Jones et al., 2014; Bhattacharjee et al., 2016; Sarkar et al., 2019). In fact the up-regulation of just two NF-kB-sensitive miRNAs - miRNA-34a and miRNA-146a - appears to promote the down-regulation of: 1) SHANK3 (leading to synaptic disorganization); 2) TREM2 and the transmembrane spanning cell surface receptor TSPAN12 (tetraspanin-12; NET-2; leading to phagocytosis deficits, amyloidogenesis and tau pathology); and 3) down-regulated complement factor H (CFH) accompanied by a defective innate-immune response and an up-regulation in inflammatory signaling (Jaber et al., 2017; Sarkar et al., 2019). As shown both in AD and related neurodegenerative disease transgenic models for AD (TgAD) many of these decreases in specifically targeted AD-relevant mRNAs are caused directly by the combination of up-regulated miRNAs and down-regulation of their target mRNAs. This is a manifold-confirmed pathological molecular-genetic process that contributes significantly to the neurodegenerative disease phenotype such as that seen in AD brain (Sethi and Lukiw 2009; Sarkar et al., 2019; Tasker et al., 2021; Roy et al., 2022; Yoon et al., 2022). Because AD is characterized in part by both increases in stress-induced miRNA and decreased and/or altered target mRNA abundance: 1) it may be advantageous to exploit the same natural strategies that the neurons of the brain and CNS have evolved in the design of functional 'remedial' circRNAs; and 2) it may be able to design and synthesize a circRNA with a series of miRNA, anti-miRNA (AM), mRNA, anti-mRNA and/or other ssRNA sequences to specifically interact with brain miRNAs and/or mRNAs to rectify their critical dysregulation.

Heterogeneity of AD and a requirement for "precision" or "individualized" medicine

AD is a distinctly heterogeneous, multifactorial neurological disorder with variation of neuropathology and patient symptoms stemming from a number of areas. These include familial genetics and family history, ethnicity, inter-current illness, multiple parameters associated with gender and aging, the environment, diet and lifestyle and other factors associated with the intrinsic complexity of the disease itself, neuro-diagnostic variation in neuroimaging, and neuropathologically *via* the pre-clinical, clinical and Braak staging of the disease, multiple interactive parameters including CSF and plasma Aβ40/Aβ42 ratios and the anatomical location of Aβ accumulation (Dong et al., 2017; DeTure and Dickson, 2019; Habes et al., 2020; Jellinger et al., 2022). Other parameters of AD diversity and heterogeneity include the abundance, speciation and inflammatory potential of different senile plaque (SP) and neurofibrillary tangle (NFT) isoforms and densities in anatomical regions of the brain implicated in behavior, cognition and memory (Dong et al., 2017; DeTure and Dickson, 2019; Habes et al., 2020; Lemercier et al., 2021; Bellenguez et al., 2022; Jellinger 2022). This significant heterogeneity in the presentation of AD, currently referred to as "the AD spectrum or continuum", requires both a highly individualized diagnostic approach and treatment strategy.

The detailed characterization of AD heterogeneity is essential for precision diagnostics, personalized predictions, and recruitment of relatively homogeneous sets of patients into clinical trials. This has also been referred to as a "precision", "personalized" or "individualized" medicinal approach to optimize the clinical management of the disease course for the individual AD patient, and in part involves a mapping of dysregulated miRNA- and mRNA-regulated gene expression patterns in individual AD patients (Dong et al., 2017; Hampel et al., 2019, 2020; Lukiw et al., 2020; Bellenguez et al., 2022; Giampietri et al., 2022; Wen et al., 2022; Wingo et al., 2022). More specifically, "precision" or "individualized" medicine is an emerging systematic undertaking that applies the most recent scientific and technological advances to overcome the limitations of Western medicine that involve sign- and symptom-based phenotypic diagnoses and the traditional "one-size-fits-all' approach. Currently the "precision medicine" approach has been to investigate early pathophysiological changes of AD to fully capture the complexity of the disease, and has been essential to develop timely screening, detection, diagnostic, prognostic and

therapeutic interventions in significantly heterogeneous AD patient populations. Importantly "precision medicine" delineates which therapeutic approach or treatment strategy would be the most effective for a specific individual, at a specified disease stage, across multiple medical research fields that include molecular-genetics, imaging technologies, neuroscience, neurology and psychiatry and the identification of miRNA- and/or mRNA-based biomarkers in the CSF and blood (Hampel et al., 2019; Lemercier et al., 2021; Giampietri et al., 2022; Jellinger 2022; Wu et al., 2022; Yamamoto et al., 2022). "Precision medicine" achieves the greatest success when multiple interdisciplinary diagnostic parameters are integrated to yield the most accurate diagnostic analysis of the AD patient who can be subsequently treated using an individualized combination therapy approach or multi-target-directed methodologies. This presents a virtually limitless opportunity: 1) of using miRNA, anti-miRNA (AM), mRNA-modifying and/or other ssRNA-containing circRNAs as a therapeutic strategy in modulating the brain's transcriptome to restore homeostatic functions to AD-affected neurons; and 2) in developing preventative disease-modifying strategies for this devastating neurological disorder.

Synthetic circRNAs containing miRNA, anti-miRNA, mRNA and/or other ssRNA sequences

Using precision medicine-derived molecular-genetic biomarker guidelines, synthetic circRNAs have a significant potential to play an important role in the improved clinical management of AD for a number of reasons. Firstly, circRNAs, as a novel topological isoform of ssRNA, are a long-lived species being up to ~5 or more times as stable than their linear ssRNA counterparts, and covalently modified or selected ribonucleotides could further enhance or modulate this longevity; for example their stability can be augmented by incorporating ribonucleotide 'instability' signals such as adenine + uridine (AU)-rich elements or endonuclease sensitive sites (Enuka et al., 2016; Lauretti et al., 2021). Secondly, ssRNAs in a covalently closed loop contains genetic regulatory information and signals that would interact directly with cellular miRNA and mRNA species. Thirdly, circRNAs appear to be themselves capable of crossing biophysical and physiological barriers including the blood-brain barrier (BBB); alternatively they may be packaged into micro-vesicles which have been shown to transverse both biophysical and physiological barriers including the BBB (Memczak et al., 2013; Lu et al., 2020; Lukiw and Pogue 2020; Wu et al., 2022). Fourthly, circRNAs containing miRNA-type sequences have potential to target multiple mRNAs and hence multiple highly interactive gene expression pathways, and several mRNAs may be targeted and down-regulated by multiple interdependent circRNA-containing miRNA or AM species (Zhao et al., 2016; Bartel, 2018; Pogue and Lukiw, 2018; Nguyen et al., 2021). Fifthly circRNAs are immunologically well-tolerated in multiple cellular systems into which they have been introduced, probably because they are a naturally-occurring form of nucleic acid and their lack of free 3′ or 5′ termini enables circRNAs to escape from RNA-mediated host immune responses (Liu et al., 2022; Wu et al., 2022).

Global miRNA and mRNA expression patterns for specific anatomical regions of the brain and CNS have already been established for AD at various stages of disease in defined groups of AD patients (Lukiw et al., 1992; Colangelo et al., 2002; Burmistrova et al., 2007; Lukiw 2007; Lukiw 2007; Cogswell et al., 2008; Sethi and Lukiw 2009; Ginsberg et al., 2012; Wilusz and Sharp 2013; Bhattacharjee et al., 2016; Zhao et al., 2016; Jaber et al., 2017; Millan et al., 2017; Pogue and Lukiw 2018; Jaber et al., 2019; Sarkar et al., 2019; Zhao et al., 2020; Lauretti et al., 2021; Nguyen et al., 2021; Rybak-Wolf and Plass 2021; Tasker et al., 2021; Walgrave et al., 2021; Wei et al., 2020; Lukiw 2022; Pogue et al., 2022; Roy et al., 2022; Yoon et al., 2022). This wealth of genetic and epigenetic information could be used as a guide to initially address and correct dysregulated miRNA patterns in the AD brain using specifically engineered circRNA. The concatenation of multiple ssRNA-based regulatory species delivered by circRNAs have therefore high potential to re-shape the pathological transcriptome of the cell as they are ubiquitous regulators of both multiple AD-relevant neurological functions and highly interactive neuropathological signaling pathways. In addition, recent findings indicate that circRNAs may be useful in peptide- or protein-based therapeutics since circRNAs can support translation *via* 5′-methyl cap-independent mechanisms that include internal ribosome entry sites (IRESs), m^6A internal ribosome entry sites (MIRESs) and novel "rolling translation" mechanisms (Prats et al., 2020; Sinha et al., 2022; Wang et al., 2022). Accordingly, circRNA-based translateomics and proteomics have attracted increasing attention as they have enormous potential in the contribution of circRNA-encoded proteins or "circ-proteins" to human health and disease, non-canonical circRNA-based proteomics and a circRNA-derived "hidden proteome" which are expanding the therapeutic potential of circRNAs for the novel treatment of multiple forms of human disease (Nisar et al., 2021; Sinha et al., 2022; Wang et al., 2022).

Although the exact mechanisms are still not fully understood, and despite considerable difficulties due to disease heterogeneity and the complexity of their regulatory roles, research in this rapidly advancing field suggests that circRNAs hold great potential as diagnostic and prognostic biomarkers, drug efficacy monitoring markers, as therapeutic targets and remedial tools for AD (D'Anca et al., 2022; Zang et al., 2022). 2022; Recent studies further indicate that highly conserved families of miRNAs and/or circRNAs are important modulators of Aβ-peptide generation and clearance, tau phosphorylation, pro-inflammatory signaling, neurotropism, synaptic plasticity and neuronal survival and gene expression in the brain and CNS, all features considered centrally involved in

the pathogenesis of AD (Bingol and Sheng 2011; Jaber et al., 2019; Lauretti et al., 2021; Jellinger 2022; Wang et al., 2022; Zhang et al., 2022). Yet another attractive feature of circRNA-containing miRNAs as a pharmacotherapeutic agent is that they appear to be involved in the regulation of highly interactive gene expression programs. As few as 10 NF-kB-sensitive miRNAs or clustered miRNA families up-regulated in abundance in AD hippocampus, including miRNA-7, miRNA-9, miRNA-23a, miRNA-29, miRNA-30b, miRNA-34a, miRNA-107, miRNA-125b, miRNA-146a and miRNA-155 can explain most of the major features of AD neuropathology in the limbic regions of multiple AD patients. These involve functionally interactive molecular networks implicated in amyloid and neurofibrillary homeostasis, amyloidogenesis, altered NF-kB (p50/p65) and innate-immune signaling, tau pathology, deficits in phagocytosis, catabolism, neurotrophism, synaptogenesis and/or neuro-inflammation (Lukiw, 2007; Millan, 2017; Pogue and Lukiw, 2018; Brennan and Henshall, 2020; Wei et al., 2020; Nguyen et al., 2021; Tasker et al., 2021; Walgrave et al., 2021; Lukiw 2022). As an initial guide, a circRNA containing AM sequences to the above mentioned 10-member pathological miRNA family could be synthesized to stabilize these altered miRNA levels. Indeed the delivery of stable, stoichiometrically designed miRNA-, AM-, mRNA- and/or anti-mRNA-containing circRNAs engineered and based on 'precision medicine' guidelines has considerable potential to ultimately promote the long-term restoration of homeostatic molecular-genetic-based signaling activities in the human brain and CNS.

Discussion

Our mechanistic and molecular-genetic understanding of the contribution of circRNA to the altered gene expression patterns and neuropathology of multiple diseases continues to advance. Since neurodegenerative disorders such as AD represent complex, dynamic, heterogeneous, polygenic syndromes involving multiple neurobiological pathways, it seems fitting that novel therapeutic strategies involving multiple regulatory components should be preferred over the classical single-target therapeutic approach (Enuka et al., 2016; Grabowska-Pyrzewicz et al., 2021; Walgrave et al., 2021). Multiple pharmaceutical companies are currently using miRNA, AM, mRNA and anti-mRNA approaches as novel drug treatments in the clinical management of neurological diseases, cancers, hepatitis C viral infection, neuroinflammation, cardiovascular disease including heart failure and anti-viral vaccines are also currently under development for targeting SARS-CoV-2 (Chakraborty et al., 2020; Deiner et al., 2022). The later two papers also thoughtfully discuss highlights and recent successes and failures of RNA-based therapeutics and clinical considerations involving side effects, dosing, administration, targeting, uptake, solubility and transmembrane penetration and other aspects of miRNA and AM-based therapeutic strategies.

Exciting studies on the regulatory roles of circRNA species and a brain- and CNS-enriched circRNA-derived '*hidden proteome*' are emerging (Yang and Wang, 2019; Nisar et al., 2021; Sinha et al., 2022; Wang et al., 2022; Zhang et al., 2022). Rapid advances in RNA sequencing, circRNA enrichment involving circularity-sensitive RNase-based screening technologies and bioinformatics have allowed the discovery and initial characterization of many thousands of different circRNAs in multiple neurological disorders (Hansen et al., 2013; Lukiw 2013; Memczak et al., 2013; Guo et al., 2014; Jeck and Sharpless 2014; Prats et al., 2020; Nisar et al., 2021; Sharma et al., 2022; Sinha et al., 2022; Zhang et al., 2022). It is our opinion that the exploitation of unique circRNA structure and function, and the proposed strategic design of synthetic ssRNA circles to contain tandem natural miRNA, AM, mRNA, anti-mRNA and/or other ssRNA sequences have provided an additional dimension to our supply of genetic regulatory agents that should be useful in the treatment of molecular-genetic-associated neurological disorders involving the brain and CNS. Emerging evidence has indicated that circRNA dysregulation can play crucial roles in multiple neurological diseases and circRNA continues to present as a suitable and useful biomarker for early diagnosis, prognosis, prediction of time-to-symptom onset, monitoring of disease progression and potential drug treatment strategies. CircRNAs, circRNA-based diagnostic tools and circRNA-mediated therapeutic strategies have a strong and currently untapped potential role in the effective treatment of neurodegenerative diseases including AD. Precision and personalized medicine approaches involving individual AD patient-tailored circRNAs containing multiple ssRNA sequences would have a significant influence on progressive, age-related diseases impacted by altered ssRNA and/or circRNA abundance, speciation and complexity. The goals of precision medicine would be to closely monitor the AD patient and the use of different circRNA designs may have to be empirically determined depending on the nature, stability and RNA-based information content of the particular therapeutic circRNA used. Further research into the transcriptomic regulatory and translational roles of circRNAs should uncover novel gene expression mechanisms during development, aging and under disease conditions and identify multiple circRNAs as important and innovative molecular and epigenetic biomarkers for age-related neurodegenerative disorders. The focused alteration of specific circRNA abundances and complexities, modification of circRNA-derived proteomes and the implementation of strategically designed and engineered synthetic circRNAs offers an exciting and innovative therapeutic prospect for the future clinical management of AD and other progressive neurological disorders (Chakraborty et al., 2020; Deiner et al., 2022). The increasing incidence of human neurodegenerative disorders such as AD and the general low rates of success of neurological drug trials to date should serve as a natural driver for the implementation of these types of

circRNA-mediated therapies, especially since no effective treatment options for AD currently exist (Lauretti et al., 2021; Nguyen et al., 2021; Walgrave et al., 2021; Roy et al., 2022).

Author contributions

YZ, VJ, and WL: collected, analyzed, distilled and summarized the current literature. WL wrote the article.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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