Chapter

The Role of Noncoding RNAs in Brain Cells during Rat Cerebral Ischemia

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Abstract

Ischemic brain stroke is one of the most serious and socially important medical conditions. Transcriptome analysis is a prospective approach to the study of the mechanisms of brain functioning, both under normal conditions and in ischemia. In addition to mRNA encoding proteins, the study of noncoding RNAs in ischemia has exceptional importance for the development of new strategies for neuroprotection. Of greatest interest are microRNAs (miRNAs) and circular RNAs (circRNAs). circRNAs have a closed structure and predominantly brain-specific expression. They can interact with miRNAs, diminish their activity, and thereby inhibit miRNA-mediated repression of mRNA. Recently, it has become clear that the analysis of circRNA-miRNA-mRNA interactions is an important requirement for the detailed study of the mechanisms of damage and regeneration during ischemia. This chapter reviews the most recent data on the role of circRNAs, miRNAs, mRNAs, and their interactions in brain cells under normal conditions and in cerebral ischemia.

Keywords: functional genomics, experimental rat brain ischemia, mRNAs, noncoding RNAs, circular RNAs, microRNAs

1. Introduction

Ischemic stroke is a serious condition and is one of the leading causes of disability and death worldwide. It arises as a consequence of a critical decrease in blood flow in the brain tissues, which leads to the death of neurons and glial cells. Therapy aimed at treating or preventing ischemic stroke is one of the most significant problems of modern medicine. Molecular genetic approaches using experimental models of ischemia based on small laboratory animals are of great importance and provide perspectives for studying the mechanisms underlying the damage to nerve cells and their ability to recover. Events occurring in ischemic stroke in humans caused by the formation of a thrombus are best reflected by the permanent middle cerebral artery occlusion (pMCAO) model. Additionally, the transient middle cerebral artery occlusion (tMCAO) model best reflects the events occurring in ischemic stroke in humans caused by subsequent treatment with thrombolytic drugs. The results of clinical studies suggest that thrombolysis is among the most effective and affordable methods of treating ischemic stroke. At the same time, it is known that reperfusion

after thrombolysis not only contributes to the restoration of penumbra cells but also causes additional damage to brain cells, including disruption of endothelial microvascular cells, the excess oxygen radicals, and activation of apoptosis.

Ischemic brain damage in combination with reperfusion damage is a complex process resulting from changes in the levels of transcripts of genes in response to pathological effects. Currently it has been shown that informational RNA and various types of noncoding RNA (ncRNA), in particular, microRNA (miRNA) and long ncRNA, are actively involved in the response to the pathology. Recently, the idea that long ncRNAs can interact with miRNAs and diminish their activity has been actively developed. Such functions are attributed to circular RNA (circRNA), which is a new and actively studied type of RNA. circRNAs can also participate in the pathogenesis of various neurodegenerative and inflammatory diseases and cancer. These properties of circRNAs can be exploited in medicine to develop technologies to correct pathological processes caused by disruption of gene expression. This chapter will examine the most recent data on the roles of circRNAs, miRNAs, mRNAs, and their interactions in brain cells under normal conditions and in cerebral ischemia.

2. Ischemic stroke

According to the latest data from the World Health Organization, ischemic stroke, which is the result of a permanent or temporary decrease in cerebral blood flow, is in most cases caused by occlusion of cerebral arteries by a thrombus or embolus and is of particular importance among vascular conditions [1–3]. This serious condition is the second most common cause of the general mortality rate of the population in Russia and is the most common cause of impaired brain function [4]. Long-term studies of ischemic stroke have proven the existence of necrosis and penumbra zones in the first hours and days after the development of ischemic stroke. The penumbra is the tissue located around the ischemic nucleus in conditions of limited access of oxygen and glucose, and cells in the penumbra are capable of recovery. The concept of a "therapeutic window" was developed in which this window is a period during which the restoration of penumbra cells is still possible and most effective. The duration of the therapeutic window may vary depending on the organism and model of ischemia, but for most cells, it is limited to 3–6 hours [4–9].

Cerebral ischemia results from biochemical changes in brain tissues after ischemic damage. During ischemia, following the occlusion of the vessel, the glutamate-calcium cascade is activated, contributing to an influx of Ca^{2+} ions, the formation of intracellular mediators (phosphoinositol and diacylglycerol), membrane depolarization, accumulation of glutamate, and further influx of Ca^{2+} leading to damage to the cell macromolecules and ultimately to cell death [4, 10]. Among the factors affecting the development of ischemic stroke, it is important to consider the effects of molecular genetic parameters. High hopes of clinicians are placed on identifying and developing systems of genetic markers, which are an important step toward the development of personalized medicine and individualized prevention. It is extremely important to study the genetic systems that determine the mechanisms underlying the events during the therapeutic window, the death of neurons during ischemic damage, and the restoration of neurological functions.

3. Transcriptomics of ischemic stroke

Recently, as a result of the rapid development of genome-wide analysis and multi-omics technologies, it has become clear that tissue damage and regeneration

during ischemia is a complex process resulting from a change in transcript levels of a significant number of genes in response to pathological effects. Thus, early-response genes such as *c-fos* and *c-jun* [11] and zinc finger genes trigger cell proliferation and differentiation [12, 13], while genes that encode heat-shock proteins are involved in the inflammatory response and cytoskeleton organization [14], and others are predominantly activated after the onset of ischemia. Of great importance and perspective in molecular genetic studies are the models based on small laboratory animals that best reflect certain features of the development of the ischemic process. Study of the molecular mechanisms of cell death using pMCAO and tMCAO models conducted by Ford et al. revealed molecular functions and biological processes unique for each model [15]. Genes unique to tMCAO were predominantly involved in the induction of inflammatory and oxidative stress, while pMCAO resulted in the expression of genes that were more associated with metabolic activity and cellular signaling [15]. A study of the dynamics of changes in gene expression in rat brain a day after pMCAO revealed a substantial number of genes that changed expression significantly and are involved in the development of ischemic damage, including those determining cell survival and death, the immune response, functioning of the vascular system, and also processes associated with hematopoiesis, immune cells, lymphocytes, leucocytes, and other cells [16].

The most frequently used tMCAO model showed a reorganization of the functioning of many genes in various areas of rodent brains, including the infarction center, during the first day after the transient occlusion [15, 17–19]. In particular, activation of the transcription factor Nf-κb was shown. An increase of the mRNA level of *Cox2*, which encodes one of the key enzymes for the synthesis of the pro-inflammatory prostaglandin E2 (PGE2), was accompanied by an increase in the level of the corresponding protein, not only at the source but also in adjacent regions, and accompanied by increased concentration of PGE2 [20–22]. At the same time, as a result of the opening of the blood-brain barrier in brain sections, extensive leucocyte infiltration was observed [21, 23, 24]. An increase of the mRNA level of the gene for INOS, encoding an enzyme for the synthesis of NO, also participating in the development of the inflammatory response in the lesion, was also noted [22, 25]. In the ischemia-reperfusion model, it was also shown that cytokines (IL-1β, IL6), adhesion molecules (ICAM1, E-selectin, MMP-9), MAPK kinase, and c-fos transcription factors were involved in the development of inflammation [17, 20, 23, 26–29]. Wang et al. studied the molecular mechanism of ischemia-reperfusion pathogenesis using genome-wide transcriptome analysis (RNA-Seq) in the hippocampus of rats at 24 h after tMCAO. These investigators detected 182 differentially expressed genes (DEGs), most of which were upregulated [17]. A Gene Ontology analysis showed that these DEGs were mainly associated with inflammation, stress, immune response, glucose metabolism, and apoptosis [17]. Our analysis of gene expression under tMCAO conditions using RNA-Seq confirmed these results. However, in the subcortical structures of the brain that contained the focus of ischemic damage and the penumbra, we identified hundreds of genes that changed expression 24 h after tMCAO using RNA-Seq. Among these, we found activation of genes involved in inflammatory and immune reactions. There were gene encoding chemokines (*Ccl2* and *Ccl3*), heat-shock proteins (*Hspa1* and *Hspb1*), macrophage receptors (*Msr1*), secreted phosphoprotein 1 (*Spp1*), cytokine 3 suppressor (*Socs3*), and other proteins. Mass suppression of genes that ensure the functioning of neurotransmitter systems (*Chrm1*, *Chrm4*, *Cplx2*, *Drd2*, *Gabra5*, and *Gng7*) was also shown [19]. A study of the dynamics of changes of gene expression in rat brain a day after tMCAO conditions revealed a significant activation of the expression of genes involved in biosynthetic cell systems (ribosome, proteasome, DNA replication, and purine metabolism functional categories). The effect obtained indicated

a large-scale reorganization of nucleic acid and protein biosynthesis that was apparently related to the adaptive response of brain cells to the damage caused by ischemia-reperfusion.

4. miRNAs in ischemic conditions

Not only coding mRNA but also various types of ncRNA, which have significant regulatory potential, are involved in the response to ischemia. Much current attention worldwide is paid to the study of the features of the functioning of mRNA, miRNA, and long ncRNA as regulators in the mechanisms of pathogenesis and neuroprotection in ischemic conditions [30–35].

miRNAs are ncRNA molecules with a length of 20–22 nt. They act by direct interaction with target sites on mRNA, which leads to the degradation of mRNA or repression of its translation [36, 37]. miRNAs are critical regulators of central nervous system plasticity and play an important role in ischemia. In particular, miRNA is actively involved in the response to ischemic brain damage [38, 39]. Following ischemic brain damage, miRNAs can play the role of both neuroprotective agents and contribute to pathological manifestations. mRNA of the AMPA receptor subunit GluA2/GluR2 (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor) is the target of miR-181a. Thus, an increase in miR-181a expression may be neuroprotective. Indeed, there are many examples of where miRNAs contribute to the development of the pathological process following ischemic brain damage. Thus, miR-132 increases the expression of the NMDA receptor, which selectively binds N-methyl-d-aspartate, increasing the risk of excitotoxicity [40, 41]. Therefore, the use of miR-132 antagonists may have a neuroprotective effect. Herzog et al. studied the role of steroid hormones 17β-estradiol (E2) and progesterone (P) in the brain as regulatory factors for miR-223-3p, miR-200c-3p, miR-375-3p, miR-199-3p, miR-214-3p, and their target genes in the tMCAO model [42]. The levels of these miRNAs are increased at 12 and 72 h after tMCAO. E2 or P selectively dampened miR-223 and miR-214 but further boosted miR-375 levels. The expression of the genes for NR2B and GRIA2, which are targets for miR-223, was reduced after tMCAO, and E2 and P canceled this effect. Steroid therapy inhibited tMCAO-induced increases in the expression of genes for BCL-2 and RAD1, which are targets for miR-375. Thus, E2 and P have a role as indirect regulators of translation of proapoptotic and pro-inflammatory genes, which leads to the weakening of ischemic damage of tissue [42].

5. Long ncRNAs and circRNAs

Long ncRNAs have lengths greater than 200 nt [30]. Analysis of GENCODE [32], LNCipedia [43], and NONCODE [44] databases indicates the number of annotated long ncRNAs reaches several tens of thousands in humans. Their number is several times greater than the number of human protein-coding genes. Long ncRNAs are classified according to the region of the genome from which they are synthesized [32, 45]. Intergenic long ncRNAs are the most common in humans (59.2%). In second place are sense long ncRNAs that overlap with protein-coding genes (24.4%). Intronic and antisense long ncRNAs account for approximately 10% each [45]. Many long ncRNAs have specific evolutionarily stable expression. In addition, long ncRNAs exhibit tissue-, sex-, developmental stage-, and disease-specific expression [34, 46]. According to Mercer et al., in mice 64% of long

ncRNAs are associated with brain tissue [47]. Cabili et al. found that long ncRNAs may have a more pronounced tissue-specific expression than protein-coding genes [48].

To date, there is evidence that a substantial part of long ncRNA exists in a circular form [49–54]. Circular RNA (circRNA) is a newly discovered and relatively poorly studied class of long ncRNA, found predominantly in mammalian cells. The mammalian circRNAs are distinguished by a variety of structural organization. A common property of all cyclic structures is their resistance to treatment with RNase R, which depletes linear forms of RNA [55, 56]. A specific feature of the structure of exonic circRNAs is the unusual order of exon connection, in which the 3′-end of the downstream exon is linked with the 5′-end of the upstream exon. The mechanism of circRNA formation is called back-splicing. circRNAs may consist of exon or intron sequences [51]. More recently, information has appeared on the existence of circRNAs containing, simultaneously with exons, sequences of un-spliced introns [57] and recursive (RS) exons [58]. We come to the study of circRNAs through the analysis of peculiarities of the structure and expression of the human *SGMS1* gene. This gene encodes the enzyme sphingomyelin synthase 1, which provides the synthesis of sphingomyelin and diacylglycerol from phosphatidylcholine and

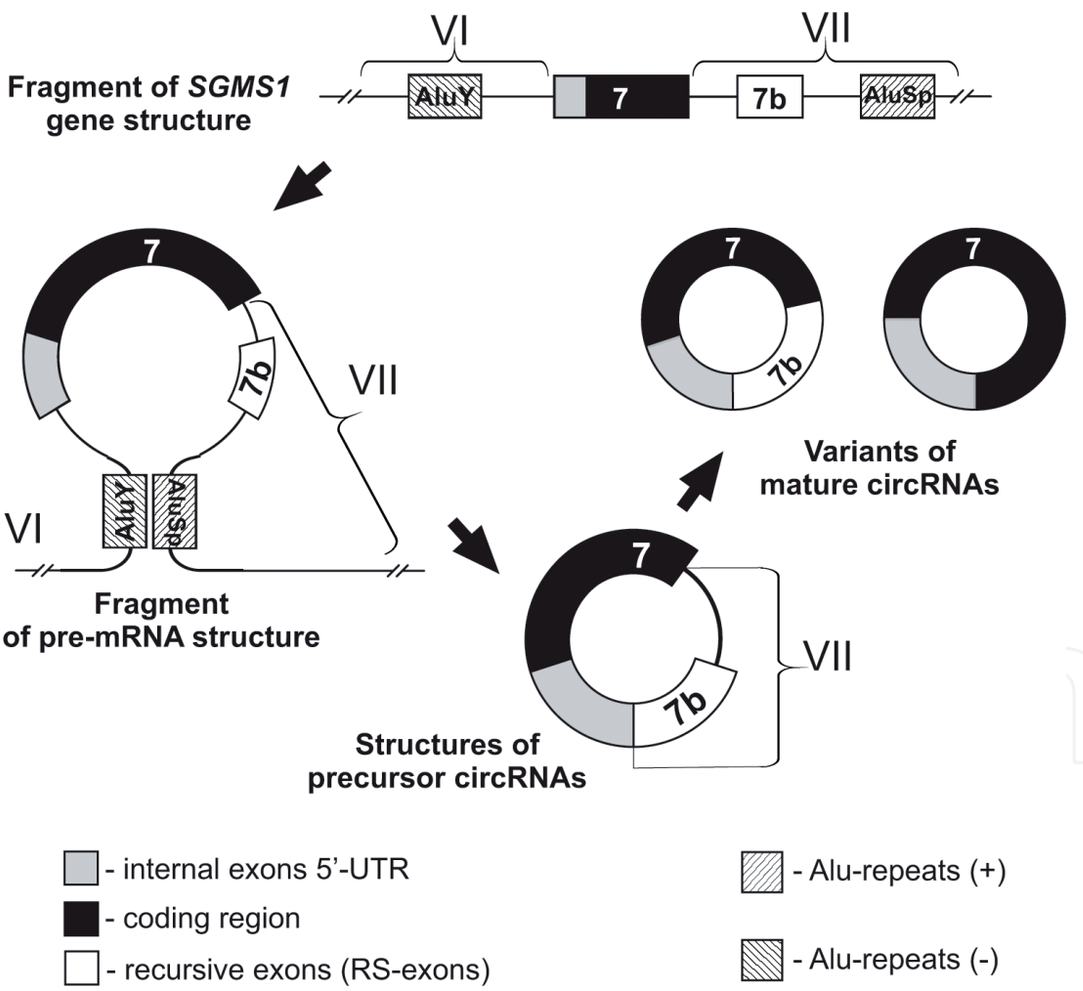

Figure 1.
A model of circRNA formation with the participation of recursive exon (RS-exon). Exons are shown as numbered blocs. Roman numerals indicate introns. Exon 7 is part of the mRNA, and RS-exon 7b is located inside the intron VII. The convergence between the 5′-end of exon 7 and the 3′-end of RS-exon 7b is effected using an interaction of highly homologous repeats of the Alu subtype, which are located near the back-splicing sites. Thus, the structure of the precursor of circRNA is formed according to the back-splicing. Next, part of intron VII is excised up to RS-exon 7b, and a linkage of the main exon with the RS-exon is formed. This leads to the formation of circRNA, which includes RS-exon 7b. Otherwise, the RS-exon 7b is excised along with the rest of intron VII and leads to the circularization of the main exon 7.

ceramide [59–63]. In addition to mRNAs providing protein synthesis, 13 circRNAs that predominantly contained sequences of the multi-exon 5′-untranslated region of the gene (5′-UTR) have been identified [54]. The RS-exons that participate in the multistep splicing of long introns of the gene were found within six circRNAs of the *SGMS1* gene. Based on the human *SGMS1* circRNAs formation from pre-mRNA with the participation of RS exons, the model of recursive back-splicing was proposed (**Figure 1**). Intronic circRNAs often have loop-like (lariat) structures with an abnormal 2′–5′ phosphodiester bond [50, 51]. More than half of circRNAs contain only protein-coding exons, while a smaller proportion contains sequences corresponding to the UTRs [64]. In related species, the circRNAs are often encoded by genes that are orthologous for human genes. So, homologous exons of these genes are detected in circRNA [64]. Most human and rodent circRNAs have predominantly brain-specific expression [54, 65–68]. In particular, it has been shown that circRNAs are predominantly localized in areas of neurons (axons and dendrites). Their level depends on the stage of development of synapses and homeostatic plasticity [69]. It is believed that the accumulation of circRNAs upon neuronal differentiation could result from the combined effect of augmented transcription of circRNA-producing genes and diverse decay rates of circRNAs and their linear counterparts [70]. The specific expression and stability of circRNAs allow them to be considered as potential biomarkers for various diseases [71].

6. Competitive endogenous RNAs

Relatively recently, it was shown that miRNA activity in the human cells can be regulated by the so-called sponge transcripts of competitive endogenous RNA (ceRNA). These transcripts compete with mRNA for binding to miRNA and diminished the effect of miRNA on the transcriptional and posttranscriptional levels of gene expression regulation [72, 73]. Long ncRNAs may act as ceRNAs in mammals. There are examples of pseudogenic and intergenic noncoding transcripts that can perform the functions of ceRNA [74]. One example is regulation of the expression of the tumor suppressor gene *PTEN* using the RNA of its pseudogene PTENP1. The 3′-terminal region of the pseudogenic RNA (PTENP1) is highly homologous to the corresponding 3′-terminal region of the mRNA of *PTEN*. Competitive binding of the 3′-terminal region of the PTENP1 pseudogenic RNA with miRNAs (miR-19b and miR20a) ensures stable transcription of *PTEN* and translation of its mRNA [75]. The expression level of PTENP1 is about 100 times higher than that of mRNA of *PTEN*. This provides a competitive advantage of PTENP1 for binding miRNAs and performing the functions of ceRNA [72]. Among the recent most important and interesting studies of the functioning of ncRNA in ischemia, it is worth mentioning the work of Li et al. [76]. Malat1 ncRNA acts as ceRNA for ULK2 when the endothelial cells of the brain capillaries are damaged. Malat1 acts as an endogenous sponge for miR-26b. This leads to an increase in the expression of ULK2 and contributes to the autophagy of the endothelial cells of the brain capillaries and to the survival of oxygen-glucose in the conditions of deprivation/reoxygenation (OGD/R). Xing et al. showed that miR-155 inhibition may play a protective role in ischemic stroke by S6K phosphorylation on the Rheb/mTOR pathway [77].

Effective ceRNAs should have multiple miRNA binding sites and a high level of expression or increased stability [73, 78]. Of particular interest are circRNAs, which have a covalently closed structure and are often formed from protein-coding genes during back-splicing [52, 58]. circRNAs are not exposed to exonucleases [51, 52], so they can more effectively act as ceRNAs because of their increased stability.

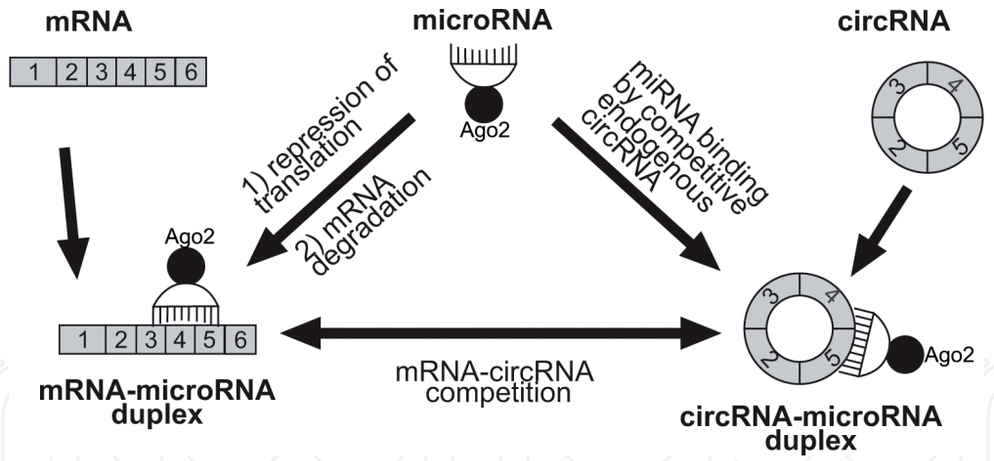

Figure 2.
Scheme of mRNA, miRNA, and circRNA interactions. Exons are shown as numbered blocs.

Currently, great attention is being paid to the function of circRNAs as miRNA sponges. CircRNA acting as ceRNA competes with mRNA for binding to miRNA and diminishes the effect of miRNA on transcriptional and posttranscriptional levels of regulation of gene expression [65, 79] (**Figure 2**). The function of several circRNAs as miRNA sponges has been investigated in various pathologies. In particular, the role of circRNA CIRs-7 in preventing models of neuropsychiatric disorders in mice is associated with its functioning as a ceRNA [79]. In addition, in Alzheimer disease [80] and various types of cancer [81–83], circRNA-miRNA-mRNA competition may be associated with regulation of pathogenesis.

7. The role of circRNA-miRNA-mRNA competition in ischemic conditions

The transcriptional profile and functional properties of circRNAs under conditions simulating brain ischemia have been investigated. Cell culture of HT22 hippocampal cells under conditions of OGD/R simulating damage during cerebral ischemia with reperfusion produced results consistent with the hypothesis that miRNA sponges are assigned to circRNA [84]. In this model, circRNA expression was associated with metabolic pathways related to apoptosis and immunity. In a tMCAO model, biological regulation, metabolism, cellular communication, and protein and nucleic acid binding were the main biological and molecular functions controlled by circRNAs, whose expression was changed during the day after occlusion [85]. Bioinformatics showed that 16 circRNAs contain binding sites for many miRNAs. In a mouse tMCAO model, microarrays detected a change in the expression of over a thousand circRNAs associated with signaling pathways regulating cell survival and death [86]. Moreover, Liu et al. predicted possible pathways of interactions between circRNA and miRNA that could provide information potentially elucidating the mechanisms of brain damage during stroke. We have investigated the expression of genes for glutamate metabotropic mGluR3 and mGluR5 receptors (*Grm3* and *Grm5*) in a tMCAO model [87]. These genes are important participants in the metabolic pathways associated with neuro-signaling. Rat *Grm3* and *Grm5* encode homologues for human and rodent circRNA. In the subcortical structures of rat brains containing a lesion, the level of such circRNAs is more stable than the corresponding mRNAs. Bioinformatics analysis revealed the distribution of miRNA binding sites along the mRNA molecules of human *GRM3* and *GRM5*, which are

homologous to the corresponding genes in rats. A sufficiently large number of binding sites are located inside the exons, which are also part of conservative circRNA. A functional role of circRNAs of the genes under study is implicated by ceRNA in the response of brain cells to ischemia. In an experimental ischemia-reperfusion model, we found numerous circRNAs that were differentially represented in the damage zone 24 h after occlusion. These circRNAs may be key modes for the regulation of the neurotransmission genetic response.

In a recent study, new important information was provided on the functioning of circRNA under ischemia conditions. Bai et al. showed that circRNA of DLGAP4 (circDLGAP4) functions as a miRNA sponge to diminish the activity of miR-143, which inhibits the expression of homologues of E6-AP C-terminal domain E3 ubiquitin ligase 1 [88]. The level of circDLGAP4 was significantly reduced in the plasma of patients with acute ischemic stroke and after tMCAO in mice. Upregulation of circDLGAP4 expression significantly reduced neurological deficit and reduced areas of infarction and damage to the blood-brain barrier in a mouse model of ischemia. Han et al. convincingly showed that circHECTD1 increases expression in the brain of mice after tMCAO, in human glioblastoma A172 cells under conditions of OGD/R, and in the blood of patients with acute ischemic stroke [89]. circHectd1 is involved in the regulation of the regenerative mechanisms of brain cells during ischemia. In particular, suppression of the expression circHectd1 was associated with a reduced infarction size in a mouse model of ischemia [89]. By interacting with MIR142, which negatively affects the mRNA level of the gene for 2,3,7,8-tetrachlorodibenzo-p-dioxin inducible poly [ADP-ribose] polymerase (TIPARP), circHECTD1 diminished the miRNA activity, with consequent circHECTD1-MIR142-TIPARP competition leading to the modulation of astrocyte activity through autophagy during cerebral ischemia.

8. Conclusion

The data presented in this review indicate that in addition to protein-coding mRNA, ncRNAs play an important role in the regulation of intracellular processes, both under normal conditions and in pathologies. An active study of the features of the functioning of ncRNAs in ischemia is of exceptional importance for the development of new strategies for neuroprotection and repair of nerve tissue and for the development of effective new drugs. circRNAs are a new class of RNAs that have enhanced resistance and preferential brain-specific expression. An analysis of circRNA-miRNA-mRNA interactions is an important component of any detailed study of the mechanisms of damage and regeneration in the case of pathological effects and the action of therapeutic agents, especially during the therapeutic window, when treatment is possible and most effective.

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Conflict of interest

The authors declare no conflict of interest.

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