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Role of Circular RNAs in Brain Development and CNS Diseases

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Abstract

In mammals, many classes of noncoding RNAs (ncRNAs) are expressed at a much higher level in the brain than in other organs. Recent studies have identified a new class of ncRNAs called circular RNAs (circRNAs), which are produced by back-splicing and fusion of either exons, introns, or both exon-intron into covalently closed loops. The circRNAs are also highly enriched in the brain and increase continuously from the embryonic to the adult stage. Although the functional significance and mechanism of action of circRNAs are still being actively explored, they are thought to regulate the transcription of their host genes and sequestration of miRNAs and RNA binding proteins. Some circRNAs are also shown to have translation potential to form peptides. The expression and abundance of circRNAs seem to be spatiotemporally maintained in a normal brain. Altered expression of circRNAs is also thought to mediate several disorders, including brain-tumor growth, and acute and chronic neurodegenerative disorders by affecting mechanisms such as angiogenesis, neuronal plasticity, autophagy, apoptosis, and inflammation. This review discusses the involvement of various circRNAs in brain development and CNS diseases. A better understanding of the circRNA function will help to develop novel therapeutic strategies to treat CNS complications.

Graphical Abstract

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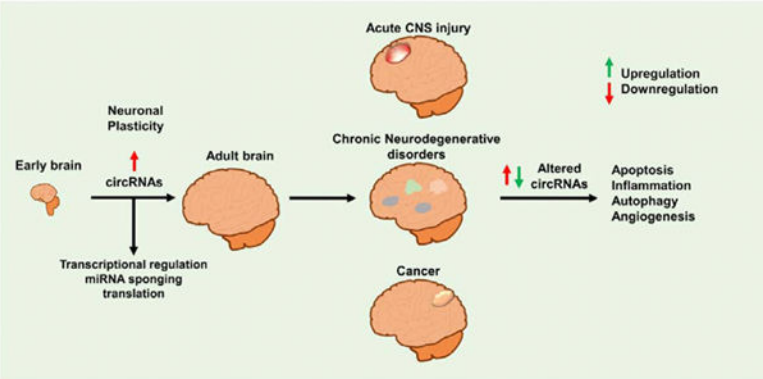

Keywords

CircRNAs; miRNAs; Brain; Development; Cancer; Acute and chronic neurodegeneration

1. Introduction

Mammalian genome is pervasively active and produces many classes of noncoding RNAs (ncRNAs) in addition to protein-coding mRNAs. Intriguingly, at any stage in time, the larger proportion of the transcriptional output (>90%) is made up of ncRNAs. Recent studies have shown that the ncRNAs are diverse in size, ranging from small RNAs like microRNAs (miRNAs) and piwi-interacting RNAs (piRNAs) which are <32 nucleotides in length, to large long noncoding RNAs (lncRNAs) which can be up to 5,000 nucleotides in length. The precise functions of many ncRNAs are still being discovered; several of them have been identified to control transcription and translation, and thus regulate various biological processes during growth, development, and disease progression (Chandran *et al.*, 2017; Czech and Hannon, 2011; Dharap *et al.*, 2009).

With well-organized regulatory checks and balances, the cellular system exists to prevent abnormalities in normal functions. For this purpose, miRNAs were considered as the guardians of the genome, but what regulate miRNAs is not known. Natural RNA circles called circular RNAs (circRNAs) were reported to function as miRNA sponges to effectively control their levels (Hansen *et al.*, 2013). CircRNAs, which are an elusive class of ncRNAs, are formed by a back-splicing process as from the same set of precursor RNAs which forms protein-coding mRNAs by canonical splicing (Jeck and Sharpless, 2014; Jeck *et al.*, 2013; Memczak *et al.*, 2013; Salzman *et al.*, 2012). As they are covalently closed continuous loops that lack defined 5’ caps and 3’ poly-A tails, making them resistant to RNase R, an enzyme with 3’-to-5’ exonuclease activity that effectively digests nearly all linear RNA species. Thus, due to the lack of free end, circRNAs are incredibly stable with a half-life of more than 48 hours compared to the corresponding linear RNAs (Jeck *et al.*, 2013; Zeng *et al.*, 2017). For several years, circRNAs were considered functionally irrelevant, cryptic viral RNAs, or storage forms of mRNAs; alternatively, they were thought to be merely splicing by-products with low abundance or transcriptional noise, due to splicing of longer mRNAs transcripts (Hsu and Coca-Prados, 1979; Sanger *et al.*, 1976). However, with the advances in the next-generation sequencing (NGS) and circRNA specific bioinformatics analysis, they

are recognized to be ubiquitously present in eukaryotic cells, plants, yeast, and viruses (Arnberg *et al.*, 1980; Hansen *et al.*, 2013; Hsu and Coca-Prados, 1979; Kos *et al.*, 1986; Sanger *et al.*, 1976).

When a protein-coding gene is transcribed under physiological conditions, the resulting precursor mRNA (pre-mRNA) undergoes canonical splicing during which the introns are cleaved, and the 3' end of one exon is joined with the 5' end of an adjacent exon to produce a mature mRNA. However, in some occasions, back-splicing of pre-mRNA can result in exon scrambling (the downstream donor end of the spliced product covalently binds to the upstream splice acceptor site) to form a circRNA (Ashwal-Fluss *et al.*, 2014; Conn *et al.*, 2015; Hansen *et al.*, 2011; Legnini *et al.*, 2017a; Meng *et al.*, 2016; Starke *et al.*, 2015). The exon-scrambling phenomenon was discovered when the spliced nonpolyadenylated exons were observed to be not always paired sequentially in order of their position in genomic DNA for the transcript of tumor suppressor gene, Deleted in Colorectal Cancer (DCC) (Nigro *et al.*, 1991). Subsequently, this pattern was also found for other transcripts such as human mixed-lineage leukemia (MLL), human E26 transformation-specific sequence-1 (ETS-1) and mouse locus sex-determining region Y (SRY) gene. The prevalence of this pattern indicates that exon scrambling might be a process that mimics partial genomic duplication resulting in the formation of excised circles (Bailleul, 1996; Caldas *et al.*, 1998; Capel *et al.*, 1993).

Recent studies have indicated that circRNAs are not the outcome of splicing errors and might be products of a well-regulated process that is potentially associated with normal physiology (Hansen *et al.*, 2013; Memczak *et al.*, 2013). Moreover, accumulating evidence also indicates that circRNAs are dynamically expressed and spatiotemporally regulated in tissue-specific and the development-dependent manner in the brain (Mahmoudi and Cairns, 2019; Memczak *et al.*, 2013; Rybak-Wolf *et al.*, 2015). These characteristics of circRNAs thus seem to be essential for normal biological functions but also could lead to disease progression if their levels are altered. This review describes the formation of circRNAs, their putative functions, role in brain development and aging, and involvement in brain cancer, acute central nervous system (CNS) injury and chronic neurodegeneration. Such an effort is needed to consolidate the present knowledge on circRNAs and define their significance for developing new approaches to treat CNS complications.

2. CircRNA Biogenesis

CircRNAs can be exon-derived, intron-derived, or both exon- and intron-derived; the exon-derived circRNAs are more abundant than the other two subtypes. The circRNA biogenesis follows specific mechanisms mediated by the spliceosomal machinery or by group I and II ribozymes (Chen and Schuman, 2016). During the canonical splicing of pre-RNAs to form mature mRNAs, a lariat with an unusual 2',5'-phosphodiester bond linkage will be hydrolyzed by the debranching enzyme (DBR1) to eliminate introns sequentially. Exon-derived circRNA formation also involves these steps, but back-splicing leads to lariat-driven and intron-pairing driven circularization (Fig. 1) (Chen and Schuman, 2016; Memczak *et al.*, 2013; Rybak-Wolf *et al.*, 2015). However, the frequency of back-splicing events is low and less efficient compared to canonical splicing. In lariat-driven circularization, pre-mRNA is

subjected to partial splicing that results in the skipping of one or more exons, due to the proximity of the exon-donor site and the acceptor site of the different exon on the same loci. It leads to the formation of circRNA intermediates with exons and introns, which will be further processed by canonical splicing machinery to produce exon-derived circRNAs. In the intron-pairing driven circularization model, introns consisting of cis-elements such as inverted *Alu* repeat sequences pair with each other to bring the downstream donor and upstream acceptor sites into close proximity, leading to circularization of exons (Chen and Schuman; Liang and Wilusz, 2014; Szabo *et al.*, 2015; Zhang *et al.*, 2016a). Importantly, if introns flanking an exon contain abundant inverted *Alu* repeats, they will be circularized (Chen and Schuman; Liang and Wilusz, 2014; Szabo *et al.*, 2015; Zhang *et al.*, 2016a). Recent evidence suggests that not all complementary sequences on either side of exons and introns necessarily promote circularization (He *et al.*, 2017; Wang *et al.*, 2014). In addition, RNA binding proteins (RBPs) such as muscleblind (Mbl), quaking, double-stranded RNA editing enzyme- adenosine deaminase acting-on RNA (ADAR), and the nuclear helicase DHX9 also control circRNA biogenesis (Aktas *et al.*, 2017; Ashwal-Fluss *et al.*, 2014; Conn *et al.*, 2015; Ivanov *et al.*, 2015). Mbl promotes RNA circularization at a second exon of the primary RNA transcript by interacting with the flanking introns (Ashwal-Fluss *et al.*, 2014). Quaking promote circularization by binding to its consensus sequences in the adjacent introns in the pre-RNA (Ivanov *et al.*, 2015). While Mbl and quaking promote circularization, ADAR prevents circularization by changing the adenine to inosine, and thereby reduces potential RNA complementarity between flanking introns by pairing inosine with guanosine. This pairing repels the downstream donor site and upstream acceptor sites, thereby preventing circRNA formation (Conn *et al.*, 2015). Similarly, DHX9 also negatively regulates circRNA biogenesis by binding specifically to inverted-repeat *Alu* elements (Aktas *et al.*, 2017). Overall, pre-mRNAs with stable 3’ ends are usually subjected to back-splicing rather than nascent RNA transcripts (He *et al.*, 2017; Liang and Wilusz, 2014).

During canonical splicing of precursor RNA to form mRNA, introns are removed by complementary binding of the consensus sequences, such as GU-rich regions and Cregions near the branching points at the 5’ and 3’ ends of the introns. However, due to two rounds of trans-esterification between the exon and intron branch points, a circular lariat with 2’,5’-phosphodiester bond linkage can be formed which will be linearized by the debranching enzyme and degraded (Fig. 1). However, if the lariat contains a conserved 7-nucleotide GU-rich motif at the 5′ splicing site and the 11-nucleotide C-rich motif at the 3′-branch site, it escapes the action of the debranching enzyme leading to the formation of an intron-derived circRNA (Ashwal-Fluss *et al.*, 2014; He *et al.*, 2017; Kopczynski and Muskavitch, 1992; Qian *et al.*, 1992; Zhang *et al.*, 2013). Furthermore, RBPs prevent linearization by binding near the unusual 2’−5’ link, allowing intron-derived circRNAs to stay stable.

In some instances, un-spliced introns are retained and persist as exon-intron-derived circRNAs during the formation of exon-derived circRNAs (Fig. 1) (Salzman *et al.*, 2012). Exon-consisting lariat precursors have also been identified in yeast following splice-site mutagenesis of the DBR1, suggesting that exon-intron-derived circRNAs are universal (Barrett *et al.*, 2015).

The majority of circRNAs are exon-derived and are highly conserved (Diederichs, 2014; Jeck and Sharpless, 2014; Memczak *et al.*, 2013; Salzman *et al.*, 2012). It has been shown that circRNAs exhibit high conservation between mammals based on the orthologous co-ordinates and having splice sites within two nucleotides (Rybak-Wolf *et al.*, 2015). A recent study also discovered that among a total of 104,388, 96,675, and 82,321 circRNAs in human, macaque, and mouse, respectively, 70,186 were evolutionarily conserved (Ji *et al.*, 2019). Notably, the regions of DNA that encode exon-derived circRNAs have been reported to be more conserved than the exon-flanking DNA (Legnini *et al.*, 2017b; Rybak-Wolf *et al.*, 2015). Although transcribed from the coding regions of genes, majority of them do not translate any proteins due to the lack of open reading frames (ORFs) and internal ribosome entry sites (IRESs) (Legnini *et al.*, 2017a; Pamudurti *et al.*, 2017b; Yang *et al.*, 2017a).

3. Detecting circRNAs

One of the reasons that eluded the detection and identification of circRNAs for long was the lack of sensitive and competent methods that could capture all the transcriptional and posttranscriptional events. Fortunately, with the advent of high throughput sequencing, it became easier to identify various types of RNAs in a cell (Fig. 2). Especially, deep sequencing with longer reads and improved bioinformatics algorithms enabled the curation of new RNA species. Surprisingly, it was found that several fragments mapped to the same gene in some instances can be arranged in the opposite order (Salzman *et al.*, 2012). As circRNAs lack poly(A) tails, they eluded detection for several years as most sequencing studies used RNAs that were isolated by their poly(A) tails. This was addressed by analysing the sequencing reads of splice junctions formed by an acceptor splice site of an exon at a 5′ end and a donor site at a downstream 3′ end (Memczak *et al.*, 2013). The specificity of circRNA detection was further increased by treating the RNA preparations with RNase R that digests the linear RNAs, but not circRNAs (Jeck and Sharpless, 2014; Memczak *et al.*, 2013). In addition, identifying reads from ribosomal RNA-depleted RNA sequencing with backsplicing improved the specificity of circRNA detection to single-nucleotide resolution (Jeck and Sharpless, 2014; Memczak *et al.*, 2013). Furthermore, circRNA microarrays that use junction-specific probes enabled systematic profiling of the circRNA expression patters in linear RNA-depleted samples (Li *et al.*, 2019b; Mehta *et al.*, 2017). Using this approach Li et al. integrated 87,935 human circRNAs sequences to identify ~80,000 circRNAs expressed in the cervical tumors (Li *et al.*, 2019b). Thus, RNA-seq and/or circRNA microarray analysis with RNA preparations in which linear RNAs and ribosomal RNAs were depleted combined with stringent algorithms refined the circRNA identification and detection in mammals (Pandey *et al.*, 2020).

PCR with convergent and divergent primers is another widely used method for circRNA identification (Fig. 2). Convergent primers are conventional primers that face toward each other that are routinely used to amplify linear DNA or RNA. These can be used for detecting circRNAs in linear RNA and ribosomal RNA depleted samples. Whereas, divergent primers which face away from each other and designed to span the circRNA junctions can be used to detect circRNAs without depleting linear or ribosomal RNAs. However, challenges like low abundance and formation of concatamers by rolling circle amplification during reverse transcription might prevent the accuracy of circRNA quantification by PCR. But, these

hindrances can be overcome by using high-throughput droplet digital PCR (ddPCR) that can precisely measure the absolute nucleic acid levels even at low abundance by using the ratio of positive and negative droplets (Fig. 2) (Chen *et al.*, 2018b; Hindson *et al.*, 2013).

Northern blot analysis with either long probes covering an entire circRNA or short probes flanking the splice junction sites can also be used to detect circRNAs (Fig. 2) (Schneider *et al.*, 2018). In the gels used for Northern blot analysis, an exonic circRNA migrates at a slower rate than a linear RNA of equal length because of the limited pore size of the gel (Schneider *et al.*, 2018; Tabak *et al.*, 1988). RNA fluorescence in situ hybridization (RNA-FISH) coupled with high-resolution microscopy using probes that are designed to flank the junction sites is a powerful method to locate the distribution and abundance of circRNAs in the cell (Zirkel and Papantonis, 2018). Further, single-molecule RNA-FISH that uses multiple singly labeled oligonucleotide probes (Stellaris® RNA FISH) can also be used to determine the subcellular localization and absolute quantification of RNA molecules in individual cells (Fig. 2) (Kocks *et al.*, 2018; Piwecka *et al.*, 2017).

4. Putative functions of circRNAs

As circRNAs are formed from many linear RNA precursors in archaea to mammals, they are thought to be functionally relevant (Danan *et al.*, 2012; Jeck *et al.*, 2013; Memczak *et al.*, 2013; Salzman *et al.*, 2012). CircRNAs are also shown to be functionally diverse (Fig. 3). Exonic circular RNAs are mainly cytosolic and thought to act as “decoys” that sponge miRNAs and RBPs, possibly to inhibit their interaction with the target mRNAs or to transport them between cell types (Chu *et al.*, 2015; Hansen *et al.*, 2013; Memczak *et al.*, 2013; You *et al.*, 2015; Zhang *et al.*, 2013). In mammalian neural tissue, the typical miRNA sponging activity was shown for several circRNAs, including cerebellar degeneration - related autoantigen 1 (CDR1), antisense (CDR1as aka ciRS-7) (Memczak *et al.*, 2013); sex determining region Y (SRY) circRNA circSRY (Hansen *et al.*, 2013); and homeodomain interacting protein kinase 3 (HIPK3) circRNA circHIPK3 (Zheng *et al.*, 2016). Interestingly, bioinformatics showed that not all circRNAs contain a sufficient number of binding sites for miRNAs and the typical miRNA sponging function seems to be is unique to only a handful of circRNAs (Jeck and Sharpless, 2014; Memczak *et al.*, 2013; You *et al.*, 2015). However, that does not seem to be the case, as several circRNAs were also shown to sponge miRNAs even with one binding site (Duan *et al.*, 2018; Lei *et al.*, 2019; Li and Diao, 2019b; Wang *et al.*, 2018a; Wu *et al.*, 2019). In addition to sequestering miRNAs, studies also showed that multiple circRNAs that are localized to the nucleus have little affinity for miRNA target sites. These circRNAs contain either introns or both exon and intron, including ci-ankrd52, circEIF3J, and circPAIP2, and control transcription mainly by interacting with the RNA polymerase II (Pol II) and U1 small nuclear ribonucleoprotein particle (U1snRNP) complex (Li *et al.*, 2015; Zhang *et al.*, 2013).

Despite being originated from the coding regions of the genes, exon-derived circRNAs are unable to translate into proteins since the ORFs in them are generally truncated. However, recent studies suggest that some exon-derived circRNAs could be translated into proteins or peptides owing to the presence of an IRES and the start codon AUG (Yang *et al.*, 2017b). In support of this theory, a cloned circRNA with GFP^{+} exons with IRESs under the

cytomegalovirus promoter was shown to be translated into GFP protein (Wang *et al.*, 2015). Accumulating evidence now demonstrates that multiple circRNAs are translated under in vivo conditions by associating with polyribosomes (Legnini *et al.*, 2017b; Pamudurti *et al.*, 2017a).

Intriguingly, adenosine in several RNAs is known to undergo methylation to form N^6-methyladenosine (m^6A) at a specific consensus motif (RRm^6ACH) (Chen *et al.*, 2019b; Liu *et al.*, 2014; Yang *et al.*, 2017b; Zhou *et al.*, 2017), mediated by a complex that consists of methyltransferase-like 3 (METTL3), METTL14, and Wilm's tumor 1-associating protein (Kobayashi *et al.*, 2018; Selberg *et al.*, 2019; Yang *et al.*, 2018c). Recent evidence indicates that m^6A modification is not limited to mRNAs, but also abundant and widespread on circRNAs that are often derived from exons not methylated in mRNAs in a cell type-specific manner (Zhou *et al.*, 2017). This epitranscriptomic modification is thought to promote the translation of circRNAs in a cap-independent manner using short sequences containing m^6A sites as IRESs (Wang *et al.*, 2015; Yang *et al.*, 2017b).

By profiling polysomes associated with circRNAs, it was shown that m^6A-driven circRNA translation is prevalent throughout the human genome (Yang *et al.*, 2017b). Human myoblasts and myotubes showed widespread differences in the expression pattern of circRNAs as a function of the stage of differentiation in Duchenne muscular dystrophy patients (Legnini *et al.*, 2017b). Mainly, circ-ZNF609 was observed to plays a role in myoblast differentiation by producing a protein. Moreover, when siRNAs that selectively targeted the exon junctions but not the parent mRNA of circRNA was used to knockdown circ-ZNF609, the protein coded by the circ-ZNF609 was not formed (Legnini *et al.*, 2017b). The protein forming potential of circRNAs can be confirmed by translating ribosome affinity purification assay, which is used to detect circRNAs associated with ribosomes. One such example circRNA that associates with the polysome is circMbl, and it was reported to translate to a ~10 kDa protein in D. *melanogaster* (Pamudurti *et al.*, 2017a). These studies thus suggest that many circRNAs can potentially produce a protein or a peptide; however, their functional significance needs to be identified.

5. CircRNAs in brain development and aging

In the mammalian brain, ~20% of the protein-coding genes are known to produce circRNAs (You *et al.*, 2015). The latest research shows that circRNAs are present in all rat tissues but enriched in brain tissue, where their levels continuously increase from 2 weeks to 104 weeks of age (Mahmoudi and Cairns, 2019). Likewise, circRNAs were revealed to be more abundant in the brain than in any other organ, including the heart, liver, and lungs in adult mice (You *et al.*, 2015). Additionally, research in the pigs also showed that circRNAs are highly expressed in the brain, and their levels in various brain structures are developmentally heterogeneous (Veno *et al.*, 2015). The abundance of circRNAs in the brain could be due to the host genes that are exclusively expressed in the brain or their relative contribution to form circRNAs is higher in the brain than any other organs. These studies thereby indicate that the mammalian brain is unique among all other organs owing in part to the abundance of circRNAs (Rybak-Wolf *et al.*, 2015; You *et al.*, 2015). Moreover, the abundance seems to depend on the circRNA's formation and functions.

Several recent studies indicate that circRNAs play an essential role in regulating CNS development (Fig. 4). Many brain-enriched circRNAs were observed to be associated with neurotransmitter function, neuron maturation, and synaptic activity (Mahmoudi and Cairns, 2019). For example, a circRNA derived from Foxo3 gene (circFoxo3) was shown to bind and impair cyclin-dependent kinase 2 (CDK2) activity, leading to disrupted cell cycle progression (Du et al., 2016). Besides, a circular RNA called circHomer 1 that originated from the Homer Scaffolding Protein 1 pre-RNA (Homer 1 is a member of the Homer family of dendritic proteins) is thought to modulate some of the structural changes at synapse during neuronal plasticity and development (You *et al.*, 2015). Many synaptically-enriched circRNAs are also reported to be derived from the genes of transformed growth factor (TGF-β) pathway, Wnt signaling pathway, and axon guidance (van Rossum *et al.*, 2016; Veno *et al.*, 2015; You *et al.*, 2015; Zhang *et al.*, 2016b). All of these studies implicate circRNAs in the neuronal plasticity.

Furthermore, studies showed that 58% of cerebral circRNAs are developmentally regulated, while only 2% of their linear isoforms have shown this trend (Mahmoudi and Cairns, 2019). A cerebral cortex transcriptomic analysis study using three-month-old and eight-year-old pigs showed the circRNAs expression in the brain is dependent on developmental age (Chen *et al.*, 2019a). This study also showed that >80% of mRNAs, miRNAs, and lncRNAs, but <22% of circRNAs, are expressed at both ages. Interestingly, many developmentally regulated circRNAs also showed sexual dimorphism in the brain and were observed to target aging-associated mRNAs (Mahmoudi and Cairns, 2019). Thus, circRNAs might dictate the development and aging process by changing the expression and availability of specific mRNAs.

In the human brain, on an average, at least three circRNAs are formed from annotated exons of the individual gene, whereas more than 2,000 genes form ten or more circRNA isoforms. Thus, the majority of the exon-derived circRNAs preferentially contain coding sequences and 5’ UTR exons (Rybak-Wolf *et al.*, 2015). The circRNA expression profiles are distinct in various regions of the brain. The prefrontal cortex, olfactory bulb, cerebellum, and hippocampus were shown to express region-specific sets of circRNAs in the mouse brain (Rybak-Wolf *et al.*, 2015). Interestingly, evidence also suggests that several of the circRNAs formed from the coding regions of the genes are expressed at a higher level than their canonical/linear isoforms, thereby indicating the existence of cell type-specific mechanisms of production and/or degradation of circRNAs (Rybak-Wolf *et al.*, 2015). The abundance of circRNAs in the brain was thought to be linked to synaptogenesis since an abrupt increase in circRNA expression levels occurs during the transition from P10 to P30 in mice (You *et al.*, 2015). A similar increase in circRNA abundance was also reported in humans, where ~6,000 circRNAs were detected during embryonic development, and their number increased significantly in fetal (~89,000) and adult (~65,000) brains (Chen *et al.*, 2018a; Rybak-Wolf *et al.*, 2015; Szabo *et al.*, 2015). The synaptogenic function of circRNAs was also supported by their host genes that are particularly enriched in synaptosomes and related to synaptic function (You *et al.*, 2015). In situ hybridization studies showed that several of the synaptic function-related circRNAs including circHomer1, circNlgn1, circElavl1, cricKlhl1, circDscam, circGigyf2, circRmst, and circNbea are abundantly present in the dendritic arbor (You *et al.*, 2015). It is also evidenced that mouse neural stem cells at various stages of

differentiation showed a set of differentially expressed circRNAs coexpressed with development-related mRNAs (Yang *et al.*, 2018a). In addition to changes in circRNA expression profiles as a function of development, aging was also shown to alter circRNA levels in the *Drosophila* CNS (Westholm *et al.*, 2014).

The abundance and/or the distribution of circRNAs can change with exposure to radiation during development. A recent study showed that the abundance of the circular transcript variants of Pvt1, Ano3, Sec14l5, and Rnf169 (p53 target genes) continuously increased in the mouse brain from embryonic day 12 until adulthood, while the increase in the respective linear RNAs ceased by postnatal day 30 (Mfossa *et al.*, 2019). Moreover, the mRNA expression of p53 target genes peaked before 6h, while the circRNAs formed form the same genes reached the highest expression at 12h or later in the embryonic brain and primary cortical neurons exposed to ionizing radiation (Mfossa *et al.*, 2019). Overall, these findings suggest that circRNAs are dynamically expressed in a development-stage specific manner in the brain.

6. CircRNAs and brain cancer

Glioma progression is mediated by cell proliferation, invasion, migration, and apoptosis. Angiogenesis and the secretion of various pro-angiogenic growth factors modulate these processes, but circRNAs also play a vital role. It is supported by many studies that showed a strong correlation between circRNA expression and glioma progression (Fig. 4)(Bian *et al.*, 2018; Duan *et al.*, 2018; Hu and Zhang, 2019; Li *et al.*, 2018b; Li and Diao, 2019a; Xie, 2018). Notably, several circRNAs, including circSMARCA5, circHIPK3, circMTO1, circTTBK2, circ007628, and circMMP9, are known to promote glioma progression by sponging miRNAs and thereby targeting their respective host genes and/or down-stream genes (Barbagallo *et al.*, 2019).

It was shown that increased levels of circSMARCA5 promote angiogenesis and glioma progression by sponging serine and arginine-rich splicing factor 1 (SRSF1) mRNA, and thus regulating VEGF-A mRNA (Barbagallo *et al.*, 2019). VEGF-A is a potent inducer of angiogenesis that leads to the progression of solid tumors. Similarly, circ_002136 (circCDK11A_001), which is formed through the looping of linear cyclin-dependent kinase 11A transcript variant 1 (CDK11A-VT1), seems to regulate the expression of SOX13. SOX13, in turn, is known to controls the expression of the spondin-2 gene that is recognized for tumor aggressiveness (He *et al.*, 2019; Jin *et al.*, 2017). It has been shown that circ_002136 but not the linear CDK11A is highly expressed in glioma cells together with SOX13. Increased circ_002136 level sponges miR-138–5p and prevents the repression of SOX13 (He *et al.*, 2019). SOX13 thus induce the expression of spondin-2 and thereby promoting glioma angiogenesis (He *et al.*, 2019). Likewise, circRNA circZNF292 (cZNF292) induced by hypoxia down-regulates various components of the Wnt/β-catenin signaling pathway, including cyclin A, CDK2, p-CDK2, β-catenin, p-STAT3 (Tyr705), p-STAT5 (Tyr694), and proline-rich protein 11 (PRR11) (Yang *et al.*, 2016). All these factors play a crucial role in cellular proliferation, migration, invasion, and angiogenesis during glioma progression. When circZNF292 was silenced in glioma cell lines (U87MG and U251), the expression of cyclin A, CDK2, p-CDK2, β-catenin, p-STAT3 (Tyr705), p-STAT5

(Tyr694), and PRR11 was decreased, indicating that circZNF292 is crucial for glioma proliferation and tube formation (Boeckel *et al.*, 2015; Yang *et al.*, 2016). Furthermore, another circRNA called circSHKBP1 (circ_0000936) that is highly abundant in human endothelial cells was also shown to regulate angiogenesis through miR-544a/FOXP1 and miR-379/FOXP2 (He *et al.*, 2018; Salzman *et al.*, 2013). Knockdown of circSHKBP1 upregulated the expression of miR-544a/miR-379 and reduced the expressions of their target mRNAs FOXP1/FOXP2. It is suggested that FOXP1/FOXP2 levels are positively associated with various types of tumors. Therefore, knockdown of circSHKBP1 suppresses the expression of FOXP1/FOXP2 by miR-544a/miR-379, which thereby prevents cell viability, migration, and tube formation in glioma (He *et al.*, 2018). Hence, all these above-listed studies point out that circRNAs are the dominant regulator of angiogenesis during glioma progression.

In addition to promoting angiogenesis and proliferation of the glioma cells, several circRNAs were also shown to play a potential regulatory role in the viability, migration, and invasion of tumor cells. For example, circHIPK3 promotes invasiveness of tumor cells by silencing miR-124–3p and thus derepressing its target STAT3 (Hu and Zhang, 2019). Similarly, sponging of miR-124 by circMMP9 was also shown to promote migration and invasion of glioma cells through cyclin-dependent kinase 4 and Aurora kinase A, both of which are the targets of miR-124 (Wang *et al.*, 2018a). Likewise, circ_007628 induces glioma oncogenesis by sponging miR-181a and thus increasing the expression of its target SIRT1 (Lei *et al.*, 2019). Similarly, circITCH downregulation promotes both migration and invasion of glioma cells and is also associated with poor prognosis of glioma patients (Li *et al.*, 2018a). By contrast, overexpression of circITCH suppressed cell proliferation and prevented apoptosis by sponging miR-214 and thus allowing the expression of miR-214 target ubiquitin-protein ligase E3. Furthermore, both circITCH and circZNF292 seems to target the Wnt/β-catenin pathway to induces glioma progression (Li *et al.*, 2018a; Yang *et al.*, 2016).

Additionally, circTTBK2 was shown to target miR-217 and the downstream hepatocyte nuclear factor-1β (HNF1β)/Derlin-1 pathway. HNF1β is a homeobox transcription factor that facilitates glucose uptake and glycolytic activity (Okamoto *et al.*, 2015; Yu *et al.*, 2015), and Derlin-1 prevents endoplasmic reticulum stress-induced apoptosis (Wang *et al.*, 2008). Intriguingly, circTTBK2 is induced in glioma and its enhanced expression prevented cell apoptosis and thereby promoted proliferation, migration, and invasion of glioma cells by targeting HNF1β/Derlin-1 pathway (Zheng *et al.*, 2017). Another circRNA called circ_0046701, which is induced in gliomas, sequester miR-143–3p and thereby derepress miR-143–3p target integrin subunit beta 8 (ITGB8). ITGB8 could promote glioma cell proliferation and invasion (Li *et al.*, 2018b). However, when silenced, circ_0046701 prevented cell proliferation and invasion by suppressing ITGB8 in glioma.

A recent study also showed that levels of CDR1 and circRNA circ_0001946 were downregulated, while miR-671 (which target CDR1 and circ_0001946) was upregulated in glioblastoma; these changes were thought to promote glioblastoma growth (Li and Diao, 2019b). When overexpressed, circ_0001946 induced apoptosis and reduced the proliferation, migration, and invasion of glioblastoma cells by sponging miR-671 and derepressing its

target CDR1 (Li and Diao, 2019b). Likewise, the miRNA miR-630 promotes chemoresistance of glioblastoma to temozolomide, whereas circMTO1 induction sponges miR-630 and thereby reverse the chemoresistance of glioblastoma to temozolomide (Rao *et al.*, 2018).

Although the translation of circRNAs is not a dominant event, circPINTexon2 formed from the exon 2 of LINC-PINT gene was shown to translate an 87 amino acid tumor-suppressive peptide (PINT87aa) (Zhang *et al.*, 2018a). PINT87aa is expressed at high levels in tissues of the brain, liver, kidney, and stomach, and at a lower level in tissues of the breast, intestine, thyroid, and pancreas (Zhang *et al.*, 2018a). PINT87aa interacts with PAF1 and inhibits the transcriptional elongation of PAF1 downstream oncogenes, including CPEB1, SOX-2, and c-Myc (Zhang *et al.*, 2018a). Down-regulation of circPINTexon2 and PINT87aa in brain tumors negatively impacts the glioma clinical prognosis (Zhang *et al.*, 2018a). The circRNA circFBXW7 formed by circularization of the transcript of exon 3 and exon 4 of the FBXW7 gene encodes a 185 aa protein called FBXW7–185aa which is abundantly expressed in the healthy human brain, but reduced in the gliomas (Yang *et al.*, 2018b). When overexpressed in glioma cells, circFBXW7 (but not the linear FBXW7), inhibited proliferation and cell cycle acceleration, whereas silencing of FBXW7 promoted glioma malignancy. Moreover, intracranial implantation of circFBXW7 overexpressing cells increased the life span of mice. These functions of circFBXW7 are mediated by FBXW7–185aa, which destabilizes c-Myc through USP28dependent ubiquitination (Yang *et al.*, 2018b). All of these studies indicate that circRNAs regulate glioma growth and invasion by sponging miRNAs and preventing the suppression of miRNA targets. Thus, circRNAs acts as a competitive endogenous RNA (ceRNA) to control miRNA availability and function during glioma progression.

7. CircRNAs in secondary brain damage following acute CNS insults

Acute CNS insults, including spinal cord injury (SCI), traumatic brain injury (TBI), and stroke, are leading causes of death and long-term disability in humans. These insults can occur in both sexes and at various ages. Acute CNS insults impair motor functions, cognitive functions, and neuropsychiatric functions in affected individuals. Decades of human and animal studies have not identified any viable therapeutic targets that can be modulated to prevent neuronal death and neurologic dysfunction after any acute CNS injury. Many studies have shown that acute CNS insults result in significant alterations in the ncRNA profiles and functions (Dharap *et al.*, 2009; Dharap *et al.*, 2011, 2012; Gaudet *et al.*, 2016; Mehta *et al.*, 2015; Mehta *et al.*, 2017; Meissner *et al.*, 2016; Paim *et al.*, 2019; Qin *et al.*, 2018; Redell *et al.*, 2010; Sabirzhanov *et al.*, 2016; Shi *et al.*, 2019; Zhang *et al.*, 2019; Zhao *et al.*, 2018).

Recent studies showed that SCI significantly alters the expression profiles of circRNAs in the adult rat brain (Qin *et al.*, 2018; Zhou *et al.*, 2019). Gene Ontology (GO) analysis showed that circRNAs altered after SCI might modulate pathways associated with AMP-activated protein kinase signaling and peroxisomes (Qin *et al.*, 2018), and might also participate in the post-SCI secondary damage via circRNA-targeted miRNA-mRNA axis (Zhou *et al.*, 2019). Recent studies showed that TBI also alters circRNA profiles in the cortex and hippocampus of adult rodents, and the altered circRNAs might be involved in modulating inflammation, cell death, and repair directly or indirectly through circRNA/

miRNA interaction (Fig. 4))(Jiang *et al.*, 2019b; Xie *et al.*, 2018). In addition to being present inside the cell, a new study showed that various circRNAs are differentially present in the exosomes isolated from the brain of a mouse after TBI. There is some possibility that altered circRNAs play a role in synaptic plasticity (Zhao *et al.*, 2018). However, the functional significance of any of the circRNAs altered after traumatic injuries to CNS has not yet been evaluated in detail.

Neonatal hypoxia-ischemia (HI) is a frequent problem that arises when blood supply to the fetus is interrupted at gestational week 36 or later. HI leads to hypoxic-ischemic encephalopathy, a condition that is characterized by long-term motor, sensory, and cognitive disabilities. A recent study showed that in a rat model of HI, expression of many circRNAs was significantly altered, and bioinformatics analysis of circRNA/mRNA networks predicted that these RNAs might be involved in brain damage as well as neural degeneration (Jiang *et al.*, 2019a). Induction of periventricular white matter damage in rats, which is a characteristic pathology of HI, also differentially induced the expression of many circRNAs that influence mechanisms such as glutamatergic synaptic function and the VEGF signaling (Zhu *et al.*, 2018).

Stroke is the leading cause of disability in the adult population all over the world. Many mechanisms, including ionic imbalance, edema, inflammation, oxidative stress, endoplasmic reticulum stress, and apoptosis, are thought to promote post-stroke secondary brain damage synergistically (Mehta *et al.*, 2007). Several studies recently showed that stroke rapidly alters the expression profiles of various classes of ncRNAs, including miRNAs, ncRNAs, transcribed ultraconserved regions (T-UCRs), and piRNAs (Dharap *et al.*, 2009; Dharap *et al.*, 2011, 2012). This research also demonstrated that altered ncRNA function leads to a compromised translation and transcription process and thereby modulates the post-ischemic functional outcome (Dharap *et al.*, 2013a; Dharap *et al.*, 2013b; Kim *et al.*, 2018; Mehta *et al.*, 2015; Pandi *et al.*, 2013). We recently reported that transient focal cerebral ischemia in adult mice induces significant changes in the expression of many circRNAs in a sustained manner between 6h to 1 day of reperfusion (Mehta *et al.*, 2017). Another study also reported a change in the expression profile of many circRNAs at two days after reperfusion in the mouse brain following focal ischemia (Liu *et al.*, 2017b). A third recent study also reported altered expression of many circRNAs in the ischemic infarct area of rats four days after focal cerebral ischemia (Duan *et al.*, 2019).

Interestingly, all of these studies show that the majority of circRNAs altered after stroke are coded from the exonic regions of the protein-coding genes (Duan *et al.*, 2019; Liu *et al.*, 2017b; Mehta *et al.*, 2017). However, it is unclear if the altered circRNA levels in the post-stroke brain are due to an altered preference to form circRNAs from linear RNAs, and if any of these circRNAs regulate parent RNA translation after stroke. At this time, the functional significance of altered circRNAs in post-ischemic pathophysiology is not known. However, bioinformatics analysis has shown that circRNAs altered after stroke might modulate mitogen-activated protein kinase signaling, Rap1 signaling, Hippo signaling, autophagy, and endocytosis all of which are known to be associated with cell survival and/or death pathways (Duan *et al.*, 2019; Liu *et al.*, 2017b; Mehta *et al.*, 2017). A recent study showed that expression of circRNA TLK1 (circTLK1) was increased in the mouse brain and plasma after

transient MCAO, as well as in the plasma of patients with acute ischemic stroke (Wu *et al.*, 2019). CircTLK1 is formed from exons 2 and 3 of the TLK1 gene, and the expression of TLK1 linear mRNA was decreased in the ischemic brain. Interestingly, when circTLK1 was knocked down, ischemic mice showed decreased apoptosis, alleviated neurological deficits, reduced infarct volume, and improved somatosensory functions (Wu *et al.*, 2019). These effects of circTLK1 were mediated by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-inducible poly (ADP-ribose) polymerase (TIPARP), a member of the poly-ADP-ribose polymerase (PARP) family that is known to participate in DNA repair and cell death (Han *et al.*, 2018). The mRNA level of TIPARP was also increased, while miRNA miR-335–3p, which binds to 3'-UTR of TIPARP, was decreased in the brain after transient MCAO. Knockdown of TIPARP resulted in reduced neurological deficits, smaller infarct volume, lowered brain atrophy volume, curtailed mortality, and improved motor function after transient MCAO (Wu *et al.*, 2019). Moreover, decreased levels of miR-335–3p were the result of sponging by circTLK1, suggesting that the circTLK1/miR-335–3p axis worsens ischemic injury through TIPARP (Wu *et al.*, 2019).

Similarly, another study also identified that TIPARP could be regulated by circRNAs circHECTD1 whose expression in significant increased in the ischemic mouse brain, as well as in the plasma of patients with acute ischemic stroke (Han *et al.*, 2018). CircHECTD1 sponges miR-142, which targets TIPARP and promotes astrocyte activation and autophagy (Han *et al.*, 2018). This study further observed that circHECTD1 knockdown leads to the loss of miR-142 sponging and thus derepresses TIPARP, resulting in curtailed autophagy and improved post-ischemic functional outcome (Han *et al.*, 2018). Together, these studies suggest that circTLK1 and circHECTD1 seem to regulate post-ischemic neurologic outcome by targeting TIPARP through sponging miR-335–3p and miR-142, respectively.

Furthermore, a recent study showed that ectopic expression of miR-29b suppresses neuronal apoptosis when exposed to conditioned medium from OGD-subjected and miR-29b mimic transfected microglia (Wang *et al.*, 2019). Our lab previously showed that post-stroke downregulation of miR-29c promotes ischemic brain damage by derepressing its target DNA methyltransferase 3a (Pandi *et al.*, 2013). Intriguingly, miR-29b could inhibit JNK2/STAT3-signaling by inducing SOCS-1, a very potent member of the suppressors of the cytokine-signaling family of proteins involved in the immune response (Wang *et al.*, 2019). JNK2/STAT3-signaling is known to regulate IL-1β production in microglial cells following OGD (Wang *et al.*, 2019). When hippocampal neurons were cultured in a microglial-conditioned medium containing IL-1β, there was significant neuronal apoptosis. Interestingly, miR-29b is a target of circRNA circPTK2, which is also induced following OGD. Therefore, overexpression of circPTK2 suppressed miR-29b and induced apoptosis. Conversely, silencing of circPTK2 preserved miR-29b levels and promoted SOCS-1 mRNA and protein expression. Additionally, circPTK2 silencing inhibited JAK2/STAT3 activation and IL-1β production and thereby prevented neuronal apoptosis (Wang *et al.*, 2019).

Down-regulation of some circRNAs after stroke was also shown to promote ischemic brain damage. For example, circDLGAP4 functions as an endogenous miR-143 sponge; in the post-ischemic mouse brain, circDLGAP4 was downregulated, leading to upregulation of miR-143 (Bai *et al.*, 2018). Plasma from acute stroke patients showed decreased levels of

circDLGAP4 (Bai *et al.*, 2018). Increased levels of miR-143 promoted Evans blue extravasation by negatively regulating the expression of tight junction proteins, including claudin-5, occludin, and ZO-1 in the control mice subjected to cerebral ischemia. By contrast, silencing of miR-143 or overexpression of circDLGAP4 ameliorated this change and improved cerebrovascular integrity by preserving the levels of tight junction proteins in mice after transient cerebral ischemia. Furthermore, miR-143 was shown to disrupt the endothelial-mesenchymal transition responsible for maintaining BBB integrity (Bai *et al.*, 2018). This study demonstrated that endothelial cells express mesenchymal cell markers such as Col I, Col III, and α-SMA upon downregulation of circDLGAP4 in mice subjected to transient focal ischemia (Bai *et al.*, 2018). Overexpression of circDLGAP4 or silencing of miR-143 curtailed the increases in Col I, Col III, and α-SMA expression, suggesting that overexpression of circDLGAP4 protects the post-stroke brain by curtailing miR-143-mediated endothelial-mesenchymal transition and BBB disruption (Bai *et al.*, 2018).

8. CircRNAs in chronic neurodegenerative diseases

8.1. Alzheimer′s disease:

Alzheimer′s disease (AD) is one of the most prevalent, irreversible, and progressive forms of dementia in the elderly population; it is caused by a combination of genetic, lifestyle, and environmental factors. Over the years, amyloid-β (Aβ) and tau proteins have been reported to play critical roles in AD pathogenesis. More specifically, the amyloid precursor protein (APP), which is converted to Aβ protein by β- and γ- secretases, clusters together to form toxic amyloid plaques and kill neurons by disrupting intracellular communication. Moreover, tau protein also accumulates to form neurofibrillary tangles that are toxic to neurons. Recent reports suggest that circRNA play a vital role in the development of AD. Notably, the accumulation of circRNAs in the cortex and hippocampus of aged mice seemed to promote an age-related decline in neural function, leading to potential susceptibility to age-related neurodegenerative diseases (Gruner *et al.*, 2016). In a mouse AD model, treatment with Panax notoginseng saponins, which is known to curtail the pathological progress of AD, led the altered expression of several circRNAs that were suggested to modulate AD pathology (Huang *et al.*, 2018). Moreover, dysregulation of hundreds of circRNAs was also shown in the hippocampus of an AD rat model (Wang *et al.*, 2018b).

A recent study identified 17 circRNAs that are derived from the Aβ coding region of the APP gene (Mo *et al.*, 2018). One of those called Aβ circRNA was observed to contain an ORF and has the potential to be translated to form Aβ-related peptide. This seems to be instrumental in increasing the levels of Aβ and Aβ plaques (Mo *et al.*, 2018). In addition to the aforementioned reason, Aβ circRNA was also shown to regulate glycogen synthase kinase-3β (GSK-3β) levels. GSK-3β is known to phosphorylate tau and promotes its aggregation into neurofibrillary tangles (Rankin *et al.*, 2007). Thus, Aβ circRNA also plays a vital role in AD pathogenesis by regulating GSK-3β and tau phosphorylation (Mo *et al.*, 2018).

Additionally, CDR1as (ciRS-7), which is an abundant circRNA in the human brain, was shown to be downregulated in the brains of AD patients (Lukiw, 2013). CDR1as has >70 binding sites for the miRNA miR-7 and thus acts as a miR-7 sponge (Hansen *et al.*, 2013;

Piwecka *et al.*, 2017). Ubiquitin protein ligase A (UBE2A), which catalyzes the proteolytic clearing of toxic amyloid peptides in the AD brain, is a robust target of miR-7 (Zhao *et al.*, 2016). It was shown that sporadic AD is associated with a misregulated CDR1as-miR-7UBE2A system (Zhao *et al.*, 2016). Additionally, CDR1as is also shown to prevent NF-κB translation. NF-κB, which one of the other targets of miR-7, regulates the expression of ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) (Shi *et al.*, 2017; Zhao *et al.*, 2015). UCHL1 is known to control the processing of APP and the degradation of β site APP - cleaving enzyme 1 (BACE1) (Zhang *et al.*, 2012). Therefore, CDR1as-dependent inhibition of NF-κB translation derepresses UCHL1 and thereby allowing the degradation of APP and BACE1 (Shi *et al.*, 2017). Moreover, upon activation by tumor necrosis factor -α (TNF-α), NF-κB negatively regulates the transcription of the UCHL1 gene by binding to its response sequences within the promoter. Interestingly, overexpression of CDR1as elevates the NF-κB inhibited UCHL1 promoter activity and thereby increases the mRNA and protein levels of UCHL1 (Shi *et al.*, 2017). By contrast, a similar effect was not observed when NF-κB was inhibited with BAY117082, a specific inhibitor of NF-κB (Shi *et al.*, 2017). Furthermore, it was also shown that overexpression of CDR1as resulted in decreased protein levels of NF-κB (p65) without altering the NF-κB mRNA levels (Shi *et al.*, 2017). Taken together, these studies suggest that circRNAs epigenetically control the gene expression leading to AD pathogenesis.

8.2. Parkinson's disease:

Parkinson's disease (PD) is a progressive neurodegenerative disorder associated with dopaminergic neuronal death in the substantia nigra that controls motor function. PD patients thus experience tremors, bradykinesia, limb rigidity, and balance problems. Presence of Lewy bodies that contain α-synuclein (α-Syn) protein aggregates is a hallmark of PD. Many ncRNAs, including miRNAs, lncRNAs and circRNAs, are known to play a role in PD progression (Idda *et al.*, 2018; Kraus *et al.*, 2017; Liu *et al.*, 2016; Majidinia *et al.*, 2016; Zhou *et al.*, 2018). Recent studies suggest that α-Syn is a major target of miR-7 (Junn *et al.*, 2009; Kim *et al.*, 2018). In the substantia nigra of PD patients, miR-7 levels were reported to be decreased, which correlates with α-Syn accumulation and aggregation (McMillan *et al.*, 2017). Interestingly, CDR1as is predominantly expressed in excitatory neurons where it regulates miR-7 stability and/or transport, and hence a loss of CDR1as leads to miR-7 deregulation, thus affecting the sensorimotor gating in PD (Piwecka *et al.*, 2017). Another circRNA called circZip-2 (derived from the Zip-2 gene) indirectly regulates α-Syn levels. Zip-2 is a bZIP transcription factor family member and mediates immune responses. CircZip-2 expression was detected in wild-type and a transgenic strain (NL5901) of *C. elegans* that expresses human α-synuclein. It was shown that circZip-2 expression decreased in PD. Interestingly, when Zip-2 was silenced with RNAi, it reduced α-Syn aggregation and reactive oxygen species (ROS) generation in a *C. elegans* PD model (Kumar *et al.*, 2018). These results indicate the possibility of competition between circRNA and its parental gene for their expression (Kumar *et al.*, 2018).

8.3. Amyotrophic lateral sclerosis:

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that causes a selective motor-neuron loss in the brainstem and spinal cord, specifically as they control

voluntary muscle movement. The cause of ALS in most patients is unknown, but mutations in the fused-in sarcoma (FUS) gene and its involvement in familial ALS was demonstrated in previous studies (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009). The normal FUS is a DNA and RNA-binding protein localized in the nucleus where it plays various roles, including DNA repair, transcription, splicing, and translation (Lagier-Tourenne *et al.*, 2012). The mutant FUS forms toxic ribonucleoprotein inclusion bodies, however, and accumulates in the cytoplasm (Sharma *et al.*, 2016; Yang *et al.*, 2015). FUS regulates circRNA biogenesis in motor neurons (MNs) by binding to the exon-intron junctions. When FUS was depleted with RNAi in neural crest-derived neuroblasts (N2A), or with MNs derived from FUS knockout mice, many circRNAs were down-regulated (Errichelli *et al.*, 2017; Verheijen and Pasterkamp, 2017).

Similarly, TAR DNA binding protein 43 (TDP-43), which is a nuclear RNA- and DNA-binding protein, regulates transcription, and RNA splicing. However, when mislocalized to the cytoplasm, TDP-43 forms toxic inclusions that cause sporadic ALS (Lagier-Tourenne *et al.*, 2012; Mackenzie *et al.*, 2010). The toxicity of TDP-43 inclusions can be reduced by inhibiting DBR1, an enzyme responsible for linearizing the circular intronic lariat during splicing events (Armakola *et al.*, 2012). Suppression of DBR1, therefore, facilitates the accumulation of circular intronic lariats and sequestration of TDP-43. Although definitive evidence is lacking about whether these circRNAs play any role in regulating the progression of ALS due to FUS deletion/mutation or DBRI inhibition, these studies indicate the possibility of circRNAs playing a role in ALS.

8.4. Multiple sclerosis:

Multiple sclerosis (MS) is a relapsing or progressive immune-mediated, inflammatory, a demyelinating disease characterized by the loss of oligodendrocytes (Dutta and Trapp, 2014). Due to the heterogeneous nature of this disease, the treatment and prognosis of MS are quite challenging. It has been proposed that various ncRNAs, including miRNAs and lncRNAs, play a role in regulating gene expression and outcomes in MS (Cardamone *et al.*, 2018; Du *et al.*, 2009). A recent study showed that >400 circRNAs are differentially expressed in the leucocytes of MS patients with the majority of them being down-regulated (Iparraguirre *et al.*, 2017). The interplay between various ncRNAs could be significant in regulating the MS-associated alternative splicing events. For instance, lncRNAs upregulated in MS patients can modulate the expression of splicing regulatory genes, thereby affecting the global splicing and back-splicing processes (Cardamone *et al.*, 2018). In particular, a lncRNA called MALAT1 was shown to modulate back-splicing and expression of 49 circRNAs, thus contributing to the heterogeneity in the pathogenesis of MS (Cardamone *et al.*, 2018).

8.5. Multiple system atrophy:

Multiple system atrophy (MSA) is a rare, sporadic, and rapidly progressive neurodegenerative disorder that affects the involuntary functions of the body, including breathing and blood pressure. Many of the symptoms of MSA, including slow movement, rigid muscles, and poor balance, are similar to PD (Brisinda *et al.*, 2014; Lipp *et al.*, 2009; Yamasaki *et al.*, 2019). Moreover, pathological α-Syn accumulation in the brains of MSA

patients seems to play a leading role in MSA disease progression (Prusiner *et al.*, 2015; Yamasaki *et al.*, 2019). Altered expression of miRNAs in the cerebellum, serum, and cerebrospinal fluid, and long intergenic ncRNAs in the frontal cortex of MSA patients has been reported (Kume *et al.*, 2018; Lee *et al.*, 2015; Marques *et al.*, 2017; Mills *et al.*, 2016). In an MSA mouse model, early changes in miRNA-mRNAs interactions were thought to precede the clinical onset of the disease (Schafferer *et al.*, 2016). Intriguingly, circRNAs IQCK, MAP4K3, EFCAB11, DTNA, and MCTP1 were overexpressed, whereas their linear transcripts were not altered in the frontal cortex of MSA patients (Chen *et al.*, 2016). This difference also suggests that in specific pathologies, expression of circRNAs and their parent linear RNAs is controlled independently (Rybak-Wolf *et al.*, 2015; Szabo *et al.*, 2015). Notably, there appear to be circRNA hotspots from which more than one circRNAs are produced, and 21 such genes in the MSA transcriptome were reported to individually form over 10 circRNAs (Chen *et al.*, 2016). However, the precise role of circRNAs in MSA pathology is yet to be identified.

9. CircRNAs and other CNS disorders:

The circRNAs have also been shown to play a role in methamphetamine addiction (Li *et al.*, 2019a). In human postmortem schizophrenia brain samples, levels of many circRNAs were observed to be lower than in healthy controls (Mahmoudi *et al.*, 2019), although the significance of these changes is not yet known. A recent study showed that high-fat, diet-induced diabetes significantly alters the circRNA expression profiles in the brain cortex of adult mice; more importantly, they found a correlated expression of circRNAs and their linear counterpart mRNAs, indicating the transcriptional control (Yoon *et al.*, 2019). In addition to these findings, circRNA levels were reported to be altered in the brains of patients with psychiatric disorders such as a major depressive disorder (MDD) (Cui *et al.*, 2016; Zhang *et al.*, 2018b). For example, levels of hsa_circRNA_103636 were significantly altered after 8weeks of antidepressant treatment in MDD patients (Cui *et al.*, 2016). Furthermore, circDYM (which is formed from the exons 4, 5, and 6 of the DYM gene and acts as a miR9 sponge) was significantly decreased in the blood of patients with MDD and chronic unpredictable stress (Zhang *et al.*, 2018b). CircDYM levels were also decreased in the blood of a lipopolysaccharide-induced depression-like mouse model (Zhang *et al.*, 2018b). The increased expression of miR-9 is linked to microglial activation and inflammation (Yao *et al.*, 2014). When overexpressed, circDYM ameliorated depression-like behavior by sponging miR-9 and inhibiting microglial activation (Zhang *et al.*, 2018b). These effects of circDYM were mediated in part by HECTD1, which is repressed by miR-9 and derepressed by circDYM overexpression (Zhang *et al.*, 2018b).

10. Advances and potential challenges in circRNA-based therapies

Altered levels of circRNAs during development or disease conditions can change the functional dynamics of the cell by regulating gene expression. Accumulated evidence suggests that manipulation of circRNAs by knockdown, overexpression, and gain- and loss-of-function mutations are potentially beneficial in alleviating the effects of a disease (Santer *et al.*, 2019). Specific circRNAs can be knocked-down with siRNAs and adenovirus or lentivirus encoded shRNAs (Du *et al.*, 2017; Holdt *et al.*, 2016; Liu *et al.*, 2017a; Shan *et al.*,

2017). One of the challenges of siRNA/shRNA-mediated circRNA knockdown is the ability to target backsplice junction sites to avoid the nonspecific targeting of linear host RNAs. An interesting much needed future strategy in circRNA based therapies is to develop methods to prevent formation of a specific circRNA in addition to knocking-down an already formed mature circRNA (Holdt *et al.*, 2018). A recent study used CRISPR/Cas9 genome-editing to delete circRNA CDR1as locus to understand the functional and biological relevance of CDR1as in the mammalian brain (Piwecka *et al.*, 2017). Using a similar approach, circGCN1L1 expression was abolished without affecting the transcription of the linear GCN1L1mRNA by excising the intronic complement sequence of the circGCN1L1-flanking introns (Zhang *et al.*, 2016b). The overexpression of a specific circRNA can be achieved with an adenoviral or a lentiviral vector carrying the circRNA sequence (Bai *et al.*, 2018; Hansen *et al.*, 2013). US FDA recently approved Zolgensma, which is an adenovirus-based gene therapy to correct bi-allelic mutations in survival motor neuron 1 (SMN1) gene and LUXTURNA (voretigene neparvovec-rzyl) to deliver a normal copy of the RPE65 gene to correct a biallelic RPE65 mutation in patients with retinal dystrophy (Domenger and Grimm, 2019; Keeler and Flotte, 2019). Hence, gene-therapy based approaches to modulate circRNAs are therapeutically feasible in future. Delivering a synthetic functional RNA circle to a cell with a goal to increase specific circRNA levels is also a viable future approach. Natural linear RNAs can be produced by in vitro transcription followed by chemical or enzymatic ring closure. With this strategy, the linear precursors can be entropically disfavored if the circle size is large (Muller and Appel, 2017; Petkovic and Muller, 2018). However, there are several challenges that need to be addressed before circRNAs are ready for the therapy, including the generation, stability, delivery and potential side effects; nevertheless, the development of circRNA-based therapies gives an opportunity to resolve the circRNA dysregulation and the resulting detrimental changes during neurological diseases.

One clinically-relevant aspect of targeted gene therapy is the cell type-specific expression and biological activity of ncRNAs. The circRNAs are found to be more predominant than linear RNAs for many mammalian genomic loci that produce both (Salzman *et al.*, 2013).

Further, the splicing pattern of genes to form circRNAs and linear RNAs is well-orchestrated to produce specific ratios of the two in certain cell-types (Salzman *et al.*, 2013). Thus, to develop cell-specific gene therapy, it is crucial to determine the transcriptome complexity of individual cells to produce a circRNA and a linear RNA from a gene locus. To answer this issue, a wide variety of single-cell RNA-seq methods, including Smart-seq, CEL-Seq, Quartz-Seq and single-cell universal poly(A)-independent RNA sequencing (SUPeR-seq) have been developed (Fan *et al.*, 2015; Tang *et al.*, 2009). Using this approach, Verboom et al have recently detected circRNAs in addition to linear RNA biotypes (Verboom *et al.*, 2019). At this time it is still in its infancy, but accumulating evidence indicates that there is a possibility to engineer lentiviral vectors to modulate circRNAs in a cell-specific manner (Kasaraneni *et al.*, 2018; Yang *et al.*, 2009; Yang *et al.*, 2006; Zhou and Buchholz, 2013).

11. Conclusions

CircRNAs are incipient ncRNAs with potential regulatory properties. CircRNAs are expressed in all tissues but are more abundant in CNS. Recent studies suggest that circRNAs are produced from several neuronal-specific genes, indicating their possible involvement in brain development and synaptic plasticity. CircRNA perturbation might be linked to neurodegenerative diseases and secondary brain damage following acute CNS injuries. A further understanding of the functions of circRNAs and their interaction with other classes of ncRNAs and transcriptional/translational mechanisms will be valuable for identifying new therapeutic paradigms to treat CNS complications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AD	Alzheimer′s disease
ADAR	adenosine deaminase acting-on RNA
ALS	Amyotrophic lateral sclerosis
APP	amyloid precursor protein
α-Syn	α-synuclein
Aβ	amyloid-β
BACE1	β-site APP-cleaving enzyme 1
BBB	blood-brain barrier
CDK2	cyclin-dependent kinase 2
CDR1	cerebellar degeneration-related autoantigen 1
CDR1as	CDR1, antisense
circRNAs	circular RNAs
CNS	central nervous system
DBR1	debranching enzyme
DCC	Deleted in Colorectal Cancer
ddPCR	droplet digital PCR

DHX9	DExD/H-box helicase 9
ETS-1	E26 transformation–specific sequence-1
FUS	fused in sarcoma
GO	Gene Ontology
GSK-3β	glycogen synthase kinase-3β
HI	hypoxia-ischemia
HIPK3	homeodomain interacting protein kinase 3
HNF1β	hepatocyte nuclear factor-1β
IRES	internal ribosome entry site
lncRNAs	long noncoding RNAs
m^{6}A	N^6-methyladenosine
MALAT1	Metastasis Associated Lung Adenocarcinoma Transcript 1
Mbl	muscleblind
MCAO	middle cerebral artery occlusion
MDD	major depressive disorder
METTL3	methyltransferase-like 3
miRNAs	microRNAs
MLL	mixed-lineage leukemia
MNs	motor neurons
MS	Multiple sclerosis
MSA	Multiple system atrophy
ncRNAs	noncoding RNAs
OGD	oxygen glucose deprivation
ORFs	open reading frames
PD	Parkinson’s disease
PINT87aa	87 amino acid tumor-suppressive peptide
PAF1	polymerase associated factor
pre mRNA	precursor mRNA
Pol II	polymerase II

PRR11	proline-rich protein 11
RBPs	RNA binding proteins
RNA-FISH	RNA fluorescence in situ hybridization
ROS	reactive oxygen species
SCI	spinal cord injury
SIRT1	Sirtuin 1
SMN1	survival motor neuron 1
SOX13	SRY-Box 13
SRSF1	serine and arginine-rich splicing factor 1
SRY	sex-determining *r*egion Y
TBI	traumatic brain injury
TDP-43	TAR DNA binding protein 43
TGF-β	transformed growth factor
TIPARP	2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)-inducible poly (ADP-ribose) polymerase
TNF-α	tumor necrosis factor-α
T-UCRs	transcribed ultraconserved regions
UBE2A	Ubiquitin protein ligase A
UCHL1	Ubiquitin C-Terminal Hydrolase L1
UTR	untranslated region
U1snRNP	U1 small nuclear ribonucleoprotein particle
VEGF-A	Vascular endothelial growth factor A
ZO-1	Zonula occludens-1

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Highlights

- Circular RNAs (circRNAs) are vastly conserved noncoding RNAs formed by back-splicing and the covalent fusion of RNA free ends into natural circles.
- They are highly abundant, dynamically expressed, and spatiotemporally regulated in the healthy brain.
- Their brain expression steadily increases from embryonic to the adult stage.
- The putative functions of circRNAs are transcription regulation, sequestration of miRNAs and ribonucleoproteins, and translation to peptides.
- Altered levels of circRNAs mediate brain diseases and degeneration.

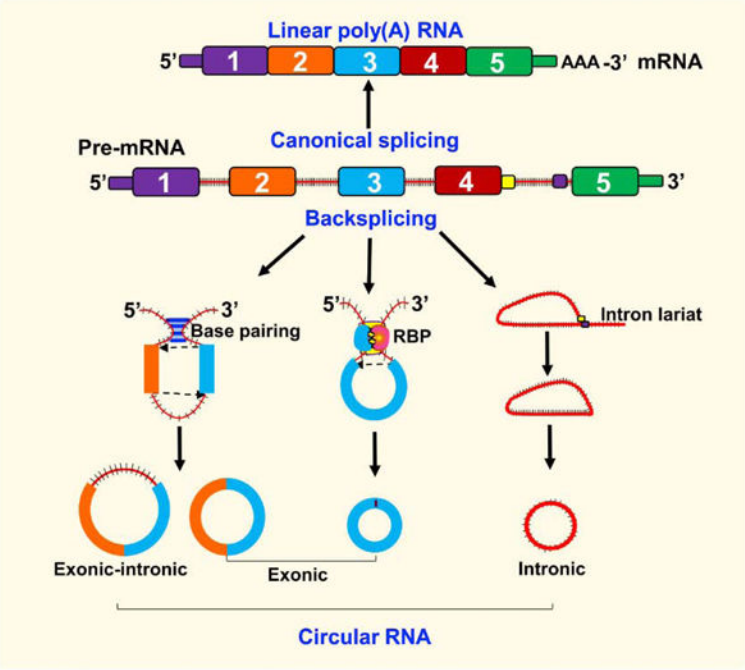

Fig. 1. Biogenesis of circRNAs.

CircRNAs are formed from either exons, introns or both exon-intron by back-splicing events and spliceosomal machinery. On the contrary, canonical splicing forms a mature mRNA after removal of introns. Various processes that form circRNAs are conventional back-splicing driven, intron-pairing-driven circularization, and lariat-driven circularization. The downstream donor and upstream acceptor sites are brought into close proximity during circularization by exon-containing lariats, direct base pairing between cis-acting regulatory elements containing reverse complementary sequences (Alu repeats) and flanking introns, trans-acting factors, such as RNA-binding proteins (RBPs, QKI, MBL) and intron lariats that escape the usual intron debranching and degradation.

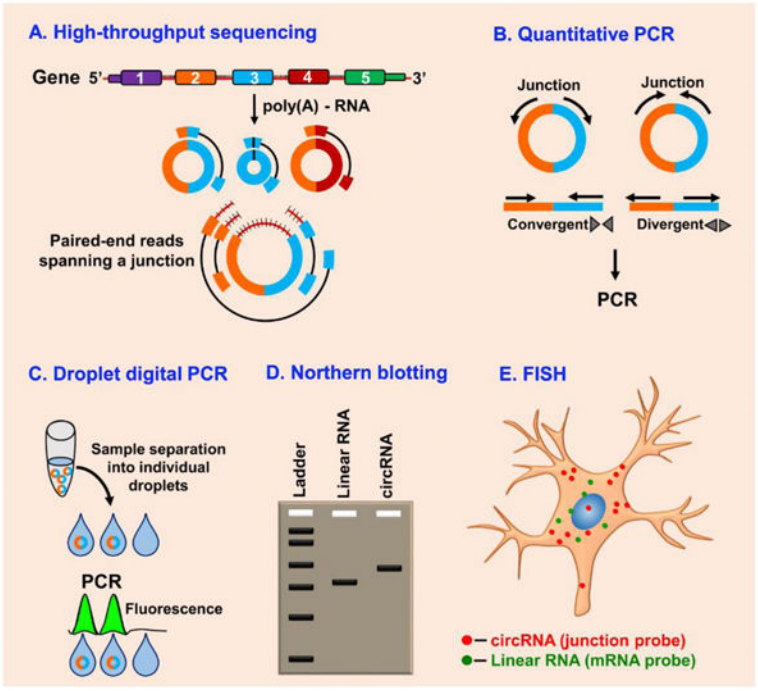

Fig. 2. Methods to detect circRNAs.

Coupled with the bioinformatics algorithms, deep sequencing and longer reads of RNAs digested with RNase R and poly(A) depleted RNAs increases the specificity of circRNAs detection (A). PCR analysis with convergent and divergent primers in RNase R-treated RNA preparations can be used to detect circRNAs (B). Droplet digital PCR quantifies absolute circRNA levels based on the single droplet molecules (C). Northern blotting can accurately detect and confirm an exact circRNA in a gel using probes that are designed to target both the circular and the linear transcript or only the circRNA with backsplice junction probe (D). Finally, RNA fluorescence in situ hybridization (RNA-FISH) coupled with high-resolution microscopy using probes flanking the junction sites can determine the distribution and abundance of circRNAs (E).

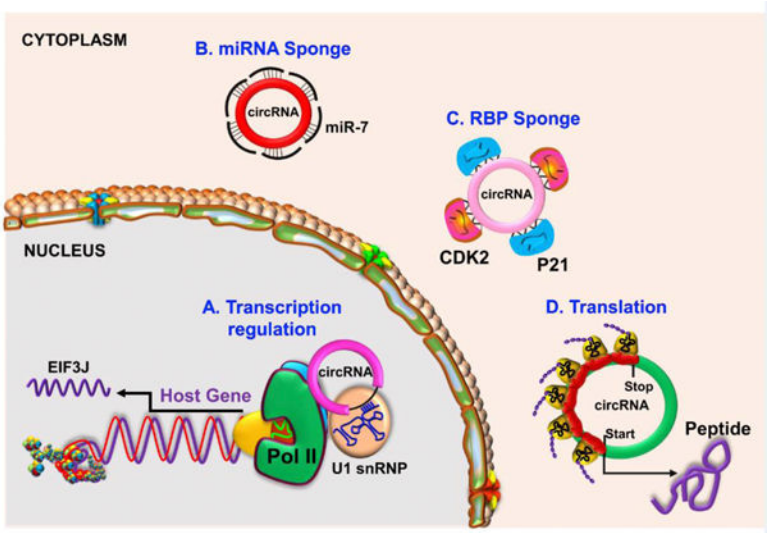

Fig. 3. Functions of circRNAs.

CircRNas plays diverse roles by regulating transcription (circEIF3J and circPAIP2) of their host genes, sponging miRNAs (CDR1as, SRY and HIPK3) and thereby derepressing miRNA-target mRNA translation, sponging RNA binding proteins (RBPs) such as CDK2 and P21, and some circRNAs also show translation potential (circMbl and circ-ZNF609) to form peptides.

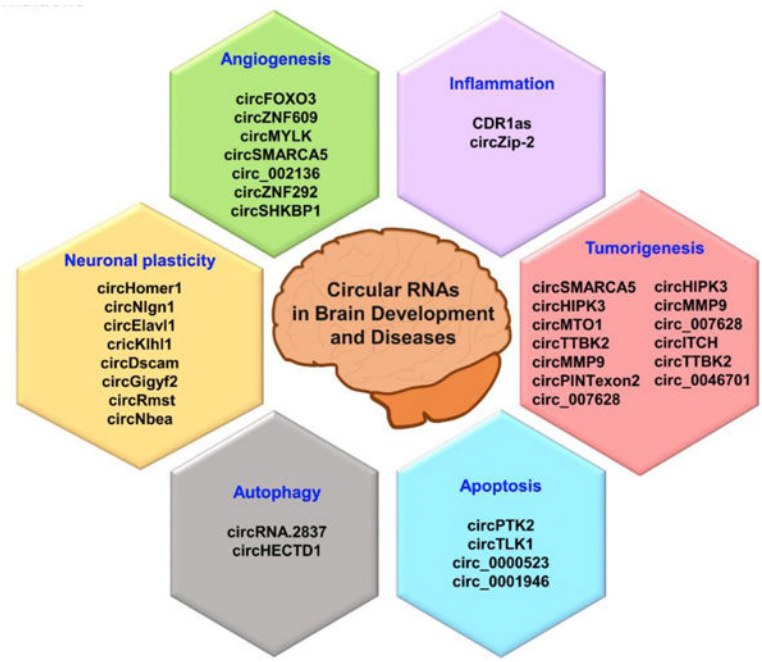

Fig. 4. Mechanisms regulated by circRNAs during brain development and disease conditions.

CircRNAs are not just abundant, but they continually increase from embryonic to adult stage in the brain to regulates functions related to neuronal plasticity. Additionally, their altered levels are engaged in brain diseases, and degeneration by various mechanisms, including angiogenesis, autophagy, apoptosis, tumorigenesis, and inflammation.