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Review

microRNA as a new agent for regulating neuronal glutathione synthesis and metabolism

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Abstract: microRNA (miRNA) is a small non-coding RNA molecule that plays a role in the post-transcriptional regulation of gene expression. Recent evidence shows that miRNAs are involved in various diseases, including neurodegenerative diseases (NDs) such as: Parkinson's disease, Alzheimer's disease, Huntington's disease, Amyotrophic lateral sclerosisand multiple system atrophy (MSA).

The initiation and progression of NDs is generally considered to be induced by oxidative stress arising from an imbalance of oxidants and antioxidants. One of the most important antioxidants against oxidative stress is glutathione (GSH), which is a tripeptide composed of cysteine, glutamate and glycine. Among these precursor amino acids, cysteine is the determinant of neuronal GSH synthesis. Cysteine uptake in the neurons is mostly mediated by excitatory amino acid carrier 1 (EAAC1), a member of the sodium-dependent excitatory amino acid transporters. Interestingly, it has been reported that one miRNA, miR-96-5p, regulates the neuroprotective effect of GSH by directly regulating EAAC1 expression. Furthermore, the expressions of miR-96-5p and its target EAAC1 are specifically deregulated in the brains of patients with MSA, suggesting that deregulated miR-96-5p induces MSA via EAAC1 down-regulation. Since miR-96-5p regulation of EAAC1 expression and GSH level is indicated to be under circadian control, a greater understanding of rhythmic miRNA regulation could lead to the use of miRNA in chronotherapy for ND.

In this review, we focus on the role of miRNA in the mechanism of GSH synthesis and metabolism; particularly with respect to a critical transport system of its rate-limiting substrate via EAAC1, as well as on the implications and chronotherapeutic potential of miRNA for NDs.

Keywords: glutathione; microRNA; oxidative Stress; EAAC1

1. Introduction

microRNAs (miRNAs), which were discovered in *Caenorhabditis elegans* in 1993, are a new class of RNA molecules with roles in the post-transcriptional modulation of gene expression [1]. Currently, 28,645 precursor and 35,828 mature miRNAs are registered in the miRBase database (release 21). These small regulatory RNAs were initially called small temporal RNAs. Later, three research groups reporting in the October 26th, 2001 issue of *Science* referred to this class of tiny RNAs as miRNAs [2,3,4]. In 2000, it was shown that one of the miRNAs, let-7, is highly conserved from flies to humans, indicating that miRNAs function across a wide range of species [5]. In fact, various miRNAs were then discovered in plants and mammals [2,3,4]. miRNA studies in humans revealed that the down-regulation and/or over-expression of miRNAs is linked to a number of clinically important diseases—such as cancer, cardiovascular disease, inflammatory disease, autoimmune disease and neurodegenerative diseases (NDs) [6]—that are also known to be closely related to oxidative stress [7-10]. Oxidative stress is defined as a persistent imbalance of redox states caused by an excess of oxidants and/or a depletion of antioxidants, resulting in cellular damage [11]. One of the endogenous antioxidants is glutathione (GSH), which plays an important role in the defense against oxidative stress initiated by reactive oxygen species (ROS) [10]. GSH was originally discovered more than 120 years ago in baker's yeast as a compound that reacts with the gluten in wheat and thereby weakens the strength of bread dough [12]. Recently, it has also been identified as a "kokumi" substance that enhances the intensities of salty, sweet and umami tastes by binding to calcium-sensing receptors [13,14]. The long history of GSH started in 1888 with the momentous discovery of a substance named "philothione" by de Rey Pailhade [12], who found that this substance had the ability to reduce sulfur to hydrogen sulfide. In 1930, its structure was revealed to be a peptide consisting of the three amino acids. One of the determinants of the intracellular GSH level is a cysteine transporter known as excitatory amino acid carrier 1 (EAAC1) [15]. Recent evidence indicates that a miRNA, miR-96-5p, regulates the GSH level via EAAC1 to control the ROS level in the brain [16]. In agreement with this finding, the expressions of miR-96-5p and its target EAAC1 have been reported to be deregulated in the brains of multiple system atrophy (MSA) patients [17], implying a strong correlation between NDs and the miRNA-associated dysregulation of GSH. In this review, we will focus on the relationships between miRNAs, oxidative stress, GSH regulation and NDs.

2. miRNA biogenesis

miRNAs comprise a class of short, non-coding, RNAs of approximately 21–23 nucleotides in length that bind to target gene transcripts and negatively regulate the expression of targets [18]. miRNAs generally target the 3'-untranslated regions (3'-UTR) of target mRNAs [19]. However, it has been reported that the 5'-UTR and amino acid coding sequences can also be targeted [20,21]. The majority of miRNAs are located in intergenic regions or in an antisense orientation to gene regions on the genome; indicating that they are transcribed using their own promoters. The remaining miRNAs are mostly located in intronic regions, indicating that they are processed from introns of protein-coding mRNAs. Clustered miRNAs can either be simultaneously transcribed from single polycistronic transcripts containing multiple miRNAs or independently transcribed.

The biogenesis of miRNAs begins from the transcription of primary transcripts, termed the

pri-miRNAs, by RNA polymerase II or, to much lesser extent, by RNA polymerase III. The pri-miRNAs are mRNAs that are usually thousands of nucleotides in length and are capped and polyadenylated. They have a stem-loop structure in which mature miRNA sequences are embedded. The pri-miRNAs can be cleaved by a complex called Microprocessor—which forms between the ribonuclease III Drosha and the RNA-binding protein DGCR8/Pasha—to generate small hairpin-shaped RNAs of approximately 70–100 nucleotides in length, called pre-miRNAs. The pre-miRNAs are then exported from the nucleus to the cytoplasm by exportin-5 in complex with RAN-GTP. Pre-miRNA is processed by a double-stranded ribonuclease III enzyme termed Dicer, which is complexed with a double-stranded RNA-binding protein (the TAR RNA-binding protein in humans). The mature miRNA duplexes are then generated; one from a 5' strand denoted with a -5p suffix and the other from a 3' strand denoted with a -3p suffix. They are subsequently loaded onto an Argonaute protein to form an effector complex called the RNA-induced silencing complex (RISC). One strand of the miRNA, called the "passenger" strand (miRNA*), is then removed from RISC to generate the mature RISC that induces translational repression. The translational regulation by the RISC complex is mediated by incomplete base-paring of miRNA-mRNA interactions, likely due to the targeting of multiple transcripts. The "seed sequence" at the 5' end of miRNAs is located at nucleotide positions 2 to 7 and is crucial for target recognition. However, recent studies have revealed that non-canonical or seedless miRNAs also bind to target transcripts, and thus the rules for the recognition of target mRNA by miRNA are complicated and still elusive [20] (for a detailed review of miRNA biogenesis, see reference [18]).

3. Oxidative stress and miRNA

The balance between oxidants and antioxidants is a key factor for normal physiological functions. The imbalance of redox states caused by an excess of oxidants and/or a depletion of antioxidants is defined as an oxidative-stress state [22]. Oxidative stress has been implicated in the etiology of various diseases, including NDs, and is associated with excess production of ROS [23]. On the other hand, ROS act as signaling molecules and play important roles in a variety of physiological functions, including the regulation of autophagy, immunity and differentiation. ROS are generated during the mitochondrial electron transport of aerobic respiration, as well as during cellular responses to xenobiotics, cytokines, and bacterial invasion [24]. ROS is a general term that includes molecules or ions formed by highly-reactive and partially reduced oxygen metabolites, such as: the superoxide anion (O_2^-), hydroxyl radicals ($^{\cdot}OH$), hydroperoxyl radicals ($HO_2^{\cdot}$) and hydrogen peroxide (H_2O_2). Superoxide rapidly reacts with the nitric oxide (NO) produced by NO synthases (NOS), producing peroxynitrite ($ONOO^-$), which is a potent cytotoxic chemical.

Several miRNAs have been shown to be induced by ROS stimulation [8,25]. Among them, it is quite interesting that H_2O_2 and NO can induce up-regulation of the miR-200 family members miR-141, miR-200a, miR-200b, miR-200c, and miR-429 [26-29], which have been extensively studied in the epithelial-to-mesenchymal transition (EMT) of cancer cells. Several reports have shown that H_2O_2-induced miR-200 family members can affect tumorigenesis by targeting the E-cadherin transcriptional repressor ZEB or p38 MAPK. In addition, H_2O_2 induces the expression of miR-21 and miR-23a, leading to apoptosis and cell death by targeting PDCD4 and Fas, respectively [30,31]. These lines of evidence indicate that miRNAs are important mediators in response to ROS.

Table1. miRNAs in regulating genes encoded antioxidant enzyme and GSH regulator SOD and catalase.

SOD and catalase					
Target gene	microRNA	Regulation	References	Associated NDs	References
SOD1	miR-24	direct	[32]	MSA	[17]
	miR-125a-3p	direct		AD	[116]
	miR-872	direct			
	miR-377	possibly direct	[33]		
SOD2	miR-17*	possibly direct	[34]		
	miR-21	TNFα-mediated	[35]		
	miR-21	unknown	[36]		
	miR-23a-3p	unknown	[37]	MSA	[17]
	miR-30a	unknown	[38]	AD	[116]
				MSA	[17]
	miR-34a	direct	[39]	AD	[115]
	miR-335	direct			
	miR-126	unknown	[40]		
	miR-146a	possibly direct	[41]	ALS	[132, 133]
				MSA	[17]
	miR-212	direct	[42]		
	miR-222	possibly direct	[43]		
	miR-377	possibly direct	[33]		
	miR-382	direct	[44]	HD	[127]
SOD3	miR-21	direct	[35]	MSA	[17]
catalase	miR-21	unknown	[45]	MSA	[17]
	miR-30b	direct	[46]	AD	[116]
	miR-146a	unknown	[47]	ALS	[132, 133]
				MSA	[17]
	miR-551b	unknown	[48]		
GSH Synthesis and Metabolism					
Target gene	microRNA	Regulation	References	Associated NDs	References
GCL	miR-433	direct	[49]	HD	[127]
GR	miR-214	direct	[50]	AD	[116]
GPx1	miR-181a	direct	[51]	AD	[116]
GPx2	miR-17*	possibly direct	[34]	HD	[126]
	miR-185	unknown	[52]		
GPx3	miR-486	neuroD6-mediated	[53]		
GSTP1	miR-133a	possibly direct	[54]		
		unknown	[55]		
		possibly direct	[56]		
	miR-133b	direct	[57]	PD	[120]
	miR-513a-3p	direct	[58]		

Txn1	miR-525-3p	possibly direct	[59]		
Txnrd2	miR-17*	possibly direct	[34]		
	miR-34a	direct	[39]	AD	[115]
	miR-335	direct			
Txnip	miR-17	direct	[60]	MSA	[17]
	miR-224	direct	[61]		
	miR-452	direct			
	miR-372	unknown	[62]		
	miR-373	direct	[63]		
Prx3	miR-23b-3p	direct	[64]	MSA	[17]
	miR-23b-3p	direct	[65]	MSA	[17]
	miR-26a-5p	direct		AD	[116]
EAATs					
Target gene	microRNA	Regulation	References	Associated NDs	References
GLAST	miR-124	unknown	[66]	HD	[126]
				MSA	[17]
GLT1	miR-107	possibly direct	[67]		
	miR-124	unknown	[66]	HD	[126]
				MSA	[17]
	miR-124	unlikely direct	[68]	HD	[126]
				MSA	[17]
	miR-181a	unknown	[69]	AD	[116]
	miR-218	unknown	[70]		
EAAC1	miR-96-5p	direct	[16]	MSA	[17]

* Regulation of miRNAs is regarded as "direct" only when 3'-UTR assays using mutations of target sequences are done, or as "possibly direct" when the target sequence is presented on 3'-UTR and 3'-UTR assays are done.

Superoxide dismutase (SOD) is an enzyme that converts superoxide to hydrogen peroxide [10]. Three types of SOD have been identified so far: SOD2 (also known as MnSOD), which requires manganese ion, is expressed in the mitochondria; SOD1 (also known as Cu/ZnSOD) and SOD3 (also known as ECSOD), which require copper and zinc ion, are respectively expressed intracellularly and extracellularly. Superoxide, which is dismutated by SOD, is subsequently converted to water and oxygen by either catalase or glutathione peroxidase (GPx) [71]. Catalase does not require any activator, while GPx requires GSH for its activity. So far, several miRNAs have been identified that target SOD and catalase (Table 1). It is of particular interest to note that miR-21 was able to modulate the expression of SOD2, SOD3 and catalase, since it has also been reported as an miRNA that is induced by H_2O_2. MiR-21 has been extensively studied and shown to be associated with various diseases, such as cancer, Alzheimer's disease (AD), diabetes and cardiac disease (the human microRNA disease database (HMDD): http://cmbi.bjmu.edu.cn/hmdd) [72]. Since all of these conditions are known to be caused by oxidative stress, miR-21 might be a key factor in the control of oxidative damage.

4. Relevance of miRNA in GSH synthesis and metabolism

GSH is a tripeptide that is composed of three amino acids, glutamate, cysteine and glycine [73], which plays important roles as an antioxidant against oxidative stress. The brain contains GSH at varying concentrations of approximately 2–3 mM [74]. GSH is one of the most important cellular thiols, also being used as a storage and transport form of cysteine. An excess amount of cysteine can be toxic to the cells because it induces free radical generation, increases extracellular glutamate production and triggers over-activation of N-methyl-D-aspartate receptors [75]; in that regard GSH plays a crucial role in the defense against cellular damage. GSH is a non-enzymatic antioxidant that acts as an important defense against all forms of ROS. It is especially important in the brain, not only because the brain exhibits lower SOD, catalase and GPx activities compared with other organs, but also because the brain contains an abundance of lipids with unsaturated fatty acids that act as a source of peroxidation [10].

Two enzymes are involved in GSH synthesis, glutamate-cysteine ligase (GCL; also known as γ-glutamylcysteinesynthetase) and glutathione synthetase (GS) [10] (Figure 1). Both enzymes require adenosine triphosphate (ATP). GCL catalyzes the rate-limiting step, which makes a bond of glutamate and cysteine to form a dipeptide, γ-glutamylcysteine (γ-GluCys) that has been recently reported to take an antioxidant function by acting as GPx cofactor in mitochondria [76]. Intracellular GSH regulates its own levels by negative feedback to GCL [77]. GCL forms a heterodimer of a catalytic (GCLc) and modulatory subunits (GCLm). GCLc possesses all of the enzymatic activity, whereas GCLm alters the kinetics of GCLc activity for GSH. Interestingly, both subunits of GCL are a direct target of miR-433, which promotes a decrease in the GSH level [49], suggesting that miR-433 is a critical GSH regulator via GCL expression. This is supported by studies involving GCLc knockout resulting in embryonic lethality in mice, and GCLm knockout resulting in a decrease in GSH levels [78,79].

GS is a second enzyme that combines γ-GluCys with glycine to generate GSH [10]. Cases of GCL or GS deficiency have been reported; these deficiencies are autosomal recessive metabolic disorders causing impaired physiological functions such as neuronal dysfunction, and can cause mortality in early life [80]. Although these disorders caused by inborn errors are very rare, disorders of GSH metabolism are common in the various diseases showing GSH depletion and increased levels of oxidative stress. Many of these diseases might be caused by alterations in miRNA expression that reduces the expression of genes related to GSH synthesis and metabolism.

Synthetized GSH reacts non-enzymatically with ROS or acts as an electron donor for the reduction of peroxides in the GPx reaction [10]. In the GPx-catalyzed reaction, the formation of a disulfide bond between two GSH molecules gives rise to oxidized glutathione (GSSG). Eight isoforms of GPx have been identified so far, from GPx1 to GPx8. GPx1-GPx4 are selenium-containing enzymes, whereas GPx5, GPx7 and GPx8 are selenium-independent [81]. Although GPx6 is a selenoprotein in humans, selenocysteine is replaced by cysteine in rodents [82]. GPx1 is the most abundant isoform in the cytosol of nearly all mammalian cells, and functions as an important antioxidative enzyme that interacts with fatty acid hydroperoxides as well as H_2O_2 in the brain [83,84]. GPx2 and GPx3 have been identified as gastrointestinal and plasma GPx, respectively. GPx4 is a phospholipid hydroperoxide Gpx that is found in the mitochondria, nucleus and cytosol, and is expressed at low levels in most mammalian cells [85,86,87]. GPx5 is known as an epididymal-specific secretory GPx [84,88], and GPx6 mRNA is only detected in embryos and the

olfactory epithelium [82]. GPx7 and GPx8 have been observed in the endoplasmic reticulum (ER) [89]. GPx1 is present in both neurons and glial cells [90], and has been reported to be directly down-regulated by miR-181a, whose expression is induced by treatment with H_2O_2 [51]. On the other hand, GPx2 has been reported to be negatively regulated by miR-17* [34] and miR-185 [52], although whether this regulation is direct or indirect is unclear. Further, miR-486 has been reported to regulate GPx3 through a pathway mediated by NeuroD6 [53], which is known to be involved in neuronal development and differentiation, and is down-regulated in AD brains [91].

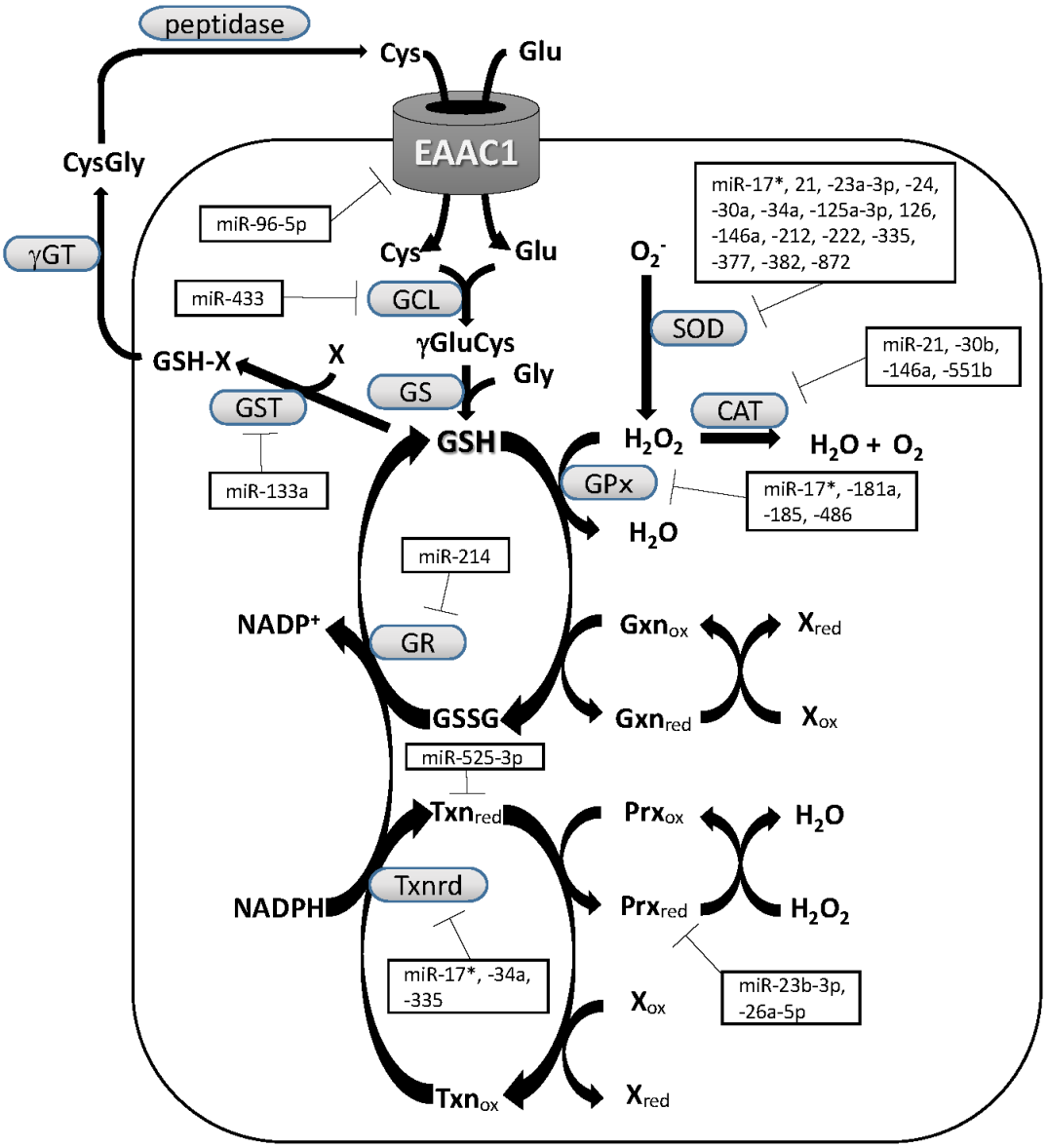

Figure 1. Redox system regulation by miRNAs. EAAC1 mediates glutamate/cysteine uptake in neurons. GSH synthesis is catalyzed by GCL and GS.GR catalyzes the reduction of GSSG to GSH. GPx reduces H_2O_2 to H_2O by gathering the needed reducing equivalents from GSH. On the other hand, catalase (CAT) reduces H_2O_2 without any activator. Both Gxns and Txns reduce substrates, and are oxidized by substrates as a result. Then, Gxns are reduced by GSH, whereas Txns are reduced by Txnrd. The

Prxfamily of enzymes is a separate node, removing H_2O_2 using reducing equivalents from Txn. Abbreviations are as follows: Glu: glutamate; Cys: cysteine; Gly: glycine; γGluCys: γ-glutamylcysteine; CysGly: cysteinylglycine; GSH: glutathione; GSSG: glutathione disulfide; GCL: γ-glutamylcysteine ligase; GS: glutathione synthase; GPx: glutathione peroxidase; GR: glutathione reductase; GST: glutathione-S-transferase; γGT: γ-glutamyltransferase; SOD: superoxide dismutase; CAT: catalase; Gxn_{ox}: oxidized form of glutaredoxin; Gxn_{red}: reduced form of glutaredoxin; Txn_{ox}: oxidized form of thioredoxin; Txn_{red}: reduced form of thioredoxin; Txnrd: thioredoxin reductase; Prx_{ox}: oxidized form of peroxiredoxin; Prx_{red}: reduced form of peroxiredoxin; X_{ox}: oxidized form of organic compound; X_{red}: reduced form of organic compound; GSH-X: GSH conjugated compound.

GSSG is a substrate of the flavoenzyme glutathione reductase (GR), which transfers an electron from nicotinamide adenine dinucleotide phosphate (NADPH) to GSSG, thereby regenerating GSH [92]. It has been reported that miR-214, which is up-regulated by alcohol-induced oxidative stress, binds specifically to the 3'-UTR of GR and represses its expression and activity [50]. Since miR-214 has also been associated with cancers, heart disease and Huntington's disease (HD) as described in the HMDD, its negative GR regulation may cause these diseases via a decrease in GSH and resulting induction of oxidative stress.

The reaction catalyzed by GPx or GR recycles GSH, whereas glutathione-S-conjugation by glutathione-S-transferases (GST) consumes GSH. Among the variety of isozymes of GST, the α-class is expressed in astrocytes, neurons and ependymal cells, the μ-class in neurons and astrocytes, and the π-class in oligodendrocytes in the CNS [93]. Only the π-class of GST, GSTP1, has been reported to be negatively regulated by miRNAs—specifically, miR-133a, 133b and 513a-3p—leading to tumor suppression or apoptosis [54-58]. GSH-conjugated compounds are subsequently excreted from the cell. Extracellular GSH is also released from glial cells, specifically astrocytes [94]. These GSH conjugates and the extracellular GSH are substrates for ectoenzymeγ-glutamyltranspeptidase (γ-GT) and undergo transpeptidation to cysteinylglycine (CysGly) or glycylcysteine (GlyCys). CysGly can be hydrolyzed by ectopeptidase to cysteine and glycine, leading neurons to take up amino acids and begin the GSH synthesis again [95].

Glutaredoxins (Gxns) and thioredoxins (Txns) have been identified as electron donors for ribonucleotide reductase, which acts as a protector of the intracellular redox state and antioxidants [96]. Both Gxns and Txns are members of a superfamily of low-molecular-mass proteins that catalyze the reduction of disulfide bonds in a variety of proteins. Gxns couple to NADPH and GR, whereas Txns couple to NADPH and thioredoxin reductase (Txnrd). Two isoforms of Gxns have been identified so far; Gxn1 is expressed in the cytosol and Gxn2 in the nucleus and mitochondria. A recent report has shown that Gxn1 is essential for the maintenance of mitochondrial complex I in the brain [97]. Further, its expression is increased in the neurons of patients with AD [98]. Decreased Txn and increased Txnrd levels have also been reported in the brains of AD patients. There are two isoforms of either Txns or Txnrds; Txn1and Txnrd1 are located in the cytosol and Txn2 and Txnrd2 are found in the mitochondria. Txn1 is one of the targets of miR-525-3p that has been reported to be involved in the ionizing radiation-induced oxidative stress response of several different cell types [59]. In addition, miR-335 and miR-34a have been shown to induce premature senescence of young mesangial cells via the suppression of Txnrd2 with a concomitant

increase in ROS [39]. Thioredoxin-interacting protein (Txnip) is known to play a critical role in inflammation, cancer progression, and diabetes, via binding with Txns [99]. Several miRNAs, such as miR-17, miR-224, miR-372, miR-373 and miR-452, have been reported to be involved in the regulation of Txnip, suggesting the importance of modulators for the antioxidant effect. Peroxiredoxins (Prxs) are peroxidase enzymes that receive electrons from NADPH by coupling with Txn and Txnrd. Although Prxs are widely detected in various neuronal populations, their levels are very low in the dopaminergic (DA) neurons of the substantia nigra pars compacta (SNc) and in the CA1/2 pyramidal cells of the hippocampus; suggesting that these neurons are vulnerable to the oxidative stress occurring in ND. Six isoforms of Prxs have been identified so far; Prx1, Prx2, and Prx6 are found mainly in the cytosol, Prx3 is found in mitochondria, and Prx4 is a secreted protein. Prx5 is widely localized to the cytosol, mitochondria, and peroxisomes. Prx3, which forms a complex with Txn2 and Txnip in mitochondria, has only been reported to be regulated directly by the miRNAs miR23b-3p and miR-26a-5p, and is involved with tumor progression and/or cell differentiation via direct regulation [64,65].

5. miRNAs and the cysteine transport system

Cysteine is the rate-limiting substrate for neuronal GSH synthesis since the other precursor amino acids, glutamate and glycine, have much higher extracellular concentrations than cysteine and therefore cannot increase intracellular GSH [100]. Cysteine uptake in neurons is mostly mediated by sodium-dependent systems, mainly the excitatory amino acid transporter (EAAT) [101]. Although there is another cysteine uptake system that is called the ASC (alanine-serine-cysteine) transport system, this system is thought to play a minor role in neurons, because cysteine uptake is not suppressed by ASC substrates but EAAT substrates [102,103]. EAATs co-transport three sodium ions and one hydrogen ion with each glutamate and counter-transport one potassium ion [104]. There are five members in EAATs, glutamate aspartate transporter (GLAST; also known as EAAT1), glutamate transporter-1 (GLT-1; also known as EAAT2), excitatory amino acid carrier-1 (EAAC1; also known as EAAT3), EAAT4 and EAAT5 [105]. GLAST and GLT-1 are expressed in astrocytes, whereas EAAC1, EAAT4 and EAAT5 are expressed in neurons. EAAC1 is widely distributed throughout the CNS, although the distribution of EAAT4 and EAAT5 is restricted to cerebellar Purkinje cells and the retina, respectively. EAATs can use not only glutamate and aspartate but also cysteine as a substrate, whereas EAAC1 is suggested to mainly transport cysteine for the following reasons. (i) EAAC1 can transport cysteine at a rate comparable to that of glutamate with an affinity that is 10- to 20-fold higher than that of GLAST or GLT-1 [106]. (ii) Cysteine uptake in the cultured neurons that knockdown EAAC1 expression resulted in approximately 20% decreases in cysteine uptake and GSH content [103]. (iii) EAAC1-defficient mice showed an approximately 40% decrease in brain GSH content and neurodegeneration at advanced age [107]. Taken together, these results suggest that abnormal expression of EAAC1 could lead to impaired neuronal GSH metabolism, and ultimately to NDs. Recently, we reported that miR-96-5p could be a critical factor for regulating GSH levels and neuroprotective activity via direct regulation of EAAC1 [16]. We showed that intracerebroventricular (i.c.v.) injection of a miR-96-5p inhibitor increased EAAC1 expression, GSH levels and neuroprotection against oxidative stress in the mouse brain. Our results suggest that manipulation of the miR-96-5p level could be a therapeutic agent for increasing GSH levels in the brain.

Our unpublished data revealed that another miRNA could regulate GTRAP3-18, which is a

negative regulator of EAAC1, through an unknown pathway. GTRAP3-18, also known as addicsin, was initially identified as a binding protein of the carboxyl-terminus of EAAC1 using a yeast two hybrid screening analysis [108]. GTRAP3-18 encodes a 188-residue hydrophobic protein with a calculated relative molecular mass of 22,500. GTRAP3-18 is widely distributed throughout the brain, including in the cerebral cortex, striatum, hippocampus and cerebellum, and appears to be coincident with the neuronal distribution of EAAC1. GTRAP3-18 is an ER-localized protein belonging to the prenylatedrab-acceptor-family interacting with small RabGTPases, which regulate intracellular trafficking events [109]. The hydrophobic domain interaction between GTRAP3-18 and EAAC1 may keep EAAC1 in the ER to inhibit EAAC1-mediated glutamate uptake; as glutamate uptake has been shown to be elevated with the reduction of the GTRAP3-18 protein level [108,110]. One characteristic of GTRAP3-18-deficient mice is an increased expression of EAAC1 on the plasma membrane and a significantly higher amount of cysteine but not glutamate, glycine, or GABA compared to the wild-type mice [111], indicating that GTRAP3-18 deficiency potentiates the ability of EAAC1 to increase cysteine uptake for GSH synthesis. Moreover, an experiment using brain slices from GTRAP3-18-deficient mice showed a significant resistance to oxidative stress, suggesting that inhibition of GTRAP3-18 function leads to neuroprotection by increasing neuronal GSH synthesis. Taken together, these results suggest that miRNA regulation of EAAC1 and GTRAP3-18 could be an important therapeutic target to increase the GSH level in neurons.

6. miRNA in neurodegenerative diseases

NDs are disorders characterized by progressive loss of neuronal function and structure, resulting in neuronal death in the CNS [112]. Age-related neurodegeneration is a common feature in various NDs, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS) and multiple system atrophy (MSA). Although gene mutations have been identified as genetic factors of NDs, many of the factors involved in disease causation and progression are still unknown, because several environmental factors including oxidative stress take part independent of genetic mutations.

AD is the most common cause of dementia, affecting 6% of the world population over 65 years old; but its pathogenic mechanisms are considerably complex, including Aβ accumulation, tau protein phosphorylation, oxidative stress, and inflammation [113]. However, the characteristic histopathologic features have been studied extensively, including senile plaques, neurofibrillary tangles, loss of neurons and synaptic connections, and glial proliferations. Several miRNAs have been shown to be over-expressed or down-regulated in the brain tissue, peripheral blood and cerebrospinal fluid [114]. It is noteworthy that miR-125a, miR-30a-5p, miR-30b, and 34a, which are involved in the regulation of SOD1, SOD2 or catalase, are over-expressed in the tissues of AD patients [115,116], suggesting that the expression of antioxidant enzymes is decreased by the abnormal expression of miRNAs in brains of patients with AD, causing neurodegeneration. On the other hand, miR-181a and miR-214, which regulate GPx1 and GR, respectively, are down-regulated in the AD tissues [116], indicating that a reciprocal mechanism may be exerted to counteract excessive oxidative stress due to the reduced expression of SODs and catalase by deregulated miRNAs. It is also of interest that miR-141, a member of the miR-200 family, is over-expressed in the brain of AD patients, suggesting that the level of ROS in the AD brain is too high, which up-regulates ROS-induced miRNAs.

PD is recognized as the second most common ND after AD and affects 1% of the population worldwide over the age of 65 years [117]. Pathologically, the disease is characterized by a progressive degeneration of DA neurons projecting from the SNc to the striatum, resulting in motor clinical symptoms such as resting tremors, rigidity, bradykinesia and postural instability [118]. The histopathological hallmark of PD is the presence of intracytoplasmic inclusions called Lewy bodies, which are mainly aggregated forms of the protein α-synuclein [119], found in the surviving DA neurons of the SNc. In the SNc of PD patients, GSTP1-regulating miR-133b is down-regulated [120], suggesting that counteraction against excessive lipid peroxidation and protein oxidation may occur in the brains of patients with PD.

HD is a neurodegenerative disorder caused by the expansion of apolyglutamine stretch within the huntingtin protein (HTT) [121,122,123]. This disease has already been shown to be caused by the expansion of a CAG repeat in exon 1 of the *HTT* gene. However, the precise mechanism underlying the selective neuronal death is still elusive. Elevated levels of oxidative damage products such as malondialdehyde, 8-hydroxydeoxyguanosine, 3-nitrotyrosine and hemeoxygenase in areas of degeneration in the HD brain, and of increased free radical production in animal models, indicates the involvement of oxidative stress either as a causative event, or as a secondary constituent of the cell death cascade in the disease [124]. Recently, increases in glutamate uptake and the expression of EAAC1 and GLT-1 were observed in an HD model cell line [125]. Although the reason for the increased levels of EAAC1 and GLT-1 in the HD model is still unknown, these increases might be associated with a change in the expression of miRNAs in HD patients. In fact, miR-124, which has been reported to regulate GLAST and GLT-1, is down-regulated in Brodmann's area 4 of HD patients [126]. It has also been shown in HD patients that miR-382, which regulates SOD2, and miR-433, which regulates GCL, is down-regulated in the frontal cortex and striatum [127], possibly counteracting oxidative stress to increase antioxidant enzyme expression and GSH content. However, miR-486, a regulator of GPx3, is over-expressed whereas miR-17*, a regulator of GPx2, is down-regulated in HD patients [126], indicating that the regulatory mechanism of GPx by miRNAs is complicated. A recent report showed that GPx activity plays an important role for clearing mutant HTT in an animal model [128], suggesting that GPx-regulating miRNAs could be a therapeutic target of HD.

ALS, also known as Lou Gehrig'sdisease, is characterized by the predominant loss of motor neurons in primary motor cortex, the brainstem, and the spinal cord [129]. In most cases, it progresses rapidly and causes death to patients within several years after diagnosis. At present, more than 180 mutations in the SOD1 gene have been found to be linked to ALS [130]. Although the mechanism of mutant-SOD1 toxicity is still unclear, most studies suggest that mutations yield misfolded proteins, leading to an unknown toxic gain of function. Furthermore, recent evidence implicates that oxidation can induce wild-type SOD1 misfolded and contribute to sporadic ALS pathogenesis [131]. It has been observed the overexpression of miR-146a which regulates SOD2 and catalasein ALS patients [132,133]. In fact, there are some reports that have shown that the activity of SOD and catalase is significantly decreased in ALS patients [134,135]. miRNA may be involved in SOD1 misfolding caused by oxidation.

Finally, MSA is a rare, late-onset and fatally progressive ND characterized by the accumulation of α-synuclein protein in the cytoplasm of oligodendrocytes, the myelin-producing support cells of the CNS [136]. The clinical features of MSA may include atypical parkinsonism, cerebellar ataxia, and pyramidal signs, and the condition is always accompanied by autonomic failure [137]. Ubhi et al.

reported that several miRNAs were up-regulated in the brains of patients with MSA, such as miR-21, miR-24, miR23a, miR-30a and miR-146a, which have been reported to be involved in SOD and/or catalase regulation (Table1) [17]. In addition, up-regulation of miR-124 was also observed. miR-124 is a possible regulator of GLAST and GLT-1 expression and an important factor for neuronal development, which is specifically expressed in the CNS. These facts suggest that the detoxification system and glutamate uptake may be altered in patients with MSA. The authors specifically focused on the up-regulation of miR-96-5p as a disease-specific miRNA dysfunction in either MSA patients or its model, and found down-regulation of EAAC1 in the MSA model mouse. This evidence agrees with the findings of our own study, in which miR-96-5p bound directly to the 3'-UTR of EAAC1 and decreased its expression, leading to a reduction in GSH [16]. Our finding that i.c.v. injection of a miR-96-5p inhibitor increased the GSH level and conferred neuroprotection against oxidative stress via an increased level of EAAC1 may contribute to the development of therapeutic approaches for MSA.

7. Chronotherapeutic potential for neurodegenerative diseases

Chronotherapy is increasingly being recognized as an important and effective therapy in cancer, hypertension, depression and asthma [138]. However, there have been no studies on the use of chronotherapy for ND progression. Chronotherapy is the science of modifying the timing of drugs in order to maximize their effectiveness and minimize side effects, based on the body's circadian system. The circadian clock is an internal timekeeping system that allows organisms to adapt physiological and behavioral processes to environmental light/dark cycles [139]. Almost all organisms harbor this system, indicating that the circadian clock developed early in the evolution of life. In mammals, the master clock is located in the suprachiasmatic nucleus (SCN). The SCN drives endogenous rhythms and controls circadian rhythms in peripheral tissues, including other brain areas [140]. The circadian system is regulated by several clock genes. One of the clock gene mutant mouse strains exhibits increased ROS levels and accelerated aging, suggesting that the circadian clock is involved in ROS regulation [141]. The facts that sleep disorders and circadian disruptions are common in ND patients, and that their symptoms display diurnal fluctuations indicate a strong correlation between circadian rhythm and ND [142]. Several reports have shown that a circadian clock regulates the GSH level and its enzymes in various organisms [143-146], and the diurnal rhythm of GSH levels in the brain is anti-phasic with the rhythm of metabolic events requiring ROS [147-150]; suggesting that rhythmic GSH regulates the diurnal rhythm of ROS activity. Interestingly, it seems that ROS production also tends to be under circadian control, which is anti-phasic with GSH rhythm and phasic with metabolic events that require ROS [151]. We have reported that the cellular protection against ROS is correlated in a time-dependent manner with the GSH rhythm [16], suggesting that GSH is a main determinant of the time-dependent protective activity against oxidative stress. In general, the system of diurnal rhythm could be considered to contribute to the efficient use of energy in the body, so that the diurnal oscillation of GSH might be necessary for accelerating the physiological events that require ROS, as well as for minimizing the damage by oxidative stress. We have also revealed that rhythmic miR-96-5p regulates GSH rhythm through the direct regulation of EAAC1 expression. This is quite interesting, because time-dependent miRNA manipulation could be an effective therapy for diseases caused by GSH depletion, including NDs. Chronotherapy using miRNA could be a new approach for the treatment of ND progression.

8. Conclusion

Several miRNAs have been reported to be involved in the regulation of GSH synthesis and metabolism in various cell lines and tissues. The expressions of some of these miRNAs are known to be induced by oxidative stress. In the brains of patients with NDs, over-expression or down-regulation of miRNAs having the ability to regulate the expression of genes involved in GSH synthesis and metabolism has been observed. These evidences suggest that altered expression of miRNAs causes oxidative stress, or oxidative stress altered expression of miRNAs, leading to various diseases, including NDs. Furthermore, our study showed that rhythmic miRNA regulates GSH rhythm via the direct regulation of EAAC1, and the treatment with a miRNA inhibitor successfully increased the GSH level in the brain and neuroprotection against oxidative stress. This is a meaningful indication that the modulation of miRNA expression could be a novel therapeutic approach for NDs via an increase in GSH levels.

Conflict of interest

All authors declare no conflicts of interest in this paper.

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